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Postharvest Oxidative Stress in Plums: Mechanism and Implications for Storage and Fruit Quality

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This thesis is presented for the Degree of
Doctor of Philosophy
of
Curtin University of Technology

June 2010

Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due the acknowledgement has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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Acknowledgements

I am sincerely thankful to the Commonwealth of Australia and Curtin University of Technology for awarding me the Endeavour International Postgraduate Research Scholarship and Curtin University Postgraduate Scholarship to pursue my PhD degree. I will forever remain indebted to my parents, Mr. Palvinder Jit Singh and Mrs. Sarbjeet Kaur, for their partial financial assistance and strengthening my morale during studies.

I would like to thank my supervisor, Prof. Zora Singh, Curtin University of Technology, and associate supervisor, Dr. Ewald E. Swinny, Senior Scientist, ChemCentre Western Australia, for competent direction, intellectual inputs and motivation during my PhD research and thesis–writing.

I greatly appreciate Dr. D. Mark Hodges from the Agriculture and Agri–Food Canada, Nova Scotia, Canada and Prof. R.K. Sairam from the Indian Agricultural Research Institute, New Delhi, India for providing me with their research protocols and helping modifying them. My past mentors, Dr. D.V. Sudhakar Rao and Prof. R.K. Pal, are acknowledged for their excellent teachings that made me capable of undertaking this research project.

Thanks Mrs. Alicia Pasznicki, ex–laboratory manager, and Ms. Susan Petersen, laboratory manager, for technical assistance and encouragement to achieve my goal. I would also like to acknowledge Dr. Soon C. Tan, Department of Agriculture and Food Western Australia (DAFWA), for providing access to controlled atmosphere storage facility. Thanks to Mr. Bob Frayne for technical help at DAFWA. Dr. Harmohinder Dhammu from DAFWA, Dr. Charanjit Kahlon from Louisiana State University, and Mr. Ismail and Mrs. Siti Zaharah from Curtin University are greatly appreciated for their help with statistical analyses.

I am very grateful to Mr. Peter Vedeniapine from AgroFresh Inc. for a gift sample of SmartFresh™ (1–methylcyclopropene) and technical advice. I would like to acknowledge the Western Australian plum growers, Mr. John Cassotti, Mr. Angelo Ghilarducci, and Mr. Mark Wilkinson, for generously providing fruit material for my research. My sincere thanks to the Australian and American Societies for Horticultural Science, Curtin University, Mr. Mark Wilkinson, and Mr. Cameron

Goodair for providing me with travel funds to present research in various conferences.

My laboratory colleagues and friends who helped me in fruit harvesting and experimental setup deserve my heartfelt thanks for their unconditional support. I thank countless hands that helped me in various ways and I could not mention them personally; I express my apology to them. Finally, I am grateful to my wife, Mrs. Rajbir Kaur, and son, Master Soham Pal Singh, for their wonderful support and patience during my PhD studies.

Sukhvinder Pal Singh

Abstract

Postharvest life and susceptibility to chilling injury (CI) in Japanese plums (*Prunus salicina* Lindl.) are greatly influenced by preharvest and postharvest factors. The phenomenon of postharvest oxidative stress has been implicated in affecting fruit quality, potential storability and susceptibility to development of physiological disorders during storage of fruits. Therefore, the investigations were carried out to understand the role of various factors, such as cultivar, harvest maturity, storage conditions (temperature and atmosphere composition), duration of storage and postharvest treatments, in the development of oxidative stress in Japanese plums, in relation to fruit quality and CI. The degree of lipid peroxidation and membrane integrity was measured by determining the activity of lipoxygenase enzyme, concentration of thiobarbituric acid–reactive substances, and amount of electrolyte leakage. The activities of antioxidant enzymes, superoxide dismutase, catalase, and peroxidase, were determined as a direct measure of the enzymatic antioxidant capacity. The activities of enzymes (ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione reductase, and glutathione–S–transferase) involved in the oxidation and regeneration of ascorbate (AA) and glutathione (GSH) were also determined to underpin the dynamics of the AA–GSH cycle. The concentrations of AA, dehydroascorbate (DHA), GSH, and oxidized glutathione (GSSG) were determined to compute the AA:DHA and GSH:GSSG ratios as the indicators of redox potential of fruit tissue. The total phenolics concentration and total antioxidant capacity were also determined as a part of the non–enzymatic antioxidants. The experimental data suggest that postharvest oxidative stress developed during fruit ripening in Japanese plums, but the rate was dependent on the climacteric behaviour of fruit. The climacteric cultivars, ‘Blackamber’ and ‘Amber Jewel’, showed a faster decline in the ability of antioxidative system to encounter the oxidative stress during fruit ripening as compared to ‘Angeleno’, a suppressed–climacteric cultivar. The delay in harvesting of ‘Amber Jewel’ plums by one week slightly improved fruit quality and the initial status of antioxidants than the commercial harvest. However, the fruit harvested at commercial maturity had better retention of antioxidative system during cold storage at 0°C for 3–4 weeks compared to the fruit from the delayed harvest. The changes in enzymatic and non–enzymatic antioxidants as a function of storage duration appear

to be more prominent in providing protection against oxidative injury expressed as CI than their at–harvest status. The response of the antioxidative system in 'Amber Jewel' plums at 5°C was significantly better than at 0°C. But, the storage temperature of 5°C was not sufficiently low to inhibit the process of fruit ripening, resulting in limited storage life of 2 weeks. The multiple–point time course analysis of lipid peroxidation and changes in enzymatic and non–enzymatic antioxidants of 'Blackamber' plums revealed that the third week of storage is the critical point beyond which the capacity of antioxidative system to cope with the increasing oxidative stress from CI and fruit ripening began to decline, resulting in increased incidence and severity of CI during the extended periods of storage. Controlled atmospheres (CA) were found beneficial to reduce the levels of oxidative stress in 'Blackamber' plums. CA containing 1% O_2 + 3% CO_2 were effective in mitigating the oxidative stress during the 5 weeks of cold storage at 0–1°C, plus 6 days of shelf life at 21±1°C. The efficacy of CA (1% or 2.5% O_2 + 3% CO_2) in alleviating CI in 'Blackamber' plums could be further enhanced by the pre–storage treatment of fruit with 1–methylcyclopropene (1–MCP, 0.6 μL L^{-1}). The combination of CA and 1–MCP exhibited synergistic effects on the alleviation of oxidative stress, resulting in enhanced storage life up to 8 weeks, plus 6 days of shelf–life. The role of nitric oxide (NO) as an antioxidant was also investigated in order to retard fruit ripening, delay the onset of senescence and development of oxidative stress in the Japanese plums. Postharvest NO fumigation (10 or 20 μL L^{-1}) delayed the fruit ripening and maintained quality for 9–12 days in 'Amber Jewel' and 'Blackamber' plums at 21±1°C. NO fumigation was also beneficial to reduce the symptoms of CI during cold storage of 'Amber Jewel' and 'Blackamber' plums for 5–6 weeks at 0°C, plus 5 days of shelf–life at 21±1°C. The positive effects of NO fumigation on the enzymatic and non–enzymatic antioxidants in addition to reduced rates of lipid peroxidation were associated with the enhanced chilling tolerance in Japanese plums. The response of 'Amber Jewel' to postharvest NO fumigation was significantly better than 'Blackamber'. In conclusion, the development of oxidative stress in Japanese plums was influenced by cultivar, harvest maturity, cold storage (temperature, duration and atmosphere composition), and postharvest treatments with NO and 1–MCP. The mitigation of oxidative stress by manipulation of postharvest storage conditions and treatments can be achieved to maintain fruit quality and reduce the incidence and severity of CI in Japanese plums.

Table of Contents

List of Figures

List of Tables

List of Symbols and Abbreviations

α	Alpha
&	And
β	Beta
°	Degree
°C	Degree Celsius
δ	Delta
γ	Gamma
%	Per cent
µg	Microgram(s)
µL	Microliter(s)
µm	Micrometer(s)
µmol	Micromole(s)
µM	Micromolar
a.i.	Active ingredient
AA	Ascorbate
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
AU	Absorbance units
BBD	Braeburn browning disorder
BHT	Butylated hydroxytoluene
c	Chroma
CA	Controlled atmosphere
CAE	Chlorogenic acid equivalent
CAT	Catalase
CDNB	1-chloro-2, 4-dinitrobenzene
C_2H_4	Ethylene
CI	Chilling injury
CIE	Commission Internationale de L'Eclairage
cm	Centimetre(s)
Co.	Company
CO_2	Carbon dioxide
Conc.	Concentration

Cyt c	Cytochrome c
DAFB	Days after full bloom
DAFWA	Department Of Agriculture and Food Western Australia
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
DPPH	1,1–diphenyl-2–picrylhydrazyl
DTNB	5,5'–dithio*bis*–(2–nitrobenzoic acid)
DTT	Dithiothreitol
EC	Electrical conductivity
EDTA	Ethylenediamine tetraacetic acid
EL	Electrolyte leakage
et al.	et alia
FAO	Food and agriculture organisation
FID	Flame ionization detector
Fig.	Figure
FW	Fresh weight
g	Gram(s)
g	Gravity
GB	Gel breakdown
GC	Gas chromatograph
GR	Glutathione reductase
GSH	Glutathione (reduced)
GSSG	Glutathione disulfide (oxidized glutathione)
GT	Glutathione–S–transferase
h	hour
h°	Hue angle
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
kg	Kilogram(s)
kPa	Kilo Pascal
L	Liter(s)
L*	Lightness
LOX	Lipoxygenase
LSD	Least significant difference

Ltd.	Limited
MA	Modified atmosphere
MAP	Modified atmosphere packaging
1–MCP	1–Methylcyclopropene
MDA	Malondialdehyde
MDHA	Monodehydroascorbate
MDHAR	Monodehydroascorbate reductase
mg	Milligram(s)
mL	Mililiter(s)
mm	Milimeter
mM	Milimolar
min.	Minute
mol	Mole(s)
mmol	Millimole(s)
N	Newton
N	Normal
N_2	Nitrogen
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NaF	Sodium fluoride
NaOH	Sodium hydroxide
nL	Nanoliter(s)
nm	Nanometer
nmol	Nanomole(s)
NO	Nitric oxide
NS	Non–significant
O_2	Oxygen
P	Probability
Pa	Pascal
PAL	Phenylalanine ammonia lyase
POD	Peroxidase
ppb	Parts per billion
ppm	Parts per million
PPO	Polyphenol oxidase

PVP	Polyvinylpyrrolidone
PUFA	Polyunsaturated fatty acids
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrrolidone
r	Correlation coefficient
RH	Relative humidity
rpm	Revolutions per min
SE	Standard error
SOD	Superoxide dismutase
SSA	Sulphosalicylic acid
SSC	Soluble solids concentration
TA	Titratable acidity
TBARS	Thiobarbituric acid–reactive substances
TCA	Trichloroacetic acid
Temp.	Temperature
U.K.	United Kingdom
U.S.A.	United States of America
UV	Ultraviolet
UV–VIS	Ultraviolet–visible
vs.	versus
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight
WA	Western Australia

Chapter 1

General Introduction

Plum is an important stonefruit crop grown in the temperate, mediterranean and subtropical climates of the world. There are mainly two types of plums: European (*Prunus domestica* L.) and Japanese (*P. salicina* Lindl.). Japanese plums are most commonly grown in the mediterranean and subtropical climates. These are highly perishable and have limited storage and shelf life. Low temperature (0°C) is recommended to maintain the fruit quality and retard the ripening process during postharvest handling, storage, transportation, distribution and marketing. However, plum fruit cannot be stored more than 2–5 weeks even under optimum storage conditions, depending on the cultivar (Crisosto et al., 1999).

There is a significant increase in the international trade of Japanese plums in the last decade (FAOSTAT, 2010). The marine transport in refrigerated containers is considered economical and practical mode of shipping for Japanese plums to overseas markets which may take 2–4 weeks, depending on the destination. The prolonged storage or transport at low temperature has been known to aggravate the development of certain physiological disorders in plums (Crisosto et al., 1999; Taylor, 1996). The symptoms of these disorders are internal and detected by the consumer at the point of consumption, and therefore can seriously affect the repurchase of the fruit. The presence of internal disorders has been linked to the recent decline in the consumption of stonefruit including Japanese plums (Crisosto et al., 2007). Given the economic consequences of the declined acceptability of plums by the consumers, there is a pressing need to understand the biological link(s) between postharvest factors and development of physiological disorders.

The biological basis of fruit quality and development of physiological disorders has been elucidated in Japanese plums (Abdi et al., 1997b, 1998; Khan and Singh, 2007a; Manganaris et al., 2008; Taylor et al., 1995). Based on the fundamental knowledge, appropriate postharvest practices have been developed and adopted by the industry to minimise the postharvest losses. The primary objective is to retard the ripening and/or delay the onset of senescence in fruit and to deliver a high quality fruit to the consumers. The processes of fruit ripening and senescence are genetically controlled and involve several degenerative changes (Giovannoni,

2004; Leshem, 1988), some of which are manifested as physiological disorders. The better understanding of the physiology of ripening and senescence can therefore lead to development of more effective postharvest practices and making the right decisions aimed at minimising qualitative and quantitative losses in fruit.

The process of senescence in plant or plant organs such as fruit involves various degenerative changes in DNA, proteins and lipids, caused by oxidative stress. The oxidative stress develops when the well–regulated balance between pro–oxidants and antioxidants is disturbed, in favour of the pro–oxidants (Apel and Hirt, 2004). The reactive oxygen species (ROS) are produced as by–products of normal metabolism for their role in signal transduction, but their proliferation in the cell can cause oxidative damage to the macromolecules (DNA, proteins and lipids). The concept of oxidative stress has emerged to a great extent to explain the anomalies in development of various chronic diseases, ageing and senescence of humans and animals (Finkel and Holbrook, 2000).

The role of oxidative stress in plants due to environmental perturbations became of significant interest to researchers in the middle to late 1980s (Hodges et al., 2004). However, the research on postharvest stress physiology in relation to oxidative stress gained impetus in the late 1990s and is still continuing. It is argued that postharvest procedures adopted to extend the potential storage or market life of a fruit can also act as stress factors (Toivonen, 2004). The development of oxidative stress in the fruit tissue has been demonstrated in response to the prolonged stress conditions and inherent metabolic changes.

Postharvest oxidative stress has been associated with processes of fruit ripening (Masia et al., 1998; Rogiers et al., 1998), senescence (Aharoni et al., 2002; Lacan and Baccou, 1998), physiological disorders (Barden and Bramlage, 1994b; Hariyadi and Parkin, 1991; Sala, 1998; Whitaker, 2004; Zhao et al., 2009) and storage procedures (Toivonen, 2003; Toivonen, 2004). Several factors have been proposed to influence the occurrence and severity of postharvest oxidative stress in fruits including: genotype (Hodges and Forney, 2003; Hodges and Lester, 2006; Łata et al., 2005; Lester and Hodges, 2008; Sala, 1998); harvest maturity (Gong et al., 2001; Lentheric et al., 1999; Masia, 1998; Rogiers et al., 1998; Zhao et al., 2009); storage conditions (Gunes et al., 2002; Hodges and Forney, 2000); storage duration (Hodges and Forney, 2000); growth regulators including ethylene (Hodges and

Forney, 2000; Larrigaudiere et al., 2004) and polyamines (Yahia et al., 2001). Postharvest oxidative stress has been associated with the development of serious physiological disorders such as chilling injury in citrus (Lafuente et al., 2004; Sala et al., 2005), superficial scald in apples (Barden and Bramlage, 1994; Whitaker, 2004), internal browning in apples (Gong et al., 2001; Toivonen, 2003) and pears (Veltman et al., 2003).

The development of oxidative stress may be influenced by the genotype of the fruit (Hodges and Forney, 2003; Hodges and Lester, 2006; Lacan and Baccou, 1998; Łata et al., 2005). The differences in the rates of fruit ripening and senescence have been related to the capacity of a cultivar to cope with the oxidative stress. The postharvest life of Japanese plum is also influenced by genetic factors (Crisosto et al., 2004; Crisosto et al., 1999). Most of the plum cultivars exhibit climacteric–type fruit ripening, while some show suppressed climacteric behaviour (Abdi et al., 1997b; Candan et al., 2008; Khan and Singh, 2007b). Currently, there is no information available to elucidate the differential rates of fruit ripening and senescence exhibited by these Japanese plum cultivars on the basis of oxidative stress.

Cold storage of Japanese plums can lead to the development of chilling injury (CI) symptoms which are expressed as flesh browning, mealiness, flesh translucency and flesh bleeding (Crisosto et al., 1999). Many factors influence susceptibility of plums to CI during and after storage, including cultivar, harvest maturity, pre–storage treatments, and storage conditions such as temperature, atmosphere composition and length of storage (Abdi et al., 1997a; Crisosto et al., 2004; Taylor et al., 1995; Ward and Melvin–Carter, 2001). Despite a significant volume of literature on the Japanese plums, no work has been reported to uncover the role of enzymatic and non–enzymatic antioxidants in the development of CI. The low temperature storage has been recognised as a causative agent of oxidative stress in horticultural commodities (Wismer, 2003). With the increase in the storage duration, there may be an increased production of ROS and a decline in the efficiency of antioxidant system, resulting in the build up of oxidative stress. The disruption of balance between pro–oxidants and antioxidants in fruit can therefore lead to the development of physiological disorders. The development of CI has been linked to the oxidative injury in fruit (Hariyadi and Parkin, 1991; Sala, 1998; Sala and Lafuente, 2000; Zhao

et al., 2009; Zhao et al., 2006). Although a causal role for oxidative stress in the CI has not been clearly established, but this does not preclude attempts to reduce oxidative injury as a means to increase the postharvest life of a fruit. As a putative aetiological factor for both fruit senescence and physiological disorders such as CI, oxidative stress is an attractive mechanism with which to explain the differences in the rates of senescence and resistance to physiological disorders in fruits.

The development of postharvest physiological disorders in fruits has been related to the antioxidant levels at harvest and the changes in their concentrations during cold storage. The accumulation of lipid–soluble antioxidants in apple peel due to delayed harvest has been shown to decrease the incidence of superficial scald during cold storage (Barden and Bramlage, 1994a; Diamantidis et al., 2002), but the relationship between antioxidant enzymes and scald resistance has remained contradictory (Du and Bramlage, 1995; Rao et al., 1998). However, the flesh browning disorder in 'Braeburn' apples increased with the advanced maturity and was associated with the declines in superoxide dismutase (SOD) and catalase (CAT) activities in flesh tissue (Gong et al., 2001). The delayed harvesting of 'Conference' and 'Passa Crassana' pears increased susceptibility to browning disorder due to decrease in the ability of antioxidant system to protect from ROS with the advanced maturation (Lentheric et al., 1999; Vanoli et al., 1995). Thus, harvest maturity of fruit appears to have a dramatic influence on the capability of the fruit to resist/tolerate the oxidative stress. At present, no information is available in the literature to explain how the harvest maturity modulates the antioxidative system in Japanese plums in relation to development of CI.

In Japanese plums, the response to chilling conditions may vary depending upon the storage temperature and duration of exposure. The storage temperatures between 2.2°C and 7.6°C (killing temperature zone) have been suggested to enhance the development of CI in plums, peaches, and nectarines (Crisosto et al., 1999; Lurie and Crisosto, 2005), and may be favouring the development of oxidative stress in fruit. Thus, storage of Japanese plums at two contrasting temperatures (0°C and 5°C) may elucidate the effect of storage temperature and storage duration on the changes in enzymatic and non–enzymatic antioxidants in relation to CI and fruit quality.

The severity of CI increases during shelf life after cold storage in Japanese plums (Crisosto et al., 2004). At present, the potential storage/market life of plums is

determined by the post–cold storage incidence of CI; the incidence of CI in excess of 25% is considered as the point of termination of storage life (Crisosto et al., 2004). It is surmised that the evaluation of the incidence of CI, fruit quality and oxidative behaviour during shelf life after each week of storage, commencing from the beginning of storage, has potential to provide an insight into the development of CI and build–up of oxidative stress. The determination of critical point during the storage period, from where onwards the efficiency of the antioxidative system tends to decrease and ultimately leading to the total collapse, may be useful to assist in decision making process on the potential shipping/market life of fruit. This can be achieved by the measurement of oxidative stress parameters in a multiple–point sampling framework during storage and shelf life (Toivonen, 2004). This comprehensive information needs to be generated for a popular plum cultivar such as 'Blackamber'.

Controlled/modified atmospheres (CA/MA) have been reported to exert several beneficial effects on fruit quality and alleviation of physiological disorders in Japanese plums (Kader, 2003; Ke and Kader, 1992; Maré et al., 2005). Decreased O_2 required for normal physiological activity of the fruit can possibly reduce the development of oxidative stress. But, the effects of CA/MA on the pro– and anti–oxidant components in relation to CI in Japanese plums have not been investigated. Furthermore, the postharvest application of 1–methylcyclopropene (1–MCP), an ethylene action inhibitor, has been commercialized for Japanese plums in many countries including Australia (Watkins, 2008). 1–MCP has also been positively implicated in influencing the antioxidative system of fruit and thus alleviating oxidative stress and its associated disorders (Fu et al., 2007; Larrigaudiére et al., 2009; Silva et al., 2010; Sivakumar and Korsten, 2010; Vilaplana et al., 2006). There is still a research gap regarding such information in case of Japanese plums. The combination of 1–MCP and CA/MA has been reported to significantly increase the storability of Japanese plums (Khan and Singh, 2008; Menniti et al., 2006). However, the oxidative behaviour of 1–MCP–treated fruit under CA/MA has not been investigated.

Nitric oxide (NO) is a free radical gas which plays significant role in plants during abiotic and biotic stress conditions (Lamattina et al., 2003; Wendehenne et al., 2004). Exogenous application of NO–donor compounds and fumigation with NO gas

have been shown to delay the process of ripening and senescence in horticultural commodities (Wills and Bowyer, 2003). However, the concentration of NO in the biological system is an important factor as it can be protective or toxic (Beligni and Lamattina, 1999). The cytoprotective role of NO as an antioxidant has been found to alleviate oxidative stress in intact and fresh–cut fruit and vegetables (Duan et al., 2007a; Duan et al., 2007b; Fan et al., 2008; Li–Qin et al., 2009; Zhu et al., 2008). The major postharvest benefits associated with NO have been related to its ability to interfere with ethylene production and act as an antioxidant. The potential effects of NO fumigation on fruit quality, CI development and antioxidative system of Japanese plums need to be elucidated.

It was therefore hypothesized that the development of postharvest oxidative stress in Japanese plums may be influenced by various factors including cultivar, harvest maturity, storage conditions, storage duration, and postharvest treatments. An understanding of these factors may lead to the development of postharvest strategies to minimise the adverse effects of oxidative stress on fruit quality and development of storage disorders in Japanese plums. Therefore, investigations were conducted with the following objectives:

1. To assess the influence of genotype on the development of postharvest oxidative stress in relation to fruit ripening in Japanese plums.
2. To study the impact of harvest maturity, storage temperature and storage duration on the antioxidative metabolism in Japanese plums cv. ‘Amber Jewel’.
3. To understand the role of enzymatic and non–enzymatic antioxidants during cold storage and shelf life of ‘Blackamber’ plums in relation to CI and fruit quality.
4. To elucidate the role of storage atmospheres, 1–MCP treatment and a combination of both on the oxidative behaviour during cold storage of Japanese plums.
5. To assess the role of nitric oxide as an antioxidant in the alleviation of oxidative stress in Japanese plums.

Chapter 2

General Literature Review

2.1 Introduction

Plums belong to the Prunoidae subfamily of the Rosaceae family (Rieger, 2006). Stonefruit such as peaches, nectarines, cherries and apricots also belong to the same family. Plums and apricots have been classified in the subgenus *Prunophora*. Among the stonefruit, plums are considered the most diverse from taxonomical perspective. There are 42 species of plums contained in two sections of the *Prunophora*, the *Euprunus* and the *Prunocerasus* (Rieger, 2006). Most of the commercially grown plums belong to the *Euprunus* ("true plums") section. In the section *Euprunus*, there are three important species of the genus *Prunus: P. domestica* L. (European plums), *P. salicina* Lindl. (Japanese plums) and *P. insititia* L. (Damson, Mirabelle, Bullace, and St. Julien). The section *Prunocerasus* ("plums–cherries") contains plums native to North America and includes *P. americana, P. augustifolia, P. hortulana, P. munsoniana* and *P. maritima*. Fruit are small, round, edible and mostly used for jams, jellies, and preserves.

European plums are native to Western Asia, in the Caucasus Mountains near to the Caspian Sea. Trees of the European plums are taller, cold hardy, long lived and later blooming than Japanese plums. Fruit are oval, smaller and more variable in colour than the Japanese plums. Japanese plums originated in China and were cultivated there for thousands of years and then domesticated in Japan about 200 to 400 years ago, from where it spread around the world. Fruit are larger, round or heart shaped and firmer than the European plums. The chilling requirement of the Japanese plums ranges from 550 to 800 hours compared to 1000 hours for the European types. The fruit belonging to *P. insititia* L. are small and oval, purple and clingstone for damsons, and yellow and freestone for mirabelles. These are mainly used for jams, jellies, and preserves. These are wild types native to the Europe, and were cultivated there prior to introduction of *P. domestica.*

2.2 Economic importance of plums

Two major species of plums, *P. domestica* L. (European plums), *P. salicina* Lindl. (Japanese plums), emerged on the separate continents, Europe and Asia, respectively. These species evolved in relatively recent time period and don't have wild

progenitors (Faust and Surányi, 1999). Modern plum industry of the world is dominated by these two species. The European plums are widely grown in temperate climates, while the Japanese types are common in warmer climates. In 2008, the world production of plums was 10.3 million tonnes from an area of 2.48 million hectares (FAOSTAT, 2010). The area and production of plums has increased by 17% to 20%, respectively, during 2001–2008. The top ten plum–producing countries in the world are— China, Serbia, the United States of America (USA), Romania, Chile, France, Turkey, Spain, the Russian Federation and Italy (Table 2.1). The production volumes and dollar value of plums produced in these countries are shown in Table 2.1. China is the world's largest producer of plums with about 46% share in the total production (Fig. 2.1). In addition to Chile, the major plum–producing countries in the Southern hemisphere are South–Africa, Argentina and Australia. Due to counter–seasonality, these countries supply plums to the markets in the Northern hemisphere to make possible the year–around availably of fruit to the consumers.

Table 2.1. Plum production in top ten producing countries in the world in 2008 (FAOSTAT, 2010)

Rank	**Country**	**Production (million tonnes)**	**Production (million US $)**
1	China	4.83	1167
2	Serbia	0.68	164
3	USA	0.68	163
4	Romania	0.37	90
5	Chile	0.30	72
6	France	0.25	60
7	Turkey	0.24	58
8	Spain	0.19	46
9	Russian Federation	0.18	44
10	Italy	0.18	42
	World	10.3	

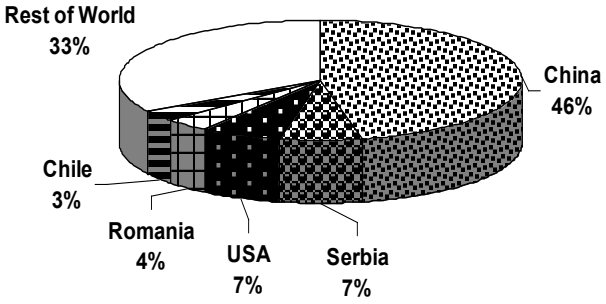


Fig. 2.1. Per cent share of the five leading plum–producing countries in total production of the world in 2008 (FAOSTAT, 2010)

According to the FAO trade statistics, 0.54 million tonnes of plums were exported from different countries in the world in 2007. Chile was the key player among major exporters and contributed about 20% to the total quantity of plum exports in 2007. Other exporters of plums included Spain (15%), USA (9%), South Africa (8%), and Italy (7%). In 2007, the United Kingdom was the largest importer of plums in the world followed by the Russian Federation, the Netherlands, Germany, and the USA. The total export and import values for plums during 2007 were 589 and 690 million USD, respectively (FAOSTAT, 2010). These statistics reflect the economic importance of plums at the global level.

Australia produced 27,294 tonnes of plums from an area of 4,000 hectares in 2008 (ABS, 2008). The current data on plum production in Australia is not available yet. Plums are commercially grown in all states of Australia except Northern Territory (NT). The share of Victoria (VIC), Western Australia (WA), New South Wales (NSW), Queensland (QLD), South Australia (SA) and Tasmania (TAS) to the total plum production in country is 46.4, 26.0, 20.6, 3.5, 0.17, and 0.02 per cent, respectively (ABS, 2008). The major plum growing regions in Australia are shown in Figure 2.2.

In 2008–09, plums contributed 3,850 tonnes to the Australian stonefruit export volume of 10,435 tonnes (Anonymous, 2009). Hong Kong, UAE, Singapore, UK, Malaysia and Thailand are the major export markets for Australian plums. In the recent years, Australian plum export industry has been crippled by a number of factors such as high value of Australian dollar, restricted access to Taiwanese markets, strong competition from Chile, South Africa and Argentina, and global

financial crisis. In 2008–09, the global financial crisis resulted in 33% reduction in demand for stonefruit in the European Union and UK markets, whilst Middle East and Hong Kong markets were least affected (Anonymous, 2009).

Fig. 2.2. Major plum growing regions in Australia
Source: www.summerfruit.com.au

Western Australia (WA) contributes about 26% to the national production of plums, but accounts, in normal seasons, for over 50% of plum exports of Australia. Plum is one of the major export commodities in WA and contributes about 25% of the earnings from total fruit exports. WA plum industry enjoys comparative advantages over other Australian states with regard to its geographic position allowing easier access and shorter distance to the South East Asian and Middle East markets.

Most of the commercial plum cultivars grown in Australia, including WA, belong to the Japanese group. The important cultivars are 'Blackamber', 'Amber Jewel', 'Angeleno', 'Tegan Blue', 'Fortune', 'Santa Rosa', 'Friar', 'Laetitia', 'Autmn Giant', 'October Sun', 'Radiant', 'Rubyred', 'Laroda', 'Ausibelle' and 'Moyer'. These are early, mid and late maturing cultivars and the period of fruit availability in Australia extends from November to April.

2.3 Postharvest management of Japanese plums

2.3.1 Harvest maturity

Harvest maturity greatly influences fruit quality and storage potential of Japanese plums (Abdi et al., 1997a; Abdi et al., 1997b; Crisosto, 1994; Taylor et al., 1995). Both early and late harvesting reduce the fruit quality. Harvesting before maturity can ensure better storage or shipping potential of fruit, but yield and fruit quality will be compromised. Late harvesting substantially reduces the storage and shelf lifewith increased susceptibility to bruising during shipment. Therefore, determining harvest maturity is about striking a balance between favourable and unfavourable outcomes of the decision.

Japanese plums offer a great diversity to the consumers in terms of fruit colour, size, texture, and flavour. The variation in size, colour, texture, and composition of fruit has impeded the efforts towards developing common harvest maturity indices for all cultivars. Therefore, these harvest indices are cultivar specific. In the USA, descriptive measures of skin colour and flesh firmness are commonly used (Crisosto, 1994), but the increasing evidence of consumer research warrants more reliance on SSC, TA and their ratio for good consumer experiences (Crisosto et al., 2003; Crisosto et al., 2004; Crisosto et al., 2007). In South Africa and Australia, the harvest maturity is determined on the basis of a combination of parameters such as skin colour, firmness and SSC. The harvest maturity standards for some commercial cultivars of plums exported from WA are listed in Table 2.2.

Table 2.2. Harvest maturity standards for export plums in Western Australia (Adapted from Ward and Melvin–Carter, 2001)

Cultivar	Colour Description	Firmness range (kg)	Minimum SSC (%)
'Blackamber'	Deep burgundy to purple	6.0–7.0	11.0
'Amber Jewel'	Dark red with remainder yellow/green	5.0–6.0	19.0
'Santa Rosa'	50% red to full red over yellow/green	4.5–7.0	10.5
'Laroda'	Dark red with remainder yellow/green to yellow	6.0–7.5	13.0
'Friar'	Dark red to burgundy	8.0–9.0	13.0
'Tegan Blue'	Deep maroon to purple	7.0–8.0	15.0
'Radiance'	Yellow with red blush	4.0–6.0	16.0
'Autumn Giant'	Bright red to dark red	7.0–8.0	13.5

2.3.2 Postharvest physiology

Fruits are broadly classified into two categories– climacteric and non–climacteric, depending upon the respiratory and ethylene production rates (Biale, 1964). A typical climacteric fruit exhibits a peak in respiration and ethylene production during ripening, while this is in a non–climacteric fruit. The response of fruit to exogenous ethylene or propylene is a criterion used to distinguish between climacteric and non–climacteric (Abdi et al., 1998). Recent studies have revealed the increasing resemblances in the ripening physiology of the climacteric and non–climacteric types at the molecular level, which render this classification oversimplified (Barry and Giovannoni, 2007). Japanese plums are, in general, climacteric. But, a few cultivars of Japanese plums belong to another category, suppressed–climacteric, that are unable to biosynthesize ethylene in a sufficient quantity to induce a climacteric rise in respiration and ethylene production (Abdi et al., 1998; Díaz–Mula et al., 2009; Khan and Singh, 2007b). The rates of respiration and ethylene production have been correlated to the potential postharvest life and rate of decay of the fresh fruit.

Japanese plums have short postharvest life and ripen very fast at ambient conditions depending on the cultivar. The most evident changes during fruit ripening

in Japanese plums include climacteric rise in respiration and ethylene production, flesh softening, changes in skin colour, decrease in titratable acidity, and minor changes in sugars. The cultivars of Japanese plums show a great variation in rates of respiration and ethylene production during fruit ripening. Some of the reported climacteric cultivars are: 'Blackamber', 'Amber Jewel', 'Gulfruby', 'Beauty' and 'Tegan Blue', while 'Angeleno', 'Shiro', 'Songold', 'Golden Japan' and 'Rubyred' have been reported to be suppressed–climacteric types (Abdi et al., 1997b; Abdi et al., 1998; Khan and Singh, 2007a; Khan and Singh, 2007b; Martínez–Romero et al., 2003b). These studies have revealed that cultivars showing suppressed–climacteric behviour have better postharvest life than their climacteric counterparts. Similar to taxonomic diversity of plums, the postharvest physiology of different cultivars of Japanese plums also appears to be diverse. The taxonomic diversity may be offering opportunities for the breeders, but the physiological diversity among different cultivars poses problems in developing common postharvest procedures for their handling, storage, and distribution.

2.3.3 Fruit texture

Fruit texture is one of the most important factors in determining the consumer acceptability of Japanese plums (Crisosto et al., 2003; Crisosto et al., 2004). Based on the flesh firmness from consumer and market perspective, Japanese plums can be segregated into three categories: mature and immature (>26 N); ready–to–buy (13–26 N); ready–to–eat (<13 N) (Valero et al., 2007). It can also serve as a guide for those involved in fruit quality management at packing house, distribution and retail. Softening of fruit is a major physical change that transforms mature hard plums into juicy–soft ones. However, the rate of fruit softening determines the potential shelf life of fruit. The presence of ethylene in the storage atmosphere and its autocatalytic production promotes fruit softening by enhancing the activities of various cell–wall hydrolytic enzymes such as exo–polygalacturonase (Exo–PG), endo–polygalacturonase (Endo–PG), pectin methylesterase (PME), and endo–1,4–β–D–glucanase (EGase) (Abdi et al., 1997b; Khan and Singh, 2007a). The activities of these enzymes cause cell–wall modifications, leading to increased pectin solubilisation (Taylor et al., 1995) and alteration in sugar composition of polysaccharides (Manganaris et al., 2008). Studies on blocking of ethylene action by postharvest application of 1–MCP and exogenous application of ethylene/propylene

revealed that ethylene is directly involved in regulation of fruit softening in Japanese plums (Abdi et al., 1997b; Khan and Singh, 2007a). Therefore, the regulation of ethylene biosynthesis and its action inhibits the rate of fruit softening during postharvest handling of Japanese plums.

2.3.4 Fruit flavour

Fruit flavour of the Japanese plums is crucial for meeting or rather exceeding the consumer expectations. Sensory evaluation studies strongly emphasize the utmost importance of taste in consumer acceptance of plums (Crisosto et al., 2007; Crisosto et al., 2004; Meredith et al., 1991; Robertson et al., 1992). Unlike many other fruits, Japanese plums emit low amounts of aroma volatile compounds (Gómez and Ledbetter, 1994), except for a few cultivars such as 'Royal Zee', 'Joanna Red', 'Fortune', and 'Flavorosa' which have a strong aroma (Crisosto et al., 2007). Concentrations of sugars, acids and their ratio are key contributors to the overall fruit flavour in the Japanese plums (Crisosto et al., 2007; Crisosto et al., 2004; Meredith et al., 1991; Robertson et al., 1992). Organoleptic studies have shown that ripe soluble solids concentration (RSSC) and ripe titratable acidity (RTA) played a significant role in consumer acceptance of 'Blackamber' plum (Crisosto et al., 2004). 'Blackamber' plum with a RSSC of higher than 12.0% had approximately 75% consumer acceptance, regardless of RTA, while RTA reduced the acceptance level when RSSC was less than 12% (Crisosto et al., 2004).

Fructose, glucose, sorbitol, and sucrose are the major soluble sugars in Japanese plums (Singh and Singh, 2008a, 2008b; Singh et al., 2009b). Malic acid is the principal organic acid in Japanese plums, and the other organic acids present in minor concentrations include shikimic, succinic, citric and fumaric acids (Robertson et al., 1992; Singh and Singh, 2008a). A significant reduction in the TA during ripening contributes to the increase in SSC:TA ratio, which in turn enhances the fruit flavour. The fruit composition of Japanese plums, like other stonefruit, varies greatly due to a number of factors such as cultivar, harvest maturity, season, crop load, and position on tree (McGlasson et al., 2008). The changes in individual sugars during ripening of 'Blackamber', 'Amber Jewel' and 'Angeleno' have been shown to be non–significant, but the profiles of individual sugars differ significantly among them (Singh and Singh, 2008a). The growing season has also strong impact on the accumulation of total sugars in Japanese plums as the early maturing cultivars have

lower sugar concentration than late season cultivars, while concentration of acids did not vary in relation to time of season (Crisosto et al., 2007). All these factors affecting the fruit composition, particularly SSC, contribute to the consumer acceptability of fruit. Poor fruit flavour has been considered as one of the factors responsible for decline in demand for Japanese plums.

2.3.5 Pre–storage treatments

Several pre–storage treatments have been tested to extend the storage life and maintain fruit quality in Japanese plums. These postharvest treatments include heat, applications of calcium, polyamines, and ethylene biosynthesis and action inhibitors such as 1–aminoethoxyvinylglycine (AVG) and 1–methylcyclopropene (1–MCP) (Dong et al., 2001; Jobling et al., 2003; Pérez–Vicente et al., 2002; Valero et al., 2003; Valero et al., 2002; Vangdal and Børve, 2002). In Australia, none of these treatments have been adopted in plums on commercial scale, except the application of 1–MCP.

2.3.5.1 1–MCP treatment

1–MCP is a gaseous molecule that can irreversibly bind to the ethylene receptors and delay the onset of respiratory and ethylene climacteric in fruits with multitude of other benefits (Sisler and Serek, 1997; Watkins, 2008). The discovery and commercial application of 1–MCP has revolutionized postharvest industries of the world (Watkins, 2008). Postharvest application of 1–MCP is permitted on the European and Japanese plums in countries such as Argentina, Australia, Canada, Chile, France, Mexico, South Africa, and the United States of America (Watkins, 2008). The beneficial effects of 1–MCP in Japanese plums include delay in fruit softening, decrease in rates of respiration and ethylene production, decrease in skin colour changes, and reduction in CI symptoms (Abdi et al., 1998; Candan et al., 2008; Dong et al., 2001; Khan and Singh, 2008; Valero et al., 2003).

Abdi et al. (1998) demonstrated, for the first time, the effectiveness of 1–MCP in reducing the rates of respiration and ethylene production in plum cultivars such as ‘Gulfruby’, ‘Beauty’, ‘Shiro’, and ‘Ruby Red’. The subsequent studies showed that 1–MCP in the concentration range of 0.5 to 1.0 $\mu L\ L^{-1}$ can be applied to obtain beneficial effects in other commercial cultivars such as ‘Blackamber’, ‘Angeleno’, ‘Fortune’, ‘Tegan Blue’, ‘Santa Rosa’, ‘Golden Japan’, ‘Red Rosa’, and

'Royal Zee'(Candan et al., 2006; Candan et al., 2008; Dong et al., 2001; Khan and Singh, 2009; Martínez–Romero et al., 2003b; Salvador et al., 2003; Valero et al., 2003). Postharvest application of 1–MCP at the rate of 0.6 μL L^{-1} for 12–24 hours is recommended for commercial use on Japanese plums by the AgroFresh, Inc. (Peter Vedeniapine, Personal Communication).

2.3.5.2 Nitric oxide treatment

Nitric oxide (NO) is a highly diffusible and reactive gas which makes it a versatile signalling molecule capable of interacting with cellular targets via either redox or additive chemistry (Lamattina et al., 2003). In plants, NO plays a role in a broad spectrum of pathophysiological and developmental processes and is a ubiquitous molecule (Lamattina et al., 2003). NO has been known to play an important role in the regulation of fruit ripening and senescence (Leshem and Wills, 1998). In fruits, its endogenous levels were reported to be higher in immature than in mature and ripe tissues of climacteric and non–climacteric fruits (Leshem and Pinchasov, 2000). The endogenous levels of ethylene and NO during fruit development and maturation have inverse and stoichiometric relationships. NO levels decrease with maturation and senescence in horticultural crops (Leshem and Pinchasov, 2000; Leshem and Wills, 1998), thereby offering an opportunity for modulation of their levels with exogenous application to exert the opposite effect.

Short–term exposure of intact and fresh–cut horticultural commodities to very low concentrations of NO retards their postharvest senescence (Pristijono et al., 2006; Wills and Bowyer, 2003; Zhu et al., 2006; Zhu et al., 2008; Zhu and Zhou, 2007). Postharvest NO application in intact and fresh–cut produce delayed ripening (Harris et al., 2003; Wills et al., 2008), inhibited ethylene biosynthesis (Eum et al., 2009; Leshem and Wills, 1998; Zhu et al., 2006; Zhu and Zhou, 2007) inhibited cut–surface browning (Pristijono et al., 2006; Wills et al., 2008), and enhanced resistance to postharvest diseases (Fan et al., 2008; Zhu and Zhou, 2007). The mechanism of action of NO in delaying senescence of postharvest horticultural produce, though not completely understood, is *via* the inhibition of ethylene biosynthesis. However, adequate evidence does not exist to ascertain the mode of action of NO.

Postharvest NO treatment has been found useful to retard fruit ripening and maintain fruit quality during cold storage in kiwifruit, longan, peach, and tomato (Duan et al., 2007a; Duan et al., 2007b; Eum et al., 2009; Zhu et al., 2009; Zhu et al.,

2010b; Zhu et al., 2006; Zhu et al., 2008; Zhu and Zhou, 2007). The increasing evidence of NO as a potential anti–senescence agent warrants further investigations to extend its application for Japanese plums. There are no published reports on the effects of NO on the delay in fruit ripening in European and Japanese plums except Singh et al. (2009) which is based on the experimental results presented in Chapter 9.

2.3.6 Cold storage

The low temperature storage is recommended for extending the shelf life and maintaining the fruit quality during prolonged market period and long–distance transport (Mitchell et al., 1974). Storage temperature is a very important factor in determining the storage or shipping potential of plums (Table 2.3). The storage or market life is mainly limited by the development of CI symptoms. However, the rate of CI development in plums depends on cultivar, storage temperature, maturity and orchard factors (Ward and Melvin–Carter, 2001). The symptoms of CI have been reported to occur during storage of stonefruit below 10°C. The temperature range of 2.2°C to 7.6°C has been called the "danger zone or killing temperature zone", during which the rate of development of CI in stonefruit is rapid compared to temperatures below and above the limit (Mitchell et al., 1974). Therefore, storage of fruit above its freezing point, –0.5°C to 2°C is recommended for plums.

Table 2.3 Effect of storage temperature on storage/shipping potential of plum cultivars (Adapted from Crisosto et al., 1999, 2004)

Cultivar	**Harvest date (Week/month)**	**Storage/shipping potential (weeks)**	
		0°C	5°C
'Fortune'	2/June	5^{+}	3
'Blackamber'	1/July	5	3
'Angeleno'	1/July	5^{+}	5
'Showtime'	1/July	5	3
'Friar'	3/July	5	3
'Howard Sun'	3/August	4	1

2.3.7 Controlled/modified atmospheres (CA/MA)

Controlled/modified atmosphere (CA/MA) storage involves the keeping of a commodity in an atmosphere containing concentrations of O_2, CO_2 and ethylene which are different from the ambient air (Kader, 2003). The supplementation of optimum storage temperature and relative humidity with CA/MA has been found useful to preserve fruit quality for longer durations and reduce postharvest losses. Under CA and MA environments, the concentration of O_2 is lowered and that of CO_2 is raised in order to reduce respiration and ethylene production, to delay fruit ripening and senescence, to alleviate certain postharvest physiological disorders and to retard the growth of decay–causing organisms (Kader, 2003). The gas composition is very precisely maintained in CA storage chambers and in shipping containers. On the other hand, MA is generally created by packaging the commodity in polymeric films with different permeabilities to O_2, CO_2 and water vapours, but the concentrations of gases cannot be maintained precisely. The response of different fruit species or cultivars to CA/MA is influenced by several other factors such as maturity stage, storage temperature, duration of exposure and interaction with other postharvest treatments (Saltveit, 2003).

The storage potential of stonefruit can be enhanced under CA conditions (Lurie and Crisosto, 2005) as it has been reported to be very useful to delay or reduce the development of CI in peaches, nectarines and plums (Crisosto et al., 1997a; Lill et al., 1989; Lurie, 1992; Murray et al., 2007; Retamales et al., 1992; Wade, 1981; Wang et al., 2005). The shipment of peaches and nectarines from Chile under CA conditions of 6% O_2 + 17% CO_2 has been found very useful to retain fruit quality (Retamales et al., 1992; Streif et al., 1992). However, in California, this CA combination has shown a limited benefit for reduction of mealiness during shipments for yellow and white flesh cultivars of peaches (Crisosto et al., 1997a). The efficacy of CA in peaches and nectarines is controlled by preharvest factors, fruit size, storage temperature, duration of storage and marketing period (Lurie and Crisosto, 2005). In general, the response of nectarines to CA storage appears to be better than peaches.

The reported beneficial effects of CA/MA in European and Japanese plums include delayed fruit ripening, reduced rates of respiration and ethylene production, inhibition of fruit softening and skin colour changes, and reduction of incidence and severity of CI (Ben and Gaweda, 1992; Brackmann et al., 2001; Elzayat and Moline, 1995; Ke and Kader, 1992; Ke et al., 1991; Menniti et al., 2006; Roelofs, 1993;

Roelofs and Breugem, 1994; Sive and Resnizky, 1979; Smith, 1939; Smith, 1967; Streif, 1989; Testoni and Eccher Zerbini, 1994; Truter and Combrink, 1992). The compositions of atmospheres reported to give these benefits in the European and Japanese plums have been summarized in Tables 2.4 and 2.5, respectively. In Japanese plums, the application of CA containing 1–5% O_2 and 2.5–10% CO_2 at near 0°C exerted favourable effects. The cultivar–specific requirements of CA have not been researched conclusively and warrant further investigations. CA is commercially used for long–distance transport of plums (Kader, 2003).

Modified atmosphere packaging has been reported to extend postharvest life and maintain fruit quality in Japanese plums. The packaging in a polymeric film of appropriate thickness and permeability can substantially reduce weight loss of fruit during storage and transport (Crouch, 1998; Khan and Singh, 2008; Kluge et al., 1999). During 7 weeks of cold storage, 'Laetitia' fruit packed in microperforated polyethylene or polypropylene bags had lower weight loss, better firmness and inhibited fruit ripening compared to control fruit (Crouch, 1998). The incidence of CI and decay has been found to be significantly lower in MA packed fruit of 'Amarelinha' cultivar compared to non–packed fruit (Kluge et al., 1999).

The application of MAP box liners (LifeSpan L316, FF–602, and FF–504) in 'Friar' plums suppressed skin colour changes, reduced weight loss and CI symptoms during cold storage at 0°C for 45 weeks; the extension of storage life to 60 days increased the incidence of CI symptoms in MA–packed fruit (Cantín et al., 2008). The MAP of 1–MCP–treated fruit of 'Tegan Blue' using LifeSpan® polyethylene bags has been reported to extend the storage life to 7 weeks followed by 8 days of fruit ripening without any adverse effects on fruit quality (Khan and Singh, 2008). The combination of 1–MCP and MAP was found more useful to prevent fruit softening and to retard changes in skin colour compared to either of alone. However, these authors reported that the rates of respiration of MA packed fruit were higher during the 10 days of shelf life after 5 and 7 weeks of cold storage than the non–packed fruit. Contrarily, ethylene production rates were suppressed in MA–packed fruit (Khan and Singh, 2008). These studies indicate that MAP has potential to overcome problems of quality losses and alleviation of CI symptoms to some extent.

Table 2.4. Summary of research on CA storage of the European plum cultivars

Cultivars(s)	Storage conditions	Remarks	Reference(s)
'Monarch'	1.1°C, 5% O_2 + 0.2% or 2.5% CO_2	Reduction in physiological and internal breakdown	Smith, 1939
'Victoria'	–0.5°C and 1.1°C, 1% O_2	Reduction in gel breakdown during 4 weeks storage. Intermittent warming for 2 days at 18.5°C after 17 days at –0.5°C extended storage life to 6 weeks with reduction in internal browning.	Smith, 1967
'Bühler Frühzwetsche'	1°C, 2% O_2 + 12% CO_2	Retarded skin colour change and fruit softening delayed under CA	Streif, 1989
'Wegierka Zwyka'	0°C, 3% O_2 + 3% CO_2	Optimum storage period of 6 weeks.	Ben and Gaweda, 1992
'Opal' and 'Victoria'	1°C, 2% O_2 + 5% CO_2	Maximum storage potential was 3 weeks. Mature unripe fruit picked with stalks intact stored better than without stalks and ripe fruit.	Roelofs, 1993; Roelofs and Breugem, 1994
'Green Gage'	2.5–3°C, 1% O_2 + 4% CO_2	Reduced fruit softening and decay during 37 days of storage	Elzayat and Moline, 1995

Table 2.5. Summary of research on CA storage of the Japanese plum cultivars

Cultivar (s)	Storage conditions	Remarks	Reference
'Red Rosa'	0°C, 3% O_2 + 2–8% CO_2	Storage life was 2–3 months	Sive and Resnizky, 1979
'Santa Rosa' and 'Songold'	–0.5°C, 4% O_2 + 5% CO_2	Partially ripened fruit having flesh firmness of 4.5kg stored in CA at –0.5°C for 1–2 weeks could be stored for additional 4 weeks at 7.5°C. CA storage alleviated internal breakdown	Truter and Combrink, 1992
'Wegierka Zwyka'	0°C, 3% O_2 + 3% CO_2	Optimum storage period of 6 weeks.	Ben and Gaweda, 1992
'Angeleno'	2°C, 3% O_2 + 10% CO_2	Better retention of firmness and acidity and reduction in internal breakdown disorders under CA.	Testoni and Eccher–Zerbini, 1993
'Laetitia' 'Casselman', and 'Songold'	3% O_2 + 5% CO_2 10 days at –0.5°C and 18 days at 7.2 °C.	Storage life could be extended to 7–8 weeks.	Truter and Combrink, 1997
'Reubenne' and 'Pluma 7'	–0.2°C, 3% O_2 + 5% CO_2	Dual temperature storage at –0.2°C for 10 days + 7.2°C for 25 days was the best for 'Reubenne'.	Brackmann et al., 2001
'Songold'	0°C, 2% O_2 + 5% CO_2	3 weeks storage at 0°C under CA and then 12 days at 0°C under normal air. CA treatment alleviated internal breakdown.	Nerya et al., 2003
'Angeleno'	0°C, 1.8% O_2 + 2.5% CO_2	Firmness retention. Fruit pre–treated with 1–MCP could be stored for 80 days.	Menniti et al., 2006

2.3.8 Physiological disorder: chilling injury

Cold storage of Japanese plums can lead to the development of CI symptoms which appear in the form of flesh browning, flesh translucency, mealiness and flesh bleeding (Candan et al., 2008; Crisosto et al., 1999; Manganaris et al., 2008; Singh et al., 2009a). The CI symptoms become evident and proliferate when the fruit are transferred to ambient conditions for ripening. Generally, flesh browning appears during cold storage or immediately after cold storage, while translucency symptoms are related to more advanced stage of development of CI during fruit ripening after cold storage (Crisosto et al., 1999). Flesh browning involves the darkening of the mesocarp tissue depending on the severity of incidence (Fig. 2.3) and is mostly found in fruit harvested before optimum maturity (Mitchell et al., 1974). The tissue senescence or deterioration leads to the changes in membrane permeability and the interaction between phenols and polyphenol oxidase, which are generally found in separate compartments in the cell, cause tissue browning (Lurie and Crisosto, 2005).

The appearance of a translucent gelatinous area in the flesh tissue surrounding the stone is called gel breakdown/ translucency or flesh translucency (Fig 2.3). The increase in its severity covers more flesh area extending outwards from the pit region and causes browning of the affected tissue in the advanced stages. It is common in mature and overmature fruit experiencing the CI (Abdi et al., 1997a; Taylor et al., 1995). Mealiness refers to the reduction in juiciness of fruit (Fig. 2.3) and is mainly related to the decreased activities of various cell wall hydrolases affecting the pectin metabolism (Manganaris et al., 2008). The alterations in pectin metabolism cause mealiness and translucency either by cell fluids forming calcium–pectate gel complexes with high molecular weight pectins in the middle lamella (Taylor et al., 1995; Zhou et al., 2000) or by decreased activities of endo–1,4–β–glucanase, endo–1,4–β–mannanase, β–galactosidase, α–arabinosidase, and expansin, leading to further alteration of cell wall metabolism and reduction in intercellular adhesion (Brummell et al., 2004; Obenland et al., 2003). Flesh bleeding is related to the release of flesh pigments due to tissue disintegration and also to the diffusion of skin pigments into the flesh tissue (Lurie and Crisosto, 2005). The appearance of flesh bleeding is the result of membrane disintegration during the fruit senescence.

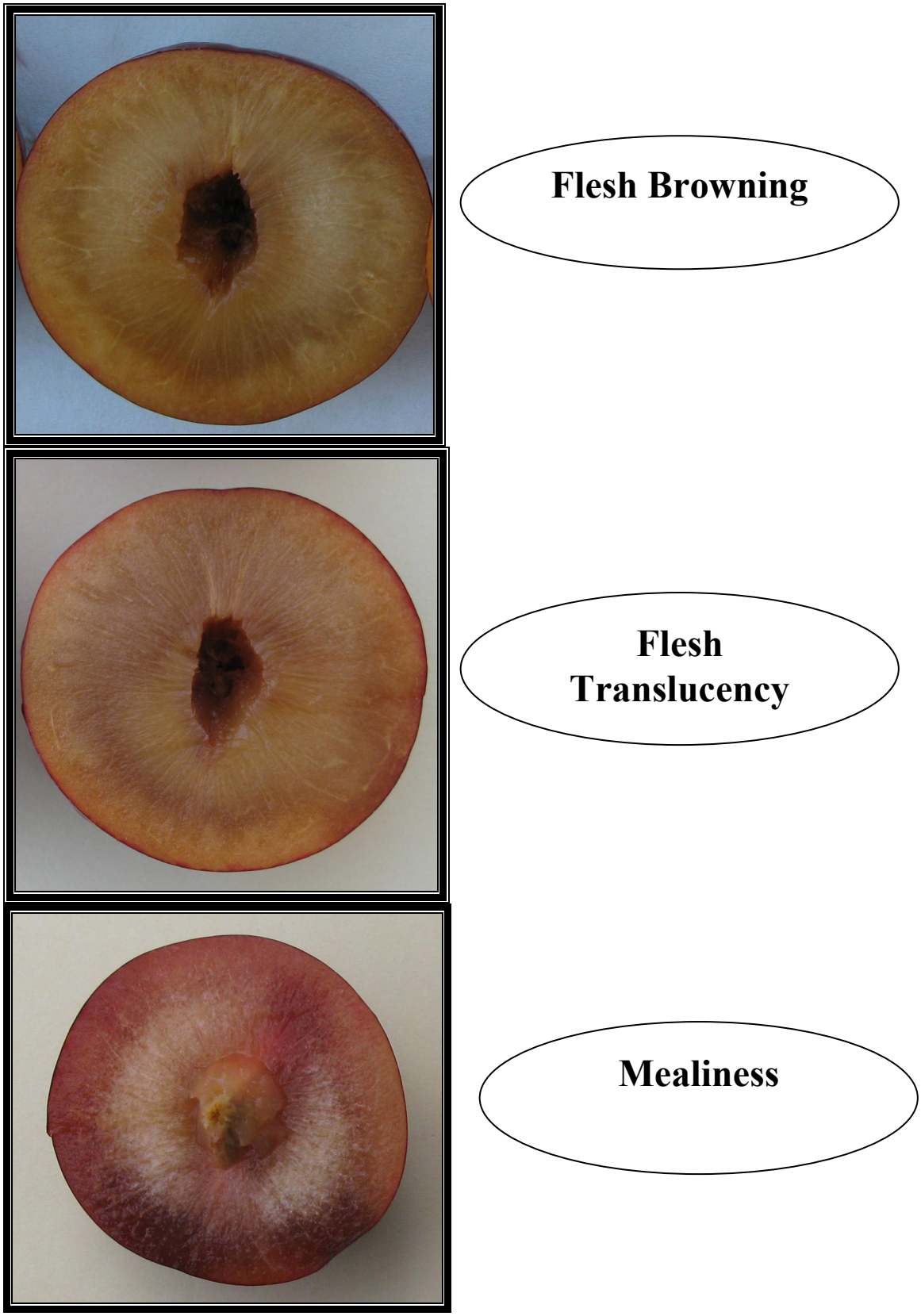


Fig. 2.3. Chilling injury symptoms on the flesh tissue of half—ripe fruit of Japanese plum.

2.3.8.1 Factors affecting development of chilling injury in Japanese plums

The factors affecting the development of CI in Japanese plums are diverse and complex. Pre and postharvest factors, such as genotype, harvest maturity, orchard factors, seasonal climactic conditions, storage time, and storage temperature, have been known to affect the incidence and severity of CI. These factors are very complicated, disabling the researchers to establish a cause–effect relationship for this disorder to any one of them. Therefore, to avoid the adversities of this disorder on fruit quality and consumer experiences, a number of precautions are recommended to be exercised by the fruit growers, packers, distributors and retailers.

Genotype/cultivar

The susceptibility to CI is genetically controlled in stonefruit (Lurie and Crisosto, 2005). The researchers in Australia, South Africa and USA have screened various cultivars of Japanese plums based on their susceptibility to CI (Crisosto et al., 1999; Taylor, 1996; Ward and Melvin–Carter, 2001). Table 2.6 presents the classification of plum cultivars based on their susceptibility to CI and potential storage/shipping life.

Table 2.6. Classification of plum cultivars based on their susceptibility to CI and potential storage/shipping life (Adapted from Crisosto et al., 1999, 2004, 2008)

Cultivar	**Fruit type**	**Storage/shipping potential (weeks)**	
		0°C	**5°C**
Category A: Non–susceptible to CI at 0°C and 5°C			
'Betty Anne'	Clingstone	5	5
'Flavorich'	Clingstone	5	5
'Joanna Red'	Freestone	5	5
'October Sun'	Semi–clingstone	5	5
Category B: Non–susceptible to CI at 0°C and susceptible at 5°C			
'Blackamber'	Freestone	5	3
'Fortune'	Semiclingstone	5^{+}	3
'Angeleno'	Semifree to freestone	5^{+}	5
'Hiromi Red'	Clingstone	5	3
'Purple Majesty'	Clingstone	5	3
Category C: Susceptible to CI at both temperatures, 0°C and 5°C			
'Howard Sun'	Freestone	4	1
'Friar'	Freestone	5	3
'Showtime'	Freestone	5	3

The knowledge of degree of susceptibility of a cultivar to CI can potentially help to reduce the postharvest quality losses. High risk cultivars include 'Amber Jewel', 'Celebration', 'Friar', 'Howard Sun', 'Showtime' and 'Songold'. 'Angeleno', 'Blackamber', 'Fortune', and 'Harry Pickstone' are the cultivars with medium risk to CI. Low risk cultivars include 'Betty Anne', 'Flavorich', 'Joanna Red', and 'October Sun'. However, this classification may be valid only under Californian conditions and may not be reliable under Australian conditions because the growing conditions have been known to affect the incidence and severity of CI in Japanese plums (Crisosto et al., 1997b; Kotze et al., 1987; Murray et al., 2005). The appearance of CI is an erratic and random event in various cultivars of Japanese plums. Even the medium risk cultivars have been known to experience severe CI symptoms, depending on the other factors. The identification of candidate genes responsible for CI in peaches have been achieved (González–Agüero et al., 2008) and a similar work on Japanese plums can open new avenues of genetic modifications to overcome this disorder.

Orchard factors

Preharvest orchard factors can strongly affect fruit quality in stonefruit and their susceptibility to storage disorders. The complex interactions among various factors such as tree vigour, crop load, plant nutrition, irrigation, canopy position, fruit size, tree age and rootstock can affect the incidence of CI in stonefruit including plums (Crisosto et al., 1997b; Kotze et al., 1987; Murray et al., 2005; Ward and Melvin–Carter, 2001; Wooldridge et al., 1995). Tree canopies of Japanese plums managed to ensure light exposure of at least 70% in all bearing positions resulted in more uniform maturity and postharvest quality of 'Laetitia' and 'Songold' cultivars (Murray et al., 2005). The fruit from shaded canopy regions ripened faster during postharvest stage with lower SSC and poor skin colour compared to those exposed to more light. Earlier reports have shown that the higher incidence of CI in Japanese plums was associated with a greater tree vigour, lower crop load, and higher level of fruit shading (Crisosto et al., 1997b). The high rates of fertilizer and irrigation administered to increase fruit size and yield have been shown to increase the incidence of CI in South Africa (Taylor and De Kock, 1995).

Among major mineral nutrients, nitrogen (N) is thought to play a crucial role in controlling the vegetative growth in Japanese plums (Wooldridge et al., 1995).

High nitrogen application rates promote tree vigour, leading to higher fruit number in the shaded area with lower SSC. The fruit with lower levels of SSC are more susceptible to CI than the fruit with higher SSC (Ward and Melvin–Carter, 2001). The incidence of CI in cultivars, 'Songold' and 'Harry Pickstone', showed a 10-fold increase when the shoot growth was vigorous (Wooldridge et al., 1995).

Calcium (Ca) deficiency can lead to development of storage disorders in fruit. Studies have shown that preharvest Ca sprays on trees did not influence the incidence of CI in plums, peaches and nectarines (Lurie and Crisosto, 2005). However, the role of concentration of magnesium (Mg) in the fruit has been correlated with the incidence of CI. Trees with poor tree vigour resulted in a decrease in Mg concentration in fruit, which decreased the incidence of CI (Kotze et al., 1987). Further investigations are required to elucidate the role of other micronutrients in the development of CI in Japanese plums. In conclusion, the orchard practices, such as summer pruning, moderate water stress, and moderate levels of nitrogen fertilization, limiting tree vigour have been shown to be effective in reducing the incidence of CI in Japanese plums.

Harvest maturity

Harvest maturity has been reported to influence the rate of development of CI in Japanese plums (Abdi et al., 1997a; Crisosto et al., 2004; Hartmann et al., 1988; Taylor and De Kock, 1995; Taylor et al., 1993; Taylor et al., 1995; Taylor, 1996; Ward and Melvin–Carter, 2001). Early and late harvesting negatively impact the fruit quality as both have been reported to be associated with CI symptoms.

CI in immature fruit caused flesh browning, leathery texture, translucent areas around the stem–end and sometimes development of cavities in the flesh (Crisosto et al., 2004; Hartmann et al., 1988; Kotze et al., 1987; Ward and Melvin–Carter, 2001). In California, early harvested 'Santa Rosa' plums showed the higher incidence of CI during cold storage (Mitchell et al., 1974). Similarly, 'Blackamber' fruit harvested on 20th June have storage potential of 2 weeks at 0°C and less than 2 weeks at 5°C compared to those harvested on 26th June which could be stored for 5 weeks at 0°C and 2 weeks at 5°C (Crisosto et al., 2004). Under WA conditions, 'Amber Jewel' plums have been shown to develop the greater incidence of CI when these were harvested before harvest maturity as determined by SSC. Fruit with lower

SSC were predisposed to higher risk of CI than those with higher SSC (Ward and Melvin–Carter, 2001).

The late–harvested fruit of Japanese plums were more prone to flesh translucency during and after cold storage (Abdi et al., 1997a; Taylor et al., 1995). The flesh translucency is also considered to be an advanced stage of flesh browning and it predominates in the late–harvested fruit (Taylor et al., 1995; Ward and Melvin–Carter, 2001). It is therefore important to critically determine the harvest maturity to minimize the incidence of CI. It has been suggested that harvest maturity though played a role in determining the severity of CI, but storage duration was more important factor in predisposing the fruit to CI (Abdi et al., 1997a).

Cooling

Cooling after harvest is recommended to remove the field heat and reduce the fruit temperature to its optimum storage conditions as soon as possible. After harvest, the delay in cooling to storage temperature has been known to significantly influence the fruit quality and incidence of CI in stonefruit (Guerra and Casquero, 2009; Lurie and Crisosto, 2005; Martínez–Romero et al., 2003a). Controlled delayed cooling in peaches and nectarines has been found useful to reduce the incidence of CI during cold storage and improving fruit quality. A delay in cooling for 24–48 hours at 20°C before cold storage at 0°C has been shown to extend the market life of peaches and nectarines cultivars susceptible to CI (Lurie and Crisosto, 2005). Contrarily, delay in cooling of Japanese plums has been shown to negatively impact the fruit quality and development of CI (Guerra and Casquero, 2009; Martínez–Romero et al., 2003a).

Forced–air cooling of 'Santa Rosa' plums after harvesting and before manipulation (transportation to packing–house, handling in packing–house, during storage or transportation) reduced rate of respiration in mechanical damaged fruit and also helped to maintain fruit quality and prolong shelf life (Martínez–Romero et al., 2003a). Delayed cooling in 'Green Gage' plums, holding fruit at 20°C for 48 hours before storage at 2°C, increased internal breakdown symptoms and also reduced the market life by 10 days (Guerra and Casquero, 2009). In Western Australia, fruit handled through the normal commercial channels took between 48 and 100 hours to reach the optimum storage temperature which increased the incidence of CI over the fruit cooled and packed within 16–24 hours of harvest (Ward and Melvin–Carter,

2001). It is therefore recommended that cooling of Japanese plums should commence within a few hours of harvest and the fruit should attain the optimum storage temperature within 18–24 hours after harvest (Mitchell, 1986). Contrary to peaches and nectarines, the susceptibility of Japanese plums to CI can be reduced and fruit quality can be maintained by the rapid postharvest cooling to the storage temperature of 0–2°C.

Storage temperature and duration

The interaction of storage temperature and duration is one of the most important factors in development of CI in Japanese plums. In California, Crisosto et al. (1999, 2004, 2008) evaluated the postharvest performance of several cultivars of Japanese plums for their susceptibility to CI at two storage temperatures, 0°C and 5°C and classified them into three categories as shown in Table 2.6. Table 2.6 also shows that there is a significant interaction among three factors: cultivar, storage temperature and storage potential. A study in Western Australia (WA) showed that 'Amber Jewel' cultivar showed flesh browning only at 0°C, but not at 5°C, during 4 or 6 weeks of storage (Ward and Melvin–Carter, 2001). The storage at 5°C for the same duration prevented the flesh browning, but overripeness was the limiting factor. These researchers defined the term 'overripeness' as follow: "Overripe plums are abnormally soft to the touch with excessive amounts of free juice. In severe cases the tissue just under the skin becomes translucent while the remainder of the tissue is normal"(Ward and Melvin–Carter, 2001). From their statements, it appears that the authors used the term 'overripeness' instead of 'flesh translucency'. Based on such studies, it is clear that 'Amber Jewel' is susceptible to CI at both temperatures, but the type of symptoms changed according to storage temperature. A similar situation of the different types of CI symptoms is also related to harvest maturity– fruit with advanced maturity show mainly flesh translucency, while those with low maturity exhibit flesh browning.

Japanese plums can therefore be safely stored at 0°C for 3–5 weeks or more depending upon the cultivar's susceptibility to CI (Crisosto et al., 1999). Storage of Japanese plums at 5°C, which falls in the 'killing temperature zone', significantly reduced the storage/shipping potential of the fruit (Table 2.6). Another conclusion is that storage duration appears to be the overriding factor in development of CI in different cultivars of Japanese plums.

Modified Storage Temperature Regimes

The researchers in South Africa manipulated the temperature regimes during cold storage to reduce the incidence of CI in Japanese plums and termed it as 'dual temperature' (DT) storage (Boyes and De Villiers, 1949). This method of DT storage involves the storage of fruit at –0.5°C for a maximum of 10 days followed by 18 days at 7.2°C and then 14 days for fruit ripening at 10°C. The strategy had been successful to reduce the incidence of CI in export cultivars, 'Songold' and 'Celebration', but the fruit ripened and softened to a greater extent than single temperature (ST) (Boyes and De Villiers, 1949; Hartmann et al., 1988; Taylor et al., 1994). Dual temperature storage (10 days at 0°C followed by 18 days or 32 days at 8 ^{o}C) has also been shown to prevent the development of flesh browning and gel breakdown in 'Amber Jewel' plums in Australia (Ward and Melvin–Carter, 2001). However, the fruit were too soft and overripe at the end of storage period, which renders DT storage strategy impractical for this cultivar. DT was further modified by raising the second stage temperature to 10°C, called modified dual temperature (MDT). The per cent incidence of CI in 'Celebration' plums has been reported to vary from 36.0%–93.4% in ST, 0.4%–13.0% in DT, and 0.0%–0.2% in MDT (Taylor and De Kock, 1995). The problem of fruit softening may be addressed through the single or multiple applications of 1–MCP to derive benefits of DT or MDT and needs further investigations. These strategies, DT and MDT, have been restricted to South Africa only, which could be attributed to the positive response of their cultivars, 'Songold' and 'Celebration', to the modified storage temperature regimes.

Intermittent warming

Intermittent warming (IW), which involves transferring the fruit to 20°C for a day every 10–14 days, has also been found to reduce the incidence of CI in stonefruit including plums (Kotze et al., 1989; Lurie and Crisosto, 2005). The objective of raising the temperature for a short period during cold storage is to recover the chilling–induced damage through removal of toxic metabolites. The increased metabolic activity in response to warming of chilled–tissue helps to repair the damage to membrane, organelles, and metabolic pathways. The increase in unsaturation of fatty acids in the membrane lipid during IW contributes to the

maintenance of membrane functionality to avoid the chilling–induced damage (Wang, 1993). In 'Victoria' plums, increase of temperature to 18°C for 2 days between the 15th and 20th day of storage at –1°C delayed the appearance of CI (Smith, 1947). Similarly, IW of 'Songold' plums to 20^{o}C for one day after 14 days storage at –0.5^{o}C extended storage life to more than 4 weeks and reduced the incidence of IB more than DT and MDT (Kotze et al., 1989). The combination of IW with postharvest treatment of nitric oxide gas (15µL L^{-1}) has been shown to reduce the incidence of CI in peaches (Zhu et al., 2010a). Further research is required to investigate the role of IW alone or its combination with other postharvest treatments in alleviation of CI in Japanese plums.

Controlled/modified atmosphere storage

Studies have shown that CI symptoms or 'internal breakdown' in European and Japanese plums can be reduced by storing the fruit under CA conditions at the optimum storage temperature (Tables 2.4 and 2.5). The positive effect of gaseous environment (5% O_2 + 0.2% or 2.5% CO_2) on the reduction of 'internal breakdown' was reported for the first time in cultivar 'Monarch' about 70 years ago (Smith, 1939). Subsequent studies have also shown that other cultivars of the European and Japanese plums also respond positively to the CA/MA storage, with the variable results (Tables 2.4 and 2.5). Unlike pome fruits, the systematic studies on the role of storage atmospheres in the development of CI in relation to physiological and biochemical changes in stonefruit are still lacking and require further investigations. This research gap has not been addressed for a long time and could be due to less importance of CA from commercial perspective. Similar to CA, the MAP has also been shown to reduce the incidence of CI to some extent in Japanese plums, but higher concentrations of CO_2 and very low O_2 concentrations have been found detrimental to induce CI rather than preventing it (Cantín et al., 2008; Crouch, 1998).

Concentration of CO_2 in the range of 0%–5% has been recommended to be ideal for plums (Kader, 2003). The concentration of CO_2 higher than 4.0% during cold storage periods longer than 45 days enhanced the development of CI symptoms in 'Friar' cultivar, which had a high ripening rate and short market life (Candan et al., 2008). The ripening rates of cultivars of Japanese plums vary greatly. It is therefore necessary to study the response of each cultivar to different types of

commercially available packaging films, storage duration and their interaction in relation to CI.

Ethylene

Ethylene is a plant hormone which regulates the process of fruit ripening, in addition to several other physiological functions (Leliévre et al., 1997). It has also been implicated in development of CI in stonefruit including plums. The capacity of fruit to biosynthesize ethylene and its presence in the storage environment are beneficial for alleviating the CI symptoms in peaches and nectarines (Lurie and Crisosto, 2005). The blocking of its action through postharvest application of 1–MCP has been shown to increase the development and severity of CI in these fruits. However, in Japanese plums, postharvest treatment with 1–MCP has been shown to alleviate the CI symptoms (Candan et al., 2008; Menniti et al., 2004). These studies indicate the involvement of ethylene in the development of CI in stonefruit. The differential responses of nectarines, peaches, and plums to 1–MCP have not been completely elucidated yet. The preliminary results from a gene expression study of these fruits have revealed significant differences in their physiological and molecular responses to 1–MCP (McGlasson, 2009; W.B. McGlasson, Personal communication, 2010). 1–MCP can therefore be used as a commercial tool for controlling the CI in Japanese plums. Other compounds, which can potentially interfere with the biosynthesis and/or action of ethylene, may also be used to alleviate CI in Japanese plums. For example, nitric oxide (NO) has been known to inhibit the biosynthesis of ethylene production in fresh fruits (Leshem et al., 1998). Therefore, postharvest application of NO in Japanese plums may be beneficial to alleviate the CI symptoms through its interference with the ethylene biosynthesis pathway and will be investigated in this thesis.

2.3.9 Basic physiology and biochemistry of chilling injury

The exposure of plants or plant organs to low or non–freezing temperatures below 10°C to 12°C cause a physiological dysfunction, called chilling injury (Lyons, 1973). The term ‘chilling injury’ was coined by a German botanist, Hans Molisch, to differentiate it from freezing damage. Low temperature storage is an important and effective way of extending the postharvest life of fresh fruits. The sensitivity of fruit to CI limits its cold storage for long–duration. Chilling sensitivity is not only

restricted to fruits of tropical and subtropical origin, but is observed in temperate fruits also (Lyons, 1973). The critical temperature below which CI occurs varies with the species or even with the cultivar. A number of mechanisms have been proposed to explain the phenomenon of development of CI in fruits. No single 'master reaction' controlling all the physiological and biochemical changes associated with the chilling injury has yet been defined.

2.3.9.1 Lipid phase transition theory

According to 'Lipid phase transition theory' proposed by Lyons (1973), the conformational and structural alterations in biological membranes are considered to be the first event at molecular level in the response to low temperature. The increase in electrolyte leakage, lipid phase transition from liquid–crystalline to solid–gel phase, and increased activation energy of membrane bound enzymes result in an imbalance in the whole physiology and metabolism of the system causing accumulation of toxic metabolites (Lyons, 1973). Significant modifications in lipid composition of the membrane include lipid peroxidation, change in saturation index of fatty acids, degradation of phospholipid and galactolipids and increase in the sterol/phospholipid ratio (Sevillano et al., 2009). The decrease in membrane fluidity caused by these changes jeopardizes the functionality of the membrane and of the proteins associated with it.

As an adaptation to the low temperature, the induction of fatty acid desaturase enzyme is involved in increasing the proportion of the unsaturated fatty acids in the membrane, which increases membrane fluidity. In the recent time, the evidences suggest that tolerance to CI can be achieved by modification of the lipid composition in the genetically modified plants, overexpressing the enzymes involved in increasing the unsaturation of the membrane lipids (Sevillano et al., 2009). A study revealed that a higher degree of unsaturation of membrane lipids and accumulation of N–acylphosphatidylethanolamine (NAPE) in peaches was beneficial for maintaining membrane fluidity, leading to enhanced tolerance to chilling stress (Zhang and Tian, 2010). It is therefore clear that the basic theory of lipid phase transition is still valid and receiving more acceptances by the researchers as the new developments occur with the advancement of molecular approaches.

2.3.9.2 Oxidative stress theory

The 'lipid phase transition theory' explains the loss of membrane integrity due to structural and compositional changes in membrane lipids. There is another theory called 'oxidative stress theory' which can explain the membrane damage caused by the reactive oxygen species (ROS) generated in response to chilling stress. The basic mechanism of oxidative stress and its effects on development of postharvest storage disorders are reviewed in the forthcoming sections in detail.

2.4 General overview of oxidative stress

2.4.1 Reactive oxygen species (ROS) and their generation

Oxygen (O_2) is the most abundant molecule in the biological system and is often a source of free radicals as its partially reduced species are generated through normal metabolic processes. Most of the O_2 consumed by non–photosynthetic plant tissues is reduced to water by the terminal oxidase(s) of the respiratory electron transport chain in the mitochondria (Apel and Hirt, 2004). Reactive oxygen species (ROS) are the partially reduced forms of molecular oxygen which result from either the excitation of O_2 to form singlet oxygen or the transfer of one, two, or three electrons to O_2 to form, respectively, superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) or hydroxyl radical ($OH^{\bullet}$) (Hodges et al., 2004). Both $O_2^{\bullet-}$ and $OH^{\bullet}$ are extremely reactive and can cause oxidative injury leading to cell death. The average life span of these ROS varies from nanoseconds (e.g., $OH^{\bullet}$) to milliseconds (e.g., $O_2^{\bullet-}$, H_2O_2).

The $OH^{\bullet}$ can also be generated by the interaction of $O_2^{\bullet-}$ and H_2O_2 in the presence of transition metal ions, so called 'Haber–Weiss reaction'. Cells don't possess detoxification mechanism for $OH^{\bullet}$ due to its very high reactivity and rely on mechanisms preventing its formation. These mechanisms include the preceding elimination of $O_2^{\bullet-}$ and H_2O_2 and/or sequestering metal ions that catalyze the Haber–Weiss reaction with metal–binding proteins such as ferritins or metallothioneins (Gechev et al., 2006). In addition to H_2O_2, $O_2^{\bullet-}$ can also react with nitric oxide radical ($NO^{\bullet}$) to form peroxynitrite ($ONOO^-$) which can rapidly protonate to peroxynitrous acid (ONOOH), a powerful oxidizing agent. The reactions among various types of ROS can therefore generate intermediates or products that are capable of causing extreme levels of oxidative injury to the cell.

There are multiple sites and sources of ROS production in a plant cell (Fig. 2.4), adding to the complexity of complete understanding of their chemistry and metabolism. As a part of normal metabolism, ROS levels are maintained at basal levels, except during stress conditions and developmental signals. The major areas of aerobic biochemistry (e.g. respiratory and photosynthetic electron transport; oxidation of glycolate, xanthine, and glucose) are involved in ROS production, in addition to their generation by several enzyme systems [e.g. plasmalemma–bound NADPH–dependent superoxide synthase and SOD (Noctor and Foyer, 1998)]. Chloroplasts are the major sites of ROS production in photosynthetic tissues (Fig. 2.4). The over–energization of photosynthetic electron transfer chains cause production of $O_2^{\bullet -}$, mainly by electron leakage from Fe–S centres of photosystem–I or reduced ferrodoxin to O_2 (Mehler reaction) (Apel and Hirt, 2004; Gechev et al., 2006). The $O_2^{\bullet -}$ is rapidly metabolized into H_2O_2 by the action of SOD. Under excess light conditions, the photoinhibition of photosystem–II also causes the drastic increase in singlet oxygen production. The importance of chloroplastic sources may however decrease in ripening fruits and commodities placed in dark storage (Hodges et al., 2004).

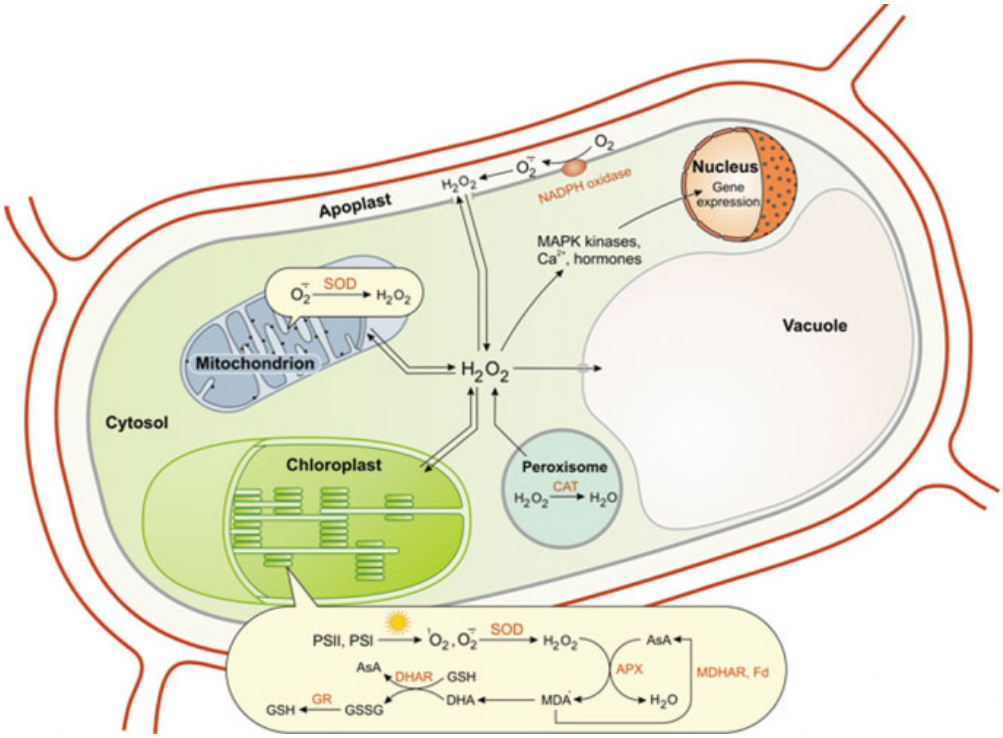


Fig. 2.4. Schematic representation of a generalized plant cell depicting major sources of ROS generation. (Adapted from Gechev et al., 2006)

Mitochondria are the major consumers of O_2 and, therefore, contribute to the production of ROS (Møller, 2001; Navrot et al., 2007). During respiration, O_2 is reduced to H_2O and different pathways are involved in this process. The respiratory electron transport chain, located on the inner membrane of the mitochondria, is made up of five multiprotein complexes and a large dynamic ubiquinone pool is also present (Fig. 2.5). During stress conditions, high O_2 concentration may build up in the cell due to reduced respiratory activities. Consequently, the high redox status of the electron transport chain (ETC) and high intra–mitochondrial O_2 concentration favour the leakage of electrons from single–electron–reduced components to molecular oxygen, resulting in the increased production of ROS. The potential sites of the leakage of single electrons to molecular O_2 are complex I and complex III (Navrot et al., 2007).

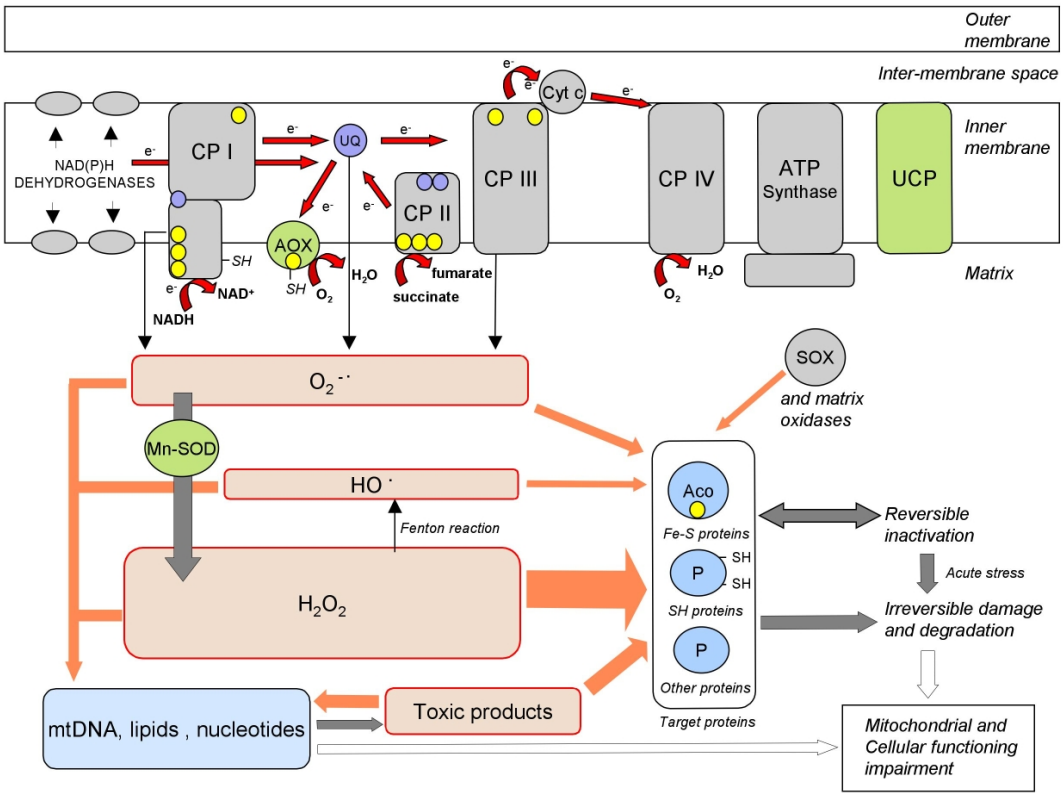


Fig. 2.5. Origins and effects of ROS produced in mitochondria I, II, III, IV respiratory complexes. (Adapted from Navrot et al., 2007)

Peroxisomes and glyoxysomes also contribute to the ROS generation in the cell during photorespiration and fatty acid oxidation, respectively. The impairment of

CO_2 fixation in chloroplasts enhances the oxygenase activity of ribulose–1,5–bisphosphate carboxylase/oxygenase leading to movement of glycolate into peroxisomes, where it is oxidized by glycolate oxidase forming H_2O_2. Fatty acid oxidation in glyoxysomes of germinating seeds generates H_2O_2 as a by–product of the enzyme acyl–CoA–oxidase (Gechev et al., 2006).

2.4.2 ROS Scavenging

Cellular homeostasis is achieved by a delicate balance among different pathways operating in various organelles. In a plant cell, ROS are produced during normal metabolism as well as stress conditions. The concentration of ROS in the cell is an important factor which determines their role as beneficial molecules in various signal transduction processes or in causing oxidative damage (Suzuki and Mittler, 2006). ROS has the ability to initiate cascade reactions and their products and intermediates result in damage to the lipids, proteins and DNA. Therefore, the level of ROS is regulated by scavenging them to avoid accumulation to toxic levels in the cell. The scavenging mechanism of these ROS is also as complex and diverse as their generation sites. It involves both enzymatic and non–enzymatic antioxidant systems to act cooperatively to mitigate the ROS levels (Hodges et al., 2004).

2.4.2.1 Enzymatic antioxidants

The enzymatic antioxidants include various enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol–peroxidase (POD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione peroxidase (GPX), and glutathione–S–transferase (GT) which are responsible for direct or indirect scavenging of ROS (Noctor and Foyer, 1998). SOD, CAT and POD are known as primary antioxidant enzymes which serve as frontline defence antioxidants. The dismutation of $O_2^{\bullet-}$ into H_2O_2 is catalysed by SOD. The activity of SOD converts a ROS into another type of ROS, H_2O_2, which is subsequently detoxified into water (H_2O) and O_2 by the CAT and APX. CAT is considered to be more efficient as it does not require any reducing power for H_2O_2 detoxification as opposed to guaiacol–POD and APX, but it has low substrate affinity (Apel and Hirt, 2004). The activity of APX requires an ascorbate (AA) and glutathione (GSH) regeneration system, which is also called ‘ascorbate–glutathione cycle’ (Fig. 2.6).

APX catalyses the conversion of H_2O_2 into H_2O using two molecules of AA as a reducing power with a concomitant production of two molecules of monodehydroascorbate (MDHA). MDHA radicals rapidly disproportionate into dehydroascorbate (DHA) and AA; the latter reaction is catalysed by MDHAR using NADPH as the electron donor. The DHA is reduced back to AA by the action of DHAR, using GSH as the reducing agent. Finally, GR can regenerate GSH from glutathione disulfide (GSSG) using NAD(P)H as a reducing agent. The efficient removal of ROS by the enzymatic antioxidants is therefore dependent on low molecular weight reductants, AA and GSH, because these supply electrons necessary for the activity of enzymes (Noctor and Foyer, 1998).

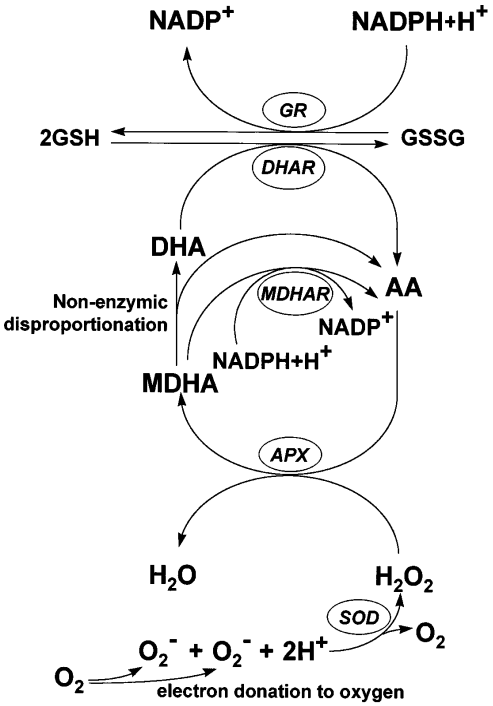


Fig. 2.6. The ascorbate–glutathione cycle. (Adapted from Noctor and Foyer, 1998)

In addition to other enzymatic antioxidants, plant mitochondria have evolved another system of mitigating the production of ROS during stress conditions (Purvis, 2004). An alternate oxidase (AOX) is located on the matrix side of the inner membrane, which oxidizes ubiquinol directly without producing a proton electrochemical gradient across the inner membrane. Therefore, electron flow through the AOX is not coupled to the synthesis of ATP and causes the reduction in redox potential of the component of ETC and lowers the concentration of O_2 in the

mitochondria. This overall process reduces the potential for leakage of electrons from the system, especially under the enhanced respiration experienced both during and after stress (Purvis, 2004). The up–regulation of AOX activity in response to various environmental stimuli can thus contribute to the reduction in ROS production in the cell.

2.4.2.2 Water–soluble non–enzymatic antioxidants

Ascorbate (AA)

AA is one of the most abundant low molecular weight antioxidants present in plant tissues (Davey and Keulemans, 2004). AA can donate electrons which makes it an effective ROS–detoxifying compound in a wide range of enzymatic and non–enzymatic reactions in the aqueous phase. AA serves as an antioxidant to scavenge ROS which oxidizes AA to MDHA and DHA. MDHA and DHA can be converted back into AA to maintain the cellular pool by the action of MDHAR and DHAR, respectively. Therefore, the amount of AA present in the cell is modulated by both its biosynthesis/regeneration and oxidation loss. The ratio of AA to DHA is an indicator of the redox potential of the cell and can also serve as a marker for the degree of oxidative stress (Davey and Keulemans, 2004). AA also regenerates tocopherols from tocopheroxyl radical providing membrane protection (Blokhina et al., 2003). It also plays important roles in resistance to a number of environmental stresses such as pathogen infection, hypoxia stress, high light and UV–B radiation (Noctor and Foyer, 1998). It has been implicated in the regulatory role in some fundamental cellular processes such as photo–protection, cell–cycle and cell expansion (Blokhina et al., 2003).

Glutathione (GSH)

GSH is a non–protein sulphur–containing tripeptide (Glu–Cys–Gly) and acts as a storage and transport form of reduced sulphur (Tausz et al., 2004). GSH is related to the sequestration of xenobiotics and heavy metals and is also an essential component of the cellular antioxidative defence system, which keeps ROS under control. Being an integral component of the AA–GSH cycle, it is involved in the regeneration of AA during the removal of excess H_2O_2 from the cell. Its role in regeneration of another antioxidant, AA, provides it a central role in the antioxidant defence system. In addition to AA–GSH cycle, GSH is possibly involved in degradation of H_2O_2 in a

reaction catalysed by GPX, but the role of GSH as a substrate of GPX is still questionable (Szalai et al., 2009). GSH plays very important role in the removal of lipid peroxides through the activity of glutathione–S–transferases (GT) (Szalai et al., 2009). The accumulation of GSSG, the oxidized form of GSH, leads to a decrease in GSH/GSSG ratio which symbolizes the oxidized environment and the reduction of antioxidant capacity of the glutathione system (Noctor and Foyer, 1998).

Polyphenolic compounds

Polyphenolic compounds, a group of plant secondary metabolites of more than 9000 individual molecules, are involved in an array of processes, including plant–pathogen interactions, pollination, light screening, seed development and allellopathy (Hernández et al., 2009). Many biosynthetic genes for polyphenolic compounds are induced under stress conditions and, accordingly, their levels increase during exposure to biotic and abiotic stresses, such as wounding, drought, metal toxicity and nutrient deprivation. *In vitro* antioxidant tests reveal that the antioxidant capacities of phenolic compounds are several–fold higher than those of ascorbate (vitamin C) or α–tocopherol (vitamin E), two well known *in planta* antioxidants (Rice–Evans et al., 1997).

Phenolic compounds have been suggested to act as antioxidants, protecting plants from oxidative stress. Phenolic compounds and peroxidases are generally localized in vacuoles of plant cells. When H_2O_2 is formed in vacuoles or tonoplasts, or when H_2O_2 formed outside of vacuoles is diffused into the organelles, vacuolar peroxidases oxidize phenolics, especially flavonols and hydroxycinnamic acids, to form phenoxyl radicals (Takahama, 2004). Phenoxyl radicals formed by peroxidase–dependent reactions are reduced by AA in vacuoles. This suggests that ascorbate/phenolics/peroxidase systems in the vacuoles can scavenge H_2O_2. Figure 2.7 shows H_2O_2 scavenging when H_2O_2, which is generated by chloroplasts, mitochondria and peroxisomes, diffuses into a vacuole.

The presence of phenolics compounds is not only restricted to vacuoles, but also to the other ROS production sites, such as chloroplast, nuclear region and plasma membrane, where they play a crucial role in defence against the ROS (Hernández et al., 2009). The spatio–temporal variation in the accumulation of oxidation products of polyphenolic compounds under oxidative stress is still not clear. There is not enough experimental evidence to explain how flavonoids and their

oxidation products are (re)distributed throughout plants and plant cells under oxidative stress? In the presence of transition metals, the prooxidant role of polyphenolic compounds is another mystery, to be proven yet. But there is increasing amount of literature in favour of *in planta* antioxidant role for different classes of polyphenolic compounds (Hernández et al., 2009).

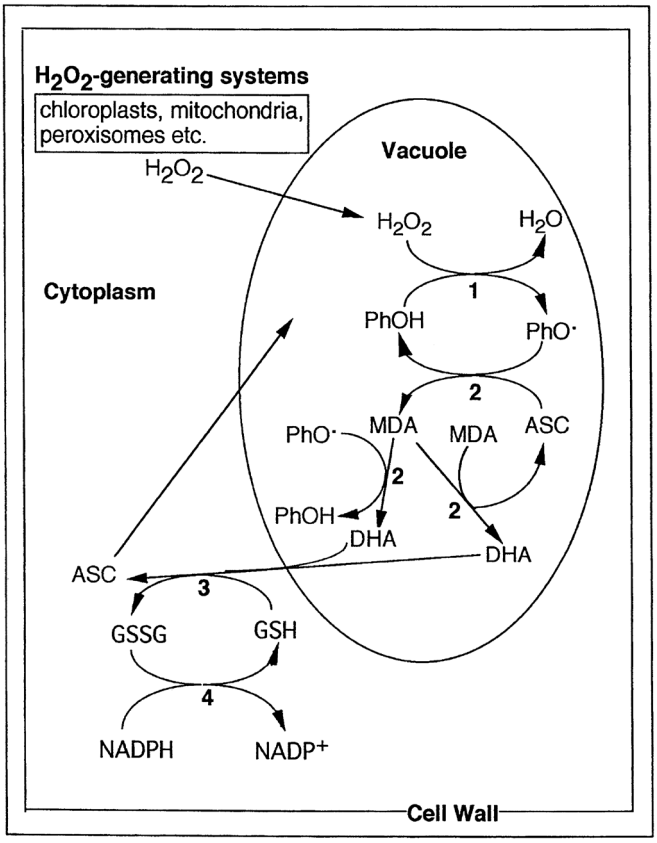


Fig. 2.6. Possible reactions of ASC/phenolics/peroxidase systems in a vacuole. 1, peroxidase; 2, chemical reactions; 3, dehydroascorbate reductase; 4, glutathione reductase. ASC, ascorbate; DHA, dehydroascorbate; GSH, glutathione; GSSG, oxidized form of glutathione; MDA, monodehydroascorbate; PhOH, phenolics; PhO., phenoxyl radicals. (Adapted from Takahama, 2004)

2.4.2.3 Lipid–soluble non–enzymatic antioxidants

Tocopherols

Tocopherols are a group of closely related phenolics benzochroman derivatives having extensive ring alkylation (Lurie, 2003). There are four tocopherols isomers (α–, β–, γ–, and δ–); α–tocopherol has the highest antioxidant activity among all. The molecule is amphipathic in nature; the hydrophobic tail is located in a membrane and

is associated with the acyl chains of fatty acids or their residues, while the polar chromanol head group lies at the membrane–cytosol interface. It acts as an antioxidant either by chemical scavenging of free–radicals or by physical deactivation, thereby preventing the proliferation of oxidative chain reactions. The major role of α–tocopherol is to protect the biological membrane by acting as a chain–reaction terminator for the removal of polyunsaturated fatty acid (PUFA) radicals generated during lipid peroxidation (Blokhina et al., 2003).

Carotenoids

Carotenoids are among the most common natural pigments in plants and play a major role in the protection against photooxidative processes (Lurie, 2003). These are very efficient antioxidants scavenging singlet molecular oxygen and peroxyl radicals, and thus prevent oxidative damage to the tissue. In plant tissues, carotenoids mostly occur in the chloroplast membranes and help prevent oxidative injury from ROS produced in the photosynthetic electron transport chain. The richness of chloroplast membranes with PUFA linolenate makes them more prone to peroxidation. Therefore, the presence of carotenoids prevents the membrane damage to the chloroplast by ROS (Lurie, 2003).

2.4.3 Oxidative stress

ROS are normally present in all aerobic cells in balance with biochemical antioxidants. Oxidative stress occurs when this critical balance is disrupted by excess of ROS, reduction in antioxidants, or both. The important homeostatic parameters must be set to counteract the oxidant effects and to restore redox balance by the cell. The changes associated with cellular oxidative damage and repair/maintenance of homeostasis often lead to up– or down–regulation of genes encoding regulatory transcription factors, antioxidant defence enzymes and structural proteins. There are several factors which augment the development of oxidative stress in plants and include, but not limited to, temperature extremes, drought and flooding, salinity, ozone exposure, UV irradiation, heavy metal toxicity, herbicides, and environmental pollutants (Apel and Hirt, 2004 and references therein). These environmental perturbations enhance the ROS production in the plant or plant organs. If ROS production exceeds the antioxidant capacity of the tissue, it causes oxidative damage to the cell, which ultimately leads to cell death.

2.5 Postharvest oxidative stress

Fresh fruits are living entities which continue to respire and transpire even after harvest. The postharvest physiological and biochemical changes have both desirable and undesirable effects on fruit quality. The most important desirable changes in climacteric fruit are brought by the ripening process which involves the changes in skin and flesh colour, texture, taste and emission of aroma–volatile compounds. The advanced stages of ripening culminate into the senescence process which eventually leads to the death of fruit, and is the most undesirable from economic viewpoint. On the other hand, non–climacteric fruit are generally harvested at the ripe stage, but there are still some desirable postharvest changes in fruit texture and flavour development. Similar to climacteric fruits, the onset of senescence in non–climacteric fruits is also a natural process which leads to death. Fruit ripening has been described as an oxidative phenomenon which involves production and removal of ROS from the tissue and the failure or reduction in the capability of antioxidant system leads to the oxidative injury coinciding with the onset/advancement of senescence.

The objective of a postharvest technology is to slow down the ripening and/or delay the onset of senescence in fruit and to deliver a high quality fruit to the consumer/processor. Low temperatures have been widely used as a mean to retard the biological activity of fruit that enables its storage, transport and distribution over longer periods and distances. Studies have shown that chilling temperatures can potentially cause oxidative injury to the fruit, depending on the storage temperature and length of exposure. Most of the postharvest practices aimed to extend the storage or shelf life of produce have been known to act as stress factors. It is therefore obvious that the development of oxidative stress in postharvest situations is indicative of the significant stress experienced by a commodity (Toivonen, 2004).

Consumers always demand for a consistently high quality fruit throughout the year. During the postharvest supply chain, some of the physiological and biochemical changes in fruit result in development of certain disorders which affect the consumer acceptability of fruit. Some of the very popular storage disorders such as superficial scald in apple, flesh and core browning in apples and pears have been attributed to the adverse effects of postharvest oxidative stress in fruit. Chilling injury is also one of such major physiological disorders which limit the long–term

cold storage of fruits below a critical threshold temperature and adversely affects the consumer experiences, especially when the symptoms are invisible or internal. Fruits of tropical and sub–tropical origin are generally more susceptible to CI than their temperate/Mediterranean counterparts. The development of CI in temperate/ Mediterranean fruits is generally a function of duration of exposure to a particular temperature. There is increasing evidence that CI in fruits is also a result of oxidative damage to the membrane which results in irregularities in the physiology and metabolism of the cell.

A large volume of literature has accumulated in the last decade on the importance of antioxidant metabolism in relation to fruit quality and durability in postharvest supply chain. The sites of ROS production and their roles in cell physiology are complex and intricate in nature, and so the antioxidant defence systems evolved by the plant or plant organs in order to cope with the environmental stresses. Postharvest oxidative stress in fruits can be detected directly and indirectly (Toivonen, 2004). The degree of oxidative stress can be directly determined by the measurements of accumulation of ROS, increases in lipid peroxidation products, enhanced membrane disintegration or accumulation of brown pigments. The indirect measurements include the determination of dynamics of non–enzymatic and enzymatic antioxidant systems. The understanding of inter–relationship of oxidative stress parameters and their association with storage disorders offer attractive alternatives to develop the innovative postharvest practices. The comprehensive measurement of various components of antioxidative protection systems is therefore central to demonstrate their role in fruit quality with regard to physiological disorders (Toivonen, 2004).

As a general rule, plants have two primary strategies of coping with oxidative stress: avoidance and tolerance (Hodges et al., 2004). Postharvest fruits are not in a situation to adopt the ‘avoidance’ strategy, but the human interventions to mitigate the conditions promoting ROS production can contribute to ‘avoidance’. However, ‘tolerance’ to oxidative stress of postharvest fruits has been associated with their inherent antioxidant potential. But there are several other factors which affect the capability of fruits to encounter postharvest oxidative stress and some of these important factors are reviewed and discussed in the following sections.

2.5 Factors affecting postharvest oxidative stress

2.5.1 Genotype/cultivar

Postharvest behaviour of a cultivar is determined by its genetic make–up. Some cultivars with a faster rate of ripening and senescence have poor postharvest storage and shelf life. The phenomena of ripening and senescence are genetically controlled, but are strongly influenced by the environmental factors. There is abundant evidence that the development of postharvest oxidative stress in fruits is greatly influenced by the cultivar factor. There are clear differences in the enzymatic and non–enzymatic antioxidant components of cultivars, differing in their susceptibility to oxidative injury. Generally, the cultivars with higher antioxidant potential have better stress resistance, nutritional quality, and storage characteristics (Davey and Keulemans, 2004; Lata et al., 2005).

2.5.1.1 Effect of cultivar on the antioxidant levels in fruits

The on–tree ripening and cold storage behaviour of two cultivars of apple differed markedly with regard to activities of antioxidant enzymes (Masia, 1998). The activities of SOD and CAT peaked with the climacteric ethylene peak during the on–tree ripening in both cultivars, ‘Fuji’ and ‘Golden Delicious’. During cold storage, SOD activity decreased in ‘Golden Delicious’, but peaked in ‘Fuji’, whereas CAT activity doubled in both cultivars (Masia, 1998). There are published reports on screening of apples cultivars for some enzymatic and non enzymatic antioxidants which revealed a great variation in the activities and concentration of these antioxidants among cultivars at harvest and following cold storage (Davey et al., 2004; Davey and Keulemans, 2004; Lata et al., 2005). As compared to variation at harvest, apple cultivars differed substantially in their ability to maintain L–AA levels during storage. Generally, cultivars that could maintain their AA and GSH pools also had better storage properties (Davey and Keulemans, 2004). For instance, ‘Sunrise’ and ‘Gravenstein’ cultivars showed maximal losses of AA and GSH by about 80% and 50% following shelf life and these cultivars had poor storability, with susceptibility to browning and rot. For cultivars such as ‘Arlet’ and ‘Angold’, there was no significant difference between AA at harvest, cold storage or shelf life, and could be stored for up to 6 months at 1°C. Some cultivars such as ‘Greenstar’ and ‘Braeburn’ were able to maintain or actually slightly increase both AA and GSH

levels during cold storage and shelf life, resulting in retention of better fruit quality for up to 6 months at 1°C (Davey and Keulemans, 2004). Other researchers have also shown a great variation in the changes in concentration of antioxidants during cold storage of different cultivars of apples (Lata et al., 2005).

The concentrations of antioxidants also differ with the fruit tissue. AA and GSH levels in the peel tissue of apples were 6.7 and 2.8 fold higher, respectively, than those in the cortical tissue (Davey et al., 2004). Similarly, in 25 cultivars of apples, on average, GR, CAT, AA, GSH, and phenolics were approximately 2.9, 1.5, 4.4, 1.7, 2.1, 2.1, and 2.5 fold higher in the apple peel than the whole apple fruit (Lata et al., 2005). A recent study also confirmed that three apple cultivars, 'Delicious', 'Golden Delicious' and 'Gala', showed higher AA content in peel than in cortical tissue as the peel tissue has to play a protective role in response to both abiotic and biotic stresses (Felicetti and Mattheis, 2010).

Recently, a vast amount of literature has accumulated on the identification and quantification of water– and lipid–soluble antioxidants in the fruits and is beyond the scope of this chapter. Studies have been conducted to screen the commercial cultivars and/or hybrids of peaches, plums and nectarines for antioxidant capacities (Chun et al., 2003; Díaz–Mula et al., 2009; Gil et al., 2002; Kim et al., 2003; Vizzotto et al., 2006). In general, the presence of various bioactive compounds, such as anthocyanins, carotenoids and phenols, and overall antioxidant activity of skin tissue is higher than flesh in stonefruit. The total phenolics concentration in these fruit had the most consistent and highest correlation with antioxidant activity. It indicates the more importance of phenolics in determining the antioxidant activity of peaches and plums compared to anthocyanins or carotenoids (Chun et al., 2003; Díaz–Mula et al., 2009; Gil et al., 2002; Vizzotto et al., 2006). In Japanese plums, at harvest, large variations in phytochemicals and antioxidant activity were found among eight cultivars. The concentration of bioactive compounds and antioxidant capacities were 2 to 40 fold higher in the peel than in the flesh tissue (Díaz–Mula et al., 2009). However, these cultivars of stonefruit including Japanese plums have not been screened for the activities of various antioxidant enzymes in the skin and flesh tissue and is worthy of investigations. The knowledge of the status of enzymatic and non-enzymatic antioxidants, which are associated with the regulation of a wide range of metabolic functions, could be useful for the genetic improvement of fruits and also for developing biochemical markers for determining

the potential storage life of various cultivars. Such comprehensive information on Japanese plums is lacking and requires detailed investigations.

2.5.1.2 Effect of cultivar on the senescence rates

The differences in senescence rates of cultivars of muskmelon (*Cucumis melo* L.) and spinach (*Spinacia oleracea* L.) have been best explained on the basis of oxidative stress theory. The comparison of senescence rates of two cultivars of non–netted muskmelon showed that 'Clipper' had a postharvest life of more than 14 days against 'Jerac' with less than 7 days (Lacan and Baccou, 1998). The higher senescence rate had been attributed to the higher extent of lipid peroxidation caused by ROS, membrane phospholipid breakdown, and a drop in the level of antioxidants, resulting in increased membrane leakiness. Higher activities of SOD and CAT and lower levels of accumulation of $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$ were implicated in the delayed senescence of 'Clipper' compared to 'Jerac' (Lacan and Baccou, 1998).

According to Hodges and Lester (2006), higher activities of antioxidant enzymes, APX, MDHAR, DHAR, CAT, POD, and SOD, were observed in a non–netted muskmelon, 'Orange Dew', compared to a netted–cultivar, 'Cruiser', when these were stored for 7 or 14 days at 7°C plus 3 days at 21°C. The accumulation of MDA, a lipid peroxidation product, was significantly lower in 'Orange Dew' than in 'Cruiser' (Hodges and Lester, 2006). In a subsequent study, these authors reported that 'Orange Delight' and 'Orange Dew' were generally superior to 'Honey Gold' and 'Temptation' as they consistently demonstrated the highest levels of AA and β–carotene (Lester and Hodges, 2008). 'Orange Delight' and 'Orange Dew' were also among the cultivars with the highest activities of APX, CAT, and SOD. These two cultivars also exhibited the least increase in MDA during storage, suggesting antioxidant levels limited oxidative stress–related senescence compared to the other genotypes. These studies indicate that there are significant differences in human health–related and storage quality–related phytochemical profiles among orange–fleshed honey dew cultivars and that the higher antioxidant levels are associated with reduced lipid peroxidation in fruit during cold storage.

Similar to muskmelon, Hodges et al. (2001) also reported that two cultivars of spinach differed greatly in their postharvest senescence rates; 'Spokane F1' showed higher senescence rate than 'BJ 412 Sponsor'. Increased H_2O_2 accumulation in 'Spokane F1' relative to 'BJ 412 Sponsor' might have contributed to an increased

rate of senescence in the harvested leaves of this cultivar. The lower activity of antioxidant enzymes such as APX and more accumulation of MDA could be attributed to the differential senescent rate shown by these two cultivars (Hodges et al., 2001).

The postharvest life of stonefruit including Japanese plums is strongly influenced by cultivar factor (Crisosto et al., 2004; Crisosto et al., 1999). Most of the plum cultivars exhibit climacteric–type fruit ripening, while some show suppressed climacteric behaviour (Abdi et al., 1997b; Candan et al., 2008; Khan and Singh, 2007b). The higher rates of respiration and ethylene production are the major contributory factors to the relatively short postharvest life of the climacteric–type plums compared to the suppressed–climacteric cultivars. The degree of lipid peroxidation and role of antioxidant system in influencing the ripening and senescence rate in different types of Japanese plums have not been studied yet.

2.5.1.3 Effect of cultivar on postharvest physiological disorders

Cultivar is one of the prime factors which determine the susceptibility of fruit to postharvest physiological disorders. For instance, apple cultivars greatly differ in their susceptibility to superficial scald, a very serious postharvest physiological disorder. The apple cultivars such as ‘Empire’, ‘Gala’, ‘Golden Delicious’ and ‘Crispin’ are resistant to scald development, whereas others such as ‘Delicious’, ‘McIntosh’, ‘Cortland’, ‘Granny Smith’, ‘Idared’, ‘Rome Beauty’ and ‘Fuji’ are susceptible to this disorder (Emongor et al., 1994). The specific mechanism of scald development is unknown although it is believed that the biosynthesis of α–farnesene and its subsequent degradation to conjugated trienes (CT) may cause the disruption, discolouration and death of surface cells (McLean et al., 2003). Scald development may also be related to ROS production either through low temperature stress or through α–farnesene metabolism. The amount of α–farnesene produced during scald development may be less important than the activity of the antioxidant enzyme system (Whitaker et al., 2000). However, no clear relationships between antioxidant enzyme activities and susceptibility/resistance to superficial scald development have been observed in different apple cultivars (Du and Bramlage, 1994; Du and Bramlage, 1995; Meir and Bramlage, 1988).

The confounding effects of cultivar, maturity, ripeness and environmental conditions may be ascribed to obstruct the development of a clear relationship

between the scald development and antioxidant systems in peel (Rao et al., 1998). Some studies have shown that the activities of POD and APX were higher in scald–resistant selections of 'White Angel' × 'Rome Beauty' apples, indicating a role for ROS in the induction of scald (Rao et al., 1998). To reduce the incidence of superficial scald in apples, the success of postharvest treatments of fruit with antioxidants and storage under low O_2 atmospheres support the notion that antioxidant metabolites and/or enzymes may influence the resistance to scald (DeLong and Prange, 2003). The studies indicate that the scald is characterized by cellular oxidation within the hypodermis, and the oxidative stress theory can partially explain the susceptibility to this disorder exhibited by different cultivars of apples.

Mandarin cultivars, 'Clemenules' and 'Clementine', are considered to be chilling–tolerant compared to 'Nova' and 'Fortune' cultivars which are chilling sensitive (Sala, 1998). The chilling tolerance in these cultivars has been attributed to the increased activities of CAT, APX and GR in response to chilling stress. Further studies on the role of antioxidant defence showed that CAT is the major antioxidant enzyme involved in the defence mechanism of mandarin fruits against chilling stress (Sala and Lafuente, 2000). In another study on pepper (*Capsicum annuum* L.) fruit, susceptibility to CI appeared to be related to the activities of SOD and CAT which were much higher in 'Buchon', a chilling–tolerant cultivar, than 'Nockgwang', a chilling–sensitive cultivar (Lim et al., 2009). These studies suggest that cultivars with tolerance to chilling stress are generally equipped with a more efficient antioxidative system.

There are many reports presenting CI as an oxidative phenomenon in different fruits (Galli et al., 2009; Hariyadi and Parkin, 1991; Lim et al., 2009; Wang et al., 2005; Zhao et al., 2009b; Zhao et al., 2006), but only a few of them have considered cultivar as an important factor (Lim et al., 2009; Sala, 1998; Sala and Lafuente, 2000; Sala and Lafuente, 2004). The lack of clear differences in the susceptibility to CI among different cultivars could be the possible reason for lesser progress in the literature on this aspect. The differences in the susceptibility to CI among cultivars of stonefruit have also been outlined (Crisosto et al., 1999). But no such work has been reported to correlate the development of this disorder with the antioxidative metabolism of different cultivars of stonefruit.

Available information in the literature prompts the viewpoint that antioxidant system in the fruit can play a greater role in providing protection against the

oxidative damage caused by ROS. The activities of various antioxidant enzymes or the concentrations of specific antioxidants may be utilized as markers for selection of elite genotypes. It may also be argued that genetic variations are exhibited by fruits in regulating or controlling postharvest oxidative stress. The preharvest and postharvest factors which can cause the augmentation or deterioration or failure of the antioxidative system should be well investigated so that appropriate postharvest strategies can be adopted to minimise losses in fruit quality.

2.5.2 Harvest maturity

Harvest maturity has been shown to affect the development of postharvest oxidative stress in fruits, thereby influencing the storage potential and susceptibility to oxidative injury (Toivonen, 2003a). The development of postharvest physiological disorders in fruits has been related to the antioxidant levels at harvest and the changes in their concentrations during cold storage. The accumulation of lipid–soluble antioxidants in apple peel due to delayed harvest has been shown to decrease the incidence of superficial scald during cold storage (Barden and Bramlage, 1994; Diamantidis et al., 2002), but a clear relationship between antioxidant enzymes and scald resistance has not been established due to contradictory reports (Du and Bramlage, 1995; Rao et al., 1998). However, the flesh related disorders in apples and pears have been shown to increase with the advanced maturity. The decrease in activities of SOD and CAT in flesh tissue increased the incidence of Braeburn browning disorder (BBD) in late–harvested ‘Braeburn’ apples (Toivonen et al., 2003).

Another study showed that the changes in activities of antioxidant enzymes, SOD, CAT and POD, in the flesh tissue of ‘Golden Smoothee’ apples were mainly influenced by the climatic conditions during the last phase of on–tree fruit maturation (Molina–Delgado et al., 2009). The cooler season resulted in a higher antioxidant potential of the fruit in terms of higher activities of these enzymes and AA concentration. In the cooler season, the delay in harvesting caused a significant decrease in the activities of SOD and POD, and AA levels in the flesh tissue. In general, the changes in quality parameters during on–tree ripening were not related to the capability of the fruit to produce ethylene, but rather to endogenous levels of antioxidants, especially CAT and AA, at the earliest picking date (Molina–Delgado et al., 2009). The decline in AA concentration in the flesh tissue of apple cultivars

during the final stages of fruit maturation has been demonstrated by various researchers (Davey et al., 2007; Felicetti and Mattheis, 2010; Molina–Delgado et al., 2009). The concentrations of AA were also found to be negatively correlated with mean preharvest daytime temperature; however, since preharvest temperature and harvest date themselves were closely linked, it was not possible to definitively separate the relative contributions of genetic and environmental factors to these components (Davey et al., 2007).

The delayed harvesting of 'Conference' and 'Passa Crassana' pears increased susceptibility to core browning due to decrease in the ability of antioxidant system to protect from ROS with the advanced maturity (Lentheric et al., 1999; Vanoli et al., 1995; Veltman et al., 1999). The behaviour of peel and flesh tissues seems to be quite different with regards to maturity and subsequent changes in respective antioxidant systems (Toivonen, 2003a). Therefore, the investigations should be directed keeping in view the specific tissue affected by a particular physiological disorder.

It is well–known that sensitivity to CI is strongly influenced by the fruit maturity. Fruit at advanced stages of maturity or ripening are chilling tolerant compared to less mature or unripe fruit. In mangoes, the activities of SOD, CAT, APX and PPO in pre–yellow and yellow fruit were reported to be higher than those of the green from the 6^{th} to 12^{th} day during cold storage (Zhao et al., 2009b). A lower content of MDA, higher levels of AA and GSH were maintained in pre–yellow and yellow fruit than that in the green fruit. These results suggest that chilling tolerance of pre–yellow and yellow mangoes compared to green fruit could be due to their higher antioxidant potential (Zhao et al., 2009b) and therefore cold storage of mangoes at advanced stages of maturity can help alleviating CI. Another typical example is pepper (*Capsicum annuum* L.) fruit. The pepper fruit harvested at mature green and breaker stages were more prone to CI than those at red–ripe stage (Lim et al., 2009; Lin et al., 1993). The higher APX and SOD activities in mitochondria from the ripe–red pepper fruit might play a role in avoiding the accumulation of ROS generated in the mitochondria (Jiménez et al., 2002b). Recently, it was confirmed that the higher activities of SOD and CAT were correlated with the chilling tolerance at the red–ripe stage in peppers (Lim et al., 2009).

Cherries harvested at advanced stages of maturity, 4 days later than commercial harvest dates, showed higher levels of phenolics and total antioxidant

activity at harvest and also after 16 days of cold storage at 2°C plus 2 days of shelf life (Serrano et al., 2009). It has been widely argued that the changes in antioxidant components during cold storage of fruit should be considered more importantly in providing protection against oxidative injury than their at–harvest antioxidant status. The information on the effect of harvest maturity on the activities of antioxidant enzymes in fruit during postharvest cold storage and shelf life is limited and there is a scope for further research in this area.

2.5.3 Storage temperature and duration

Storage temperature and duration are important factors which govern the development of postharvest oxidative stress in fruits (Hodges et al., 2004; Toivonen, 2004). Despite the inhibitory effects of certain postharvest procedures on fruit ripening and senescence, the storage potential of each fruit cultivar is definite. Storage at optimum temperature is essential to maintain fruit quality. However, storage at temperatures below and above the optimal limit accelerates the loss of fruit quality, either by inducing chilling injury or by faster rate of ripening and senescence. Even storage under optimum conditions is also possible for a limited time period, depending on the cultivar and several other factors such as fruit maturity.

Antioxidant metabolism has been shown to play a very important role in determining the storage potential of fruit. The accumulation of ROS continues during postharvest phase of fruit to variable extent depending upon the environmental conditions and the antioxidant potential of the fruit. The production and accumulation of ROS beyond the antioxidant capability of fruit tissue causes the oxidative damage, resulting in senescence and appearance of visible injuries on the fruit. The impact of storage duration on the development of oxidative stress has been widely studied in fruits, but there is relatively less information about the role of storage temperature.

Studies have shown that with the increase in storage duration, there is an increased accumulation of ROS in the fruit tissue (Cao et al., 2009a, 2009b; De Castro et al., 2008; Huang et al., 2008; Zheng et al., 2007). The increase in storage duration of non–netted muskmelons caused a progressive increase in the concentration of ROS, $O_2^{\bullet -}$, H_2O_2 and $OH^{\bullet}$, in the fruit tissue and determined the senescence rate of cultivars differing in their accumulation patterns (Lacan and

Baccou, 1998). The increase in concentration of H_2O_2 has been reported to occur during 16 weeks of cold storage at 0.5°C in different apple selections derived from a cross between 'White Angel' and 'Rome Beauty' (Rao et al., 1998). The higher levels of H_2O_2 have been associated with the occurrence of oxidative damage and related to the development of flesh browning in 'Pink Lady'™ apples (De Castro et al., 2008) and watercore in 'Fuji' apples (Kasai and Arakawa, 2010). On the other hand, only a transient increase in concentration of H_2O_2 was observed in 'Golden Smoothee' apples during the first 3 days of storage, and then it declined continuously during the subsequent 90 days of storage period (Vilaplana et al., 2006). Contrary to H_2O_2, the accumulation of lipid peroxidation products continued during the entire storage duration which indicated the development of oxidative stress with the progression of storage (Vilaplana et al., 2006). The cultivar differences do exist in the storage behaviour of fruits, especially with regard to accumulation of ROS, depending on tissue type. The increase in ROS concentration and accumulation of lipid peroxidation products as a function of storage duration have been reported in loquats (Cao et al., 2009a, 2009b), peaches (Zheng et al., 2007), oranges (Huang et al., 2008), and mangoes (Singh and Dwivedi, 2008; Wang et al., 2009)

The activities of primary antioxidant enzymes, SOD, CAT and POD, have been known to change with respect to storage duration. The activities of CAT and POD increased significantly in the skin tissues of apple cultivars, 'Empire', 'Cortland', and 'Delicious' during the cold storage at 0°C for 12 or 24 weeks, while no significant change in SOD activity was reported (Du and Bramlage, 1995). Similarly in the flesh tissue, CAT activity has been reported to increase during cold storage of 'Golden Delicious', 'Golden Smoothee' and 'Fuji' cultivars, with less impact on SOD activity (Masia, 1998; Vilaplana et al., 2006). CAT activity increased only gradually in 'Golden Delicious' while peaking in 'Fuji' after 49 days of cold storage. The decrease in CAT after 84 days of storage was coincident with the appearance of watercore disorder in 'Fuji' apples (Masia, 1998). Another study has shown the decreases in activities of CAT and POD and increase in SOD activity during 16 weeks of cold storage of apple selections derived from a cross between 'White Angel' and 'Rome Beauty' (Rao et al., 1998). On the other hand, POD activity has been reported to increase significantly in the peel tissue of 'Jonagold' and 'Šampion' apples during 120 days of cold storage at 1°C in air or CA (Leja et

al., 2003). These studies show the mixed results in terms of changes in the activities of various enzymes in the same fruit tissue.

The changes in activities of various antioxidant enzymes have been studied in other fruits such as mandarins (Sala, 1998; Sala and Lafuente, 2000; Sala and Lafuente, 2004), mangoes (Wang et al., 2009; Zhao et al., 2009b; Zhao et al., 2006), oranges (Huang et al., 2008; Huang et al., 2007), pears (Larrigaudiére et al., 2001b; Larrigaudiére et al., 2004), peaches (Wang et al., 2004, 2005, 2006), and raspberries (Chanjirakul et al., 2006). These studies indicate that the increased activities of SOD, CAT and POD enzymes are important to regulate the ROS levels in the fruit tissue. The decreases in the activities of these enzymes were generally coincident with the failure of the overall antioxidant system resulting in loss of membrane integrity, appearance of physiological disorders and termination of storage life.

Regarding non-enzymatic antioxidants, AA concentrations generally decrease during cold storage of fruits. The decrease in concentration of AA in apples has been reported to be a function of storage duration (Barden and Bramlage, 1994; Davey et al., 2007; Davey et al., 2004; Davey and Keulemans, 2004; De Castro et al., 2008; Fawbush et al., 2009; Felicetti and Mattheis, 2010; Lata et al., 2005) and a subsequent increase in the number of physiological disorders (Davey et al., 2004; Davey and Keulemans, 2004; De Castro et al., 2008; Kasai and Arakawa, 2010). The role of AA in better postharvest storage performance of pears has been widely studied and accepted (Franck et al., 2003; Lentheric et al., 1999; Veltman et al., 2000; Veltman et al., 1999). The factors which contribute to the core breakdown in pears have been closely related to the loss of AA in fruit (Franck et al., 2003).

Similar to AA, a significant decrease in GSH levels has been reported to occur during long–term cold storage of fruits such as mangoes (Zhao et al., 2009b), oranges (Huang et al., 2008), and pawpaws (Galli et al., 2009). High AA levels in some apple cultivars have been linked to the high GSH levels, resulting in better storage properties (Davey and Keulemans, 2004). Apple cultivars such as ‘Greenstar’ and ‘Braeburn’ were able to maintain or actually slightly increase GSH levels under both cold storage and shelf life and could retain quality for up to 6 months at 1°C (Davey and Keulemans, 2004). A comprehensive study on the changes in different antioxidants in apple fruit during cold storage revealed that GSH levels were significantly higher in the fruit skin after 45 days of storage in air and then declined after 90 days to harvest levels (Łata, 2008). The study supports the notion that

concentration of GSH increases in response to chilling stress as a mechanism for acclimatization, but prolonged storage duration may result in decrease in its concentration to original levels or below. At the same time, the increase in GSSG concentration has been reported to occur causing a decrease in ratio of GSH:GSSG, which has been considered to be a marker of oxidative stress in the tissue (Tausz et al., 2004). The decrease in glutathione concentration has been associated with internal browning in methyl bromide–fumigated 'Thompson Seedless' grapes (Liyanage et al., 1993) and methyl–iodide–induced phytotoxicity in lemons (Ryan et al., 2007). These studies reflect that most of the changes in different antioxidant compounds including GSH are cultivar–specific. The role of GSH as an antioxidant has not been widely studied in fruits and needs further investigations.

The concentrations of other antioxidant compounds such as phenolics and flavonoids are strongly influenced by the storage temperature and storage duration. Strawberry fruit stored at 10°C or 5°C showed higher antioxidant capacity, total phenolics, and anthocyanins than those stored at 0°C (Ayala–Zavala et al., 2004). Strawberries stored at 0°C retained an acceptable overall quality for the longest storage duration; however, berries stored at temperatures higher than 0°C showed higher content of aroma compounds and antioxidant capacity during the postharvest period.

2.5.4 Storage atmospheres

Storage atmospheres containing low O_2 and/or high CO_2 can retard the fruit metabolism through reduced rates of respiration and ethylene production (Kader, 2003). The modification of storage atmospheres also provides other benefits such as alleviation of certain physiological disorders and suppression of microbial and insect activity. The presence of low O_2 atmospheres surrounding the fruit can decrease inter– and intra–cellular O_2 levels in the flesh tissue (Whitaker, 2004). Therefore, there is a potential for reducing the rates of oxidative processes in the fruit kept in low O_2 environments.

2.5.4.1 Role of CA/MA in alleviation/ aggravation of oxidative stress in apples and pears

Researchers have shown that both beneficial and detrimental effects can be expected from storage under CA/MA conditions depending on the concentrations of gases, the physiological maturity of fruit and the preharvest environmental conditions. Control of superficial scald in apples is a classical example of the suppression of oxidative reactions in the fruit by CA/MA storage. Low O_2 atmospheres have been known to reduce the incidence and severity of superficial scald in apples (Patterson and Workman, 1962). The storage of 'Starkrimson Delicious' apples in atmospheres containing 0.7% O_2 for 6 to 8 months markedly suppressed scald incidence compared with air–storage (Lau et al., 1998). 'Braeburn' fruit held in atmospheres containing 1.2% or 1.5% O_2 + 1.0% or 1.2% CO_2 for 6 months had significantly less core browning and superficial scald than fruit held in air for the same period (Lau, 1998). However, CA–stored fruit were highly susceptible to BBD and internal cavities (IC) after cool growing seasons. And the susceptibility of fruit to BBD and IC was greatest in late–harvested fruit stored in 3.0% CO_2 and 1.5% O_2. Therefore, seasonal environmental conditions coupled with fruit maturity stage can override the benefits of low O_2 storage atmospheres in providing protection against the superficial scald (Emongor et al., 1994; Lau, 1998). CA storage in combination with postharvest treatment with an antioxidant such as diphenylamine (DPA) or ethylene action inhibitor, 1–MCP, can reduce the incidence of scald in apples and pears (Rizzolo et al., 2005; Watkins et al., 2000). These studies suggest that CA storage alone can not provide protection against the incidence of scald in apple and pear, without the supplementation of a postharvest treatment with an antioxidant or 1–MCP. The combination of CA storage and 1-MCP will be investigated to alleviate CI in Japanese plums.

The role of high CO_2 during CA storage in development of postharvest oxidative stress has also been studied in fruits. 'Pink Lady'™ apples held in CA containing 1.5 kPa O_2 and 5 kPa CO_2 accumulated more H_2O_2 than those stored in air, indicating stress from the high–CO_2 concentrations in storage and causing flesh browning symptoms (De Castro et al., 2008). A direct comparison of the effects of CO_2 concentration in CA on the oxidative stress during storage of 'Blanquilla' pears was investigated (Larrigaudiére et al., 2001b). During 6 months of storage in regular air or CA containing either 2% O_2 + 0.7% CO_2, or 2% O_2 + 5% CO_2, the incidence

of core browning occurred in pears stored at 5% CO_2 which was associated with the accumulation of lipid peroxidation products (ethane and TBARS) and lowest concentration of AA after 6 months of storage. The effectiveness of antioxidative defence system might have decreased causing lipid peroxidation and finally browning. The study suggested that oxidative damage was involved in the CO_2–related physiological damage occurring during CA storage in pears (Larrigaudiére et al., 2001b).

It is believed that most of the changes in the antioxidant metabolites and enzymes occur during the initial stages of CA storage of apples and pears, which subsequently affect the long–term storage behaviour of fruit. Some of these changes may have detrimental effects on fruit quality as reported in 'Conference' pears. 'Conference' pears, subjected to CA containing 2% O_2 and 5% CO_2 or regular air for 21 days at –1°C, showed a rapid decrease in total ascorbate content and increase in DHA under CA conditions (Larrigaudiére et al., 2001a). A sharp increase in the activities of SOD, APX, GR and LOX was coupled with the accumulation of H_2O_2 and lipid peroxidation products, when the fruit were exposed to CA. These changes marked a significant amount of oxidative stress in 'Conference' pears exposed to CA containing 5% CO_2 which can further lead to development of core browning during extended storage (Larrigaudiére et al., 2001a). It is generally accepted that decrease in AA concentration caused by late–harvesting and 5% CO_2 concentration in the storage atmosphere are mainly associated with the appearance of brown heart in 'Conference' pears (Franck et al., 2003; Zerbini et al., 2002).

Metabolomics– and proteomics–based approaches have been followed to explicate the physiological and biochemical aspects of browning in 'Conference' pears (Pedreschi et al., 2007, 2008, 2009). The metabolic profiling was carried out on the flesh tissue of late–harvested 'Conference' pears stored in CA containing 1% O_2 + 10% CO_2 at –1°C for 4 or 6 months, conditions suitable for inducing browning (Pedreschi et al., 2009). The flesh tissue browning in pears was mainly related to a disturbed energy metabolism, alteration in concentrations of metabolites dependent on energy metabolism pathways, collapsed antioxidant system and cell wall architecture. The brown tissue exhibited a decrease of malic acid and an increase in fumaric acid and gamma aminobutyric acid (GABA), which indicated a reduced metabolic activity at the level of the Krebs cycle and a putative block of the GABA shunt pathway. Increased gluconic acid concentration might be related to AA

degradation due to insufficient reducing equivalents or to an impaired pentose phosphate pathway. The concentrations of other compounds such as trehalose and putrescine were also considerably higher in brown tissue than in sound tissue, suggesting hypoxic stress. The concentration of some sugars which are typically found in xyloglucans also increased during brown development, possibly indicating cell wall breakdown due to enzymatic processes or chemical reactions of hydroxyl radicals (Pedreschi et al., 2009). Proteomics study has also shown that down–regulation of APX, GT and MDHAR in brown tissue was indicative of total impairment of the ascorbate–glutathione cycle (Pedreschi et al., 2007). These studies indicate that core breakdown development is a consequence of an imbalance between oxidative and reductive processes caused by too low oxygen or too high carbon dioxide conditions which lead to a deficiency of reducing equivalents for defensive mechanisms, cell damage repair processes and biosynthesis reactions.

A study on apple cultivars, 'Šampion', 'Jonagold', 'Topaz', 'Sawa' and the clone U 633, showed that pool of antioxidants such as AA, thiols, and phenolic compounds, in the skin tissues of fruit showed a pronounced increase under CA conditions (1.5% O_2 + 1.5% CO_2) during the initial 45 days of storage and should be considered as an acclimation response (Łata, 2008). After 90 days of storage, the antioxidant status was kept more efficiently in the CA as compared to storage in regular air.

2.5.4.2 Role of CA/MA in alleviation/aggravation of oxidative stress in fruits other than apples and pears

Cranberries (*Vaccinium macrocarpon* Aiton) could be stored for 2 months at 3°C under storage atmosphere containing 30% CO_2 + 21% O_2 (Gunes et al., 2002). The storage atmosphere did not affect the content of total phenolics or flavonoids. However, the total antioxidant activity of the fruit increased overall by about 45% in fruits stored in air, while this increase was prevented by storage in atmospheres containing 30% CO_2 + 21% O_2.

Longan (*Dimocarpus longan* Lour.) fruit stored in a 5% O_2 atmosphere (balance N_2) for 6 days at 28°C markedly delayed pericarp browning in association with maintenance of high total phenolics concentration and reduced activities of PPO, POD and PAL (Cheng et al., 2009). Moreover, the fruit stored in a 5% O_2

atmosphere exhibited a lower relative leakage rate and pulp breakdown and higher DPPH radical scavenging activity than fruit stored in air.

The short–term exposure to MA has also been reported to be beneficial for inhibiting lipid peroxidation and enhancing the antioxidant potential of fruit. Postharvest exposure of kiwifruit cv. 'Xuxiang' to pure N_2 gas for 6 hours maintained a high level of firmness within 14 days of storage at 1±1°C and reduced the decrease in the firmness during shelf life (Song et al., 2009). A similar study was conducted on loquat, in which fruit exposed to 100% N_2 for 6 hours could retain their quality during 35 days storage at 5°C (Gao et al., 2009). In kiwifruit and loquat, the short–term anoxic treatment reduced the increases in membrane permeability and lipid peroxidation, delayed the increases in both O_2^- production rate and H_2O_2 content, increased activities of SOD and POD, but reduced LOX activity throughout storage period (Gao et al., 2009; Song et al., 2009). These studies show that the beneficial effects obtained from anoxic treatment could be due to reduced lipid peroxidation, enhanced antioxidant ability and membrane integrity maintenance.

Loquat (*Eriobotrya japonica* Lindl.) fruit held in atmospheres containing 10% O_2 + 1% CO_2 at 1°C for 50 days or more retained their fruit quality better than MAP and regular air (Ding et al., 2006). CA storage was more effective than MAP in reducing the adverse effects of oxidative stress as the activities of antioxidant enzymes such as SOD, CAT and POD remained significantly higher than the MAP and regular air.

The non–enzymatic and enzymatic antioxidants in mangoes have also been studied with respect to CA storage (Kim et al., 2007; Niranjana et al., 2009). According to Kim et al. (2007), gallic acid, total hydrolysable tannins, total soluble phenolics, and antioxidant capacity significantly decreased throughout mango fruit ripening from mature–green to full ripe stages, but CA storage can possibly delay the decrease through delayed fruit ripening (Kim et al., 2007). Higher concentration of phenolics compounds and activities of CAT and POD and lower levels of carotenoids were found in 'Alphonso' mangoes stored in CA containing 5% O_2 and 5% CO_2 compared to those kept in regular air after 45 days storage at 8°C , which could be attributed to the effect of CA on fruit ripening. CA storage was also effective in alleviating CI symptoms in mangoes (Niranjana et al., 2009).

Peach fruit cv. Okubao stored at 0°C for 60 days under CA of 5% O_2 + 5% CO_2, or CA with high O_2 concentration (70% O_2 + 0% CO_2 for 15 days, then in CA

with 5% O_2 + 5% CO_2) showed reduced CI compared to those held in regular air (Wang et al., 2005). CA storage delayed the reduction in activities of SOD, CAT and POD, which have been associated with the alleviation of CI in peaches. High O_2 treatment also induced the activities of SOD and CAT, but no significant effect on alleviating CI was found compared to CA storage. The extent of lipid peroxidation was greatly reduced under CA conditions, resulting in the maintenance of membrane integrity (Wang et al., 2005). This study demonstrates that storage of peaches under ideal atmospheres can alleviate the adverse effects of oxidative stress.

Post–hypoxic and/or post–anoxic conditions are among the stresses in which ROS are implicated as the principle cause of injury (Ioannidi et al., 2009). When aerobic conditions are re–established, a burst of ROS takes place, resulting in post–hypoxic or post–anoxic injury to the tissues. A study was conducted on the monitoring of gene expression of enzymes involved in AA biosynthesis, oxidation, and recycling in tomato, in response to hypoxia, and post–anoxic stress (Ioannidi et al., 2009). To create hypoxic conditions, mature green tomato fruit were subjected to atmospheres containing 0%, 0.5% and 3% O_2 (balance N_2) for 1, 3, 6, 12, 24, 48, and 72 hours at 22°C. For the post–hypoxic stress, mature green fruit were subjected to 100% N_2 for 48 h and then were exposed to air. Post–anoxic stress caused mature green tomatoes to respond by inducing the transcript accumulation of all AA biosynthetic genes as early as 3–6 h after return to air, coinciding with elevated levels of AA. Similarly, enzymes involved in AA recycling responded to the post–anoxic stress by increasing their mRNA steady–state levels upon return to air. This was an indication of the magnitude of the oxidative damage and the crucial role of AA in scavenging ROS under these conditions. The induction of AA recycling genes suggest that this massive activation of transcription is needed to increase the reduced AA pool in order to compensate for the oxidative stress.

A study on the effects of anaerobic stress (exposure to N_2 atmospheres for 24 h) on the proteome of mandarins and grapefruit revealed that the majority of the identified citrus anaerobic proteins (ANPs) in both mandarins (8 out of 10 proteins) and grapefruit (5 out of 9 proteins) were stress–related proteins, such as SOD, APX and LOX (Shi et al., 2008). The activation of SOD and APX in response to anaerobiosis might be crucial to preserve the redox status of the cells. Furthermore, LOX is one of the main enzymes known to be involved in generation of ROS and, thus, the observed suppression of the LOX protein in mandarin flavedo may be part

of the cellular efforts to cope with the undesired accumulation of ROS. The increase in the abundance of two oxidoreductases– zinc oxidoreductase and quinine oxidoreductase in flavedo may contribute to the elevated anaerobic stress tolerance of 'StarRuby' grapefruit (Shi et al., 2008).

It is therefore evident that storage of fruit under optimal CA conditions can potentially maintain or slightly increase the antioxidant potential of the fruit. The short–term hypoxia or anoxia can also stimulate the antioxidant defence system in the fruit. But, there is absolute lack of information on the transcript levels of various antioxidant enzymes involved in ROS scavenging and AA–GSH cycle in fruit subjected to CA/MA. Most of the studies have been restricted to measuring the activities of SOD, CAT and POD and lipid peroxidation. The emerging tools of proteomics and metabolomics have provided a great insight into the development of browning disorders in pears in relation to CA storage (Pedreschi et al., 2007, 2008, 2009) and development of anaerobic stress in mandarin and grapefruit (Shi et al., 2008). The application of these tools has also been extended to study the CI in peaches (Dagar et al., 2010). There is a vast scope for research in this area to explicate the processes involved in developing oxidative stress leading to physiological disorders in various fruits.

2.5.5 Postharvest treatments

Postharvest oxidative stress has been associated with the enhanced rates of ripening and senescence, development of physiological disorders and reduction in nutritional quality of fruits (Hodges et al., 2004). Therefore, the regulation of oxidative stress can be very important in improving shelf life and quality maintenance during postharvest handling, storage and distribution of fruits (Toivonen, 2003b).

According to Toivonen (2003b), postharvest treatments to control oxidative stress–related injury in fruits involve two approaches: (a) the use of antioxidant dips and coatings to directly prevent oxidation reactions, and (b) postharvest treatments, such as low or high temperature, exposure to CA/MA and growth regulators, to enhance endogenous resistance or tolerance to oxidative stress. The postharvest treatments such as low temperature conditioning (cold shock treatments), heat treatments, and antioxidant dips etc. also play an important role in strengthening the antioxidant system of fruit (Toivonen, 2003b); but these treatments will not be reviewed as these are not pertinent to my research project. The role of storage

temperature, storage duration and CA/MA in alleviating oxidative stress in fruits has been reviewed in previous sections. In this section, the role of postharvest treatments with ethylene action inhibitor, 1–MCP, and putative ethylene biosynthesis inhibitor, nitric oxide, in relation to oxidative stress in fruits will be reviewed.

2.5.5.1 1–MCP

Ethylene is a plant hormone which regulates growth and developmental processes in plants including fruit ripening and senescence (Leliévre et al., 1997). The role of ethylene in promotion of fruit ripening and senescence is well–known. Therefore, modulation of ethylene production and its action has been used as a postharvest tool to extend the shelf life of fresh produce. Among different strategies to control ethylene, the postharvest exposure of fresh produce to 1–methylcyclopropene (1–MCP) has emerged as the greatest tool of commercial importance all over the world (Blankenship and Dole, 2003; Watkins, 2006, 2008). 1–MCP is an ethylene action inhibitor, which can irreversibly bind to ethylene receptors (Sisler and Serek, 1997) and thereby preventing ethylene–dependent responses. 1–MCP has been used as a supplement to molecular approaches for identifying and understanding the spectrum of senescence and ripening processes under the direct control of ethylene perception (Huber, 2008). The major beneficial effects of 1–MCP in fresh horticultural produce included suppression of ethylene production and respiration rates resulting in delayed fruit ripening and senescence, retardation of changes in fruit softening and skin colour, and alleviation of certain physiological disorders such as chilling injury and superficial scald. The details of benefits of 1–MCP in a range of fruit, vegetables and ornamentals have been reviewed elsewhere (Blankenship and Dole, 2003; Watkins, 2006, 2008).

Most of 1–MCP–based studies have been focused on the inhibition of fruit ripening and senescence, and alleviation of storage disorders. Recently, there is more interest in studying the effects of 1–MCP on the antioxidants in the fruit. 1–MCP treatment has the potential to improve the resistance of fruit to oxidative processes. Studies have shown that 1–MCP treatment can reduce the lipid peroxidation in different fruits during postharvest storage and shelf life (Table 2.7). The inhibition of accumulation of lipid peroxidation products in the skin and flesh tissues of various fruits such as apples, pears, loquats, mangoes and papayas (Ali et al., 2008; Cao et al., 2009b; Singh and Dwivedi, 2008; Vilaplana et al., 2006) indicates a positive role

for 1–MCP in alleviation of oxidative stress and maintenance of membrane integrity. The maintenance of membrane structural integrity has been demonstrated by the reduced ion leakage from the skin or flesh tissue of 1–MCP–treated fruits such as apricot, litchi, loquat, and pear (Cao et al., 2009b; Egea et al., 2010; Larrigaudiére et al., 2004; Sivakumar and Korsten, 2010).

1–MCP treatment has been shown to affect the activities of primary antioxidant enzymes, SOD, CAT and POD. A significant increase in the activities of SOD, CAT, POD and APX has been observed in flesh tissues of fruits, such as apricot, loquat, mango and pear, in response to 1–MCP treatment (Cao et al., 2009b; Egea et al., 2010; Fu et al., 2007; Martino et al., 2006; Singh and Dwivedi, 2008) which might have helped to regulate the concentration of ROS in the fruit tissue, resulting in lesser oxidative damage. Some researchers have shown that the concentrations of ROS in 1–MCP–treated fruit were significantly lower than in the untreated fruit (Cao et al., 2009b; Larrigaudière et al., 2008; Larrigaudiére et al., 2004; Singh and Dwivedi, 2008; Vilaplana et al., 2006; Wang et al., 2009).

There are contradictory reports on the effects of 1–MCP on the non–enzymatic antioxidants and antioxidant capacity of fruits. 1–MCP treatment has been shown to reduce the levels of AA in the flesh tissues of apples and pears (Larrigaudiére et al., 2004; Vilaplana et al., 2006). Another study showed that there was no consistent effect on the concentrations of AA and GSH in 1–MCP treated pears (Silva et al., 2010). The antioxidant capacity in apples has been reported to increase in the skin tissue (Larrigaudiére et al., 2004; MacLean et al., 2003; MacLean et al., 2006; Shaham et al., 2003; Vilaplana et al., 2006) and remain unaffected in the flesh tissue (Vilaplana et al., 2006). The beneficial effects of 1–MCP in retention of AA in the fruit have been mainly attributed to the retardation of fruit ripening and senescence (Egea et al., 2010; Singh and Pal, 2008; Sivakumar and Korsten, 2010; Wang et al., 2010). The stimulation of phenylpropanoid pathway by 1–MCP treatment has been associated with increase or maintenance of phenolics and flavonoids in the skin or flesh tissue of fruits (Egea et al., 2010; Fawbush et al., 2009; Sivakumar and Korsten, 2010).

The effects of 1–MCP are influenced by the fruit, cultivar, 1–MCP dose, exposure duration, and postharvest conditions (Blankenship and Dole, 2003; Watkins, 2006; Watkins, 2008). However, the response of antioxidant system to 1–MCP treatment has been tissue–specific in fruits. For instance, 1–MCP treatment of

'Golden Smoothee' apples increased the susceptibility of skin tissue to browning by increased activity of PPO and POD (Larrigaudière et al., 2008), while it was beneficial for the flesh tissue due to decreased lipid peroxidation and lower accumulation of H_2O_2 (Vilaplana et al., 2006). Total phenolics in 'Empire' apples showed a significant increase in the skin, but a decrease in the flesh tissue during cold storage for 5 months (Fawbush et al., 2009). The control of superficial scald in 'Granny Smith' apples has been attributed to the increase in lipid soluble antioxidants in the skin of 1–MCP–treated fruit, while activities of CAT and APX were suppressed by 1–MCP treatment (Shaham et al., 2003).

The exposure of fruit to higher doses of 1–MCP has some detrimental effects on the antioxidant system of fruit. In strawberries, exposure to 1 μL L^{-1} 1–MCP increased the disease susceptibility through reduced activity of PAL and lower concentration of phenolics (Jiang et al., 2001). Similarly, in limes, the higher dose of 1 μL L^{-1} 1–MCP increased the activity of POD, enhanced the skin yellowing as compared to lower doses which had inhibitory effect on the loss of chlorophyll (Win et al., 2006). The levels of AA, total carotenoids and total antioxidant capacity were also reduced by higher doses (1.0 and 2.0 μL L^{-1}) of 1–MCP treatment during 6 weeks cold storage of 'Tegan Blue' plums (Khan and Singh, 2009).

The impact of postharvest conditions on the efficacy of 1–MCP in influencing the antioxidant potential of fruit has been demonstrated in 'Sunrise' apples (Qiu et al., 2009). The study showed that the phenolics fraction of the flesh tissue digestate showed higher Folin Ciocalteu reaction reducing capacity in response to 1–MCP treatment only if the fruit were stored at ≥15°C for three weeks. The effects of 1–MCP treatment on the retention of non–enzymatic antioxidants during storage of 'Empire' apples were also influenced by the storage atmospheres, but most of the results were inconsistent (Fawbush et al., 2009).

The postharvest application of 1–MCP in Japanese plums is a commercial practice in many countries (Watkins, 2008). A vast amount of information is available on the effects of 1–MCP on the postharvest fruit quality and storage aspects of Japanese plums (Khan and Singh, 2007a, 2008; Martínez–Romero et al., 2003b; Menniti et al., 2006; Menniti et al., 2004; Valero et al., 2003), but is limited about the antioxidant components of the fruit (Khan and Singh, 2008; Larrigaudière et al., 2008). Larrigaudiére et al. (2008) studied the effects of 1–MCP on the activities of SOD, CAT and POD during cold storage of 'Larry Ann' plums for 2 weeks only and

the results were inconsistent and inconclusive. 1–MCP treatment has been shown to have slightly positive effect on AA and total antioxidant capacity of 'Tegan Blue' plums during 5 or 7 weeks of cold storage (Khan and Singh, 2008). But in a subsequent report, the levels of AA, carotenoids and total antioxidant capacity in the flesh tissue of 1–MCP–treated plums of the same cultivar have been reported to be lower than the untreated fruit after the same duration of cold storage (Khan et al., 2009). Further research is required to comprehensively investigate the effects of 1–MCP on the enzymatic and non–enzymatic antioxidants in the fruit in relation to development of storage disorders.

Table 2.7. Responses of antioxidant system of fruits to postharvest treatment with 1–MCP.

Fruit	**Cultivar/1–MCP dose**	**Storage Conditions**	**Antioxidant response (↑:Increase; ↓: Decrease; ↔: Unaltered)**	**Remarks**	**Reference**
Apples	'Empire' and 'Delicious' 0.6 μL L^{-1}	0–1°C, 90–95% RH for 120 days	*Skin*: total oxyradical scavenging activity↑	First study to show the effect of 1–MCP on antioxidants	(MacLean et al., 2003)
	'Granny Smith' 1 μL L^{-1}	0°C for 16 weeks	*Skin*: APX↓, CAT↓, POD↔, lipid soluble antioxidants↑	Complete inhibition of superficial scald	Shaham et al., 2003)
	'Golden Smoothee' 0.625 μL L^{-1}	0.5°C for 3 months in air	*Flesh*: SOD↔, CAT↓, POD↑, H_2O_2↓, AA↓, DPPH scavenging activity↔, Lipid peroxidation↓	1–MCP inhibited lipid peroxidation and enhanced POD activity	(Vilaplana et al., 2006)
	'Golden Smoothee' 0.625 μL L^{-1}	1°C, 90% RH, 90 days	*Skin*: H_2O_2↔, SOD↔, CAT↑, POD↑, PPO↑	1–MCP increased skin browning potential	(Larrigaudière et al., 2008)
	'Empire' 1 μL L^{-1}	0.5°C for 5 months in air or 9 in CA (2/3% O_2 + 2% CO_2)	*Skin*: total phenolics↑, antioxidant activity↑, anthocyanins↔ *Flesh*: total phenolics↓, antioxidant activity↑, flavonoids↑	A consistent pattern of changes in antioxidants was not reported.	(Fawbush et al., 2009)

Table 2.7. Continued

Fruit	**Cultivar/1–MCP dose**	**Storage Conditions**	**Antioxidant response (↑:Increase; ↓: Decrease; ↔: Unaltered)**	**Remarks**	**Reference**
Apples	'Sunrise' 0.25 µL L^{-1}	5, 13, 15, 18 and 22 °C for 3 weeks	*Phenolic fraction of flesh digestate*: Folin–Ciocalteu reaction reducing capacity↑ (only at ≥15°C),	Non–phenolics fraction contributed more to antioxidant capacities in *in vitro* digestates	(Qiu et al., 2009)
Apricot	'Marietta' 0.5 µL L^{-1}	20°C, 85% RH for 6 days	*Flesh*: SOD↑, POD↑	Increased tolerance to impact damage in fruit	(Martino et al., 2006)
	'Búlida', 1.0 µL L^{-1}	2°C, 90% RH for 21 days	*Flesh*: Ion leakage↓, SOD↑, POD↑, AA↑, β–carotene↑, antioxidant activity↑	Alleviated oxidative stress	(Egea et al., 2010)
Lime	'Paan' 0.25, 0.75, 0.5 and 1.0 µL L^{-1}	24–31°C, 73–81% RH for 21 days	*Skin*: 0.25–0.75 µL L^{-1} : POD↓, AA↔ 1.0 µL L^{-1} : POD↑, AA↓	1–MCP treatment (0.25–0.75 µL L^{-1}) delayed fruit yellowing	(Win et al., 2006)
Litchi	'MacLean's Red' 0.5 µL L^{-1}	2°C, 90% RH, 17% O_2 + 6% CO_2 for 21 days	*Skin*: Ion leakage↓, PPO↓, POD↓, anthocyanins↑ *Flesh*: AA↑	Inhibition of pericarp browning	(Sivakumar and Korsten, 2010)
Loquat	'Fuyang' 0.05 µL L^{-1}	1°C, 95% RH for 35 days	*Flesh*: Ion leakage↓, TBARS↓, $O_2^{\cdot-}$↓, H_2O_2↓, LOX↓, SOD↑, CAT↑, APX↔,	Alleviation of chilling injury	(Cao et al., 2009b)

Table 2.7. Continued

Fruit	**Cultivar/ 1–MCP dose**	**Storage Conditions**	**Antioxidant response (↑:Increase; ↓: Decrease; ↔: Unaltered)**	**Remarks**	**Reference**
Loquat	'Fuyang' 0.05 μL L^{-1}	1°C, 95% RH for 35 days	*Flesh*: Ion leakage↓, TBARS↓, $O_2^{\cdot-}$↓, H_2O_2↓, LOX↓, SOD↑, CAT↑, APX↔,	Alleviation of chilling injury	(Cao et al., 2009b)
Mango	'Dashehari' (2 mg kg^{-1})	(Temp. not specified) for 11 days	*Flesh*: H_2O_2↓, TBARS↓, SOD↑, CAT↑, APX↑	Delayed fruit ripening	(Singh and Dwivedi, 2008)
	'Tainong' 1.0 μL L^{-1}	20 ± 1°C, 85–90% RH for 12 days	*Flesh*: $O_2^{\cdot-}$↓, H_2O_2↓, SOD↓, CAT↓, APX↓, AA↑	Delayed fruit softening	(Wang et al., 2009)
Papaya	'Sekaki' 0.27 μL L^{-1}	10°C for 4 weeks under MAP	*Flesh*: TBARS↓, SOD↑, CAT↔, APX↔	Inhibition of fruit softening	(Ali et al., 2008)
Peach	'Jiubao' 0.5 μL L^{-1}	22°C for 12 days	*Flesh*: PAL↑, POD↑, PPO↔	Enhanced disease resistance	(Liu et al., 2005)
Pears	'Blanquilla' 0.1 μL L^{-1}	0.5°C, 90% RH for 5 months	*Flesh*: Ion leakage↓, H_2O_2↓, SOD↑, CAT↑, POD↑, APX↑, AA↓	Retained fruit quality	(Larrigaudiére et al., 2004)
	'Yali' 0.2 μL L^{-1}	20°C, 85–95% RH for 32 days	*Flesh*: SOD↑, CAT↑, POD↑	Reduced core browning	(Fu et al., 2007)

Table 2.7. Continued

Fruit	**Cultivar/1–MCP dose**	**Storage Conditions**	**Antioxidant response (↑:Increase; ↓: Decrease; ↔: Unaltered)**	**Remarks**	**Reference**
Pear	'Rocha' 0.5 µL L^{-1}	0.5°C, 90–95% RH, 2.5% O_2 + 0.7% CO_2 for 8 months	*Skin & flesh*: Free radical scavenging activity↔**,** AA↔, GSH↔	Inhibition of superficial scald	(Silva et al., 2010)
Plum	'Larry Ann' 0.625 µL L^{-1}	0°C for 14 days	*Flesh*: H_2O_2↔, SOD↔, CAT↔, POD↓	Retained fruit quality	(Larrigaudiére et al., 2009)
	'Tegan Blue' 0.5, 1.0, and 2.0 µL L^{-1}	0°C for 6 weeks	*Flesh*: AA↓, total carotenoids↓, antioxidant capacity↓	Antioxidants levels decreased with the increase in 1–MCP dose	(Khan et al., 2009)
Strawberry	'Everest' 0.01, 0.1, and 1.0 µL L^{-1}	20°C, 95–100% RH for 3 days	PAL↓, anthocyanins↓, phenolics↓	Enhanced susceptibility to disease	(Jiang et al., 2001)
Tomato	1.0 µL L^{-1}	20°C, 85–95% RH for 20 days	*Flesh*: lycopene↓, AA↑, phenolics↑, DPPH and FRAP activity↑	Improved nutritional quality	(Wang et al., 2010)

2.5.5.2 Nitric oxide

NO is an important signalling molecule which plays an important role in ROS metabolism during normal and stress conditions in plants (Lamattina et al., 2003). NO is a free radical molecule with very high reactivity. The presence of an unpaired electron in the NO molecule makes it a reactive species allowing a high reactivity with oxygen (O_2), superoxide ($O_2^{\bullet -}$), N derivatives, and transition metals, which accounts for direct sources of both cytoprotection and cytotoxicity in the biological system. The cytoprotection is provided by NO through its ability to regulate the levels and toxicity of ROS. Oxidative stress, which is provoked by increased concentrations of $O_2^{\bullet -}$, H_2O_2, and alkyl peroxides, can therefore be alleviated through protective effects of NO (Beligni et al., 2002).

Additionally, NO possesses antioxidant properties (Beligni et al., 2002; Hung and Cao, 2004). The reaction of NO with lipid alcoxyl (LO·) and peroxyl (LOO·) radicals is rapid and beneficial to prevent the propagation of ROS mediated lipid peroxidation. The transition metal scavenging ability of NO makes it a beneficial molecule to interfere with the Fenton–type reaction which produces hydroxyl radicals. Consequently, the presence of NO can attenuate the Fenton oxidative damage preventing the formation of strong oxidants (Lamattina et al., 2003). The interaction between NO and other cellular antioxidants provides indirect protection from the ROS damage.

The toxic effects of NO are generated when it reacts with $O_2^{\bullet -}$ to form peroxynitrites ($ONOO^-$) (Lamattina et al., 2003). Peroxynitrites can further react with thiol groups of proteins and polyunsaturated radicals of fatty acid lipids of membrane, causing serious damage to proteins, lipids and DNA, and is also capable of generating the most damaging hydroxyl radicals through its protonation. Therefore, NO can be cytotoxic and cytoprotective depending on the local concentration of NO as an effect of the rate of synthesis, translocation, effectiveness of removal of this reactive nitrogen species, as well as its ability to directly interact with other molecules and signals (Arasimowicz and Floryszak–Wieczorek, 2007; Beligni and Lamattina, 1999).

NO has been suggested to function in mediating responses to biotic and abiotic stresses in the plants, such as cold (Zhao et al., 2009a), drought (Mata and Lamattina, 2001), salt (Shi et al., 2007), UV–B radiation (Shi et al., 2005), disease resistance and apoptosis (Wendehenne et al., 2004). The NO–dependent signalling

during stresses is often closely related to the plant hormones such as abscisic acid and ethylene, and other signalling molecules such as H_2O_2 and Ca^{2+} (Arasimowicz and Floryszak–Wieczorek, 2007). The tolerance to various types of environmental stresses in plants has been primarily attributed to the role of NO in preventing the oxidative injury during the stress conditions (Beligni et al., 2002; Shi et al., 2007; Shi et al., 2005; Zhao et al., 2007; Zhao et al., 2009a).

Similarly, the role of NO in delaying fruit ripening and senescence has been established. NO is thought to be involved in interfering with ethylene biosynthetic pathway in fruit (Eum et al., 2009; Leshem and Wills, 1998; Zhu et al., 2006; Zhu et al., 2008; Zhu and Zhou, 2007) which helps to inhibit ethylene–dependent responses in the fruit. In the past few years, the role of NO in alleviation of postharvest oxidative stress has been reported in kiwifruit, longan, peach and tomato (Duan et al., 2007a; Zhu et al., 2006; Zhu et al., 2008). The antioxidant responses of different fruits to NO treatment have been summarized in Table 2.8. The concentrations of NO that were higher than optimum increased the oxidative stress in the fruit tissue as shown by increased lipid peroxidation and accumulation of ROS (Zhu et al., 2008). Therefore, the cytoprotective effects of NO can only be obtained at certain concentrations of NO, which are required to be optimized for different fruits.

In general, the postharvest treatment of fruit with either donors compounds of NO or NO gas resulted in decreased levels of lipid peroxidation, reduced accumulation of $O_2^{\bullet-}$ and H_2O_2, and increased activities of antioxidant enzymes favourable for delay in initiation and reduction of oxidative stress (Duan et al., 2007a; Duan et al., 2007b; Zhu et al., 2009; Zhu et al., 2006; Zhu et al., 2008). Currently, there is no information on the effects of NO fumigation on the enzymatic and non–enzymatic antioxidant systems of Japanese plums. The published reports on other fruits have mainly focused on lipid peroxidation and primary antioxidant enzymes, while a detailed account of the antioxidant system is still lacking and warrants further investigations.

Table 2.8. Responses of the antioxidant systems of fruit to postharvest treatment with nitric oxide (NO)

Fruit	**Cultivar/ NO Concentration**	**Storage Conditions**	**Antioxidant response (↑:Increase; ↓: Decrease; ↔:Unaltered)**	**Remarks**	**Reference(s)**
Kiwifruit	'Xuxiang' 1 μmolL^{-1} SNP	4°C, 90% RH for 80 days	*Flesh*: $O_2^{-\cdot}$↓, H_2O_2↓, LOX↓, TBARS↓, SOD↑, CAT↑, POD↓, AA↑, vitamin E↑	2 μmol L^{-1} SNP caused oxidative damage to fruit	(Zhu et al., 2008)
Longan	'Shixia' 1.0 mM SNP	28°C for 6 days under MAP using 0.03mm PE film	*Skin*: Ion leakage↓, LOX↓, TBARS↓, PPO↓, PAL↓, SOD↓, CAT↑, POD↓, APX↑, phenols↑, DPPH scavenging activity↑	Reduced pericarp browning and pulp breakdown	(Duan et al., 2007a; Duan et al., 2007b)
Peach	'Rojo Rito' 5 μL L^{-1} NO gas	20°C for 14 days	Ion leakage↓, LOX↔, TABRS↔, PPO↓, SOD↑, CAT↑, POD↓, AA↔, DHA↔, total carotenoids↔	Delayed fruit senescence	(Flores et al., 2008)
Peach (Fresh–cut)	'Zhonghuashoutao' 0.5 μM NO	10°C, 95% RH for 10 days	Ion leakage↓, PPO↓, POD↓, phenols↑, PAL↑,	Reduced surface browning	(Li–Qin et al., 2009)

Table 2.8. Continued

Fruit	**Cultivar, NO Concentration and donor**	**Storage Conditions**	**Antioxidant response (↑:Increase; ↓: Decrease; ↔: Unaltered)**	**Remarks**	**Reference**
Tomato	'Lichun' 0.2 mmol L^{-1} SNP	25±1°C, 80–85% RH for 8 days	*Pericarp*: H_2O_2↓, SOD↑, CAT↑, APX↑, PAL↑, chitinase↑, GT↑	Enhanced disease resistance	(Fan et al., 2008)

Chapter 3

General Materials and Methods

3.1 Fruit material

Japanese plum cultivars, 'Blackamber', 'Amber Jewel' and 'Angeleno', were harvested in the early morning hours (6 am to 9 am) at commercial maturity, unless otherwise specified, from either the Casuarina Valley Orchard or Red Valley Orchard, Karagullen, Perth Hills (lat. 31° 57'S; long. 115° 50'E), Western Australia (WA). At Casuarina Valley Orchard, 'Blackamber' and 'Amber Jewel' plum trees were planted in 1996 in the north–south row direction (4.25 m between rows and 2.5 m within rows), while 'Angeleno' trees were planted in 1995 in south–east direction with the same planting distances. At Red Valley Orchard, 'Amber Jewel' plantation was done in 1988 in the north–south direction (4.25 m between rows and 1.8 m within rows). At both the properties, all cultivars were trained on a palmate system. All cultivars were grafted on myrobalan (*Prunus cerasifera* Ehrh.) rootstock and were under integrated orchard management practices. Fruit maturity for each cultivar was determined on the basis of soluble solids concentration (SSC) and fruit firmness as recommended by the Department of Agriculture and Food WA (DAFWA). Fruit were randomly harvested from all parts of the tree canopy up to the height of about 2 m. Fruit of uniform size and maturity, free from visual blemishes and disease were selected for the experiments. After picking, fruit were placed into standard plastic crates (~15 kg) lined with polyethylene liners and were transported in an air–conditioned station wagon to the Curtin Horticultural Research Laboratory located at Bentley Campus of Curtin University of Technology, Perth, WA.

3.2 Storage and ripening environment monitoring

The temperature and relative humidity data during all experiments were recorded at 15 min intervals using TinyTag data loggers (Gemini Data Loggers Ltd., West Sussex, UK). The air samples from cold storage and laboratory were regularly monitored for the presence of ethylene using gas chromatography as discussed in the next section. The controlled atmospheres were also regularly monitored using an

infrared gas analyser with automatic sampling (ADC 7000; Analytical Development Co., Ltd., Hoddesdon, UK) for the concentrations of O_2 and CO_2 to estimate any deviation from the set concentrations.

3.3 Fruit physiology

3.3.1 Determination of ethylene (C_2H_4) production rate

Two fruit per experimental unit were enclosed in an airtight glass jar (1050 mL) fitted with a septum (SubaSeal®, Sigma–Aldrich Co., St. Louis, USA) for 1 h at room temperature (21±1°C). One millilitre of headspace gas sample from the glass jar containing fruit was injected into a gas chromatograph (6890N Network GC system; Agilent Technologies, Palo Alto, CA, USA) fitted with a 2–m–long stainless steel column (Porapak–Q, 3.18 mm, mesh size 80/100; Supelco, Bellefonte, PA, USA) and a flame ionization detector (FID). Nitrogen (N_2) was used as carrier gas with a flow rate 20 mL min^{-1}. The column, injector, and detector were maintained at 110, 150, and 250°C, respectively. A blank injection from the headspace of an empty jar was also run under similar conditions to check the possibility of ethylene emission from the septum. Ethylene was identified by comparing the retention time with its authentic standard (0.98 and 8.0 µL L^{-1} of ethylene in N_2; BOC Gases, Sydney, NSW, Australia). The amount of ethylene production was calculated on the basis of peak areas of these standards. The ethylene production rate was calculated using the following formula:

$$\text{Ethylene production rate } (\mu L\ kg^{-1}\ h^{-1}) = \frac{C_2H_4 \text{ concentration } (\mu L\ L^{-1}) \times \text{Void volume of container (L)}}{\text{Fruit weight (kg)} \times \text{Incubation period (h)}}$$

The units of ethylene production rate (µL kg^{-1} h^{-1}) were converted into µmol kg^{-1} h^{-1} following the ideal gas law, PV = *n*RT, where V represent volume (mL), P represents pressure (kPa), n represents the number of moles, R = 8.134 (Ideal gas constant) and T is temperature (k).

3.3.2 Determination of respiration rate

Respiration rate of plum fruit was determined on the basis of amount of CO_2 evolved. Two millilitres of gas sample was taken from the headspace of the same jar, which was used for ethylene measurements, and injected into an infra–red gas analyser (Servomex, Gas Analyser, Analyser Series 1450; Servomex Ltd., East Sussex, UK). The respiration rate of each sample was calculated based on the peak area of a 2 ml CO_2 standard (8.52% CO_2 in N_2; BOC Gases, Sydney, NSW, Australia). The respiration rate was calculated as mL CO_2 kg^{-1} h^{-1} by using the following formula:

$$\text{Respiration rate (mL kg}^{-1}\text{ h}^{-1}\text{)} = \frac{\text{Changes in CO}_2\text{ conc. (\%) x Void volume of container (mL)}}{\text{100 x Fruit weight (kg) x Incubation period (h)}}$$

The respiration rate (mL CO_2 kg^{-1} h^{-1}) was converted into mmol CO_2 kg^{-1} h^{-1} using the ideal gas law as described in Section 3.3.1.

3.4 Fruit quality assessment

3.4.1 Flesh firmness

3.4.1.1 Determination of firmness using electronic pressure tester

During 2006–07, flesh firmness was determined using an electronic pressure tester (Lake City Technical Products, Kelowna, BC, Canada) fitted with an 8–mm spherical probe. A small slice (~2 mm thick) of fruit skin was removed and the firmness was recorded on the opposite sides of equatorial region of individual fruit and mean values were expressed as Newtons (N).

3.4.1.2 Determination of firmness using texture analyser

During 2007–09, flesh firmness was determined using a texture analyser (TA Plus, AMETEK Lloyd Instruments Ltd, Hampshire, UK) interfaced to a personal computer with Nexygen® software. A 5/16" Magness–Taylor probe, with a 500 N load cell on, punctured the peeled fruit at a crosshead speed of 100 mm min^{-1} to 7.5 mm depth. Each fruit was punctured on both the sides at equatorial region. The firmness was expressed as Newton (N).

3.4.2 Fruit colour

The changes in fruit colour were measured in Commission Internationale de L'Eclairage units as L*, a*, and b* colour coordinates using a HunterLab ColorFlex 45°/0° spectrophotometer (Hunter Associates Inc., Reston, VA, USA) with the 15–mm diameter port. L* represents lightness: 0 refers to absolute black and 100 is the maximum lightness (i.e. absolute white). The negative a* value indicates green and the positive a* refers to red. The positive and negative b* represent yellow and blue, respectively. Chroma (c) represents the saturation and was calculated using the following formula: $c= [(a^{*2} + b^{*2})]^{½}$ (McGuire, 1992). C value ranges from 0, which is completely unsaturated (i.e. a neutral grey, black or white), to 100 for maximum saturation. The hue angle was calculated as $\tan^{-1}$ b*/a* (McGuire, 1992). Hue angle refers to the line from the origin to the intercept of a* (x–axis) and b* (y–axis) coordinates, where 0° = red, 90° = yellow, 180° = green, and 270° = blue.

To measure skin colour, four readings were taken along the equatorial region of fruit. For flesh colour measurements, fruit were cut around the equatorial axis, and readings were taken from four positions on the mesocarp tissue of each half of the fruit.

3.4.3 Soluble solids concentration (SSC), titratable acidity (TA), and SSC:TA ratio

The juice was extracted from peeled fruit using a juice extractor. To determine the SSC of fruit juice, a digital refractometer (Atago– Palette PR 101; Atago Co., Tokyo, Japan) was used and SSC was expressed as °Brix. To determine the TA, juice was titrated against 0.1 N NaOH solution using phenolphthalein as an indicator to pH 8.2, and was expressed as % malic acid. SSC : TA ratio was calculated by dividing SSC with the corresponding TA value.

3.4.4 Chilling injury (CI)

3.4.4.1 CI incidence

Fruit were cut around the equatorial axis, the two halves of each fruit twisted in opposite directions, and the mesocarp was examined for CI symptoms such as flesh

browning, translucency, mealiness and bleeding. These symptoms have been described in detail in Section 2.3.7. Irrespective of the amount of area affected and the type of symptoms, the number of affected fruit was counted, and expressed as a percentage of the total number of fruit examined per treatment.

3.4.4.2 CI index

The severity of CI was determined by calculating the CI index as described by Singh et al. (2009). The area of the fruit flesh affected by CI symptoms was compared to the total flesh area. The CI index was determined using a five point hedonic scale based on the surface area of fruit flesh affected The scale used was: 0, 0% area affected; 1, 1%–20% area affected; 2, 21%–40% area affected; 3, 41%–60% area affected; 4, 61%–80% area affected; 5, >80% area affected. The scale was used to classify fruit into five categories according to the severity of CI: absent (0), very low (1), low (2), moderate (3), high (4), and very high (5). CI index was calculated by multiplying the number of fruit scored with the same value of the hedonic scale with the corresponding scale number. Finally, the resultant number was divided by the total number of fruit.

3.5 Lipid peroxidation analysis

3.5.1 Extraction and assay of lipoxygenase (LOX: EC 1.13.11.12) enzyme

LOX catalyzes the addition of oxygen to *cis*–1, 4 unsaturated fatty acids producing conjugated linoleate hydroperoxides. The activity of LOX was determined by measuring the increase in absorbance at 234 nm due to formation of a conjugate diene from linoleic acid as described by Maalekuu et al. (2006). The enzyme extract was prepared by homogenizing 10 g of flesh tissue with 30 mL ice cold 50 mM phosphate buffer (pH 7.0) followed by centrifugation (Eppendorf 5810R, Hamburg, Germany) at 15,000 *g* for 20 min at 4°C and supernatant collected for assay. Linoleic acid (70 mM) emulsified with 2% (w/v) Tween–20 was used as a substrate. The reaction was initiated with addition of 20 µL of 70 mM linoleic acid to 3.0 mL of 0.2 M phosphate buffer (pH 6.5) and 10 µL of enzyme extract. The rate of formation of conjugated dienes was measured as an increase in absorbance at 234 nm for 2 min. The auto

decomposition of substrate was corrected with a control assay to eliminate the non–enzymatic increase in absorbance. LOX activity was calculated using the extinction coefficient of 25, 000 M^{-1} cm^{-1} using the following formula:

$$\text{LOX activity} = \frac{\Delta A_{234}\,\text{min}^{-1} \times \text{Total volume in cuvette}}{\text{Extinction coefficient} \times \text{Volume of enzyme extract} \times \text{Protein conc.}}$$

LOX activity was expressed as μmol linoleate hydroperoxides formed min^{-1} mg^{-1} protein.

3.5.2 Determination of thiobarbituric acid–reactive substances (TBARS)

Malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids, reacts with two molecules of thiobarbituric acid (TBA) *via* an acid–catalyzed nucleophilic–addition reaction yielding a pinkish–red chromagen with an absorbance maximum at 532 nm (Hodges et al., 1999). Thiobarbituric acid–reactive substances (TBARS) including MDA are considered good indicators of lipid peroxidation in tissue. For extraction of TBARS, 1g of flesh tissue was homogenized in 10 mL of 80:20 (v/v) ethanol: water along with 0.5 g inert sand (–50 to +70 mesh particle size; Sigma–Aldrich Pty. Ltd., Castle Hill, NSW, Australia) using a mortar and pestle followed by centrifugation at 10,000 *g* for 15 min at 4°C. The supernatants were collected and diluted 10–fold. A 1 mL aliquot of the diluted sample was either added to 1 mL of 0.65% TBA solution containing 20.0% (w/v) trichloroacetic acid (TCA) and 0.01% butylated hydroxytoluene (BHT) or added to a solution containing 20.0% (w/v) TCA and 0.01% BHT. The samples were then mixed vigorously, incubated at 95°C for 25 min, cooled in an ice bath immediately, and centrifuged at 3,000 *g* for 10 min at 4°C. Absorbances at 532 nm, 440 nm, and 600 nm were recorded using a UV–Vis spectrophotometer. MDA equivalents were calculated using an extinction coefficient of 157 $mM^{-1}cm^{-1}$ as follow:

1. $[(\text{Abs } 532_{+TBA}) - (\text{Abs } 600_{+TBA}) - (\text{Abs } 532_{-TBA} - \text{Abs } 600_{-TBA})] = A$
2. $[(\text{Abs } 440_{+TBA} - \text{Abs } 600_{+TBA}) \, 0.0571 = B$
3. MDA equivalents (nmol mL^{-1}) $= \{A–B/ 157\,000\}/ 10^6$

3.5.3 Determination of electrolyte leakage (EL)

The electrolyte leakage (EL) as a measure of membrane integrity was determined by following the method of Lafuente et al. (1991) with some modifications. From each of six randomly selected fruit, six discs (10 mm in diameter and 4 mm thick) were removed from the mesocarp tissue, washed with deionised water, and then placed in 30 mL of 0.4 M mannitol in 50–mL plastic centrifuge tubes. The tubes were shaken at 120 cycles per min for 1.5 h at 21±1°C and initial electrical conductivity (EC) readings of the solutions were taken as a measure of EL from the discs, using a conductivity meter (HI 8733, Hanna Instruments Pty Ltd, Melbourne, Australia). Preliminary experiments showed that the EL increased up to 1.5 h. After 1.5 h, tubes were frozen at –20°C, thawed, and weighed. The content was boiled for 30 min in a 50–mL flask, allowed to cool to room temperature, and transferred back to plastic tubes. Deionised water was added to make their initial weight and the total conductivity was measured after an additional 0.5 h of shaking, and regarded as the final EC. The EL was calculated using the formula: (Initial EC/Final EC)*100. The data were expressed as percentage of the total EL.

3.6 Enzymatic antioxidants analysis

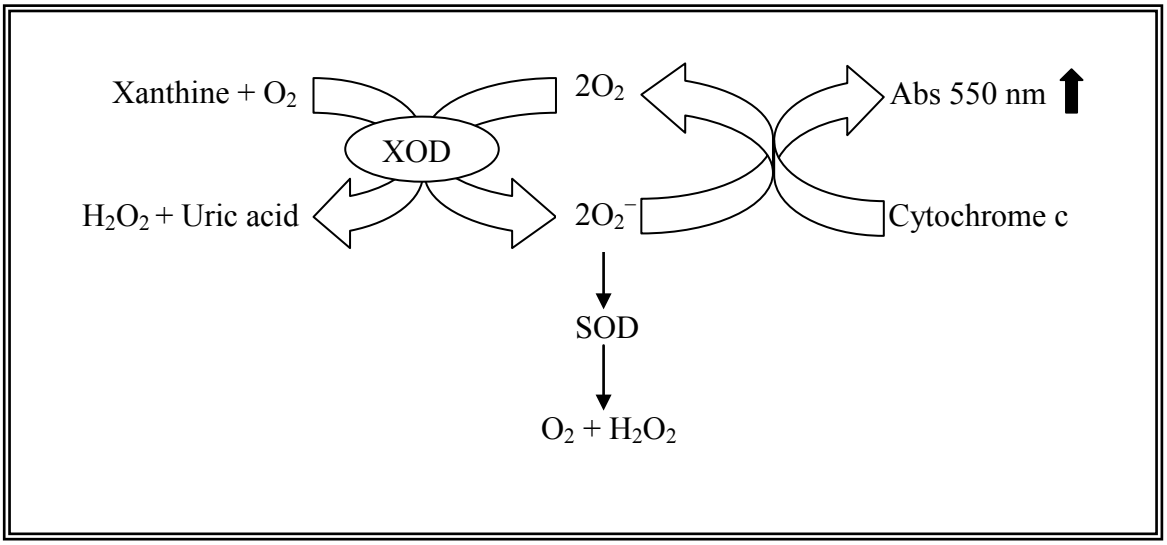
3.6.1 Extraction of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) enzymes

Enzyme extracts of SOD, CAT, and POD were prepared by homogenizing 5 g of flesh tissue in a prechilled mortar and pestle at 4°C along with 0.5 g PVPP, 0.5 g inert sand and 15 mL of chilled 0.1 M potassium phosphate buffer (pH 7.5) containing 0.5 mM EDTA, 0.5% (w/v) Triton X–100 and 1 mM dithiothreitol. The extracts were passed through two layers of cheesecloth and then centrifuged at 15,000 *g* for 30 min at 3°C. For desalting and removal of low–molecular weight compounds from the extract, an aliquot of 2.5 mL was loaded on a Sephadex^{TM} G–25 column (PD–10 , GE Healthcare Bio–Sciences Pty Ltd, NSW, Australia) pre–equilibrated with 10 mL of 0.1 M potassium phosphate buffer (pH 7.5). The elution of enzyme extract was carried out with 3.5 mL of the same buffer and used for determining the activities of SOD, CAT, and POD.

3.6.2 Enzyme assays of SOD, CAT, and POD

3.6.2.1 SOD (EC 1.15.1.1) assay

SOD activity was assayed following the method of McCord and Fridovich (1969) with slight modifications of Hodges and Forney (2000). The assay involved the measurement of cytochrome c (Cyt c) reduction by superoxide anions (O_2^-) generated in the xanthine–xanthine oxidase (XOD) system, and of the inhibition of Cyt *c* reduction by SOD as shown below in the box:



The reaction mixture consisted of 65 mM potassium phosphate (pH 7.5), 0.13 mM cyt c, 0.5 mM xanthine, 0.01 mM EDTA, 0.025 units xanthine oxidase (units as defined by Sigma–Aldrich Pty. Ltd., Castle Hill, NSW, Australia), and 40 µL of enzyme extract. The reaction was started by the addition of xanthine oxidase and the change in absorbance at 550 nm was recorded for 3 min. The rate of control reaction (without protein) was in the range of 0.015 to 0.025 *A*/min. SOD activity was calculated using the extinction coefficient of 28 mM^{-1} cm^{-1} and expressed as µmol Cyt c conserved min^{-1} mg^{-1} protein.

$$\text{SOD activity} = \frac{\Delta A_{550}\, min^{-1} \times \text{Total volume in cuvette}}{28\ mM^{-1}\ cm^{-1} \times \text{Volume of enzyme extract} \times \text{Protein conc.}}$$

3.6.2.2 CAT (EC 1.11.1.6) assay

CAT activity was assayed by measuring the rate of decomposition of H_2O_2 using the method of Aebi (1984). The reaction mixture consisted of 50 mM potassium

phosphate buffer (pH 7.0), 12 mM H_2O_2, and 50 µL of enzyme extract. The rate of disappearance of H_2O_2 was followed by observing the rate of decrease in the absorbance at 240 nm for 3 min. One unit of CAT was defined as the amount of enzyme which decomposes 1 µmol H_2O_2 min^{-1} at pH 7.0 at 25°C. An extinction coefficient of 43.6 M^{-1} cm^{-1} was used for calculating the CAT activity (µmol min^{-1} mg^{-1} protein).

$$\text{CAT activity} = \frac{\Delta A_{240}\, min^{-1} \times \text{Total volume in cuvette}}{43.6\ M^{-1}\ cm^{-1} \times \text{Volume of enzyme extract} \times \text{Protein conc.}}$$

3.6.2.3 POD (EC 1.11.1.7) assay

POD activity was determined by measuring the increase in absorbance at 470 nm due to formation of tetra–guaiacol, an oxidation product of guaiacol (Castillo et al., 1984). The reaction mixture contained 33.0 mM potassium phosphate buffer (pH 6.1), 16 mM guaiacol, 2 mM H_2O_2, and 200 µL of enzyme extract. The increase in absorbance at 470 nm was monitored for 3 min with and without addition of enzyme extract. One unit of POD was defined as the amount of enzyme which consumes 1 µmol of H_2O_2 per min at pH 6.1 at 25°C. POD activity (µmol H_2O_2 decomposed min^{-1} mg^{-1} protein) was calculated using the extinction coefficient of 26.6 mM^{-1} cm^{-1}.

$$\text{POD activity} = \frac{\Delta A_{470}\, min^{-1} \times \text{Total volume in cuvette}}{26.6\ mM^{-1}\ cm^{-1} \times \text{Volume of enzyme extract} \times \text{Protein conc.}}$$

3.6.3 Extraction of ascorbate–glutathione cycle enzymes

The extracts of ascorbate–glutathione cycle enzymes were prepared by homogenizing 10 g of flesh tissue in 20 mL of chilled extraction buffer containing 100 mM potassium phosphate (pH 6.5), 1 mM ascorbate (added fresh) and 1mM EDTA along with 0.5 g PVPP and 0.5 g inert sand in a pre–chilled mortar and pestle at 4°C. The homogenates were passed through two layers of cheesecloth and then centrifuged at 15,000 *g* for 25 min at 3°C. The supernatants were gel–filtered using PD–10 columns as described in Section 3.6.1 and the eluants were collected for determining the enzyme activities.

3.6.4 Assays of ascorbate–glutathione cycle enzymes

3.6.4.1 Ascorbate peroxidase (APX: EC 1.11.1.11) assay

APX activity was determined by following the method of Nakano and Asada (1981) with slight modifications. This method involved the H_2O_2 dependent oxidation of ascorbate followed by monitoring the decrease in absorbance at 290 nm. The assay reaction mixture containing 90 mM potassium phosphate buffer (pH 7.0), 0.65 mM ascorbate, 1.0 mM H_2O_2, and 0.1 mM EDTA, was incubated for 3 minutes to observe non–specific ascorbate degradation and then the reaction was initiated by the addition 100 µL of protein extract. The decrease in absorbance at 290 nm was observed for 5 min and the APX activity was calculated assuming an absorption coefficient of 2.8 mM^{-1} cm^{-1}. One unit of APX activity was defined as the amount of enzyme necessary to oxidize 1 µmol of ascorbic acid per minute per mg of protein.

$$\text{APX activity} = \frac{\Delta A_{290}\,\text{min}^{-1} \times \text{Total volume in cuvette}}{2.8\ \text{mM}^{-1}\ \text{cm}^{-1} \times \text{Volume of enzyme extract} \times \text{Protein conc.}}$$

3.6.4.2 Monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) assay

MDHAR activity was measured by a method adapted from Hodges and Forney (2000). The method involved a decrease in A_{340} due to the oxidation of NADH to NAD^+ due to generation of monodehydroascorbate via the action of ascorbate oxidase. The reaction mixture contained 90 mM potassium phosphate (pH 7.5), 0.25 units of ascorbate oxidase (unit as defined by Sigma–Aldrich Pty. Ltd., Castle Hill, NSW, Australia), 0.01 mM EDTA, 0.2 mM NADH, 2.5 mM ascorbate, and 30 µL of extract. The reaction rates were monitored by following a decrease in the absorbance at 340 nm for 5 min. The rate of conversion of NADH to NAD^+ was determined using an extinction coefficient for NADH at 340 nm of 6.2 mM^{-1} cm^{-1}. One unit of MDHAR was defined as the amount of enzyme necessary for oxidation of 1 µmol NADH per minute at conditions described above.

$$\text{MDHAR activity} = \frac{\Delta A_{340}\,\text{min}^{-1} \times \text{Total volume in cuvette}}{6.2\ \text{mM}^{-1}\ \text{cm}^{-1} \times \text{Volume of enzyme extract} \times \text{Protein conc.}}$$

3.6.4.3 Dehydroascorbate reductase (DHAR; EC 1.8.5.1) assay

The activity of DHAR was determined by monitoring the glutathione–dependent reduction of dehydroascorbate (Hodges and Forney, 2000). The reaction mixture consisted of 90 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, 5.0 mM reduced glutathione (GSH), and 30 µL of enzyme extract. The reaction was initiated with the addition of 0.2 mM dehydroascorbate (freshly made) and the increase in the reaction rate was monitored at 265 nm for 5 min. The non–enzymatic reduction of dehydroascorbate by GSH was corrected by a reaction in the absence of the enzyme extract. An extinction coefficient of 14.7 mM^{-1} cm^{-1} was used for calculation of DHAR activity. One unit of DHAR was defined as the amount of enzyme required to oxidize 1 µmol dehydroascorbate per min at pH 7.0 under above conditions.

$$\text{DHAR activity} = \frac{\Delta A_{265}\, \text{min}^{-1} \times \text{Total volume in cuvette}}{14.7\ \text{mM}^{-1}\ \text{cm}^{-1} \times \text{Volume of enzyme extract} \times \text{Protein conc.}}$$

3.6.4.4 Glutathione reductase (GR; EC 1.6.4.2) assay

GR activity was determined as described by Foyer and Halliwell (1976) and modified by Hodges and Forney (2000). The method involved the NADPH–dependent reduction of glutathione disulfide (GSSG) to glutathione (GSH).

$$GSSG + NADPH + H^{+} \xrightarrow{GR} 2GSH + NADP^{+}$$

GR activity was assayed by addition of 100 µL of protein extract into a reaction mixture containing 80 mM potassium phosphate buffer (pH 7.0), 2.5 mM oxidized glutathione, 1.5 mM EDTA, 0.5 mM NADPH prepared in (w/v) 1% $NaHCO_3$. The rate of oxidation of NADPH was monitored as the decrease in absorbance at 340 nm for 4 min. The GR activity (nmol min^{-1} mg^{-1} protein) was calculated using the extinction coefficient of 6.2 $mM^{-1}cm^{-1}$. One unit of GR causes the oxidation of 1.0 nmol NADPH per min at 25°C at pH 7.0.

$$\text{GR activity} = \frac{\Delta A_{340}\, \text{min}^{-1} \times \text{Total volume in cuvette}}{6.2\ \text{mM}^{-1}\ \text{cm}^{-1} \times \text{Volume of enzyme extract} \times \text{Protein conc.}}$$

3.6.4.5 Glutathione–s–transferase (GT; EC 2.5.1.18) assay

GT activity was determined following the method of Rogiers et al. (1998). The method involved the GT catalysed conjugation of 1–chloro–2, 4–dinitrobenzene (CDNB) with reduced glutathione (GSH), which is accompanied by an increase in the absorbance at 340 nm due to formation of S–2,4–dinitrophenylglutathione.

$$\text{GSH + CDNB} \xrightarrow{\text{GT}} \text{S–2,4–dinitrophenylglutathione + HCl}$$

The assay reaction mixture consisting of 90 mM potassium phosphate buffer (pH 6.5), 2 mM each of GSH and CDNB, 3.8% (v/v) ethanol was incubated at 25°C and the reaction was initiated by addition of 100 µL of enzyme extract. The increase in absorbance at 340 nm was monitored for 4 min. GT activity was calculated using the molar extinction coefficient of G–SCDNB conjugate, 9.6 $mM^{-1}cm^{-1}$. One unit of GT was defined as amount of enzyme necessary for conjugation of 1.0 nmol of CDNB with GSH per min at pH 6.5 at 25°C.

$$\text{GT activity} = \frac{\Delta A_{340}\, \text{min}^{-1} \text{ x Total volume in cuvette}}{9.6\ \text{mM}^{-1}\ \text{cm}^{-1} \text{ x Volume of enzyme extract x Protein conc.}}$$

3.7 Determination of protein concentration

Protein concentrations in the enzyme extracts were determined following the method of Bradford (1976). The protein reagent used in assay consisted of 0.1 mg mL^{-1} Coomassie Blue G, 5% methanol, and 8.5% *o*–phosphoric acid. A 100 µL of enzyme extract was added to 5 mL of protein reagent, the mixture vortexed vigorously, and allowed to stand for 5 min. The absorbance at 595 nm was measured using a UV–VIS spectrophotometer. The protein concentration was calculated using bovine serum albumin as a standard and expressed as mg of protein per mL of enzyme extract.

3.8 Non–enzymatic antioxidants analysis

3.8.1 Determination of ascorbate (AA), dehydroascorbate (DHA), and total ascorbate (AA + DHA)

Extraction procedure for ascorbate analysis was followed as described by Davey et al (2003). Ten grams of flesh tissue was homogenized with a chilled extraction buffer consisting of 6% (w/v) metaphosphoric acid (MPA), 2 mM EDTA and 1% PVPP. The inclusion of PVPP is important to help prevent oxidation of AA by complexing phenolic compounds (Davey et al., 2003). A high speed homogenization was carried out using a tissue homogenizer (Heidolph DIAX 900, Cinnaminson, NJ, USA) for 1 min at in a dark cool room maintained at 4°C. The duration of homogenization up to 2 min neither influences the recoveries nor the oxidation status of AA under the above described conditions (Davey et al., 2003). The homogenate was filtered through two layers of a cheese cloth and the filtrate was collected and centrifuged at 15 000 *g* in an Eppendorf centrifuge for 15 min at 3°C. After centrifugation, 10 mL of each supernatant was flushed through a Sep–Pak C–l8 cartridge (Waters Assoc., Milford, MA), previously conditioned with 10 mL HPLC–grade methanol (Mallinckrodt Baker, Inc. Phillipsburg, NJ, USA) and 10 mL Milli–Q water. The first 5 mL of eluent was discarded and the next 5 mL was retained for HPLC analysis. Finally, the sample extract was filtered through 0.22 μm True™ syringe filter (LabServ Filtration, BioLab Australia) and loaded into the 1mL glass injection vial (Alltech Assoc Australia, Baulkham Hills, NSW).

3.8.1.1 HPLC analysis of AA

The reverse phase–liquid chromatography was performed for determination of L–AA using a HPLC system (Waters, Milford, MA, USA). An aliquot (20 μl) of the extract was injected using an autosampler (Waters 717plus, Milford, MA, USA) maintained at 4°C. The samples were isocratically eluted through Aminex® 87 X–H column, 300 mm x 7.8 mm i.d., (Bio Rad Laboratories, Hercules, CA, USA) at 25°C with a mobile phase consisting of 0.005N H_2SO_4 + 16% HPLC grade acetonitrile (Mallinckrodt Baker, Inc. Phillipsburg, NJ, USA) as described by Yuan and Chen (1999) and were detected by an absorbance detector (Waters 2487) at 243 nm. Chromatographic peak was identified by comparing its retention time with that of authentic standard and also by co–chromatography involving spiking with the AA standard of known concentration. L–AA was quantified from integrated areas of the sample and its standard. Stock solution of L–AA standard was prepared in 3% MPA/1mM EDTA, stabilized with 2.5 mM DTT. The data from HPLC were collected and processed

with Breeze® 3.30 software (Waters, Milford, MA, USA). The concentration of AA was expressed as nmol AA g^{-1} FW.

3.8.1.2 HPLC determination of total ascorbate (AA + DHA)

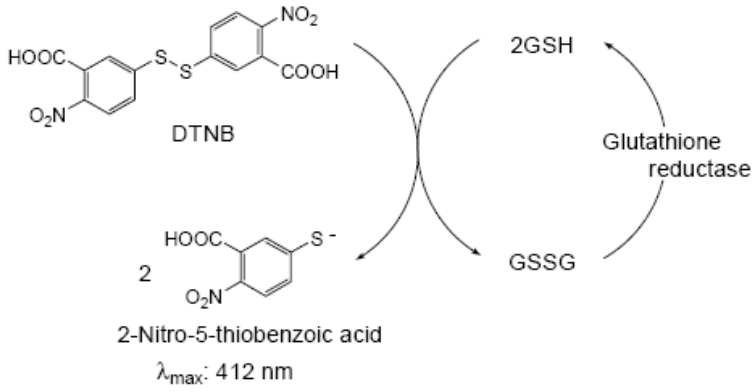
Total ascorbate concentration was determined by the reduction of DHA into AA using dithiothreitol (DTT) as a reducing agent (Davey et al., 2003). Briefly, 500 µL of flesh extract was added to 250 µL of 0.2 M DTT, which was prepared in 0.4 M Tris base. This generated a final pH of 6–6.8. The reaction was allowed to occur for 15 min at room temperature at 21±1°C and was stopped by acidification with a further addition of 250 µL of 8.5% o–phosphoric acid, resulting in a two–fold dilution of the original sample. The reduced samples were then directly analysed by HPLC under similar conditions as for AA and the concentration was expressed as nmol total ascorbate g^{-1} FW.

3.8.1.3 Determination of DHA concentration

DHA concentration was determined following the 'subtractive' technique as described by Davey et al. (2003). The concentration of DHA was calculated from the difference between the total L–AA (L–AA +DHA) and the L–AA content and was expressed as nmol DHA g^{-1} FW.

3.8.2 Determination of reduced glutathione (GSH), oxidized glutathione (GSSG), and total glutathione (GSH + GSSG)

Glutathione (Total and GSSG) was assayed spectrophotometrically by the 5,5'–dithio*bis*–(2–nitrobenzoic acid) (DTNB)–glutathione reductase (GR) recycling method as described by Hodges and Forney (2000). This method involved the reaction of DTNB, known as Ellman's Reagent, with GSH to generate 2–nitro–5–thiobenzoic acid, a yellow colour product with λmax at 412 nm, and GSSG. GR regenerates GSH from GSSG, which then reacts with DTNB again to produce 2–nitro–5–thiobenzoic acid. The whole principle of GSH recycling process is shown below in the box:



Source: http://www.dojindo.com/newimages/TGQKTechnicalInformation.pdf

3.8.2.1 Extraction procedure for glutathione

Five gram of plum flesh tissue was extracted in a pre–chilled pestle and mortar at 3°C along with 0.5 g of white quartz sand and 15 mL ice–cold freshly made 5% (w/v) sulphosalicylic acid (SSA). The extract was centrifuged at 10,000 *g* at 3 °C for 15 min and the supernatant was used for glutathione determination.

3.8.2.2 Assays for total glutathione (GSH + GSSG) and GSSG

The supernatant (200 µL) was neutralized with 0.5 M potassium phosphate buffer (pH 7.0) resulting into 1:25 dilution. Two solutions, A and B, were then prepared separately. Solution A (pH 7.2) consisted of 100 mM Na_2HPO_4, 40 mM Na H_2PO_4. $2H_2O$, 1.8 mM DTNB, 0.04% BSA and 15 mM EDTA. Solution B (pH 7.2) consisted of 50 mM imidazole, 0.2% BSA, 2 units mL^{-1} GR (units as defined by Sigma–Aldrich Pty. Ltd., Castle Hill, NSW, Australia), and 1 mM EDTA. For determination of total glutathione (GSH + GSSG), the reaction mixture consisted of 400 µL of solution A, 320 µL of solution B, 80 µL of 3mM of NADPH and 400 µL of the neutralized extract. The increase in absorbance due to formation of 2–nitro–5–thiobenzoic acid was recorded at 412 nm for 5 min.

For determination of GSSG, an aliquot (500 µL) of supernatant was neutralized with 0.5 M potassium phosphate buffer (pH 6.5) leading to 1: 10 dilution. GSH was first sequestered by incubating 1mL of diluted and neutralized extract with 20 µL of 2–vinylpyridine at 25°C for 1 h and then subjected to a similar reaction as described above for total glutathione. Stock solutions of GSH (2.5 mM) and GSSG

(0.5 mM) standards were prepared in 5% SSA and were used to prepare standard curves for calculating the glutathione concentrations (total or GSSG). For each sample, GSH levels were obtained by subtracting GSSG from total glutathione. The concentrations of total glutathione, GSH, and GSSG were expressed as nmol g^{-1} FW.

3.8.3 Determination of total phenolics

3.8.3.1 Extraction procedure for total phenolics

The plum flesh tissue (5 g) was extracted with 5 mL of 80% aqueous methanol containing 2 mM sodium fluoride (NaF) to avoid oxidation (Gil et al., 2002) using a mortar and pestle. The extraction procedure was repeated with another 5 mL of extraction medium, and the extracts were combined. The extract was centrifuged at 15,000 *g* for 15 min at 3–4°C and the clear supernatant was decanted, and brought to a final volume of 15 mL with methanol.

3.8.3.2 Assay procedure for total phenolic compounds

Total phenolic compounds were quantified by following the Folin–Ciocalteu reagent (FCR) based colorimetric method (Singleton et al., 1999), with slight modifications. A 0.1 mL of appropriately diluted extract was added to 0.9 mL of distilled deionized water (ddH_2O). A 0.1 mL of FCR was added to the reaction mixture, vortexed for 5 s and allowed to stand for 5 min before addition of a 1 mL of 7% Na_2CO_3. The resultant solution was diluted to the volume (2.5 mL) with ddH_2O, then, allowed to stand for 90 min. The absorbance was measured at 750 nm using a UV/VIS spectrophotometer (Jenway Ltd, Essex, UK). In blank, the extract was replaced with the ddH_2O. To calculate the concentration of total phenolics, the calibration curve was made with different concentrations of 5–*O*–caffeoylquinic (chlorogenic) acid (0.02–0.2 mg/mL). Total phenolics were expressed in terms of mg chlorogenic acid equivalent (CAE) per 100g fresh weight.

Gallic acid equivalent (GAE), most commonly used to express the total phenolics in fruits, was not followed due to following reasons: 1) 3–*O*–Caffeoylquinic acid (neochlorogenic acid) is a major hydroxycinnamate in the plum flesh tissue (Tomás–Barberán et al., 2001). 2) The total phenolics concentration in

chlorogenic– or neochlorogenic–rich fruits is underestimated by 0.6 times if expressed in terms of GAE. 3) Similar absorption spectra of chlorogenic and neochlorogenic acids allow the use of former in the preparation of calibration curve (Chun and Kim, 2004).

3.8.4 Determination of DPPH· radical scavenging activity

3.8.4.1 Extraction procedure for DPPH· radical scavenging activity

The extract used for determination of total phenolics (refer to 3.8.3.1) was used for measuring the DPPH· radical scavenging activity.

3.8.4.2 Assay procedure for DPPH· radical scavenging activity

The DPPH· radical scavenging activity was determined using a modified method of Brand–Williams et al. (1995). A stock solution (60 µM) of DPPH (1,1–diphenyl–2–picrylhydrazyl), which was used as a source of free radical, was prepared in 80% aqueous methanol. An aliquot (50 µL) of the appropriately diluted extract was mixed with 950 µL of the freshly prepared methanolic DPPH (12 µM). The mixture was vortexed for 5 s and allowed to stand in the dark at 21±1°C for 15 min. The decrease in absorbance of DPPH was measured at 515 nm. A mixture containing 50 µL of extraction buffer (80% aqueous methanol and 2 mM NaF) and 950 µL of methanolic DPPH· served as the control. The DPPH· radical scavenging activity was calculated from a standard curve made with known concentrations of ascorbic acid (0.02–0.2 mg/mL) and expressed in terms of mg ascorbic acid equivalent per 100 g fresh weight.

3.9 Statistical analysis

The data were subjected to analysis of variance (ANOVA) using GenStat Release 11.1 (VSN International Ltd., Hemel Hempstead, UK). The treatment effects on various parameters were assessed within ANOVA and the least significant differences (LSD) were calculated following significant F–test at $P \leq 0.05$.

Chapter 4

Climacteric Levels during Fruit Ripening Influence Lipid Peroxidation and Dynamics of Antioxidants Levels in Japanese Plums[1]

Summary

Depending upon the climacteric or non–climacteric nature of the fruit or cultivar, the oxidative behaviour may also differ due to differences in the respiration and ethylene production rates. The objective of this experiment was to assess the significance of antioxidant metabolism in regulation of fruit ripening in three cultivars of Japanese plums differing in climacteric levels. To achieve this objective, plum cultivars, 'Blackamber', 'Amber Jewel', and 'Angeleno', harvested at commercial maturity were allowed to ripen at 21±1°C for 8 days, and on the basis of measurement of respiration and ethylene production rates, these were categorized into, highly–climacteric, moderately–climacteric, and suppressed–climacteric, respectively. Fruit ripening in all cultivars involved flesh softening, increase in SSC:TA ratio, and skin colour darkening as evident from the decrease in L* and hue angle values. The extent of lipid peroxidation, indicated by the increase in lipoxygenase (LOX) activity and thiobarbituric acid reactive substances (TBARS), increased during fruit ripening according to cultivar; it was lower in 'Angeleno' than in 'Blackamber' and 'Amber Jewel' during the first 6 days of ripening period. During fruit ripening, SOD activity decreased in all cultivars and remained lower in 'Angeleno' than in the other cultivars. In contrast, POD activity was significantly higher in 'Blackamber' and 'Amber Jewel' than in 'Angeleno' during 4–8 days of fruit ripening. The role of ascorbate–glutathione cycle as a potential mechanism for scavenging ROS was also examined. The advancement of fruit ripening caused an oxidation of the redox buffers such as AA and GSH resulting in increased concentrations of DHA and GSSG leading to a decline in the ratios of AA: DHA and GSH:GSSG, respectively. Depending upon the cultivar, the reduction in the activities of key enzymes regulating

[1] The additional data on the changes in concentrations of individual sugars and organic acids from this experiment has been published in the following article: Singh, S.P. and Z. Singh. 2008. Major flavor components in some commercial cultivars of Japanese plum. *Journal of the American Pomological Society*. 62(4):185–190.

the ascorbate–glutathione cycle, APX, DHAR, DHAR, and GR suggested the failure of ascorbate–glutathione mediated ROS scavenging system leading to accumulation of DHA and GSSG on the 8th day of fruit ripening. The changes in total phenolics and antioxidant capacity in three cultivars were also related to the changes in overall ascorbate/phenolics/peroxidase system during fruit ripening. The data suggest that climacteric–type Japanese plum cultivars, 'Blackamber' and 'Amber Jewel' showed a faster decline in the ability of components of enzymatic and non–enzymatic antioxidative protection systems as compared to the suppressed–climacteric cultivar, 'Angeleno'.

4.1 Introduction

Fruit ripening is a highly regulated and irreversible phenomenon involving a series of physiological, biochemical, and structural changes in fruit leading to an attractive, edible, and ripe fruit. The fruits are broadly classified into two categories– climacteric and non–climacteric (Biale, 1964). A typical climacteric fruit exhibits a peak in respiration and ethylene evolution during ripening, while this is lacking in a non–climacteric fruit. There is another category of suppressed–climacteric fruits and/or cultivars that are unable to biosynthesize ethylene in a sufficient quantity to induce a climacteric rise in respiration and ethylene production, which leads to a delay and suppression of climacteric (Abdi et al., 1998). Japanese plums are climacteric in nature, but a few cultivars show suppressed–climacteric behavior (Abdi et al., 1998; Díaz–Mula et al., 2009; Khan and Singh, 2007b).

Fruit ripening is an aerobic metabolic process generating ROS, causing tissue damage (Brennan and Frenkel, 1977). The ROS action has been known to initiate and enhance the degenerative processes associated with fruit ripening and senescence (Brennan and Frenkel, 1977; Du and Bramlage, 1994; Lacan and Baccou, 1998; Rogiers et al., 1998). The fruit ripening is considered a protracted form of senescence that is characterized by membrane disintegration due to a change in membrane composition induced by LOX among other enzymes. The peroxidation of free polyunsaturated fatty acids (PUFA) by LOX causes accumulation of lipid–hydroperoxides which further decompose into oxy–radicals, ethane, and TBARS that are considered markers for peroxidative damage to the tissue (Rogiers et al., 1998).

Under normal conditions, ROS are rapidly scavenged by various cellular enzymatic and non–enzymatic mechanisms. The enzymatic antioxidant defense in plants includes enzymes such as SOD, CAT, and POD (Apel and Hirt, 2004). SOD catalyzes the dismutation of $O_2^{\bullet -}$ to H_2O_2, which is subsequently detoxified by CAT and POD. Non–enzymatic antioxidant system has a cellular pool of water–soluble compounds such as ascorbate, glutathione, and phenolic compounds and lipid–soluble compounds such as carotenoids and tocopherols, which are capable of quenching ROS (Apel and Hirt, 2004; Noctor and Foyer, 1998). AA is oxidized by ROS to MDHA and DHA, whereas GSH is converted into GSSG. An ascorbate–glutathione cycle involving enzymes such as APX, DHAR, MDHAR, and GR operates to maintain the levels of AA and GSH (Noctor and Foyer, 1998). The phenolic compounds have redox properties and may also play a co–operative role in the protection against ROS in the plant tissue (Kahkonen et al., 1999). A gradual decrease in the activities of antioxidant enzymes and a lower level of antioxidants in the reduced state can lead to the accumulation of the ROS to toxic levels and also the disturbance of the equilibrium of ROS production and removal (Apel and Hirt, 2004).

The development of oxidative stress during fruit ripening has been reported in a range of climacteric and non–climacteric fruits (Jiménez et al., 2002a, 2002b; Larrigaudiére et al., 2009; Masia, 1998; Qin et al., 2009; Rogiers et al., 1998; Wang et al., 2004; Yahia et al., 2001; Zheng et al., 2007). Previous studies have also shown that cultivars differing in antioxidant metabolism also differed in their potential storage or shelf life and susceptibility to various physiological disorders (Hodges et al., 2001; Lacan and Baccou, 1998; Lata et al., 2005). Furthermore, the antioxidant levels in apple peel and flesh have been linked to the susceptibility of fruit to the storage disorders such as superficial scald and flesh browning (DeLong and Prange, 2003; Du and Bramlage, 1995; Toivonen et al., 2003; Whitaker, 2004). Given the importance of antioxidant metabolism in postharvest fruit ripening, it is imperative to understand the dynamics of enzymatic and non–enzymatic antioxidant components in plum fruit cultivars ‘Blackamber’ and ‘Amber Jewel’ that behave as climacteric type, and ‘Angeleno’ as suppressed–climacteric type (Candan et al., 2008; Díaz–Mula et al., 2009; Khan and Singh, 2007b). Since the electron transport during respiration is one of the major sources contributing to the ROS production in the plant tissue (Apel and Hirt, 2004); it was therefore hypothesized that fruit cultivars differing in their

respiratory behaviours may also differ in their oxidative and antioxidative potentials during fruit ripening. The objective of this study was to understand the significance of antioxidant metabolism in regulation of fruit ripening in Japanese plum cultivars in relation to their climacteric behaviour.

4.2 Materials and methods

4.2.1 Fruit material and experimental design

Japanese plum (*Prunus salicina* Lindell) cultivars, 'Blackamber', 'Amber Jewel' and 'Angeleno', were harvested at commercial maturity on 22 Dec 2006, 22 Jan 2007, and 27 Feb 2007, respectively, from the Casuarina Valley Orchard, Karragullen, Perth Hills (latitude 31º 57' S; longitude 115º 50' E), Western Australia. Fruit maturity was judged on the basis of soluble solids concentration (SSC) and fruit firmness. All plum cultivars were grafted on myrobalan (*Prunus cerasifera* Ehrh.) rootstock and were trained on a palmate system. Fruit of uniform size and maturity, free from visual blemishes and disease were harvested from the orchard. The fruit were placed into the standard plastic crates (~15 kg) lined with polyethylene liners, transported to the laboratory immediately after harvesting, and allowed to ripen at 21±1°C. The evaluation of fruit quality, enzymatic and non–enzymatic components was carried out at two–day intervals commencing from the day of harvest until 8th day of ripening, i.e. 0, 2, 4, 6, and 8 day. The experiment was laid out by following a completely randomized design with two factors including cultivar and ripening period.

4.2.2 Respiration and ethylene production rates

Respiration and ethylene production rates of plums were determined during 8 days (10 days for 'Angeleno' cultivar) of the ripening period at 20±1°C. The detailed procedures for estimation of ethylene production and respiration rates have been described in Sections 3.3.1 and 3.3.2, respectively. Two fruit enclosed in an air–tight container were treated as an experimental unit and replicated four times. The respiration and ethylene production rates were expressed as mmol of CO_2 kg^{-1} h^{-1} and μmol kg^{-1} h^{-1}, respectively.

4.2.3 Fruit quality evaluation

Fruit quality was evaluated during ripening on 0, 2, 4, 6, and 8 days after harvest. Flesh firmness was measured using an electronic pressure tester (Model: EPT–1, Lake City Technical Products, Kelowna, BC, Canada) fitted with an 8–mm spherical probe as described in Section 3.4.1.1. Ten fruit per replication were subjected to firmness testing with each fruit punctured on both sides at equatorial region. The firmness was expressed as Newtons. The changes in fruit colour parameters were measured and calculated as described in 3.4.2. Ten fruit constituted one replication unit for skin colour measurements. Juice obtained from 10 fruit was used for determination of SSC, TA and SSC: TA ratio as described in Section 3.4.3.

4.2.4 Lipid peroxidation

The extraction and assay for LOX were carried out following the method described in Section 3.5.1. LOX activity was expressed as µmol linoleate hydroperoxides formed min^{-1} mg^{-1} protein. The method for estimation of TBARS content has been described in Section 3.5.2. TBARS concentration was expressed as nmol MDA equivalents g^{-1} FW. The method for estimation of EL as a measure of membrane integrity has been described in Section 3.5.3. The data were expressed as percentage of the total electrolyte leakage.

4.2.5 Extraction and assays of antioxidant enzymes (SOD, CAT, and POD)

The detailed procedures for extraction and assays of SOD, CAT, and POD are described in details in Sections 3.6.1 and 3.6.2. SOD activity was calculated and expressed as µmol Cyt c conserved min^{-1} mg^{-1} protein. CAT activity was expressed as µmol H_2O_2 decomposed min^{-1} mg^{-1} protein. POD activity was expressed as µmol tetra–guaiacol formed min^{-1} mg^{-1} protein.

4.2.6 Extraction and assays of ascorbate–glutathione cycle enzymes

The extraction and assay procedures of enzymes involved in the ascorbate–glutathione cycle are described in Sections 3.6.3 and 3.6.4, respectively. The activities of APX, MDHAR and DHAR were expressed as µmol AA oxidized min^{-1}

mg^{-1} protein, µmol NADH oxidized min^{-1} mg^{-1} protein and µmol DHA oxidized min^{-1} mg^{-1} protein, respectively. GR activity was expressed as nmol NADPH oxidized min^{-1} mg^{-1} protein, whereas GT activity was expressed as nmol S–2,4–dinitrophenylglutathione formed min^{-1} mg^{-1} protein.

4.2.7 Determination of protein concentration

Protein concentrations in the enzyme extracts were determined following the method described in Section 3.7.

4.2.8 Analysis of non–enzymatic antioxidants, ascorbate and glutathione

The detailed procedure for extraction and liquid chromatography analysis of AA, DHA and total ascorbate (AA + DHA) are described in Section 3.8.1. The procedures for determination of GSH, GSSG and total glutathione (GSH + GSSG) have been detailed in Section 3.8.2. The concentrations of ascorbate and glutathione were expressed as nmol g^{-1} FW.

4.2.9 Determination of total phenolics and DPPH· radical scavenging activity

The extraction and assay procedures for total phenolics and DPPH· radical scavenging activity have been described in previous Sections 3.8.3 and 3.8.4, respectively. Total phenolics were expressed in terms of mg chlorogenic acid equivalent (CAE) $100g^{-1}$ FW. DPPH· radical scavenging activity was expressed in terms of mg ascorbic acid equivalents (AAE) $100g^{-1}$ FW.

4.2.10 Statistical analysis

The data were subjected to a two–way analysis of variance (ANOVA) using GenStat Release 11.1 (VSN International Ltd., Hemel Hempstead, UK). The effects of factors, cultivar and ripening period, on different parameters were assessed within ANOVA and the least significant differences (LSD) were calculated following a significant F–test at $P \leq 0.05$. The validity of statistical analysis was ensured by checking all the assumptions of analysis.

4.3 Results

4.3.1 Respiration and ethylene production rates

The respiratory behaviours of three cultivars under study were significantly different during fruit ripening at 21±1°C (Fig. 4.1A). The respiratory rates of ‘Amber Jewel’ and ‘Blackamber’ continued to increase during fruit ripening achieving climacteric peaks on days 6 and 7, respectively, whereas ‘Angeleno’ showed a consistent rate of respiration up to day 7, showing a minor climacteric rise on day 8. The climacteric rise in respiration during fruit ripening of ‘Blackamber’ was higher than of ‘Amber Jewel’ and ‘Angeleno’ by about 1.9 and 3.3 fold, respectively.

Similarly to respiration rates, ethylene production rates of three cultivars differed significantly (Fig. 4.1B). Ethylene evolution in ‘Amber Jewel’ and ‘Angeleno’ cultivars was not observed up to 3 and 5 days after harvest, respectively, whilst ‘Blackamber’ produced ethylene in detectable amount from the first day of harvest. The peaks of ethylene production rates in both, ‘Blackamber’ and ‘Amber Jewel’ cultivars were observed on day 7; the former showing about three times higher rate than the later. ‘Angeleno’ exhibited the lowest rate of ethylene production with a continuous rise showing a peak on day 10. The data on respiration and ethylene production rates suggest that these cultivars can be categorized into highly–climacteric–‘Blackamber’, moderately–climacteric– ‘Amber Jewel’ and suppressed–climacteric– ‘Angeleno’.

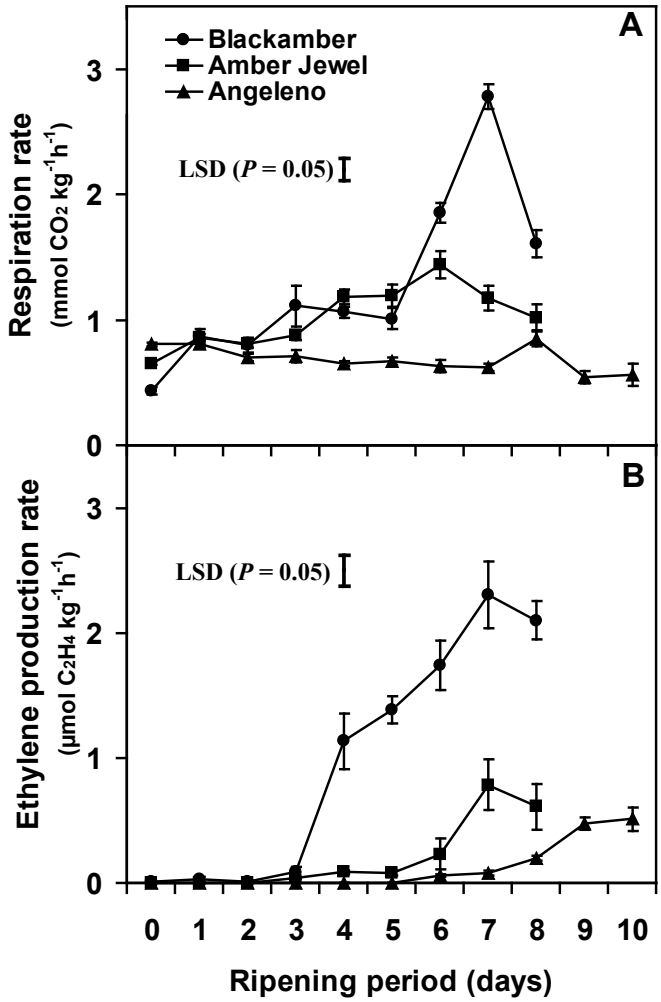


Fig. 4.1. Respiration (A) and ethylene production (B) rates of three cultivars of Japanese plums during fruit ripening at 21±1°C. Vertical bars represent S.E. of means (n = 4). LSD (P = 0.05) values for Respiration: Cultivar (C) = 0.05, Ripening period (RP) = 0.10, C × RP = 0.18. Ethylene: C = 0.08, RP = 0.15, C × RP = 0.25. LSD values for C × RP are shown as separate bars.

4.3.2 Fruit firmness, colour, SSC, TA and SSC:TA ratio

The characteristic skin colours of 'Blackamber', 'Amber Jewel', and 'Angeleno' are purple–black, red on cream to yellow background, and dark–purple, respectively. The chromaticity value L* also decreased significantly during fruit ripening leading to skin darkening (Fig. 4.2A). The decrease in hue angle was also significant during the ripening process in all cultivars (Fig. 4.2B). A great variation in the L* and hue angle values among cultivars was noticed due to a variation in the characteristic skin colours of these cultivars. The flesh firmness decreased during fruit ripening in all three cultivars (Fig 4.2C). However, a significant variation in the rates of fruit softening existed among these cultivars. The loss of firmness in 'Blackamber',

'Amber Jewel', and 'Angeleno' was, respectively, 3.4, 2.6, and 1.7 fold during 8 days of fruit ripening.

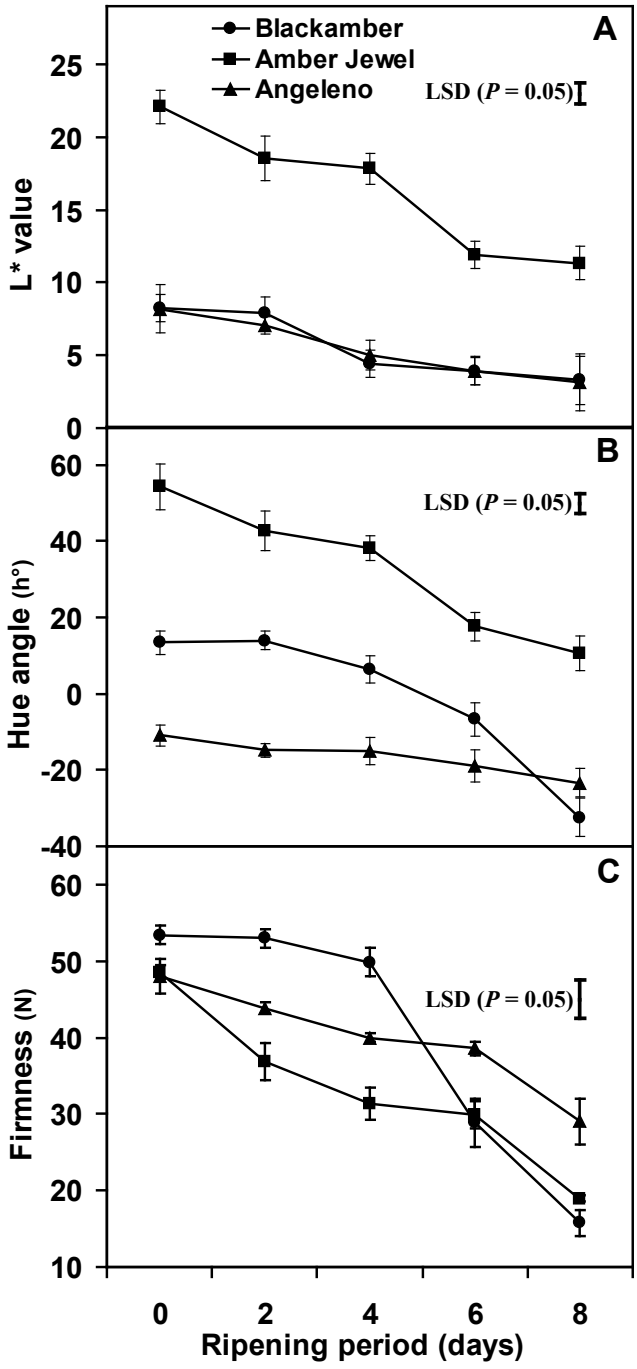


Fig. 4.2. Changes in L* (A), hue angle (B), and flesh firmness (C) of three cultivars of Japanese plums during fruit ripening at 21±1°C. Vertical bars represent S.E. of means. All measurements were made in triplicate. LSD (P = 0.05) values for L*: Cultivar (C) = 0.62, Ripening period (RP) = 0.80, C × RP = 1.39. Hue angle: C = 2.30; RP = 2.96, C × RP = 5.13. Firmness: C = 2.27, RP = 2.94, C × RP = 5.09. LSD values for C × RP are shown as separate bars.

The flesh firmness decreased during fruit ripening in all three cultivars (Fig 4.2C). However, a significant variation in the rates of fruit softening existed among these cultivars. The loss of firmness in 'Blackamber', 'Amber Jewel', and 'Angeleno' was, respectively, 3.4, 2.6, and 1.7 fold during 8 days of fruit ripening.

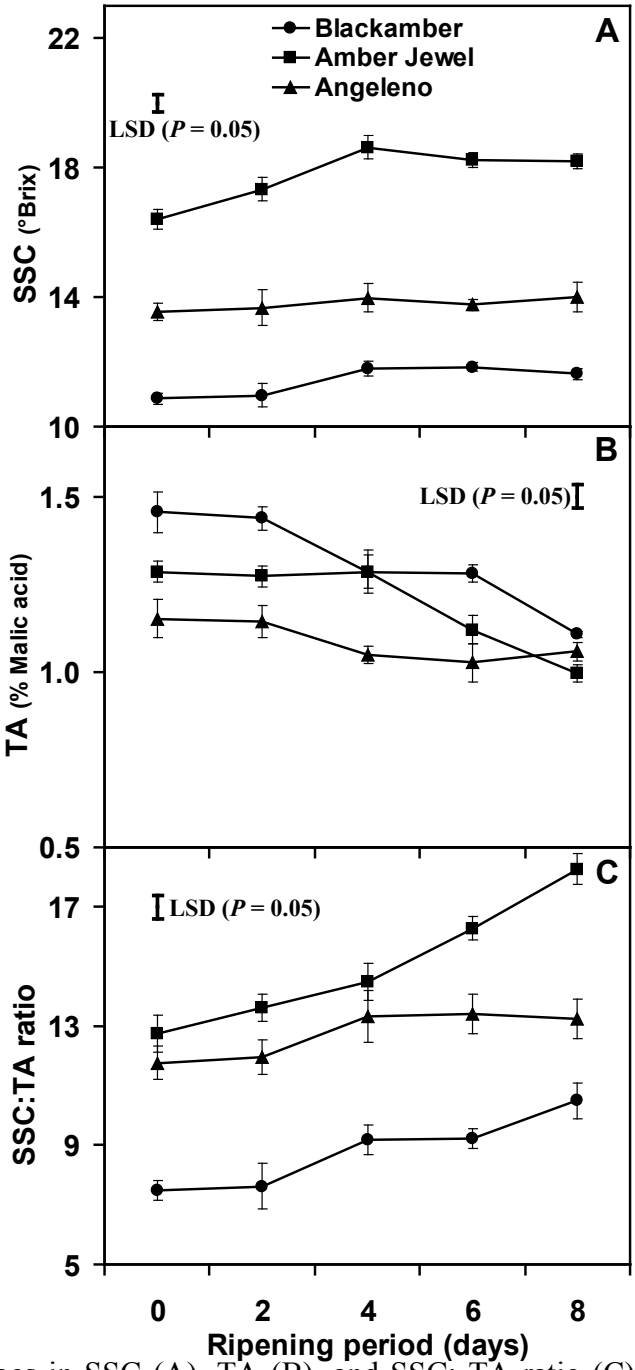


Fig. 4.3. Changes in SSC (A), TA (B), and SSC: TA ratio (C) of three cultivars of Japanese plums during fruit ripening at 21±1°C. Vertical bars represent S.E. of means. All measurements were made in triplicate. LSD (P = 0.05) values for SSC: Cultivar (C) = 0.24, Ripening period (RP) = 0.31, C × RP = 0.54. TA: C = 0.03, RP = 0.04, C × RP = 0.07. SSC:TA ratio: C = 0.36, RP = 0.46, C × RP = 0.80. LSD values for C × RP are shown as separate bars.

The SSC increased during fruit ripening depending upon the cultivar. The increase in SSC of 'Amber Jewel' was significantly higher than of 'Blackamber' and 'Angeleno' (Fig. 4.3A). The cultivar type significantly influenced the decrease in TA (Fig. 4.3B). 'Blackamber', 'Amber Jewel', and 'Angeleno' showed the decrease in TA by about 27%, 23%, and 8%, respectively, during fruit ripening period of 8 days. The decrease in TA improved the SSC: TA ratio in all cultivars (Fig. 4.3C).

4.3.3 Lipid peroxidation

LOX activity was strongly influenced by the cultivar and ripening factors (Fig. 4.4A). The increase in LOX was noticed parallel to the climacteric rise of respiration and ethylene production in all cultivars. A higher LOX activity was observed in 'Blackamber' cultivar during initial stages (0, 2, and 4 days) of fruit ripening compared to 'Amber Jewel' and 'Angeleno'. The peak LOX activities in 'Blackamber' and 'Amber Jewel', observed on day 6, were about two times higher than the peak in 'Angeleno' that was observed on day 8.

The TBARS content significantly increased during fruit ripening of three cultivars (Fig. 4.4B). In 'Blackamber and 'Amber Jewel', a consistent increase in the TBARS content occurred during fruit ripening, whereas such increase was noticed only after 4 days of ripening period in 'Angeleno'. After 8 days of fruit ripening, the maximum accumulation of TBARS was observed in 'Blackamber' followed by 'Amber Jewel' and 'Angeleno'.

The electrolyte leakage increased during fruit ripening, irrespective of the cultivar (Fig. 4.4C). 'Amber Jewel', in general, exhibited the highest amount of electrolyte leakage compared to other two cultivars. After 8 days of fruit ripening, the electrolyte leakage was found highest in 'Amber Jewel', followed by 'Blackamber' and 'Angeleno'.

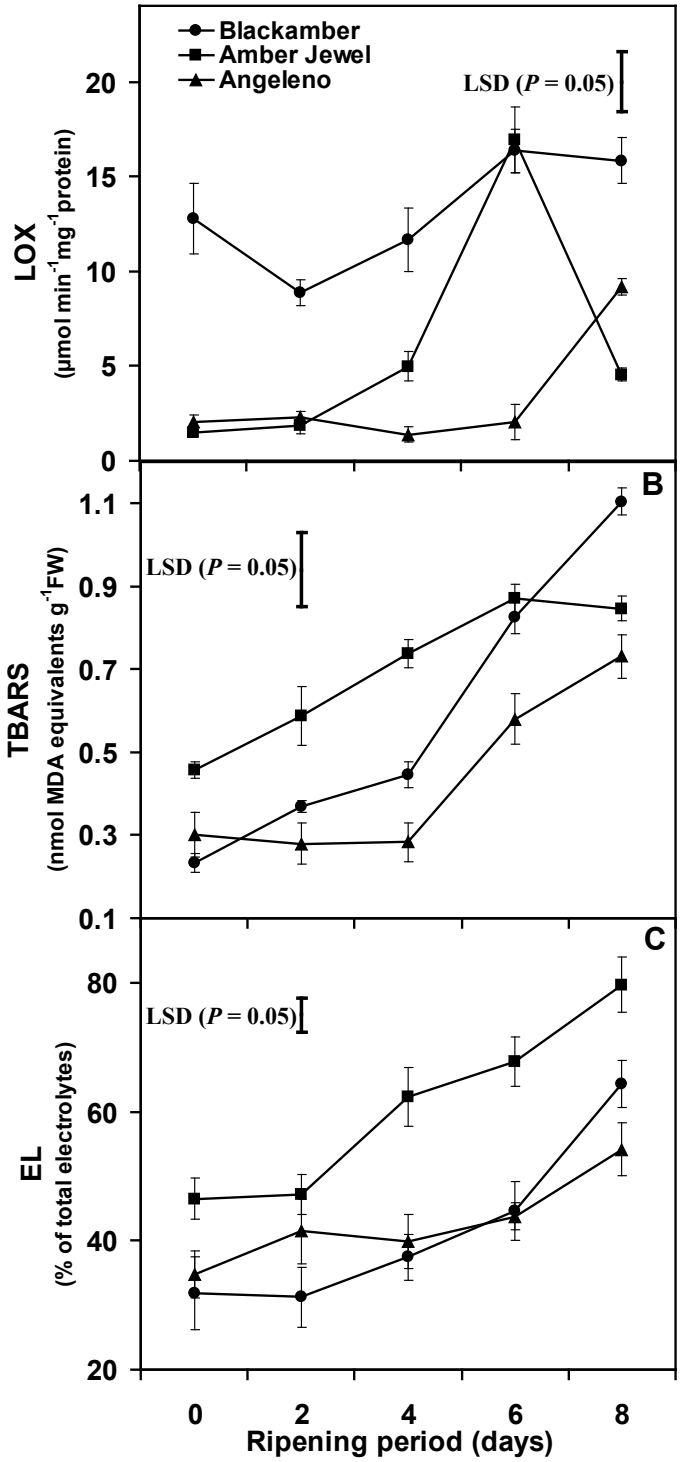


Fig. 4.4. Changes in LOX activity (A), TBARS concentration (B), and EL (C) in the flesh tissue of three cultivars of Japanese plums during fruit ripening at 21±1°C. Vertical bars represent S.E. of means. All measurements were made in triplicate. LSD (P = 0.05) values for LOX: Cultivar (C) = 1.43, Ripening period (RP) = 1.85, C × RP = 3.20. TBARS: C = 0.08, RP = 0.10, C × RP = 0.18. EL: C = 2.37, RP = 3.07, C × RP = 5.31. LSD values for C × RP are shown as separate bars.

4.3.4 Antioxidant enzyme activities (SOD, CAT, and POD)

The SOD activity decreased during fruit ripening, regardless of the cultivar (Fig. 4.5A).

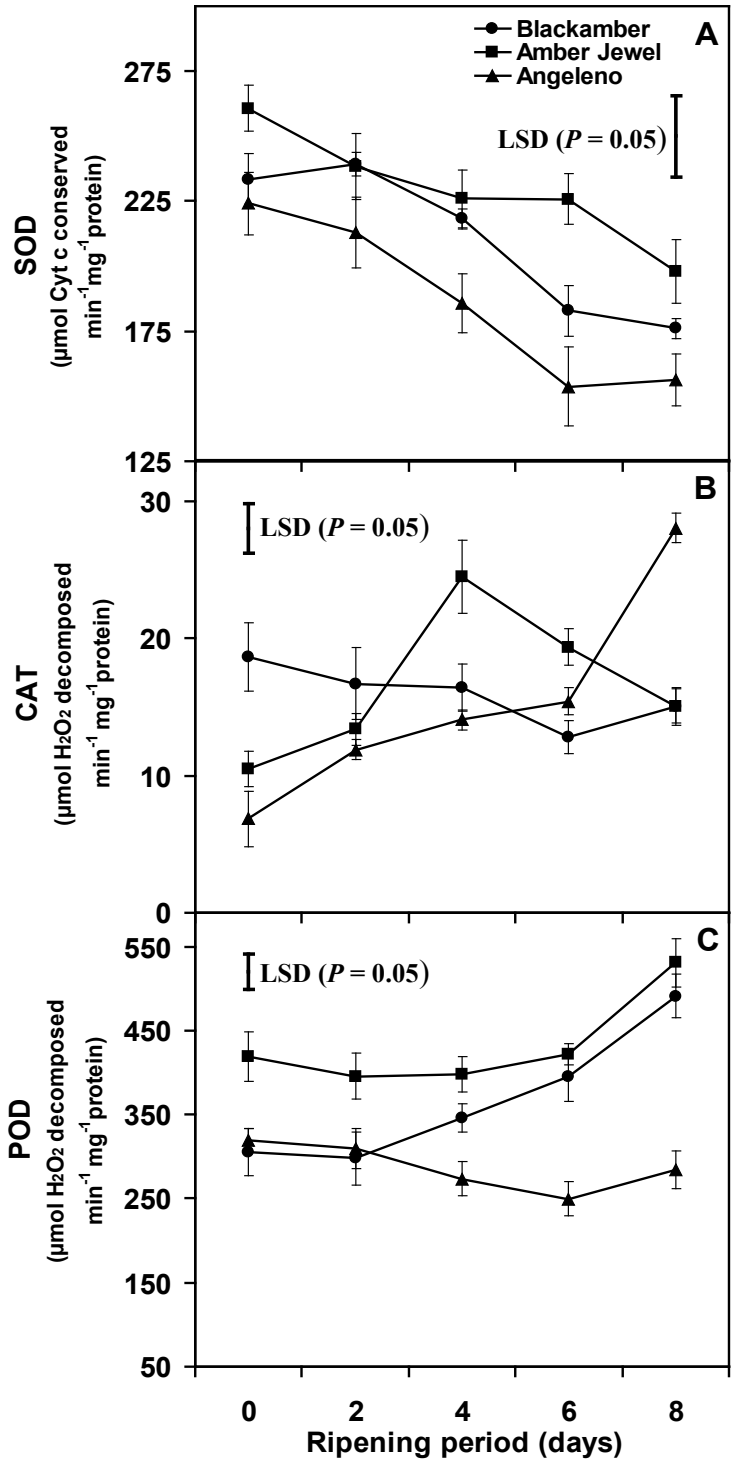


Fig. 4.5. Changes in activities of SOD (A), CAT (B), and POD (C) in the flesh tissue of three cultivars of Japanese plums during fruit ripening at 21±1°C. Vertical bars represent S.E. of means. All measurements were made in triplicate. LSD (P = 0.05) values for SOD: Cultivar (C) = 13.90, Ripening period (RP) = 17.94, C × RP = NS. CAT: C = NS, RP = 2.08, C × RP = 3.60. POD: C = 4.71, RP = 6.08, C × RP = 10.52. LSD values for C × RP are shown as separate bars.

The extent of decrease in SOD activities during fruit ripening of 8 days in 'Blackamber', 'Amber Jewel', and 'Angeleno' cultivars was about 24%, 24%, and 30%, respectively. The SOD activity, however, remained significantly higher during late stages of fruit ripening (6 and 8 days) in the climacteric cultivars, 'Blackamber' and 'Amber Jewel' than in the suppressed climacteric cultivar, 'Angeleno'.

CAT activity showed a peak on the 4th and 8th day of fruit ripening in Amber Jewel' and 'Angeleno', respectively. The CAT activity in 'Angeleno' on the 8th day of fruit ripening was about 1.9–fold higher than in 'Blackamber' and 'Amber Jewel' on the same day. Contrarily, the CAT activity showed an overall decrease during fruit ripening in 'Blackamber' except with a minor increase on the 8th day.

The fruit ripening in 'Blackamber' and 'Amber Jewel' commenced with a continuous increase in POD activities, whereas, in 'Angeleno', it declined during the first 6 days with a non–significant increase on the 8th day (Fig. 4.5C). In general, POD activity increased during the advanced stages of fruit ripening. Among three cultivars, 'Amber Jewel' showed the highest POD activity followed by 'Blackamber' and 'Angeleno' during fruit ripening.

4.3.5 Ascorbate–glutathione cycle

4.3.5.1 Ascorbate, dehydroascorbate, and related enzymes

Total ascorbate (AA + DHA) levels were influenced by fruit ripening, depending upon the cultivar (Fig. 4.6A). During the fruit ripening, no significant change in total AA was observed in 'Blackamber' and 'Amber Jewel', but it increased significantly in 'Angeleno'. After the 8 days of fruit ripening, the concentration of AA significantly decreased in 'Amber Jewel' cultivar to half of its original concentration at the harvest (Fig. 4.6B). The decrease in AA during the same period was to the extent of 17% and 12% in 'Blackamber' and 'Angeleno' cultivars, respectively.

In contrast to AA, the concentrations of DHA increased significantly during fruit ripening in all cultivars (Fig. 4.6C). The increase in concentrations of DHA in 'Blackamber', 'Amber Jewel', and 'Angeleno' cultivars was about 5–, 1.7–, and 2.8–fold higher on the 8th day of fruit ripening compared to the concentrations on the day of harvest. The AA: DHA ratio dropped significantly with the advancement of fruit ripening, irrespective of the cultivar (Fig. 4.6D). The differences in AA: DHA ratio

among three cultivars became non–significant from the 4th day of fruit ripening. However, the extent of decline in ratio was variable; it was 4.4 fold in 'Blackamber', 3.5 fold in 'Amber Jewel', and 3.2 fold in 'Angeleno'.

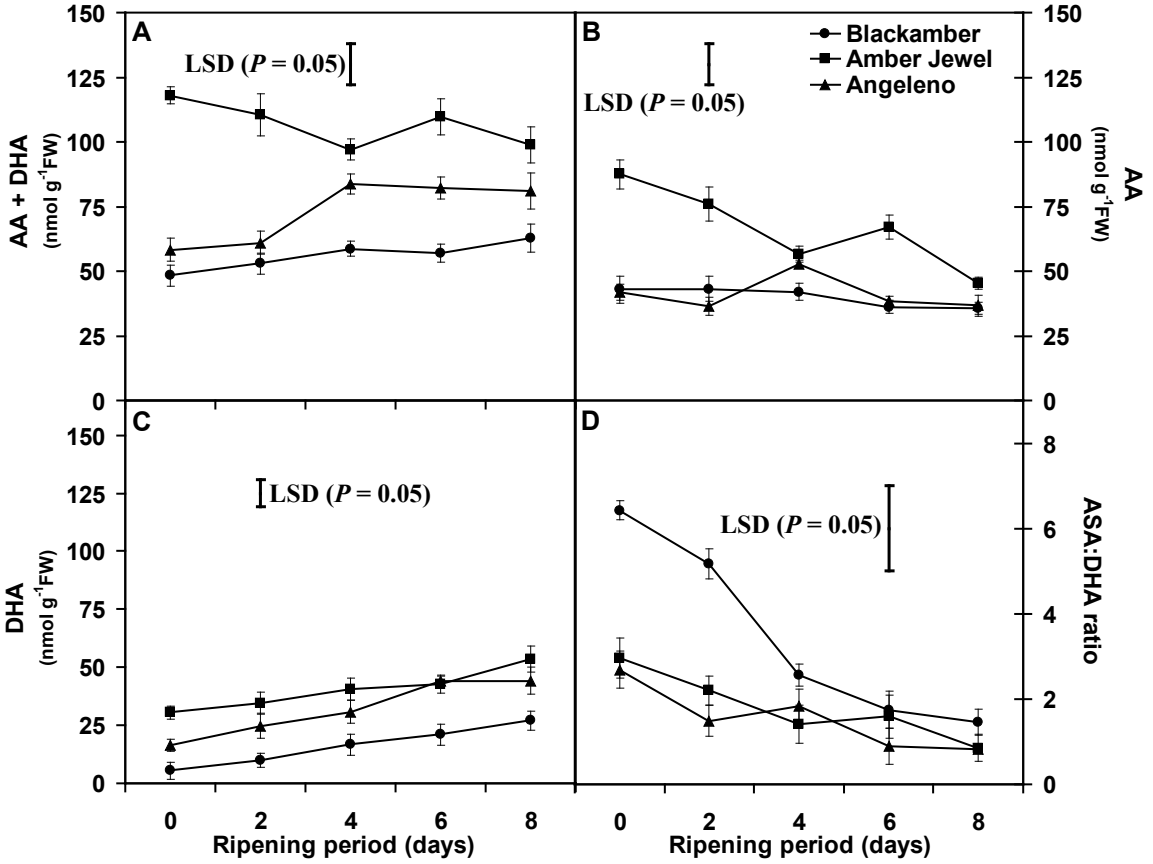


Fig. 4.6. Changes in concentrations of total ascorbate (AA + DHA) (A), AA (B), DHA (C), and AA:DHA ratio (D) in the flesh tissue of three cultivars of Japanese plums during fruit ripening at 21±1°C. Vertical bars represent S.E. of means. All measurements were made in triplicate. LSD (P = 0.05) values for AA + DHA: Cultivar (C) = 7.15, Ripening period (RP) = 9.23, C × RP = 15.99. AA: C = 4.59, RP = 5.93, C × RP = 10.27. DHA: C = 5.11, RP = 6.60, C × RP = 11.44. AA:DHA ratio: C = 0.88, RP = 1.14, C × RP = 1.98. LSD values for C × RP are shown as separate bars.

The activities of APX and MDHAR increased during the first 4 days of fruit ripening in 'Blackamber' and 'Amber Jewel' cultivars, and then decreased, leading to these enzymes activities on the 8th day even lower than those on the day of harvest (Fig. 4.7A & B). The MDHAR activity in 'Angeleno' remained almost constant during the same period. However, the DHAR activities in 'Blackamber' and 'Angeleno' showed a peak on the 4th day of fruit ripening, while in case of 'Amber Jewel', it continuously decreased during the entire period of ripening and were at the

lowest level on the 8th day without any statistically significant difference from the other two cultivars (Fig. 4.7C).

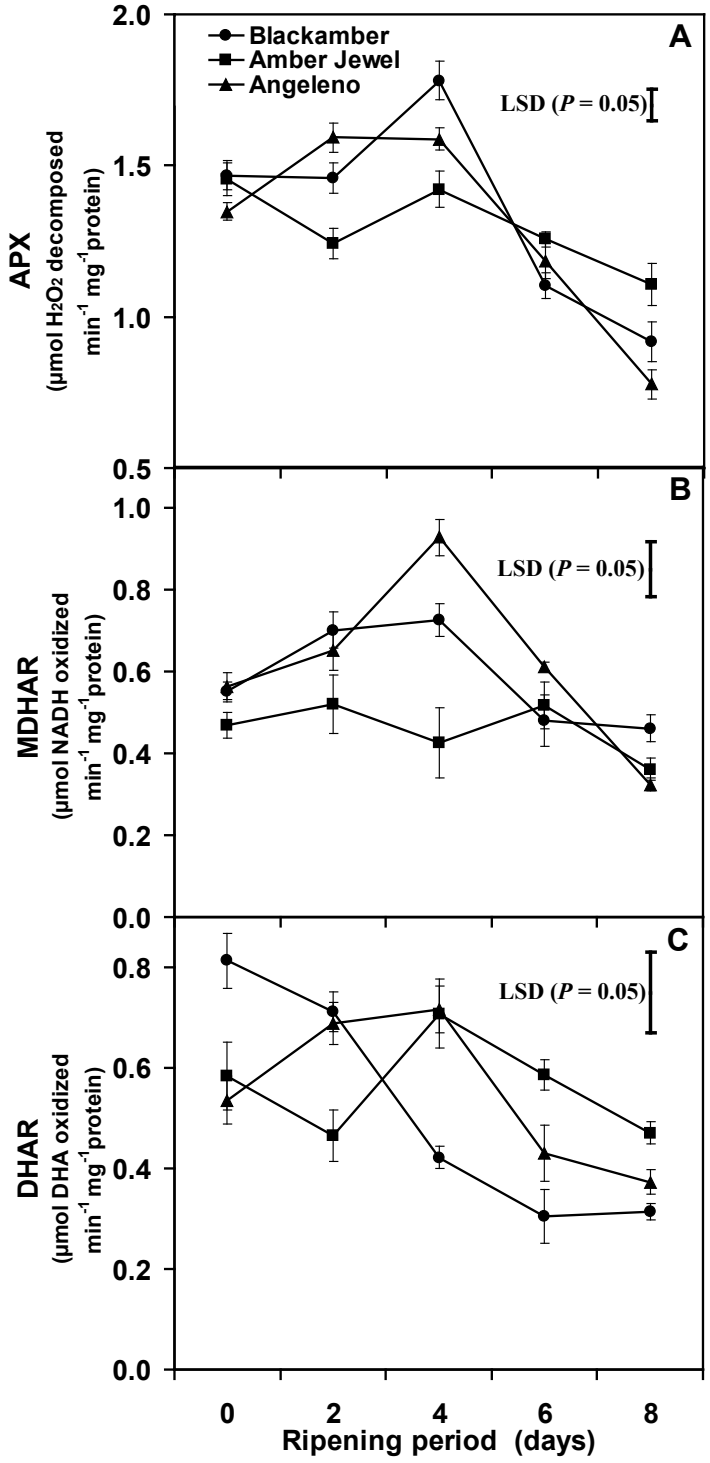


Fig. 4.7. Changes in activities of APX (A), MDHAR (B), and DHAR (C) in the flesh tissue of three cultivars of Japanese plums during fruit ripening at 21±1°C. Vertical bars represent S.E. of means. All measurements were made in triplicate. LSD (P = 0.05) values for APX: Cultivar (C) = NS, Ripening period (RP) = 0.06, C × RP = 0.10. MDHAR: C = 0.06, RP = 0.08, C × RP = 0.13. DHAR: C = NS, RP = 0.09, C × RP = 0.16. LSD values for C × RP are shown as separate bars.

4.3.5.2 Glutathione, glutathione disulfide, and related enzymes

Concentrations of total glutathione (GSH + GSSG) were significantly affected by the cultivar type and ripening period (Fig. 4.8A). Regardless of the cultivar, total glutathione, GSH, and GSH:GSSG ratio decreased during the fruit ripening (Fig. 4.8). 'Angeleno' had a significantly higher concentration of total glutathione than 'Amber Jewel' and 'Blackamber' during all the stages of fruit ripening. The decrease in GSH concentrations was noticed in all cultivars to variable extents; the loss of GSH during 8 days of fruit ripening was about 50% in 'Blackamber', 41% in 'Amber Jewel', and 32% in 'Angeleno' (Fig. 4.8B).

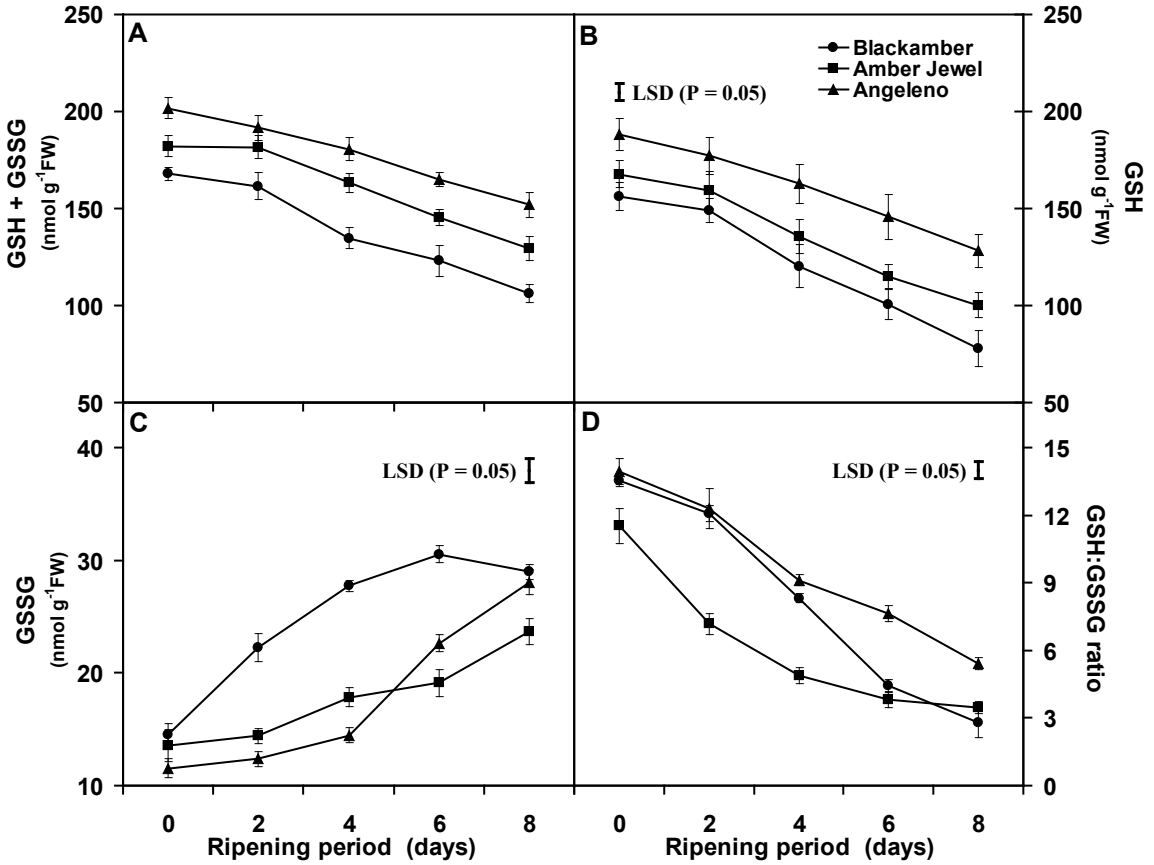


Fig. 4.8. Changes in concentrations of total glutathione (GSH + GSSG) (A), GSH (B), GSSG (C), and GSH:GSSG ratio (D) in the flesh tissue of three cultivars of Japanese plums during fruit ripening at 21±1°C. Vertical bars represent S.E. of means. All measurements were made in triplicate. LSD (P = 0.05) values for GSH + GSSG: Cultivar (C) = 4.03, Ripening period (RP) = 5.20, C × RP = NS. GSH: C = 3.90, RP = 5.04, C × RP = 8.72. GSSG: C = 0.95, RP = 1.23, C × RP = 2.13. GSH:GSSG ratio: C = 0.33, RP = 0.43, C × RP = 0.74. LSD values for C × RP are shown as separate bars.

GSSG levels showed a significant increase during fruit ripening in all cultivars. However, the accumulation of GSSG in 'Blackamber' was at a significantly faster rate than the other two cultivars, reaching to a maximum concentration on the 6^{th} day of fruit ripening (Fig. 4.8C). As a general consequence of GSSG increase and GSH decrease, GSH: GSSG ratio also showed a decreasing trend. 'Angeleno' retained significantly higher ratio even after 8 days of fruit ripening than the other two cultivars (Fig. 4.8D).

GR activity showed a decreasing trend during the advanced stages of fruit ripening in all three cultivars (Fig. 4.9A). But the higher levels (>20 nmol NADPH oxidized min^{-1} mg^{-1} protein) of GR activities were observed up to 4 and 6 days of fruit ripening in 'Amber Jewel and 'Angeleno' cultivars, respectively. The differences in GR activities on the 8^{th} day of fruit ripening were statistically non–significant among these cultivars. The activities of GT were found significantly higher in 'Blackamber' and 'Amber Jewel' than in 'Angeleno' (Fig. 4.9B). The highest GT activity was observed during fruit ripening in 'Blackamber' followed by 'Amber Jewel' and 'Angeleno'.

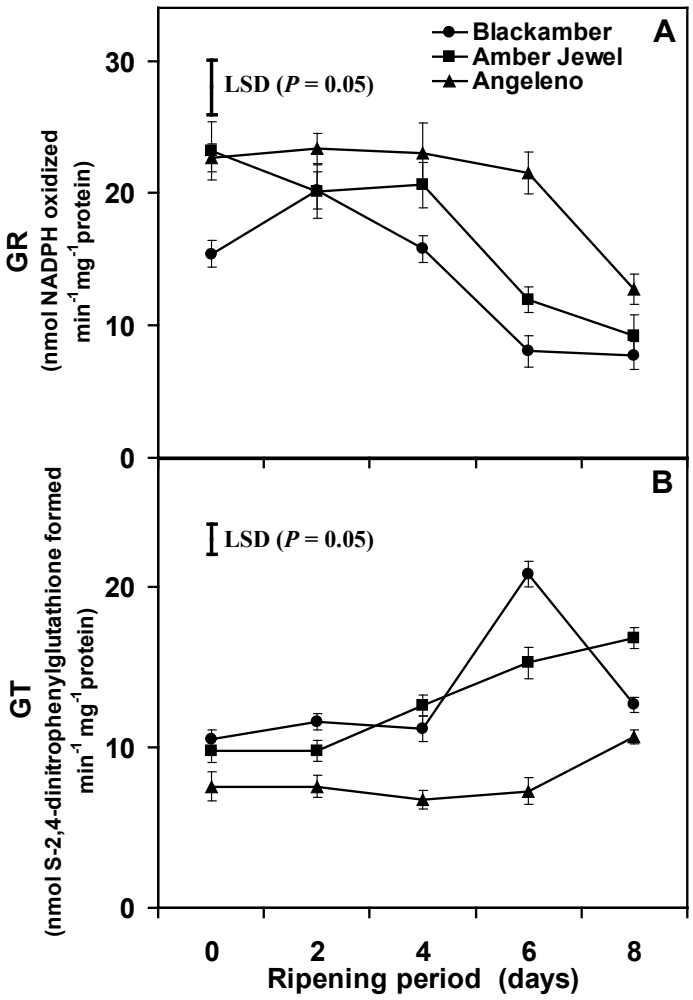


Fig. 4.9. Changes in activities of GR (A) and GT (B) in the flesh tissue of three cultivars of Japanese plums during fruit ripening at 21±1°C. Vertical bars represent S.E. of means. All measurements were made in triplicate. LSD (P = 0.05) values for GR: Cultivar (C) = 1.85, Ripening period (RP) = 2.39, C × RP = 4.14. GT: C = 0.82, RP = 1.06, C × RP = 1.83. LSD values for C × RP are shown as separate bars.

4.3.6 Phenols and DPPH· radical scavenging activity

A significant difference in the total phenolics concentration and the antioxidant capacity of flesh tissues of three cultivars was observed (Fig. 4.10). The concentrations of total phenolics increased significantly during the first 6 days of fruit ripening in 'Blackamber' and 'Amber Jewel' cultivars followed by a decline. A net decrease in the total phenolics was observed during 8 days of ripening period in 'Blackamber' and 'Angeleno', whereas it increased in 'Amber Jewel'. The trends in changes of the DPPH· radical scavenging activity followed very closely to those of the total phenolics.

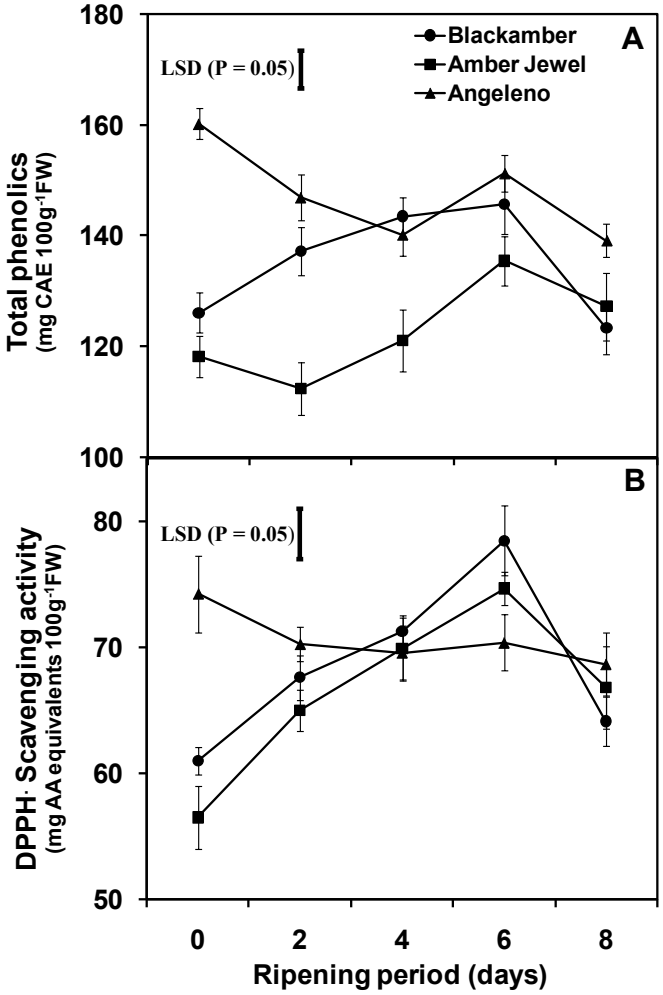


Fig. 4.10. Changes in total phenolics concentration (A) and DPPH scavenging activity (B) in the flesh tissue of three cultivars of Japanese plums during fruit ripening at 21±1°C. Vertical bars represent S.E. of means. All measurements were made in triplicate. LSD (P = 0.05) values for total phenolics: Cultivar (C) = 3.07, Ripening period (RP) = 3.96, C × RP = 6.85. DPPH scavenging activity: C = 1.80, RP = 2.33, C × RP = 4.03. LSD values for C × RP are shown as separate bars.

4.4 Discussion

4.4.1 Fruit ripening

The different levels of ethylene production and respiration rates (Fig. 4.1) achieved during fruit ripening in 'Blackamber', 'Amber Jewel', and 'Angeleno' cultivars observed in this study confirmed the previous reports (Candan et al., 2008; Díaz–Mula et al., 2009; Khan and Singh, 2007b). For discussion purpose, these cultivars are placed into three categories: highly–climacteric, moderately–climacteric, and suppressed–climacteric, respectively. A few other cultivars of Japanese plums have

also been reported to exhibit suppressed–climacteric behaviour during fruit ripening (Abdi et al., 1998; Díaz–Mula et al., 2009). The changes during fruit ripening were evident from flesh softening and decrease in L* and hue angle values for skin colour in all cultivars (Fig. 4.2). These changes, however, progressed in 'Angeleno' cultivar even in the absence or low amounts of ethylene in the initial stages of ripening. These observations support the findings of Abdi et al. (1997) and Díaz–Mula et al. (2009) that the changes occurring during fruit ripening of suppressed–climacteric cultivars of Japanese plums are, to some extent, independent of ethylene. These cultivars can not be classified as non–climacteric because they respond to exogenous application of propylene, an analogue of ethylene, like climacteric fruits (Abdi et al., 1997). The increase in SSC:TA ratio is mainly due to the loss of organic acids during fruit ripening in plums (Singh and Singh, 2008a), but the decrease in TA in 'Angeleno' was minor. The lower rate of respiration in 'Angeleno' might have helped conservation of malic acid, which is the principal organic acid and may act as a respiratory substrate (Singh et al., 2009).

4.4.2 Lipid peroxidation

Fruit ripening has been described as an oxidative phenomenon involving the production of ROS, leading to senescence (Brennan and Frenkel, 1977; Rogiers et al., 1998). Membrane lipid peroxidation has been suggested to be a characteristic of the truly senescing systems, and is initiated by either enzymatic activities or ROS (Shewfelt and del Rosario, 2000; Shewfelt and Purvis, 1995). In enzymatic pathway, LOX, acting in concert with other hydrolytic enzymes, is responsible for the peroxidation of PUFA in membrane systems resulting in the accumulation of lipid hydroperoxides. It is still unresolved whether the accumulation of peroxidative products is a primary cause or a secondary effect, but these are further metabolized through various pathways in a complex manner (Shewfelt and Purvis, 1995). The increased LOX activities were noticed in three plum cultivars which corresponded with the increase in ethylene production rates during fruit ripening (Figs. 4.4A & 4.1B). LOX has been implicated in diverse functions associated with fruit ripening and its activity has been reported to be up–regulated by ethylene during fruit ripening in apple (Schaffer et al., 2007), kiwifruit (Zhang et al., 2009), peach (Zheng et al., 2007) and saskatoon (Rogiers et al., 1998). The data also suggest that LOX activity

may be regulated by ethylene in plums, and may be helpful in explaining the extent of lipid peroxidation occurring in cultivars showing different rates of ethylene evolution. Ethylene has been implicated in enhanced lipid peroxidation during senescence of carnation petals (Bartoli et al., 1996).

The lipid hydroperoxides can be metabolized to various products including TBARS such as malondialdehyde (Hodges et al., 1999). The estimation of TBARS can, therefore, indicate the extent of lipid peroxidation and hence oxidative stress. The results showed that TBARS content increased during fruit ripening reflecting the degradation of membrane lipids that might be caused by the increased LOX activity (Jiménez et al., 2002a; Rogiers et al., 1998; Zheng et al., 2007). However, the rate of TBARS accumulation in suppressed–climacteric cultivar showing lower LOX activity was less than in highly– and moderately–climacteric cultivars with higher LOX activities (Fig. 4.4B). Previous studies have also shown that the cultivars belonging to the same plant species differing in their senescence rates also showed this pattern of TBARS accumulation (Hodges and Lester, 2006; Lacan and Baccou, 1998; Lester and Saftner, 2008). The ROS–mediated membrane lipid damage might also be contributing to the increased concentrations of TBARS and finally membrane disintegration and dysfunction of membrane bound enzymes (Shewfelt and Purvis, 1995).

The increase in EL due to membrane disruption is another indicator of lipid peroxidation. A higher LOX activity coupled with a higher TBARS concentration indicate the cell membrane dissociation resulting in electrolyte leakage and fruit softening (Lester and Saftner, 2008). The percent increase in EL during 8 days of fruit ripening period was significantly higher in ‘Blackamber’ and ‘Amber Jewel’ than in ‘Angeleno’ (Fig. 4.4C). Earlier, a similar increase in EL during fruit ripening in peaches has been reported (Zheng et al., 2007). In addition to LOX and TBARS, the increase in EL provides an additional evidence of the increasing oxidative stress developing during fruit ripening in Japanese plums depending upon the cultivar.

4.4.3 Antioxidant enzymes (SOD, CAT, and POD)

The generation of ROS such as $O_2^{\cdot -}$, H_2O_2 and $OH^{\cdot}$ during fruit ripening and senescence is natural and unavoidable. The primary line of defense involving

antioxidant enzymes such as SOD, CAT, POD, and APX operate to scavenge these ROS to avoid their accumulation to toxic levels. During early stages of fruit ripening in three cultivars of Japanese plums, the SOD activity remained higher, but followed a significant decline as the ripening progressed (Fig. 4.5A). The reports on changes in the SOD activity during fruit ripening and senescence are inconclusive. The increase in SOD activity has been reported during senescence of apple (Du and Bramlage, 1994), but it decreased during fruit ripening of blackberry (*Rubus* sp.) (Wang and Jiao, 2001), orange (Huang et al., 2007), plum (Larrigaudiére et al., 2009), mango (Singh and Dwivedi, 2008), peach (Zheng et al., 2007), and saskatoon (*Amelanchier alnifolia* Nutt.) (Rogiers et al., 1998). The decrease in SOD activity indicates a decline in the capacity of fruit to neutralize the $O_2^{\bullet-}$ radicals produced during climacteric stages of fruit ripening.

It was hypothesized that the ROS production may increase during climacteric rise in respiration as the mitochondrial electron transport chain is a major source of ROS in non–photosynthetic tissues (Apel and Hirt, 2004). SOD catalyses the dismutation of $O_2^{\bullet-}$ into H_2O_2 which is further metabolized by CAT, APX, and POD. CAT is considered more efficient as it does not require any reducing power for H_2O_2 detoxification as opposed to guaiacol–POD and APX, but it has low substrate affinity (Apel and Hirt, 2004). CAT activity remained higher during initial stages of fruit ripening in 'Blackamber' and 'Amber Jewel' and then declined, which is in contrast to 'Angeleno' which showed a progressive increase over the entire ripening period (Fig. 4.5B). The highest CAT activity in 'Angeleno' on the 8th day of fruit ripening points towards the functioning of a highly efficient system of H_2O_2 removal, whereas high POD activities in 'Blackamber' and 'Amber Jewel' cultivars during the same period showed that H_2O_2 removal is occurring at the expense of cellular reductants. A similar trend in the activities of CAT and POD has also been reported during fruit ripening in climacteric–type 'Larry Ann' plums (Larrigaudiére et al., 2009). Decreased CAT activity as the fruit ripens results in decreased H_2O_2–scavenging capacity (Wang and Jiao, 2001). Higher POD activity may also be considered a reflection of the extent of tissue damage that might have occurred during late stages of ripening in highly– and moderately–climacteric cultivars as the higher POD activity has been linked to more tissue damage in strawberry (Vicente et al., 2006) and over–ripeness in blackberry (Wang and Jiao, 2001). The data on the dynamics of

primary antioxidant enzymes, SOD, CAT, and POD, implicate the development of oxidative stress to a greater extent in climacteric type 'Blackamber' and 'Amber Jewel' cultivars compared with suppressed cultivar, 'Angeleno'.

4.4.4 Ascorbate–glutathione cycle

AA and GSH are redox buffers crucial to the maintenance of a strong antioxidant system in the cell (Noctor and Foyer, 1998). The data show a significant difference in the concentrations of AA and GSH among three cultivars at harvest (Fig. 4.6B & 4.8B). At harvest, AA concentration was significantly higher in 'Amber Jewel' than in 'Blackamber' and 'Angeleno' plums. Davey and Keulemans (2004) proposed a hypothesis that early maturing apple cultivars are low in AA than the late maturing ones. It appears to be partially true in plums as 'Blackamber', an early cultivar, had lower AA than 'Amber Jewel', a mid–season cultivar. However, the late season cultivar, 'Angeleno', showing AA levels lower than 'Amber Jewel' poses a contradiction to the above hypothesis. The variation in the antioxidant concentrations in different genotypes of fruits exists as it is influenced by multiple extrinsic and intrinsic factors (Davey et al., 2004; Davey and Keulemans, 2004; Hodges and Lester, 2006). AA and GSH are very important components of the ascorbate–glutathione cycle, which operates for the removal of H_2O_2 from the cell involving oxidation of AA and GSH, and their regeneration through enzymatic or non–enzymatic pathways (Noctor and Foyer, 1998).

No significant change in the concentrations of AA was observed during fruit ripening in 'Blackamber' and 'Angeleno', whereas a significant loss of AA was observed in 'Amber Jewel' (Fig. 4.6B). The decrease in AA levels during fruit ripening has also been reported in apples (Davey and Keulemans, 2004) and blackberries (Wang and Jiao, 2001). The concentrations of DHA increased during 8 days of fruit ripening period; 'Blackamber' showed the highest percent increase in DHA during this period (Fig. 4.6C). However, transient increases in AA during 4^{th} and 6^{th} day of fruit ripening in 'Angeleno' and 'Amber Jewel', indicate the possibility of simultaneous biosynthesis of AA during fruit ripening in plums as in case of tomato (Jiménez et al., 2002a). In relation to the amount of AA oxidized, the disproportionate accumulation of DHA in 'Blackamber' and 'Angeleno' also

provides another evidence of AA biosynthesis. The higher AA levels have been related to increased capacity to cope with the oxidative stress (Davey et al., 2004).

APX catalyses the conversion of H_2O_2 into water using two molecules of AA as a reducing power with a concomitant production of two molecules of MDHA. MDHA radicals rapidly disproportionate into DHA and AA; the latter reaction is catalysed by MDHAR using NADPH as the electron donor. The DHA is reduced back to AA by the action of DHAR, using GSH as the reducing agent. The decreased concentrations of AA during fruit ripening might be attributed to the increased APX activity (Fig. 4.7A). In 'Blackamber' and 'Amber Jewel', APX activity increased in the initial stages of fruit ripening, that might be in response to increasing need for H_2O_2 removal utilizing AA. It declined significantly on the 8^{th} day of fruit ripening, but remained higher in 'Angeleno', indicating its antioxidant potential was better than the other two. A minor increase in APX activity during initiation of fruit ripening and a significant decline during 3 days of ripening at 20°C after 28 days of cold–storage at 0°C in peaches has also been reported earlier (Wang et al., 2006; Zheng et al., 2007). Higher APX activity has been linked to confer a greater resistance to postharvest oxidative stress in 'Orange Dew' and 'Honey Brew' melons (Hodges and Lester, 2006).

The increased concentrations of DHA during fruit ripening resulted in an overall decrease in the AA:DHA ratio, which differed non–significantly among three cultivars on the 8^{th} day (Fig. 4.6C & D). 'Blackamber' showed the highest decrease in AA:DHA ratio as compared with 'Amber Jewel' and 'Angeleno'. The decrease in AA:DHA ratio in these plum cultivars suggests that the cellular environment would presumably be in more oxidative state on the 8^{th} day of fruit ripening which coincided with the decrease in MDHAR and DHAR activities. (Fig. 4.7B & C) The transient increases in MDHAR and DHAR activities reflect the regeneration of AA towards restoration of the antioxidant potential of fruit, and consequently resulting into lower levels of DHA during initial stages of fruit ripening. But, the lower levels of AA during later stages of fruit ripening might be due to a decline in the regeneration capacity, possibly resulting from the lower activities of MDHAR and DHAR. The MDHAR and DHAR activities have been reported to decrease during fruit ripening in blackberries (Wang and Jiao, 2001).

Concentrations of GSH and ratio of GSH:GSSG consistently decreased during fruit ripening in all three cultivars (Fig. 4.8). The decrease in GSH during fruit ripening in apples, blackberries, oranges, and peaches has been reported (Davey and Keulemans, 2004; Huang et al., 2007; Wang et al., 2006; Wang and Jiao, 2001), which is in contrast to increase in GSH and GSSG levels during late stages of fruit ripening in saskatoon (Rogiers et al., 1998). The increase in glutathione levels has been directed towards the acclimatory response of fruit to oxidative stress (Rogiers et al., 1998; Wang et al., 2006). The accumulation of GSSG and decline in GSH during fruit ripening in plums could be attributed to the decrease in GR and increase in GT activities, respectively (Fig. 4.9). GR is responsible for conversion of GSH into GSSG, whereas GT catabolises the lipid hydroperoxides formed during lipid peroxidation using GSH as a reducing agent (Rogiers et al., 1998). GSH:GSSG ratio remained higher in suppressed–climacteric than in climacteric cultivars during later stages of ripening. The higher GSH levels in 'Angeleno' may also be explained on the basis of possibility of GSH biosynthesis exceeding its degradation and also higher GR activity during fruit ripening. The activation of GSH biosynthesis and up–regulation of GR activity appear to be essential components of the oxidative stress tolerance (Davey et al., 2004; Wang et al., 2006). The GSH levels and GSH:GSSG ratio have been reported to be influenced by the cultivar type in apples and blackberries (Davey and Keulemans, 2004; Wang and Jiao, 2001), maturity stage in oranges and saskatoon (Huang et al., 2007; Rogiers et al., 1998), and cold–storage in peaches (Wang et al., 2006). The data show that despite the possibility of AA and GSH biosynthesis, the redox status of the AA and GSH pools was significantly shifted towards oxidized state during fruit ripening in plums suggesting the increasing levels of oxidative stress developing to a variable extent.

4.4.5. Total phenolics and DPPH· scavenging activity

Phenolic compounds play an important role in ROS scavenging from the vacuole in the cell in concert with AA and peroxidase, and this system has been termed as ascorbate/phenolics/peroxidase system (Takahama, 2004). Total phenolics concentrations increased during the first 6 days of fruit ripening in 'Blackamber' and 'Amber Jewel' followed by a decrease on the 8th day, and is contrary to 'Angeleno' showing a decrease until 4th day followed by a transient increase on the 6th day (Fig.

4.10A). The increase in phenolics in flesh of several cultivars of Japanese plums, including 'Blackamber' and 'Angeleno' has been reported to occur during cold storage and subsequent shelf life at 20°C (Díaz–Mula et al., 2009). However, the decrease in total phenolics in these cultivars was not observed by Diaz–Mula et al. (2009) as these authors did not evaluate the fruit beyond 4 days of shelf life after cold storage. The reason for the decrease in total phenolics on the 8th day of ripening is uncertain. Phenolics can be oxidized by the peroxidases in the presence of H_2O_2 because of the co–existence of phenolics and peroxidase in vacuoles (Takahama, 2004). A very high POD activity observed on the 8th day of fruit ripening in 'Blackamber' and 'Amber Jewel' plum cultivars may be linked to the degradation of the phenolics and ascorbate for H_2O_2 removal. It could be surmised that phenolic compounds in co–operation with other antioxidative processes might have contributed to cope with the increasing oxidative stress towards late stages of fruit ripening.

The observed pattern of changes in phenolics was closely related to the changes in antioxidant capacity, measured as DPPH· scavenging activity, during fruit ripening of three cultivars of plums (Fig. 4.10B). The total antioxidant capacity in plum flesh tissue is mainly contributed by the phenolics and to very little extent by ascorbate and carotenoids (Díaz–Mula et al., 2009; Gil et al., 2002). A very strong correlation between total phenolics and antioxidant capacity measured by DPPH and ferric reducing ability of plasma (FRAP) methods has been reported in the flesh tissues of plums, peaches and nectarines evaluated at the ripe stage (Gil et al., 2002).

During adaptation or response to stress conditions, the spatial and temporal variation in the enzymatic and non–enzymatic antioxidant components is prevalent at the subcellular level (Jiménez et al., 2002b). This study determined the dynamics of antioxidant enzymes and metabolites in total extracts of the flesh tissue of ripening plums. Further research is required to understand the changes in antioxidant systems occurring at the subcellular level that regulate fruit ripening process in plums. The three cultivars of Japanese plums differing in their climacteric levels during fruit ripening exhibit differences in their oxidative behaviour. Further work is required to explore whether the differences in antioxidant metabolism of these cultivars influence their long–term cold storage and susceptibility to physiological disorders. It may be concluded that increasing levels of oxidative stress are related to the levels of

climacteric during fruit ripening in Japanese plums. The suppressed–climacteric cultivars can, therefore, retain their postharvest quality longer than the climacteric cultivars.

Chapter 5

Harvest Maturity in Relation to Antioxidative Metabolism during Postharvest Chilling Stress in Japanese Plums[2]

Summary

Harvest maturity is an important factor influencing the dynamics of enzymatic and non–enzymatic antioxidants in relation to the development of postharvest physiological disorders in fruits. The objective was to determine if the levels of antioxidants at harvest and during cold storage at 0°C for 7 weeks can influence the development of CI and other biochemical changes in the fruit of Japanese plums cv. 'Amber Jewel' harvested at commercial maturity and one week after commercial maturity. Fruit quality, lipid peroxidation, enzymatic and non–enzymatic antioxidant components in fruit flesh were determined immediately after storage at weekly intervals. The incidence and severity of CI, which appeared in the form of flesh browning after 3 weeks of storage, did not differ significantly for the first 5 weeks of storage in fruit from both harvests, but was significantly higher in the form of flesh translucency during the last 2 weeks of storage in delayed harvested fruit than in commercial harvest. The extent of membrane lipid peroxidation, indicated by the increase in LOX activity, concentrations of TBARS, and enhanced EL, increased with the increase in storage duration, and was significantly higher during storage in late harvested fruit compared with the commercial harvest. In commercially harvested fruit, SOD increased significantly during the first two weeks of storage followed by a decline, but remained significantly higher during most of the storage period than in delayed harvested fruit. CAT activity also increased significantly in fruit from both maturities during the first 2 weeks of storage, remained at a higher level for further 4 weeks in commercial harvest, but declined thereafter in delayed harvested fruit. A significant decrease in AA:DHA and GSH:GSSG ratios was observed with the prolonged storage, irrespective of harvest maturity. The severity of CI symptoms was linked to the predominance of the oxidized state of the tissue as reflected by lower

[2] The additional data on the changes in concentrations of individual sugars and organic acids from this experiment has been published in the following article: Singh, S.P., Z. Singh, and E.E. Swinny. 2009. Sugars and organic acids in Japanese plums (*Prunus salicina* Lindell) as influenced by maturation, harvest date, storage temperature and period. *International Journal of Food Science and Technology*. 44:1973–1982.

ratios of AA:DHA and GSH:GSSG during the last 3–4 weeks of storage. The possible roles of enzymes, such as APX, MDHAR, DHAR, GR, and GT, were examined with regard to the levels of antioxidant pools of AA and GSH and development of CI symptoms. The increased lipid peroxidation, decrease in levels of AA and GSH, and lower activities of SOD, GR, MDHAR and DHAR indicated the development of oxidative stress due to prolonged cold storage. In conclusion, the acclimatory response to chilling stress in Japanese plums as indicated by the activities of various antioxidant enzymes, levels of AA and GSH, and ascorbate–glutathione cycle enzymes was too weak and slow to compensate for the presumed increased production of ROS during prolonged cold storage. Therefore, the changes in antioxidant components during cold storage of Japanese plums seem to be more important in providing protection against oxidative injury expressed as CI than their at–harvest antioxidant status.

5.1 Introduction

Japanese plums (*Prunus salicina* Lindl.) have short postharvest life depending upon the cultivar and supply–chain conditions. The low–temperature storage at 0°C is recommended for extending the shelf life and maintaining the fruit quality during prolonged market period and long–distance transport (Mitchell et al., 1974). The storage potential of Japanese plums is influenced by several factors such as cultivar, harvest maturity, storage conditions, and susceptibility to postharvest physiological disorders and diseases (Abdi et al., 1997; Crisosto et al., 1999; Taylor et al., 1995). The long–term cold storage of plums results in development of CI symptoms, which appear in the form of flesh browning, mealiness and flesh translucency (Candan et al., 2008; Crisosto et al., 1999; Manganaris et al., 2008; Singh et al., 2009a). The CI symptoms proliferate during shelf life or marketing period after cold storage, and can have detrimental effects on fruit quality and consumer experiences. Many factors influence susceptibility of plums to CI during and after storage, including cultivar, harvest maturity, and storage duration and conditions (Abdi et al., 1997; Crisosto et al., 2004; Taylor et al., 1995; Ward and Melvin–Carter, 2001).

Oxidative stress has been proposed to be an early response to development of CI during cold storage of many fruits (Hariyadi and Parkin, 1991; Mao et al., 2007;

Sala, 1998; Sala and Lafuente, 2000; Zhao et al., 2009). The oxidative stress develops as a consequence of ROS generation exceeding the capability of the antioxidant system in the cell (Hodges et al., 2004). The chilling stress has been implicated in activation of the antioxidant defense system in response to increasing pro–oxidant levels (Sala, 1998; Sala and Lafuente, 2000). However, the reduction or failure of the enzymatic and non–enzymatic antioxidants to protect against ROS can cause oxidative damage leading to enhanced lipid peroxidation and loss of membrane integrity in the tissue (Hariyadi and Parkin, 1991; Hodges et al., 2004; Mao et al., 2007). However, it is still unclear whether the oxidative stress is the cause or effect of CI.

In addition to CI, the development of other postharvest physiological disorders in fruit crops has been related to the antioxidant levels at harvest and the changes in their concentrations during cold storage. The incidence of superficial scald in apples has been reported to be reduced by delayed harvesting due to accumulation of lipid–soluble antioxidants in the skin (Barden and Bramlage, 1994; Diamantidis et al., 2002). However, the delayed harvesting in 'Conference' and 'Passa Crassana' pears (Lentheric et al., 1999; Vanoli et al., 1995) and 'Braeburn' apples (Gong et al., 2001; Toivonen et al., 2003) increased their susceptibility to internal browning disorders. The development of internal browning disorders in apples and pears has been linked to the decrease in concentrations of AA during storage (Davey and Keulemans, 2004; De Castro et al., 2008; Veltman et al., 1999). Similarly, the decreased activity of SOD enzyme in flesh tissue increased the incidence of Braeburn browning disorder (BBD) in late–harvested 'Braeburn' apples (Toivonen et al., 2003). It has been widely argued that the initiation or aggravation of various physiological disorders in fruit was due to an inefficient antioxidant system in the skin or flesh tissue, but the cause–effect relationship is still unclear in many cases.

Given the importance of harvest maturity in relation to antioxidative systems in fruits, it has not been investigated whether the differences in enzymatic and non–enzymatic antioxidants at harvest persisted or changed during the cold storage of Japanese plums and their relation to the development of CI symptoms. In previous studies (Abdi et al., 1997; Taylor et al., 1993; Taylor et al., 1995), the changes during cold storage of plums of different maturity groups have shown that the delayed harvesting increases the susceptibility to CI expressed as flesh translucency.

However, no information is available on the oxidative behaviour of Japanese plums during prolonged cold storage in relation to harvest maturity. It was therefore hypothesized that the dynamics of antioxidant components may explain the development of CI during cold storage of Japanese plums harvested at commercial and delayed maturities.

5.2 Materials and methods

5.2.1 Fruit material, storage conditions, and experimental design

In Western Australia, Japanese plums cv. 'Amber Jewel' is considered the most susceptible to CI and may be used as a model cultivar for investigating the mechanisms of CI development (Ward and Melvin–Carter, 2001). Therefore, this experiment was conducted on 'Amber Jewel' cultivar. The fruit were harvested at commercial maturity (129 days after full bloom; firmness = 48.6 N, SSC = 16.4°Brix, TA = 1.29%) and one week after commercial maturity (delayed–harvesting; firmness = 31.1 N, SSC = 17.9°Brix, TA = 1.14%) in the early morning hours from the Casuarina Valley Orchard, Karragullen, Perth Hills (latitude 31° 57' S; longitude 115° 50' E), WA. Fruit of uniform size and maturity, free from visual blemishes and disease were harvested from the orchard, transported to the laboratory, and stored in plastic crates lined with 30 µm thick low density polyethylene (LDPE) film (AMCOR Packaging, Pvt. Ltd., Melbourne, Australia) at 0°C and 86.5±5.5% RH for 7 weeks.

Lots of 20 fruit from each of the three replicates from both maturity groups were transferred from cold store at weekly intervals, allowed to stay at 20±1°C for about 4 h, and were analysed for changes in fruit quality. Fruit flesh tissue was cut into small cubes and immediately frozen with liquid nitrogen and stored at –80°C until further analyses. The experiment was laid out by following a completely randomised design with two factors including harvest maturity and storage period.

5.2.2 Fruit firmness, colour, SSC, TA, and SSC:TA

The fruit quality was evaluated at weekly interval during 7 weeks of cold storage at 0°C. Fruit firmness, colour, SSC, and TA were determined as described in the Section 4.2.3 of the previous chapter.

5.2.3 Chilling injury (CI)

The incidence and severity of CI was evaluated 4 h after transfer from cold storage to 21±1°C. Twenty plums per replication were cut around the equatorial axis, the two halves of each fruit twisted in opposite directions, and the mesocarp was examined for symptoms such as flesh browning, mealiness, and translucency. The incidence and severity of CI were calculated as described in the Section 3.4.4.

5.2.4 Oxidative stress parameters

The determinations of lipid peroxidation, enzymatic and non–enzymatic antioxidants, and protein concentration were carried out as described in the Chapter 3 on general materials and methods (Sections 3.5 to 3.8).

5.2.5 Statistical analysis

The data were subjected to a two–way analysis of variance (ANOVA) using GenStat Release 11.1 (VSN International Ltd., Hemel Hempstead, UK). Before statistical analysis, the data on CI incidence were subjected to arcsine transformation to reduce heteroscedasticity. The effects of harvest maturity and storage duration on different parameters were assessed within ANOVA and the least significant differences (LSD) were calculated following a significant F–test at $P \leq 0.05$.

5.3 Results

5.3.1 Fruit quality

Harvest maturity significantly affected the fruit quality parameters that included flesh firmness, skin colour, SSC, TA and SSC: TA ratio (Fig. 5.1). The flesh firmness

decreased by 1.5–fold in fruit harvested one week after commercial maturity (Fig. 5.1A). The skin colour changes, measured as L* and hue angle, also declined significantly between two harvest dates (Fig. 5.1). The delayed harvesting also resulted in a significant increase in SSC and decrease in TA that improved the SSC:TA ratio (Fig. 5.2).

The increase in storage duration caused a significant reduction in the flesh firmness of fruit, but the extent of reduction over 7 weeks of cold storage was about 37% and 50% in fruit harvested at commercial and delayed maturities, respectively (Fig. 5.1A). The decrease in L* and hue angle values was observed with the increase in storage duration and the fruit from delayed harvest showing changes in skin colour to a greater extent than those harvested at commercial maturity (Figs. 5.1B & C). The SSC increased during the first 3 and 4 weeks of storage in fruit harvested at commercial and delayed maturities, respectively, and was followed by a decrease in the next 3–4 weeks of storage leading to the differences in the SSC that existed at harvest to a non–significant level (Fig. 5.2A).

During 7 weeks of storage, the decrease in TA was relatively more following 4 and 5 weeks of cold storage in delayed and commercially harvested fruit, respectively, and did not differ significantly on the 7^{th} week of storage (Fig. 5.2B). The pattern of changes in SSC:TA ratio was based on the changes in SSC and TA (Fig. 5.2C).

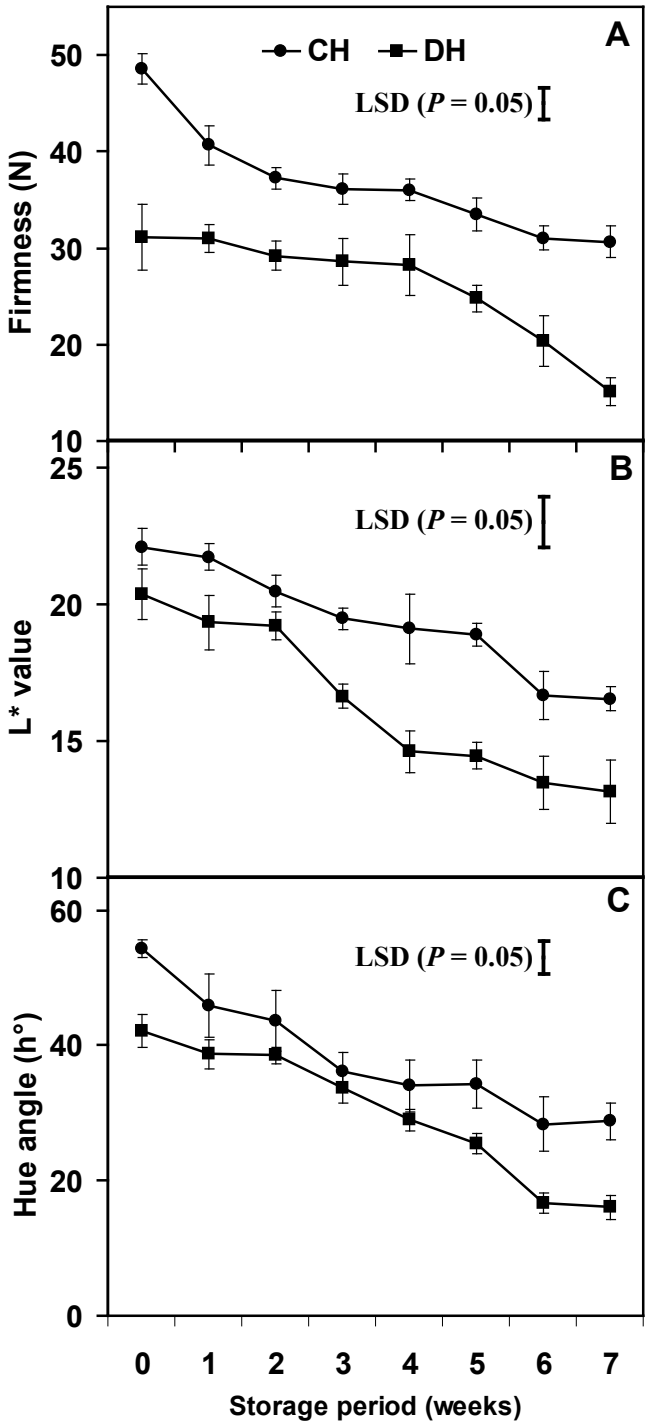


Fig. 5.1. Changes in flesh firmness (A), L* (B), and hue angle (C) values of 'Amber Jewel' plums as influenced by harvest maturity during cold storage at 0±0.3°C plus 4 h at 21±1°C. The legends, CH and DH, refer to the commercial and delayed harvests, respectively. Vertical bars represent S.E. of means. All measurements were made in triplicate. LSD (P = 0.05) values for firmness: Maturity (M) = 1.17, Storage period (SP) = 2.35, M × SP = 3.32. L*: M = 0.65, SP = 1.31, M × SP = 1.85. Hue angle: M = 1.67, SP = 3.33, M × SP = 4.71. LSD values for M × SP are shown as separate bars.

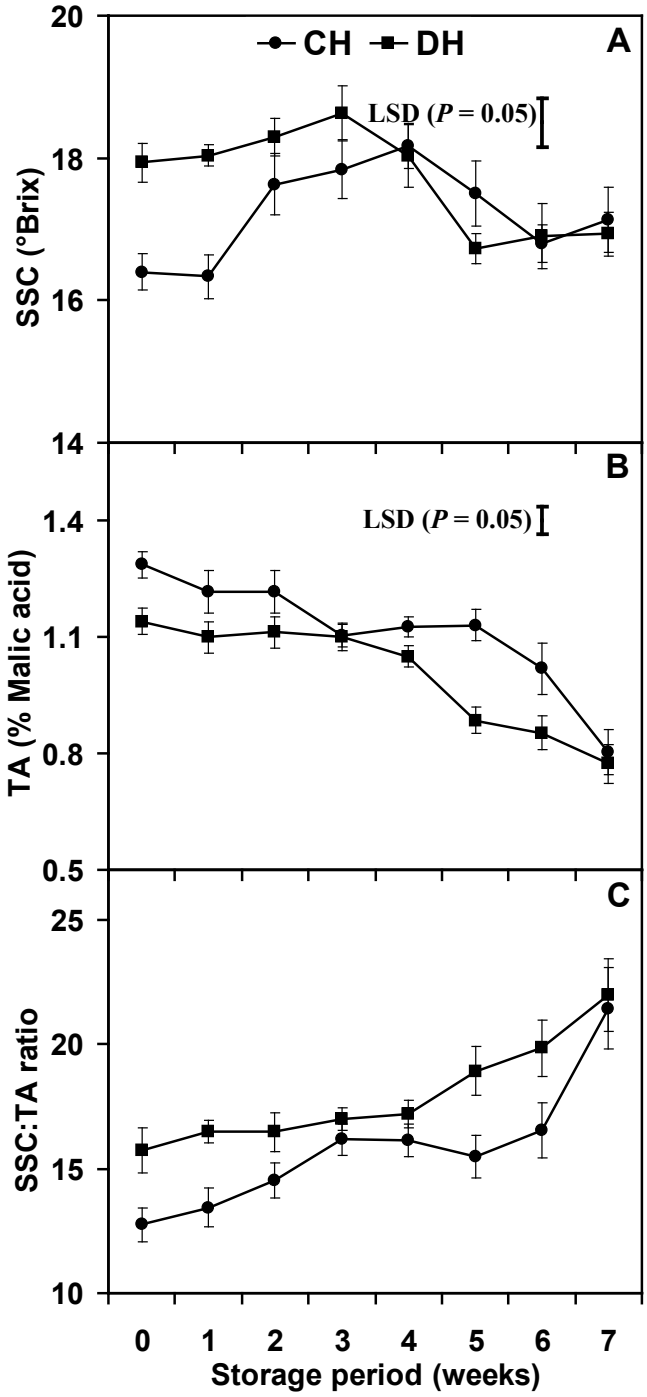


Fig. 5.2. Changes in SSC (A), TA (B), and SSC: TA ratio (C) of 'Amber Jewel' plums as influenced by harvest maturity during cold storage at 0±0.3°C plus 4 h at 21±1°C. The legends, CH and DH, refer to the commercial and delayed harvests, respectively. Vertical bars represent S.E. of means. All measurements were made in triplicate. LSD (P = 0.05) values for SSC: Maturity (M) = 0.24, Storage period (SP) = 0.48, M × SP = 0.69. TA: M = 0.03, SP = 0.05, M × SP = 0.07. SSC:TA ratio: M = 0.60, SP = 1.20, M × SP = NS. LSD values for M × SP are shown as separate bars.

5.3.2 Chilling injury (CI)

Harvest maturity and storage duration had significantly affected the development of CI symptoms (Fig. 5.3).

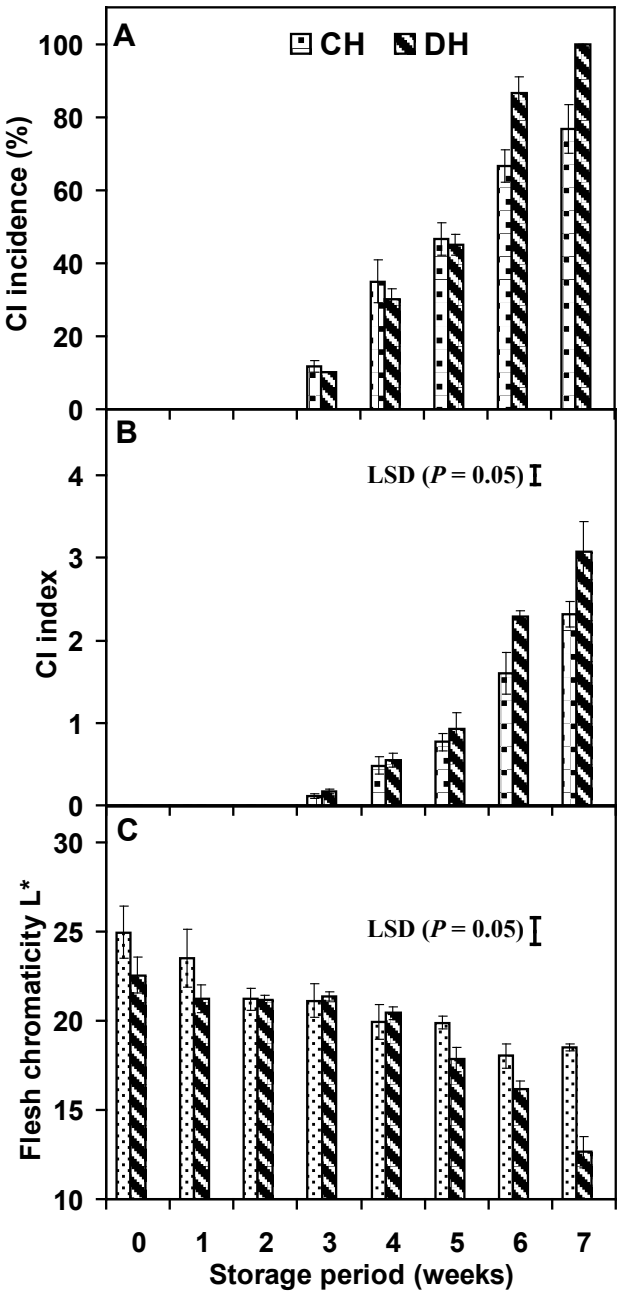


Fig. 5.3. Effects of harvest maturity and storage duration on the CI incidence (A), CI index (B), and flesh chromaticity L* values (C) of 'Amber Jewel' plums as influenced by harvest maturity during cold storage at 0±0.3°C plus 4 h at 21±1°C. The legends, CH and DH, refer to the commercial and delayed harvests, respectively. Data on CI incidence were arcsine transformed, but back–transformed data are presented for simplifying comparisons. Vertical bars represent S.E. of means. LSD (*P* = 0.05) values for CI incidence (transformed data): Maturity (M) = 0.04, Storage period (SP) = 0.08, M × SP = 0.11. CI index: M = 0.08, SP = 0.16, M × SP = 0.23. Flesh chromaticity: M = 0.52, SP = 1.04, M × SP = 1.48. LSD values for M × SP are shown as separate bars.

The first CI symptoms manifested as browning of the outer part of mesocarp were observed after 3 weeks of storage in fruit from both harvests. As the duration of cold storage progressed, the severity and incidence of CI increased in fruit from both harvests (Figs. 5.3A & B). The incidence and severity of CI did not differ significantly during the first 5 weeks of storage, regardless of fruit maturity. However, the delayed harvested fruit showed significantly higher incidence and severity of CI than the commercial harvest when the storage duration was extended to more than 5 weeks. The symptoms were mainly in the form of flesh browning in commercially harvested fruit throughout the storage, whilst these symptoms further developed to form a gel–like texture in delayed harvested fruit and predominated during the last 2 weeks of storage.

The decrease in flesh chromaticity (L*) was observed as the storage duration increased (Fig. 5.3C). The extent of decrease was significantly greater in delayed harvested fruit than in commercial harvest, especially during the last 3 weeks of storage. The delayed harvested fruit showed significantly lower L* values than the commercial harvest after 5 weeks and onwards of storage.

5.3.3 Lipid peroxidation

A one–week delay in harvesting caused a non–significant increase in LOX activity, TBARS concentration, and EL in fruit when compared with the commercial harvest (Fig. 5.4). As the storage period increased, the TBARS concentration and EL increased in fruit from both maturity groups. However, increase in LOX activity was observed only during the first 4–5 weeks of storage followed by a decline (Fig. 5.4A). The peaks in LOX activities were observed after 4 and 5 weeks of storage in fruit from the delayed and commercial harvests, respectively. The average LOX activity remained higher in fruit from the delayed harvest compared with the commercial harvest. The TBARS concentration in fruit from the delayed harvest did not significantly differ from the commercial harvest throughout the storage period, except after 7 weeks of storage (Fig. 5.4B). The EL in the commercially harvested fruit increased significantly after 3 and 6 weeks of storage in contrast to the fruit from the delayed harvested showing a significant increase during the first two weeks, followed by another increase between 4 and 6 weeks of storage (Fig. 5.4C).

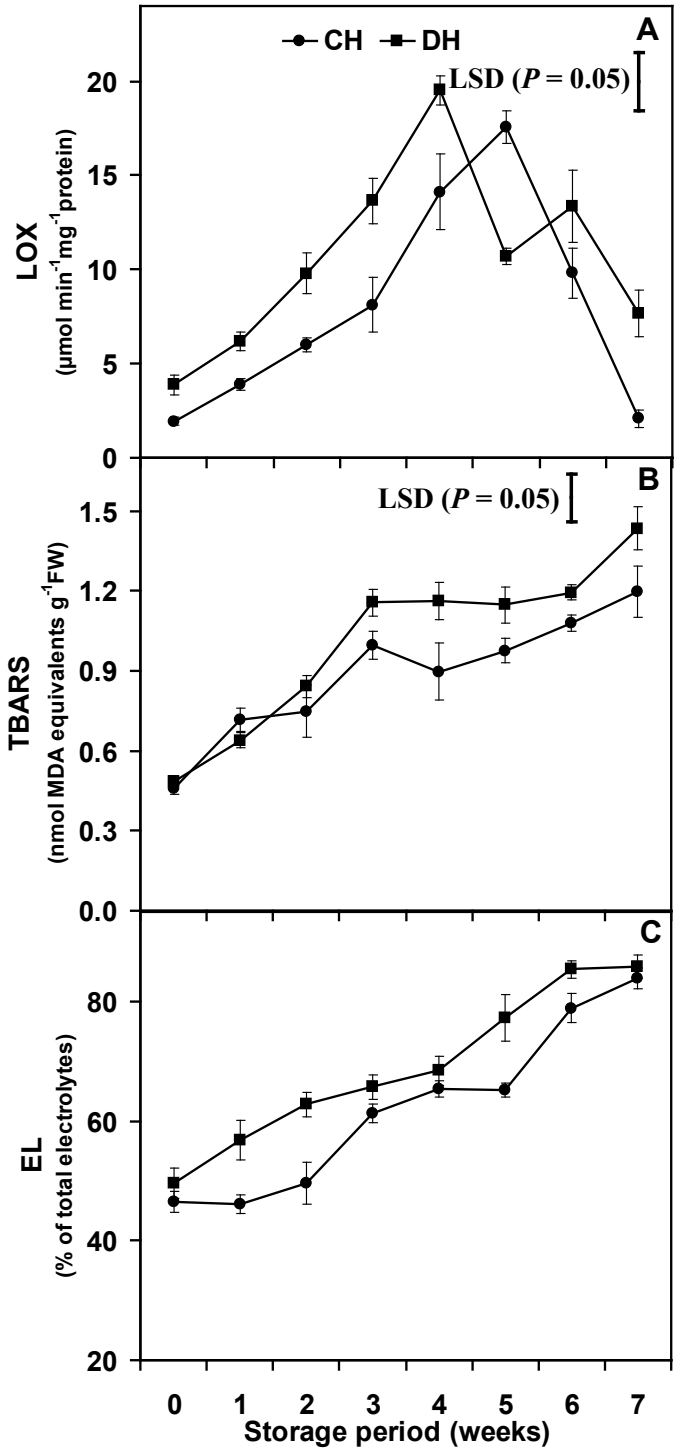


Fig. 5.4. Changes in LOX activity (A), TBARS concentration (B), and EL (C) in the flesh tissue of 'Amber Jewel' plums as influenced by harvest maturity during cold storage at 0±0.3°C plus 4 h at 21±1°C. The legends, CH and DH, refer to the commercial and delayed harvests, respectively. Vertical bars represent S.E. of means. All measurements were made in triplicate. LSD (P = 0.05) values for LOX: Maturity (M) = 1.09, Storage period (SP) = 2.18, M × SP = 3.09. TBARS: M = 0.06, SP = 0.13, M × SP = 1.85. EL: M = 2.18, SP = 4.36, M × SP = NS. LSD values for M × SP are shown as separate bars.

5.3.4 Antioxidant enzyme activities (SOD, CAT, and POD)

The data on changes in activities of SOD, CAT and POD are shown in Fig. 5.5.

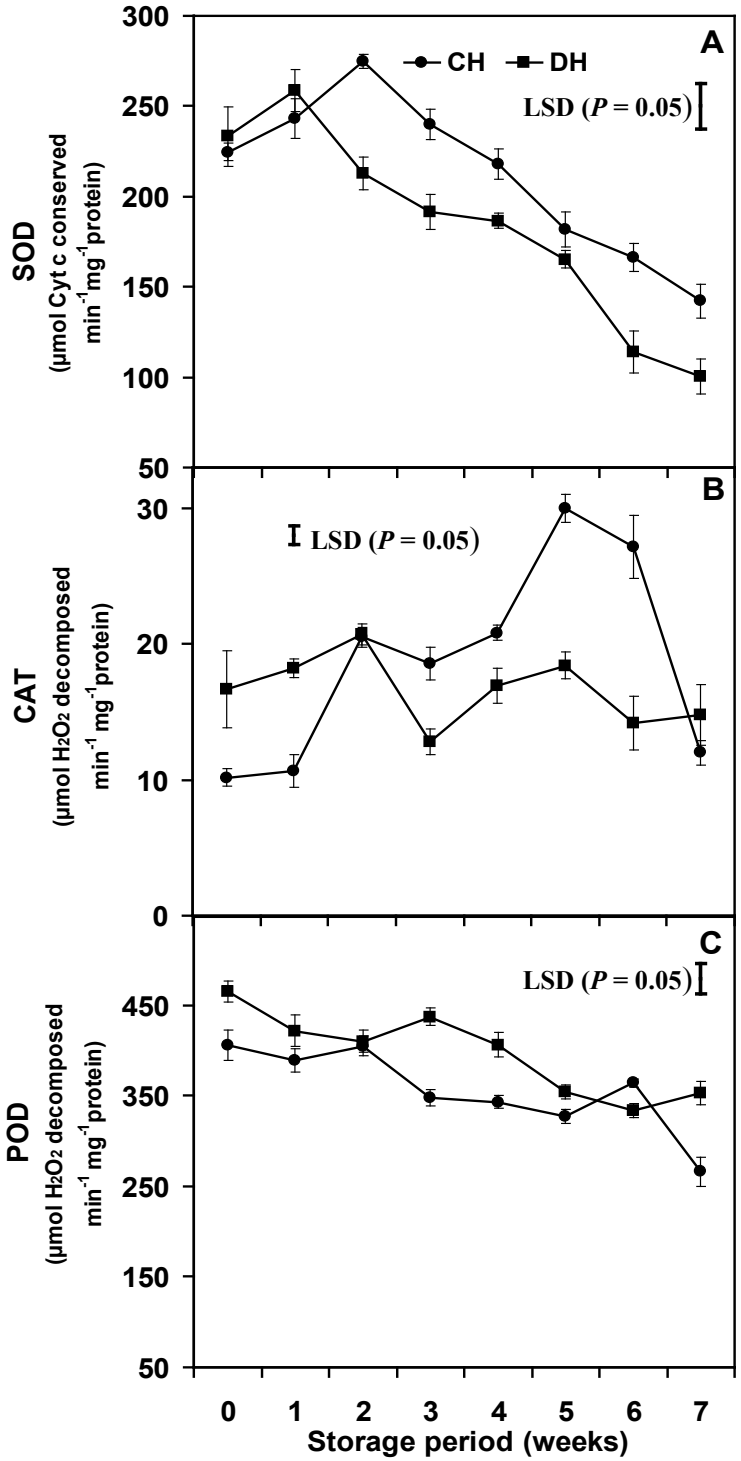


Fig. 5.5. Changes in activities of SOD (A), CAT (B), and POD (C) in the flesh tissue of 'Amber Jewel' plums as influenced by harvest maturity during cold storage at 0±0.3°C plus 4 h at 21±1°C. The legends, CH and DH, refer to the commercial and delayed harvests, respectively. Vertical bars represent S.E. of means. All measurements were made in triplicate. LSD (P = 0.05) values for SOD: Maturity (M) = 8.88, Storage period (SP) = 17.76, M × SP = 25.12. CAT: M = 1.46, SP = 2.91, M × SP = 4.12. POD: M = 2.94, SP = 5.87, M × SP = 8.30. LSD values for M × SP are shown as separate bars.

The delay in harvesting increased the activities of SOD, CAT and POD over the commercial harvest, but the increase was significant only for CAT and POD enzymes (Fig. 5.5). During the first two weeks of storage, SOD activity increased significantly in fruit from the commercial harvest compared with the delayed harvest that showed a non–significant increase only during the first week of storage (Fig. 5.5A). This increase in SOD activity during the initial storage period was followed by a consistent decrease irrespective of the harvest maturity. In general, SOD activity was observed to be significantly higher during 2–7 weeks of storage in commercially harvested fruit than in delayed–harvested fruit except on week 5.

In commercially harvested fruit, CAT activity increased to about 3–fold after 5 weeks of storage, followed by a significant decline during the last two weeks of storage (Fig. 5.5B). It showed a significant increase during the first 2 weeks of storage in delayed harvested fruit, followed by another marginal increase after 5 weeks of storage. But the overall increase in the CAT activity during storage in delayed harvested fruit was much lower than in commercially harvested fruit.

In commercially harvested fruit, POD activity showed a decreasing trend after 2 weeks of storage with a transient increase observed after 6 weeks of storage (Fig. 5.5C). POD activity remained higher during the first 4 weeks of storage in delayed harvested fruit, and then decreased significantly during the last 3 weeks of storage. The overall decrease in POD activity during 7 weeks of storage was to the extent of 1.5 and 1.3 fold in commercially and delayed harvested fruit, respectively.

5.3.5 Ascorbate–glutathione cycle

5.3.5.1 Ascorbate

At harvest, concentration of AA was significantly higher in delayed–harvested fruit than in commercial harvest (Fig. 5.6B). With the advancement of storage, the concentration of AA decreased in fruit from both maturity groups. The delayed harvested fruit showed a significantly higher AA concentration during the first 3 weeks of storage than the commercial harvest, but these differences became non–significant during the later period of storage.

During the first 3–4 weeks of storage, no consistent pattern of changes in concentrations of DHA was observed (Fig. 5.6C). However, a marked increase in

DHA was noticed after 5 weeks of storage in the fruit harvested at both maturities and it declined during the subsequent storage period. As the concentrations of AA and DHA determined their ratio (AA: DHA), it remained in the range of 2.1 to 3.4 during the first three weeks of storage in both harvests (Fig. 5.6D). But, this ratio significantly declined after 4 and 5 weeks of storage in fruit from the delayed and commercial harvests, respectively.

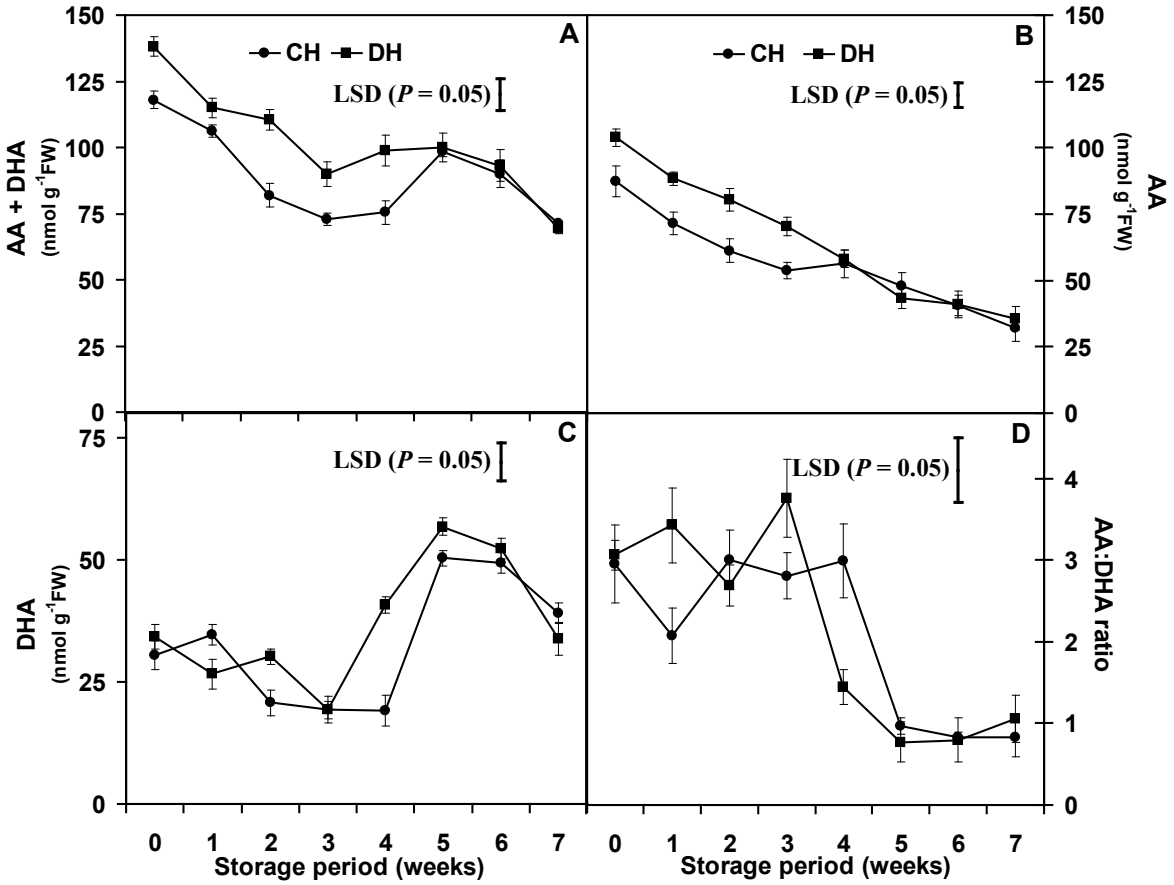


Fig. 5.6. Changes in concentrations of total ascorbate (AA + DHA) (A), AA (B), DHA (C), and AA:DHA ratio (D) in the flesh tissue of 'Amber Jewel' plums as influenced by harvest maturity during cold storage at 0±0.3°C plus 4 h at 21±1°C. The legends, CH and DH, refer to the commercial and delayed harvests, respectively. Vertical bars represent S.E. of means. All measurements were made in triplicate. LSD (P = 0.05) values for AA + DHA: Maturity (M) = 4.15, Storage period (SP) = 8.30, M × SP = 11.74. AA: M = 3.26, SP = 6.52, M × SP = 9.21. DHA: M = 2.79, SP = 5.58, M × SP = 7.89. AA:DHA ratio: M = NS, SP = 0.56, M × SP = 0.80. LSD values for M × SP are shown as separate bars.

5.3.5.2 Glutathione

Similar to AA, the concentration of GSH increased in fruit harvested one week after the commercial harvest (Fig. 5.7B). A decreasing trend in GSH concentration was

observed throughout the storage, independent of harvest maturity. The decrease in GSH over the 7 weeks of storage period was to the extent of about 1.2 and 1.4 fold in commercially and delayed harvested fruit, respectively. The differences in GSH concentrations at harvest in two maturity groups did not persist even after 1 week of storage and were non–significant during the first 4 weeks of storage in fruit from both harvests. But, the commercially harvested fruit showed higher GSH concentrations during the last 3 weeks of storage than the delayed harvest.

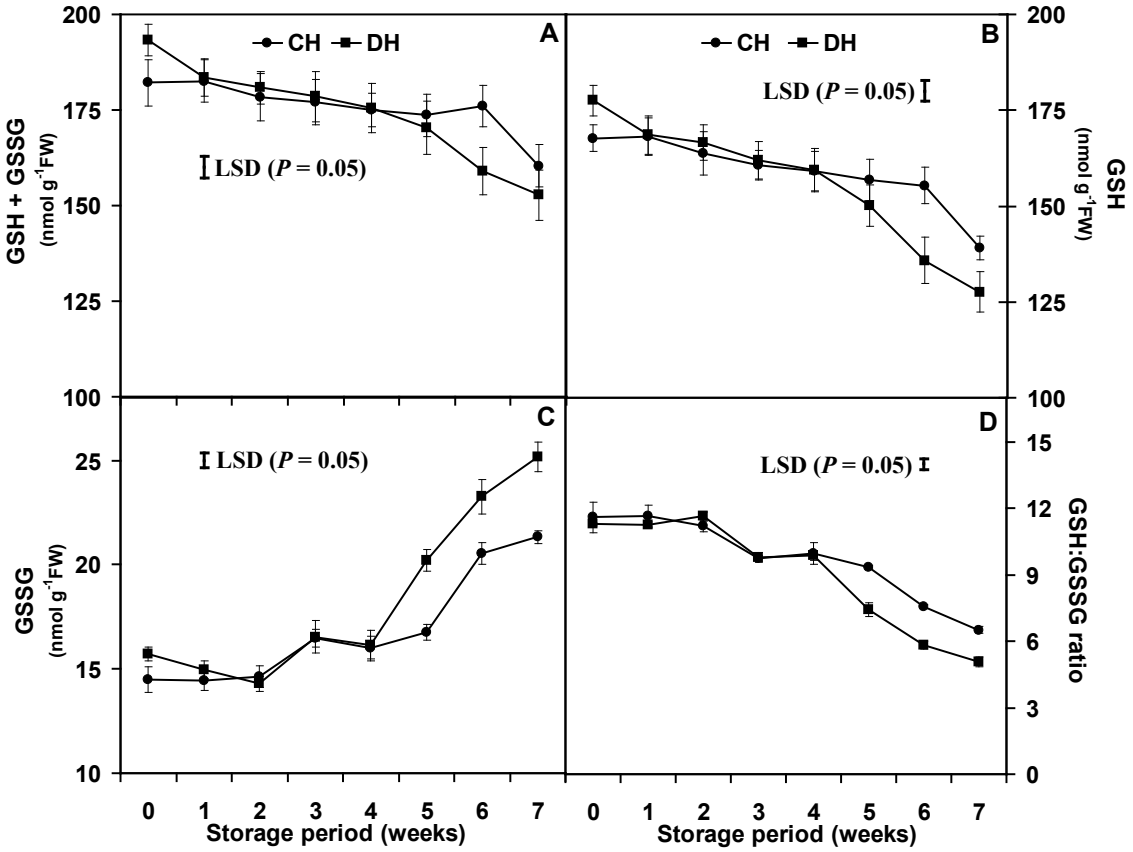


Fig. 5.7. Changes in concentrations of total glutathione (GSH + GSSG) (A), GSH (B), GSSG (C), and GSH:GSSG ratio (D) in the flesh tissue of 'Amber Jewel' plums as influenced by harvest maturity during cold storage at 0±0.3°C plus 4 h at 21±1°C. The legends, CH and DH, refer to the commercial and delayed harvests, respectively. Vertical bars represent S.E. of means. All measurements were made in triplicate. LSD (P = 0.05) values for GSH + GSSG: Maturity (M) = NS, Storage period (SP) = 3.97, M × SP = 5.62. GSH: M = 1.93, SP = 3.87, M × SP = 5.47. GSSG: M = 0.26, SP = 0.51, M × SP = 0.72. GSH:GSSG ratio: M = 0.17, SP = 0.35, M × SP = 0.49. LSD values for M × SP are shown as separate bars.

GSSG levels were not significantly different at two harvest maturities, but increased with the increase in storage duration for more than 2 weeks (Fig. 5.7C). In

both maturity groups, GSSG concentrations were almost similar during the first 4 weeks of storage followed by a sharp increase during the last 3 weeks of storage, leading to accumulation of GSSG at significantly higher levels in delayed–harvested fruit than in commercial harvest. The increase in GSSG and decrease in GSH levels led to an overall decrease in the GSH:GSSG ratio during storage (Fig. 5.7D). The GSH:GSSG ratio in fruit from both harvests did not significantly differ during the first 4 weeks of storage, but it was significantly lower in the delayed harvested fruit than in the commercially harvested fruit during the last three weeks of storage.

5.3.5.3 Ascorbate–glutathione cycle enzymes

The delay in harvesting significantly decreased APX activity compared with the commercial harvest (Fig. 5.8A). In both commercially and delayed harvested fruit, APX activity reached a maximum value after 4 weeks of storage. The amount of increase in APX activity leading to a peak was significantly greater in delayed harvested fruit than in commercially harvested fruit.

MDHAR activity showed a non–significant decrease during the one–week of delay in harvest (Fig. 5.8B). The MDHAR activities increased by 1.4 and 2 fold after 3 and 2 weeks of storage of commercially and delayed harvested fruit, respectively. The delayed harvested fruit showed a significantly higher MDHAR activity after 2 weeks of storage than the commercially harvested fruit, but the differences were statistically non–significant afterwards.

The delay in harvesting resulted in a significant decrease in DHAR activity as compared with the commercial harvest (Fig. 5.8C). A clear trend in the DHAR activities was not observed during storage to compare the fruit from two maturity groups. DHAR activity increased during the first 3 weeks of storage in delayed harvested fruit, followed by a significant decrease in the next 4 weeks of storage and reached to a value about 2–fold lower than at peak. A significant transient increase in DHAR activity was noticed only after 3 weeks of storage in commercially harvested fruit.

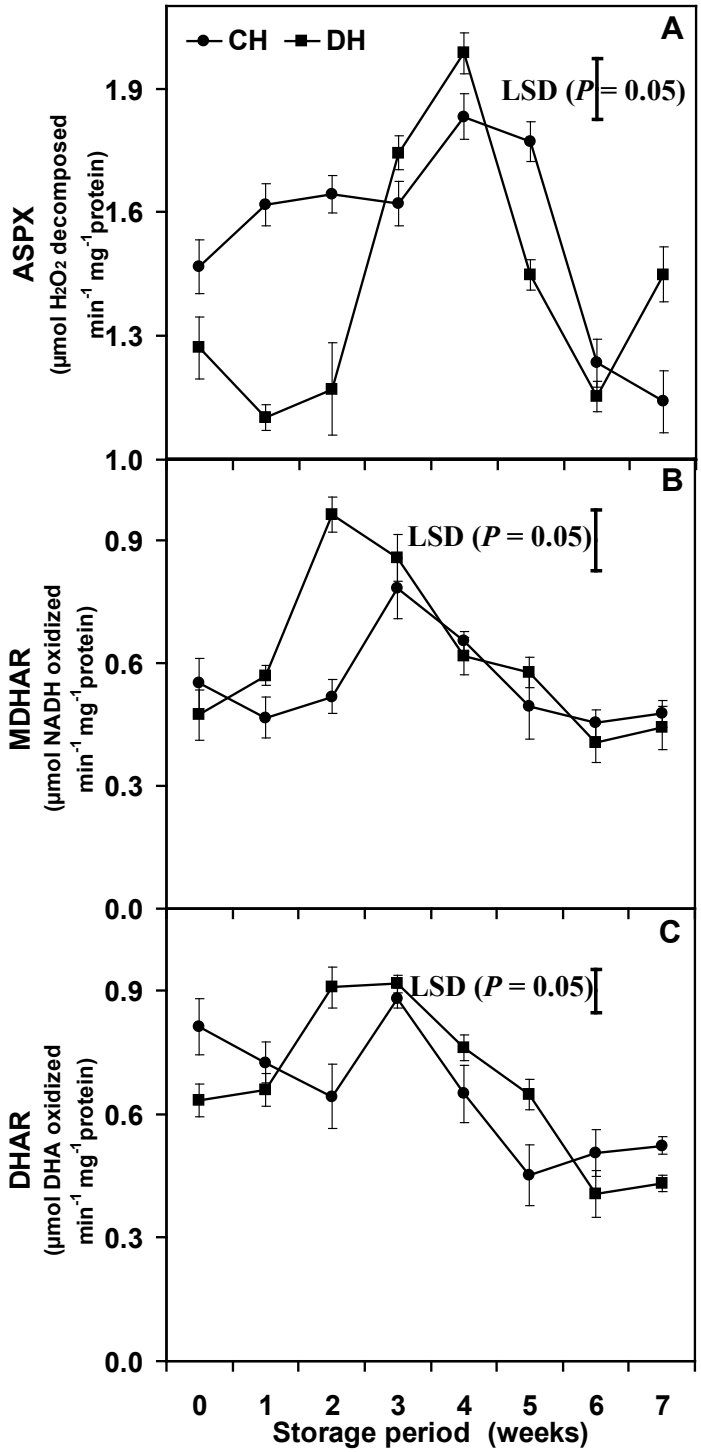


Fig. 5.8. Changes in activities of APX (A), MDHAR (B), and DHAR (C) in the flesh tissue of 'Amber Jewel' plums as influenced by harvest maturity during cold storage at 0±0.3°C plus 4 h at 21±1°C. The legends, CH and DH, refer to the commercial and delayed harvests, respectively. Vertical bars represent S.E. of means. All measurements were made in triplicate. LSD (P = 0.05) values for APX: Maturity (M) = 0.05, Storage period (SP) = 0.10, M × SP = 0.15. MDHAR: M = 0.05, SP = 0.11, M × SP = 0.15. DHAR: M = NS, SP = 0.07, M × SP = 0.11. LSD values for M × SP are shown as separate bars.

At harvest, GR activity was higher in the delayed–harvested fruit than in the commercially–harvested fruit (Fig. 5.9A). The GR activity persisted through the first 5 weeks of storage without a significant decrease regardless of the harvest maturity. However, an abrupt and significant decline in GR activity was observed during the last 2 weeks of storage in fruit from both maturities. In general, during the first 4 weeks of storage the GR activity remained at significantly higher levels in delayed harvested fruit than in the commercial harvest.

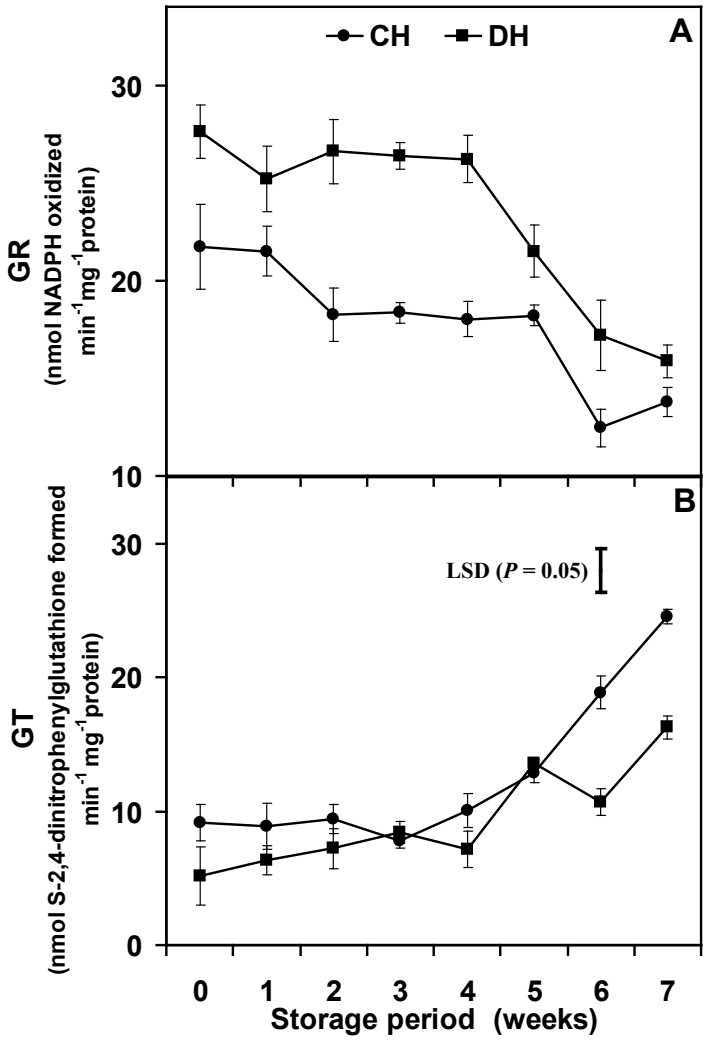


Fig. 5.9. Changes in activities of GR (A) and GT (B) in the flesh tissue of 'Amber Jewel' plums as influenced by harvest maturity during cold storage at 0±0.3°C plus 4 h at 21±1°C. The legends, CH and DH, refer to the commercial and delayed harvests, respectively. Vertical bars represent S.E. of means. All measurements were made in triplicate. LSD (P = 0.05) values for GR: Maturity (M) = 1.46, Storage period (SP) = 2.92, M × SP = NS. GT: M = 1.14, SP = 2.28, M × SP = 3.22. LSD value for M × SP is shown as a separate bar.

The delay in harvesting by one–week beyond commercial maturity caused a significant decrease in GT activity. Fig. 5.9B shows that GT activity remained almost

constant with non–significant changes during the first 4 weeks of storage. In both maturity groups, the increase in GT activity was observed after 5 weeks of storage and onwards, and the maximum GT activity was found after 7 weeks of storage. However, the overall extent of increase in GT activities during storage of fruit from both maturities was almost similar when compared to the values at harvest.

5.3.6 Phenols and DPPH· radical scavenging activity

The data on concentration of total phenolics and DPPH· radical scavenging activity are presented in Figure 5.10.

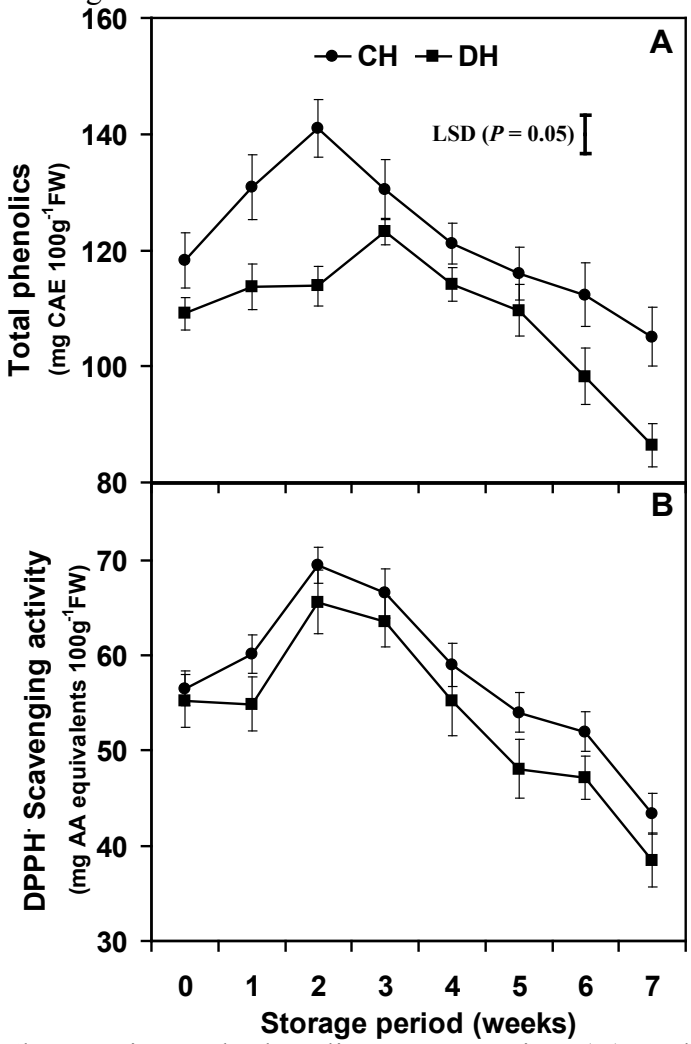


Fig. 5.10. Changes in total phenolics concentration (A) and DPPH scavenging activity (B) in the flesh tissue of 'Amber Jewel' plums as influenced by harvest maturity during cold storage at 0±0.3°C plus 4 h at 21±1°C. The legends, CH and DH, refer to the commercial and delayed harvests, respectively. Vertical bars represent S.E. of means. All measurements were made in triplicate. LSD (P = 0.05) values for total phenolics: Maturity (M) = 2.35, Storage period (SP) = 4.71, M × SP = 6.66. DPPH· scavenging activity: M = 1.42, SP = 2.85, M × SP = NS. LSD value for M × SP is shown as a separate bar.

The total phenolics concentration significantly decreased during the one–week period after commercial–harvest (Fig. 5.10A). Storage duration had a significant impact on the concentrations of total phenolics as these increased during the first 2–3 weeks of storage followed by a consistent decrease up to 7th week of storage in both maturity groups. In commercially–harvested fruit, a significant increase in the total phenolics was noticed during the first 2 weeks of storage followed by a decrease leading to the values that were similar to at harvest after 5 and 6 weeks of storage. A similar trend of changes in the concentration of total phenolics was also observed in the delayed–harvested fruit. Unlike total phenolics, harvest maturity did not affect the DPPH· radical scavenging activity (Fig. 5.10B). However, the changes in DPPH· radical scavenging activity during storage corresponded with the changes in the concentrations of total phenolics in both maturity groups. The DPPH· radical scavenging activity decreased to values lower than at harvest after 5 and 4 weeks of storage in commercially– and delayed–harvested fruit, respectively.

5.4 Discussion

5.4.1 Fruit quality

In addition to SSC and skin colour, flesh firmness is considered an important maturity index in Japanese plums (Crisosto, 1994; Crisosto et al., 2004). As expected, a significant reduction in flesh firmness (Fig. 5.1A) observed in delayed harvested fruit at harvest compared with commercial harvest may be attributed to the cell–wall modifications by the action of hydrolytic enzymes, including PG, PE, and EGase, leading to increased pectin solubilisation (Taylor et al., 1995) and alteration in sugar composition of polysaccharides (Manganaris et al., 2008). The changes in skin colour marked by decrease in L* and hue angle due to delayed harvesting are also indicative of advanced fruit maturity (Abdi et al., 1997). Similar results on fruit softening and skin colour changes due to delayed harvesting have been reported in many cultivars of Japanese and European plums (Abdi et al., 1997; Casquero and Guerra, 2009; Crisosto et al., 2004; Taylor et al., 1993; Taylor et al., 1995). During storage, the higher levels of increase in fruit softening and decrease in L* and hue angle values in delayed harvested fruit compared with commercial harvest could be due to initiation of ripening before harvesting in the former and it might have continued at a slower

rate during cold storage (Fig. 5.1). The results are consistent with the findings in other cultivars of the European and Japanese plums harvested at different maturities (Casquero and Guerra, 2009; Khan and Singh, 2008; Taylor et al., 1993). The increased activities of PG, PE, and EGase are reportedly responsible for fruit softening during cold storage in 'Tegan Blue' plums harvested at commercial maturity (Khan and Singh, 2008). In this experiment, delayed harvesting might have triggered the activities of these enzymes before harvest which persisted during storage at higher levels, causing more softening compared with commercial harvest. In contrast to observed decrease in flesh firmness during storage, Abdi et al. (1997) reported a slight increase in flesh firmness due to CI in 'Gulfruby' and 'Shiro' cultivars.

Concurrent to fruit softening and skin colour development, the increase in SSC and decrease in TA in delayed harvest indicates on–tree–advancement of fruit maturity (Fig. 5.2). The increase in SSC:TA ratio resulting from delayed harvest is generally favourable for improving fruit flavour and consumer acceptability of Japanese plums (Crisosto et al., 2004). Crisosto et al. (2004) reported that consumer acceptance of 'Blackamber' plums increased with the increase in SSC and was not influenced by SSC:TA ratio when SSC was equal or higher than 12 °Brix. The delayed harvesting in 'Amber Jewel' plums is therefore likely to increase its consumer acceptance marginally as the SSC at commercial harvest was 16.4 °Brix, which is much higher than the critical value of 12 °Brix. But it can possibly limit the storage potential due to a substantial fruit softening occurred during one week after commercial harvest. The increase in SSC during the first 3–4 weeks of storage followed by decline is contrary to previous reports showing either no change (Casquero and Guerra, 2009) or decrease in SSC (Abdi et al., 1997) throughout the storage in plums. However, the decrease in TA in both harvests during storage agrees with Abdi et al. (1997) and Casquero and Guerra (2009). The decrease in TA during storage improved SSC:TA ratio as the storage progressed.

5.4.2 Chilling injury

The storage potential of Japanese plums, as limited by CI, is strongly influenced by the genetic factors, amongst others (Abdi et al., 1997; Candan et al., 2008; Crisosto et

al., 1999). 'Amber Jewel' plums could be stored for 2 weeks without any CI symptoms at 0°C which agrees with the findings of Ward and Melvin–Carter (2001). A similar storage life has also been reported for 'Shiro' and 'Gulfruby' cultivars (Abdi et al., 1997). The data suggest that harvest maturity had a significant effect on the incidence and severity of CI only if the fruit were stored beyond 5 weeks at 0°C (Fig. 5.3). The increase in CI incidence even up to 100% was observed with the increase in storage duration in the delayed–harvested fruit as reported previously by Abdi et al. (1997), Taylor et al. (1995), and Ward and Melvin–Carter (2001). As observed in this study, early harvested fruit have been reported to show CI mainly in the form of flesh browning than the late harvested fruit exhibiting flesh translucency (Abdi et al., 1997; Crisosto et al., 2004; Taylor et al., 1995).

The appearance of flesh browning due to CI could be attributed to the oxidation of phenolics by PPO and peroxidases into semiquinones and quinones which polymerize into brown–pigmented polymers. Generally, phenolics and PPO are separately located in vacuoles and plastids, respectively (Nguyen et al., 2003). The membrane disruption in these subcellular organelles as indicated by increase in LOX, TBARS concentration and EL (Fig. 5.4) was observed with the increase in storage duration, and might have enhanced the interaction of phenols and PPOs to make browning reaction possible. The increase in activities of PPO has been associated with the development of CI symptoms in other fruits (Galli et al., 2009; Nguyen et al., 2003).

The flesh translucency is considered to be an advanced stage of flesh browning and predominates in late–harvested fruit (Taylor et al., 1995; Ward and Melvin–Carter, 2001). The loss of membrane permeability in delayed–harvested fruit might have increased the availability of cell fluids to form gel complexes around the stone earlier than the commercial–harvest. That is why the flesh translucency symptoms appeared earlier in delayed–harvested fruit compared with commercial–harvest as previously reported by Taylor et al. (1995). Even though, harvest maturity played a role in determining the severity of CI, but storage duration appears to be more critical factor in predisposing the fruit to CI (Abdi et al., 1997).

5.4.3 Lipid peroxidation

Lipid peroxidation can be initiated enzymatically by a sequential action of LOX, which catalyses the peroxidation of free PUFA in membrane system to form fatty acid hydroperoxides (Shewfelt and del Rosario, 2000). These hydroperoxides are capable of initiating free–radical chain reactions destabilizing the membrane structures. The peroxidative damage in membrane lipids by the action of LOX may result in loss of membrane integrity and dysfunction of several membrane bound enzymes, vital for maintaining various physiological processes (Shewfelt and del Rosario, 2000). Lipid peroxidation has been considered an early response to low temperature stress in chilling–sensitive commodities (Hariyadi and Parkin, 1991; Hodges et al., 2004; Imahori et al., 2008; Mao et al., 2007; Rivera et al., 2007).

The increased activities of LOX during the initial stages of storage might be responsible for increase in TBARS concentration and EL, and gradual loss of membrane integrity (Fig. 5.4). LOX activity has been positively correlated with the EL resulting in enhanced membrane damage in pepper fruit (Maalekuu et al., 2006). However, the increases in TBARS concentration and EL were observed to continue even after the decline in LOX activity. The peroxidative damage to plasma membrane to a greater extent during the late period of storage might have resulted in decreased concentrations of LOX substrate (Mao et al., 2007) which decreased the activity of membrane–bound LOX. With the extension of storage period to 6 or 7 weeks, the levels of TBARS were at the maximum. Zhuang et al. (1995) proposed that lipid hydroperoxide–derivative free radicals and MDA have potential in modifying LOX protein. And this could be the possible explanation for the self–destructive nature of LOX during the advanced stages of senescence due to increased lipid peroxidation. The membrane disintegration due to lipid peroxidation can also continue even in the absence of high LOX activity because lipid peroxidation is a self–propagating process requiring LOX for initiation only (Song et al., 2009; Zhuang et al., 1995). Chilling–induced increase in LOX activity followed by a decline during cold storage has been reported in many horticultural commodities (Ding et al., 2007; Mao et al., 2007; Promyou et al., 2008; Rivera et al., 2007).

The increase in concentration of TBARS, which is often utilized as a suitable biomarker for lipid peroxidation and oxidative damage, has been found to increase in response to chilling stress and senescence in various fruits such as apples (Rao et al.,

1998), bananas (Promyou et al., 2008), kiwifruit (Song et al., 2009), mangoes (Ding et al., 2007; Zhao et al., 2009), melons (Lacan and Baccou, 1998), mumes (Imahori et al., 2008), pears (Larrigaudiére et al., 2001), and plums (Eum et al., 2009). In response to chilling, ROS production may exceed the antioxidant potential of the system leading to an oxidative stress. The role of ROS as a mediating factor in initiation and propagation of lipid peroxidation is well documented (Lacan and Baccou, 1998; Shewfelt and del Rosario, 2000). The higher levels of TBARS in delayed harvested fruit reflect the development of oxidative stress over the storage period to a greater degree than in commercial harvest (Fig. 5.4B).

The increase in EL preceded the appearance of CI symptoms in delayed–harvested fruit, while it coincided in case of commercial harvest (Fig. 5.4C). Accumulated evidence shows that increase in EL, an indicator of the extent of membrane damage, across cell membrane may occur due to CI, senescence or a combination of both (Maalekuu et al., 2006; Mao et al., 2007; Song et al., 2009; Zhao et al., 2009). Both the reasons for increase in EL are valid in this study as the fruit showed various changes associated with ripening and senescence during storage. The concentration of TBARS and EL also showed a significantly positive correlation with CI in ‘Amber Jewel’ plums (Table 5.1). Consistent with the previous hypothesis that lipid peroxidation is involved in chilling stress; biochemical evidence is presented here that the prolonged cold storage increased lipid peroxidation in Japanese plums. The results also demonstrate that fruit harvested at delayed maturity undergo lipid peroxidation to a greater extent than those harvested at commercial maturity.

Table 5.1. Correlations of CI index and CI incidence with harvest maturity, storage period, lipid peroxidation, enzymatic and non-enzymatic antioxidants in 'Amber Jewel' plums during 7 weeks of cold storage at 0°C.

CI index *vs.*	**Pearson's correlation coefficient**	**CI incidence *vs.***	**Pearson's correlation coefficient**
Harvest maturity	NS	Harvest maturity	NS
Storage period	0.88***	Storage period	0.93***
TBARS	0.73***	TBARS	0.77***
LOX	NS	LOX	NS
EL	0.86***	EL	0.89***
SOD	–0.88***	SOD	–0.88***
CAT	NS	CAT	NS
POD	–0.63***	POD	–0.67***
AA	–0.76***	AA	–0.82***
DHA	0.47***	DHA	0.53***
AA:DHA	–0.71***	AA:DHA	–0.76***
GSH	–0.92***	GSH	–0.90***
GSSG	0.95***	GSSG	0.93***
GSH:GSSG	–0.94***	GSH:GSSG	–0.95***
APX	–0.29*	APX	NS
MDHAR	–0.46**	MDHAR	–0.44**
DHAR	–0.72***	DHAR	–0.74***
GR	–0.62***	GR	–0.63***
GT	0.72***	GT	0.71***
Phenols	–0.76***	Phenols	–0.71***
DPPH scavenging activity	–0.82***	DPPH scavenging activity	–0.80***

NS, *, **, *** = non-significant, $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, respectively

5.4.4 Antioxidant enzymes (SOD, CAT, and POD)

To counteract the increased ROS production in response to abiotic and biotic stresses, plant tissues have evolved an antioxidant defense system that can scavenge the ROS

and repair oxidative damage. SOD, CAT, and POD are part of the repertoire of antioxidant defence system that provide protection against the ROS (Apel and Hirt, 2004). The data suggest that SOD significantly increased during the first two weeks of storage in commercial harvest compared with a non–significant change in delayed harvest. The increase in SOD could be to dismutate the $O_2^{\bullet-}$ generated in response to chilling stress (Bowler et al., 1992). The enhanced SOD activity was also coupled with a significant increase in CAT activity, which is essential for the removal of H_2O_2 from the cell to avoid its accumulation to toxic levels (Figs. 5.5A & B). As the storage duration increased, decline in SOD activity might have contributed to the accumulation of $O_2^{\bullet-}$ as reported during cold storage of kiwifruit and mango (Ding et al., 2007; Song et al., 2009). The abundance of $O_2^{\bullet-}$ due to lower SOD activity may increase the Haber–Weiss reaction rate which involves production of the most reactive and damaging hydroxyl radicals (Bowler et al., 1992). Therefore, SOD is likely to be an enzyme central to the antioxidant defense mechanism as its activity determines the levels of H_2O_2 and $O_2^{\bullet-}$, which are both substrates of the Haber–Weiss reaction (Bowler et al., 1992). Another possible explanation for decrease in SOD activity is that in chilling–sensitive cultivars, such as 'Amber Jewel' (Ward and Melvin–Carter, 2001), increased H_2O_2 production may irreversibly inactivate SOD enzymes during prolonged chilling stress (Bowler et al., 1992).

Despite a continuous decrease in SOD activity beyond 2 weeks of storage, CAT activity in fruit from commercial harvest remained at significantly higher level than in delayed harvest between 3 and 6 weeks of storage. The differences in CI incidence were not significant in both harvests during most of the storage time when CAT activity was elevated. It is evident that decline in SOD activity preceded the visibility of CI symptoms after 3 weeks of storage (Fig. 5.3A & B) and showed negative correlation with CI (Table 5.1). There was an overall decrease, except a few transient increases, in POD activity during cold storage in fruit of both harvests, but POD levels were higher in delayed–harvested fruit during most of the storage time compared with commercial–harvest. The decrease in POD has been associated with the reduction of antioxidant potential in some fruits, such as apples, kiwifruit, mangoes, pears, and peaches and development of CI symptoms (Ding et al., 2007; Larrigaudiére et al., 2004; Rao et al., 1998; Song et al., 2009; Wang et al., 2005).

The increase in activities of SOD and CAT in response to low temperature stress has been associated with enhanced chilling tolerance in limes (Rivera et al., 2007), mangoes (Zhao et al., 2009), mumes (Imahori et al., 2008) and peaches (Wang et al., 2004), and resistance against browning in 'Braeburn' apples (Gong et al., 2001; Toivonen et al., 2003) and 'Conference' pears (Lentheric et al., 1999). In the peel tissues of chilling–sensitive cultivars of apples and mandarins, SOD activity did not differ from the chilling–tolerant cultivars, in contrast to higher CAT activity which has been implicated in providing chilling tolerance in mandarins (Sala, 1998) and scald resistance in apples (Rao et al., 1998). Based on the previous studies in other fruits and observations of this experiment, it is presumable that the acquisition of chilling tolerance in Japanese plums may be possible with the concerted action of both SOD and CAT in the flesh tissue. The primary antioxidant system, in terms of activities of SOD and CAT, in delayed harvested fruit was less efficient than in commercial harvest.

The data also suggest that, initially higher levels of SOD, CAT, and POD activities in delayed harvested fruit compared with commercial harvest did not persist longer during cold storage to provide a strong antioxidant system required to compensate for the increasing oxidative stress (Fig. 5.5). Vanoli et al. (1995) proposed that the SOD and CAT activities at harvest in 'Passa Crassana' pears were not influenced by harvest dates, but after 5 months of storage, higher SOD activity was associated with the lower incidence of internal browning in the late–harvested fruit, whereas CAT activity was found significantly lower in fruit severely affected with browning. It is therefore important to note that the changes in primary antioxidant enzymes, not the actual levels at harvest, are likely to affect the response of tissue to oxidative stress developing during cold storage.

5.4.5 Ascorbate and glutathione

The capacity of ascorbate–glutathione cycle to regenerate AA utilizing GSH is dependent on the concentrations of these antioxidants and the related enzyme activities, which indicates that these parameters would be suitable markers for the oxidative stress (Tausz et al., 2004). AA and GSH can be considered important markers for the fruit quality from nutritional perspective, and their concentrations at

harvest and during storage are also important determinants of fruit's capability to withstand oxidative stress presumed to be developing due to suboptimal conditions prevailing postharvest (Davey and Keulemans, 2004). Therefore, the changes in concentrations of AA, GSH and their oxidized forms at harvest and during storage were determined in 'Amber Jewel' plums harvested at two maturity stages. The delay in harvesting by one week resulted in a significant increase in concentrations of AA and GSH without affecting their redox status as reflected by AA:DHA and GSH:GSSG ratios, at harvest (Figs. 5.6 & 5.7).

The increase in AA during the one–week delay in harvesting was also accompanied by a significant increase in sucrose concentration, with a slight decrease in glucose, fructose and sorbitol during the same period (Singh et al., 2009b). The published results on changes in concentrations of sugars during fruit maturation in 'Amber Jewel' (Singh et al., 2009b) and also based on the observations in developing pears (Franck et al., 2003), a close relationship between sugars influx/interconversion and AA metabolism could be speculated. Another explanation could be the on–tree initiation of fruit ripening process. The post–cold storage fruit ripening has been reported to increase the concentrations of AA and GSH in pawpaw and tomato (Galli et al., 2009; Jiménez et al., 2002a). On the other hand, the decrease in AA levels has been observed during final maturation phase in apples, oranges, and pears (Davey et al., 2007; Huang et al., 2007; Lentheric et al., 1999; Molina–Delgado et al., 2009). Davey et al. (2004) reported that *de novo* synthesis of AA was not possible in exocarp and mesocarp tissues of apples, and further suggested that fruit tissue was dependent upon phloem transport of AA. The changes in AA and GSH concentrations in a developing fruit are influenced by multiple factors, such as cultivar, tissue type, soil and climatic conditions, and canopy position of fruit (Davey et al., 2004; Davey and Keulemans, 2004; Lentheric et al., 1999) and might have led to the contradictory results in different fruits or even inter– and intra–varietal variations (Davey and Keulemans, 2004).

During the 7 weeks of cold storage, concentrations of AA and GSH decreased significantly in fruit from both harvests (Figs. 5.6 & 5.7). The decrease in concentrations of AA and GSH of 'Amber Jewel' plums could be attributed to their utilization as reductants to encounter the oxidative stress during cold storage. A significant decrease in AA and GSH levels has been reported to occur during long–

term cold storage of other fruits such as mangoes (Zhao et al., 2009), oranges (Huang et al., 2008), and pawpaws (Galli et al., 2009). In response to chilling, GSH levels have been reported to increase in apples (Davey and Keulemans, 2004) and remain unaltered in peaches (Wang et al., 2006). There are contradictory reports on the response of GSH to various kinds of stresses; GSH levels may or may not increase or may even decrease upon exposure to stress (Tausz et al., 2004). GSH concentration was significantly higher after the 6^{th} and 7^{th} weeks of storage in commercial harvest than in delayed harvest, indicating a possible role of GSH as an antioxidant in reducing the CI symptoms in plums. Higher levels of GSH have been implicated in providing chilling tolerance in plants (Tausz et al., 2004). The lower levels of glutathione have been linked to the internal browning in methyl bromide–fumigated 'Thompson Seedless' grapes (Liyanage et al., 1993) and methyl–iodide induced phytotoxicity in lemons (Ryan et al., 2007). However, the protective role of GSH as a cellular antioxidant in various postharvest disorders of fruits has not been conclusively studied. Apple cultivars with higher total AA have been reported to have higher GSH levels, and thus possess better storage characteristics (Davey and Keulemans, 2004).

The flesh browning symptoms of CI in fruit from harvests appeared after 3 weeks of storage and intensified during the subsequent period of storage (Figs. 5.3A & B). The fruit tissue affected with browning in apples and pears is known to be depleted of AA due to prolonged cold storage in air or controlled atmospheres (De Castro et al., 2008; Veltman et al., 1999). Healthy tissue surrounding the brown tissue contained lower concentration of AA in flesh–browning–damaged 'Pink Lady' apples during cold storage (De Castro et al., 2008). The hypothesis of Veltman et al. (1999) that AA levels below a critical threshold value induces internal browning in pears has been found valid in apples by De Castro et al. (2008) and Davey and Keulemans (2004). The increased losses of AA and GSH during cold storage in some early maturing apple cultivars, 'Sunrise' and 'Gravenstein', have been associated with their poor storage characteristics and susceptibility to internal browning and rots (Davey and Keulemans, 2004). The concentrations of AA and AA:DHA ratio in 'Amber Jewel' plums also showed a significant negative correlation with CI (Table 5.1).

The concentrations of DHA and GSSG increased towards the end of storage indicating a shift in the equilibrium towards more oxidized state in the tissue, which

is an indication of the failure of the ascorbate–glutathione cycle (Figs. 5.6C & 5.7C). It is interesting to note that the redox status of fruit tissue, AA:DHA and GSH:GSSG, was worse affected after 3–4 weeks of storage when incidence and severity of CI was higher (Figs. 5.6D & 5.7D). The transient increases in AA:DHA redox state during the first 3–4 weeks of chilling exposure of fruit from both maturities suggest that there was an acclimation reaction to anticipated increasing load of ROS. However, a greater shift in AA:DHA and GSH:GSSG ratios towards more oxidized state during the late stages of storage reflects that acclimatory responses during the initial weeks of storage were too weak and slow to prevent the collapse of the antioxidant system during prolonged exposure to chilling stress (Tausz et al., 2004).

Though harvest maturity influenced the concentrations of both AA and GSH at harvest, the decrease in their levels, particularly for AA, during storage appears to be independent of harvest maturity. The manifestation of CI as flesh browning and translucency in 'Amber Jewel' plums is, perhaps, due to decrease in AA and GSH concentrations below a certain level (Table 5.1). The results indicate that the response or maintenance of the AA and GSH pools during cold storage is more important than their levels at harvest.

5.4.6 Ascorbate–glutathione cycle enzymes

The activities of ascorbate–glutathione cycle enzymes, APX, MDHAR, DHAR, and GR, which are responsible for the oxidation and recycling of AA and GSH pools in the flesh tissue, were determined (Figs. 5.8 & 5.9). The increase in APX activity during the first two weeks of storage in commercially harvested fruit was significantly higher than in delayed harvested fruit, but a significant reduction in APX activity was noticed in the last 2–3 weeks of storage (Figs. 5.8A). The increase in APX activity, utilizing AA as a substrate in the oxidation reaction to prevent the accumulation of H_2O_2, has been involved in providing chilling tolerance in several fruits subjected to different postharvest treatments (Ding et al., 2007; Sala and Lafuente, 2004; Wang et al., 2008; Wang et al., 2006; Zhao et al., 2009).

APX–catalysed oxidation of AA generates the unstable radical MDHA, which rapidly dissociates into DHA and AA. MDHAR and DHAR are two reductases catalysing the reduction of MDHA and DHA, respectively, to maintain the AA pool.

In response to increased APX activity that might have generated MDHA and DHA, the activities of MDHAR and DHAR increased significantly in the initial weeks of storage followed by a great decline in the last 2–3 weeks of storage (Figs. 5.8B & C). The sudden increase in DHA levels observed after 4 or 5 weeks of storage in fruit from both harvests coincided with a significant reduction in MDHAR and DHAR activities. Therefore, a role for MDHAR and DHAR is indicated in the maintenance of AA:DHA ratio at a higher level during chilling stress. The increase in MDHAR enzyme activity and transcript levels has been linked to increase in AA levels and chilling tolerance in tomatoes exposed to 4°C for 40 days (Stevens et al., 2008). The increase in activities of MDHAR and DHAR indicates an acclimatory response to chilling stress, while decrease in their activities is a reflection of the failure of the antioxidative system which resulted into increased concentrations of DHA and lower levels of AA in the tissue.

GR activity sustained for the first 4–5 weeks of storage without a significant change, but declined sharply after 6 and 7 weeks of storage and vice–versa in case of GT (Figs. 5.8 & 5.9). The down–regulation of GR and up–regulation of GT may explain the accumulation of GSSG in the final weeks of storage. GR is responsible for NADPH–dependent reduction of GSSG into GSH (Apel and Hirt, 2004), whereas GT utilizes GSH as a substrate to detoxify lipid hydroperoxides accumulated as a result of increased lipid peroxidation (Rogiers et al., 1998). The higher activity of GR can lead to a strong pool of GSH in order to maintain the H_2O_2–scavenging ascorbate–glutathione cycle. With the increase in storage duration, GR activity has also been reported to decrease in response to chilling stress in oranges (Huang et al., 2008) and peaches (Wang et al., 2006). The postharvest treatments that were reported to enhance chilling tolerance in some fruits also showed higher levels of GR (Sala and Lafuente, 2004; Wang et al., 2006).

Despite the increase in MDHAR and DHAR activities during initial stages of storage, GR activity either remained stable during the first 5 weeks of storage or decreased during the late stages. Consequently, the recovery of the antioxidant pools of AA and GSH was not observed in fruit from both harvests. These observations imply that ascorbate–glutathione cycle, in response to chilling stress, did not operate efficiently to maintain antioxidants pools in more reductive state in flesh tissues of plum. There are reports (Davey et al., 2004; Galli et al., 2009; Huang et al., 2008;

Huang et al., 2007) showing that the oxidation of AA and GSH did not always yield a proportionate increase in DHA and GSSG, respectively. The activities of recycling enzymes did not correspond with the increase in the levels of their reductant forms, AA and GSH. It further suggests a complexity in the regulation of antioxidant mechanism in response to a variety of stresses in different plants. The results show that activities of the AA–GSH recycling enzymes, MDHAR, DHAR, and GR, could not consistently remain at higher levels which were required to provide adequate protection through maintenance of the redox status of plum flesh tissue in response to chilling stress imposed for 7 weeks.

5.4.7 Phenols and DPPH· radical scavenging activity

The increase in concentration of total phenolics during initial storage period could be the result of acclimatization response of fruit to chilling conditions as previously reported in plums and pawpaws (Díaz–Mula et al., 2009; Galli et al., 2009). Phenylalanine ammonia lyase (PAL) activity, which is the initial regulatory enzyme in biosynthesis of phenolics, has been reported to increase in response to CI in bananas (Nguyen et al., 2003) and the same might have contributed to increase in phenolics concentrations in flesh tissue of plum during the first 2–3 weeks of storage. The increase in phenolics concentration is an indication of the activation of defense mechanism against chilling stress (Galli et al., 2009). The prolonged storage resulted in decrease in total phenolics concentration which could be due to their utilization as substrate of PPO in the browning reactions. Flesh browning appeared after 3 weeks of storage and its severity increased as the storage progressed (Fig. 5.10A). Another possible explanation for decrease in phenols is that these may be oxidized by peroxidases in the presence of H_2O_2 because of the co–existence of phenolics and peroxidases in vacuoles (Takahama, 2004). The decrease in phenolics has been reported to occur during 12 days at 2 °C storage of mangoes, irrespective of harvest maturity (Zhao et al., 2009).

Phenolic compounds are the major contributors to the total antioxidant capacity in plum flesh tissue, whereas a very little contribution by other compounds has been reported (Díaz–Mula et al., 2009; Gil et al., 2002). Apparently, the pattern of changes in DPPH radical scavenging activity during cold storage of fruit from both

harvests was almost similar to the changes in total phenolics (Fig. 5.10B). The data suggest that phenolic compounds in co–operation with other antioxidative components during initial stages of storage might have contributed to protection against oxidative injury to the tissue. The increase in storage duration accompanied by the decrease in total phenols and reduced efficiency of other antioxidative systems might have contributed to the build up of oxidative stress to potentially damaging levels. It may be concluded that the changes in antioxidant components during cold storage of Japanese plums seem to be more important in providing protection against oxidative injury expressed as CI than their at–harvest antioxidant status.

Chapter 6

Storage Temperature Influences Fruit Quality, Chilling Injury and Antioxidative Metabolism of Japanese Plums[3]

Summary

The objective was to investigate the effect of storage temperature and storage duration on lipid peroxidation, enzymatic and non–enzymatic antioxidants of fruit in relation to fruit quality and chilling injury (CI). To achieve this objective, commercially mature fruit were stored at 0°C (recommended storage temperature) and 5°C (considered lethal temperature for stonefruit) for 6 weeks, and evaluations for various parameters were carried out at 2–week intervals immediately after storage. Over–ripeness, which was indicated by fruit softening, skin colour development and loss of titratable acidity, was the major problem during storage at 5°C after 4 and 6 weeks of storage, and fruit could not have any potential market–life after cold storage. The magnitude of fruit softening and skin colour changes during the first 2 weeks of storage at 5°C was similar to that occurring during 6 weeks storage at 0°C. CI symptoms in the form of flesh browning and flesh translucency were noticed after 4 weeks of storage at 0°C and 5°C, respectively. The degree of lipid peroxidation as reflected in TBARS concentration was significantly higher at 5°C beyond 2 weeks of storage than at 0°C. The activities of SOD, CAT, and POD were significantly higher during storage at 5°C than at 0°C. At 5°C, the activities of ascorbate–glutathione cycle enzymes, MDHAR, DHAR and GR increased during the first 2 weeks of storage at 5°C and helped to maintain AA pool better than at 0°C. However, redox state of GSH pool, indicated by GSH:GSSG ratio, was significantly better at 0°C than at 5°C. Overall, the response of antioxidant system to chilling stress at 5°C was comparatively faster and stronger than at 0°C, but it could not sustain for 6 weeks of storage as the increasing lipid peroxidation, and oxidative stress might have resulted in failure of the antioxidant protection system. In conclusion, 'Amber Jewel' plums showed CI susceptibility to both storage temperatures, 0°C and 5°C, and could be

[3] The additional data on the changes in concentrations of individual sugars and organic acids from this experiment has been published in the following article: Singh, S.P., Z. Singh, and E.E. Swinny. 2009. Sugars and organic acids in Japanese plums (*Prunus salicina* Lindell) as influenced by maturation, harvest date, storage temperature and period. *International Journal of Food Science and Technology*. 44:1973–1982.

stored for 2 weeks at 5°C and for 2–3 weeks at 0°C. The extent of lipid peroxidation and changes in enzymatic and non–enzymatic antioxidants could partially explain that oxidative stress developed during storage at both temperatures and increased with the increase in storage duration.

6.1 Introduction

Optimum storage temperature is a basic requirement for postharvest handling and quality maintenance in Japanese plums. Japanese plums can be stored at 0°C for 3–5 weeks depending upon the cultivar's susceptibility to CI (Crisosto et al., 1999). The cold storage of Japanese plums can cause CI in the form of flesh browning, mealiness, and flesh translucency (Crisosto et al., 1999; Singh et al., 2009a; Taylor et al., 1995). In California, Crisosto et al. (1999, 2004, 2008) tested Japanese plum cultivars for their susceptibility to CI at two storage temperatures, 0°C and 5°C, and classified them into three categories: A) non–susceptible to CI at both temperatures; B) susceptible to CI at 5°C, but not at 0°C; C) susceptible to CI at both temperatures. A study in Western Australia showed that 'Amber Jewel' cultivar showed more sensitivity to CI at 0°C than at 5°C, while over–ripeness was the limiting factor during storage at 5°C (Ward and Melvin–Carter, 2001). Some cultivars of peaches and nectarines can also develop CI symptoms at both storage temperatures (Crisosto et al., 1999; Lurie and Crisosto, 2005). These studies clearly showed that susceptibility to CI in stonefruits including Japanese plums is a phenomenon under the control of genetic factors. 'Amber Jewel' is a very popular cultivar for its demand in export markets, and is known to be highly susceptible to CI or internal breakdown limiting its export potential.

Plants have evolved an efficient antioxidant system to encounter the increased ROS levels in response to a variety of environmental stresses, such as extreme temperatures, salinity, drought, ozone exposure, and UV irradiation (Hodges et al., 2004 and references therein). In response to stress conditions, ROS are produced and rapidly removed or detoxified by various cellular enzymatic and non–enzymatic systems. The slow and weak response of the antioxidative system can cause accumulation of ROS to damaging levels leading to enhanced lipid peroxidation and loss of membrane integrity in the tissue (Shewfelt and del Rosario, 2000; Wismer,

2003). Like other stresses, chilling conditions can alter the equilibrium between ROS production and removal, and can result in oxidatively induced CI (Hodges et al., 2004). Oxidative stress has been implicated in development of CI in many fruits exposed to chilling conditions (Hariyadi and Parkin, 1991; Mao et al., 2007; Sala, 1998; Sala and Lafuente, 2000; Wismer, 2003; Zhao et al., 2009).

It is argued that chilling–tolerant species/cultivars are known to have a strong antioxidant system in response to stress and/or produce fewer ROS than chilling–susceptible counterparts (Sala and Lafuente, 2000; Wismer, 2003). The response to chilling conditions may vary depending upon the storage temperature and duration of exposure. The storage temperatures between 2.2 and 7.6 °C (killing temperature zone) have been considered to be responsible for development of CI in plums, peaches, and nectarines (Crisosto et al., 1999; Lurie and Crisosto, 2005), and may be favouring the development of oxidative stress in fruit. It was hypothesized that the oxidative behaviour of Japanese plums during storage at optimum temperature may differ from that occurring during storage in the 'killing/danger temperature zone'. Therefore, the effects of storage at two contrasting temperatures (0°C and 5°C) on the changes in enzymatic and non–enzymatic antioxidants in relation to CI and fruit quality were investigated in Japanese plums.

6.2 Materials and methods

6.2.1 Experimental material and design

The experiment was conducted on 'Amber Jewel' cultivar of Japanese plums. 'Amber Jewel', also known as 'Teak Gold', is a patented Japanese plum cultivar which originated as a chance seedling in WA. The fruit were harvested at commercial maturity (129 days after full bloom) in the early morning hours from the Casuarina Valley Orchard, Karragullen, Perth Hills (latitude 31° 57' S; longitude 115° 50' E), WA. Fruit of uniform size and maturity, free from visual blemishes and disease were harvested from the orchard, transported to the laboratory, and stored in plastic crates lined with 30 µm thick low density polyethylene (LDPE) film (AMCOR Packaging, Pvt. Ltd., Melbourne, Australia) at 0°C and 5°C for 6 weeks.

Aliquots of 20 fruit from each of the three replicates from both maturity groups were transferred from cold store at 2–week intervals, allowed to stay at 20±1

°C for about 4 h, and were analysed for changes in fruit quality. Fruit flesh tissue was frozen in liquid nitrogen and stored at –80°C until further analyses of enzymatic and non–enzymatic antioxidants. The experiment was laid out by following a completely randomised design with two factors including storage temperature and storage period.

6.2.2 Fruit quality evaluation

Fruit quality was evaluated at 2–week intervals during 6 weeks of cold storage at 0°C and 5°C. Fruit firmness, colour, SSC, and TA were determined as described in the Sections, 3.4.1.1, 3.4.2, and 3.4.3, of Chapter 3. For all these quality parameters, ten fruit constituted an experimental unit, with three replications.

6.2.3 Chilling injury (CI)

The incidence and severity of CI was evaluated 4 h after transfer from cold storage to 21±1°C as described in the Section 5.2.3 of the previous chapter. The calculations were made as described in the Section 3.4.4 of the Chapter 3.

6.2.4 Oxidative stress parameters

The determinations of lipid peroxidation, enzymatic and non–enzymatic antioxidants, and protein concentration were carried out as described in the Chapter 3 (Sections 3.5 to 3.8).

6.2.5 Statistical analysis

Data were subjected to a two–way ANOVA using GenStat Release 11.1 (VSN International Ltd., Hemel Hempstead, UK). The data on CI incidence were subjected to arcsine transformation to reduce heteroscedasticity. The effects of storage temperature and storage duration and their interactions on different parameters were assessed within ANOVA and the LSD values were calculated at 5% level of significance following a significant F–test.

6.3 Results

6.3.1 Fruit quality

Table 6.1 shows that storage temperature and duration had a significant impact on fruit quality attributes such as firmness, skin colour, SSC, TA, and SSC:TA ratio. The magnitude of firmness loss over 6 weeks of storage at 0°C was similar to that occurring during 2 weeks storage at 5°C. In general, the rate of fruit softening was fast during the first 2 weeks of storage at both temperatures. The changes in skin colour marked by decreases in L* and hue angle were also significantly higher during storage at 5°C than at 0°C. Similar to firmness, the amount of changes in skin colour that occurred during 6 weeks at 0°C were achieved in 2 weeks storage at 5°C. No significant change in SSC was observed in fruit stored for 6 weeks at 5°C, while a significant increase in SSC was noticed during the first 4 weeks of storage at 0°C followed by a decline to the values similar to at harvest. The decrease in TA for fruit stored at 5°C was significantly higher than at 0°C. With the advancement of storage period, the decrease in TA favoured the improvement in SSC:TA ratio that was significantly greater after 6 weeks of storage at 5°C than at 0°C.

Table 6.1. Fruit quality attributes (Firmness, L*, hue angle, SSC, TA, and SSC:TA ratio) of 'Amber Jewel' plums at harvest (0 week) and after 2, 4, and 6 weeks of storage at 0°C and 5°C. Means for each parameter followed by the same letter(s) are not significantly different ($P \leq 0.05$).

Storage (weeks)	**Firmness (N)**		**L***		**Hue angle (° h)**		**SSC (°Brix)**		**TA (%)**		**SSC:TA**	
	0°C	**5°C**	**0°C**	**5°C**	**0°C**	**5°C**	**0°C**	**5°C**	**0°C**	**5°C**	**0°C**	**5°C**
0	48.6 a		22.1 a		54.3 a		16.4 a		1.29 a		12.8 a	
2	37.2 b	30.5 c	19.5 b	17.3 bc	43.7 b	31.7 cd	17.6 b	16.5 a	1.21 b	1.24 a	14.5 b	13.3 ab
4	36.0 b	16.9 d	19.1 b	11.1 d	34.0 c	17.1 e	18.2 b	16.6 a	1.13 c	0.96 d	16.2 c	17.2 c
6	31.0 c	17.1 d	16.0 c	9.6 e	28.4 d	17.0 e	16.8 a	16.5 a	1.02 d	0.65 e	16.5 c	25.4 d
Least significant differences of means at 5% level and levels of significance for a two–factor ANOVA												
Storage temp. (ST)	2.52***		1.53***		3.73***		0.42***		0.04***		1.04***	
Storage period (SP)	1.78***		1.08***		2.64***		0.30***		0.03***		0.73***	
ST X SP	3.56***		2.16***		5.28**		0.60**		0.06***		1.47***	

*** = $P \leq 0.001$; ** = $P \leq 0.01$

6.3.2 Chilling injury (CI)

Regardless of storage temperature, fruit were observed to be healthy without any CI symptoms for 2 weeks of storage. After 4 weeks of storage at 0°C, CI symptoms were observed in the form of flesh browning with low severity, whereas these symptoms were observed in the form of flesh translucency at 5°C. After 4 weeks of storage, incidence of CI was about 35% and 40% at 0°C and 5°C, respectively, and it increased to 66% and 100% after 6 weeks (Fig. 6.1A). CI symptoms intensified during the last 2 weeks of storage at both temperatures and were observable in the form of flesh browning and translucency (Fig. 6.1B). On the other hand, it was very difficult to distinguish the CI symptoms and over–ripeness in fruit stored at 5°C for 6 weeks; most of the fruit showed flesh disintegration and translucency. The flesh chromaticity L* also decreased with the increase in storage duration at both temperatures (Fig. 6.1C).

6.3.3 Lipid peroxidation

LOX activity increased with the increase in storage duration, but storage temperatures had no significant effect on it (Fig. 6.2A). In general, LOX activity increased to a peak during the first 2–4 weeks of storage and then followed a significant decrease in the last phase of storage. Depending on the storage temperature, increase in TBARS concentration was observed with the increase in storage duration (Fig. 6.2B). The differences in TBARS concentrations in fruit stored at 0°C and 5°C were statistically significant and large after 4 and 6 weeks of storage; fruit stored at 5°C had higher TBARS concentration than those stored at 0°C. EL during the first 2 weeks of storage was stable and continued to increase thereafter (Fig. 6.2C). Though the EL was significantly higher in fruit stored at 5°C after 4 weeks of storage than at 0°C, but these differences were non–significant after 6 weeks of storage.

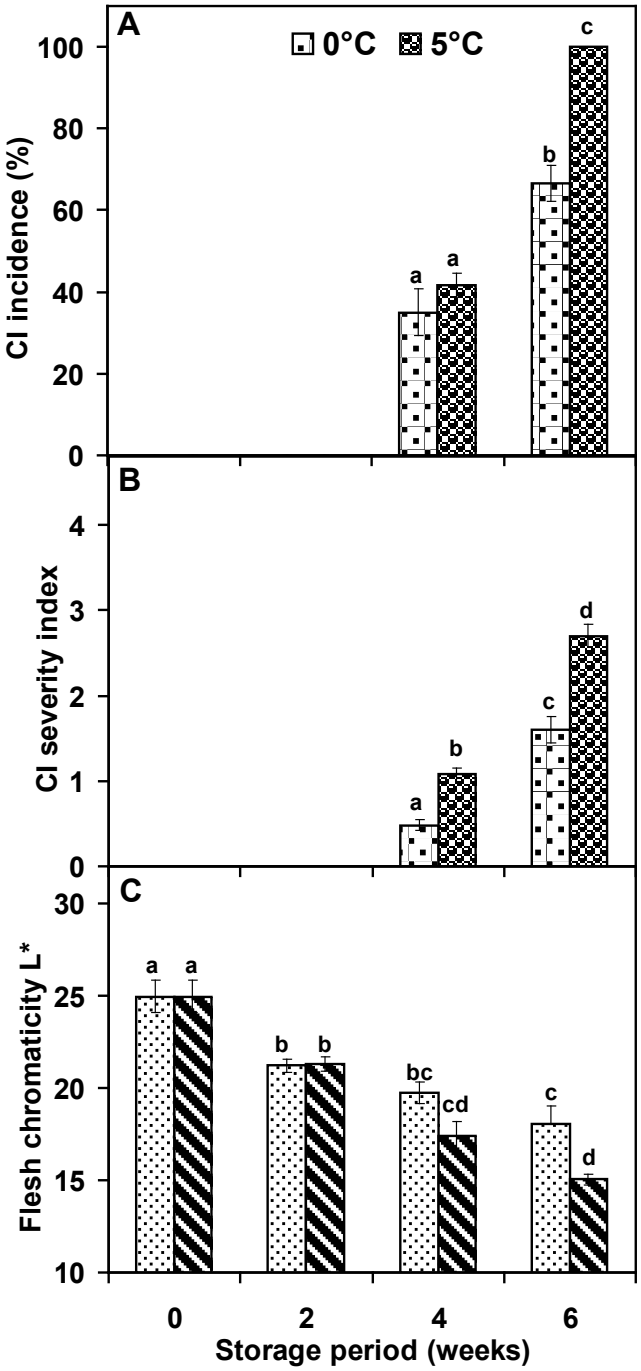


Figure 6.1. Effects of storage temperature and storage duration on the CI incidence (A), CI severity index (B), and flesh chromaticity L* values (C) of 'Amber Jewel' plums during 6 weeks of cold storage at 0°C and 5°C plus 4 h at 21±1°C. Vertical bars represent S.E. of means. All measurements were made in triplicate. Data on CI incidence were arcsine transformed, but back–transformed data are presented for simplifying comparisons. The bars, showing mean values, bearing the same letter(s) are not significantly different ($P \leq 0.05$).

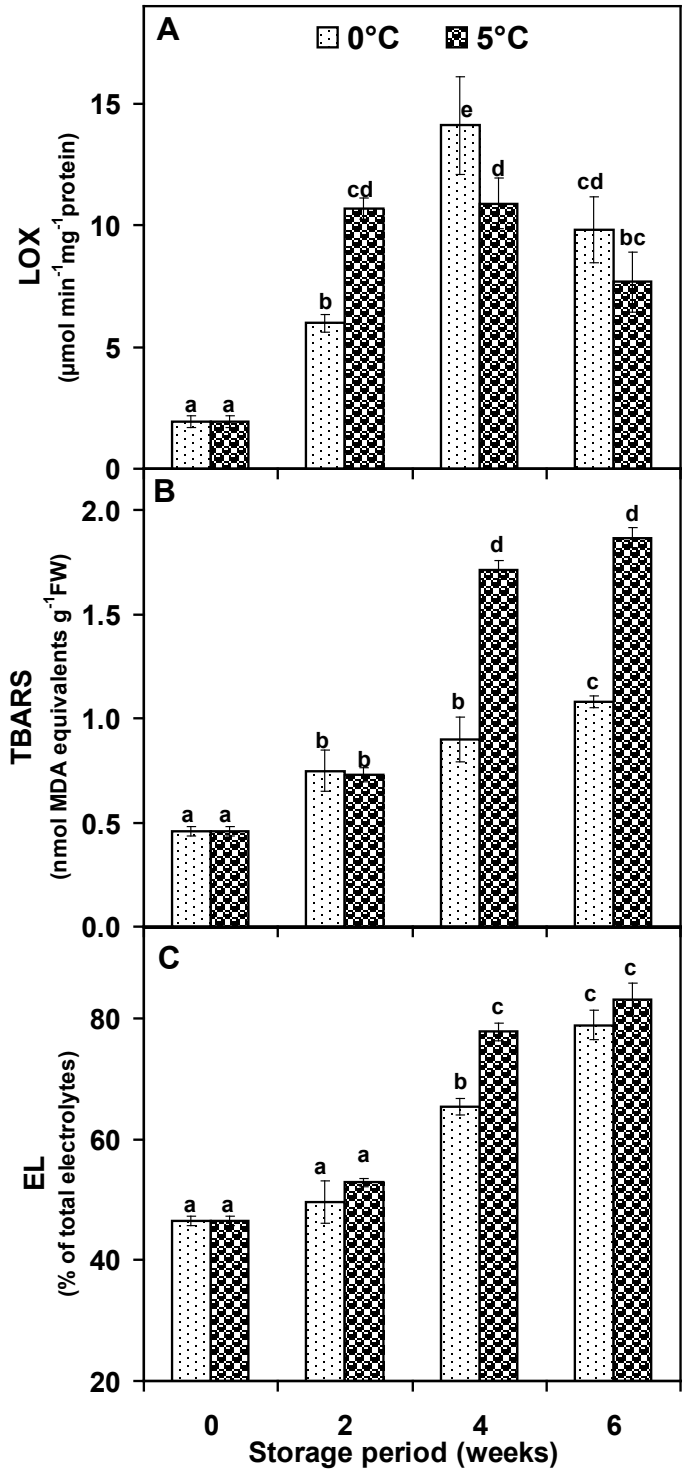


Figure 6.2. Changes in LOX activity (A), TBARS concentration (B), and electrolyte leakage (C) in the flesh tissue of 'Amber Jewel' plums during 6 weeks of cold storage at 0°C and 5°C plus 4 h at 21±1°C. Vertical bars represent S.E. of means. All measurements were made in triplicate. The bars, showing mean values, bearing the same letter(s) are not significantly different ($P \leq 0.05$).

6.3.4 Antioxidant enzyme activities (SOD, CAT, and POD)

SOD activity was significantly higher in fruit stored at 5°C compared with those stored at 0°C (Table 6.2). SOD activity increased during the first 2 weeks of storage at both temperatures; the increase was significantly greater in fruit stored at 5°C than those stored at 0°C. The increase in SOD activity continued until 4 weeks of storage at 5°C followed by a decline in the last 2 weeks of storage, whereas it declined in the last 4 weeks of storage at 0°C. A significant increase in CAT activity was observed during storage at both temperatures. The increase in CAT activity was about 5–fold during the first 4 weeks of storage at 5°C compared with about 2–fold increase during the same period of storage at 0°C. POD activity showed a significant increase during storage at 5°C which is contrary to a significant decline noticed during storage at 0°C.

6.3.5 Ascorbate–glutathione cycle

6.3.5.1. Ascorbate

Changes in ascorbate pool and its redox status were significantly influenced by storage temperature and duration (Table 6.3). Concentration of AA decreased significantly with the progression of storage at 0°C, whereas it remained unaffected at 5°C despite the increase in storage duration up to 6 weeks. DHA concentrations decreased during the first 2 weeks of storage at both temperatures. A significant increase in DHA concentration was noticed during the last 2 weeks of storage at 0°C. After 6 weeks of storage, DHA concentration was about 2.5–fold higher in fruit stored at 0°C than those stored at 5°C. The increase in AA:DHA ratio was significant during the first 2 weeks of storage at 5°C, while it did not change during the first 4 weeks of storage at 0°C. The AA:DHA ratio declined significantly in fruit stored at 0°C after 6 weeks of storage and a transient decline in this ratio was also observed after 4 weeks of storage at 5°C which then recovered after 6 weeks of storage.

Table 6.2. SOD, CAT, and POD activities in the flesh tissue of 'Amber Jewel' plums at harvest (0 week) and after 2, 4, and 6 weeks of storage at 0°C and 5°C. Means for each parameter followed by the same letter(s) are not significantly different ($P \leq 0.05$).

Storage (weeks)	**SOD[x]**		**CAT[x]**		**POD[x]**	
	0 °C	**5 °C**	**0 °C**	**5 °C**	**0 °C**	**5 °C**
0	224.7 d		10.2 a		406.4 d	
2	274.8 c	299.4 b	20.5 b	27.5 d	405.2 d	426.4 cd
4	218.1 d	323.3 a	20.8 bc	50.2 f	343.2 e	461.2 b
6	166.1 f	197.6 e	27.1 cd	41.5 e	364.4 e	504.4 a
Least significant differences of means at 5% level and levels of significance for a two-factor ANOVA						
Storage temp. (ST)	13.1***		4.6***		22.8***	
Storage period (SP)	9.3***		3.3***		20.0*	
ST X SP	18.6***		6.6***		32.4***	

*** = $P \leq 0.001$; ** = $P \leq 0.01$; * = $P \leq 0.05$

[x] Units of SOD, CAT and POD are µmol cytochrome c conserved min^{-1} mg^{-1} protein, µmol H_2O_2 decomposed min^{-1} mg^{-1} protein, and µmol H_2O_2 decomposed min^{-1} mg^{-1}protein, respectively.

Table 6.3. Concentrations of AA, DHA, AA:DHA ratio, and activities of APX, MDHAR, and DHAR in the flesh tissue of 'Amber Jewel' plums at harvest (0 week) and after 2, 4, and 6 weeks of storage at 0°C and 5°C. Means for each parameter followed by the same letter(s) are not significantly different ($P \leq 0.05$).

	Ascorbate pool (nmol g^{-1} FW)						**Ascorbate oxidation and recycling enzymes** [x]					
Storage (weeks)	**AA**		**DHA**		**AA:DHA**		**APX**		**MDHAR**		**DHAR**	
	0°C	**5°C**	**0°C**	**5°C**	**0°C**	**5°C**	**0°C**	**5°C**	**0°C**	**5°C**	**0°C**	**5°C**
0	87.4 a		30.5 b		3.0 c		1.47 c		0.55 c		0.81 bc	
2	61.3 b	86.5 a	20.7 cd	16.2 d	3.0 c	5.4 a	1.64 b	1.70 ab	0.52 c	0.69 ab	0.64 cd	1.06 a
4	56.4 b	85.8 a	19.2 cd	24.9 bc	3.0 c	3.7 bc	1.83 a	1.50 c	0.65 b	0.75 a	0.65 cd	0.86 b
6	40.8 c	87.8 a	49.3 a	20.0 cd	0.8 d	4.6 ab	1.23 d	1.33 d	0.45 d	0.55 c	0.51 d	0.60 d
Least significant differences of means at 5% level and levels of significance for a two–factor ANOVA												
Storage temp. (ST)	10.7***		5.6**		1.1***		NS		0.05***		0.13***	
Storage period (SP)	7.6**		3.9***		NS		0.06***		0.04***		0.09***	
ST X SP	15.1**		7.9***		1.6**		0.13***		0.08*		0.18*	

*** = $P \leq 0.001$; ** = $P \leq 0.01$; * = $P \leq 0.05$; NS = non–significant. [x] Units of APX, MDHAR and DHAR are µmol H_2O_2 decomposed min^{-1} mg^{-1} protein, µmol NADH oxidized min^{-1} mg^{-1} protein, , and µmol DHA oxidized min^{-1} mg^{-1}protein, respectively.

6.3.5.2. Glutathione

Concentrations of GSH increased in response to storage for 2 and 4 weeks at 5°C followed by a decline after 6 weeks; but a consistent decrease in GSH levels was observed throughout storage at 0°C (Table 6.4). GSSG concentrations were not influenced by the storage temperature during the first 2 weeks of storage. The increase in GSSG was significantly greater during the subsequent weeks of storage at 5°C than at 0°C. GSH:GSSG ratio was unaffected during the first two weeks of storage, but it declined significantly during the later storage period as the concentrations of GSSG increased.

6.3.5.3. Ascorbate–glutathione cycle enzymes

The activity of APX, an enzyme responsible for oxidation of AA, showed a significant increase during the first 2 and 4 weeks of storage at 5°C and 0°C, respectively (Table 6.3). After 4 weeks of storage, APX activity was found significantly higher in fruit stored at 0°C than at 5°C. A decrease in APX activity was observed in the last 2 weeks of storage at both temperatures and it led to the differences in APX activity to non–significant levels after 6 weeks of storage. The activities of AA–recycling enzymes, MDHAR and DHAR, were significantly influenced by the storage temperature and storage period. In response to low temperature storage, the increase in MDHAR activity was significantly higher during the first 2 weeks of storage at 5°C than at 0°C, and the higher MDHAR activity sustained during the first 4 weeks of storage in fruit stored at 5°C. The increase in MDHAR at 0°C was noticed only after 4 weeks of storage followed by a significant decline during the last 2 weeks of storage. During storage at 0°C, DHAR activity decreased during storage, whereas a transient increase in DHAR activity after 2 weeks of storage at 5°C was observed followed by a continuous decrease until 6 weeks of storage. GR activity did not show any significant change during the first 4 weeks of storage at both temperatures, but it decreased drastically after 6 weeks of storage (Table 6.4). No significant differences in GT activities at two storage temperatures were noticed after 2 weeks of storage. GT activity was significantly higher after 4 weeks of storage at 5°C than at 0°C where it increased significantly only after 6 weeks of storage.

Table 6.4. Concentrations of GSH + GSSG, GSH, GSSG, GSH:GSSG ratio, and activities of GR and GT in the flesh tissue of 'Amber Jewel' plums at harvest (0 week) and after 2, 4, and 6 weeks of storage at 0°C and 5°C. Means for each parameter followed by the same letter(s) are not significantly different ($P \leq 0.05$).

	Glutathione pool (nmol g^{-1} FW)								**Glutathione–related enzymes** [x]			
Storage (weeks)	**GSH + GSSG**		**GSH**		**GSSG**		**GSH:GSSG ratio**		**GR**		**GT**	
	0°C	**5°C**	**0°C**	**5°C**	**0°C**	**5°C**	**0°C**	**5°C**	**0°C**	**5°C**	**0°C**	**5°C**
0	182.2 c		167.7 c		14.5 a		11.6 a		21.7 ab		9.2 a	
2	178.4 cd	189.8 b	163.8 cd	174.7 b	14.6 a	15.1 a	11.2 a	11.6 a	18.2 bc	23.0 a	9.5 a	12.6 a
4	175.0 d	204.9 a	159.0 de	181.6 a	16.0 b	23.3 d	10.0 b	7.8 c	18.0 bc	21.8 ab	10.1 a	23.4 c
6	175.9 d	176.7 cd	155.4 ef	150.4 f	20.5 c	26.3 e	7.6 c	5.7 d	12.5 d	16.2 cd	18.9 b	21.4 bc
Least significant differences of means at 5 % level and levels of significance for a two–factor ANOVA												
Storage temp. (ST)	4.3***		4.3***		0.6***		0.5***		3.2**		2.5***	
Storage period (SP)	3.1***		3.0***		0.4***		0.4***		2.2***		1.7***	
ST X SP	6.1***		6.1***		0.8***		0.7***		NS		3.5***	

*** = $P \leq 0.001$; ** = $P \leq 0.01$; NS = non–significant. [x] Units of GR and GT are nmol NADPH oxidized min^{-1} mg^{-1} protein and nmol S–2,4–dinitrophenylglutathione formed min^{-1} mg^{-1} protein, respectively.

6.4 Discussion

6.4.1 Fruit quality

As expected, storage temperature had a dramatic effect on fruit quality of 'Amber Jewel' plums. Flesh softening and skin colour changes in fruit stored at 5°C were much faster than those stored at 0°C (Table 6.1). In addition to other quality attributes, flesh firmness is a critical factor in determining the consumer acceptability of Japanese plums (Crisosto et al., 2003, 2004). According to Valero et al. (2007), based on flesh firmness, plums can be segregated into three categories: mature and immature (>26 N); ready–to–buy (13–26 N); ready–to–eat (<13 N). A shelf life period of 4–5 days is generally required under commercial marketing conditions, and it might not have been achieved after 4 or 6 weeks of storage at 5°C as the average flesh firmness was about 17 N which can be considered optimum for 'ready–to–buy'. The potential storage life of 'Amber Jewel' plums based on fruit firmness and skin colour changes appeared to be not more than 2 weeks at 5°C as the fruit could have 2–3 days of shelf life. The faster rate of fruit ripening at 5°C as observed in this study could be due to increased ethylene production rates compared with storage at 0°C. It has been reported that ethylene production rates of mature unripe and ripe plums during storage at 5°C (0.02–15 μL C_2H_4 kg^{-1} h^{-1}) were about 2–3–fold higher than at 0°C (0.01–5 μL C_2H_4 kg^{-1} h^{-1}) (Kader and Mitchell, 1989). Ethylene has been implicated in promoting fruit softening in plums; the treatment of plums with 1–MCP, an ethylene action inhibitor, has been reported to delay fruit softening through reduced activities of cell–wall hydrolysing enzymes (Khan and Singh, 2007a).

SSC increased during the first 4 weeks of storage at 0°C and then decreased to the levels at harvest after 6 weeks of storage (Table 6.1). On the other hand, there was no significant change in SSC during storage at 5°C. There are often contradictory reports on the changes in SSC in the European and Japanese plums during storage. Abdi et al. (1997) reported a slight increase in SSC in 'Gulfruby', 'Radiant', and 'Shiro' cultivars of Japanese plums during the first 2 weeks of storage at 0°C which then declined with the increasing periods of storage; these observations confirm the results during storage at 0°C. Casquero and Guerra (2009) reported that there was no change in SSC during storage of 'Oullins Gage' cultivar, the European plums, and it happened during storage of 'Amber Jewel' plums at 5°C. The decrease in TA at both storage temperatures agrees well with Abdi et al. (1997) and Casquero

and Guerra (2009). The extent of TA decrease was more during storage at 5°C than at 0°C (Table 6.1) and it could be related to the faster ripening process at the former as evident from the changes in flesh firmness and skin colour. The decrease in TA during storage improved SSC:TA ratio as the storage progressed.

6.4.2 Chilling injury

The storage potential and susceptibility to CI are highly cultivar–specific phenomena and this fact has been repeatedly confirmed by research investigations on stonefruit in California during the past 2–3 decades (Crisosto et al., 1999; Crisosto et al., 2004; Kader and Mitchell, 1989; Lurie and Crisosto, 2005). It is widely accepted that storage of stonefruit including Japanese plums at near 5°C induces CI symptoms more severely than at 0°C. Flesh browning symptoms, though in very low severity, were observed in fruit stored at 0°C after 4 weeks of storage, while flesh translucency predominated in fruit stored at 5°C (Fig. 6.1). 'Amber Jewel' plum showed chilling sensitivity at both temperatures, 0°C and 5°C, and can therefore be placed into category C. Japanese plum cultivars such as, 'Showtime', 'Friar' and 'Howard Sun' have also been placed under the same category (Table 2.6). The loss of more than 25% fruit due to CI has been considered to be a benchmark to determine the storage potential in Japanese plums (Crisosto et al., 2004). Under both storage temperatures in this study, the incidence of CI exceeded the benchmark level of 25% which suggests that 'Amber Jewel' plums can be stored for 2 weeks at 5°C and 2–3 weeks at 0°C. As described in the Chapter 5, the potential storage life of 'Amber Jewel' plums harvested at commercial maturity is 3 weeks because CI symptoms begin to appear after 3 weeks of storage and the CI severity increases with the increase in storage duration. The incidence of CI in 'Amber Jewel' plums appears to be very erratic in nature because several factors, such as crop load, canopy position of fruit, tree mineral nutrition, delay in cooling have been reported to influence the susceptibility of 'Amber Jewel' plums to CI (Ward and Melvin–Carter, 2001). Moreover, 'Amber Jewel' cultivar has not been researched in other countries, but it is a very popular cultivar of Australia prized for exports due to its high sweetness and low acidity which are highly desirable plum characteristics in the South–east Asian markets.

It is clear that storage temperature of 5°C was not sufficiently low to retard the ripening process in this cultivar and also increased flesh translucency after 4 weeks of storage. On the other hand, the recommended storage temperature of 0°C caused CI after 4 weeks of storage. After 4 weeks of storage, the flesh firmness of fruit at 5°C was favourable to present these fruit as 'ready–to–buy', but the losses due to CI exceeded the benchmark proposed by Crisosto et al. (2004). Therefore, based on incidence of CI and fruit quality attributes, particularly flesh firmness, the potential storage life of 'Amber Jewel' plums is 2 weeks at 5°C while 2–3 weeks at 0°C.

6.4.3 Lipid peroxidation

Lipid peroxidation can cause membrane disintegration and inactivation of several membrane bound enzymes responsible for various physiological processes (Shewfelt and del Rosario, 2000). Low–temperature stress has been proposed to induce lipid peroxidation as an early event during chilling injury (Hodges et al., 2004; Shewfelt and del Rosario, 2000). After 2 weeks of storage, LOX activity was considerably higher in fruit stored at 5°C compared with those at 0°C (Fig. 6.2A). Surprisingly, despite the differences in LOX activity, TBRAS concentration and EL were not found to differ after 2 weeks of storage at two temperatures (Figs. 6.2B & C).

The early appearance of peak in LOX activity might be indicative of initiation of fruit ripening as LOX has been reported to be up–regulated during fruit ripening, and is a key enzyme regulating several pathways involved in aroma–volatiles synthesis and jasmonic–acid based plant defense systems (Gardner, 1995). A significant increase in TBARS concentration and EL was observed during the first 2 weeks of storage. The increased activities of LOX might be responsible for increase in TBARS concentration and EL, and gradual loss of membrane integrity. The decline in LOX activity during the last 2 weeks of storage did not affect the increases in TBARS concentration and EL. Lipid peroxidation is a self–propagating process that requires LOX for initiation and therefore causes membrane disintegration even in the absence of high LOX activity (Song et al., 2009; Zhuang et al., 1995). An increase in LOX activity during cold storage followed by a decline has

been reported in many chilling–sensitive horticultural commodities (Ding et al., 2007; Mao et al., 2007; Promyou et al., 2008; Rivera et al., 2007).

Table 6.5. Correlations of CI index and CI incidence with storage temperature, storage period, lipid peroxidation, enzymatic and non-enzymatic antioxidants in 'Amber Jewel' plums during 6 weeks of cold storage at 0°C and 5°C.

CI index *vs.*	**Pearson's correlation coefficient**	**CI incidence *vs.***	**Pearson's correlation coefficient**
Storage temp.	NS	Storage temp.	NS
Storage period	0.86***	Storage period	0.91***
TBARS	0.85***	TBARS	0.84***
LOX	NS	LOX	NS
EL	0.89***	EL	0.92***
SOD	–0.42*	SOD	–0.47*
CAT	0.67	CAT	0.65
POD	0.47*	POD	NS
AA	NS	AA	NS
DHA	NS	DHA	NS
AA:DHA	NS	AA:DHA	NS
GSH	–0.54**	GSH	–0.57**
GSSG	0.93***	GSSG	0.90***
GSH:GSSG	–0.96***	GSH:GSSG	–0.95***
APX	–0.59**	APX	–0.51**
MDHAR	NS	MDHAR	NS
DHAR	–0.48*	DHAR	–0.53**
GR	–0.53**	GR	–0.59**
GT	0.77***	GT	0.75***
Phenols	–0.76***	Phenols	–0.73***
DPPH scavenging activity	–0.79***	DPPH scavenging activity	–0.76***

NS, *, **, *** = non-significant, $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, respectively

The higher levels of TBARS in fruit stored at 5°C after 4 and 6 weeks of storage reflect the development of oxidative stress over the storage period to a greater degree than in those stored at 0°C (Fig. 6.2B). In response to chilling stress and senescence, TBARS concentration has been reported to increase in various fruits such as bananas (Promyou et al., 2008), mangoes (Ding et al., 2007; Zhao et al., 2009), and plums (Eum et al., 2009). ROS are known to mediate the initiation and propagation of lipid peroxidation (Lacan and Baccou, 1998; Shewfelt and del Rosario, 2000). During storage at 5°C, which is considered to be the lethal storage temperature for stonefruits, the ROS production might have exceeded the antioxidant potential of the system leading to enhanced lipid peroxidation. As outlined in the Chapter 4, TBARS concentration in 'Amber Jewel' plums also increased during fruit ripening at ambient conditions. Chilling stress and ripening process could therefore have additive effect on the accumulation of TBARS in the flesh tissue.

A substantial increase in EL was noticed after 4 weeks of storage at both temperatures; fruit at 5°C showed more EL than those at 0°C (Fig. 6.2C). The increase in EL, a marker of the extent of membrane damage, may occur due to CI, senescence or a combination of both (Maalekuu et al., 2006; Mao et al., 2007; Song et al., 2009; Zhao et al., 2009). Both the reasons for increase in EL are valid in our study as the fruit showed various changes associated with ripening and senescence during storage, particular at 5°C. The data demonstrate that fruit stored at 5°C undergo lipid peroxidation to a greater extent than those stored at 0°C. EL and TBARS concentration had a significant positive correlation with the incidence and severity of CI (Table 6.5).

6.4.4 Antioxidant enzyme activities (SOD, CAT, and POD)

SOD, CAT, and POD are the primary constituents of the antioxidant defense system that provide protection against the ROS (Apel and Hirt, 2004). The data suggest that SOD significantly increased during the first two weeks of storage at both temperatures, but to a greater extent at 5°C (Table 6.2). As the storage duration increased, decline in SOD activity was noticed at 0°C, whereas it further increased until after 4 weeks of storage at 5°C and then declined. SOD is responsible for dismutation of the $O_2^{\cdot-}$ produced in response to chilling stress (Bowler et al., 1992).

The enhanced SOD activity was also accompanied by a significant increase in CAT activity during the first 2 weeks of storage. CAT activity, in general, was significantly higher during storage at 5°C than at 0°C. The combined action of SOD and CAT is thought be necessary to scavenge $O_2^{\bullet -}$ and H_2O_2, respectively, and reduce the oxidative load on the cell (Apel and Hirt, 2004).

Chilling tolerance in several fruit crops, such as limes (Rivera et al., 2007), mangoes (Zhao et al., 2009), mumes (Imahori et al., 2008) and peaches (Wang et al., 2004), and resistance against browning in 'Braeburn' apples (Toivonen et al., 2003) and 'Conference' pears (Lentheric et al., 1999) have been associated with the increase in SOD and CAT activities in response to low temperature storage. Higher CAT activity has been proposed to impart chilling tolerance in mandarins (Sala, 1998) and scald resistance in apples (Rao et al., 1998), while SOD activity did not differ among chilling–sensitive and chilling–tolerant cultivars. POD activity showed significant increase during most of the storage time at 5°C, while it showed a significant decrease during storage at 0°C. The decrease in POD has been associated with the development of CI symptoms in fruits, such as apples, kiwifruit, mangoes, pears, and peaches (Ding et al., 2007; Larrigaudiére et al., 2004; Rao et al., 1998; Song et al., 2009; Wang et al., 2005). Higher POD activity may also be an indicator of the extent of tissue damage and over–ripeness that occurred during storage at 5°C as the higher POD activity has previously been linked to over–ripeness in blackberries (Wang and Jiao, 2001).

As evident from the data, the response of primary antioxidant enzymes, SOD, CAT, and POD, was significantly greater during the initial 2–4 weeks of storage at 5°C than at 0°C (Table 6.2). There could be two possibilities: 1) the production of ROS might be lower due to slower metabolism at 0°C than at 5°C, resulting in a weaker response of these enzymes at 0°C; 2) the storage at 5°C might have resulted in increased ROS production than at 0°C that caused strong antioxidant enzymatic response at 5°C. Despite the higher activities of these enzymes (Table 6.2), TBARS concentration clearly shows that oxidative stress level after the first 4 weeks of storage was significantly greater at 5°C than at 0°C (Fig. 6.2B), and therefore validates the second possibility of more ROS production at 5°C. The up–regulation of SOD, CAT, and POD in response to fruit storage at 5°C, which is in the killing temperature range for stonefruit, compared with 0°C appeared not to be sufficient to

encounter the increased ROS production and could not sustain for longer storage duration of 6 weeks.

6.4.5 Ascorbate–glutathione cycle

AA and GSH can be considered important markers for determining the fruit's capability to withstand postharvest oxidative stress (Davey and Keulemans, 2004). Furthermore, their oxidized forms, DHA and GSSG, and AA:DHA and GSH:GSSG ratios indicate the redox status of the cell, and may serve as an indirect measure of the responsiveness of the cell to oxidative stress. The concentrations of AA decreased significantly in fruit stored at 0°C, while it showed no significant change in fruit stored at 5°C (Table 6.3). The decrease in concentrations of AA has been reported to occur during long–term cold storage of other fruits such as mangoes (Zhao et al., 2009), oranges (Huang et al., 2008), and pawpaws (Galli et al., 2009). The decrease in AA concentration during storage at 0°C was also coincident with the appearance of flesh browning symptoms in fruit stored for 4 weeks at 0°C. The prolonged cold storage in air or controlled atmospheres caused depletion of AA in apples and pears resulting in internal browning (De Castro et al., 2008; Veltman et al., 1999). The increased losses of AA during cold storage in some early maturing apple cultivars, 'Sunrise' and 'Gravenstein' have been linked to their poor storage characteristics and susceptibility to internal browning (Davey and Keulemans, 2004).

The concentration of DHA increased towards the end of storage at 0°C indicating a shift in the equilibrium towards more oxidized state in the tissue, which is an indication of the failure of the ascorbate–glutathione cycle. It is interesting to note that AA:DHA ratio was less affected during storage at 5°C and also showed a transient increase during the first 2 weeks of chilling exposure of fruit suggesting that there was a strong acclimation reaction to anticipated increasing load of ROS (Table 6.3).

The increase in APX activity during the first two weeks of storage at both temperatures was noticed, but a significant reduction in APX activity was also observed during the subsequent storage period at 5°C (Table 6.3). The APX activity showed a significant negative correlation with the development of CI in 'Amber Jewel' plums (Table 6.5). The increase in APX activity has also been associated with

acquisition of chilling tolerance in several fruits during cold storage (Ding et al., 2007; Sala and Lafuente, 2004; Wang et al., 2008; Wang et al., 2006; Zhao et al., 2009). The lower APX activity after 4 weeks of storage at 5°C than at 0°C may in part explain the higher concentration of AA at 5°C as APX utilizes AA as a reductant to remove H_2O_2 from the tissue. The other possible explanation for higher concentration of AA in the fruit flesh during storage at 5°C could be due to higher activities of MDHAR and DHAR during the most of storage period at 5°C compared with 0°C (Table 6.3). MDHAR and DHAR are two reductases which catalyse the reduction of MDHA and DHA, respectively, into AA during the recycling process to maintain the reduced redox state in the cell (Apel and Hirt, 2004). The increase in DHA levels observed after 6 weeks of storage in fruit stored at 0°C coincided with a significant reduction in MDHAR and DHAR activities. The data suggest that MDHAR and DHAR play a role in the maintenance of AA:DHA ratio at a higher level during chilling stress (Stevens et al., 2008). The increase in activities of MDHAR and DHAR indicates an acclimatory response to chilling stress, while decrease in their activities reflects the failure of the antioxidative system which resulted into increased concentrations of DHA and lower levels of AA in the tissue.

GSH concentration may increase or decrease depending upon the stress level (Tausz et al., 2004). The increase in GSH during the first 4 weeks of storage at 5°C indicates that the stress level may be higher at 5°C than at 0°C (Table 6.4). In response to chilling, GSH levels have been reported either to increase in apples (Davey and Keulemans, 2004) or remain unaltered in peaches (Wang et al., 2006). The reduced levels of glutathione have been linked to the internal browning in methyl bromide–fumigated 'Thompson Seedless' grapes (Liyanage et al., 1993). The decrease in GSH during storage at 0°C might be associated with the decrease in GR activity during the first 2 weeks of storage (Table 6.4). GR activity was significantly higher after 2 weeks of storage at 5°C than at 0°C and could be associated with the higher GSH levels. GR is responsible for NADPH–dependent reduction of GSSG into GSH (Apel and Hirt, 2004), and is an integral component of the ascorbate–glutathione system. GR activity has also been reported to decrease with the storage duration in response to chilling stress in oranges (Huang et al., 2008) and peaches (Wang et al., 2006).

Though a significant increase in GSH concentration was observed during storage at 5°C, but a concomitant increase in GSSG resulted in lower GSH:GSSG ratio after 4 and 6 weeks of storage (Table 6.4). The up–regulation of GT to a greater extent may be related to more accumulation of GSSG at 5°C than at 0°C. GT utilizes GSH as a substrate to detoxify lipid hydroperoxides accumulated as a result of increased lipid peroxidation (Rogiers et al., 1998). GSH:GSSG ratio has been considered to be more important in antioxidant protection system rather than the GSH concentration. The data suggest that glutathione pool is adversely affected at both storage temperatures either through decrease in GSH concentration at 0°C or through decrease in GSH:GSSG ratio at 5°C. A very strong negative correlation was observed between GSH:GSSG and CI (Table 6.5). On the other hand, the changes in AA pool were unfavourable to the antioxidant protection system during storage at 0°C compared to 5°C.

To my knowledge, this is the first report on comparison of the enzymatic and non–enzymatic antioxidant components during storage at two contrasting temperatures (0°C and 5°C) which have been generally known to exert the opposite effects on CI incidence in stonefruits. The responses of antioxidant protective systems in 'Amber Jewel' plums were quite different under these storage temperatures. It is therefore evident that storage temperature had a direct influence on the antioxidant components in the fruit during prolonged storage. Like a few other plum cultivars, 'Amber Jewel' showed CI symptoms during storage at both temperatures, 0°C and 5°C, and a clear–cut sequence of changes in lipid peroxidation and antioxidant components was not evolved as expected under the proposed hypothesis.

The components of the enzymatic (SOD, CAT, MDHAR, DHAR, and GR) and non–enzymatic (ASH and GSH) antioxidants were favoured by the storage at 5°C than at 0°C, but 5°C appears not to be sufficiently low temperature to retard the metabolic processes such as ethylene production and respiration in 'Amber Jewel' plums, and it resulted in faster fruit ripening than 0°C. The data on fruit quality attributes, CI incidence, lipid peroxidation and antioxidants clearly indicate that storage potential of 'Amber Jewel' plums was 2–3 weeks at 0°C and 2 weeks at 5°C. The increase in storage duration under these temperatures can possibly induce

undesirable physiological changes leading to poor fruit quality which may affect the consumer experiences.

Chapter 7

A Comprehensive Biochemical Analysis of the Metabolites, Enzymatic and Non–enzymatic Antioxidants in Response to Cold Storage and Shelf life in Japanese plums[4]

Summary

In a multiple–point sampling framework, the dynamics of antioxidative systems in the flesh tissue of Japanese plums cv. 'Blackamber' in relation to CI and fruit quality during 5 weeks of cold storage at 0°C (86.5±5.5% RH) were investigated. Samplings were carried out at 2–day intervals (0, 2, 4, 6, and 8) during the 8 days of shelf life (SL) at 21±1°C (60.4±7.3% RH) after each week of storage and the fruit that were allowed to ripen without storage served as the control. Ethylene production and respiration rates were higher during the SL after 4 and 5 weeks of storage in comparison with the first 3 weeks of storage, but the number of days to reach the ethylene peak was not affected by the storage period. The extension of storage period to 4 or 5 weeks enhanced the rate of fruit ripening during SL as shown by the changes in fruit softening, skin colour, and SSC:TA ratio. CI symptoms in the form of flesh browning were visible during the SL after 3 weeks of storage, but the incidence and severity increased in the last 2 weeks of storage with diverse symptoms such as flesh browning, mealiness, translucency, and bleeding. The concentration of TBARS, a marker for lipid peroxidation, showed a significant positive correlation (r = 0.83) with the severity of CI. The extent of lipid peroxidation and membrane disintegration increased with the increase in storage period and SL. The activities of primary antioxidant enzymes, SOD, CAT, and POD, increased during the first 3 weeks in response to chilling stress, but declined significantly during SL after 4 or 5 weeks of storage. The increase in total ascorbate and glutathione during the initial phase of storage represented an acclimatory response of the fruit to chilling conditions. The activities of enzymes responsible for

[4] The additional data on the changes in concentrations of individual sugars and organic acids from this experiment has been published in the following article: Singh, S.P., Z. Singh, and E.E. Swinny. 2009. Sugars and organic acids in Japanese plums (*Prunus salicina* Lindell) as influenced by maturation, harvest date, storage temperature and period. *International Journal of Food Science and Technology*. 44:1973–1982.

the oxidation and recycling of AA and GSH were also determined to explain the levels of reduced and oxidized forms of these two cellular redox buffers. The increase in storage period caused a shift in the redox status of the fruit flesh towards more oxidized state because of the increase in concentrations of DHA and GSSG, leading to the decreased ratios of AA:DHA and GSH:GSSG. Furthermore, the concentration of total phenolics and DPPH scavenging activity increased during the middle of cold storage, but their levels declined towards the end of storage and also during SL. The data revealed that in response to chilling stress, enzymatic and non–enzymatic antioxidative systems were activated during the first 3 weeks of storage, but the extended period of storage resulted in an overall decrease in the capacity of antioxidative system to ameliorate increasing levels of oxidative stress. It appears that the declining ability of the antioxidative systems during the prolonged chilling stress resulted in oxidative injury in the form of CI, limiting the storage potential of 'Blackamber' plums.

7.1 Introduction

Postharvest oxidative stress is a phenomenon caused by an imbalance in the production and scavenging of ROS in the fruit during postharvest handling and storage (Hodges et al., 2004). ROS such as $O_2^{\bullet -}$, H_2O_2, nitric oxide ($NO^{\bullet}$), and peroxynitrite ($ONOO^{\bullet}$) are produced in plants under normal and stress conditions (Apel and Hirt, 2004). If these are not removed or detoxified by the enzymatic and non–enzymatic antioxidants, they accumulate to toxic levels in the cell and can also serve as the precursors for the generation of more toxic ROS such as $OH^{\bullet}$. The presence of ROS at higher levels and reduction in the capability of antioxidant protection system can lead to oxidative damage in the tissue (Hodges et al., 2004). The study of dynamics of antioxidants in the fruits is important for mainly three reasons. Firstly, the presence of higher levels of antioxidants protects the fruit from potentially toxic ROS and thereby providing stress tolerance (Davey et al., 2004). Secondly, the nutritional value of fruit attributable to the antioxidants is important from human consumption perspective (Gil et al., 2002). Thirdly, it has been widely argued that the higher concentrations of antioxidants in the peel and flesh tissues of fruit reduced the incidence of senescence–related disorders (Barden and Bramlage, 1994; De Castro et al., 2008; Veltman et al., 1999).

There are many external and internal factors which contribute to the initiation and propagation of ROS production in the fruit tissues (Hodges et al., 2004 and references therein). The development of postharvest oxidative stress has serious implications in fruit quality and development of several physiological disorders. Cold storage is known to extend the storage life and maintain fruit quality, but it can also be a stress factor responsible for production of ROS in the tissue if the exposure of a commodity to low temperature exceeds the threshold leading to the development of CI. The development of CI is a function of storage temperature and storage duration (Hariyadi and Parkin, 1991). Earlier reports indicate that CI is an oxidative phenomenon linked to the production of ROS causing lipid peroxidation, membrane damage, and overall reduced response of antioxidative systems (Hariyadi and Parkin, 1991; Zhang and Tian, 2009; Zhao et al., 2009). A revelation of the sequence and timing of events in the development of postharvest oxidative stress during chilling stress can help better understanding of the development of CI in fruits. Under normal and mild stresses conditions, ROS have been known to act as signalling molecules in a variety of metabolic processes such as enhancement of antioxidant protection systems and gene regulation to affect metabolic responses to ameliorate the stress response (Toivonen, 2004). On the other hand, in response to the prolonged or severe stress conditions, the ROS production may exceed the capacity of the system to scavenge them, and subcellular and cellular damage can occur.

Fruit of Japanese plums are chilling–sensitive in nature (Candan et al., 2008; Crisosto et al., 1999, 2004; Singh et al., 2009a). However, the sensitivity to chilling depends on several factors including genotype, harvest maturity, storage temperature, and storage period and shelf life (Crisosto et al., 1999; Singh et al., 2009a). The effects of the harvest maturity, storage temperature and storage period have been described in the previous chapters. This chapter is attributed to the comprehensive analysis of the effects of storage period and subsequent shelf life on the enzymatic and non–enzymatic antioxidants in flesh tissues of Japanese plums cv. ‘Blackamber’. Cold storage at 0°C is recommended to extend its storage life and maintain fruit quality for 3–5 weeks (Mitchell et al., 1974). Like many other Japanese plum cultivars, ‘Blackamber’ is also susceptible to chilling injury (CI) during prolonged cold storage (Candan et al., 2008; Crisosto et al., 2004; Crisosto et al., 1999). The symptoms of CI aggravate when the fruit are transferred to ambient conditions after

cold storage. No attempt has been made to study the influences of storage period and subsequent SL on the development of CI in Japanese plums and fruit quality in relation to lipid peroxidation, enzymatic and non–enzymatic antioxidants.

Toivonen (2004) emphasized the importance of the dynamic nature of the oxidative processes in the plant system and suggested to have time course analysis with multiple–point samplings to improve the understanding of the role of enzymatic and non–enzymatic antioxidants during postharvest stress conditions. Currently, such comprehensive information on the dynamics of antioxidants during chilling stress in many fruits including Japanese plums is not available. Therefore, the objective was to understand the dynamics of antioxidative protection systems in the flesh tissue of 'Blackamber' plums during cold storage and shelf life in a multiple point sampling framework.

7.2 Materials and methods

7.2.1 Fruit material

Japanese plum cv. 'Blackamber' were manually harvested at commercial maturity (as detailed in Section 4.2.1) on 22 December 2006 in the early morning hours from the Casuarina Valley Orchard, Karragullen, Perth Hills (lat. 31° 57' S; long. 115° 50' E), WA. Fruit were placed in plastic crates lined with 30 µm thick LDPE film folded over them and stored at 0°C for 5 weeks. Lots of 100 fruit from each replication were removed from cold storage at weekly intervals and allowed to ripen at 21±1°C for 8 days. Twenty fruit as a single replicate were analysed at 2–day intervals (0, 2, 4, 6, and 8) for various parameters. Similarly, 100 fruit, treated as control, were allowed to ripen at 21±1°C for 8 days without any storage and were analysed at 2–day intervals. The fruit were peeled and the flesh tissue was cut into small cubes and immediately frozen with liquid nitrogen and stored at –80°C for further analyses. The experiment was laid out by following a completely randomised design with two factors including storage duration and SL.

7.2.2 Respiration and ethylene production rates

Respiration and ethylene production rates of plums were determined on daily basis during 8 days of SL at 21±1°C after each week of storage. The detailed procedures for estimation of ethylene production and respiration rates have been described in Sections 3.3.1 and 3.3.2, respectively. Two fruit enclosed in an air–tight container were treated as an experimental unit and replicated four times. The respiration and ethylene production rates were expressed as mmol of CO_2 kg^{-1} h^{-1} and μmol kg^{-1} h^{-1}, respectively.

7.2.3 Fruit quality evaluation

The fruit quality was evaluated immediately after storage at weekly intervals during 5 weeks of cold storage at 0°C and also at 2–day intervals during SL period of 8 days at 21±1°C after each week of storage. Fruit firmness, colour, SSC, and TA were determined as described in Sections, 3.4.1.1, 3.4.2, and 3.4.3, of Chapter 3. For all these quality parameters, ten fruit constituted an experimental unit, with three replications.

7.2.4 Chilling injury (CI)

The incidence and severity of CI was evaluated immediately after transfer from cold storage and also during SL for 8 days at 21±1°C. Twenty fruit per replication were cut around the equatorial axis, the two halves of each fruit twisted in opposite directions, and the mesocarp was examined for symptoms such as flesh browning, mealiness, and translucency. The incidence and severity of CI were calculated as described in Section 3.4.4.

7.2.5 Oxidative stress parameters

The determinations of lipid peroxidation, enzymatic and non–enzymatic antioxidants, and protein concentration were carried out as described in Chapter 3, Sections 3.5 to 3.8.

7.2.6 Statistical analyses

The data were subjected to two–way ANOVA using GenStat Release 11.1 (VSN International Ltd., Hemel Hempstead, UK). Before statistical analysis, the data on CI incidence were subjected to arcsine transformation to reduce heteroscedasticity. The effects of storage period and SL and their interactions on different parameters were assessed within ANOVA and the LSD were calculated following a significant F–test at $P \leq 0.05$.

7.3 Results

7.3.1 Respiration and ethylene production rates

The peaks in respiratory rates were observed during fruit ripening after each storage interval, irrespective of the storage duration (Fig. 7.1A). However, the maximum rates of respiration during SL after 1, 2 and 3 weeks of storage were significantly lower than the control fruit, which were allowed to ripen directly after harvest. As the storage period increased to 4 or 5 weeks, the rates of respiration during climacteric differed non–significantly from the control fruit. The respiratory peaks were observed on the 7^{th} day of SL after each week of storage for 4 weeks, but the peak was advanced by 2 days when the storage period was extended to 5 weeks. Ethylene production rates were generally higher during SL after cold storage compared with the control fruit (Fig. 7.1B). The peak ethylene production rates were also higher in fruit held in cold storage than in control fruit. The differences in peak ethylene production rates of control and cold–stored fruit were significant after 3, 4, and 5 weeks of storage. In general, higher rates of respiration and ethylene production were observed during SL after 4 and 5 weeks of storage.

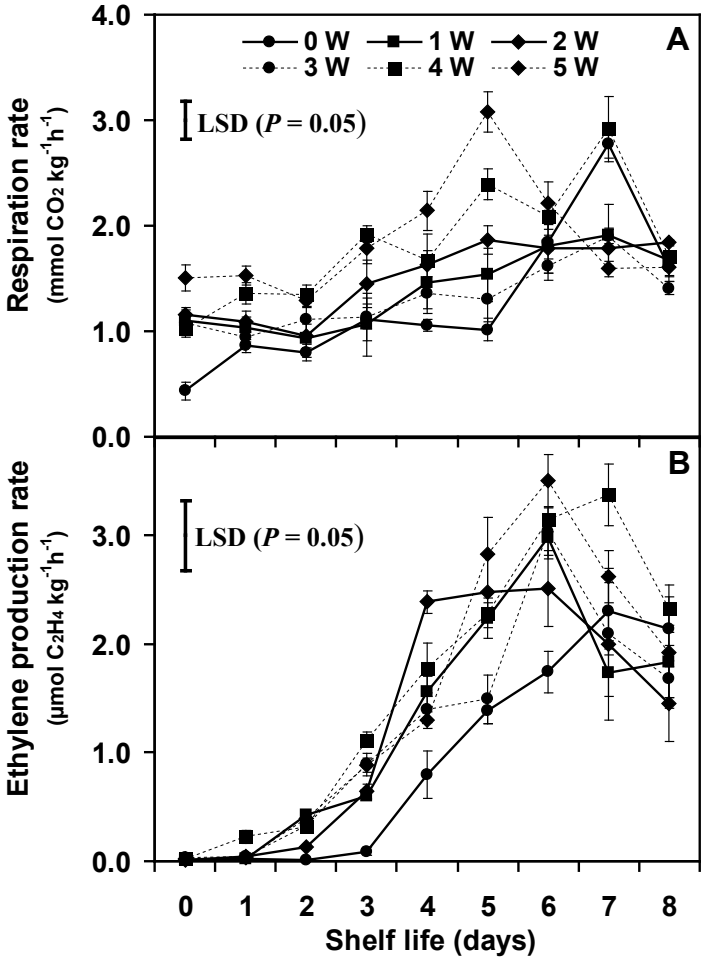


Fig. 7.1. The rates of respiration (A) and ethylene production (B) in 'Blackamber' plums as influenced by cold storage at 0°C for 5 weeks (W) and 8 days of shelf life at 21±1°C. Measurements were made on daily basis for 8 days after each week of storage (n = 4; 2 fruit/replicate). Error bars represent S.E. of means. LSD (P = 0.05) values for respiration: Storage period (SP) = 0.12, Shelf life (SL) = 0.14, SP × SL = 0.35. Ethylene: SP = 0.22, SL = 0.27, SP × SL = 0.65. LSD values for SP × SL are shown as separate bars.

7.3.2 Fruit firmness, skin colour, SSC, TA, and SSC: TA ratio

No significant loss of flesh firmness was observed during 4 weeks of storage at 0°C, but it was significant during the last week of storage (Fig. 7.2). The rate of fruit softening during SL after 0, 1, 2, and 3 weeks of storage were almost similar. As the storage period was extended to 4 and 5 weeks, the rate of fruit softening increased to some extent. However, the flesh firmness values after 8 days of SL during each storage interval were not significantly affected by the storage period indicating a remarkable storage potential of 'Blackamber' plums for 5 weeks.

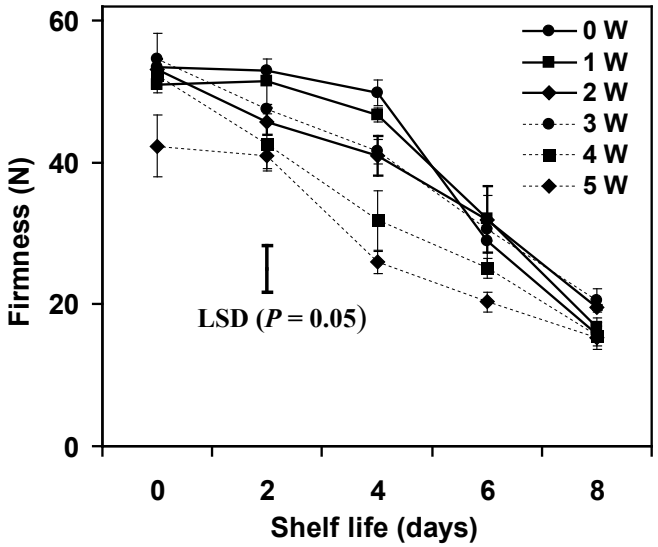


Fig. 7.2. Changes in flesh firmness of 'Blackamber' plums as influenced by cold storage at 0°C for 5 weeks (W) and 8 days of shelf life at 21±1°C. Measurements were made (n = 3; 10 fruit/replicate) on 2–day intervals for 8 days after each week of storage. Error bars represent S.E. of means. LSD ($P = 0.05$) values for flesh firmness: Storage period (SP) = 2.90, Shelf life (SL) = 2.65, SP × SL = 6.49. LSD value for SP × SL is shown as a separate bar.

Table 7.1 shows that significant changes in skin colour, as evident from L* and hue angle values, were observed during cold storage of 'Blackamber' plums. The decrease in L* values and hue angle continued during the SL after each storage week interval. The rate of changes in skin colour during SL was found parallel to the changes in flesh firmness. SSC did not change significantly during cold storage for 5 weeks, but a decrease in TA was observed as the storage period increased (Table 7.2). When the fruit were allowed to ripen at 21±1°C for 8 days after each storage week, a minor increase (< 1%) in SSC was noticed only up to the 6th day of SL followed by a slight decrease on the 8th day. This trend persisted mostly during SL after each week of storage. As expected, a significant decrease in TA was also observed during SL period. With the extended storage period, the magnitude and rate of TA loss also increased. The decrease in TA during SL and a slight increase in SSC contributed to the increased SSC:TA ratio.

Table 7.1. Changes in skin colour chromaticity (L*) and hue angle (°h) of 'Blackamber' plums during 5 weeks of storage at 0°C and during 8 days of shelf life at 21±1°C after each week of storage.

	L*					**Hue angle (°h)**				
Storage period	**Shelf life (days)**					**Shelf life (days)**				
(weeks)	0	2	4	6	8	0	2	4	6	8
0	8.36	7.93	4.46	3.92	3.28	13.4	13.9	6.3	–6.70	–32.4
1	7.62	6.88	5.54	5.24	5.45	9.4	–3.5	–10.8	–26.1	–34.3
2	6.00	5.20	5.40	4.74	4.57	9.2	–4.1	–9.5	–28.9	–40.1
3	5.74	4.67	4.82	5.60	4.45	7.7	–3.4	–9.6	–27.9	–48.0
4	5.81	4.39	5.85	6.79	4.60	7.1	–6.9	–27.0	–29.2	–50.1
5	6.37	5.24	5.13	6.35	3.97	4.0	–6.6	–27.2	–39.2	–54.5
Least significant differences of means at 5% level and levels of significance for a two–factor ANOVA										
Storage period (SP)	0.46 ***					3.2 ***				
Shelf life (SL)	0.42 ***					2.9 ***				
SP X SL	1.03 ***					7.1 ***				

*** = $P \leq 0.001$

Table 7.2. Changes in soluble solids concentration (SSC) and titratable acidity (TA) of 'Blackamber' plums during 5 weeks of storage at 0°C and during 8 days of shelf life at 21±1°C after each week of storage.

	SSC (°Brix)					**TA (%)**				
Storage period	**Shelf life (days)**					**Shelf life (days)**				
(weeks)	0	2	4	6	8	0	2	4	6	8
0	10.9	11.0	11.8	11.6	10.9	1.46	1.44	1.29	1.28	1.11
1	11.0	11.2	11.2	11.5	11.0	1.42	1.42	1.21	1.08	0.96
2	10.8	10.7	10.6	11.4	10.8	1.40	1.24	1.21	1.21	0.95
3	10.9	10.8	10.7	10.7	10.9	1.29	1.12	1.09	1.06	0.80
4	10.9	10.9	11.0	11.3	10.9	1.17	1.07	0.84	0.72	0.70
5	10.9	10.8	10.9	11.5	10.9	0.88	0.78	0.82	0.77	0.78
Least significant differences of means at 5% level and levels of significance for a two–factor ANOVA										
Storage period (SP)	0.1 ***					0.02 ***				
Shelf life (SL)	0.1 ***					0.02 ***				
SP X SL	0.3 ***					0.05 ***				

*** = $P \leq 0.001$

7.3.3 Chilling injury (CI)

CI symptoms in the form of flesh browning and mealiness appeared during SL after 3 weeks of storage, but the incidence and severity of CI were very low (Fig. 7.3). After 4 and 5 weeks of storage at 0°C, the incidence of CI was about 12% and 18% which increased to 22% and 35% after 8 days of SL period, respectively. With the advancement of storage period and SL, the incidence and severity of CI increased; the CI index was very low (< 1) after 4 weeks and low (< 2) after 5 weeks of storage. Flesh bleeding was also observed during SL after 5 weeks of storage as the fruit ripening progressed.

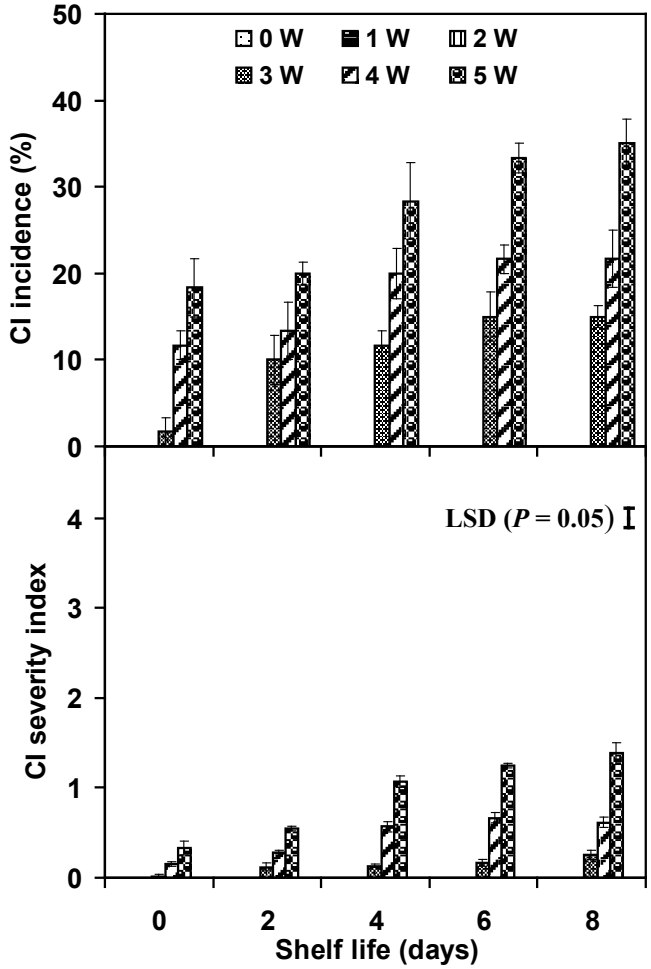


Fig. 7.3. CI incidence (A) and CI severity index (B) in 'Blackamber' plums as influenced by cold storage at 0°C for 5 weeks (W) and 8 days of shelf life at 21±1°C. Observations were recorded (n=3; 20 fruit/replicate) on 2–day intervals for 8 days after each week of storage. Error bars represent S.E. of means. Data on CI incidence were arcsine transformed, but back–transformed data are presented. LSD (P = 0.05) values for CI incidence (transformed data): Storage period (SP) = 0.03, Shelf life (SL) = 0.03, SP × SL = 0.08. CI index: SP = 0.05, SL = 0.04, SP × SL = 0.11. LSD value for SP × SL is shown as a separate bar.

7.3.4 Lipid peroxidation

The data on changes in LOX activity, TBARS concentration and EL during storage and SL of 'Blackamber' plums are presented in Figure 7.4.

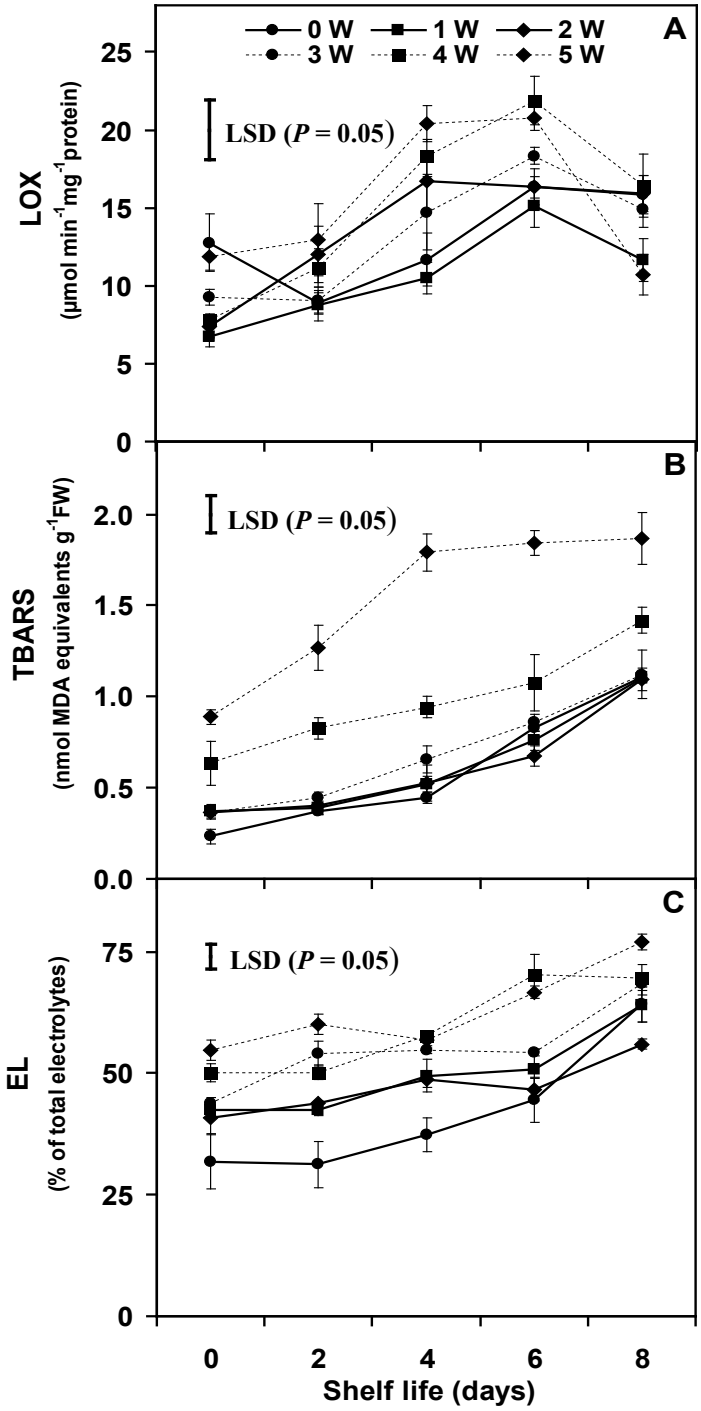


Fig. 7.4. Changes in LOX activity (A), TBARS concentration (B), and EL (C) in the flesh tissue of 'Blackamber' plums as influenced by cold storage at 0°C for 5 weeks (W) and 8 days of shelf life at 21±1°C. Error bars represent S.E. of means (n = 3). LSD (P = 0.05) values for LOX: Storage period (SP) = 1.73, Shelf life (SL) = 1.58, SP × SL = 3.86. TBARS: SP = 0.09, SL = 0.08, SP × SL = 0.21. EL: SP = 2.37, SL = 2.16, SP × SL = 5.29. LSD values for SP × SL are shown as separate bars.

A significant decrease in LOX activity in flesh tissue was observed during the first week of storage and it remained stable during the subsequent 3 weeks followed by a sharp increase on the 5th week of storage (Fig. 7.4A). The increase in LOX activity reached a peak followed by a decline during SL period after each storage interval. The maximum LOX activity was observed during the 6th day of fruit ripening after 4 and 5 weeks of storage. Fig. 7.4B shows that TBARS concentration increased significantly during cold storage and SL of 'Blackamber' plums. The increase in TBARS concentration was about 3 and 4 fold higher after 4 and 5 weeks of storage, respectively, compared with the concentrations at harvest. After each storage interval, the increase in TBARS concentration during SL was in the range of 2 to 4 fold. The trends in accumulation of TBARS were quite similar during the first 3 weeks of storage, but the highest amount of TBARS accumulation was observed during the SL after 5 weeks of storage at 0°C. A significant increase in EL was observed during cold storage and SL of fruit (Fig. 7.4C). The increase in EL progressed as the storage period was extended and the amount of EL immediately after 5 weeks of storage was about 1.7 fold higher than its initial concentration at harvest. A significant increase in EL was also observed during fruit ripening after each storage interval.

7.3.5 Antioxidant enzymes activities (SOD, CAT, and POD)

Storage period and SL had a significant effect on the activities of SOD, CAT, and POD in flesh tissue of fruit (Fig. 7.5). SOD activity remained stable for the first 2 weeks of storage and showed a significant increase after 3 weeks of storage followed by a substantial decrease during the subsequent storage period (Fig. 7.5A). During the SL, SOD activity in general showed a decreasing trend except a non–significant increase during the first 2 days in some cases. The amount of decrease in SOD activity during the 8 days of SL after 4 and 5 weeks of storage was significantly greater than that occurred after the first 3 weeks of storage. CAT activity also showed a significant increase with the increase in storage period and reached a peak value after 3 weeks followed by a significant decline during the last 2 weeks of storage (Fig. 7.5B). CAT activity showed a decreasing trend during 8 days of SL after each week of storage interval. No significant differences in the CAT activities

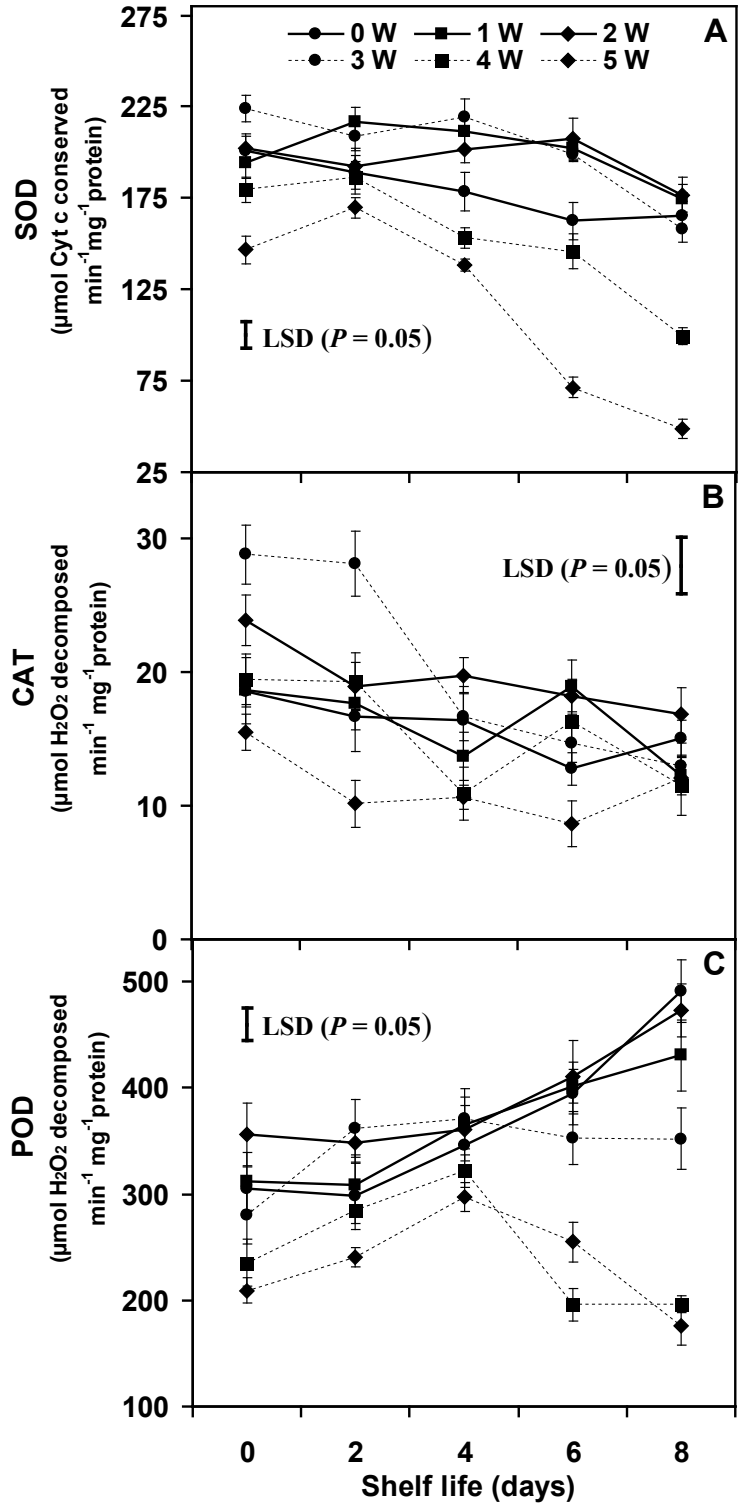


Fig. 7.5. Changes in activities of SOD (A), CAT (B), and POD (C) in the flesh tissue of 'Blackamber' plums as influenced by cold storage at 0°C for 5 weeks (W) and 8 days of shelf life at 21±1°C. Error bars represent S.E. of means (n = 3). LSD (P = 0.05) values for SOD: Storage period (SP) = 6.50, Shelf life (SL) = 5.93, SP × SL = 14.53. CAT: SP = 1.90, SL = 1.73, SP × SL = 4.24. POD: SP = 3.50, SL = 3.20, SP × SL = 7.83. LSD values for SP × SL are shown as separate bars.

were observed on the 8th day of SL irrespective of the storage period. POD activity increased significantly after 2 weeks of storage and exhibited a decrease during the last 3 weeks (Fig. 7.5C). A continuous increase in POD activity was observed during the SL after 0, 1, and 2 weeks of storage. However, POD activity showed a transient increase on the 4 day of SL after 3, 4, and 5 weeks of storage followed by a decline.

7.3.6 Ascorbate–glutathione cycle

7.3.6.1 Ascorbate

A significant increase in total ascorbate (AA + DHA) concentration in the flesh tissue was observed during the first 3 weeks followed by a slight decrease during the last 2 weeks of storage (Fig. 7.6A). An increase in total ascorbate concentration was observed in fruit during SL period after each week of storage except in control, when fruit were allowed to ripen without any storage. The increase in total ascorbate concentration during SL after cold storage was to the extent of 1.6 to 2.3 fold compared with the control showing 1.3 fold increase over the same period. Contrary to a significant increase in the concentration of total ascorbate during cold storage, no significant change in the concentration of ascorbate (AA) was observed during the first 4 weeks of storage and a minor decrease in AA was noticed during the last week (Fig. 7.6B). However, a significant increase in the concentration of AA was observed during fruit ripening after each week of cold storage, while a decrease in AA was noticed in fruit which were allowed to ripen without storage. The per cent increase in AA concentration in fruit during SL after 4 and 5 weeks of storage was comparatively lower than that occurred after the first 3 weeks of storage.

The concentration of DHA increased by about 7 fold during the first week of storage (Fig. 7.6C). With the prolonged storage period, the concentrations of DHA showed minor fluctuations with no clear trend. A noticeable increase in DHA concentration was also observed during SL period after each week of storage. It is evident that the contribution of DHA to the total ascorbate concentration increased during the SL after each storage week. As a consequence of it, AA:DHA ratio decreased significantly during SL after each week of storage (Fig. 7.6D). As the storage period increased, the decrease in AA:DHA ratio was to a greater extent. For

example, decrease in AA:DHA ratio during 8 days of SL was from 1.1 to 0.9 and from 1.0 to 0.4 after 1 and 5 weeks of storage, respectively.

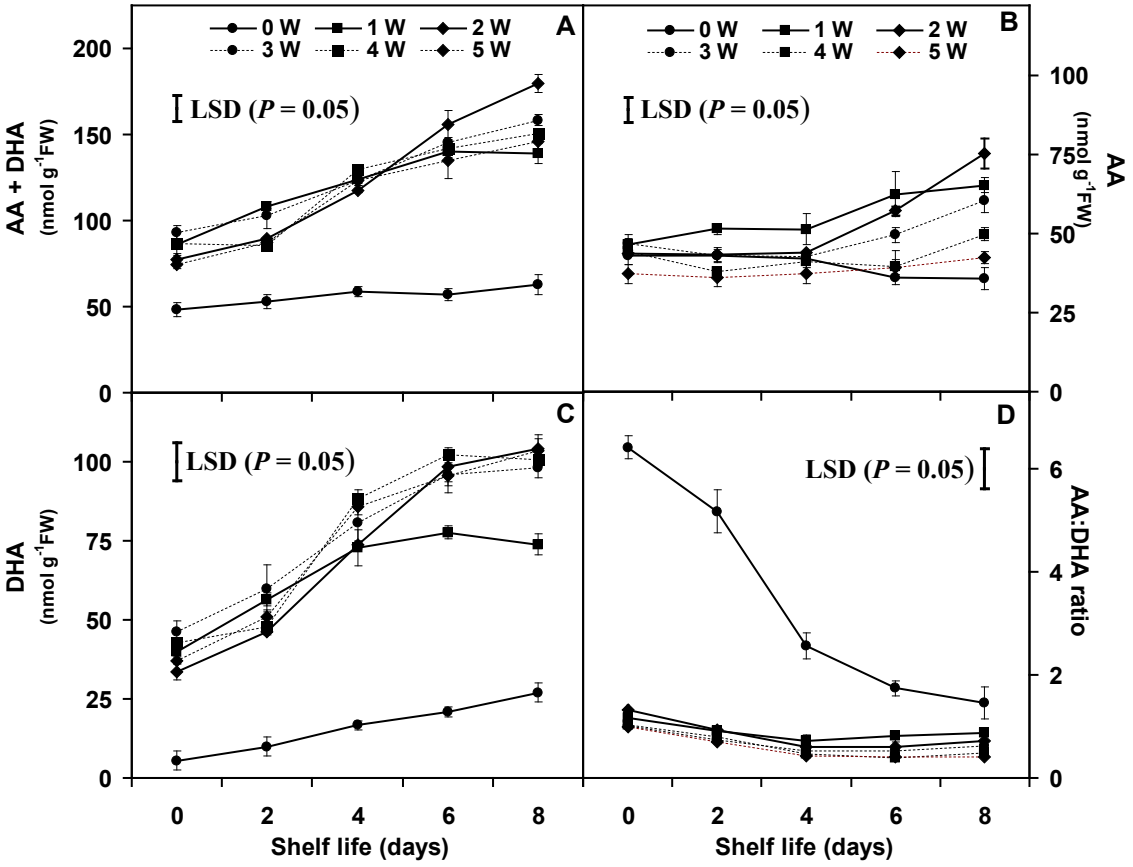


Fig. 7.6. Changes in concentrations of total ascorbate (AA + DHA) (A), AA (B), DHA (C), and AA:DHA ratio (D) in the flesh tissue of 'Blackamber' plums as influenced by cold storage at 0°C for 5 weeks (W) and 8 days of shelf life at 21±1°C. Error bars represent S.E. of means (n = 3). LSD (P = 0.05) values for AA + DHA: Storage period (SP) = 6.78, Shelf life (SL) = 6.19, SP × SL = 15.17. AA: SP = 3.56, SL = 3.25, SP × SL = 7.96. DHA: SP = 5.39, SL = 4.92, SP × SL = 12.06. AA:DHA ratio: SP = 0.35, SL = 0.32, SP × SL = 0.78. LSD values for SP × SL are shown as separate bars.

7.3.6.2 Glutathione

Similar to total ascorbate, the concentration of total glutathione (GSH + GSSG) increased significantly during the first 3 weeks of storage followed by a significant decrease to the values almost equal to at harvest (Fig. 7.7A). In response to fruit ripening during SL period, a general trend of decrease in total glutathione was observed after each week of storage. However, the concentration of total glutathione

in cold–stored fruit during all samplings of SL remained significantly higher than in control fruit that were not exposed to low temperature. GSH concentration increased in response to cold storage for 3 weeks, followed by a significant decrease during the subsequent 2 weeks (Fig. 7.7B). The decrease in concentration of GSH during fruit ripening paralleled the decrease in total glutathione. Concentration of GSSG increased with the increase in storage period; GSSG concentration was almost doubled during 5 weeks of cold storage (Fig. 7.7C). The increase in GSSG concentration was further noticed after during the SL period. The overall increase in GSSG concentration during SL was greater (1.5 to 2.4 fold) after the first 3 weeks of storage compared with the last 2 weeks (1.3 fold). As a consequence of increase in GSSG concentration during storage and SL, the GSH:GSSG ratio declined significantly (Fig. 7.7D).

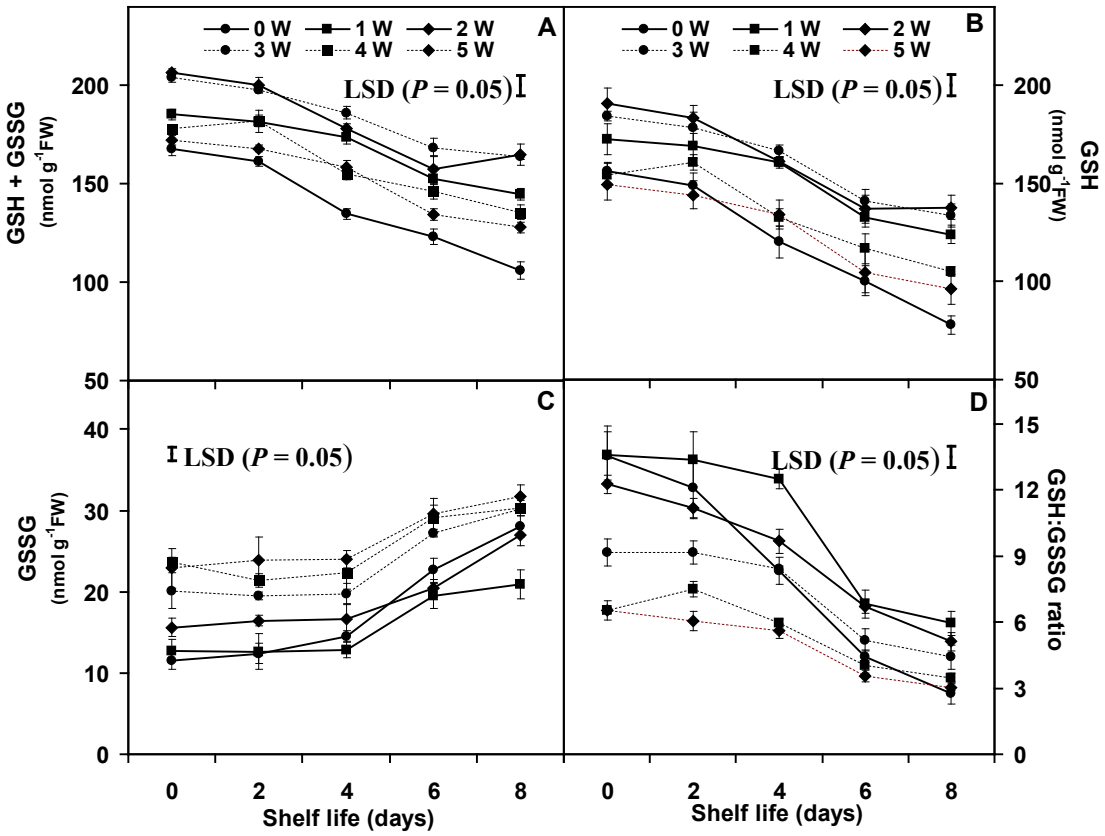


Fig. 7.7. Changes in concentrations of total glutathione (GSH + GSSG) (A), GSH (B), GSSG (C), and GSH:GSSG ratio (D) in the flesh tissue of 'Blackamber' plums as influenced by cold storage at 0°C for 5 weeks (W) and 8 days of shelf life at 21±1°C. Error bars represent S.E. of means (n = 3). LSD (P = 0.05) values for GSH + GSSG: Storage period (SP) = 2.42, Shelf life (SL) = 2.21, SP × SL = 5.41. GSH: SP = 2.38, SL = 2.18, SP × SL = 5.33. GSSG: SP = 0.37, SL = 0.34, SP × SL = 0.84. GSH:GSSG ratio: SP = 0.22, SL = 0.20, SP × SL = 0.48. LSD values for SP × SL are shown as separate bars.

7.3.6.3 Ascorbate glutathione cycle enzymes

APX activity remained stable for the first 2 weeks of storage followed by a significant increase during the last 3 weeks of storage (Fig. 7.8A).

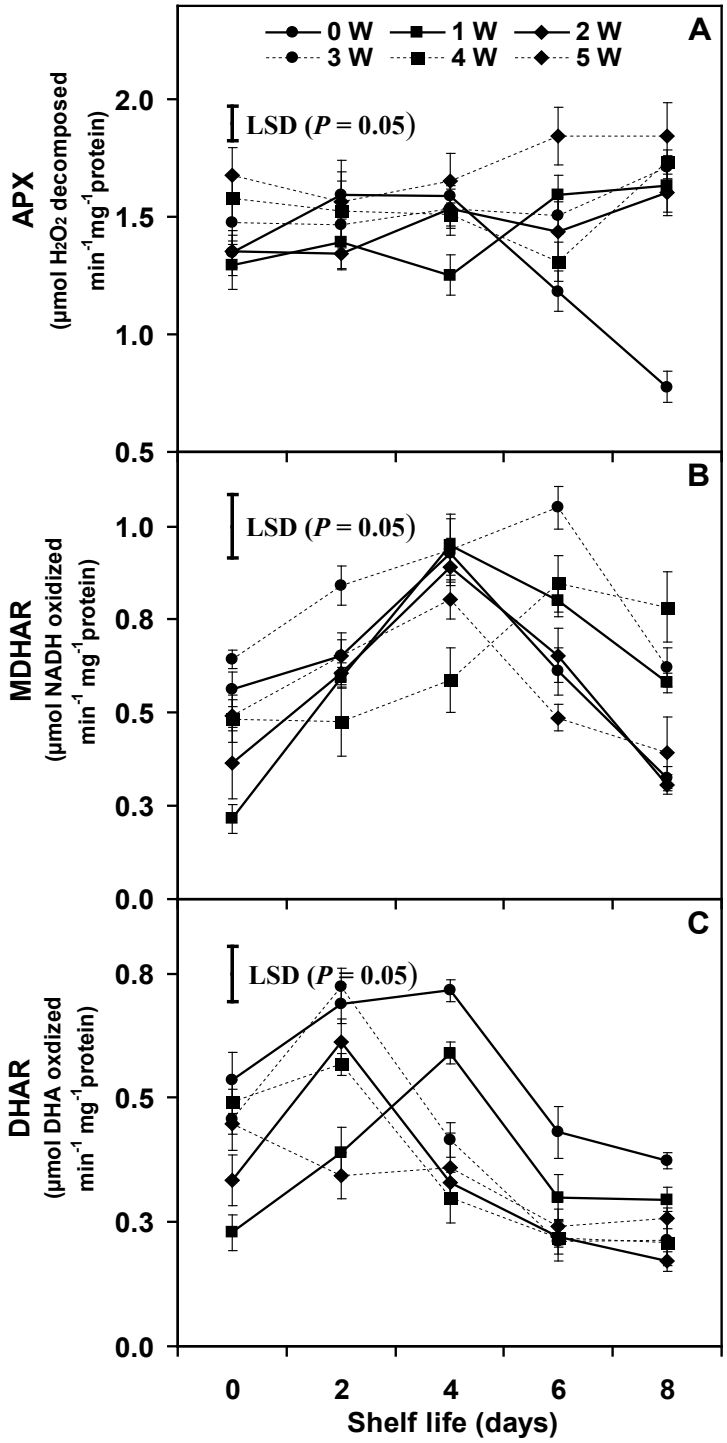


Fig. 7.8. Changes in activities of APX (A), MDHAR (B), and DHAR (C) in the flesh tissue of 'Blackamber' plums as influenced by cold storage at 0°C for 5 weeks (W) and 8 days of shelf life at 21±1°C. Error bars represent S.E. of means (n = 3). LSD (P = 0.05) values for APX: Storage period (SP) = 0.07, Shelf life (SL) = 0.06, SP × SL = 0.15. MDHAR: SP = 0.08, SL = 0.07, SP × SL = 0.17. DHAR: SP = 0.05, SL = 0.05, SP × SL = 0.11. LSD values for SP × SL are shown as separate bars.

APX activity was also strongly influenced by the SL period. A transient increase in APX was observed in control fruit during the first 4 days of SL followed by a decline. However, the APX activity showed a continuous increase during SL after each week of cold storage. With the increase in storage period, the peak APX activities also increased and the maximum activity was exhibited during the SL of fruit stored for 5 weeks. A clear trend in MDHAR activity was not noticed in relation to the storage period, but it decreased during the initial 2 weeks of storage followed by an increase on the 3rd week of storage, and then finally decreased (Fig. 7.8B). MDHAR activity declined during the advanced stages of SL after each week of storage; the differences in magnitude of peak activities did not vary greatly and followed the same trend except with minor diversions in case of weeks 3 and 4. Similar to MDHAR, the activity of DHAR decreased significantly during the first 2 weeks of storage which recovered to the initial level during the last 3 weeks of storage (Fig. 7.8C). Interestingly, a peak in DHAR activity was also observed during SL after each week of storage up to 4 weeks, but it showed a decreasing trend without any peak after 5 weeks of storage. The increase in DHAR continued up to 4th day after 0 and 1 weeks of storage, while peak DHAR activity was noticed on the 2nd day after 2, 3, and 4 weeks.

Storage period and SL had a significant influence on GR activity. In response to cold storage, the GR activity increased during the first 3 weeks of storage and then decreased later on (Fig. 7.9A). Depending on the storage period, the GR activity either showed a peak or declined during SL of 8 days. GR activity showed an increase during SL after 1 week of storage and this increase was significantly higher and remained stable compared to that occurring in control fruit without storage. The stability in GR activity was generally observed during the first 4 days of SL after 2 to 5 weeks of cold storage. GR activity during the advanced stages (6 and 8 days) of SL after 4 and 5 weeks was significantly lower than after 1, 2, and 3 weeks. No significant change in GT activity was observed during the first 4 weeks of storage, but it increased significantly during the last week (Fig. 7.9B). GT activity showed an increase with the advancement of SL period and the peak activity was observed on the 8th day of SL after each week of storage except in control showing peak activity on the 6th day. The maximum activity of GT was observed during the SL period after 5 weeks of storage.

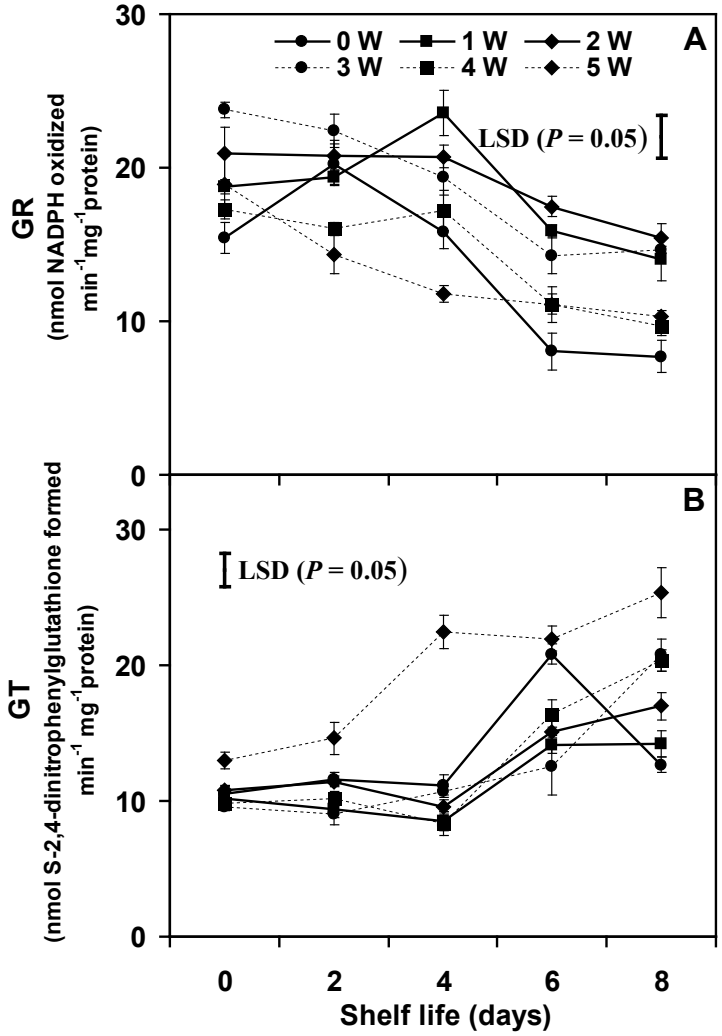


Fig. 7.9. Changes in activities of GR (A) and GT (B) in the flesh tissue of 'Blackamber' plums as influenced by cold storage at 0°C for 5 weeks (W) and 8 days of shelf life at 21±1°C. Error bars represent S.E. of means (n = 3). LSD (P = 0.05) values for GR: Storage period (SP) = 1.26, Shelf life (SL) = 1.15, SP × SL = 2.83. GT: SP = 1.07, SL = 0.98, SP × SL = 2.40. LSD values for SP × SL are shown as separate bars.

7.3.7 Total phenolics and DPPH˙ radical scavenging activity

The concentration of total phenolics in fruit flesh was significantly influenced by storage period and SL (Fig. 7.10A). An increase in total phenolics was observed after 3 weeks of storage followed by a marked decrease in the last 2 weeks. A significant increase in total phenolics concentration also occurred during the SL period after each week of storage up to 3 weeks. This trend discontinued as the storage progressed into the 4 and 5 weeks and a decreasing trend was observed throughout the SL period. Similar to total phenolics, a slight increase in DPPH˙ radical

scavenging activity in fruit flesh was also noticed after 3 weeks of cold storage (Fig. 7.10B). The increase in DPPH· radical scavenging activity was also noticed during the SL of cold stored fruit after each week of storage. In general, the peak DPPH· radical scavenging activity was slightly higher during the SL period after the first 3 weeks of storage than those stored for 4 or 5 weeks.

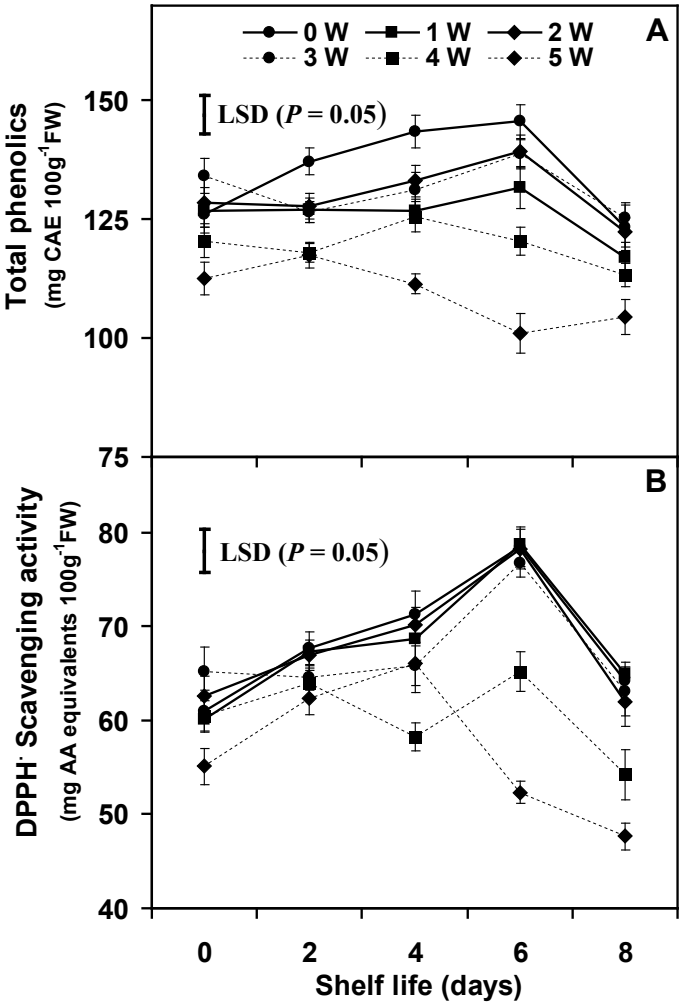


Fig. 7.10. Changes in total phenolics concentration (A) and DPPH scavenging activity (B) in the flesh tissue of 'Blackamber' plums as influenced by cold storage at 0°C for 5 weeks (W) and 8 days of shelf life at 21±1°C. Error bars represent S.E. of means (n = 3). LSD (P = 0.05) values for total phenolics: Storage period (SP) = 3.63, Shelf life (SL) = 3.32, SP × SL = 8.13. DPPH· scavenging activity: SP = 2.20, SL = 2.01, SP × SL = 4.92. LSD values for SP × SL are shown as separate bars.

7.4 Discussion

7.4.1 Respiration and ethylene production

'Blackamber' is a climacteric–type plum cultivar (Khan and Singh, 2007b) and its storage potential is, therefore, influenced by physiological processes such as rates of respiration and ethylene production. The increase in storage duration to 4 or 5 weeks stimulated the respiration rates and advanced the peaks to some extent (Fig. 7.1A). The prolonged cold storage also increased the ethylene production rates during SL without much effect on the number of days to reach the peak (Fig. 7.1B). Candan et al. (2008) also reported that cold storage increased ethylene production in 'Larry Ann' plums, but the number of days to reach the ethylene peak was independent of the storage period. Cold storage of Japanese plums cv. 'Tegan Blue' for 3 and 6 weeks at 0°C has been reported to promote the accumulation of 1–aminocyclopropane–1–carboxylic acid (ACC) and increase the activities of ACC synthase and ACC oxidase (Khan and Singh, 2009). This could explain the rise in ethylene production, triggered by the exposure to ambient conditions (21±1°C) during SL, to a greater level after 4 and 5 weeks of cold storage at 0°C.

7.4.2 Fruit quality and chilling injury

Fruit softening was not significant in 'Blackamber' plums during the first 4 weeks of cold storage and no significant differences in the flesh firmness existed after 8 days of SL in each week of storage (Fig. 7.2). The firmness losses in other cultivars, such as 'Tegan Blue' and 'Amber Jewel', over the same storage period have been reported to be significantly greater (Khan and Singh, 2009; Singh et al., 2009a). After the first 3 weeks of storage, the rate of fruit softening during SL was almost similar, while it increased after 4 and 5 weeks. The increased rate of softening after prolonged storage could be related to the enhanced respiration and ethylene production rates. Ethylene promotes fruit softening in Japanese plums through increased activities of cell–wall hydrolysing enzymes such as PG, PE, and EGase (Khan and Singh, 2009). The blocking of ethylene action by postharvest application of 1–MCP, an ethylene action inhibitor, has been found to decrease the activities of various enzymes involved in ethylene biosynthesis and cell–wall modifications (Khan and Singh, 2009).

Therefore, the presence of ethylene at higher levels might have promoted fruit softening and other ripening–related processes.

The changes in skin colour as indicated by decrease in L* and hue angle were significant during 5 weeks of cold storage and SL (Table 7.1). These changes in skin colour during storage were not associated with other fruit ripening processes such as fruit softening, and thus appear to be independent of ethylene action as previously suggested in Japanese plums (Abdi et al., 1997). There is another possible explanation that the darkening of skin colour during cold storage might have been caused by the activation of phenylpropanoid metabolism resulting in enhanced biosynthesis of anthocyanins during cold storage as reported in grapes (Sanchez–Ballesta et al., 2007). The major genes of the anthocyanin biosynthetic pathway, such as phenylalanine ammonia–lyase (PAL) and chalcone synthase (CHS), can be considered 'cor' (cold–regulated) genes (Christie et al., 1994). The increase in concentration of total phenolics after 3 weeks of storage is a direct evidence of the stimulation of this pathway which might have resulting in the accumulation of increased levels of anthocyanin pigments in the skin. Storage period has no significant influence on SSC, but TA decreased with the increase in storage period. Casquero and Guerra (2009) also reported similar observations on the changes in skin colour, SSC, and TA during 40 days cold storage of 'Oullins Gage' cultivar of European plums. The loss of TA was mainly responsible for increase in SSC:TA ratio and thus improving fruit flavour in 'Blackamber' plums. Crisosto et al. (2004) found that the consumer acceptance of 'Blackamber' plums, which are low in sugar and high in acid, was not influenced by SSC:TA ratio when SSC was≥ 12°Brix, but the consumer liking for plums with SSC of 10.0–11.9°Brix increased with the decrease in TA from 0.99% to less than 0.6%. The data suggest that the storage of 'Blackamber' plums for 5 weeks at 0°C and 8 days of SL at 21±1°C had no detrimental effect on fruit quality in terms of flesh firmness, skin colour and SSC:TA ratio, but the rate of ripening was slightly accelerated when storage was extended to 4 or 5 weeks. The fruit were in ready–to–buy (13–26 N) stage after 8 days of SL in each week of storage at 0°C.

In this experiment, the CI symptoms were noticed in very low severity in the form of flesh browning during SL after 3 weeks of storage (Fig. 7.3B). With the advancement of storage period and SL, the incidence and severity of CI increased;

but the CI index was very low (< 1) even after 4 weeks and low (< 2) after 5 weeks of storage. The loss of more than 25% fruit due to CI has been considered to be a benchmark to determine the storage potential in Japanese plums (Crisosto et al., 2004) and therefore the storage life of 'Blackamber' plums was 4 weeks at 0°C. According to Crisosto et al. (2004), 'Blackamber' can be stored at 0°C for 5 weeks, and its market life is limited beyond this period due to CI symptoms expressed as flesh browning, mealiness, and translucency. The development of CI in Japanese plums is influenced by multiple factors such as cultivar, crop load, canopy position of fruit, tree mineral nutrition, and delay in cooling (Crisosto et al., 1999; Ward and Melvin–Carter, 2001).

7.4.3 Lipid peroxidation

The increase in concentration of TBRAS in fruit flesh during cold storage and SL is an indication of the peroxidative damage caused to the fruit tissue; the extent of damage increased significantly during the last 2 weeks of storage (Fig. 7.4B). The peroxidation of free PUFA by LOX causes accumulation of lipid hydroperoxides which can further decompose into oxy–radicals, ethane, and TBARS (Shewfelt and Purvis, 1995). The increase in LOX activity during SL may be related to the changes during fruit ripening (Fig. 7.4A) as LOX is a multifunctional and key enzyme involved in the biosynthesis of aroma–volatile compounds during fruit ripening and also defense–related pathways (Gardner, 1995). In addition to the LOX, the membrane lipids may also be damaged by the ROS in response to chilling stress (Shewfelt and Purvis, 1995). The higher concentration of TBARS towards the advanced stages of storage indicates the higher levels of oxidative stress which might have developed due to increased concentration of the ROS or decreased capability of the antioxidant protection system or both. The concentration of TBARS was found to be positively correlated ($r = 0.83$) with the severity of CI (Table 7.3). Earlier, it has been reported that TBARS concentration increases in response to chilling stress in fruits such as bananas, mangoes, and plums (Ding et al., 2007; Promyou et al., 2008; Zhao et al., 2009). A higher LOX activity coupled with a higher TBARS concentration indicate the cell membrane dissociation resulting in EL and fruit softening during post–storage SL. The higher levels of EL after 4 and 5 weeks of storage compared with previous weeks reflect that the membrane permeability had

increased substantially with the increase in CI incidence and the relationship between EL and CI index was positively correlated (Table 7.3). Lipid peroxidation indicated by TBARS concentration and membrane degradation as marked by increase of EL could possibly be due to both chilling injury and fruit senescence (Du and Bramlage, 1995). In conclusion, the extent of lipid peroxidation in 'Blackamber' plums seems to be a function of both storage period and SL.

Table 7.3. Correlations of CI index and CI incidence with lipid peroxidation, enzymatic and non–enzymatic antioxidants in 'Blackamber' plums during 5 weeks of cold storage plus 8 days of shelf life at 21±1°C.

CI index *vs.*	**Pearson's correlation coefficient**	**CI incidence *vs.***	**Pearson's correlation coefficient**
TBARS	0.83***	TBARS	0.78***
LOX	0.39***	LOX	0.38**
EL	0.65***	EL	0.68***
SOD	–0.85***	SOD	–0.76***
CAT	–0.51***	CAT	–0.47***
POD	–0.65***	POD	–0.61***
AA	–0.32**	AA	–0.33**
DHA	0.49***	DHA	0.50***
AA:DHA	–0.32**	AA:DHA	–0.37**
GSH	–0.45***	GSH	–0.37**
GSSG	0.63***	GSSG	0.68***
GSH:GSSG	–0.55***	GSH:GSSG	–0.58***
APX	0.50***	APX	0.52***
MDHAR	NS	MDHAR	NS
DHAR	–0.32***	DHAR	–0.31***
GR	–0.47**	GR	–0.42***
GT	0.65***	GT	0.55***
Phenols	–0.70***	Phenols	–0.66***
DPPH scavenging activity	–0.56***	DPPH scavenging activity	–0.55***

NS, *, **, *** = non–significant, $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, respectively

7.4.4 Antioxidant enzymes (SOD, CAT, and POD)

The objective of multiple samplings during cold storage and SL of 'Blackamber' plums was to identify the critical stages of antioxidant responses and also to determine the point of their decline. It has been proposed that in response to low temperature storage, the demand for adenosine triphosphate (ATP) decreases in plants or plant parts, resulting in an excess or overflow of electrons during electron transport chain in mitochondria and increased formation of ROS (Wismer, 2003). The exposure of fruit to prolonged chilling conditions can enhance the production of ROS that are required to be quenched from the system to maintain the cellular redox homeostasis. The antioxidant enzymes such as SOD, CAT, and POD are the primary line of defence against the ROS to avoid their accumulation to toxic levels (Apel and Hirt, 2004). SOD causes dismutation of $O_2^{\bullet-}$ into H_2O_2 which is further metabolized by CAT, POD and APX. These enzymes act in a concerted manner to maintain ROS concentration at a low steady–state level.

The increases in SOD, CAT, and POD activities were observed after 3 weeks of cold storage and it could be attributed to the stimulation of the antioxidant system to scavenge the increasing levels of ROS. However, a decline in the activities of SOD, CAT and POD during the last 2 weeks of storage (Fig.7.5) suggests that antioxidant protection system might have degraded and failed to cope with the increasing levels of stress due to prolonged chilling exposure. The activities of all these enzymes in the fruit flesh were notably lower during the SL period after 4 and 5 weeks of storage in comparison with previous weeks. The lower SOD activity can promote the abundance of $O_2^{\bullet-}$ which indirectly may increase the Haber–Weiss reaction rate, leading to production of the most reactive and damaging hydroxyl radicals (Bowler et al., 1992). The decreased activities of these enzymes might have led to the accumulation of ROS to a greater extent resulting in oxidative stress in the fruit tissue. The level and duration of stress which is being imposed also determines the level of ROS and the response of antioxidant system (Leshem et al., 1998).

The decreased activities of SOD, CAT and POD were coincident with the higher incidence of CI after 5 weeks of storage (Fig. 7.5 and 7.3A). The correlation analysis also shows that the severity of CI was negatively correlated with the activities of SOD, CAT, and POD and the correlation coefficients (r) were –0.85, –0.51, and –0.65, respectively (Table 7.3). These observations imply that chilling

damage occurred during decreased activities of these antioxidant enzymes. Previous studies have shown that higher activities of SOD and CAT in response to cold storage imparted chilling tolerance in several fruit crops such as limes (Rivera et al., 2007), mandarins (Sala, 1998), mangoes (Zhao et al., 2009) and peaches (Wang et al., 2004), and also conferred resistance against internal browning and skin scald in apples (Rao et al., 1998; Toivonen et al., 2003). Furthermore, the development of CI symptoms in fruits such as apples, kiwifruit, mangoes, pears, and peaches has been attributed to the decrease in POD activity (Ding et al., 2007; Song et al., 2009; Wang et al., 2004). It is clear from the data that duration of chilling stress had a remarkable influence on the activities of antioxidant enzymes which sustained at higher levels during the initial 3 weeks of storage.

7.4.5 Ascorbate–glutathione cycle

AA and GSH are very important components of the ascorbate–glutathione cycle, which operates to remove H_2O_2 from the cell involving oxidation of AA and GSH, and their regeneration through enzymatic or non–enzymatic pathways (Noctor and Foyer, 1998). The increase in concentration of total ascorbate in fruit flesh during the first 3 weeks of storage may be attributed to an acclimatization response to the chilling stress (Fig. 7.6B). Interestingly, the increase in total ascorbate was mainly due to increase in the concentration of DHA as the AA levels showed non–significant changes during the same period. These observations show that AA biosynthesis and oxidation were occurring simultaneously during storage and SL of 'Blackamber' plums. The increase in AA was also noticed during fruit ripening after the first 3 weeks of storage, while such increase was lacking after 4 and 5 weeks. The increase in AA during fruit ripening and chilling stress has also been reported in tomatoes (Ioannidi et al., 2009; Jiménez et al., 2002a; Stevens et al., 2008). The increased transcript levels of L–galactose–1–phosphate phosphatase (GPP), a regulatory enzyme in AA biosynthetic pathway, have been closely related to the increased AA production in response to ethylene, fruit ripening and chilling stress in tomatoes (Ioannidi et al., 2009). Possibly, a similar mechanism of AA biosynthesis in ripening plums may be operating under the control of GPP, when increased AA concentration is required in response to chilling stress.

The increased AA concentration in ripening fruit may also be the result of the combined action of biosynthetic and recycling enzymes. The accumulation of DHA in fruit tissue could be due to increased activity of APX and decreased activities of MDHAR and DHAR (Figs. 7.6C & 7.8). The increase in APX activity during storage and SL might have caused oxidation of AA into MDHA. MDHA is very unstable and thus spontaneously dismutates into DHA and AA. MDHAR activity also increased and reached a peak during initial stages of SL and then decreased (Fig. 7.8B). It is likely that decreased activity of MDHAR could be one of the factors responsible for the accumulation of DHA towards the end of SL. The increase in MDHAR enzyme activity and transcript levels has been linked to increase in the concentration of AA and chilling tolerance in tomatoes exposed to 4°C for 40 days (Stevens et al., 2008). The data show that the increased levels of DHA in fruit during storage and SL corresponded with the reduced activities of DHAR (Fig. 7.8C) as a negative correlation ($r = –0.64$) existed between them. The increase in activities of MDHAR and DHAR might have helped the regeneration of AA towards restoration of the antioxidant potential of the fruit. The lower AA:DHA ratio during SL after the last 2 weeks of storage shows that the equilibrium shifted towards more oxidized state in the tissue in response to extended storage period. However, the levels of AA and DHA could not be completely explained on the basis of activities of these oxidizing and recycling enzymes because of weak correlations observed among these parameters. A recent study on expression profiling of the AA biosynthesis, oxidation and recycling genes in tomatoes has also shown difficulties in explaining the levels of AA in response to various types of stresses due to biological complexities involved in various pathways (Ioannidi et al., 2009). Overall, no significant loss of AA was observed during 5 weeks of cold storage and SL of ‘Blackamber’ plums which signifies better storage potential of this cultivar. Previously, studies have also shown that the retention of AA during cold storage was linked to good storage characteristics and resistance to internal browning in some cultivars of apples and pears (Davey and Keulemans, 2004; De Castro et al., 2008; Veltman et al., 1999).

Concentrations of total glutathione (GSH+GSSG) and GSH increased during the first 2 weeks of storage followed by a continuous decline and reached the value at harvest (Fig. 7.7). In response to chilling stress, transient increase in GSH levels has been reported in apples (Davey and Keulemans, 2004) which is a good indication of

the activation of the antioxidant system to combat the increasing production of ROS (Tausz et al., 2004). In general, a significant decrease in GSH levels has been reported to occur during long–term cold storage of other fruits such as mangoes (Zhao et al., 2009), oranges (Huang et al., 2008), and pawpaws (Galli et al., 2009). The decrease in glutathione concentration has also been associated with internal browning in methyl bromide–fumigated 'Thompson Seedless' grapes (Liyanage et al., 1993) and methyl–iodide–induced phytotoxicity in lemons (Ryan et al., 2007). Higher levels of GSH have been implicated in providing chilling tolerance in plants (Tausz et al., 2004).

The regeneration of AA by the action of DHAR is driven by oxidation of GSH into GSSG (Apel and Hirt, 2004). The lower levels of DHAR activity were coincident with the increased levels of GSH during the first 2 weeks of storage compared with values at harvest (Figs. 7.7B & 7.8C). Furthermore, the increased activities of DHAR during SL for the first 2–4 days of SL might have contributed to the decrease in GSH levels. In addition to DHAR, GR activity also determines the concentration of GSH in the pool as it catalyses the reduction of GSSG into GSH. The increase in GR in response to first 2–3 weeks of cold storage might have helped to regenerate the GSH (Fig. 7.9A). As the storage duration progressed, the capacity of GR to recycle GSH was observed to be diminishing and the similar trend was observed during SL after each week of storage. The GR activity and GSH were found to be positively correlating ($r = 0.84$) with each other. The decline in the activity of GR also corresponded with the increase in concentration of GSSG and showed a negative correlation ($r = –0.66$). Chilling stress has been implicated in decreased GR activity in oranges (Huang et al., 2008) and peaches (Wang et al., 2006). GT is also involved in providing protection against oxidative stress by detoxifying lipid hydroperoxides through the utilization of GSH as a reducing power. It is therefore likely that decrease in GSH and increase in GSSG could also be related to the increased activity of GT to counteract increasing lipid peroxidation products during advanced stages of storage and SL (Fig. 7.9B). The accumulation of GSSG and decrease in GSH led to the substantial decrease in the GSH:GSSG ratio in the fruit flesh tissue as reported in pawpaw (Galli et al., 2009).

7.4.6 Phenols and DPPH· radical scavenging activity

The ROS–scavenging properties of flavonoids resulted in characterization of the major phenolic components of naturally occurring phytochemicals as antioxidants. The increase in concentration of total phenolics during initial storage period could be due to activation of phenylpropanoid metabolism in response to chilling stress as reported in grapes, plums and pawpaws (Díaz–Mula et al., 2009; Galli et al., 2009; Sanchez–Ballesta et al., 2007). The increased production of phenolic compounds in the tissue imparts resistance against different abiotic and biotic stresses (Sanchez–Ballesta et al., 2007). The gene expression of phenylalanine ammonia lyase (PAL), which is a key enzyme in phenylpropanoid pathway, was up–regulated during low temperature storage of grapes (Sanchez–Ballesta et al., 2007). On the other hand, the prolonged storage for 4 and 5 weeks resulted in decrease in total phenolics concentration (Fig. 7.10A) which could possibly be due to their utilization as a substrate of PPO in the browning reactions involved in CI (Galli et al., 2009) and also as antioxidants in peroxidases–mediated removal of H_2O_2, because of the co–existence of phenolics and peroxidases in the vacuoles (Takahama, 2004).

The data show that severity of CI showed a negative correlation ($r = –0.70$) with the concentration of total phenolics (Table 7.3). The prolonged storage has been shown to cause decrease in the levels of phenolics in grapes, mangoes, and pawpaws (Galli et al., 2009; Zhao et al., 2009). The antioxidant capacity in the flesh tissue has been mainly due to the presence of phenolic compounds, whereas a very little contribution by other compounds has been reported (Díaz–Mula et al., 2009; Gil et al., 2002). The pattern of changes in DPPH radical scavenging activity during cold storage and SL was almost similar to the changes in total phenolics (Fig. 7.10B) as a positive correlation ($r = 0.76$) was observed between them. It could be argued that phenolic compounds in co–operation with other antioxidative components during initial stages of storage might have contributed to maintaining ROS at low levels, and thus protected the tissue from oxidative injury. The increase in storage duration accompanied by the decrease in total phenols and reduced efficiency of other antioxidative systems might have contributed to the build up of oxidative stress to potentially damaging levels.

This experiment has shown the dynamics of various antioxidant systems in response to cold storage and subsequent SL in ‘Blackamber’ plums. The potential

storage and SL based on the assessment of fruit quality including CI can be considered as 4 weeks in this case. If the response of antioxidant system is taken into account, it would be considered safer to store these fruit for 3 weeks. The possibility of increasing storage duration can increase the risk of expression of CI. Therefore, the 3rd week of storage for 'Blackamber' was the critical point from where onwards the degeneration of the antioxidant system proceeded and led to near collapse after 5 weeks. It is also clear that the storage duration of 3 weeks does not seem to be sufficient to regulate the fruit marketing and long–distance marine transport. From this standpoint, postharvest strategies have to be adopted to reduce the lipid peroxidation and strengthen the antioxidative systems in order to reduce the risk of development of CI in Japanese plums.

Chapter 8

Effects of 1–MCP Treatment and Storage Atmospheres on Fruit Quality, Lipid Peroxidation, and Enzymatic and Non–enzymatic Antioxidants in 'Blackamber' plums

Summary

Experiments were conducted to investigate the effects of storage atmospheres and 1–MCP treatment on fruit quality, lipid peroxidation, and enzymatic and non–enzymatic antioxidants in 'Blackamber' plums. In the first experiment, commercially mature 'Blackamber' plums were stored at 0–1°C in different storage atmospheres, viz. normal air, 1% O_2 + 3% CO_2, 2.5% O_2 + 3% CO_2 and modified atmospheres (~10% O_2 and 3.8% CO_2) generated through packaging in low density polyethylene bags. In the second experiment, the fruit were treated with 1–MCP (0.6 µL L^{-1} for 12 h) and then stored at 0–1°C under different atmospheres similar to the first experiment. The results showed that storage under CA containing either 1% O_2 or 2.5% O_2 was beneficial in terms of retention of flesh firmness and titratable acidity, reduction in CI, and retarding skin colour changes. A combination of 1–MCP and CA provided synergistic effects in maintaining fruit quality and reducing CI during 5 and 8 weeks of storage. CA storage was more effective than MAP and normal air in preserving fruit quality and alleviation of oxidative stress in 1–MCP–treated and untreated fruit. 1–MCP treatment alone was also effective to some extent in reducing the oxidative stress, but its combination with CA yielded more favourable results. The changes in enzymatic and non–enzymatic antioxidants in fruit held in different storage atmospheres in relation to development of CI were examined and are discussed in this chapter. In conclusion, 'Blackamber' plums pre–treated with 1–MCP stored under CA containing either 1% or 2.5% O_2 and 3% CO_2 could be stored for 8 weeks with acceptable changes in fruit quality and antioxidant system.

8.1 Introduction

Chilling injury (CI) and fruit softening are the major postharvest constraints which limit long–term cold storage and shipping of Japanese plums. CI symptoms, such as flesh browning, translucency and mealiness appear, as a consequence of interaction between storage temperature and duration (Crisosto et al., 2004; Crisosto et al., 1999). Supplementation of low temperature with controlled/modified atmospheres (CA/MA) containing low O_2 and high CO_2 have been known to extend storage life, maintain quality and alleviate certain postharvest disorders in fruits (Prange and DeLong, 2006). CA storage has been reported to reduce the physiological and internal breakdown in European plums (Smith, 1939; Smith, 1967). In Japanese plums, the application of CA containing 1%–5% O_2 and 2.5%–10% CO_2 at near 0°C also exerted favourable effects such as reduced rates of respiration and ethylene production, retention of flesh firmness, retardation of changes in skin colour, and alleviation of CI (Ke et al., 1991; Maré et al., 2005; Menniti et al., 2006). CA storage has also been reported to be beneficial to delay or reduce the development of CI in peaches and nectarines (Crisosto et al., 1997; Lill et al., 1989; Lurie, 1992; Murray et al., 2007; Retamales et al., 1992; Wade, 1981; Wang et al., 2005).

CA requirements are commodity– and cultivar–specific (Saltveit, 2003), especially when the physiological behaviour of cultivars is diverse as in case of Japanese plums. For example, ‘Angeleno’ cultivar exhibits suppressed–climacteric behaviour, while ‘Blackamber’ is a typical climacteric–type cultivar which respires and produces ethylene at a faster rate. Thus, the physiological and biochemical responses of ‘Angeleno’ under CA could be different to ‘Blackamber’. Currently, no information is available on the response of ‘Blackamber’ plums to CA. Furthermore, modified atmosphere packaging (MAP) has also been reported to give some beneficial effects in Japanese plums (Cantín et al., 2008; Khan and Singh, 2008). But, no attempt has been made to directly compare the effects of CA and MAP on fruit quality and CI in Japanese plums. Therefore, the first objective was to determine the effects of CA storage and MAP on fruit quality and CI in Japanese plums.

As outlined in the previous chapters, CI is an oxidative phenomenon in Japanese plums. The reduction in the capability of enzymatic and non–enzymatic antioxidant protection systems leads to the development of CI symptoms and faster senescence in fruit. CA/MA storage under optimal O_2 and CO_2 levels, if

administered correctly, can reduce or suspend the oxidative stress, decrease sensitivity to ethylene, and delay postharvest senescence (Toivonen, 2004). However, the effects of CA on development of oxidative stress are commodity and cultivar specific. For instance, storage of 'Conference' pears under CA containing 2% O_2 and 5% CO_2 resulted in increased levels of oxidative stress than under normal air (Larrigaudiére et al., 2001a). To my knowledge, the effects of CA/MA on development of oxidative stress in the European and Japanese plums have not been investigated. It was hypothesized that CA/MA storage may be useful to retard fruit metabolism, reduce sensitivity to ethylene, and effectively maintain the enzymatic and non–enzymatic antioxidants which can possibly reduce the CI symptoms in Japanese plums. Therefore, second objective was to determine if the changes in enzymatic and non–enzymatic antioxidants in response to CA and MAP relate to the development of CI.

CA storage reduces the sensitivity of fruit to ethylene (Saltveit, 2003), while 1–methylcyclopropene (1–MCP) blocks the perception of ethylene (Sisler and Serek, 1997). 1–MCP is registered for use on the European and Japanese plums in countries such as Argentina, Australia, Canada, Chile, France, Mexico, South Africa, and the United States of America (Watkins, 2008). From ethylene perspective, the favourable effects of CA and 1–MCP on postharvest life and fruit quality are expected to be quite similar. 1–MCP can potentially replace short–duration CA storage where adequate facilities are not available (Fawbush et al., 2009). The major beneficial effects of 1–MCP in Japanese plums include retarded fruit softening, decrease in rates of respiration and ethylene production, decrease in skin colour changes, and reduction in CI symptoms (Candan et al., 2008; Khan and Singh, 2008; Valero et al., 2003). Postharvest treatment with 1–MCP has been implicated in enhancing or maintaining the antioxidant potential in some fruits such as apple, apricot, loquat, mango, and pear (Cao et al., 2009b; Fawbush et al., 2009; Larrigaudiére et al., 2004; Martino et al., 2006; Wang et al., 2009). A combination of CA/MA and 1–MCP has been reported to overcome superficial scald in apples (Watkins et al., 2000) and to extend the storage life and maintain quality of Japanese plums than either of alone (Khan and Singh, 2008; Menniti et al., 2006). Menniti et al. (2006) reported that 1–MCP treated 'Angeleno' plums could be stored for 80 days at 0°C under CA containing 1.8% O_2 and 2.5% CO_2. The effects of 1–MCP on fruit physiology of

Japanese plums have been investigated (Khan and Singh, 2008; Larrigaudiére et al., 2009; Valero et al., 2003), but a comprehensive analysis of lipid peroxidation, enzymatic and non–enzymatic antioxidants during cold storage under CA/MA in 1–MCP–treated Japanese plums is still lacking. Therefore, it was aimed to investigate the oxidative behaviour of 1–MCP–treated and –untreated 'Blackamber' plums during cold storage under normal air, CA and MAP.

8.2 Material and methods

8.2.1 Fruit material

Japanese plum cv. 'Blackamber' fruit were harvested at commercial maturity (SSC 11.8 °Brix, titratable acidity 1.17%, and firmness 33.2 N) on 29 December 2007 from the Casuarina Valley Orchard, Karragullen, Perth Hills (lat. 31° 57'S; long. 115° 50'E), WA. The fruit were transported to the laboratory immediately after harvest. The fruit free from visible symptoms of disease, blemish, and bruising were used in experiments.

8.2.2 Experiments

8.2.2.1 Experiment 1: Effects of storage atmospheres on fruit quality, lipid peroxidation, and enzymatic and non–enzymatic antioxidants in 'Blackamber' plums kept in cold storage

After receiving in laboratory, fruit were subjected to different storage atmospheres at 0–1°C. The storage atmospheres comprised of regular air, controlled atmospheres (CA) and modified atmospheres (MA) passively generated inside the low density polyethylene (LDPE) packs. During storage in regular air, the fruit were placed in fibre board boxes (Visy Pty., Ltd., Southbank, Victoria, Australia) lined with 30 µm thick LDPE film folded over them and stored at 0–1°C for 8 weeks. Each box containing 60 fruit served as an experimental unit and replicated three times. CA–storage consisted of combinations of two concentrations of O_2 (1% and 2.5%) and one level of CO_2 (3%) at 0–1°C. Concentrations of O_2 and CO_2, adjusted with N_2, were determined using an infrared gas analyser with automatic sampling (ADC 7000; Analytical Development Co., Ltd., Hoddesdon, UK) and are shown in Table 8.1. The

flow rate of the humidified CA–gas mixtures was 350 mL min^{-1}. The CA chamber containing 60 fruit served as an experimental unit and replicated three times. For MAP, 60 fruit were packed in a LifeSpan® (AMCOR Packaging, Pty. Ltd., Melbourne, Australia) polyethylene bag (71 cm long, 50 cm wide, and 30 μm thick) and sealed with a unique clip system following the instructions provided by the manufacturer. LifeSpan® MAP system has been commercially used for fresh fruits for over 10 years in Australia. The MAP bag was then placed in a fibre board box and stored at 0–1°C for 8 weeks. The gas composition inside the MAP bags was determined by withdrawing 2 mL of headspace air and injecting into an infrared gas analyser (Series 1450; Servomex Ltd., East Sussex, UK) at weekly intervals commencing 1 week after storage. The concentrations of O_2 and CO_2 inside MAP bags containing fruit are presented in Table 8.1. A MAP bag containing 60 fruit represented an experimental unit and replicated three times.

Table 8.1. Concentrations of O_2 and CO_2 either at the inlets of the CA containers or inside MAP bags

	O_2 (%)			**CO_2 (%)**		
	Mean ± SD	Max.	Min.	Mean ± SD	Max.	Min.
CA–1	1.02 ± 0.028	1.05	0.95	3.05 ± 0.049	3.13	2.95
CA–2	2.49 ± 0.021	2.50	2.45	3.01 ± 0.048	3.13	2.95
MAP*	9.98 ± 2.29	14.44	7.81	3.78 ± 0.31	4.36	3.44

* During the first week of storage, maximum, minimum and average concentrations of O_2 and CO_2 may not be the same as shown in the table because gas composition was determined at weekly intervals commencing after 1 week of storage.

8.2.2.2 Experiment 2: Effects of storage atmospheres in combination with 1–MCP on fruit quality, lipid peroxidation, and enzymatic and non–enzymatic antioxidants in 'Blackamber' plums kept in cold storage

1–MCP was obtained as SmartFresh™ SmartTabs from AgroFresh, Inc. (Rohm and Hass Australia Pty., Ltd., Victoria, Australia). SmartFresh™ SmartTabs are tablets that, when mixed with the blue activator tablets in a proprietary activator solution,

volatilize the active ingredient, 1–MCP. 1–MCP treatment was carried out following the instructions provided by the manufacturer. Fruit were treated with 0.6 μL L^{-1} 1–MCP for 12 h at 0°C in an air–tight plastic tent (4000 L; LDPE 0.004 inch thickness). Four mini-electric fans were used in the tent to evenly distribute 1–MCP gas around the fruit. 1–MCP–treated fruit were subjected to different storage atmospheres at 0–1°C. The experimental set up for 1–MCP–treated fruit was similar to the untreated fruit as described in Experiment 1.

8.2.3 Fruit quality evaluation

Fruit firmness, colour, SSC, and TA were determined as described in Sections 3.4.1.2, 3.4.2, and 3.4.3, respectively. Lots of 30 fruit from each replication of the treatment were removed after 5 and 8 weeks of storage in both experiments. For all these quality parameters, 15 fruit constituted an experimental unit, with three replications. The fruit were peeled and the flesh tissue was cut into small cubes and immediately frozen with liquid nitrogen and stored at –80°C for further analyses of oxidative stress related parameters.

8.2.4 Chilling injury (CI)

The incidence and severity of CI was evaluated immediately after transfer from cold storage and also after SL of 6 days at 21±1°C. Fifteen fruit per replication were cut around the equatorial axis, the two halves of each fruit twisted in opposite directions, and the mesocarp was examined for symptoms such as flesh browning, mealiness, and translucency. The incidence and severity of CI were calculated as described in Section 3.4.4.

8.2.5 Oxidative stress parameters

The determinations of lipid peroxidation, enzymatic and non–enzymatic antioxidants, and protein concentration were carried out as described in the general materials and methods (Sections 3.5 to 3.8).

8.2.4 Statistical analyses

The data were subjected to a two–way ANOVA using GenStat Release 11.1 (VSN International Ltd., Hemel Hempstead, UK). Storage atmosphere and duration of storage were treated as two factors. Before statistical analysis, the data on CI incidence were subjected to arcsine transformation to reduce heteroscedasticity. The effects of storage atmosphere and storage period on different parameters were assessed within ANOVA and the LSD were calculated following a significant F–test at $P \leq 0.05$.

8.3 Results

8.2.2.1 Experiment 1: Effects of storage atmospheres on fruit quality, lipid peroxidation, and enzymatic and non–enzymatic antioxidants in 'Blackamber' plums kept in cold storage

8.3.1.1 Fruit skin colour, firmness, SSC, TA, and SSC:TA)

Storage atmosphere significantly influenced fruit quality of 'Blackamber' plums during cold storage for 5 and 8 weeks. The changes in skin colour showed by decreases in chromaticity value L* and hue angle were significantly retarded in fruit held in CA compared to those stored in regular air and MA packs (Figs. 8.1A & B). The increase in storage duration from 5 to 8 weeks further caused a decrease in L* and hue angle values. After removal from cold storage, a significant increase in darkening of skin colour occurred during 6 days at 21±1°C. However, after 5 weeks of storage plus 6 days at 21±1°C, the hue angles of fruit held in CA were significantly higher than those held in air and MAP, but these differences became non–significant when storage duration was extended to 8 weeks plus 6 days at 21±1°C.

Softening was significantly lower in fruit stored in CA and MAP compared to regular air (Fig. 8.1C). The loss of flesh firmness during the first 5 weeks of cold storage was to the extent of 60% in regular air, 35% in CA–1 (1% O_2 + 3% CO_2), 47% in CA–2 (1% O_2 + 3% CO_2), and 50 % in MAP; these losses further increased to, respectively, 66%, 45%, 50%, and 51% after 8 weeks of storage. The fruit stored

in CA for 5 and 8 weeks were significantly firmer than those stored in regular air or MAP even after 6 days of SL at 21±1°C.

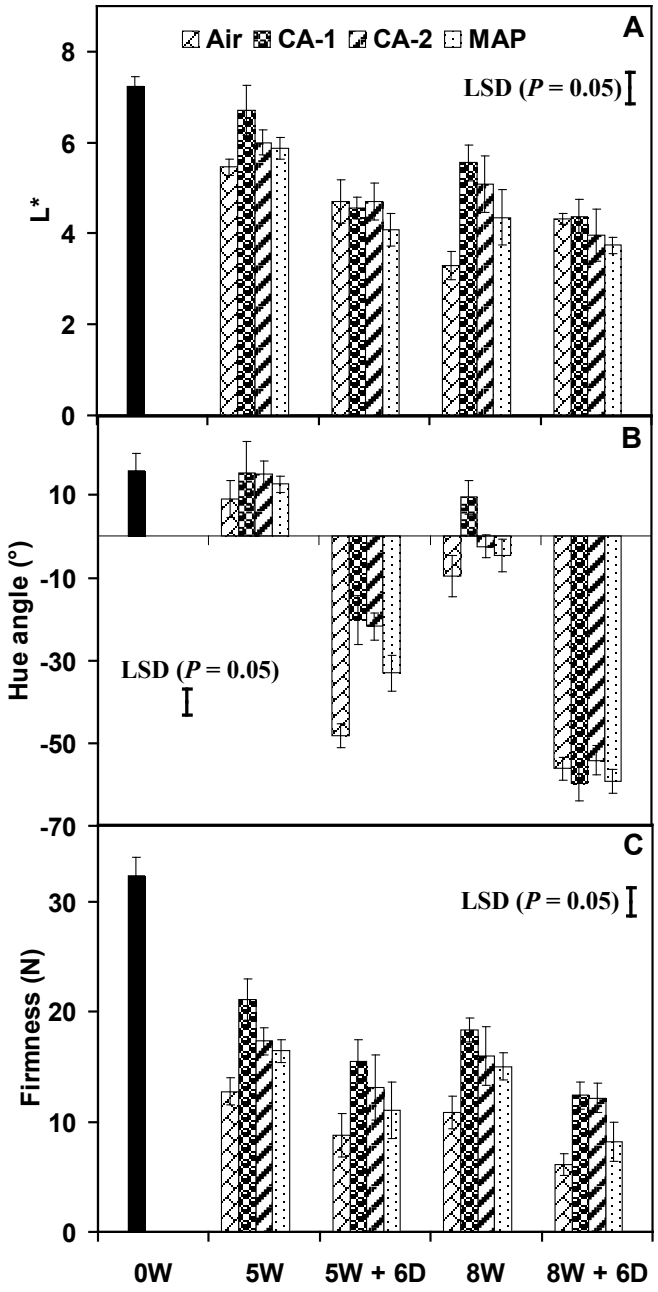


Fig. 8.1. Changes in skin colour (L* [A] and hue angle [B]) and flesh firmness (C) of 'Blackamber' plums as influenced by cold storage in different atmospheres at 0–1°C for 5 and 8 weeks (W), plus 6 days (D) of shelf life at 21±1°C after each storage interval. Vertical bars represent S.E. of means. LSD (P = 0.05) values for L*: Storage atmosphere (SA) = 0.32, Storage period (SP) = 0.35, SA x SP = 0.71. Hue angle: SA = 2.82, SP = 3.15, SA x SP = 6.29. Flesh firmness: SA = 1.10, SP = 1.23, SA x SP = 2.46. LSD values for SA x SP are shown as separate bars.

Table 8.2 shows the changes in SSC, TA, and SSC:TA of fruit during cold storage in different atmospheres. The amount of change in SSC during cold storage was of minor practical importance. The retention of TA was significantly higher in CA–stored fruit compared with MAP and regular air. With the extension in storage duration and during SL, TA decreased significantly depending on the storage atmosphere. The loss of TA in MA packed fruit was even greater than in fruit kept in regular air. The decrease in TA caused a significant increase in SSC:TA ratio. The higher retention of TA in CA–stored fruit was responsible for a lower SSC:TA ratio in these fruit.

8.3.1.2 Chilling injury

Storage atmosphere and duration of storage had a significant impact on incidence and severity of CI symptoms in fruit (Fig. 8.2). CA storage significantly reduced the incidence and severity of CI symptoms during 5 and 8 weeks of storage. Fruit held in atmospheres containing 1% O_2 + 3% CO_2 showed the minimum incidence and severity of CI. MAP was not effective to reduce the incidence of CI, but the severity of CI was significantly lower in MA packed fruit than in those held in regular air. CI symptoms were in the form of flesh browning and translucency when fruit were evaluated immediately after 5 or 8 weeks of storage. After a SL period of 6 days, the severity increased and symptoms were more diverse in the form of flesh browning, translucency, and bleeding.

Table 8.2. Fruit quality attributes (SSC, TA, and SSC:TA ratio) of 'Blackamber' plums at harvest (0 week) and after 5 and 8 weeks (W) of storage in different atmospheres at 0–1°C, plus 6 days of shelf life at 21±1°C after each storage interval.

Storage period	**SSC (°Brix)**				**TA (%)**				**SSC:TA**			
	Air	**CA–1**	**CA–2**	**MAP**	**Air**	**CA–1**	**CA–2**	**MAP**	**Air**	**CA–1**	**CA–2**	**MAP**
0 W + 0 days	11.8				1.17				10.1			
5 W + 0 days	12.8	11.8	12.3	12.3	0.76	0.79	0.83	0.72	16.9	14.9	14.9	17.0
5 W + 6 days	12.8	12.1	12.4	12.4	0.64	0.69	0.68	0.59	20.0	17.4	18.3	21.0
8 W + 0 days	12.0	12.0	12.3	11.7	0.65	0.80	0.75	0.58	18.6	14.9	16.4	20.4
8 W + 6 days	11.3	11.9	12.1	11.3	0.30	0.46	0.41	0.31	37.8	25.9	30.1	37.0
Least significant differences of means at 5% level and levels of significance for a two–factor ANOVA												
Storage atmosphere (SA)	0.15***				0.02***				1.44***			
Storage period (SP)	0.16***				0.02***				1.61***			
SA X SP	0.33***				0.05***				3.22***			

*** = $P \leq 0.001$

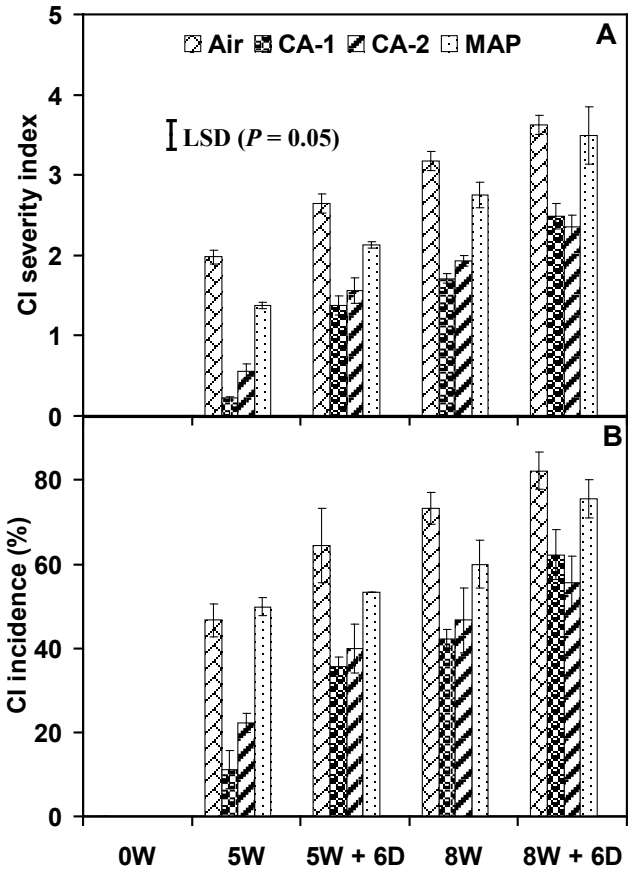


Fig. 8.2. CI index (A) and CI incidence (B) in 'Blackamber' plums as influenced by cold storage in different atmospheres at 0–1°C for 5 and 8 weeks (W), plus 6 days (D) of shelf life at 21±1°C after each storage interval. Vertical bars represent S.E. of means. LSD (P = 0.05) values for CI index: Storage atmosphere (SA) = 0.16, Storage period (SP) = 0.18, SA x SP = 0.36. LSD value for SA x SP is shown as a separate bar. Data on CI incidence were arcsine transformed, but back–transformed data are presented for simplifying comparisons. LSD values for transformed data on CI incidence are: SA = 0.04, SP = 0.05, SA x SP = 0.09.

8.3.1.3 Lipid peroxidation

Table 8.3 shows the data on LOX activity, TBARS concentration and EL during cold storage in different atmospheres and SL of 'Blackamber' plums. LOX activity was significantly influenced by storage atmosphere and duration of storage. After 5 and 8 weeks of storage, LOX activity in fruit stored in CA and MAP did not differ significantly, but was significantly lower than fruit stored in regular air. LOX activity showed about 2–fold increase during SL period after each storage interval.

Table 8.3. Lipoxygenase (LOX) activity, concentration of thiobarbituric acid–reactive substances (TBARS), and electrolyte leakage (EL) in 'Blackamber' plums at harvest (0 week) and after 5 and 8 weeks (W) of storage in different atmospheres at 0–1°C, plus 6 days of shelf life at 21±1°C after each storage interval.

Storage period	**LOX** (µmol min^{-1} mg^{-1} protein)				**TBARS** (nmol MDA equivalents g^{-1} FW)				**EL** (%)			
	Air	**CA–1**	**CA–2**	**MAP**	**Air**	**CA–1**	**CA–2**	**MAP**	**Air**	**CA–1**	**CA–2**	**MAP**
0 W + 0 days	6.5				0.25				34.5			
5 W + 0 days	12.9	8.1	8.2	9.4	0.73	0.49	0.42	0.60	53.3	38.3	38.9	47.0
5 W + 6 days	21.0	14.4	15.7	18.7	1.74	1.14	1.37	1.41	74.9	59.6	58.4	69.3
8 W + 0 days	14.3	7.8	8.2	8.7	0.97	0.64	0.50	0.87	63.7	48.0	50.8	54.0
8 W + 6 days	26.3	11.5	16.4	17.1	1.85	1.77	1.78	1.82	80.7	63.3	64.0	78.2
Least significant differences of means at 5% level and levels of significance for a two–factor ANOVA												
Storage atmosphere (SA)	2.0***				0.08***				1.53***			
Storage period (SP)	2.3***				0.09***				1.71***			
SA X SP	4.6*				0.18*				3.43***			

*** = $P \leq 0.001$; * = $P \leq 0.05$

The accumulation of TBARS during 5 and 8 weeks of storage was significantly lower in CA–stored fruit compared to air–stored ones. Concentration of TBARS increased considerably during the SL period and the differences in these concentrations after 8 weeks of storage were non–significant, irrespective of the storage atmosphere. EL increased by 10–12% during the first 5 weeks of storage under CA, while it was 23% and 35% in fruit held in MAP and air, respectively. With the increase in storage duration and also during SL, further increases in EL were observed. In general, LOX activity, TBARS concentration and EL did not differ much in fruit stored in either CA–1 or CA–2 (Table 8.3).

8.3.1.4 Antioxidant enzymes (SOD, CAT, and POD)

After 5 and 8 weeks of storage, SOD activity was observed to be significantly higher in fruit held in CA–1 and CA–2 compared to those held in MAP and regular air (Fig. 8.3A). A further decrease in SOD activity was noticed during 6 days of SL in fruit, irrespective of storage atmosphere. A comparison of the SOD activities immediately after 5 and 8 weeks of storage for each storage atmosphere showed that these differences were non–significant. However, after 8 weeks of storage plus 6 days of SL, SOD activities were significantly lower than after 5 weeks of storage plus 6 days of SL. No significant differences in CAT activities were observed after 5 and 8 weeks of storage, regardless of storage atmosphere (Fig. 8.3B). After 6 days of SL following 5 weeks of storage, CAT activity was significantly higher in CA–stored fruit compared to those held in MAP and regular air. Storage atmosphere had no significant effect on POD activities while these showed an increasing trend in response to cold storage and SL (Fig. 8.3C).

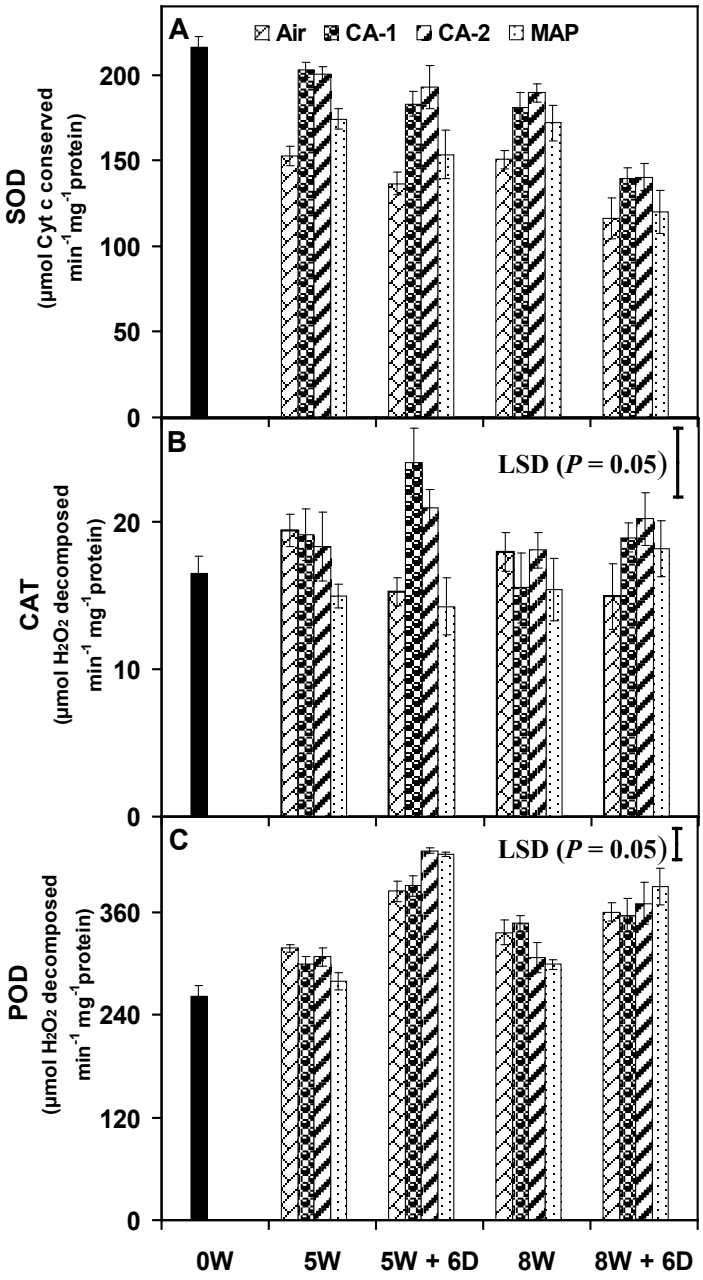


Fig. 8.3. Changes in activities of SOD (A), CAT (B), and POD (C) in the flesh tissue of 'Blackamber' plums as influenced by cold storage in different atmospheres at 0–1°C for 5 and 8 weeks (W), plus 6 days (D) of shelf life at 21±1°C after each storage interval. Vertical bars represent S.E. of means. LSD (P = 0.05) values for SOD: Storage atmosphere (SA) = 10.53, Storage period (SP) = 11.77, SA x SP = NS. CAT: SA = 2.09, SP = NS, SA x SP = 4.66. POD: SA = NS, SP = 4.69, SA x SP = 9.39. LSD values for SA x SP are shown as separate bars.

8.3.1.5 Ascorbate–glutathione cycle

Ascorbate

Storage duration had a pronounced effect on concentrations of total ascorbate (AA + DHA) as these declined significantly with the progression of storage (Fig. 8.4A). During 8 weeks of storage, total ascorbate concentrations decreased by about 5 fold and no significant differences were observed among fruit stored in different atmospheres. However, these concentrations increased during SL by about 1.5 fold in air–stored and about 2 fold in CA– or MAP–stored fruit.

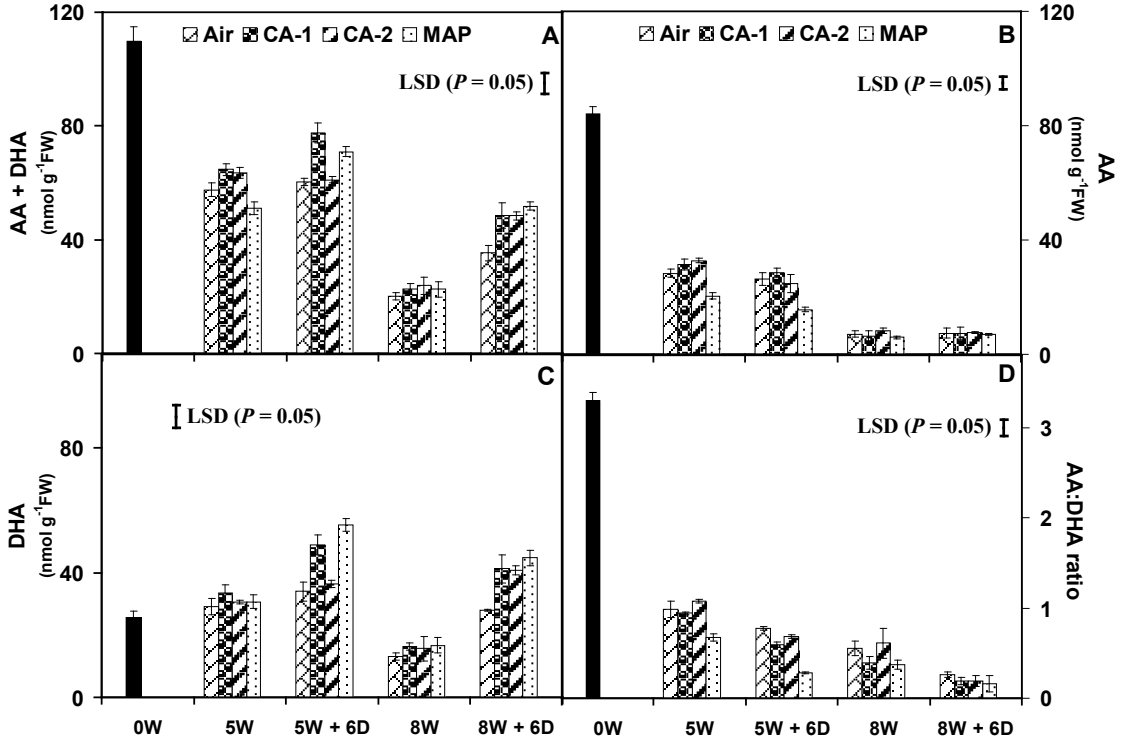


Fig. 8.4. Changes in concentrations of total ascorbate (AA + DHA) (A), AA (B), DHA (C), and AA:DHA ratio (D) in the flesh tissue of 'Blackamber' plums as influenced by cold storage in different atmospheres at 0–1°C for 5 and 8 weeks (W), plus 6 days (D) of shelf life at 21±1°C after each storage interval. Vertical bars represent S.E. of means. LSD (P = 0.05) values for AA + DHA: Storage atmosphere (SA) = 3.63, Storage period (SP) = 3.24, SA x SP = 7.25. AA: SA = 0.57, SP = 0.51, SA x SP = 2.28. DHA: SA = 3.70, SP = 3.31, SA x SP = 7.39. AA:DHA ratio: SA = 0.09, SP = 0.08, SA x SP = 0.18. LSD values for SA x SP are shown as separate bars.

The concentrations of AA were significantly higher in CA–stored fruit compared to MAP and air–stored ones after 5 weeks of storage; the decrease in AA was to the extent of 2.6 fold in CA, 3 fold in air, and 4.2 fold in MAP during this storage period (Fig. 8.4B). The extension of storage period to 8 weeks caused about 10 to 14 fold

decrease in AA concentration. A slight decrease in AA was observed during SL after 5 weeks, while no such change in AA concentration was observed after 8 weeks. Moreover, the differences in AA concentrations among fruit stored in different atmospheres were non–significant after 6 days of SL following 8 weeks of storage.

A significant increase in concentration of DHA was observed during the first 5 weeks in all storage atmospheres and there were no significant differences in DHA levels among fruit, irrespective of the storage atmosphere (Fig. 8.4C). In comparison to the 5 weeks storage, the concentration of DHA decreased by about 50% in the last 3 weeks of storage. On the other hand, a significant increase in DHA occurred during SL after 5 and 8 weeks of storage. The levels of DHA were significantly higher in fruit kept in CA and MAP than in air–stored fruit after 6 days of SL following 8 weeks of storage. AA:DHA ratio showed a significant decrease by about 3 to 5 fold during 5 weeks of storage in different atmospheres (Fig. 8.4D). The differences in AA:DHA ratio after 5 weeks of storage in regular air, CA–1, and CA–2 were non–significant, but this ratio was significantly higher under these atmospheres than in MAP. The AA:DHA ratio further decreased with the increase in storage duration to 8 weeks and also during SL of 6 days at 21±1°C.

Glutathione

Glutathione levels in flesh tissue of fruit were significantly influenced by storage atmosphere and duration of storage. The decrease in total glutathione in CA–stored fruit was significantly lower than fruit stored in air or MAP (Fig. 8.5A). The decrease in total glutathione continued even during SL period after cold storage in different atmospheres. GSH constituted a major proportion of the total glutathione, and thus followed the changes during storage in a pattern similar to total glutathione (Fig. 8.5B). Concentration of GSSG increased significantly during storage and SL in all storage atmospheres (Fig. 8.5C). The amount of increase in GSSG was significantly higher in air–stored fruit than in CA– and MAP–stored fruit. The differences in concentrations of GSSG in fruit stored in CA–1 and CA–2 were small and mostly non–significant. As a result of increase in concentration of GSSG and decrease in GSH during storage, a significant decline in the GSH:GSSG ratio was observed during cold storage and shell–life (Fig. 8.5D). With the increase in storage duration

from 5 to 8 weeks, a significant decrease in GSH:GSSG ratio was observed. However, the ratio was significantly higher in CA–and MAP–stored fruit than in air–stored fruit.

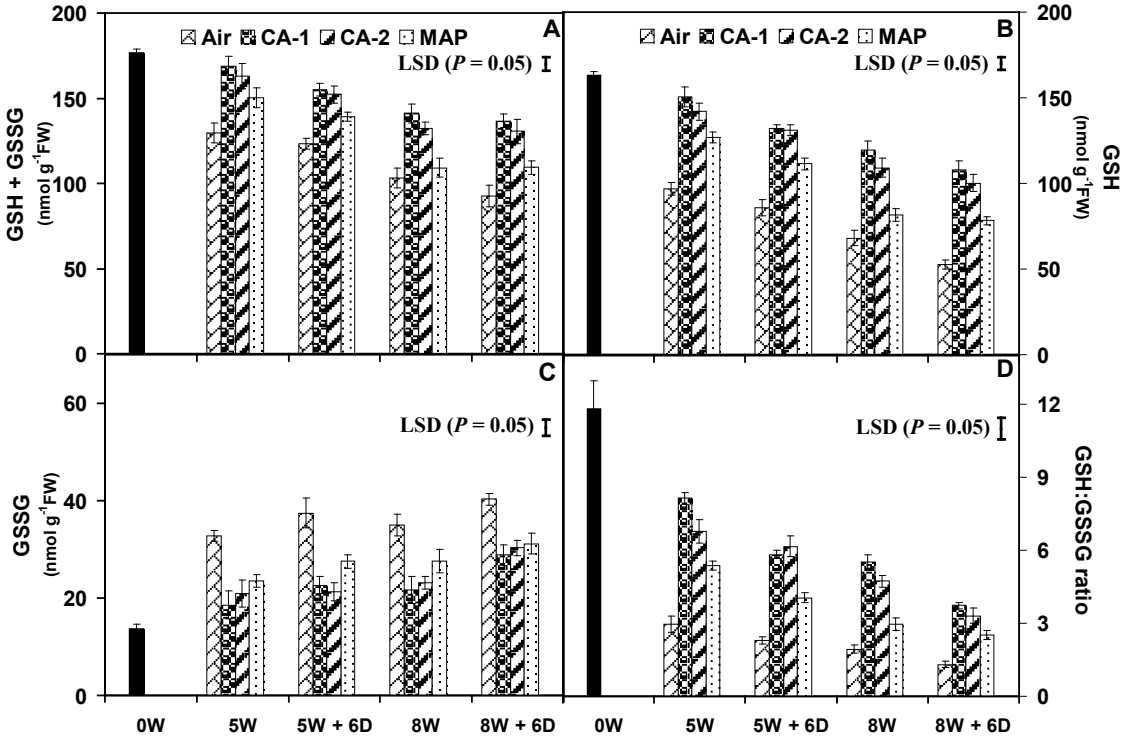


Fig. 8.5. Changes in concentrations of total glutathione (GSH + GSSG) (A), GSH (B), GSSG (C), and GSH:GSSG ratio (D) in the flesh tissue of 'Blackamber' plums as influenced by cold storage in different atmospheres at 0–1°C for 5 and 8 weeks (W), plus 6 days (D) of shelf life at 21±1°C after each storage interval. Vertical bars represent S.E. of means. LSD (P = 0.05) values for GSH + GSSG: Storage atmosphere (SA) = 1.93, Storage period (SP) = 1.72, SA x SP = 3.86. GSH: SA = 1.97, SP = 1.76, SA x SP = 3.93. GSSG: SA = 0.37, SP = 0.33, SA x SP = 0.74. GSH:GSSG ratio: SA = 0.21, SP = 0.19, SA x SP = 0.43. LSD values for SA x SP are shown as separate bars.

Ascorbate–glutathione cycle enzymes

APX activity increased significantly during cold storage for 5 or 8 weeks depending on the storage atmosphere (Fig. 8.6A). After 5 weeks of storage, the maximum APX activity was observed in fruit held in MAP followed by air, CA–1, and CA–2. The changes in APX activity during SL did not show a clear trend. There were no significant differences in the APX activities of fruit held in different atmospheres after 6 days of SL following 8 weeks of storage.

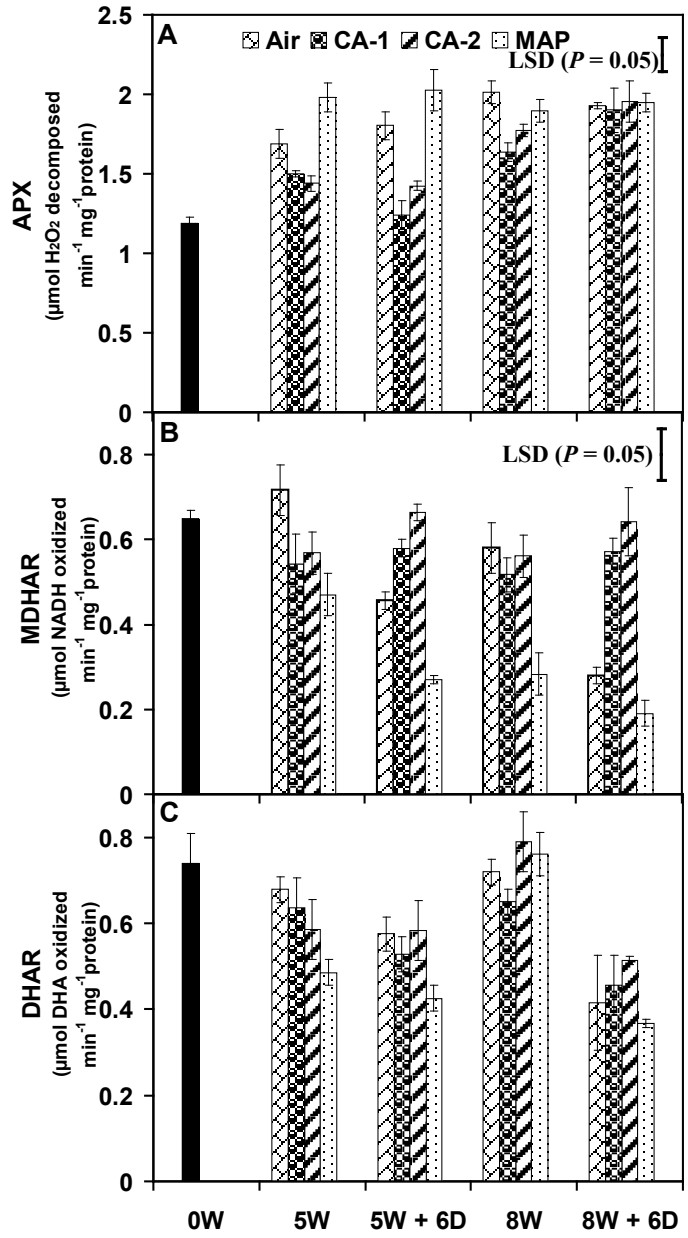


Fig. 8.6. Changes in activities of APX (A), MDHAR (B), and DHAR (C) in the flesh tissue of 'Blackamber' plums as influenced by cold storage in different atmospheres at 0–1°C for 5 and 8 weeks (W), plus 6 days (D) of shelf life at 21±1°C after each storage interval. Vertical bars represent S.E. of means. LSD (P = 0.05) values for APX: Storage atmosphere (SA) = 0.10, Storage period (SP) = 0.11, SA x SP = 0.22. MDHAR: SA = 0.05, SP = 0.06, SA x SP = 0.12. DHAR: SA = NS, SP = 0.08, SA x SP = NS. LSD values for SA x SP are shown as separate bars.

MDHAR activity did not show a clear relationship with storage atmosphere and length of storage (Fig. 8.6B). After 5 weeks of storage in MAP, MDHAR activity decreased to the extent of 1.4 and 2.3 fold during 5 and 8 weeks of storage, respectively. This decrease was significantly higher than in CA– and air–stored fruit. Interestingly, MDHAR increased during 6 days of SL in CA–stored fruit, while it decreased in MAP and air–stored fruit after 5 and 8 weeks of storage. The decrease

in DHAR activity during the first 5 weeks of storage was significant in fruit held in MAP, and was non–significant in other storage atmospheres (Fig. 8.6C). But, it increased during the last 3 weeks of storage in all atmospheres resulting in the highest activity in CA–2, and then declined significantly during the SL.

Storage duration had a pronounced effect on GR activity depending on storage atmosphere (Fig. 8.7A). Fruit stored in CA–2 retained the highest GR activity after 5 and 8 weeks of storage, while it decreased significantly in other storage atmospheres which showed no significant differences among themselves.

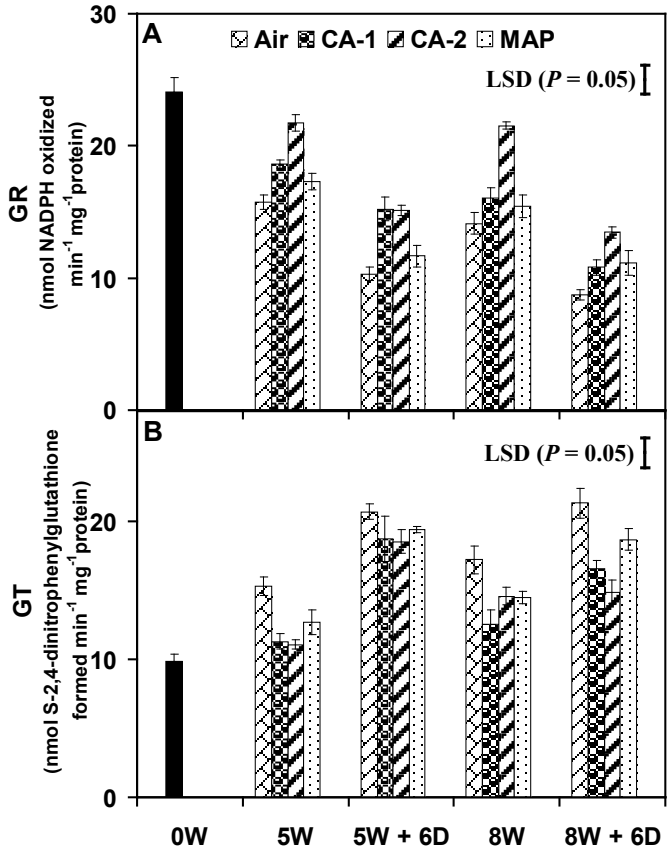


Fig. 8.7. Changes in activities of GR (A) and GT (B) in the flesh tissue of 'Blackamber' plums as influenced by cold storage in different atmospheres at 0–1°C for 5 and 8 weeks (W), plus 6 days (D) of shelf life at 21±1°C after each storage interval. Vertical bars represent S.E. of means. LSD (P = 0.05) values for GR: Storage atmosphere (SA) = 0.97, Storage period (SP) = 1.08, SA x SP = 2.16. GT: SA = 0.98, SP = 1.09, SA x SP = 2.19. LSD values for SA x SP are shown as separate bars.

A significant decrease in GR activity was further noticed during SL after both storage intervals. On the other hand, increase in GT activity was observed with the increase in storage duration and the higher amount of increase was noticed in air–

stored fruit (Fig. 8.7B). The GT activity after 5 and 8 weeks of storage in CA– and MAP–stored fruit did not differ significantly. Regardless of storage atmosphere, GT activity also increased significantly during 6 days of SL following both storage intervals.

8.3.1.6 Total phenolics and DPPH$^{\cdot}$ scavenging activity

Concentration of total phenolics remained stable during the first 5 weeks of storage in CA–stored fruit, while it declined significantly in air– and MAP–stored fruit (Fig. 8.8A).

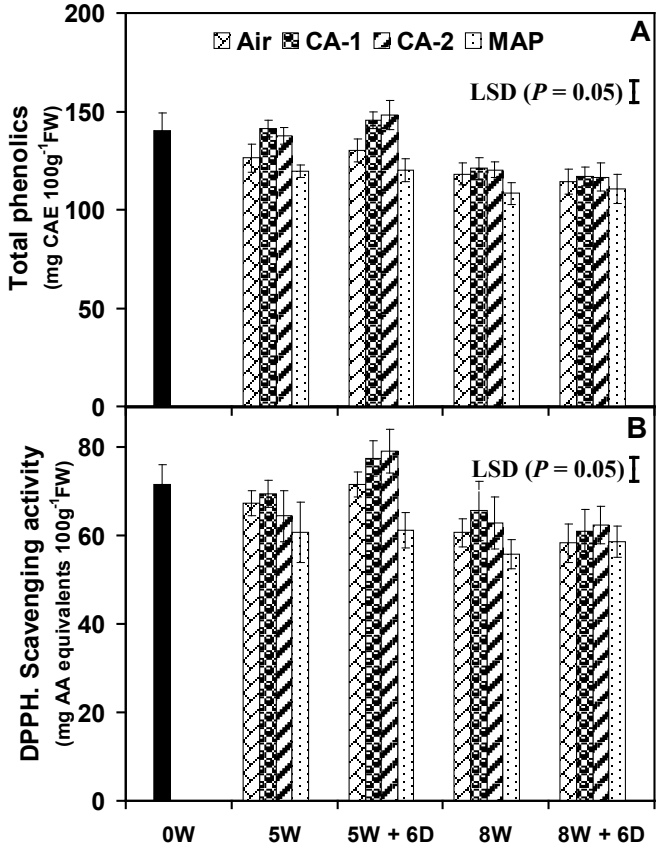


Fig. 8.8. Changes in total phenolics concentration (A) and DPPH scavenging activity (B) in the flesh tissue of 'Blackamber' plums as influenced by cold storage in different atmospheres at 0–1°C for 5 and 8 weeks (W), plus 6 days (D) of shelf life at 21±1°C after each storage interval. Vertical bars represent S.E. of means. LSD (P = 0.05) values for Total phenolics: Storage atmosphere (SA) = 2.77, Storage period (SP) = 2.48, SA x SP = 5.54. DPPH scavenging activity: SA = 1.33, SP = 1.19, SA x SP = 2.65. LSD values for SA x SP are shown as separate bars.

The extension of storage period to 8 weeks caused a significant decrease in concentration of total phenolics in CA–stored fruit. The changes in total phenolics

concentration during 6 days of SL were mostly non–significant, except fruit held in CA–2 showed an increase after 5 weeks of storage. $DPPH^{\cdot}$ scavenging activity showed no significant change during the first 5 weeks of storage in air and CA–1, but decreased in CA–2 and MAP (Fig. 8.8B). As the storage progressed to 8 weeks, $DPPH^{\cdot}$ scavenging activity further decreased in all storage atmospheres to variable extent. A significant increase in the $DPPH^{\cdot}$ scavenging activity was observed in fruit held in air and CA during SL after 5 weeks of storage, while such increase was not noticed after 8 weeks.

8.3.1.7. Correlation analysis

The correlation coefficients of CI index and CI incidence with the lipid peroxidation, enzymatic and non-enzymatic antioxidants in 'Blackamber' plums during cold storage under different atmospheres at 0–1°C plus 6 days of shelf life at 21±1°C are presented in Table 8.4. The CI incidence and severity index had a significantly positive correlation with TBARS, LOX, EL, APX, POD, GSSG and GT, whereas negative correlations with SOD, AA, AA:DHA ratio, GSH, GSH:GSSG ratio, GR, MDHAR, DHAR, phenols and DPPH scavenging activity were observed.

Table 8.4. Correlations of CI index and CI incidence with lipid peroxidation, enzymatic and non-enzymatic antioxidants in 'Blackamber' plums during 5 and 8 weeks of cold storage under different atmospheres at 0–1°C plus 6 days of shelf life at 21±1°C.

CI index *vs.*	**Pearson's correlation coefficient**	**CI incidence *vs.***	**Pearson's correlation coefficient**
TBARS	0.78***	TBARS	0.78***
LOX	0.67***	LOX	0.68***
EL	0.90***	EL	0.90***
SOD	–0.86***	SOD	–0.87***
CAT	NS	CAT	NS
POD	0.60***	POD	0.60***
AA	–0.87***	AA	–0.83***
DHA	NS	DHA	NS
AA:DHA	–0.84***	AA:DHA	–0.79***
GSH	–0.95***	GSH	–0.96***
GSSG	0.92***	GSSG	0.91***
GSH:GSSG	–0.95***	GSH:GSSG	–0.93***
APX	0.84***	APX	0.80***
MDHAR	–0.56***	MDHAR	–0.57***
DHAR	–0.46***	DHAR	–0.42***
GR	–0.86**	GR	–0.84***
GT	0.80***	GT	0.79***
Phenols	–0.76***	Phenols	–0.77***
DPPH scavenging activity	–0.61***	DPPH scavenging activity	–0.61***

NS, *, **, *** = non-significant, $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, respectively

8.3.2 Experiment 2: Effects of storage atmospheres in combination with 1–MCP on fruit quality, lipid peroxidation, and enzymatic and non–enzymatic antioxidants in 'Blackamber' plums kept in cold storage

8.3.2.1 Fruit skin colour, firmness, SSC, TA, and SSC:TA

The fruit quality of 1–MCP–treated 'Blackamber' plums was significantly influenced by storage atmosphere and duration of storage. L* values showed a minor decrease with the storage, and the effect of storage atmosphere on L* values was also relatively small (Fig. 8.9A). The changes in skin colour as shown by decreased hue angle were significantly higher in MAP than in CA– and air–stored fruit (Fig. 8.9B). A substantial decrease in hue angle occurred during SL period after 5 and 8 weeks of storage and the decrease was significantly greater after 8 weeks of storage compared to 5 weeks in all storage atmospheres. The hue angle values after 6 days of SL at 21±1°C following 5 weeks of storage were significantly lower in CA–stored fruit compared to MAP– and air–stored fruit which differed non–significantly. But, the differences in these values after 8 weeks of storage were non–significant among fruit stored in different atmospheres.

The retention of flesh firmness in 1–MCP–treated fruit kept under CA was significantly higher than air– and MAP–stored fruit during 5 and 8 weeks of cold storage (Fig. 8.9C). Despite a significant decline in the flesh firmness during the 6 days of SL at 21±1°C, CA–stored fruit were significantly firmer than air– and MAP–stored fruit. Similar to CA–1 and CA–2, the differences in flesh firmness of air– and MAP–stored fruit were non–significant.

The changes in SSC, TA, and SSC:TA of 1–MCP–treated fruit during cold storage in different atmospheres are presented in Table 8.5. The interaction effect of storage atmosphere and duration of storage on SSC was non–significant. During the 6 days of SL after 5 weeks of storage, the SSC increased significantly, but there were no significant differences among different atmospheres. Contrarily, no significant change in SSC occurred during the same time period after 8 weeks of storage. The retention of TA was significantly greater in CA–stored fruit compared with MAP and regular air. No significant differences in TA of air– and MAP–stored fruit were found after 5 and 8 weeks of storage and SL. With the increase in storage duration and during SL, TA decreased significantly depending on the storage atmosphere.

SSC:TA ratio was mainly influenced by the changes in TA. As a consequence of decrease in TA, a significant increase in SSC:TA was observed during cold storage and SL.

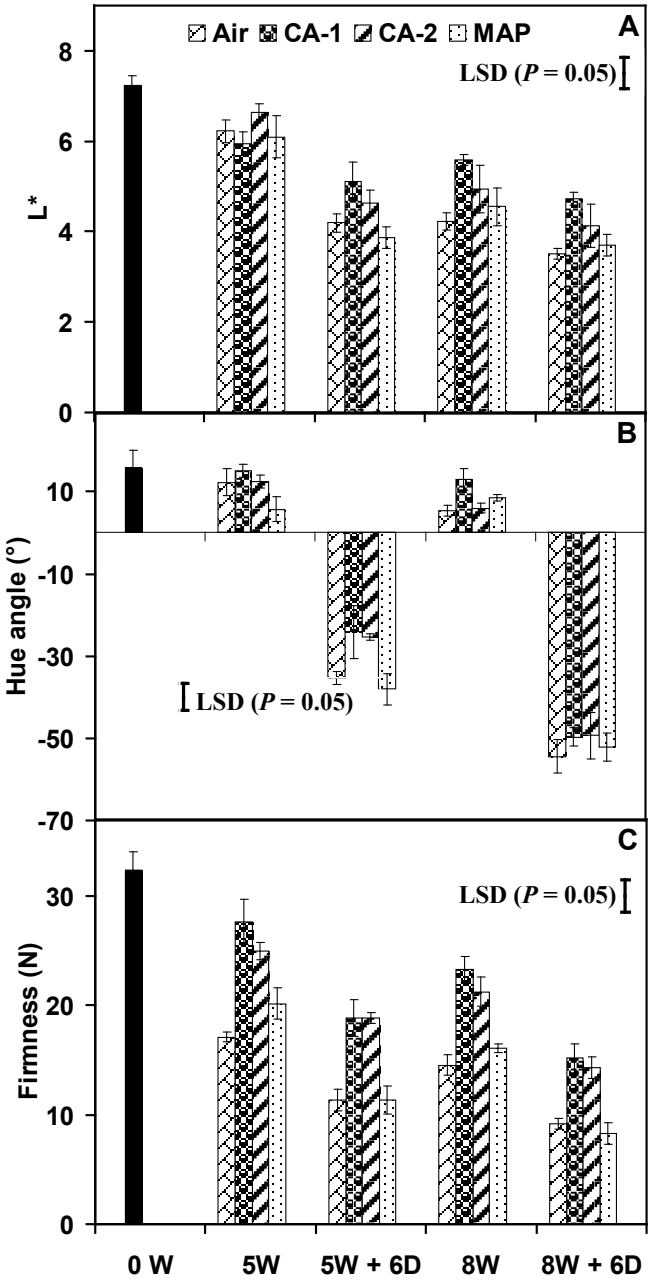


Fig. 8.9. Changes in skin colour (L* [A] and hue angle [B]) and flesh firmness (C) of 1–MCP–treated 'Blackamber' plums as influenced by cold storage in different atmospheres at 0–1°C for 5 and 8 weeks (W), plus 6 days (D) of shelf life at 21±1°C after each storage interval. Vertical bars represent S.E. of means. LSD (P = 0.05) values for L*: Storage atmosphere (SA) = 0.35, Storage period (SP) = 0.31 SA x SP = 0.70. Hue angle: SA = 3.26, SP = 2.92, SA x SP = 6.51. Flesh firmness: SA = 1.44, SP = 1.29, SA x SP = 2.89. LSD values for SA x SP are shown as separate bars.

Table 8.5. Fruit quality attributes (SSC, TA, and SSC:TA ratio) of 1–MCP–treated 'Blackamber' plums at harvest (0 week) and after 5 and 8 weeks (W) of storage in different atmospheres at 0–1°C, plus 6 days of shelf life at 21±1°C after each storage interval.

Storage period	**SSC (°Brix)**				**TA (%)**				**SSC:TA**			
	Air	**CA–1**	**CA–2**	**MAP**	**Air**	**CA–1**	**CA–2**	**MAP**	**Air**	**CA–1**	**CA–2**	**MAP**
0 W + 0 days	11.8				1.17				10.1			
5 W + 0 days	12.1	11.9	11.9	12.1	0.80	1.00	0.89	0.80	15.2	12.0	13.4	15.0
5 W + 6 days	12.6	12.6	12.4	12.4	0.68	0.81	0.72	0.67	18.5	15.6	17.2	18.4
8 W + 0 days	12.2	12.0	12.4	11.8	0.68	0.91	0.86	0.69	17.8	13.2	14.3	17.0
8 W + 6 days	12.1	12.1	12.3	11.7	0.39	0.58	0.49	0.40	30.9	20.7	25.0	29.2
Least significant differences of means at 5% level and levels of significance for a two–factor ANOVA												
Storage atmosphere (SA)	0.2***				0.02***				0.86***			
Storage period (SP)	0.2**				0.02***				0.77***			
SA X SP	NS				0.05***				1.72***			

*** = $P \leq 0.001$; ** = $P \leq 0.01$; NS = non–significant

8.3.2.2 Chilling injury

CI symptoms included flesh browning and translucency, when evaluations were conducted immediately after 5 and 8 weeks of storage. After transfer from cold storage to ambient conditions, severity of these symptoms increased, and flesh bleeding was also observed. Storage atmosphere and duration of storage had a significant impact on the incidence and severity of CI symptoms in 1–MCP–treated fruit (Fig. 8.10).

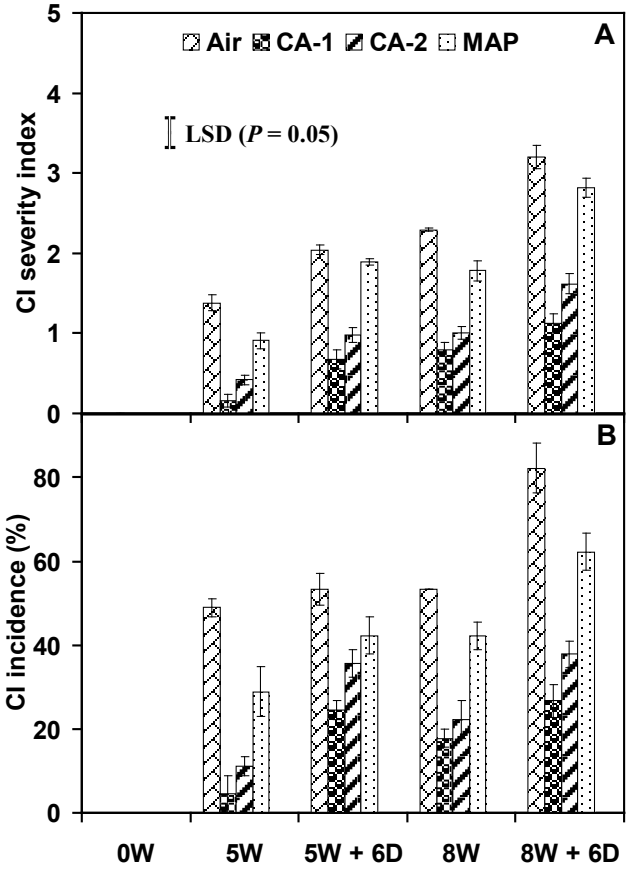


Fig. 8.10. CI severity index (A) and CI incidence (B) in 1–MCP–treated 'Blackamber' plums as influenced by cold storage in different atmospheres at 0–1°C for 5 and 8 weeks (W), plus 6 days (D) of shelf life at 21±1°C after each storage interval. Vertical bars represent S.E. of means. LSD (P = 0.05) values for CI index: Storage atmosphere (SA) = 0.16, Storage period (SP) = 0.18, SA x SP = 0.36. LSD value for SA x SP is shown as a separate bar. Data on CI incidence were arcsine transformed, but back–transformed data are presented for simplifying comparisons. LSD values for transformed data on CI incidence are: SA = 0.04, SP = 0.05, SA x SP = 0.09.

The incidence and severity of CI symptoms was greatly reduced during 5 and 8 weeks of storage under CA compared to MAP and regular air. Fruit held in atmospheres containing 1% O_2 plus 3% CO_2 showed the minimum incidence and severity of CI. MAP was also effective to some extent to reduce the chilling damage in 1–MCP–treated fruit. A comparison between 5 and 8 weeks of storage plus 6 days of SL showed that the CI incidence did not differ significantly under both CA conditions, while it increased by about 1.5 fold in MAP– and air–stored fruit after 8 weeks of storage (Fig. 8.10).

8.3.2.3 Lipid peroxidation

Table 8.6 presents the data on LOX activity, TBARS concentration and EL during cold storage in different atmospheres and SL of 1–MCP–treated 'Blackamber' plums. After the first 5 weeks of storage, an increase in LOX activity was observed in air– and MAP–stored fruit compared with CA–stored fruit. LOX activity did not change significantly during the last 3 weeks of storage in all atmospheres except CA–2 in which it increased significantly. In general, LOX activity increased significantly during the SL after 5 and 8 weeks of storage in all atmospheres.

The accumulation of TBARS during cold storage was higher in air– and MAP–stored fruit than in CA–stored fruit. A significant increase in TBARS concentration was further noticed during the SL after both storage intervals. Noteworthy, the interaction effect of storage atmosphere and duration of storage was non–significant on TBARS concentration. EL increased during cold storage depending on the storage atmosphere. EL was observed to be significantly lower in fruit held in CA than in MAP– and air–stored fruit after 5 and 8 weeks of storage. After transfer to 21±1°C, the EL increased in fruit held in all storage atmospheres, and the differences in EL were even non–significant after the SL following 8 weeks of storage.

Table 8.6. Lipoxygenase (LOX) activity, concentration of thiobarbituric acid–reactive substances (TBARS), and electrolyte leakage (EL) in 1–MCP–treated 'Blackamber' plums at harvest (0 week) and after 5 and 8 weeks (W) of storage in different atmospheres at 0–1°C, plus 6 days of shelf life at 21±1°C after each storage interval

Storage period	**LOX (µmol min^{-1} mg^{-1} protein)**				**TBARS (nmol MDA equivalents g^{-1} FW)**				**EL (%)**			
	Air	**CA–1**	**CA–2**	**MAP**	**Air**	**CA–1**	**CA–2**	**MAP**	**Air**	**CA–1**	**CA–2**	**MAP**
0 W + 0 days	6.5				0.25				34.5			
5 W + 0 days	11.9	7.0	8.1	11.0	0.65	0.48	0.41	0.52	48.6	39.5	40.1	46.1
5 W + 6 days	19.0	14.3	16.8	21.8	1.62	1.30	1.28	1.42	67.9	54.6	62.4	64.3
8 W + 0 days	12.1	7.6	11.2	10.2	0.72	0.66	0.59	0.71	52.1	43.1	45.2	50.0
8 W + 6 days	16.3	20.3	17.7	26.0	1.50	1.43	1.38	1.54	76.3	74.3	73.7	74.2
Least significant differences of means at 5% level and levels of significance for a two–factor ANOVA												
Storage atmosphere (SA)	1.76***				0.08***				1.55***			
Storage period (SP)	1.97***				0.09***				1.73***			
SA X SP	3.95**				NS				3.47***			

*** = $P \leq 0.001$; ** = $P \leq 0.01$; NS= non–significant

8.3.2.4 Antioxidant enzymes (SOD, CAT, and POD)

SOD activity decreased significantly during the first 5 weeks of storage in 1–MCP–treated fruit held in normal air and MAP, while it did not change significantly in CA–stored fruit (Fig. 8.11A). With the increase in storage period to 8 weeks, the decrease in SOD activity was significant for fruit stored in all atmospheres except CA–1. No significant change in SOD activity was noticed during SL after 5 weeks of storage in all atmospheres, while MAP stored fruit showed a significant decline after 6 days of SL following 8 weeks of storage.

In general, SOD activity was found maximum in fruit stored in CA–2 following 6 days of SL after both sampling intervals. CAT activity showed an increasing trend during 5 and 8 weeks of storage in all atmospheres, but the degree of increase was statistically non–significant (Fig. 8.11B). SL period of 6 days also did not induce any significant change in CAT activity, regardless of storage atmosphere. Storage atmosphere and storage period had a significant influence on POD activity (Fig. 8.11C).

POD activity increased to variable extent in air– and CA–stored fruit, but remained unchanged in fruit held in MAP during the fist 5 weeks of storage. A marked increase in POD was noticed in the last 3 weeks of storage in all atmospheres including MAP. The increase in POD activity continued during SL after 5 and 8 weeks of storage in all atmospheres. The differences in POD activities of fruit held in different atmospheres were not statistically significant after 6 days of SL following 5 and 8 weeks of storage except fruit held in CA–2 showed significantly higher activity than others after 5 weeks plus 6 days at 21±1°C.

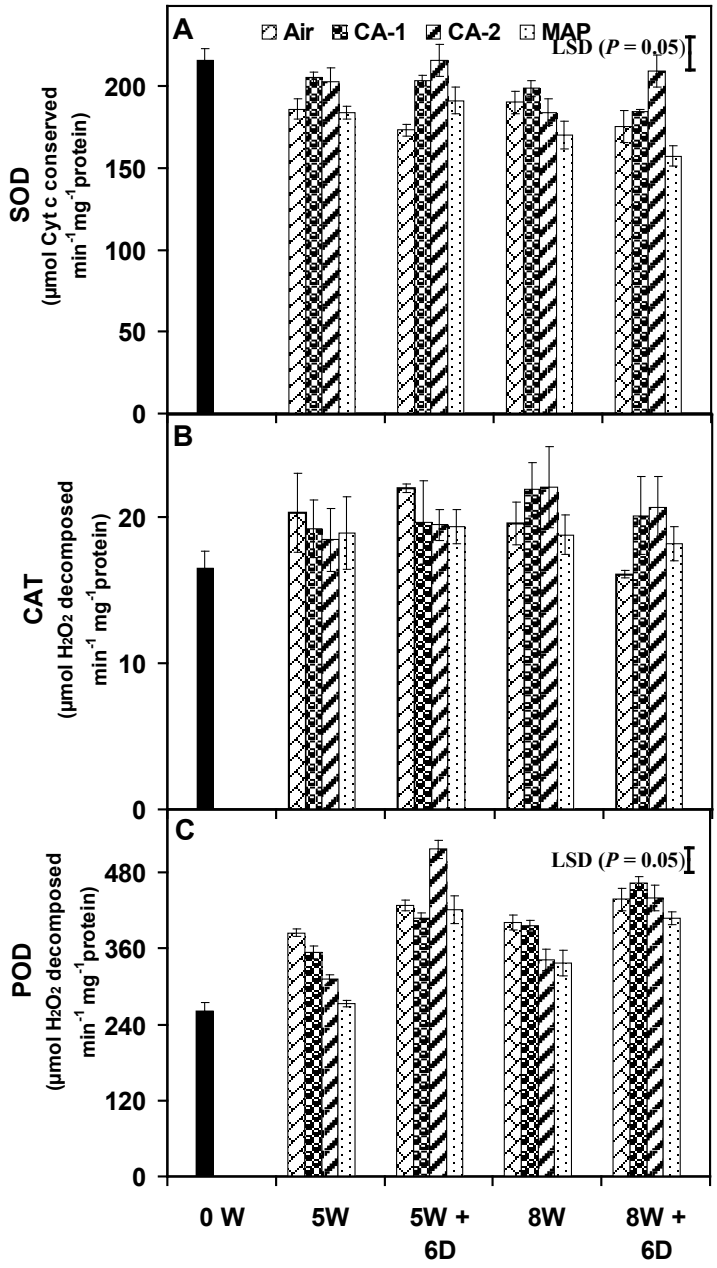


Fig. 8.11. Changes in activities of SOD (A), CAT (B), and POD (C) in the flesh tissue of 1–MCP–treated 'Blackamber' plums as influenced by cold storage in different atmospheres at 0–1°C for 5 and 8 weeks (W), plus 6 days (D) of shelf life at 21±1°C after each storage interval. Vertical bars represent S.E. of means. LSD (P = 0.05) values for SOD: Storage atmosphere (SA) = 8.80, Storage period (SP) = 9.84, SA x SP = 19.67. CAT: SA = NS, SP = 2.60, SA x SP = NS. POD: SA = 4.21, SP = 4.70, SA x SP = 9.41. LSD values for SA x SP are shown as separate bars.

8.3.2.5 Ascorbate–glutathione cycle

Ascorbate

Concentrations of total ascorbate (AA + DHA) in 1–MCP–treated fruit were significantly influenced by storage atmosphere and storage duration (Fig. 8.12A). The losses of total ascorbate during 5 and 8 weeks of storage were about 45%–52% and 75%–85% depending upon the storage atmosphere, respectively. The highest amount of decrease in total ascorbate was observed in fruit held in MAP. The fruit held in CA and normal air showed decrease in concentration of total ascorbate to similar extent after 5 weeks of storage, while the decrease was slightly higher in CA–stored fruit after 8 weeks.

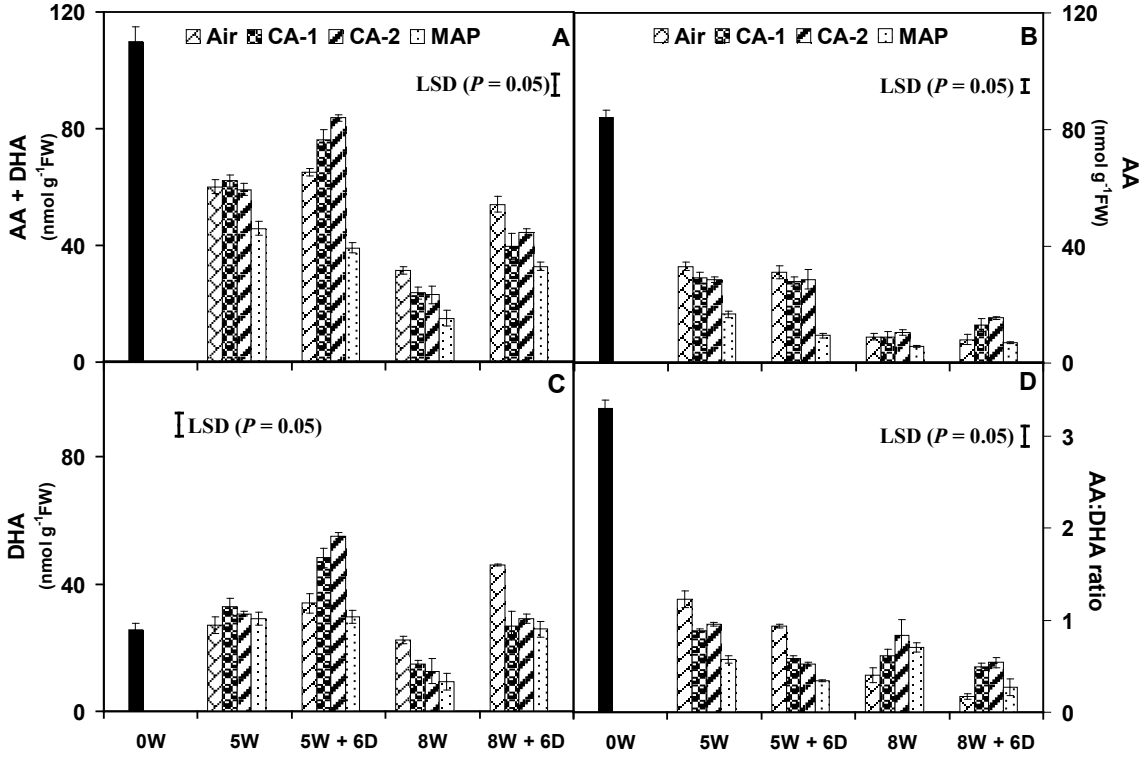


Fig. 8.12. Changes in concentrations of total ascorbate (AA + DHA) (A), AA (B), DHA (C), and AA:DHA ratio (D) in the flesh tissue of 1–MCP–treated 'Blackamber' plums as influenced by cold storage in different atmospheres at 0–1°C for 5 and 8 weeks (W), plus 6 days (D) of shelf life at 21±1°C after each storage interval. Vertical bars represent S.E. of means. LSD (P = 0.05) values for AA + DHA: Storage atmosphere (SA) = 2.68, Storage period (SP) = 2.40, SA x SP = 5.37. AA: SA = 0.85, SP = 0.76, SA x SP = 1.69. DHA: SA = 2.75, SP = 2.46, SA x SP = 5.50. AA:DHA ratio: SA = 0.11, SP = 0.10, SA x SP = 0.22. LSD values for SA x SP are shown as separate bars.

The concentrations of total ascorbate increased greatly in CA–stored fruit after 6 days of SL following 5 and 8 weeks of storage. The trend of changes in AA was almost similar to changes in total ascorbate (Fig. 8.12B). The extent of decrease in AA concentration was 2.5, 3.0 and 5.0 fold in air–, CA–, and MAP–stored fruit during the first 5 weeks of storage, respectively. The decrease in AA concentration continued during the last 3 weeks of storage and led to about 8.4 to 10 fold decrease in normal air and CA–stored fruit and about 15–fold in MAP compared to AA concentration at harvest. No clear trend of changes in AA concentration was noticed during the SL period after 5 and 8 weeks of storage; these concentrations either remained almost stable or slightly increased or decreased.

During the first 5 weeks of storage, DHA concentration increased slightly in all storage atmospheres, but the increase was significant only in case of CA–1 (Fig. 8.12C). Interestingly, during the last 3 weeks of storage, the concentrations of DHA declined to variable extent. No significant differences in DHA concentration of CA– and MAP–stored fruit were recorded after 8 weeks of storage and were significantly lower than in fruit stored in normal air. A tremendous increase in DHA concentration was observed in fruit held in all storage atmospheres after 6 days of SL following 5 and 8 weeks of storage, except in MAP after 5 weeks of storage. AA:DHA ratio declined significantly during the entire storage period depending on the storage atmosphere. The highest decrease in this ratio during 8 weeks of storage was observed in fruit held in air, while those stored in CA and MAP did not differ significantly. In general, a decrease in AA:DHA ratio was observed after 6 days of SL following 5 and 8 weeks of cold storage in all atmospheres, but fruit held in CA storage exhibited higher ratio than those in air and MAP.

Glutathione

Glutathione levels in flesh tissue of 1–MCP–traeted fruit were significantly influenced by storage atmosphere and duration of storage (Fig. 8.13). Concentrations of total glutathione decreased significantly in fruit kept in all storage atmospheres during 5 and 8 weeks of storage, but the decrease was significantly lower in fruit held in CA than those in air or MAP (Fig. 8.13A). The decrease in total glutathione continued even during SL period after cold storage in different atmospheres. Overall,

the highest levels of total glutathione were observed in CA–stored fruit after 6 days of SL following 5 and 8 weeks of storage. GSH was the major constituent of the total glutathione and the changes in its concentration during storage and SL followed a pattern similar to total glutathione (Fig. 8.13B).

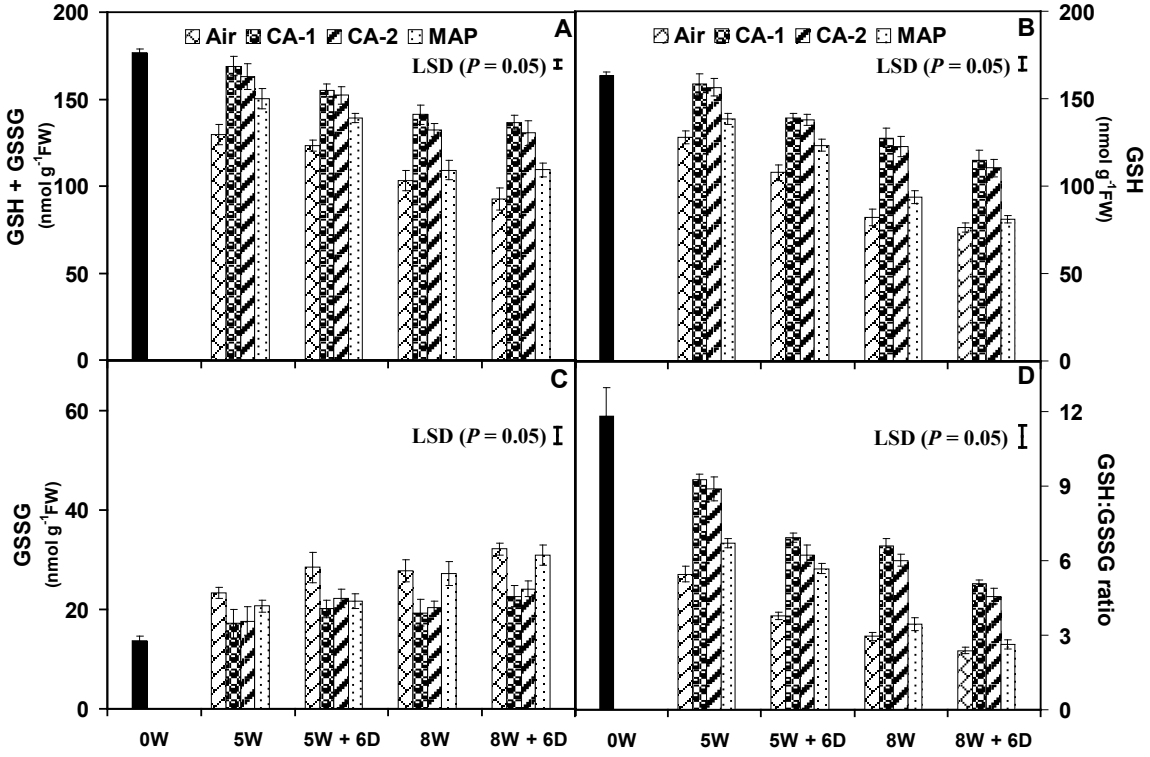


Fig. 8.13. Changes in concentrations of total glutathione (GSH + GSSG) (A), GSH (B), GSSG (C), and GSH:GSSG ratio (D) in the flesh tissue of 1–MCP–treated 'Blackamber' plums as influenced by cold storage in different atmospheres at 0–1°C for 5 and 8 weeks (W), plus 6 days (D) of shelf life at 21±1°C after each storage interval. Vertical bars represent S.E. of means. LSD (P = 0.05) values for GSH + GSSG: Storage atmosphere (SA) = 2.30, Storage period (SP) = 2.06, SA x SP = 4.60. GSH: SA = 2.27, SP = 2.03, SA x SP = 4.53. GSSG: SA = 0.457, SP = 0.40, SA x SP = 0.90. GSH:GSSG ratio: SA = 0.21, SP = 0.24, SA x SP = 0.47. LSD values for SA x SP are shown as separate bars.

The concentration of GSSG increased significantly during storage and SL in all storage atmospheres (Fig. 8.13C), but the extent of increase in GSSG was significantly higher in air–stored fruit than in CA– and MAP–stored fruit. During 8 weeks of storage plus 6 days at 21±1°C, the concentrations of GSSG increased by 1.6 and 2.3 fold in CA– and air– or MAP–stored fruit, respectively. A significant decline in the GSH:GSSG ratio was observed during cold storage and SL (Fig.

8.13D). With the increase in storage duration from 5 to 8 weeks, a significant decrease in GSH:GSSG ratio was observed. However, the ratio was maintained at a significantly higher level in CA–stored fruit than in air– and MAP–stored fruit.

Ascorbate–glutathione cycle enzymes

The activities of enzymes involved in AA–GSH cycle were significantly influenced by storage atmosphere and duration of storage. APX activity increased significantly during cold storage for 5 or 8 weeks and the highest amount of increase was noticed in fruit held in MAP, while no statistically significant differences in APX activities of air and CA–stored fruit were observed following 5 and 8 weeks of storage (Fig. 8.14A). After 6 days of SL, CA–and MAP–stored fruit did not show a significant change in APX activity and those in normal air showed an increase. During 5 and 8 weeks of storage, MDHAR activity remained either stable or increased slightly in air– and CA–stored fruit, but it showed a significant decrease in MAP–stored fruit (Fig. 8.14B). Mostly, there were no significant changes in MDHAR activities during 6 days of SL following 5 and 8 weeks of storage, regardless of storage atmosphere. DHAR activity did not show a significant change during 5 and 8 weeks of storage, irrespective of storage atmosphere (Fig. 8.14C). No further significant change in DHAR activity was noticed in fruit after 6 days of SL following 5 weeks of storage in different atmospheres. DHAR activities decreased significantly during SL after 8 weeks of storage in all atmospheres, except CA–2.

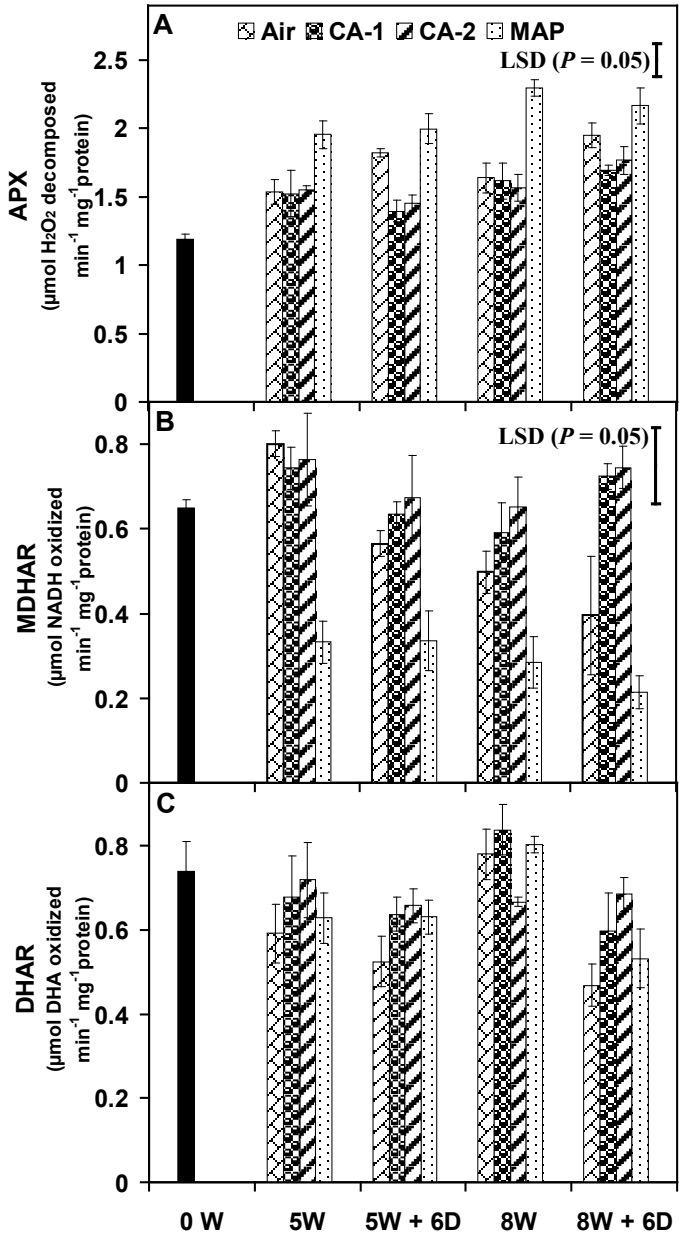


Fig. 8.14. Changes in activities of APX (A), MDHAR (B), and DHAR (C) in the flesh tissue of 1–MCP–treated 'Blackamber' plums as influenced by cold storage in different atmospheres at 0–1°C for 5 and 8 weeks (W), plus 6 days (D) of shelf life at 21±1°C after each storage interval. Vertical bars represent S.E. of means. LSD (P = 0.05) values for APX: Storage atmosphere (SA) = 0.11, Storage period (SP) = 0.12, SA x SP = 0.24. MDHAR: SA = 0.08, SP = 0.09, SA x SP = 0.18. DHAR: SA = NS, SP = 0.09, SA x SP = NS. LSD values for SA x SP are shown as separate bars.

The enzymes involved in oxidation and recovery of glutathione in the AA–GSH cycle were also influenced by storage atmosphere and duration of storage. Fruit held in normal air and MAP showed non–significant differences in GR activity (Fig. 8.15A). Similarly, the differences in concentration of O_2 in CA–1 and CA–2 had no significant impact on the activity of GR. During the first 5 weeks of storage, GR

activity remained unchanged in CA–stored fruit compared to those held in normal air and MAP.

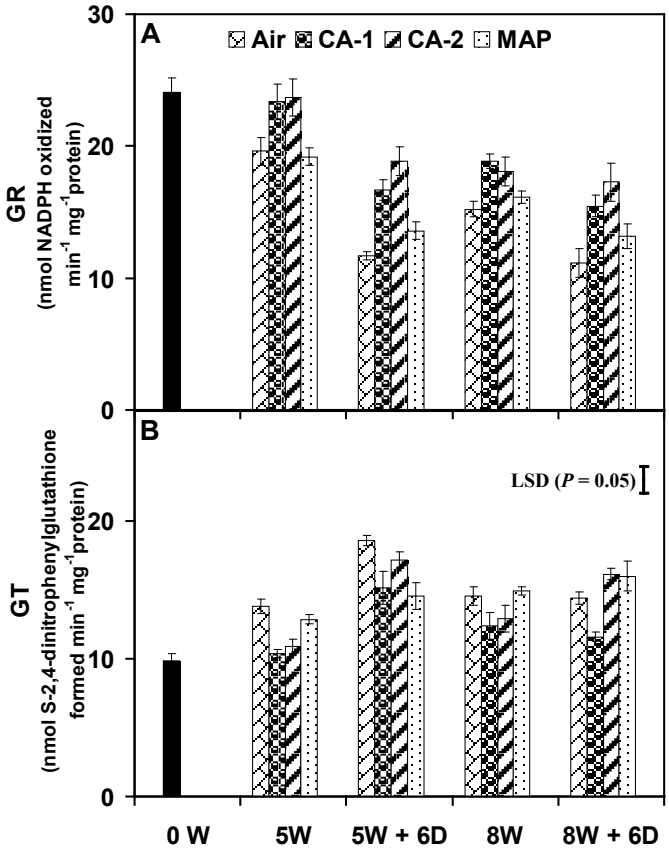


Fig. 8.15. Changes in activities of GR (A) and GT (B) in the flesh tissue of 1–MCP–treated 'Blackamber' plums as influenced by cold storage in different atmospheres at 0–1°C for 5 and 8 weeks (W), plus 6 days (D) of shelf life at 21±1°C after each storage interval. Vertical bars represent S.E. of means. LSD (P = 0.05) values for GR: Storage atmosphere (SA) = 1.25, Storage period (SP) = 1.40, SA x SP = NS. GT: SA = 0.84, SP = 0.94, SA x SP = 1.88. LSD value for SA x SP is shown as a separate bar.

The increased storage duration caused a further decrease in GR activity in all storage atmospheres which led to the differences among different atmospheres to non–significant status after 8 weeks. The trend of decrease in GR activity also continued during the SL period after both storage intervals, and CA–stored fruit showed higher GR activity than MAP– and air–stored fruit. In contrast to the GR activity, the activity of GT showed an increasing trend during storage and SL depending on the storage atmosphere (Fig. 8.15B). The determination of GT activity immediately after 5 or 8 weeks of storage showed that fruit held in normal air and MAP had higher GT activity than those stored in CA. However, GT activity showed a significant increase

during SL and no clear pattern could be observed for fruit held in different atmospheres. In general, fruit held in CA–1 showed the minimum GT activity.

8.3.2.6 Total phenolics and DPPH$^{\cdot}$ scavenging activity

Storage atmosphere and storage duration had a significant influence on concentrations of total phenolics and DPPH$^{\cdot}$ scavenging activity (Fig. 8.16). The concentrations of total phenolics remained stable during the first 5 weeks of storage in CA, while it declined significantly in air– and MAP–stored fruit (Fig. 8.16A).

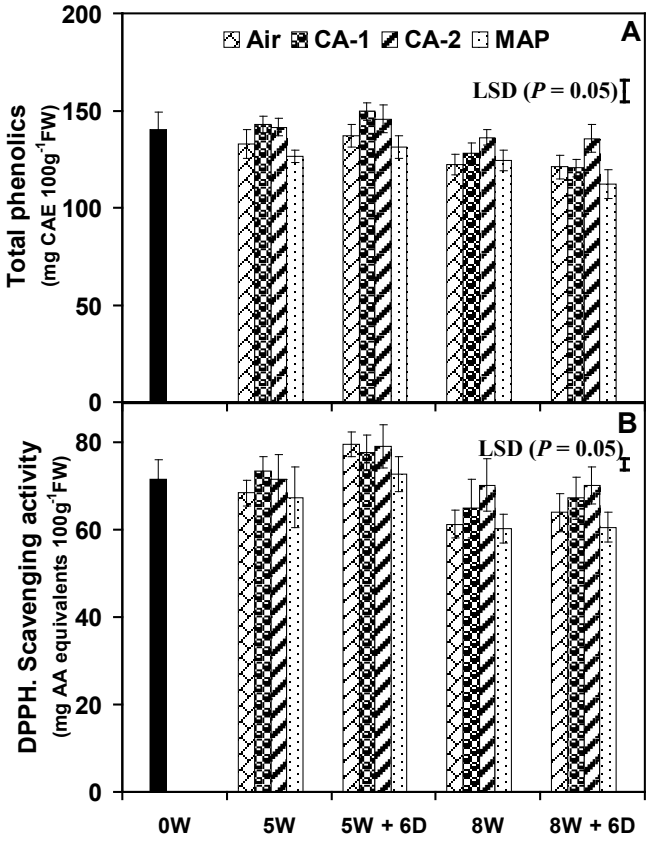


Fig. 8.16. Changes in total phenolics concentration (A) and DPPH scavenging activity (B) in the flesh tissue of 1–MCP–treated 'Blackamber' plums as influenced by cold storage in different atmospheres at 0–1°C for 5 and 8 weeks (W), plus 6 days (D) of shelf life at 21±1°C after each storage interval. Vertical bars represent S.E. of means. LSD (P = 0.05) values for Total phenolics: Storage atmosphere (SA) = 2.32, Storage period (SP) = 2.59, SA x SP = 5.19. DPPH scavenging activity: SA = 1.51, SP = 1.69, SA x SP = 3.38. LSD values for SA x SP are shown as separate bars.

The total phenolics concentration decreased significantly during the last 3 weeks of storage and the differences among different atmospheres were of minor importance. A slight increase in total phenolics was observed after 6 days of SL following 5 weeks of storage in all atmospheres, while an opposite trend of slight decrease was observed after 8 weeks of storage. $DPPH^{\cdot}$ scavenging activity is primarily contributed by the phenolics in Japanese plums. Therefore, the pattern of changes in $DPPH^{\cdot}$ scavenging activity resembled the changes in total phenolics during 5 and 8 weeks of storage and subsequent 6 days of SL at 21±1°C (Fig. 8.16B).

8.3.2.7 Correlation analysis

The correlation coefficients of CI index and CI incidence with the lipid peroxidation, enzymatic and non-enzymatic antioxidants in 1–MCP–treated 'Blackamber' plums during cold storage under different atmospheres at 0–1°C plus 6 days of shelf life at 21±1°C are presented in Table 8.7. The CI incidence and severity index had a significantly positive correlation with TBARS, LOX, EL, APX, POD, GSSG and GT, whereas negative correlations with SOD, AA, AA:DHA ratio, GSH, GSH:GSSG ratio, GR, MDHAR, DHAR, phenols and DPPH scavenging activity were observed.

Table 8.7. Correlations of CI index and CI incidence with storage period, lipid peroxidation, enzymatic and non-enzymatic antioxidants in 1–MCP–treated 'Blackamber' plums during 5 and 8 weeks of cold storage under different atmospheres at 0–1 °C plus 6 days of shelf life at 21±1 °C

CI index *vs.*	**Pearson's correlation coefficient**	**CI incidence *vs.***	**Pearson's correlation coefficient**
TBARS	0.73***	TBARS	0.73***
LOX	0.67***	LOX	0.70***
EL	0.79***	EL	0.80***
SOD	–0.66***	SOD	–0.69***
CAT	NS	CAT	NS
POD	0.63***	POD	0.59***
AA	–0.68***	AA	–0.71***
DHA	NS	DHA	NS
AA:DHA	–0.69***	AA:DHA	–0.69***
GSH	–0.89***	GSH	–0.94***
GSSG	0.95***	GSSG	0.96***
GSH:GSSG	–0.91***	GSH:GSSG	–0.91***
APX	0.69***	APX	0.74***
MDHAR	–0.48***	MDHAR	–0.55***
DHAR	–0.47***	DHAR	–0.41***
GR	–0.85**	GR	–0.87***
GT	0.72***	GT	0.68***
Phenols	–0.60***	Phenols	–0.69***
DPPH scavenging activity	–0.34*	DPPH scavenging activity	–0.43*

NS, *, **, *** = non-significant, $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, respectively

8.4 Discussion

8.4.1 Fruit quality

There were four levels of O_2 under different atmospheres in these experiments: 20.8% in normal air, ~10.0% in MAP, 2.5% in CA–2 and 1.0% in CA–1. CO_2 concentration was 3% under both CA conditions, while its range was 3%–4% under MAP (Table 8.1). In general, the physiological and biochemical effects on fruit quality and oxidative stress could be related to the differences in O_2 concentrations because the differences in CO_2 concentrations between CA and MAP were less than 1%. CA containing either 1% O_2 or 2.5% O_2 did not differ much in influencing fruit quality. As expected, CA was more effective than MAP and normal air in retarding changes in skin colour, firmness, and SSC:TA of 1–MCP–treated and –untreated fruit during 5 and 8 weeks of storage at 0–1°C (Table 8.2 & Fig. 8.1). The results are in agreement with previous reports suggesting the beneficial effects of CA and MAP in preserving fruit quality in the European and Japanese plums (Khan and Singh, 2008; Maré et al., 2005; Menniti et al., 2006; Truter and Combrink, 1992; Truter and Combrink, 1997; Wang and Vestrheim, 2003). The reduction in O_2 levels and increase in CO_2 concentrations in storage atmospheres might have reduced the rates of respiration and ethylene production in 'Blackamber' plums as reported for 'Songold' and 'Angeleno' plums (Maré et al., 2005). The flesh firmness of CA–stored 'Blackamber' plums was retained at a higher level even after 6 days of SL following 5 or 8 weeks of cold storage (Fig. 8.1C). This could possibly be related to retarded fruit ripening due to the residual effects of CA storage (Maré et al., 2005). As the levels of CO_2 were almost similar under CA and MAP, the differences in the effects on fruit quality could be attributed to the low O_2 concentrations.

It is generally accepted that low O_2 atmospheres reduce the sensitivity of fruit to ethylene (Saltveit, 2003) and this might have caused a significant reduction in the initiation of ethylene–mediated responses such as fruit softening (Khan and Singh, 2008). Furthermore, pre–storage application of 1–MCP might have caused a significant reduction in the action of ethylene and also its autocatalytic production. The results show that the retention of fruit quality in terms of flesh firmness, skin colour changes, and TA was better in 1–MCP–treated fruit compared with their untreated counterparts (Fig. 8.9 and Table 8.5). For instance, the firmness of 1–MCP–treated fruit held in normal air after 5 and 8 weeks of storage was comparable

to the firmness of 1–MCP–untreated fruit held in CA–2. A similar observation was also reported by Menniti et al. (2006) suggesting that 1–MCP–treatment could be an alternative to short–term CA storage in Japanese plums. The mechanism of action of CA and 1–MCP are similar from ethylene perspective as both reduce the ethylene action and its autocatalytic production. A combination of CA and 1–MCP can have synergistic effects on maintaining fruit quality than either alone. Although, MAP of 1–MCP–treated fruit has been reported to be beneficial to increase the storage life of 'Tegan Blue' plums (Khan and Singh, 2008), but data suggest that the effects of CA storage on 1–MCP–treated fruit were more favourable than MAP.

8.4.2 Chilling injury (CI)

CI symptoms in the form of flesh browning, translucency and mealiness were observed in fruit, irrespective of storage atmosphere. However, the severity and incidence of CI was greatly influenced by the storage atmosphere. CI incidence and severity were the lowest in fruit held in CA containing 1% O_2 and it increased with the increase in concentration of O_2 in the storage atmosphere (Fig. 8.2). The data strongly support the fact that low O_2 atmospheres were primarily responsible for reducing the CI symptoms during storage as the CO_2 concentrations were almost at the same level under CA and MAP. The delay or prevention of CI symptoms in the form of mealiness, browning and flesh reddening in plums, peaches and nectarines has been associated with the low O_2 and high CO_2 concentrations in the storage atmospheres (Crisosto et al., 1997; Lurie, 1992; Retamales et al., 1992; Sive and Resnizky, 1979; Streif et al., 1992; Wang et al., 2005; Zhou et al., 2000). The high CO_2 concentration (10%–17%) in CA has been reported to confer more benefits in terms of delay in appearance of CI symptoms in peaches and nectarines (Crisosto et al., 1997; Lurie, 1992; Retamales et al., 1992; Zhou et al., 2000). Earlier reports have shown that the presence of CO_2 in CA was essential (Kajiura, 1975; Lill et al., 1989; Wade, 1981) and more important than O_2 (Lurie, 1992) to reduce CI in peaches and nectarines. The potential of CA in ameliorating CI in Japanese plums may be further enhanced by testing the increased concentrations of CO_2 (≥10%) in the storage atmospheres as reported in peaches and nectarines.

1–MCP treatment reduced the severity of CI in fruit held in normal air compared with untreated fruit (Fig. 8.10). The combination of 1–MCP and CA containing 1% O_2 and 3% CO_2 was the best in ameliorating CI in 'Blackamber' plums for 5 and 8 weeks of storage plus 6 days of SL at 21±1°C. The effectiveness of 1–MCP treatment in maintaining fruit quality and preventing internal breakdown in 'Angeleno' plums has been demonstrated to be equal to the 1–MCP–untreated fruit held in CA storage for 40 or 60 days (Menniti et al., 2006). The data suggest that the same was not true in case of 'Blackamber' plums during cold storage for 35 and 56 days plus 6 days of SL. The possible reason could be a sharp contrast in the postharvest physiology of two cultivars. Ethylene has been implicated in development of CI in climacteric type cultivars of Japanese plums which produce more ethylene than suppressed–climacteric type cultivars (Candan et al., 2008). Previously, the effectiveness of 1–MCP in alleviating CI in climacteric type 'Larry Ann' and suppressed–climacteric type 'Angeleno' plums has been demonstrated (Candan et al., 2008; Menniti et al., 2006). The results show that synergistic effects of 1–MCP and CA were prevalent in reducing the incidence and severity of CI in 'Blackamber' plums.

8.4.3 Lipid peroxidation

It is widely accepted that CI involves the membrane damage or dysfunction which is primarily caused by the lipid peroxidation. Membrane lipid peroxidation is initiated either enzymatically or by ROS (Shewfelt and del Rosario, 2000; Shewfelt and Purvis, 1995). LOX activity increases in response to chilling stress (Table 8.3) and may be involved in occurrence of CI through enhanced lipid peroxidation as reported in loquat (Cao et al., 2009b). LOX also contributes to the production of superoxide radicals (Gardner, 1995) which are among the ROS contributing to accumulation of TBARS and increased oxidative stress. The data show that LOX activity was greatly reduced under CA storage compared to MAP and regular air, but increased significantly during SL period after storage. In 'Conference' pears, LOX activity in fruit under optimum CA (2% O_2 + 0.7% CO_2) was significantly lower than under stressful atmospheres high in CO_2 (2% O_2 + 5% CO_2) (Larrigaudiére et al., 2001b). The LOX activity during postharvest storage can be triggered by stresses such as low temperature, stressful atmospheres and a combination of both, which caused

enhanced lipid peroxidation leading to initiation of certain physiological disorders and senescence–related processes (Ding et al., 2006; Larrigaudiére et al., 2001a; Larrigaudiére et al., 2001b; Mao et al., 2007; Rivera et al., 2007). The lower LOX activity under CA was coincident with the reduced incidence and severity of CI in 'Blackamber' plums. Similarly, the lower LOX activity was also associated with the lower core browning in 'Conference' pears held under optimum CA (Larrigaudiére et al., 2001b). These observations support the role of LOX in occurrence of physiological disorders in fruits. 1–MCP treatment reduced the LOX activity in fruit held in normal air, and no remarkable differences were observed in the LOX activities of CA–stored fruit which were either treated with 1–MCP or untreated (Table 8.6). 1–MCP treatment has been shown to reduce the LOX activity in loquat and pears during cold storage and alleviate CI and superficial scald, respectively (Cao et al., 2009b; Li and Wang, 2009).

TBARS is a marker for the degree of lipid peroxidation and oxidative stress (Hodges and Forney, 2000). The accumulation of TBARS was also lower in CA stored fruit compared to MAP and normal air (Table 8.3). The data suggest that low O_2 atmospheres reduced the accumulation of TBARS which indicates the lower level of lipid peroxidation in CA–stored fruit. CA storage under optimal conditions have been known to inhibit the increase in TBARS concentration in spinach (Hodges and Forney, 2000) and pearLarrigaudiére et al., 2001b). Surface coating of 'Sapphire' plums with a carbohydrate based formulation also delayed the accumulation of TBARS (Eum et al., 2009), thus extended postharvest life. 1–MCP treatment has also been implicated in inhibiting the postharvest accumulation of TBARS in apples (Vilaplana et al., 2006), mangoes (Singh and Dwivedi, 2008) and pears (Li and Wang, 2009), symbolizing the role of 1–MCP in reducing the level of postharvest oxidative stress in these commodities. The measurement of EL provides an indirect measure of the membrane permeability. Membrane integrity was maintained to a greater extent under CA storage than under MAP or regular air as shown by the data on EL (Table 8.3). Low O_2 atmospheres have been reported to reduce EL during cold storage in longan fruit (Cheng et al., 2009). 1–MCP has also been shown to delay the onset of membrane disintegration and thus reduced EL during cold storage of fruits such as loquat, pear and Japanese plums (Cao et al., 2009b; Larrigaudiére et al., 2004). EL showed significantly positive correlations with TBARS, LOX and CI

index (Tables 8.4 & 8.7). Therefore, the degree of lipid peroxidation could explain the incidence and severity of CI in Japanese plums. In conclusion, CA storage comprising of low O_2 atmospheres was more effective in reducing the lipid peroxidation and oxidative stress which develops when the levels of ROS production exceed the removal efficiency of the system.

8.4.4 Antioxidant enzymes (SOD, CAT, and POD)

Antioxidant enzymes play a crucial role in protecting fruit against the increased production of ROS in response to various environmental stimuli (Hodges et al., 2004). The generation of O_2^- in response to chilling stress has been noticed in fruits. The action of SOD further converts these anionic radicals into H_2O_2. The accumulation of H_2O_2 to toxic levels is prevented by the action of peroxidases. The increased concentrations of superoxide anions, H_2O_2 and both reflect the failure of the primary antioxidant system. The effects of CA/MA on the activities of antioxidant enzymes in the European and Japanese plums have not been investigated before. The data show that SOD activity in CA–stored fruit was significantly higher after 5 weeks of cold storage than in MAP and normal air and these differences were non–significant among different atmospheres after 8 weeks of storage (Fig. 8.3A). SOD activities were sustained in CA–stored fruit during SL period in contrast to air–stored in which it decreased. The higher SOD activities indicate the efficient removal of the superoxide free radicals from the system. On the other hand, CAT either remained stable or showed an increase with the storage, and did not differ much among different storage atmospheres (Fig. 8.3B). No clear pattern of POD activity was evolved that could assist in meaningful interpretations (Fig. 8.3C). However, in most cases, a continuous increase in POD activity was observed during storage and SL of fruit. The increased activities of CAT and POD coupled with SOD might have caused increased quenching of ROS in CA–stored fruit.

Previously, it has been reported that SOD and CAT activities of ‘Conference’ pears were significantly higher in CA during long–term storage for 90 or 180 days compared to storage in normal air (Larrigaudiére et al., 2001b). The delay in reduction of activities of SOD, CAT, and POD was also observed during CA storage of peaches (Wang et al., 2005). Interestingly, 1–MCP treatment also resulted in

significant improvement in SOD and POD activities in fruit held in normal air (Fig. 8.11). Similar observations on increase in SOD, CAT and POD in response to 1–MCP treatment have been reported in apricots (Martino et al., 2006), mangoes (Singh and Dwivedi, 2008), and pears (Larrigaudiére et al., 2004). In 'Larry Ann' plums, the suppression of POD has been observed during cold storage at 0°C for 14 days in 1–MCP–treated fruit compared with control (Larrigaudiére et al., 2009). This report is not conclusive as the storage duration was limited to 14 days only which might not have been enough to induce the oxidative stress to levels where 1–MCP effects were measurable. In general, CA storage and its combination with 1–MCP resulted in an efficient system of primary antioxidant enzymes that might have resulted in reduced levels of ROS production.

Previous studies have shown that the increased activities of these enzymes were closely associated with the increased acquisition of chilling tolerance in fruits such as limes (Rivera et al., 2007), mangoes (Zhao et al., 2009), and peaches (Wang et al., 2004), and resistance against browning in 'Braeburn' apples (Toivonen et al., 2003) and 'Conference' pears (Lentheric et al., 1999). Higher CAT activity has been proposed to impart chilling tolerance in mandarins (Sala, 1998) and scald resistance in apples (Rao et al., 1998), while SOD activity did not differ among chilling–sensitive and –tolerant cultivars. The accumulation of H_2O_2 to greater levels occurred during storage of apples and pears in CA containing CO_2 concentration higher than optimal which has been associated with the increased incidence of flesh browning and core browning, respectively (De Castro et al., 2008; Larrigaudiére et al., 2001a; Larrigaudiére et al., 2001b).

The lower incidence and severity of CI in CA–stored fruit, and particularly those treated with 1–MCP and then held in CA, suggest that the atmospheres containing 1% or 2.5% O_2 and 3% CO_2 had favourable effects on reducing the oxidative stress in Japanese plums. CI index showed significantly negative correlation with SOD, but the correlation with CAT was non–significant (Tables 8.4 & 8.7). The activities of antioxidant enzymes could not be explained completely on the basis of O_2 concentration in the storage atmospheres. Because O_2 concentration inside MA packs were much lower than in regular air, but the activities of these enzymes were occasionally lower in the former. Such observations prompt that the

antioxidant defence mechanisms in fruits are complex and influenced by multiple factors both external and internal.

8.4.5 Ascorbate–glutathione cycle

Ascorbate and glutathione are water–soluble antioxidants present in small quantities in fruit, but play a major role in providing protection against ROS. The ratio of the reduced to oxidized forms of these compounds are determinants of the redox status of the tissue. The storage duration was the principal factor which determined the concentrations of AA in Japanese plums. The data suggest that a significant reduction in total ascorbate and AA occurred during storage of Japanese plums irrespective of the storage atmospheres (Fig. 8.4B). In general, the loss in AA was greater in fruit held in MAP compared to other storage atmospheres. There is ample evidence in literature suggesting that AA is very labile to losses in fruit during cold storage under normal air and CA conditions. The decrease in AA concentration in 'Conference' and 'Rocha' pears under CA storage has been widely studied and correlated with the core browning disorder (Franck et al., 2003; Galvis–Sánchez et al., 2006; Larrigaudiére et al., 2001b; Veltman et al., 1999, 2000). In 'Conference' pears, the threshold of AA concentration has been defined and the reduction in AA below that level enhanced the susceptibility to core browning (Veltman et al., 1999). The depletion of AA in 'Pink Lady' apples stored in CA containing high CO_2 has also been associated with the appearance of flesh browning symptoms during cold storage (De Castro et al., 2008). Several reports suggested that AA concentration decreased during long–term cold storage of mangoes (Zhao et al., 2009), oranges (Huang et al., 2008), and pawpaws (Galli et al., 2009). The data show that in most cases, fruit held in normal air and CA differed in CI symptoms, but the differences were non–significant with regard to AA. The decrease in AA in CA–stored fruit might have been compensated by other components of the antioxidant defence to provide protection against the oxidative stress. The retention of AA is, therefore, important for maintaining fruit quality, nutritional value and preventing storage disorders.

A significant increase in DHA was also observed during the first 5 weeks of storage and then it showed a decreasing trend during the last 3 weeks of storage

(Figs. 8.4C & 8.12C). DHA concentrations increased significantly during SL period after both storage intervals which is in agreement with Galvis–Sánchez et al. (2006) who reported a similar increase in DHA during SL of 'Rocha' pears following 4 months of CA storage. The activities of enzymes involved in oxidation and recovery of AA were determined to explain the dynamics of oxidized and reduced forms of ascorbate. The decrease in AA could be related to the increase in APX activity (Figs. 8.6A & 8.14A) during storage as these showed a significant negative correlation ($r = -0.80$) between them. The degree of increase in APX after 5 weeks of storage in MAP was significantly higher than those stored under normal air and CA. The increased APX activity might have contributed to the oxidation of AA into DHA, and its higher activity could explain the lower levels of AA. The increased loss of AA in fruit held in MAP is unexpected and difficult to explain, thus warrants further investigations. APX utilizes AA as a reducing agent to detoxify H_2O_2 from the tissue. 'Conference' pear fruit held under CA for 90 and 80 days exhibited higher APX activity than those under normal air (Larrigaudiére et al., 2001b). The increase in APX activity has been reported to occur during cold storage in normal air in different fruits and it has also been related to increased tolerance to chilling stress (Ding et al., 2007; Sala and Lafuente, 2004; Wang et al., 2008; Wang et al., 2006; Zhao et al., 2009).

MDHAR and DHAR activities were also influenced by storage atmospheres to some extent (Fig. 8.6B & C). The lower MDHAR activity in MAP compared to normal air and CA could be related to the lower concentration of AA because MDHAR is a critical enzyme in restoring the AA pool in AA–GSH cycle. The increased activity of DHAR after 8 weeks of storage could be related to lower levels of DHA compared to the levels after 5 weeks of storage. In most cases, the activities of MDHAR and DHAR either remained stable or increased in response to storage under different atmospheres. MDHAR activity during most cases was higher in CA stored fruit compared to air–stored fruit. The maintenance of higher activity of MDHAR has also been reported previously during CA storage of spinach compared to storage in air or air containing ethylene (Hodges and Forney, 2000). It is clear that the activities of enzymes involved in recovery of AA were not sufficient to maintain total ascorbate and AA pool in the fruit tissue. The data also indicate the extreme sensitivity of AA to cold storage under all storage atmospheres.

Glutathione is an important component of the AA–GSH cycle and helps scavenging ROS from the system. The data show that total glutathione and GSH decreased significantly with the increase in storage time, but the decrease was significantly lower in CA–stored fruit than in normal air and MAP (Fig. 8.5A & B). The concentration of GSSG showed an increasing trend during storage and the level of increase was significantly higher in MAP and air–stored fruit than in CA (Fig. 8.5C). The decrease in GSH and accumulation of GSSG could be related to the decrease in GR activity and increase in GT activity during storage, respectively (Fig. 8.7A & B), because concentrations of GSH and GSSG showed significant and positive correlations with GR ($r = 0.80$) and GT ($r = 0.79$) activities. The storage atmospheres low in O_2 were effective in reducing the loss of GSH during storage and the losses increased with the increase in O_2 concentration in MAP and normal air. The decrease in glutathione concentration in 'Conference' pear fruit was significantly higher during the initial 22 days of storage in normal air than in CA; however, the concentrations of glutathione increased significantly in all storage atmospheres during 90 and 180 days of storage (Larrigaudiére et al., 2001b). In another report on spinach, the glutathione concentration was significantly lower during CA storage as compared to storage in normal air (Hodges and Forney, 2000). The data on 'Blackamber' plums suggest that CA storage was not helpful to maintain total ascorbate and AA components, whereas the total glutathione and GSH components were preserved to a greater extent under similar storage atmospheres. In spinach, the ascorbate concentrations were maintained during 21 days of CA storage in contrast to the loss of glutathione (Hodges and Forney, 2000). Such contradictory results may arise because the responses of these antioxidants to various types and levels of stresses are commodity and tissue specific. The results also show that postharvest treatment with 1–MCP before storage slightly increased the retention of GSH in 'Blackamber' plums, regardless of storage atmosphere (Fig. 8.13B). 1–MCP has been implicated in enhancing the enzymatic antioxidant potential in different fruits such as loquat, pears, and mangoes (Cao et al., 2009b; Larrigaudiére et al., 2009; Larrigaudiére et al., 2004; Singh and Dwivedi, 2008); the higher levels of GSH could be another effect contributing to some benefits to the non–enzymatic antioxidants in 'Blackamber' plums. A slight reduction in GT and increase in GR activities in response to 1–MCP treatment could be one of the factors contributing to the higher GSH levels.

GSH concentrations may increase or decrease depending upon the stress levels, but the higher GSH concentrations have been known to provide tolerance against chilling stress in several commodities (Tausz et al., 2004). There is a possibility that higher concentration of GSH in CA stored fruit led to the lesser CI symptoms compared to those held in normal air and MAP. The role of glutathione in development of various physiological disorders in fruits has not been defined clearly. In grapes, the reduced levels of glutathione have been linked to the internal browning in berries fumigated with methyl bromide (Liyanage et al., 1993). The experimental results indicate that glutathione could be playing important role in acquisition of chilling tolerance in Japanese plums leading to lower CI incidence and severity. This role could be quite similar to the one ascribed to ascorbate in 'Conference' pears. More research is required to elucidate the role of glutathione in development of various physiological disorders in fruits.

8.4.6 Total phenolics and DPPH$^{\cdot}$ scavenging activity

Total phenolics concentration was maintained at significantly higher level in CA–stored fruit compared to normal air and MAP for the first 5 weeks of storage (Fig. 8.8A). The increase in storage time to 8 weeks caused further reduction in concentration of total phenolics in fruit irrespective of the storage atmosphere. Contrarily, an increase in phenolics in flesh of several cultivars of Japanese plums including 'Blackamber' has been reported to occur during cold storage and subsequent shelf life at 20°C (Díaz–Mula et al., 2009). Generally, concentration of total phenolics remains stable or increase slightly during storage in apples (Fawbush et al., 2009). The data show that the increase in total phenolics concentration was minor and observed only after 6 days of SL following 5 weeks of CA storage. The increase in concentration of total phenolics during some stages might be due to activation of phenylpropanoid metabolism in response to chilling stress as reported in grapes, plums and pawpaws (Díaz–Mula et al., 2009; Galli et al., 2009; Sanchez–Ballesta et al., 2007). 1–MCP treatment caused a slight improvement in the retention of total phenolics during storage and SL (Fig. 8.16A) which could likely be due to higher antioxidant potential of fruit attributed to the effects of 1–MCP (McLean et al., 2003). Studies have shown that phenolic compounds play an important role in ROS scavenging from the vacuole in the cell in concert with AA and peroxidase, and

this system has been termed as ascorbate/phenolics/peroxidase system (Takahama, 2004). The appearance of CI symptoms also involved flesh browning which might have occurred due to the oxidation of phenols by PPO which caused a reduction in their concentrations (Galli et al., 2009). CI index and total phenolics also showed a negative and significant correlation with phenols (Tables 8.4 & 8.7). It may also be argued that the utilization of phenolics as antioxidants in peroxidases–mediated removal of H_2O_2 because of the co–existence of phenolics and peroxidases in the vacuoles (Takahama, 2004). These factors might have contributed to the decrease in concentration of total phenolics during storage of 'Blackamber' plums for 8 weeks.

A significant positive correlation existed between total phenolics and DPPH scavenging activity of the flesh tissue of 'Blackamber' plums. The antioxidant capacity in the flesh tissue of Japanese plums has been mainly due to the presence of phenolic compounds, whereas a very little contribution by other compounds has been reported (Díaz–Mula et al., 2009; Gil et al., 2002). Therefore, the pattern of changes in $DPPH^{\cdot}$ scavenging activity could be explained on the basis of changes in concentration of total phenolics (Figs. 8.8B & 8.16B). The data reflect that combination of 1–MCP with any of the storage atmospheres did not yield any favourable results in terms of antioxidant activity. The total antioxidant activity in the skin tissues of 1–MCP treated 'Empire' and 'Delicious' apples has been reported to be significantly higher than the untreated fruit (McLean et al., 2003), while in another report, 1–MCP treatment and CA–storage have been shown to inconsistently affect the concentration of total phenolics and antioxidant activity in the peel and flesh tissues of 'Empire' apples (Fawbush et al., 2009). Such variable results are produced due to the multiple effects of different factors on the antioxidants in fruit and their complex interactions with the environment.

It may be concluded that either 1–MCP treatment (under regular air) or CA storage (1% O_2 + 3% CO_2) alone may be useful in maintaining fruit quality in 'Blackamber' plums during cold storage for 5 weeks plus 6 days of SL at 21±1°C. The combination of pre–storage treatment with 1–MCP and CA storage (1.0% or 2.5% O_2 + 3% CO_2) has potential to yield more favourable results than either of alone for 5 and 8 weeks of cold storage plus 6 days of SL. The effectiveness of MAP alone or its combination with 1–MCP to control the CI symptoms could not be confirmed in 'Blackamber' plums, and is therefore not recommended.

Chapter 9

Effects of Postharvest Nitric Oxide Fumigation on Fruit Quality and Antioxidative Systems in Japanese plums[5]

Summary

Effects of nitric oxide (NO) fumigation on fruit ripening, chilling injury (CI), and antioxidative system were investigated in 'Amber Jewel' and 'Blackamber' cultivars of Japanese plums. Mature fruit were fumigated with 0, 5, 10, and 20 μL L^{-1} NO gas at 20°C for 2 h. Post–fumigation, fruit were either kept at 21±1°C for 12 days or were stored at 0°C for 5, 6, and 7 weeks followed by SL of 5 days at 21±1°C. NO fumigation with 10 μL L^{-1} was effective to delay loss of fruit firmness in both cultivars during SL of 12 days at 21±1°C. In general, the response of 'Amber Jewel' cultivar to NO fumigation was better than 'Blackamber' with regards to delay in fruit ripening. The incidence and severity of CI was significantly lower in fruit fumigated with 20 μL L^{-1} NO during 5 and 6 weeks of cold storage at 0°C and 5 days of subsequent SL at 21±1°C. Different concentrations of NO tested to study its protective or toxic effects on lipid peroxidation and antioxidant system revealed that the lower concentration of 5 and 10 μL L^{-1} had either no effect or slightly positive effect and the increase in concentration to 20 μL L^{-1} enhanced the protective effects. NO fumigation inhibited the lipid peroxidation as marked by the concentration of TBARS during ambient and cold storage. The delay in loss of SOD activity was also observed in response to fumigation with 10 or 20 μL L^{-1} NO under both storage conditions, while no significant effects were observed on activities of CAT and POD. In the ascorbate–glutathione cycle, the glutathione component was least influenced by NO fumigation, while a consistent trend in ascorbate was not evident. NO fumigation with 10 or 20 μL L^{-1} delayed the decrease in concentration of total phenolics and $DPPH^{\cdot}$ scavenging activity during storage under both conditions. In conclusion, postharvest NO fumigation seems to play an important role in delaying

[5] A part of this chapter has been published in the following article: Singh, S.P., Z. Singh, and E.E. Swinny. 2009. Postharvest nitric oxide fumigation delays fruit ripening and alleviates chilling injury during cold storage of Japanese plums (*Prunus salicina* Lindell). *Postharvest Biology and Technology*. 53: 101–108.

fruit ripening, alleviating CI symptoms through augmenting the antioxidant potential of Japanese plums.

9.1 Introduction

Nitric oxide (NO), a highly reactive free radical gas, acts as a multifunctional signalling molecule in various physiological processes in animal and plants (Wendehenne et al., 2001). NO modulates hormonal, wounding, and defence responses in plant tissues (Wendehenne et al., 2004) and its endogenous levels are higher in immature than in mature and ripe tissues of climacteric and non–climacteric fruits (Leshem and Pinchasov, 2000). The endogenous levels of ethylene and NO during fruit development and maturation have inverse and stoichiometric relationships. NO levels decrease with maturation and senescence in horticultural crops (Leshem and Pinchasov, 2000; Leshem and Wills, 1998), thereby offer an opportunity for modulation of their levels with exogenous application to exert the opposite effect.

Short–term exposure of intact and fresh–cut horticultural commodities to very low concentrations of NO is known to retard their postharvest senescence (Pristijono et al., 2006; Wills and Bowyer, 2003; Zhu et al., 2006; Zhu et al., 2008; Zhu and Zhou, 2007). The benefits of NO application in fresh produce included delayed fruit ripening (Harris et al., 2003; Wills et al., 2008), inhibited ethylene biosynthesis (Eum et al., 2009; Leshem and Wills, 1998; Zhu et al., 2006; Zhu and Zhou, 2007) inhibited cut–surface browning (Pristijono et al., 2006; Wills et al., 2008), and enhanced resistance to postharvest diseases (Fan et al., 2008; Zhu and Zhou, 2007). The mechanism of action of NO in delaying senescence at postharvest of horticultural produce, though not completely understood, is *via* the inhibition of ethylene biosynthesis. However, adequate evidence does not exist to ascertain the mode of action of NO.

The postharvest life of plums is limited due to fast rate of ripening, which is regulated by the endogenous and exogenous levels of ethylene (Abdi et al., 1998; Khan and Singh, 2007a). The regulation of ethylene biosynthesis and/or its action through postharvest application of 1–methylcyclopropene (1–MCP) delays fruit ripening and alleviates CI during cold storage of Japanese plums (Candan et al.,

2008; Khan and Singh, 2007a). The overall effects of the NO on fruit ripening and quality are considered to be similar to the 1–MCP.

NO is a free radical gas with high diffusivity in the cell system. There are increasing pieces of evidence that NO plays an important role in ROS metabolism and signalling network during normal and stress conditions in plants (Lamattina et al., 2003). NO has been reported to play a mediatory role in responses to biotic and abiotic stresses in the plants such as cold (Zhao et al., 2009a), drought (Mata and Lamattina, 2001), salt (Shi et al., 2007), UV–B radiation (Shi et al., 2005), disease resistance and apoptosis (Wendehenne et al., 2004). Depending on the local concentration of NO, it can be cytotoxic and cytoprotective (Arasimowicz and Floryszak–Wieczorek, 2007; Beligni and Lamattina, 1999).

There is increasing evidence in favour of a protective role of NO at lower concentration in alleviation of postharvest oxidative stress in fruits. The postharvest treatment of fruit with either donors compounds of NO or NO gas has been reported to inhibit lipid peroxidation, reduce accumulation of superoxides and H_2O_2, and increase activities of antioxidant enzymes, favourable for delay in initiation and reduction of oxidative stress in peaches, kiwifruit and longan (Duan et al., 2007a; Zhu et al., 2006; Zhu et al., 2008). In the previous chapters, the development of oxidative stress during postharvest storage and ripening of Japanese plums has been discussed. Currently, no information is available on the effects of NO fumigation on the enzymatic and non–enzymatic antioxidants of Japanese plums. Whilst, the earlier reports on other fruits mainly focus on lipid peroxidation and primary antioxidant enzymes. To my knowledge, the response of ascorbate–glutathione cycle to NO fumigation has not been revealed conclusively in fruits. It was therefore hypothesized that fumigation of Japanese plums with low concentrations of NO may alter their physiological response to augment antioxidative system to cope with the increasing oxidative stress during fruit ripening and long–term cold storage. To prove the hypothesis, the investigations were carried out on the effects of NO fumigation on the dynamics of enzymatic and non–enzymatic antioxidants during fruit ripening at ambient conditions and during cold storage of Japanese plums.

9.2 Materials and Methods

9.2.1 Fruit material

Experiments were conducted on two cultivars of Japanese plums, 'Amber Jewel' and 'Blackamber'. In 2008, 'Amber Jewel' fruit were harvested (firmness: 24.80±0.45 N; SSC: 13.0±0.21 °Brix; TA: 1.19±0.05%) from the Red Valley Orchard, Karagullen, Perth Hills, Western Australia (WA). In 2009, 'Blackamber' fruit were harvested from the Sunpick Orchard, Mundaring, Perth Hills, WA. At harvest, the firmness, SSC, and TA in 'Blackamber' fruit were 39.2±2.97 N, 12.4±0.5 °Brix, and 1.05 ± 0.13%, respectively. Fruit were transported to the laboratory immediately after harvesting, and were subjected to various treatments. Fruit of uniform size and maturity, free from visual blemishes and disease were used for the experiments.

9.2.2 NO fumigation procedure

Fruit were fumigated with different concentrations of NO (0, 5, 10, and 20 μL L^{-1}) in sealed plastic containers (90 L). The desired concentrations of NO were obtained from a cylinder containing 4810±100 μL L^{-1} NO in nitrogen (BOC Gases Ltd, Sydney, NSW, Australia) and injected into the container through an injection port fitted in the lid of the container. Fruit were held in an atmosphere containing NO for 2 h at 20°C. NO has been reported to be sufficiently stable at low concentrations and short treatment times, required for fruit to be treated in normal air (Soegiarto et al., 2003). Therefore, fruit were fumigated with NO in containers having normal air without depletion of O_2. Control fruit were sealed in a plastic container for the same duration except without addition of NO. After 1.5 h of fumigation, the average concentrations of CO_2 in the headspace of treatment containers injected with 0, 5, 10, and 20 μL L^{-1} NO were less than 0.8%.

9.2.3 Experimental layout

In 2008, the experiments were conducted on 'Amber Jewel' cultivar and the same set of experiments under similar conditions was conducted on 'Blackamber' in 2009. Both cultivars have been known to exhibit distinct postharvest physiology and storage behaviours as outlined in the previous chapters. To study the response of

cultivar factor to NO fumigation, the data from both years for each experiment were collectively analysed and discussed in this chapter.

9.2.3.1 Effects of NO fumigation on fruit ripening and enzymatic and non–enzymatic antioxidants of Japanese plums at ambient conditions (21 ± 1 °C)

The fruit of both cultivars fumigated with different concentrations of NO (0, 5, 10, and 20 μL L^{-1}) were kept at 21 ± 1°C, RH 60.4±7.3% for ripening. The experimental design was completely randomized including three factors— cultivar, NO fumigation , and SL period. All treatments were replicated three times, and ten fruit were treated as an experimental unit. The fruit were assessed for various quality parameters (flesh firmness, skin colour, SSC, individual sugars, and TA) at 3–day intervals during fruit ripening commencing from day 0.

9.2.3.2 Effects of NO fumigation on development of CI symptoms, fruit quality and antioxidant system of Japanese plums during cold storage (0°C)

The fruit fumigated with different concentrations of NO (0, 5, 10, and 20 μL L^{-1}) were kept in plastic crates lined with 30 μm thick LDPE film (AMCOR Packaging, Pvt. Ltd., Melbourne, Australia) at 0°C for 7 weeks. During cold storage, 30 fruit per replication of each treatment (15 fruit per replication for immediate analysis, fruit allowed to warm to ambient before assessment and 15 fruit per replication following ripening at 21±1°C for 5 days) were transferred from cold store after 5, 6, and 7 weeks to ripen at 21±1°C for 5 days. The experimental design was completely randomized including the three–factors of cultivar, NO fumigation, and storage/SL period. Fifteen fruit were treated as an experimental unit.

9.2.4 Fruit quality evaluation

Fruit quality parameters such as flesh firmness, SSC, TA, and SSC:TA were evaluated as described in Sections 3.4.1.2 and 3.4.3 of Chapter 3.

9.2.5 Chilling injury (CI)

The incidence and severity of CI was evaluated immediately after transfer from cold storage after 5, 6 and 7 weeks and also after SL of 5 days at 21±1°C. Fifteen fruit per replication were cut around the equatorial axis, the two halves of each fruit twisted in opposite directions, and the mesocarp was examined for symptoms such as flesh browning, mealiness, and translucency. The incidence and severity of CI were calculated as described in Section 3.4.4.

9.2.6 Oxidative stress parameters

The determinations of lipid peroxidation, enzymatic and non–enzymatic antioxidants, and protein concentration were carried out as described in Chapter 3, Sections 3.5 to 3.8.

9.2.7 Statistical analyses

The experimental data were subjected to a three–way ANOVA including cultivar, treatment and storage/ripening period as factors, using GenStat Release 11.1 (VSN International Ltd., Hemel Hempstead, UK). Before statistical analysis, the data on CI incidence were subjected to arcsine transformation to reduce heteroscedasticity. The effects of cultivar, treatment and storage/ripening period and their interactions were assessed using the three–way ANOVA and the LSD values were calculated following a significant ($P \leq 0.05$) F–test. All the assumptions of ANOVA were checked to ensure validity of statistical analysis.

9.3 Results

9.3.1 Experiment 1: Effects of NO fumigation on fruit ripening and enzymatic and non–enzymatic antioxidants of Japanese plums at ambient conditions (21 ± 1 °C)

9.3.1.1 Fruit quality (flesh firmness, SSC, TA, and SSC:TA)

NO fumigation significantly reduced the rate of fruit softening in both cultivars of Japanese plums, ‘Amber Jewel’ and ‘Blackamber’ (Fig. 9.1). During the first 9 days

of SL at 21±1°C, NO–fumigated fruit had significantly higher flesh firmness than non–fumigated fruit. 'Amber Jewel' fruit fumigated with 0, 5, 10, and 20 µL L^{-1} NO concentrations retained 8.4%, 26.5%, 39.4%, and 41.5% of their initial firmness values on the 12th day, whereas the firmness retention during the same time in 'Blackamber' fruit fumigated with similar concentrations of NO was 13.7%, 16.0%, 18.4%, and 27.8%, respectively. The differences in the flesh firmness of fruit exposed to different concentrations of NO were practically significant and broad on the 12th day in contrast to the differences on the 3rd day. The data also show that NO fumigation was more effective in retaining flesh firmness in 'Amber Jewel' than in 'Blackamber' cultivar during 12 days of SL at 21±1°C.

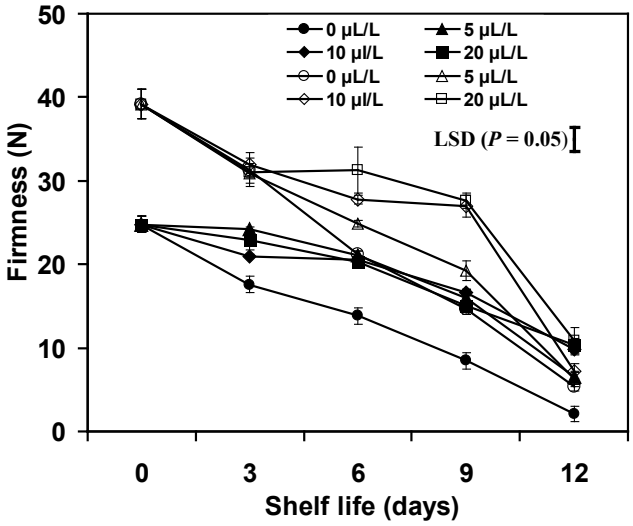


Fig. 9.1. Effects of nitric oxide fumigation on the changes in flesh firmness of Japanese plums during 12 days of SL at 21±1°C. Solid symbols represent 'Amber Jewel' cultivar, while empty symbols represent 'Blackamber'. Vertical bars indicate the S.E. of means. All measurements were made in triplicate (n=3). LSD (P = 0.05) values for firmness: Cultivar (C) = 0.64, Fumigation (F) = 0.90, Storage/shelf life period (SP) =1.01, C x F = 1.28, C x SP = 1.43, F x SP = 2.02, C x F x SP = 2.86. LSD value for C x F x SP is shown as a separate bar.

NO fumigation did not significantly affect the changes in SSC during SL at 21±1°C (Fig. 9.2A). SSC either increased slightly in 'Amber Jewel' or remained unchanged in 'Blackamber' during 6–9 days of SL and then showed a minor decrease. The increase in the SSC was restricted to some extent in the NO–fumigated fruit of 'Amber Jewel' cultivar, while such increase was non–significant in

'Blackamber'. NO fumigation significantly reduced the decrease in TA during fruit ripening in 'Amber Jewel' plums (Fig. 9.2B).

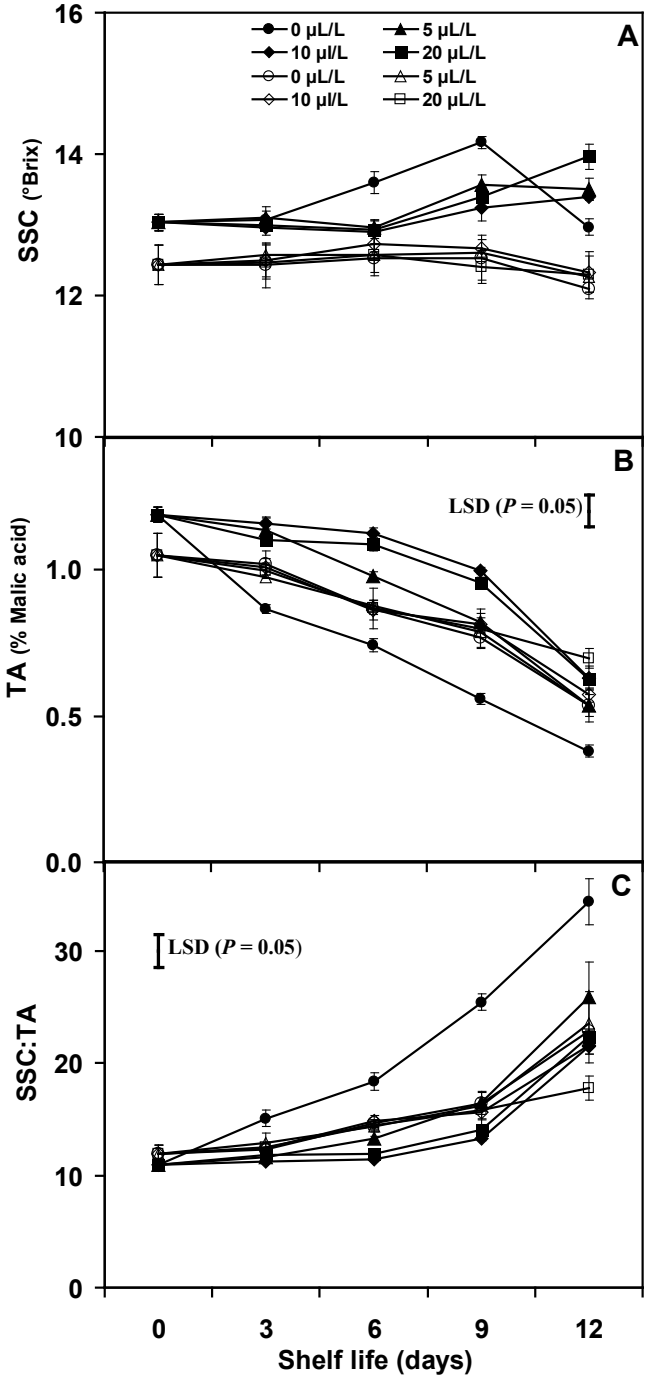


Fig. 9.2. Effects of nitric oxide fumigation on the changes in soluble solids concentration (SSC; A), titratable acidity (TA; B), and SSC:TA ratio (C) of Japanese plums during 12 days of SL at 21±1°C. Solid symbols represent 'Amber Jewel' cultivar, while empty symbols represent 'Blackamber'. Vertical bars indicate the S.E. of means. All measurements were made in triplicate (n=3). LSD (P = 0.05) values for SSC: Cultivar (C) = 0.13, Fumigation (F) = NS (non–significant), Storage/shelf life period (SP) = 0.21, C x F = NS, C x SP = 0.29, F x SP = NS, C x F x SP = NS. TA: C = 0.03, F = 0.04, SP = 0.04, C x F = 0.05, C x SP = 0.06, F x SP = 0.08, C x F x SP = 0.11. SSC:TA ratio: C = 0.66, F = 0.93, SP = 1.04, C x F = 1.31, C x SP = 1.47, F x SP = 2.07, C x F x SP = 2.93. LSD values for C x F x SP are shown.

'Blackamber' fruit exposed to NO concentration of 20 µL L^{-1} showed significantly higher TA than those exposed to 0, 5, and 10 µL L^{-1}. 'Amber Jewel' fruit fumigated with NO gas had significantly lower SSC: TA ratio than non–fumigated ones (Fig. 9.2C). The increase in SSC:TA ratio was not influenced by the NO fumigation in 'Blackamber' fruit, except on the 12th day.

9.3.1.2 Lipid peroxidation

The degree of lipid peroxidation was determined by the concentration of TBARS in the flesh tissue of fruit. The concentration of TBARS increased consistently during SL period of 12 days at 21±1°C (Fig. 9.3). The accumulation of TBARS during the first 3 days of SL was not influenced by the NO fumigation in both cultivars. But, the TBARS concentration in all NO–fumigated fruit was significantly lower than in non–fumigated fruit on the 6 and 9th days of SL in both cultivars. On the 12th day, the lowest concentration of TBARS was noticed in fruit exposed to 10 µL L^{-1} compared to other treatments including control.

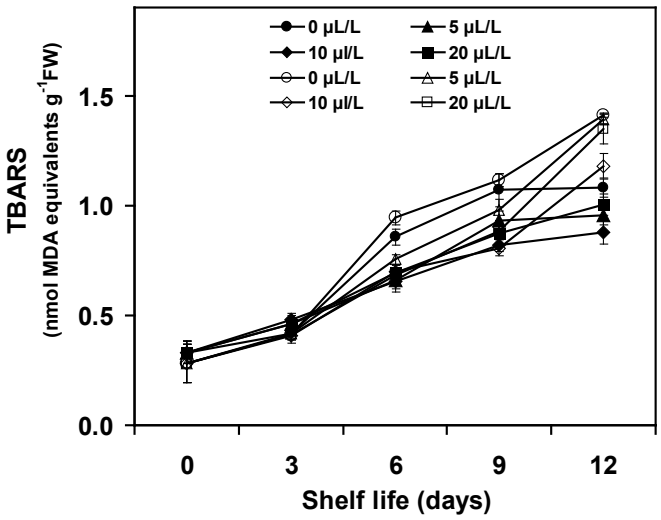


Fig. 9.3. Effects of nitric oxide fumigation on the accumulation of thiobarbituric acid reactive substances (TBARS) in the flesh tissue of Japanese plums during 12 days of SL at 21±1°C. Solid symbols represent 'Amber Jewel' cultivar, while empty symbols represent 'Blackamber'. Vertical bars indicate the S.E. of means. All measurements were made in triplicate (n=3). LSD (P = 0.05) values for TBARS: Cultivar (C) = 0.02, Fumigation (F) =0.03, Storage/shelf life period (SP) = 0.04, C x F = NS (non–significant), C x SP = 0.05, F x SP = 0.08, C x F x SP = NS.

9.3.1.3 Antioxidant enzymes (SOD, CAT, and POD)

Cultivar factor did not significantly influence SOD activity in the flesh tissue of fruit. The main effects of NO fumigation and SL period and their interaction were statistically significant for SOD activity. The SOD activity did not change significantly during the first 6 days of SL in both cultivars, except in 'Amber Jewel' fruit fumigated with 20 μL L^{-1} NO showed a significant increase on the 6^{th} day (Fig. 9.4A). A decrease in SOD activity was observed after 9^{th} and 12^{th} day of SL compared to initial values. On the 9^{th} day, SOD activity, though statistically non–significant, was higher in all NO–fumigated fruit compared to non–fumigated ones. The decrease in SOD activity continued during the last 3 days of SL and no clear differences among NO–fumigated and non–fumigated were observed on the 12^{th} day.

The main effects of cultivar and NO fumigation were significant on CAT activity, while the effect of SL period was non–significant. The interaction among three factors, cultivar, NO fumigation and SL, was also non–significant. In 'Amber Jewel', CAT activity increased significantly during the SL period between 3 and 9 days, whereas a decreasing trend was observed in 'Blackamber' throughout the SL period (Fig. 9.4B). CAT activity in NO–fumigated and non–fumigated fruit did not differ significantly during the SL period in 'Blackamber' and during the first 6 days of SL in 'Amber Jewel'. After 9 and 12 days, the NO–fumigated fruit of 'Amber Jewel' showed significantly higher CAT activity than control; the maximum activity was observed in fruit fumigated with 20 μL L^{-1} NO.

NO fumigation had no statistically significant effect on POD activity in the flesh tissue of fruit. A significant increase in POD activity was observed after 6 days of SL in both cultivars; however, the increase in non–fumigated 'Amber Jewel' fruit was greater than the fumigated ones and no such differences were noticed in 'Blackamber' on the same day (Fig. 9.4C). In 'Amber Jewel' cultivar, the peak POD activity was observed on the 6^{th} day in non–fumigated fruit against the 9^{th} day in fumigated ones. No marked differences in POD activity of 'Blackamber' fruit were observed in relation to NO fumigation. On the 12^{th} day, a significant decrease in POD activity in fruit was observed in comparison with 9^{th} day, irrespective of the cultivar and NO fumigation.

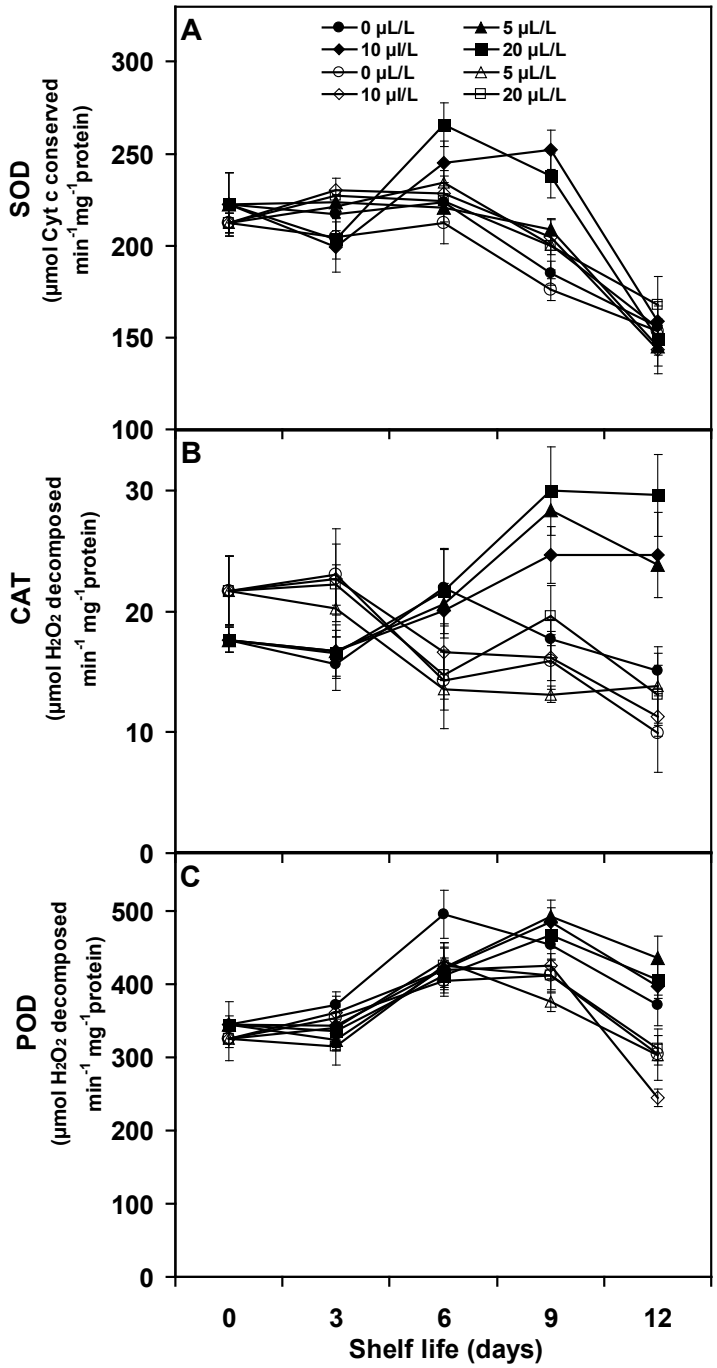


Fig. 9.4. Effects of nitric oxide fumigation on the changes in activities of SOD (A), CAT (B), and POD (C) in the flesh tissue of Japanese plums during 12 days of SL at 21±1°C. Solid symbols represent 'Amber Jewel' cultivar, while empty symbols represent 'Blackamber'. Vertical bars indicate the S.E. of means. All measurements were made in triplicate (n=3). LSD (P = 0.05) values for SOD: Cultivar (C) = NS (non–significant), Fumigation (F) = 10.74, Storage/shelf life period (SP) = 12.01, C x F = NS, C x SP = 16.98, F x SP = NS, C x F x SP = NS. CAT: C = 1.53, F = 2.17, SP = NS, C x F = NS, C x SP = 3.43, F x SP = NS, C x F x SP = NS. POD: C = 3.94, F = NS, SP = 6.23, C x F = NS, C x SP = 8.81, F x SP = NS, C x F x SP = NS.

9.3.1.4 Ascorbate–glutathione cycle

Ascorbate

Concentrations of total ascorbate (AA + DHA) were significantly influenced by cultivar, NO fumigation and SL period, and interactions among them. At harvest, the flesh tissue of 'Blackamber' cultivar had about 2.5–fold higher total ascorbate concentration than 'Amber Jewel' (Fig. 9.5A).

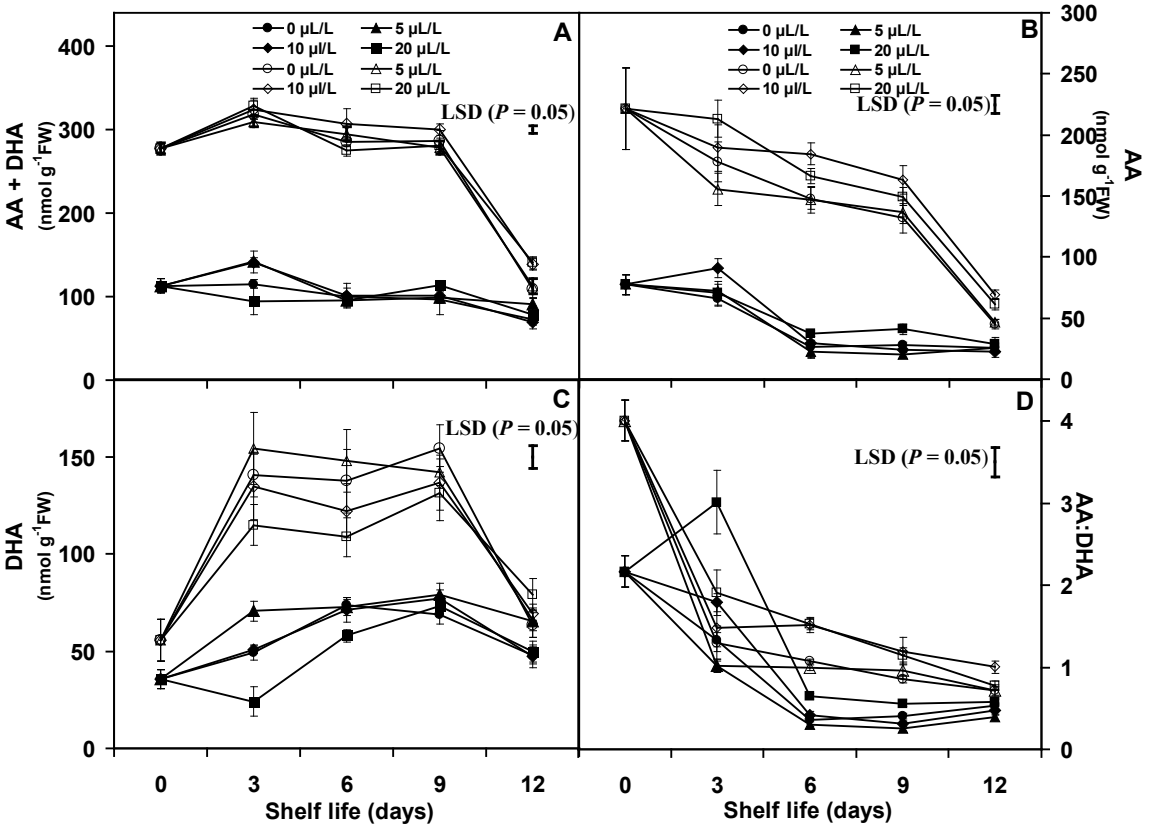


Fig. 9.5. Effects of nitric oxide fumigation on the changes in concentrations of total ascorbate (AA + DHA) (A), AA (B), DHA (C), and AA:DHA ratio (D) in the flesh tissue of Japanese plums during 12 days of SL at 21±1°C. Solid symbols represent 'Amber Jewel' cultivar, while empty symbols represent 'Blackamber'. Vertical bars indicate the S.E. of means. All measurements were made in triplicate (n=3). LSD (*P* = 0.05) values for AA + DHA: Cultivar (C) = 2.09, Fumigation (F) = 2.96, Storage/shelf life period (SP) = 3.31, C x F = 4.18, C x SP = 4.68, F x SP = 6.61, C x F x SP = 9.35. AA: C = 3.21, F = 4.55, SP = 5.08, C x F = 6.43, C x SP = 7.19, F x SP = 10.17, C x F x SP = 14.38. DHA: C = 2.65, F = 3.74, SP =4.19, C x F = NS, C x SP = 5.92, F x SP = 8.37, C x F x SP = 11.84. AA:DHA ratio: C = 0.08, F = 0.11, SP = 0.13, C x F = 0.16, C x SP = 0.18, F x SP = 0.25, C x F x SP = 0.36.

During the first 3 days of SL, the concentration of total ascorbate increased significantly in 'Amber Jewel' fruit fumigated with 5 or 10 µL L^{-1} and this increase

occurred in all NO–fumigated and non–fumigated 'Blackamber' fruit. During the following 9 days of SL, a general decrease in total ascorbate concentration was observed in both cultivars. A clear trend in changes in total ascorbate in relation to NO fumigation was not observed in both cultivars. However, 'Blackamber' fruit fumigated with 10 or 20 μL L^{-1} NO showed significantly higher total ascorbate concentration compared to those fumigated with 0 or 5 μL L^{-1} NO after 12 days of SL.

Similar to total ascorbate, the effects of various factors under study and interactions among them had significant effects on concentrations of AA during 12 days of SL. At harvest, the concentration of AA in 'Blackamber' was about 3–fold higher than in 'Amber Jewel' (Fig. 9.5B). In 'Amber Jewel' cultivar, the concentrations of AA decreased by 2 to 3 fold between 3 and 6 days of SL, irrespective of treatment; only minor changes in their levels were observed during the last 6 days of SL. In general, the concentrations of AA in 'Blackamber' fruit fumigated with 10 μL L^{-1} NO were greater than others fumigated with 0, 5 and 20 μL L^{-1} NO during the last 6 days of SL. Such differences in 'Amber Jewel' fruit fumigated with different levels of NO were mostly non–significant after 6, 9, and 12 days of SL.

During 12 days of SL at 21±1°C, concentrations of DHA were significantly influenced by cultivar, NO fumigation, SL period and their interactions. During the first 3 days of SL, a significant increase in concentrations of DHA was observed in both cultivars, except in 'Amber Jewel' fruit fumigated with 20 μL L^{-1} NO (Fig. 9.5C). After 3 days of SL, the concentrations of DHA were significantly lower in fruit of both cultivars fumigated with 20 μL L^{-1} NO than in other treatments. The trend of increase in concentrations of DHA in all fruit continued up to 9 days of SL, and then a significant decrease was noticed after 12 days of SL in fruit of both cultivars, regardless of NO fumigation.

'Blackamber' fruit exhibited significantly higher AA:DHA ratio than 'Amber Jewel' (Fig. 9.5D). A significant decrease in AA:DHA ratio was observed after 3 days of SL in both cultivars, except 'Amber Jewel' fruit fumigated with 20 μL L^{-1} NO showed an increase. In general, AA:DHA ratio remained higher in 'Amber Jewel' fruit fumigated with 20 μL L^{-1} NO than in other treatments throughout the SL period of 12 days; but the differences among all NO levels were significant only after

the first 3 days. In 'Blackamber', the AA:DHA ratio was comparatively higher in fruit fumigated with 10 or 20 μL L^{-1} NO than in those fumigated with 0 or 5 μL L^{-1} NO during the SL.

Ascorbate–related enzymes (APX, MDHAR, and DHAR)

APX activity was not significantly influenced by cultivar and NO fumigation, but SL period significantly affected the changes in APX activity. A significant increase in APX activity was observed during 12 days of SL in both cultivars, regardless of fumigation with different concentrations of NO (Fig. 9.6A). NO fumigation and SL period had statistically significant effect on activity of MDHAR. A general decrease in MDHAR activity was observed after 3 days of SL in fruit of both cultivars fumigated with 0, 5, and 10 μL L^{-1} NO, while those fumigated with 20 μL L^{-1} NO showed either no change in 'Amber Jewel' or significant increase in 'Blackamber' (Fig. 9.6B). In 'Amber Jewel', the MDHAR activity remained almost stable after 6 and 9 days of SL, similar to after 3 days, without any significant differences among fruit fumigated with different levels of NO; the activity increased greatly after 12 days of SL. In 'Blackamber', no regular pattern of changes in APX activity was observed in response to NO fumigation, except after 6 days of SL it was significantly higher in NO–fumigated fruit than in control. Contrary to 'Amber Jewel, no increase in MDHAR activity was noticed during the last 3 days of SL in 'Blackamber'.

DHAR activity was not significantly influenced by NO fumigation, but cultivar and SL period had significant effects on it. Both cultivars differed to each other with regard to changes in DHAR activity (Fig. 9.6C). In 'Amber Jewel', DHAR increased slightly after 3 days of SL in all fruit, and the increase sustained only in fruit fumigated with 10 or 20 μL L^{-1} NO up to 9th day; then, it declined to almost half the value at harvest in all treatments. In 'Blackamber', fruit fumigated with 10 or 20 μL L^{-1} NO showed higher DHAR activity compared to those fumigated with 0 or 5 μL L^{-1} NO after 3, 6, and 9 days of SL, but the differences among different levels of NO were mostly non–significant.

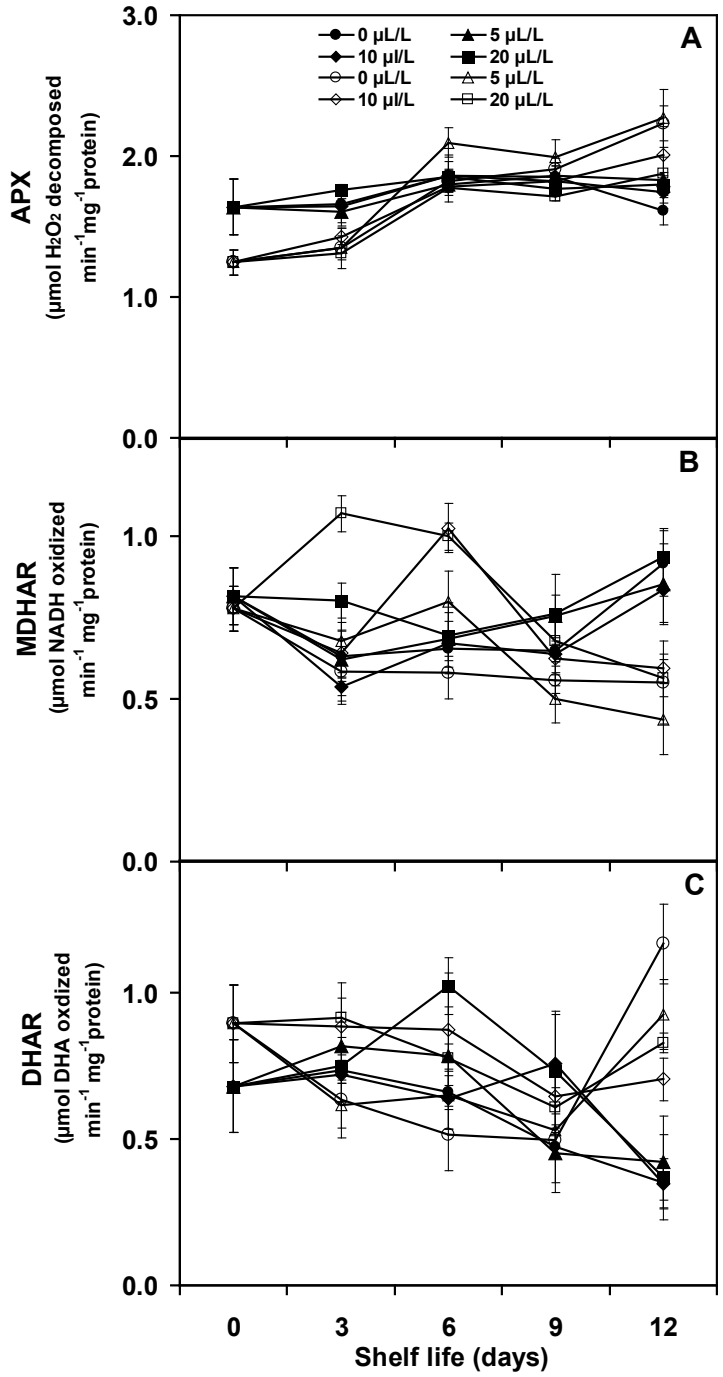


Fig. 9.6. Effects of nitric oxide fumigation on the changes in activities of APX (A), MDHAR (B), and DHAR (C) in the flesh tissue of Japanese plums during 12 days of SL at 21±1°C. Solid symbols represent 'Amber Jewel' cultivar, while empty symbols represent 'Blackamber'. Vertical bars indicate the S.E. of means. All measurements were made in triplicate (n=3). LSD (P = 0.05) values for APX: Cultivar (C) = NS (non–significant), Fumigation (F) = NS, Storage/shelf life period (SP) = 0.10, C x F = NS, C x SP = 0.14, F x SP = NS, C x F x SP = NS. MDHAR: C = NS, F = 0.07, SP = 0.08, C x F = NS, C x SP = 0.11, F x SP = 0.16, C x F x SP = NS. DHAR: C = 0.07, F = NS, SP = 0.12, C x F = NS, C x SP = 0.16, F x SP = NS, C x F x SP = NS.

Glutathione

NO fumigation had non–significant effects on concentration of total glutathione, GSH, GSSG, and GSH:GSSG ratio. However, the changes in their concentrations and GSH:GSSG ratio were significantly influenced by cultivar and SL period. A significant decrease in total glutathione was observed after 6 and 9 days of SL in 'Blackamber' and 'Amber Jewel' cultivars, respectively (Fig. 9.7A).

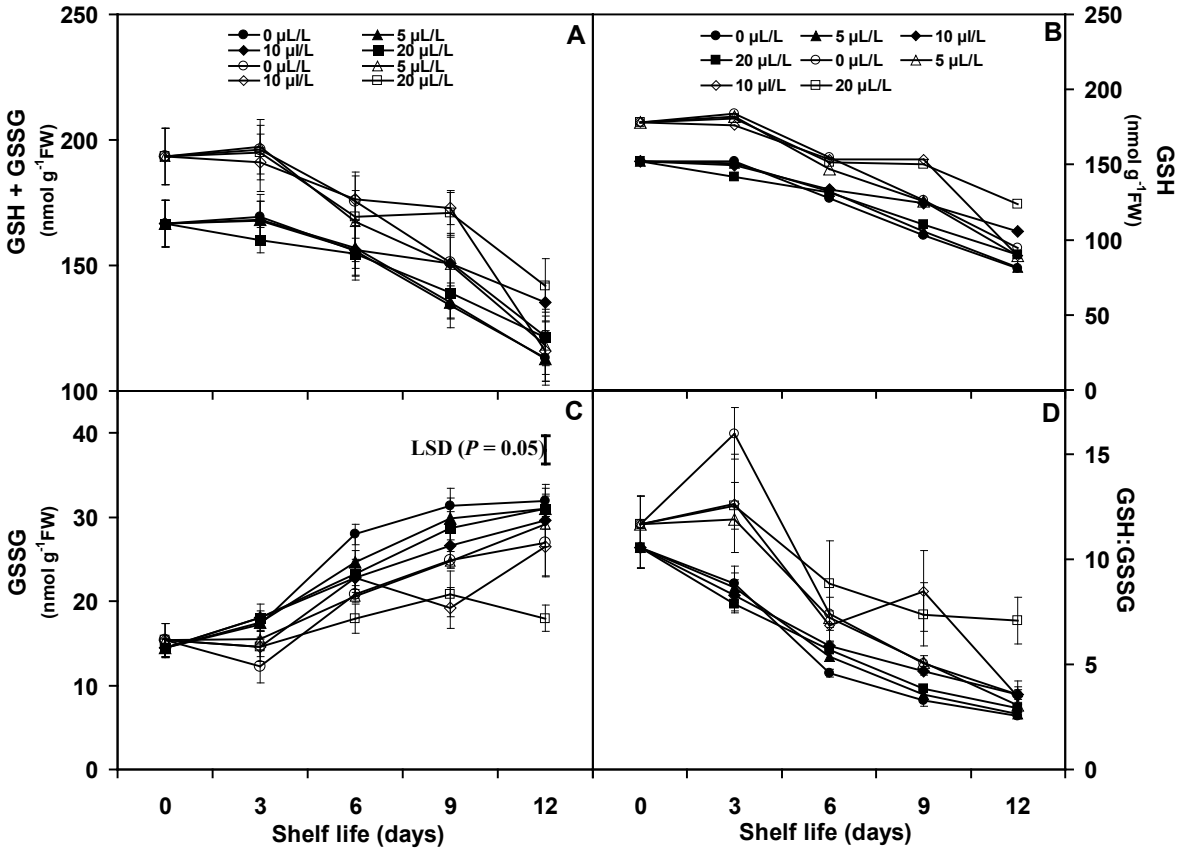


Fig. 9.7. Effects of nitric oxide fumigation on the changes in concentrations of total glutathione (GSH + GSSG) (A), GSH (B), GSSG (C), and GSH:GSSG ratio (D) in the flesh tissue of Japanese plums during 12 days of SL at 21±1°C. Solid symbols represent 'Amber Jewel' cultivar, while empty symbols represent 'Blackamber'. Vertical bars indicate the standard error of mean and the LSD ($P < 0.05$) values are shown. All measurements were made in triplicate (n=3). Vertical bars indicate the S.E. of means. All measurements were made in triplicate (n=3). LSD ($P = 0.05$) values for GSH + GSSG: Cultivar (C) = 4.73, Fumigation (F) = NS (non–significant), Storage/shelf life period (SP) = 7.48, C x F = NS, C x SP = 10.58, F x SP = NS, C x F x SP =NS. GSH: C = 4.90, F = NS, SP = 7.75, C x F = NS, C x SP = NS, F x SP = NS, C x F x SP = NS. GSSG: C = 0.73, F = NS, SP =1.15, C x F = 1.46, C x SP = 1.63, F x SP = 2.31, C x F x SP = 3.26. GSH:GSSG ratio: C = 0.57, F = NS, SP = 0.90, C x F = NS, C x SP = 1.27, F x SP = NS, C x F x SP = NS.

The decrease in concentration of GSH was noticed parallel to decrease in total glutathione in fruit of both cultivars as the SL period progressed beyond 3 days (Fig. 9.7B). 'Amber Jewel' and 'Blackamber' fruit exposed to 10 and 20 μL L^{-1} NO, respectively, showed relatively higher concentrations of GSH compared to other treatments after 12 days of SL. Contrary to GSH, the concentrations of GSSG increased significantly with the increase in SL period in both cultivars (Fig. 9.7C). Consequently, a significant decrease in GSH:GSSH ratio was observed in both cultivars during 12 days of SL (Fig. 9.7D). 'Blackamber' fruit fumigated with 20 μL L^{-1} NO showed greater GSH:GSSG ratio than the other treatments after 12 days of SL.

Glutathione–related enzymes (GR and GT)

The effect of NO fumigation was not statistically significant on activities of GR and GT during 12 days of SL. GR and GT activities were mainly affected by the duration of SL. A significant decrease in GR (Fig. 9.8A) and increase in GT activity (Fig. 9.8B) was observed in both cultivars and the patterns of changes in the activities of both enzymes were consistent. The impairment of GR activity was remarkable during the last 3 days of SL in both cultivars.

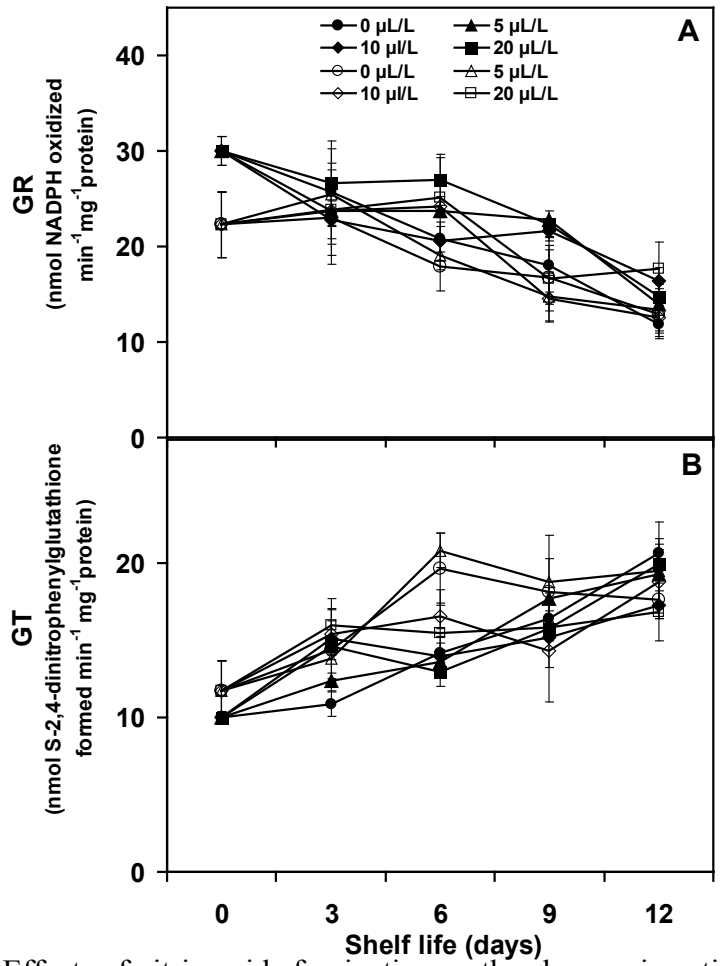


Fig. 9.8. Effects of nitric oxide fumigation on the changes in activities of GR (A) and GT (B) in the flesh tissue of Japanese plums during 12 days of SL at 21±1°C. Solid symbols represent 'Amber Jewel' cultivar, while empty symbols represent 'Blackamber'. Vertical bars indicate the S.E. of means. All measurements were made in triplicate (n=3). LSD (P = 0.05) values for GR: Cultivar (C) = 1.72, Fumigation (F) = NS (non–significant), Storage/shelf life period (SP) = 2.72, C x F = NS, C x SP = 3.84, F x SP = NS, C x F x SP = NS. GT: C = 1.02, F = NS, SP = 1.62, C x F = NS, C x SP = 2.28, F x SP = NS, C x F x SP = NS.

9.3.1.5 Total phenolics and DPPH$^{\cdot}$ scavenging activity

The main effects of cultivar, NO fumigation and SL period were significant on concentration of total phenolics and DPPH$^{\cdot}$ scavenging activity. A general trend of slight decrease in concentration of total phenolics and DPPH$^{\cdot}$ scavenging activity was observed in non–fumigated fruit during the SL of both cultivars, particularly during the last 6 days of SL (Fig. 9.9). 'Amber Jewel' fruit fumigated with 10 or 20 µL L^{-1} NO showed a significant increase in total phenolics and DPPH$^{\cdot}$ scavenging activity after 6 days of SL compared to other treatments. The data show that the

concentration of total phenolics and DPPH· scavenging activity in 'Blackamber' fruit fumigated with 10 or 20 μL L^{-1} NO remained stable during the first 9 days of SL.

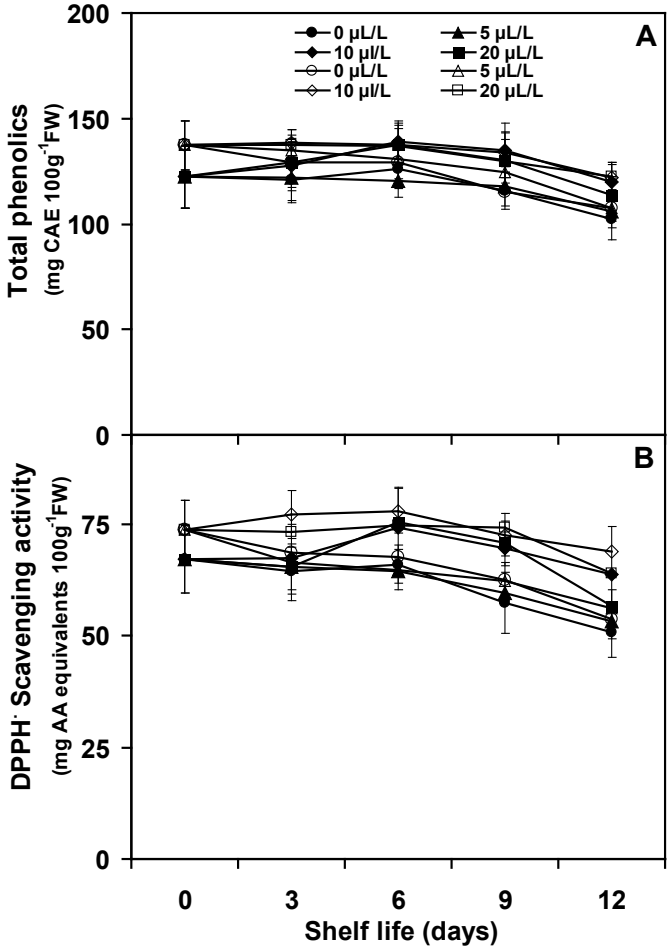


Fig. 9.9. Effects of nitric oxide fumigation on the changes in concentration of total phenolics (A) and DPPH· scavenging activity (B) in the flesh tissue of Japanese plums during 12 days of SL at 21±1°C. Solid symbols represent 'Amber Jewel' cultivar, while empty symbols represent 'Blackamber'. Vertical bars indicate the S.E. of means. All measurements were made in triplicate (n=3). LSD (P = 0.05) values for total phenolics: Cultivar (C) = 1.78, Fumigation (F) = 2.51, Storage/shelf life period (SP) = 2.81, C x F = NS (non–significant), C x SP = 3.97, F x SP = 5.62, C x F x SP = NS. DPPH· scavenging activity: C = 1.10, F = 1.55, SP = 1.73, C x F = NS, C x SP = 2.45, F x SP = 3.47, C x F x SP = NS.

9.3.2. Experiment 2: Effects of NO fumigation on fruit quality, CI symptoms, and antioxidative system of Japanese plums during cold storage

9.3.2.1. Fruit quality (flesh firmness, SSC, TA, and SSC:TA)

The main effect of cultivar as a factor in response to NO fumigation was non–significant on flesh firmness during cold storage and subsequent SL. After 5 and 6 weeks of cold storage, the fruit fumigated with 20 µL L^{-1} NO showed the highest retention of flesh firmness in both cultivars; the fruit softening was also retarded during the SL of 5 days at 21±1°C in these fruit (Fig. 9.10).

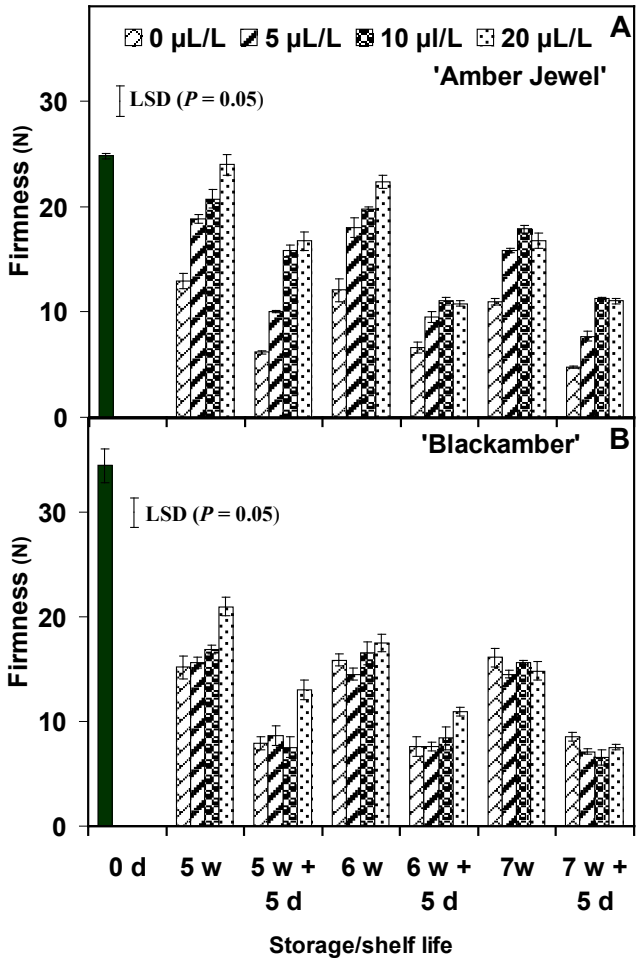


Fig. 9.10. Effects of nitric oxide fumigation on the changes in flesh firmness of 'Amber Jewel' and 'Blackamber' plums during 5, 6, and 7 weeks of cold storage at 0°C and plus 5 days of SL at 21±1°C. Black bar indicates the value at harvest. Vertical bars indicate the S.E. of means. All measurements were made in triplicate (n=3). LSD (P = 0.05) values for firmness: Cultivar (C) = NS (non–significant), Fumigation (F) =0.56, Storage/shelf life period (SP) = 0.74, C x F = 0.80, C x SP = 1.05, F x SP = 1.49, C x F x SP = 2.11.

After each storage interval, 5, 6, and 7 weeks, flesh firmness further decreased during SL at 21±1°C for 5 days. All NO treatments significantly reduced the fruit softening in 'Amber Jewel' plums during ripening subsequent to storage for 5, 6, and 7 weeks. However, the effect of NO fumigation diminished with the progression of storage time, particularly in case of 'Blackamber'. No significant differences in the flesh firmness of NO–fumigated or non–fumigated 'Blackamber' fruit existed after 7 weeks of storage and 5 days of SL.

The changes in SSC during cold storage and SL were not significantly influenced by NO fumigation and storage/SL period (Table 9.1). The interactions among various factors, cultivar, NO fumigation and storage/SL period were also non–significant. The amount of changes in SSC during cold storage and subsequent SL was not practically important, irrespective of the treatment. The main effects of cultivar, NO fumigation, and storage/SL period as factors on retention of TA were statistically significant, but the interaction among them was non–significant (Table 9.2). In 'Amber Jewel', NO–fumigated fruit retained TA better than non–fumigated fruit during storage for 5, 6, and 7 weeks. There were small differences in the TA concentration of fumigated and control fruit of 'Blackamber'. TA concentration decreased during 5 days of SL after cold storage; however, in most cases the fumigated fruit still had comparatively higher TA than non–fumigated fruit. As a consequence of reduction in TA, an increase in SSC: TA ratio was observed both during storage and SL (Table 9.3). The data show that the changes in SSC, TA, and SSC:TA ratio of 'Amber Jewel' fruit were influenced by NO fumigation to a greater extent compared to 'Blackamber'.

Table 9.1. Changes in soluble solids concentration (SSC, °Brix) of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	Soluble solids concentration (°Brix)							
Storage/	**'Amber Jewel'**				**'Blackamber'**			
Shelf life	**NO fumigation (µL L^{-1})**				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0			13.0				12.4	
5 W	12.9	12.8	13.0	12.9	12.4	12.4	12.4	12.2
5 W + 5 D	13.0	13.1	13.3	12.7	12.5	12.1	12.1	12.2
6W	12.5	12.9	13.2	12.8	12.1	12.4	12.2	12.1
6 W + 5 D	13.0	13.1	13.3	12.8	12.6	12.3	12.7	12.5
7 W	12.5	13.0	13.1	12.7	12.0	12.8	12.3	12.6
7 W + 5 D	12.6	13.0	13.0	12.7	12.3	12.6	12.3	12.3
Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA								
Cultivar (C)	0.14***		C x F	NS	C x F x SP	NS		
Fumigation (F)	NS		C x SP	NS				
Storage/shelf life period (SP)	NS		F x SP	NS				

*** = $P \leq 0.001$; NS = non–significant

Table 9.2. Changes in titratable acidity (TA, % malic acid) of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	TA (% malic acid)							
Storage/ Shelf life	**'Amber Jewel'**				**'Blackamber'**			
	NO fumigation (µL L^{-1})				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0			1.19				1.16	
5 W	0.89	0.94	1.00	0.96	0.87	0.86	0.89	0.92
5 W + 5 D	0.63	0.70	0.77	0.71	0.70	0.74	0.70	0.81
6W	0.86	0.94	1.00	0.98	0.87	0.85	0.80	0.79
6 W + 5 D	0.61	0.74	0.76	0.73	0.65	0.68	0.65	0.80
7 W	0.81	0.93	0.96	0.96	0.81	0.80	0.80	0.84
7 W + 5 D	0.48	0.58	0.66	0.63	0.59	0.66	0.63	0.62
Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA								
Cultivar (C)		0.02***	C x F	0.04***	C x F x SP	NS		
Fumigation (F)		0.03***	C x SP	0.05***				
Storage/shelf life period (SP)		0.04***	F x SP	NS				

*** = $P \leq 0.001$; NS = non–significant

Table 9.3. Changes in SSC:TA ratio of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	SSC:TA ratio							
Storage/ Shelf life	**'Amber Jewel'**				**'Blackamber'**			
	NO fumigation (µL L^{-1})				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0	11.0				10.7			
5 W	14.6	13.7	12.9	13.4	14.3	14.4	14.0	13.3
5 W + 5 D	20.9	18.8	17.2	17.8	17.8	16.5	17.4	15.1
6W	14.6	13.8	13.2	13.1	14.0	14.6	15.4	15.5
6 W + 5 D	21.5	17.9	17.4	17.6	19.6	18.5	19.8	15.6
7 W	15.4	14.0	13.7	13.3	14.9	16.1	15.7	15.0
7 W + 5 D	26.6	22.4	19.9	20.4	21.1	19.1	20.8	19.8
Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA								
Cultivar (C)	NS		C x F	1.1**	C x F x SP	NS		
Fumigation (F)	0.8***		C x SP	1.4**				
Storage/shelf life period (SP)	1.0***		F x SP	NS				

*** = $P \leq 0.001$; ** = $P \leq 0.01$; NS = non–significant

9.3.2.2 Chilling injury (CI)

CI symptoms in the form of flesh browning and translucency were manifested immediately after cold storage and also after 5 days of fruit ripening at 21±1°C. The incidence and severity of CI was greater in 'Amber Jewel' than in 'Blackamber' plums. NO fumigation (10 or 20 µL L^{-1}) had a significant effect on the reduction in incidence and severity of CI in both cultivars (Fig. 9.11).

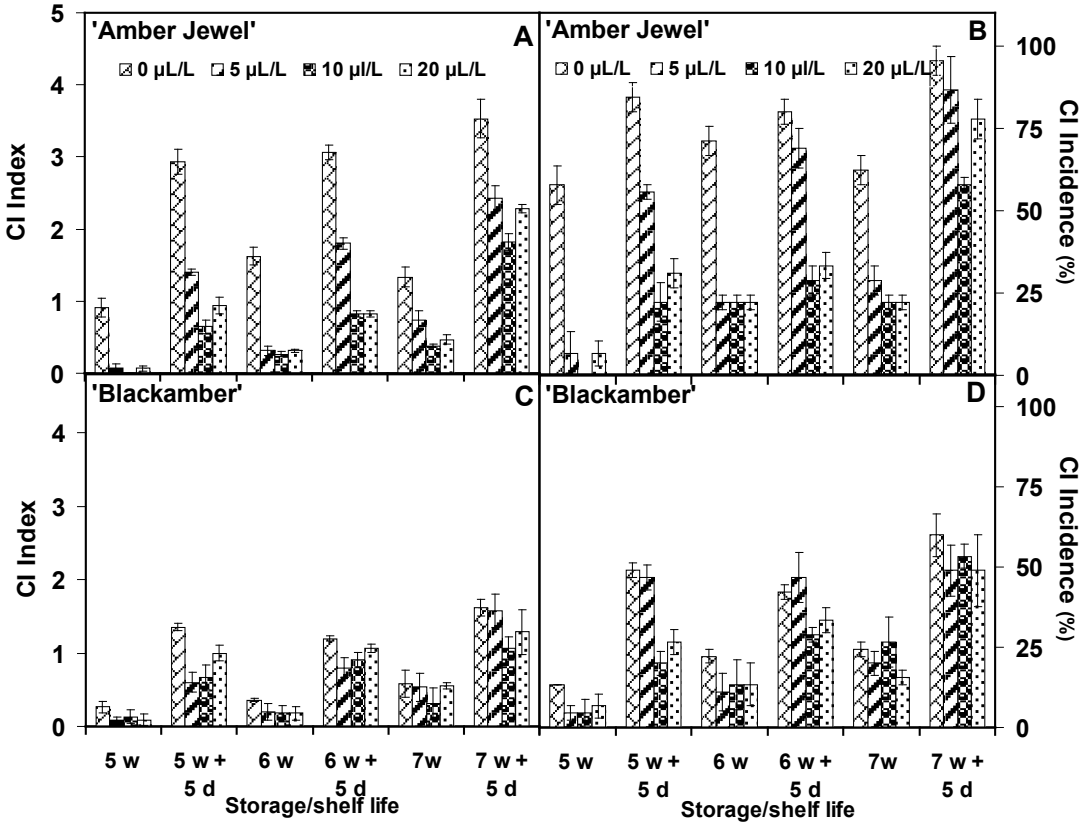


Fig. 9.11. Effects of nitric oxide fumigation on the severity and incidence of CI in 'Amber Jewel' (A & B) and 'Blackamber' (C & D) plums during 5, 6, and 7 weeks of cold storage at 0°C and plus 5 days of SL at 21±1°C. Vertical bars indicate the S.E. of means. All measurements were made in triplicate (n=3). LSD (P = 0.05) values for CI index: Cultivar (C) = 0.07, Fumigation (F) = 0.10, Storage/shelf life period (SP) = 0.13, C x F = 0.13, C x SP = 0.18, F x SP = 0.25, C x F x SP = 0.36. LSD (P = 0.05) values for transformed data of CI incidence: C = 0.04, F = 0.05, SP = 0.07, C x F = 0.08, C x SP = 0.10, F x SP = 0.14, C x F x SP = NS.

NO fumigation was more effective in reducing the severity of CI in 'Amber Jewel' than in 'Blackamber' after 5, 6, and 7 weeks of storage at 0°C. However, the

effectiveness of NO in alleviating CI declined with the increase in storage duration from 5 to 7 weeks. The severity of CI increased during SL after cold storage, irrespective of the treatment (Fig. 9.11).

9.3.2.3 Lipid peroxidation

The increase in lipid peroxidation during cold storage and subsequent SL was marked by the increase in the concentration of TBARS in the flesh tissue of fruit (Fig. 9.12).

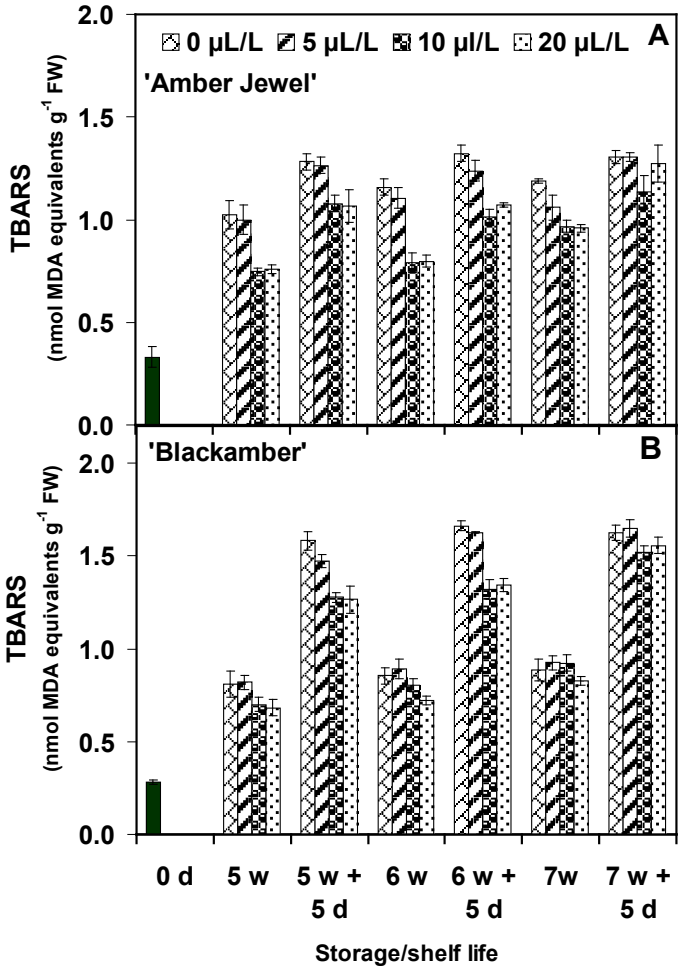


Fig. 9.12. Effects of nitric oxide fumigation on the accumulation of TBARS in the flesh tissue of 'Amber Jewel' (A) and 'Blackamber' (B) plums during 5, 6, and 7 weeks of cold storage at 0°C and plus 5 days of SL at 21±1°C. Black bar indicates the value at harvest. Vertical bars indicate the S.E. of means. All measurements were made in triplicate (n=3). LSD (P = 0.05) values for TBARS: Cultivar (C) = 0.02, Fumigation (F) = 0.03, Storage/shelf life period (SP) = 0.04, C x F = 0.05, C x SP = 0.06, F x SP = 0.09, C x F x SP = NS.

The main effects of cultivar, NO fumigation and storage period were significant on the accumulation of TBARS in the flesh tissue, but their interaction was non–significant. In general, fruit of both cultivars exposed to 10 or 20 μL L^{-1} NO showed significantly lower TBARS levels after 5 and 6 weeks of storage and plus 5 days of SL (Fig. 9.12). However, the differences in TBARS concentrations of NO–fumigated and non–fumigated fruit of 'Blackamber' cultivar were non–significant following 7 weeks of storage, and also after 5 days of SL.

9.3.2.4 Antioxidant enzymes (SOD, CAT, and POD)

SOD activity was significantly influenced by cultivar, NO fumigation and storage/SL period, but all interactions among them were non–significant (Table 9.4). After 5 weeks of storage, the highest SOD activity was observed in fruit of both cultivars fumigated with 20 μL L^{-1} NO. However, the data did not present a clear trend to observe any differences in the SOD activity caused by NO fumigation in a dose–dependent manner. In general, the SOD activity showed a slight decrease after 7 weeks of storage in comparison with 5 and 6 weeks. SL period of 5 days resulted in a significant decrease in SOD activity in fruit. SOD activity did not follow a pattern of decrease during the SL with regard to NO fumigation. In general, the fruit of both cultivars fumigated with 20 μL L^{-1} NO had slightly higher SOD activity than other treatments including control following 5, 6, and 7 weeks of cold storage plus 5 days of SL.

NO fumigation did not significantly affect CAT activity during cold storage and subsequent SL of 5 days at 21±1°C. The main effects of cultivar and storage/SL period and their interaction were significant on CAT activity. The increase in CAT activity during 5, 6, and 7 weeks of cold storage was greater in 'Amber Jewel' than in 'Blackamber'. The change in CAT activity during 5 days of SL did not follow a regular trend in 'Amber Jewel' cultivar, whereas the CAT activity decreased consistently in 'Blackamber' fruit.

Table 9.4. Changes in activity of SOD enzyme in the flesh tissue of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	SOD (µmol Cyt c conserved min^{-1} mg^{-1} protein)							
Storage/ Shelf life	**'Amber Jewel'**				**'Blackamber'**			
	NO fumigation (µL L^{-1})				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0	222.2				212.4			
5 W	202.1	188.0.	196.6	241.6	173.0	163.2	167.8	180.1
5 W + 5 D	155.3	175.1	175.3	203.6	165.1	142.5	185.8	183.6
6W	218.4	200.3	202.3	226.7	170.0	184.0	187.7	181.4
6 W + 5 D	161.1	157.3	170.6	214.2	162.9	145.6	170.1	187.4
7 W	182.6	202.3	190.2	205.7	161.0	154.3	165.4	163.7
7 W + 5 D	159.4	168.8	158.7	182.4	129.4	138.7	166.4	167.4
Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA								
Cultivar (C)	6.4***		C x F	NS	C x F x SP	NS		
Fumigation (F)	9.1***		C x SP	NS				
Storage/shelf life period (SP)	12.0***		F x SP	NS				

*** = $P \leq 0.001$; NS = non–significant

Table 9.5. Changes in activity of CAT enzyme in the flesh tissue of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	CAT (µmol H_2O_2 decomposed min^{-1} mg^{-1} protein)							
Storage/ Shelf life	**'Amber Jewel'**				**'Blackamber'**			
	NO fumigation (µL L^{-1})				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0			17.7				21.8	
5 W	29.8	28.5	28.0	23.3	25.5	26.8	25.4	21.4
5 W + 5 D	20.3	27.9	29.6	23.1	16.7	15.7	12.7	14.8
6W	24.3	27.3	26.8	27.2	23.9	24.9	25.5	29.7
6 W + 5 D	19.8	20.91	24.3	31.7	16.1	13.9	14.7	16.7
7 W	24.7	23.4	25.6	31.1	17.9	22.8	22.9	21.4
7 W + 5 D	18.2	18.6	18.6	19.	11.4	11.9	12.6	17.5
Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA								
Cultivar (C)		1.5***	C x F	NS	C x F x SP		NS	
Fumigation (F)		NS	C x SP	4.0***				
Storage/shelf life period (SP)		2.8***	F x SP	NS				

*** = $P \leq 0.001$; NS = non–significant

Table 9.6. Changes in activity of POD enzyme in the flesh tissue of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	POD (µmol H_2O_2 decomposed min^{-1} mg^{-1} protein)							
Storage/ Shelf life	**'Amber Jewel'**				**'Blackamber'**			
	NO fumigation (µL L^{-1})				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0	344.8				325.6			
5 W	256.4	236.8	268	247.2	390.8	396.8	386	431.6
5 W + 5 D	297.6	344.4	327.6	336.4	380.0	414.4	365.6	468.0
6W	269.6	260.0	265.2	276.0	406.0	373.6	409.6	448.4
6 W + 5 D	354.4	364.4	348.0	396.4	388.8	421.6	476.0	434.4
7 W	269.2	259.6	234.0	264.4	355.6	369.2	408.4	420.4
7 W + 5 D	312.0	310.4	323.6	335.2	286.8	315.2	302.0	350.0

Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA

Cultivar (C)	4.0***	C x F	NS	C x F x SP	NS
Fumigation (F)	5.7*	C x SP	10.7***		
Storage/shelf life period (SP)	7.5***	F x SP	NS		

*** = $P \leq 0.001$; * = $P \leq 0.05$; NS = non–significant

In contrast to CAT, the changes in POD activity were significantly influenced by NO fumigation during cold storage and SL of both cultivars. Both cultivars showed opposite behaviour with regard to change in POD activity during cold storage; it decreased in 'Amber Jewel' and increased in 'Blackamber' with the advancement of storage period. POD activity increased during the 5 days of SL of 'Amber Jewel' fruit, without any clear pattern in response to NO fumigation. In 'Blackamber', it either increased or decreased slightly during the SL after 5 and 6 weeks of storage, but showed a significant decrease during the same period after 7 weeks of storage.

9.3.2.5 Ascorbate–glutathione cycle

Ascorbate

Concentrations of total ascorbate (AA+DHA), AA, DHA, and AA:DHA ratio were significantly influenced by cultivar, NO fumigation, storage/SL period and their interactions. A significant decline in concentrations of total ascorbate and AA was observed during the first 5 weeks of storage in both cultivars. In 'Amber Jewel' cultivar, the concentrations of total ascorbate (Table 9.7) and AA (Table 9.8) were significantly higher in fruit fumigated with 20 μL L^{-1} NO compared to other treatments after 5, 6 and 7 weeks of storage. In 'Blackamber', no clear pattern of changes in total ascorbate and AA emerged in response to NO fumigation and duration of cold storage. The concentrations of total ascorbate and AA, in general, increased significantly in both cultivars during 5 days of SL at 21±1°C after 5, 6, and 7 weeks of storage. Fruit of both cultivars fumigated with 20 μL L^{-1} NO showed significantly higher concentrations of AA after 5 days of SL than non–fumigated fruit (Table 9.8).

Table 9.7. Changes in concentration of total ascorbate (AA + DHA) in the flesh tissue of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	AA + DHA (nmol g^{-1} FW)							
Storage/ Shelf life	**'Amber Jewel'**				**'Blackamber'**			
	NO fumigation (µL L^{-1})				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0	113.1				277.3			
5 W	71.0	70.23	70.7	90.1	96.9	81.8	88.8	86.8
5 W + 5 D	133.2	129.1	136.1	140.1	124.4	145.8	147.8	136.9
6W	69.9	70.7	65.8	84.9	69.9	76.5	90.4	79.0
6 W + 5 D	133.1	132.1	144.1	140.6	136.5	139.7	146.2	148.3
7 W	52.7	52.6	59.1	76.9	100.1	88.5	79.5	81.3
7 W + 5 D	68.4	77.9	93.6	111.5	128.5	99.1	128.2	124.0
Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA								
Cultivar (C)	1.7***		C x F	3.3***	C x F x SP	8.8***		
Fumigation (F)	2.4***		C x SP	4.4***				
Storage/shelf life period (SP)	3.1***		F x SP	6.2***				

*** = $P \leq 0.001$

Table 9.8. Changes in concentration of ascorbate (AA) in the flesh tissue of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	AA (nmol g^{-1} FW)							
Storage/ Shelf life	**'Amber Jewel'**				**'Blackamber'**			
	NO fumigation (µL L^{-1})				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0			77.4				221.6	
5 W	33.0	25.5	42.7	52.5	49.9	44.4	51.2	48.9
5 W + 5 D	40.5	42.4	44.6	57.4	41.6	58.9	74.1	68.8
6W	28.1	26.7	39.6	50.8	37.7	41.4	51.1	44.6
6 W + 5 D	42.6	45.1	47.7	54.1	52.9	52.4	59.9	71.1
7 W	19.2	17.1	30.2	35.8	33.5	32.6	41.3	42.7
7 W + 5 D	17.0	17.0	29.1	25.1	41.1	38.8	54.7	53.4
Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA								
Cultivar (C)	0.9***		C x F	1.9***	C x F x SP	5.0***		
Fumigation (F)	1.3***		C x SP	2.5***				
Storage/shelf life period (SP)	1.8***		F x SP	3.5***				

*** = $P \leq 0.001$

In ‘Amber Jewel’, the concentration of DHA remained lower in fruit fumigated with 10 μL L^{-1} NO compared to other treatments during 7 weeks of storage (Table 9.9). ‘Blackamber’ fruit fumigated with 5, 10 or 20 μL L^{-1} NO showed significantly lower concentrations of DHA after 5 and 7 weeks of storage compared to non–fumigated fruit. However, the concentrations of DHA did not differ among fruit subjected to different levels of NO after 6 weeks of storage. A significant increase in concentration of DHA was noticed during 5 days of SL following each storage interval. In ‘Amber Jewel’ the concentrations of DHA did not present a clear trend in response to NO fumigation; the differences among different treatments were non–significant in most cases after 5 and 6 weeks of storage. After 7 weeks of storage plus 5 days of SL, the concentrations of DHA increased with the increase in concentration of NO. In ‘Blackamber’, the concentrations of DHA, after 5 weeks of storage plus 5 days of SL, were significantly lower in fruit fumigated with 10 or 20 μL L^{-1} NO compared to 0 and 5 μL L^{-1} NO.

AA:DHA ratio declined greatly during the first 5 weeks of storage in both cultivars (Table 9.10). In general, the AA:DHA ratio was observed to be higher in fruit of both cultivars fumigated with 10 and 20 μL L^{-1} NO compared to 0 and 5 μL L^{-1} NO after 5, 6 and 7 weeks of storage. Due to increase in the concentrations of DHA, the AA:DHA ratio showed a significant decrease during 5 days of SL following each storage interval. As a general observation in both cultivars, fruit fumigated with 20 μL L^{-1} NO showed higher AA:DHA ratio than in those fumigated with 0 and 5 μL L^{-1} NO.

Table 9.9. Changes in concentration of dehydroascorbate (DHA) in the flesh tissue of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	DHA (nmol g^{-1} FW)							
Storage/	**'Amber Jewel'**				**'Blackamber'**			
Shelf life	**NO fumigation (µL L^{-1})**				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0			35.7				55.7	
5 W	38.0	44.8	28.0	37.6	47.0	37.3	37.7	37.9
5 W + 5 D	92.7	86.7	91.5	82.8	82.7	86.9	73.6	68.2
6W	41.8	44.0	26.2	34.1	32.2	35.1	39.4	34.4
6 W + 5 D	90.6	87.0	96.4	86.6	83.6	87.3	86.4	77.2
7 W	33.5	35.5	28.9	41.2	66.6	55.9	38.2	38.6
7 W + 5 D	51.4	60.9	64.6	86.5	87.4	60.2	73.5	70.4
Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA								
Cultivar (C)		1.7***	C x F	3.3***	C x F x SP		8.8***	
Fumigation (F)		2.4***	C x SP	4.4***				
Storage/shelf life period (SP)		3.1***	F x SP	6.2***				

*** = $P \leq 0.001$

Table 9.10. Changes in AA:DHA ratio in the flesh tissue of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	AA:DHA ratio							
Storage/ Shelf life	**'Amber Jewel'**				**'Blackamber'**			
	NO fumigation (µL L^{-1})				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0			2.17				4.00	
5 W	0.87	0.57	1.54	1.45	1.06	1.19	1.37	1.32
5 W + 5 D	0.44	0.49	0.49	0.70	0.51	0.69	1.01	1.02
6W	0.68	0.61	1.54	1.52	1.17	1.18	1.31	1.30
6 W + 5 D	0.47	0.52	0.50	0.63	0.63	0.60	0.69	0.92
7 W	0.57	0.48	1.12	0.89	0.51	0.59	1.08	1.11
7 W + 5 D	0.33	0.28	0.45	0.29	0.47	0.71	0.74	0.76

Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA

Cultivar (C)	0.05***	C x F	NS	C x F x SP	0.29***
Fumigation (F)	0.08***	C x SP	0.14***		
Storage/shelf life period (SP)	0.10***	F x SP	0.20***		

*** = $P \leq 0.001$; NS = non–significant

Ascorbate–related enzymes (APX, MDHAR, and DHAR)

The activities of APX, MDHAR and DHAR were not significantly affected by NO fumigation during 7 weeks of cold storage (Tables 9.11–9.13). The storage/SL period had statistically significant effect on the activities of all these enzymes. A significant increase in activity of APX enzyme was noticed during the first 5 weeks of storage in both cultivars and the activity did not change significantly during the last 2 weeks of storage (Table 9.11). Regardless of NO fumigation , a decrease in APX activity was observed after 5 days of SL following 5, 6, and 7 weeks of storage. In most cases, the activity of MDHAR increased significantly in both cultivars during the first 5 weeks of storage and then remained almost stable in the last 2 weeks, but it showed a decreasing trend after 5 days of SL following each storage interval (Tables 9.12). However, the increase in DHAR activity during cold storage was significant only for 'Amber Jewel'; it either decreased slightly or remained unchanged in 'Blackamber' (Table 9.13). A general trend of decrease in activity of DHAR was observed during SL in both cultivars.

Table 9.11. Changes in activity of ascorbate peroxidase (APX) enzyme in the flesh tissue of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	APX (µmol H_2O_2 decomposed min^{-1} mg^{-1} protein)							
Storage/ Shelf life	**'Amber Jewel'**				**'Blackamber'**			
	NO fumigation (µL L^{-1})				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0			1.64				1.25	
5 W	2.01	2.00	2.11	2.14	2.06	1.95	1.96	1.93
5 W + 5 D	1.81	1.88	1.75	1.81	1.98	2.07	1.96	2.17
6W	1.92	2.08	1.96	2.08	2.06	1.97	1.91	1.81
6 W + 5 D	1.48	1.64	1.48	1.56	1.92	1.64	1.81	1.82
7 W	1.94	2.13	1.98	2.16	2.02	2.14	1.94	1.92
7 W + 5 D	1.77	1.58	1.68	1.75	2.03	2.20	2.02	2.09
Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA								
Cultivar (C)	NS		C x F	NS		C x F x SP	NS	
Fumigation (F)	NS		C x SP	0.15***				
Storage/shelf life period (SP)	0.10***		F x SP	NS				

*** = $P \leq 0.001$; NS = non–significant

Table 9.12. Changes in activity of monodehydroascorbate reductase (MDHAR) enzyme in the flesh tissue of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	MDHAR (µmol NADH oxidized min^{-1} mg^{-1} protein)							
Storage/ Shelf life	**'Amber Jewel'**				**'Blackamber'**			
	NO fumigation (µL L^{-1})				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0			0.82				0.78	
5 W	0.95	1.03	0.94	1.02	1.09	0.92	0.94	0.96
5 W + 5 D	0.65	0.74	0.62	0.72	0.63	0.71	0.71	0.82
6W	1.02	1.08	1.07	1.06	0.89	0.92	0.81	0.84
6 W + 5 D	0.77	0.81	0.80	0.99	0.42	0.39	0.54	0.69
7 W	1.16	1.22	1.10	1.01	0.82	0.85	0.76	0.82
7 W + 5 D	0.60	0.77	0.68	0.80	0.53	0.49	0.75	0.61

Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA

Cultivar (C)	0.05***	C x F	NS	C x F x SP	NS
Fumigation (F)	NS	C x SP	0.13***		
Storage/shelf life period (SP)	0.09***	F x SP	NS		

*** = $P \leq 0.001$; NS = non–significant

Table 9.13. Changes in activity of dehydroascorbate reductase (DHAR) enzyme in the flesh tissue of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	MDHAR (µmol NADH oxidized min^{-1} mg^{-1} protein)							
Storage/ Shelf life	**'Amber Jewel'**				**'Blackamber'**			
	NO fumigation (µL L^{-1})				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0			0.68				0.89	
5 W	0.97	0.82	0.96	0.80	0.76	0.87	0.90	0.75
5 W + 5 D	0.60	0.58	0.52	0.56	0.61	0.57	0.75	0.74
6W	1.02	0.94	0.96	0.97	0.75	0.81	0.75	0.90
6 W + 5 D	0.41	0.36	0.40	0.61	0.51	0.50	0.56	0.56
7 W	0.71	0.81	0.67	0.89	0.79	0.82	0.82	0.84
7 W + 5 D	0.52	0.48	0.51	0.69	0.41	0.45	0.45	0.66

Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA

Cultivar (C)	NS	C x F	NS	C x F x SP	NS
Fumigation (F)	NS	C x SP	NS		
Storage/shelf lifeperiod (SP)	0.15***	F x SP	NS		

*** = $P \leq 0.001$; NS = non–significant

Glutathione

Concentration of total glutathione (GSH + GSSG) was not significantly influenced by NO fumigation, but the main effects of cultivar and storage/SL period were significant (Table 9.14). A general trend of decrease in concentration of total glutathione was observed in both cultivars during cold storage and the trend of decrease continued with the progression of storage. A significant reduction in total glutathione levels was further noticed after 5 days of SL following 5, 6, and 7 weeks of cold storage (Table 9.14). In contrast to total glutathione, the concentration of GSH was significantly affected by NO fumigation, but the interaction effect of cultivar × fumigation × storage/SL period was non–significant (Table 9.15). In general, 'Amber Jewel' fruit fumigated with 10 or 20 μL L^{-1} NO showed higher concentrations of GSH compared to non–fumigated fruit after 5, 6, and 7 weeks of storage and plus 5 days of SL. In 'Blackamber' cultivar, the similar trend was noticed only for fruit fumigated with 20 μL L^{-1} NO.

Concentration of GSSG was significantly affected by NO fumigation. Table 9.16 shows that fumigation of fruit with 20 μL L^{-1} NO concentration significantly inhibited the increase in concentration of GSSG during cold storage and SL of both cultivars. The effectiveness of lower concentrations of NO, such as 5 and 10 μL L^{-1}, was less in 'Blackamber' cultivar compared to 'Amber Jewel'. The effect of NO fumigation was also significant on GSH:GSSG ratio (Table 9.17). A significant decrease in GSH:GSSG ratio occurred during cold storage and SL of both cultivars. A trend of changes in GSH:GSSG ratio was similar to that obtained for GSSG concentrations in response to fumigation with different concentrations of NO.

Table 9.14. Changes in concentration of total glutathione (GSH + GSSG) in the flesh tissue of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	GSH + GSSG (nmol g^{-1} FW)							
Storage/ Shelf life	**'Amber Jewel'**				**'Blackamber'**			
	NO fumigation (µL L^{-1})				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0	166.6				193.5			
5 W	152.3	150.8	161.3	159.3	190.5	189.8	179.5	192.0
5 W + 5 D	125.7	126.0	131.3	127.9	172.2	168.4	166.4	164.9
6W	140.7	143.8	154.5	158.0	181.1	184.9	184.1	183.0
6 W + 5 D	113.7	126.2	128.0	124.7	166.4	160.1	157.9	171.7
7 W	132.2	135.7	148.7	139.8	174.4	162.3	164.9	181.8
7 W + 5 D	96.7	116.0	116.5	104.5	150.1	147.0	146.1	167.5
Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA								
Cultivar (C)	3.8***		C x F	7.5*	C x F x SP	NS		
Fumigation (F)	NS		C x SP	NS				
Storage/shelf life period (SP)	7.0***		F x SP	NS				

*** = $P \leq 0.001$; * = $P \leq 0.05$; NS = non–significant

Table 9.15. Changes in concentration of reduced glutathione (GSH) in the flesh tissue of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	GSH (nmol g^{-1} FW)							
Storage/ Shelf life	**'Amber Jewel'**				**'Blackamber'**			
	NO fumigation (µL L^{-1})				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0	152.2				178.2			
5 W	134.4	132.5	144.3	144.2	165.7	166.0	155.5	172.2
5 W + 5 D	97.5	96.6	106.9	104.72	139.1	132.4	131.6	133.1
6W	121.2	124.3	137.4	139.8	153.5	158.6	156.9	161.3
6 W + 5 D	85.5	95.2	99.2	95.7	136.7	128.3	129.4	146.6
7 W	109.5	115.2	131.9	123.0	143.5	134.9	138.5	153.1
7 W + 5 D	63.2	85.3	86.9	75.8	120.0	118.3	114.9	135.7
Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA								
Cultivar (C)	3.6***		C x F	7.2**		C x F x SP	NS	
Fumigation (F)	5.1**		C x SP	9.6**				
Storage/shelf life period (SP)	6.8***		F x SP	NS				

*** = $P \leq 0.001$; ** = $P \leq 0.01$; NS = non–significant

Table 9.16. Changes in concentration of glutathione disulfide (GSSG) in the flesh tissue of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	GSSG (nmol g^{-1} FW)							
Storage/ Shelf life	**'Amber Jewel'**				**'Blackamber'**			
	NO fumigation (µL L^{-1})				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0	14.45				15.34			
5 W	17.91	18.38	16.94	15.08	24.78	23.82	24.01	19.80
5 W + 5 D	28.19	29.47	24.37	23.14	33.12	36.01	34.72	31.87
6W	19.54	19.42	17.10	18.30	27.58	26.31	27.17	21.73
6 W + 5 D	28.23	31.00	28.91	29.06	29.70	31.79	28.53	25.10
7 W	22.66	20.53	16.81	16.77	30.92	27.43	26.43	28.72
7 W + 5 D	33.55	30.71	29.52	28.67	30.14	28.71	31.23	31.75

Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA

Cultivar (C)	0.7***	C x F	NS	C x F x SP	NS
Fumigation (F)	1.1***	C x SP	2.0***		
Storage/shelf life period (SP)	1.4***	F x SP	NS		

*** = $P \leq 0.001$; NS = non–significant

Table 9.17. Changes in GSH:GSSG ratio in the flesh tissue of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	GSH:GSSG ratio							
Storage/ Shelf life	**'Amber Jewel'**				**'Blackamber'**			
	NO fumigation (µL L^{-1})				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0			10.53				11.69	
5 W	7.50	7.21	8.53	9.57	6.71	7.01	6.48	8.73
5 W + 5 D	3.46	3.28	4.39	4.52	4.19	3.67	3.83	4.17
6W	6.21	6.40	8.04	7.64	5.60	6.21	5.78	7.49
6 W + 5 D	3.03	3.07	3.44	3.30	4.67	4.10	4.57	5.86
7 W	4.84	5.66	7.91	7.54	4.64	5.03	5.25	5.35
7 W + 5 D	1.88	2.79	2.97	2.66	4.04	4.22	3.78	4.32

Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA

Cultivar (C)	NS	C x F	0.43***	C x F x SP	NS
Fumigation (F)	0.30***	C x SP	0.57***		
Storage/shelf life period (SP)	0.40***	F x SP	0.80**		

*** = $P \leq 0.001$; * = $P \leq 0.05$; NS = non–significant

Glutathione–related enzymes (GR and GT)

The activity of GR varied significantly with regard to cultivar and storage/SL period; the main effect of NO fumigation on GR activity was statistically non–significant (Table 9.18). In 'Amber Jewel', a significant decline in GR activity was observed during the first 5 weeks of storage, with minor changes in the last 2 weeks; the change in GR activity in 'Blackamber' was minor during 7 weeks of cold storage. In both cultivars, a significant decline in GR activity was noticed after 5 days of SL following 5, 6, and 7 weeks of storage. The activity of GT was significantly affected by main effects of cultivar, fumigation, and storage/SL period, but the interaction among three factors was non–significant (Table 9.19). A general trend of increase in GT activity was observed during cold storage in both cultivars. The fumigation of fruit with 10 or 20 μL L^{-1} NO concentrations decreased the activity of GT to some extent during storage and SL.

9.3.2.6 Total phenolics and DPPH$^{\cdot}$ scavenging activity

Concentration of total phenolics and DPPH$^{\cdot}$ scavenging activity were significantly affected by cultivar, NO fumigation and storage/SL period, but three–way interactions among these factors were non–significant (Tables 9.20 & 9.21). Fruit of both cultivars fumigated with 10 or 20 μL L^{-1} NO, in general, showed higher concentrations of total phenolics and DPPH$^{\cdot}$ scavenging activity than those fumigated with 0 or 5 μL L^{-1} NO after cold storage and SL. A slight decrease in total phenolics concentration was observed during cold storage, but the extent of decrease was significant during 5 days of SL in both cultivars.

9.3.2.7 Correlation analysis

The correlation coefficients of CI index and CI incidence with NO fumigation, storage period, lipid peroxidation, enzymatic and non-enzymatic antioxidants in NO–fumigated fruit of Japanese plums, 'Blackamber' and 'Amber Jewel', during 7 weeks of cold storage at 0 °C plus 5 days of shelf life at 21±1 °C are presented in Table 9.22.

Table 9.18. Changes in activity of glutathione reductase (GR) in the flesh tissue of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	GR (nmol NADPH oxidized min^{-1} mg^{-1} protein)							
Storage/ Shelf life	**'Amber Jewel'**				**'Blackamber'**			
	NO fumigation (µL L^{-1})				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0	30.0				22.30			
5 W	17.04	16.33	18.99	18.28	22.54	24.28	21.79	24.39
5 W + 5 D	13.08	13.44	16.57	17.0	11.49	13.82	18.38	17.53
6W	17.13	18.45	15.62	17.54	24.09	24.39	23.75	29.12
6 W + 5 D	11.19	11.24	14.79	11.52	14.84	14.44	10.41	17.07
7 W	14.78	14.34	14.42	17.24	21.94	20.1	22.21	22.49
7 W + 5 D	13.82	16.51	15.44	15.31	12.86	10.91	11.80	13.51

Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA

Cultivar (C)	1.21**	C x F	NS	C x F x SP	NS
Fumigation (F)	NS	C x SP	3.20***		
Storage/shelf life period (SP)	2.26***	F x SP	NS		

*** = $P \leq 0.001$; ** = $P \leq 0.01$; NS = non–significant

Table 9.19. Changes in activity of glutathione–S–transferase (GT) in the flesh tissue of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	GT (nmol S–2,4–dinitrophenylglutathione formed min^{-1} mg^{-1} protein)							
Storage/ Shelf life	**'Amber Jewel'**				**'Blackamber'**			
	NO fumigation (µL L^{-1})				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0			9.97				11.72	
5 W	15.56	14.50	13.71	13.91	18.83	18.88	17.86	18.65
5 W + 5 D	19.17	15.98	16.25	14.04	21.38	17.85	18.67	15.69
6W	15.81	12.13	13.83	13.95	17.73	22.35	16.24	21.20
6 W + 5 D	18.87	16.90	15.10	16.47	22.40	21.77	20.25	18.29
7 W	17.31	15.83	13.29	13.73	20.53	17.57	15.98	19.24
7 W + 5 D	20.35	18.12	15.84	17.29	23.82	19.02	24.25	18.30

Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA

Cultivar (C)	1.06***	C x F	NS	C x F x SP	NS
Fumigation (F)	1.50*	C x SP	NS		
Storage/shelf life period (SP)	1.98***	F x SP	NS		

*** = $P \leq 0.001$; * = $P \leq 0.05$; NS = non–significant

Table 9.20. Changes in concentration of total phenolics in the flesh tissue of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	Total phenolics (mg cholorogenic acid equivalents 100g^{-1} FW)							
Storage/ Shelf life	**'Amber Jewel'**				**'Blackamber'**			
	NO fumigation (µL L^{-1})				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0	122.4				137.3			
5 W	109.3	108.6	123.7	121.6	125.2	123.4	134.6	131.6
5 W + 5 D	99.4	97.3	113.3	115.4	117.7	119.0	133.1	135.4
6W	103.1	104.1	116.6	109.8	119.6	115.7	130.4	139.2
6 W + 5 D	97.0	97.9	111.3	105.5	109.0	104.9	119.4	122.8
7 W	100.6	106.3	112.5	109.9	120.0	117.0	122.8	126.2
7 W + 5 D	88.3	100.0	107.4	103.4	101.4	97.2	117.0	108.8

Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA

Cultivar (C)	1.5***	C x F	3.0*	C x F x SP	NS
Fumigation (F)	2.1***	C x SP	3.9***		
Storage/shelf life period (SP)	2.8***	F x SP	5.5***		

*** = $P \leq 0.001$; * = $P \leq 0.05$; NS = non–significant

Table 9.21. Changes in DPPH scavenging activity in the flesh tissue of Japanese plums fumigated with NO during 5, 6 and 7 weeks (W) of storage at 0°C and during 5 days (D) of shelf life at 21±1°C after each storage interval.

	DPPH scavenging activity (mg ascorbic acid equivalents 100g^{-1} FW)							
Storage/ Shelf life	**'Amber Jewel'**				**'Blackamber'**			
	NO fumigation (µL L^{-1})				**NO fumigation (µL L^{-1})**			
	0	**5**	**10**	**20**	**0**	**5**	**10**	**20**
0	67.1				73.7			
5 W	60.0	55.6	69.0	68.1	66.3	64.6	71.5	73.8
5 W + 5 D	55.6	56.2	65.3	64.1	64.9	62.7	71.8	75.9
6W	55.1	52.0	64.1	65.6	63.0	60.9	69.3	71.7
6 W + 5 D	52.7	51.4	62.0	63.5	61.9	57.2	70.8	71.5
7 W	50.7	47.8	57.7	60.7	60.2	63.7	65.3	62.8
7 W + 5 D	48.0	46.4	56.0	56.6	59.7	54.9	63.3	59.7

Least significant differences of means at 5% level and levels of significance for a three–factor ANOVA

Cultivar (C)	0.86***	C x F	NS	C x F x SP	NS
Fumigation (F)	1.21***	C x SP	NS		
Storage/shelf life period (SP)	1.61***	F x SP	3.21***		

*** = $P \leq 0.001$; NS = non–significant

Table 9.22. Correlations of CI index and CI incidence with NO fumigation , storage period, lipid peroxidation, enzymatic and non-enzymatic antioxidants in NO–fumigated fruit of Japanese plums, 'Blackamber' and 'Amber Jewel', during 7 weeks of cold storage at 0°C plus 5 days of shelf life at 21±1°C.

CI index *vs.*	**Pearson's correlation coefficient**	**CI incidence *vs.***	**Pearson's correlation coefficient**
TBARS	0.65***	TBARS	0.72***
LOX	0.67***	LOX	0.70***
EL	0.79***	EL	0.80***
SOD	–0.40***	SOD	–0.41***
CAT	–0.24***	CAT	–0.19***
POD	NS	POD	NS
AA	–0.37***	AA	–0.45***
DHA	0.54***	DHA	0.50***
AA:DHA	–0.56***	AA:DHA	–0.62***
GSH	–0.76***	GSH	–0.76***
GSSG	0.57***	GSSG	0.57***
GSH:GSSG	–0.71***	GSH:GSSG	–0.73***
APX	NS	APX	NS
MDHAR	–0.30*	MDHAR	–0.24*
DHAR	–0.37***	DHAR	–0.32***
GR	–0.54**	GR	–0.59***
GT	0.34***	GT	0.35***
Phenols	–0.68***	Phenols	–0.72***
DPPH scavenging activity	–0.63***	DPPH scavenging activity	–0.69***

NS, *, **, *** = non-significant, $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, respectively

9.4 Discussion

9.4.1 Fruit quality

Postharvest fumigation with NO has been known to delay ripening and senescence in many fruits such as tomato, peach, strawberry and kiwifruit (Zhu et al., 2006; Zhu and Zhou, 2007; Flores et al., 2008). Respiration and ethylene production rates in fruit decrease in response to postharvest fumigation with very low concentrations of NO. Postharvest exposure of Japanese plums to NO gas significantly reduced the respiration and ethylene production rates during SL at 21±1°C (data not shown). The suppression of respiration during fruit ripening in NO–fumigated peaches and strawberries has been reported earlier (Zhu et al., 2006; Zhu and Zhou, 2007; Flores et al., 2008). A strong inhibition of ethylene production was observed in NO–fumigated fruit, particularly in 'Amber Jewel', leading to a delay in the onset of ripening and senescence. The inhibition of ethylene biosynthesis has been reported in NO–fumigated fruits such as kiwifruit, peach, strawberry, and tomato (Zhu et al., 2006; Zhu and Zhou, 2007; Flores et al., 2008; Eum et al., 2009). Anti–senescence action of NO on plant tissues has been proposed to take place *via* the inhibition of ethylene biosynthesis (Leshem et al., 1998; Zhu et al., 2006). The reduction in ethylene production during fruit ripening in NO–fumigated fruit may be due to binding of NO with ACC oxidase and ACC to form a stable ternary complex, thus limiting the ethylene production (Tierney et al., 2005). Zhu et al. (2006) supported the proposed mechanism of action of NO in peach fruit and showed that ethylene biosynthesis was mainly due to decreased activity of ACC oxidase and accumulation of ACC and 1–malonylaminocyclopropane–1–carboxylic acid (MACC) without any significant effect on ACC synthase activity. The inhibition of ethylene biosynthesis in NO–fumigated tomatoes was reportedly due to decreased and delayed expression of ACC oxidase genes (Eum et al., 2008). However, another study on strawberry revealed that NO decreased the activity of ACC synthase, but not ACC oxidase (Zhu and Zhou, 2007). Therefore, either one of these proposed or any other mechanism of action of NO *via* ethylene inhibition may exist in Japanese plums. Exploring the possible mode of action of NO in ethylene inhibition in climacteric and suppressed–climacteric type plums is worthy of further study.

Fruit softening was significantly reduced by NO fumigation (Figs. 9.1 & 9.11) which might be due to decreased activities of fruit softening enzymes caused

by inhibition of ethylene production. Ethylene has been shown to increase the activities of fruit softening enzymes in the Japanese plums (Khan and Singh, 2007a). It is, therefore, possible that inhibition of ethylene production through NO fumigation as observed in our study and blocking of its action though 1–MCP (Abdi et al., 1998; Khan and Singh, 2007a) helps to retard the fruit softening process. Fumigation of peaches with 5 or 10 μL L^{-1} NO (Zhu et al., 2006; Flores et al., 2008) and kiwifruit with 1 μmol L^{-1} NO (Zhu et al., 2008) has been reported to retard the fruit softening during storage and ripening, but a higher concentration of 15 μL L^{-1} NO in peaches and 2 μmol L^{-1} NO in kiwifruit enhanced the fruit softening. The increase in NO concentrations from 5 to 20 μL L^{-1} did not significantly increase fruit softening in plums during fruit ripening for 12 days. Higher concentration (20 μL L^{-1}) instead helped to retain higher firmness during storage at 0°C for 5 and 6 weeks. More research is required to examine if the enhanced fruit softening effect in plums may be obtained by applying higher concentrations of NO.

The changes in other quality parameters such as, SSC, TA and SSC: TA ratios were restricted, to some extent, in NO–fumigated fruit during storage and ripening (Fig. 9.2). The results on 'Amber Jewel' cultivar are in agreement with Zhu et al. (2006, 2008) who reported a delay in the increase of SSC in peaches and kiwifruit fumigated with 5 or 10 μL L^{-1} NO and 0.5 or 1 μmol L^{-1} NO, respectively, during fruit ripening. The increase in SSC: TA ratio, also called ripening index, was significantly reduced in NO–fumigated fruit during fruit ripening and storage. Previously, 1–MCP treatment has been reported to delay the increase in the SSC: TA ratio during cold storage and ripening of 'Santa Rosa' (Martínez–Romero et al., 2003). Plums fruit ripening involves a slight increase in sugars concentration and a significant loss of malic acid, the major organic acid (Singh and Singh, 2008a), which result into an increase in the SSC: TA ratio.

9.4.2 Chilling injury (CI)

The occurrence of CI and increase in its severity during SL at 21±1°C after cold storage were observed (Fig. 9.11). These observations are consistent with the findings of Candan et al. (2008) in 'Larry Ann' plums. Crisosto et al. (1999) also described similar CI symptoms in the various cultivars of plums stored for 5 weeks at

0°C. The flesh browning may be related to the disintegration of tissue membrane resulting into the enzymatic oxidation of phenolic compounds by PPO to *o*–quinones, which are brown coloured polymers. NO fumigation had a significant effect on reduction in incidence and severity of CI in Japanese plums during cold storage for 5 and 6 weeks and subsequent SL of 5 days at 21±1°C (Fig. 9.11). The alleviation of CI symptoms in plums could be attributed to the inhibition of ethylene production during cold storage of NO–fumigated fruit as reported in case of kiwifruit and peaches (Zhu et al., 2010; Zhu et al., 2006; Zhu et al., 2008). A reduction in CI symptoms during cold storage of 1–MCP– treated 'Larry Ann' plums was observed by Candan et al. (2008), which suggests a significant role of ethylene in the series of events associated with the initiation and development of CI symptoms in plums during cold storage. The data also suggest that the beneficial effect of NO fumigation on alleviation of CI decreased in fruit stored beyond 6 weeks. The cross–talk of NO with other plant hormones including ethylene is very complex and not fully understood.

9.4.3 Lipid peroxidation

The extent of postharvest lipid peroxidation was determined by the concentration of TBRAS in the flesh tissue of Japanese plums. The data revealed that the concentration of TBARS increased with the increase in SL for 12 days at 21±1°C (Fig. 9.3) and also with the increase in storage time during cold storage and subsequent 5 days of SL at 21±1°C (Fig. 9.12). NO can possibly react with lipid alcoxyl (LO·) and peroxyl (LOO·) radicals to prevent the propagation of ROS–mediated lipid peroxidation. The decrease in lipid peroxidation by NO fumigation has also been reported in other fruits such as peach, kiwifruit and longan (Duan et al., 2007a; Duan et al., 2007b; Zhu et al., 2008). The higher concentration of NO has been found to increase the lipid peroxidation in these fruits, which implies that the toxic or protective effects depend on the concentration of NO. The effectiveness of NO in decreasing lipid peroxidation in Japanese plums improved with the increase in NO concentration from 5 to 20 μL L^{-1}. The increased levels of lipid peroxidation as reported in other fruits might occur with the exposure of Japanese plums to NO concentrations more than 20 μL L^{-1} and warrants further investigation. The protective effects of NO as revealed by lower levels of TBARS indicate that it can be

helpful to reduce the postharvest oxidative stress in Japanese plums. The lower levels of CI in NO–fumigated fruit might have resulted from the lower oxidative stress and better maintenance of cell membrane structure (Fig. 9.11) as the incidence and severity of CI were positively correlated with TBARS, LOX and EL (Table 9.22).

9.4.4 Antioxidant enzymes (SOD, CAT, and POD)

The primary antioxidant enzymes, SOD, CAT, and POD constitute the first line of defence against the ROS produced during normal and stress conditions. The activities of these enzymes in a concerted manner are crucial in preventing the accumulation of O_2^- and H_2O_2 which can potentially initiate a cascade of reactions involving the damage to lipids, proteins and DNA, resulting in loss of membrane integrity and oxidative stress. The increased activities of SOD, CAT and POD, in response to generation of ROS, have been associated with the efficiency of the system to immediately scavenge the ROS before these accumulate to toxic levels. NO fumigation with 10 or 20 μL L^{-1} showed higher SOD activity in fruit after 6 and 9 days of SL at 21±1°C (Fig. 9.4A) and also during cold storage (Table 9.4) which indicates that NO played an important role in delaying the decrease in SOD activity. Higher SOD activity might have resulted in prevention and delay of accumulation of O_2^- in the flesh tissue of NO–fumigated fruit.

The enhanced SOD activity in response to NO treatment has been observed to be associated with delayed fruit senescence in kiwifruit and peaches (Flores et al., 2008; Zhu et al., 2008) and enhanced tolerance to abiotic stresses in plants (Shi et al., 2007; Shi et al., 2005). The activities of CAT and POD showed a significant increase during the first 6 to 9 days of fruit ripening at 21±1°C, without any significant effect of NO fumigation . POD activity was significantly affected by NO fumigation during cold storage and remained at higher level in fruit exposed to 20 μL L^{-1} NO. The enhanced activities of CAT and POD have been reported in NO–treated plants or plant organs (Shi et al., 2007; Shi et al., 2005), but there are reports showing that NO inhibited and suppressed activity of either CAT or POD and also both in some cases (Clark et al., 2000; Duan et al., 2007a; Zhu et al., 2008). Apparently, there is a contradiction between the conditions in which NO appears to counteract ROS actions through enhanced activities of antioxidant enzymes and those in which it acts

synergistically with ROS. This remains a dilemma in NO research in both plants and animals.

NO also acts as an antioxidant by scavenging O_2^- from the cell (Beligni et al., 2002; Hung and Cao, 2004) and converting it into $ONOO^-$ that can produce NO_2^- and NO_3^-. However, with the advancement of senescence process in fruit, the activities of antioxidant enzymes declined. This could possibly be due to the degradation of proteins and membrane structures which might have occurred as a result of fruit senescence. The data suggest that NO fumigation exerted positive effects on the capability of the antioxidant protection system in Japanese plums through both enhancing the SOD activity and limiting lipid peroxidation.

9.4.5 Ascorbate–glutathione cycle

Ascorbate–glutathione cycle operates in conjunction with other antioxidant systems to regulate the concentration of ROS in the cell (Apel and Hirt, 2004). AA and GSH are important cellular redox buffers which are essential to be maintained in the reduced states to act as reducing agents. The data show that concentrations of total ascorbate (AA+DHA) and AA declined during 12 days of SL at 21±1°C and were significantly influenced by NO fumigation, but a consistent trend in the changes to their concentration was lacking to draw any valid conclusion with regard to NO fumigation (Fig. 9.5). The decrease in concentration of AA and a sharp increase in concentrations of DHA during the first 9 days at 21±1°C reflect that AA was being utilized as an antioxidant during the fruit ripening in both cultivars of Japanese plums. The decrease in AA concentration was inhibited to some extent in fruit fumigated with 20 μL L^{-1} NO during cold storage which could be related to the lower concentration of ROS in these fruit due to higher SOD activity and lower degree of lipid peroxidation. Fruit in cold storage showed an increase in concentrations of AA and DHA during SL of 5 days at 21±1°C (Tables 9.8 & 9.9). Such increase was not noticed in fruit directly allowed to ripen at 21±1°C without any cold storage. This could be explained on the basis of stimulation of AA biosynthesis as an acclimatization response to counteract the increasing oxidative stress which might have occurred during the post–cold storage phase.

The increase in activity of APX (Fig. 9.6A) could be related to the decrease in AA concentration because APX activity increases to counteract the increasing levels of H_2O_2 from the system by using AA as a reducing power. Interestingly, fruit fumigated with 20 μL L^{-1} NO showed lower levels of DHA after 3 and 6 days compared to other treatments, but the differences became non–significant during the advanced stages of fruit ripening. The lower concentrations of DHA in the fruit flesh were closely related to the increased activities of MDHAR (Fig. 9.6B) and DHAR (Fig. 9.6C) in these fruit during the initial stages. The general decline in the activities of MDHAR and DHAR could be another possible reason to support the decline in the concentration of AA and increase in DHA levels. This observation supports that NO fumigation might have helped to maintain the AA pool in the reduced state during the initial stages of fruit ripening, but as the time progressed, the effects of NO were marginalized.

NO fumigation also helped to positively maintain the redox status of AA pool during cold storage when fruit were exposed to 20 μL L^{-1} concentration. The beneficial effects of NO fumigation during cold storage of Japanese need to be further investigated by application of NO concentrations more than 20 μL L^{-1}. The activities of APX, MDHAR and DHAR were not significantly affected by NO fumigation . The data on the activities of these enzymes could not entirely explain the changes in concentrations of AA and DHA. Several other researchers have previously reported the same difficulty in studying the ascorbate biosynthesis and regeneration cycle in fruits (Davey et al., 2004; Huang et al., 2008; Ioannidi et al., 2009; Stevens et al., 2008).

The changes in concentrations of GSH, GSSG and GSH:GSSG ratio were not significantly influenced by NO fumigation during SL at 21±1°C for 12 days (Fig. 9.7). However, these results were significant in fruit kept in cold storage for 5, 6, and 7 weeks and subsequently held for 5 days at 21±1°C (Tables 9.15 to 9.17). The decline in concentration of GSH could be attributed to its oxidation during the conversion of DHA into AA by DHAR, utilization as substrate by GT and also due to the lesser recovery through the activity of GR which consistently declined during SL at 21±1°C for 12 days. Consequently, GSSG concentration increased significantly during the same period. The upsurge in activity of DHAR in

'Blackamber' (Fig. 9.6C) after 12 days of SL might have resulted in substantial decrease in GSH levels as the GR activity was then at the lowest level (Fig. 9.8A).

Similar to GSH and GSSG, no significant effect of NO fumigation on the activities of GR and GT was observed during 12 days of SL (Fig. 9.8). The GR is responsible for NADPH–dependent reduction of GSSG into GSH (Apel and Hirt, 2004). The maintenance of GR activity is important in maintaining the concentration of GSH in the glutathione pool. The efficiency of ascorbate–glutathione cycle to counteract the increasing levels of ROS declined as the time progressed. Contrary to GR, the activity of GT was found to be significantly influenced by NO fumigation during cold storage (Tables 9.18 & 9.19) as the fruit fumigated with 20 μL L^{-1} NO showed significantly lower GT activity compared to other treatments. This could possibly explain slightly higher concentration of GSH in these fruit during cold storage because GT utilizes GSH as a substrate to detoxify lipid hydroperoxides accumulated as a result of increased lipid peroxidation (Rogiers et al., 1998).

The concentrations of AA and GSH have been reported to decrease during long–term cold storage of other fruits, such as mangoes (Zhao et al., 2009), oranges (Huang et al., 2008), and pawpaws (Galli et al., 2009). Little information is available on the effects on NO treatment on glutathione component in plants. It has been postulated that NO can react with GSH to form S–nitrosoglutathione (GSNO) which can function as a mobile reservoir of NO bioactivity or a S–nitrosylating agent (Feechan et al., 2005). The binding of a NO group to the thiol side chain of a cystein residue is called S–nitrosylation which is involved in a large part of the almost ubiquitous influence of NO on the cellular signal transduction. The characterization of mechanisms regulating *S*–nitrosylation/de–nitrosylation is still not completely understood in plants in relation to NO (Lindermayr et al., 2005). There are no published reports on the role of NO in such mechanisms in fleshy fruits.

9.4.6 Total phenolics and DPPH$^{\cdot}$ scavenging activity

Total antioxidant capacity in plum flesh tissue is primarily due to the presence of phenolics compounds, in addition to a few other phytochemicals (Díaz–Mula et al., 2009; Gil et al., 2002). The data on changes in concentration of total phenolics is closely related to the changes in DPPH$^{\cdot}$ radical scavenging activity. Total phenolics

and DPPH$^{\cdot}$ scavenging activity showed a significant increase in 'Amber Jewel' fruit fumigated with 10 or 20 μL L^{-1} NO after 6 days of SL compared to other treatments, while in 'Blackamber' fruit, these parameters remained stable during the first 9 days of SL (Fig. 9.9). The stimulatory effect of NO on the concentration of total phenolics could be attributed to the enhanced activity of PAL, which is a key regulatory enzyme in biosynthesis of phenolics, and also due to inhibition of PPO activity. The increased activity of PAL and decrease in activity of PPO in response to treatment with NO in peaches has been previously reported (Zhu et al., 2009). The maintenance of higher concentration of total phenolics may also be related to the inhibition in their decrease due to retardation of fruit ripening caused by NO fumigation.

During cold storage, fruit of both cultivars fumigated with 10 or 20 μL L^{-1} NO, in general, showed higher concentrations of total phenolics and DPPH$^{\cdot}$ scavenging activity than those fumigated with 0 or 5 μL L^{-1} NO (Tables 9.20 & 9.21). A slight decrease in total phenolics concentration was observed during cold storage, but the extent of decrease was significantly great during 5 days of SL in both cultivars. The presence of higher concentration of total phenolics and greater DPPH radical scavenging activity indicate the viability of the antioxidant protection system of the fruit against the oxidative injury (Galli et al., 2009). The increase in storage duration accompanied by the decrease in total phenols and reduced efficiency of other antioxidative systems might have contributed to the build up of oxidative stress to potentially damaging levels.

In conclusion, postharvest application of NO (10 μL L^{-1}) has potential to delay the fruit ripening in Japanese plums. NO fumigation with 20 μL L^{-1} may be useful in improving the storage potential of plums at 0°C through alleviation of CI symptoms to some extent. Further work is required to understand the mechanisms of action of NO gas in the climacteric and suppressed–climacteric type plums that may allow its commercial use in the future.

Chapter 10

General Discussion, Conclusions, Recommendations, and Future Research

10.1 Introduction

Postharvest oxidative stress is a phenomenon in which ROS production in response to inherent physiological processes and external environmental stimuli exceeds the capability of antioxidant protection system in fruits (Toivonen, 2004). The activation of antioxidant system is essential to regulate ROS levels required for their roles as signalling molecules in various physiological processes (Apel and Hirt, 2004). The inadequacy of the antioxidant system may result into accumulation of ROS in the fruit tissue which can potentially damage the components of cellular membranes and organelles, causing an array of physiological and metabolic imbalances. The oxidative damage resulting from the toxic levels of ROS and failure or inadequate antioxidant system in the fruit can have serious implications for fruit quality and its storage/SL potential (Hodges et al., 2004). Postharvest fruit ripening and senescence rates, which determine the potential storage/SL, are strictly controlled by the oxidative processes. Furthermore, the development of various storage disorders in fruits has been attributed to the oxidative damage resulting from either the enhanced ROS production or the reduced capability of antioxidant system in the fruit and/or both. Therefore, it is imperative to understand the role of internal and external factors which trigger the pro–oxidant and antioxidant systems during postharvest handling, storage, distribution and retailing of fruits.

10.2 Fruit ripening in Japanese plums: an oxidative phenomenon

Fruit ripening is an oxidative process generating ROS which cause tissue damage (Brennan and Frenkel, 1977). Japanese plums are highly perishable and exhibit faster rates of fruit ripening and senescence due to higher rates of respiration and ethylene production. Previous studies (Abdi et al., 1997b; Candan et al., 2008; Khan and Singh, 2007b) and the data presented in Chapter 4 show that depending on the rates of respiration and ethylene production, Japanese plum cultivars can be categorized

into highly–climacteric, moderately–climacteric, and suppressed–climacteric types. There are reportedly huge differences among cultivars of the same fruit species with regard to the status of enzymatic and non–enzymatic antioxidants and susceptibility to lipid peroxidation (Davey et al., 2004; Davey and Keulemans, 2004; Felicetti and Mattheis, 2010; Łata, 2008; Łata et al., 2005). These differences have partially explained the differences in the potential storage life and susceptibility to various storage disorders. Postharvest senescence rates in fresh horticultural produce such as melons and spinach have been clearly correlated with the capacity of antioxidant system to counteract the increasing oxidative stress during postharvest phase (Hodges et al., 2001; Lacan and Baccou, 1998). No attempt has been made to elucidate the role of oxidative stress in the differential display of the ripening and senescence rates in Japanese plums. Therefore, Chapter 4 presented the information on how the climacteric levels during fruit ripening influence lipid peroxidation and dynamics of antioxidants levels in Japanese plums.

The lower rate of increase in lipid peroxidation, marked by TBARS accumulation, during fruit ripening in 'Angeleno', a suppressed–climacteric cultivar, compared to 'Blackamber' and 'Amber Jewel' may be attributed to the lower activity of LOX and the lower level of oxidative stress (Fig. 4.4). The activity of LOX has been reported to be up–regulated by ethylene during fruit ripening (Schaffer et al., 2007). Therefore, the delay in appearance of quantifiable amounts of ethylene (Fig. 4.4) in 'Angeleno' might be the possible reason for lower LOX activity during the initial 4–5 days of fruit ripening. Consequently, the loss of membrane integrity as shown by EL was also delayed in this cultivar compared to others.

The reduced levels of ROS production may be expected in 'Angeleno' cultivar due to the lower rate of respiration compared to others as the mitochondrial respiration is the primary source of ROS in the non–photosynthetic plant tissues (Purvis, 2003). The lower activity of SOD in this cultivar during the entire ripening process may be an indication of the decreased production of O_2^- which did not necessitate the increase in SOD to dismutate them (Fig. 4.5A). CAT activity remained higher during initial stages of fruit ripening in 'Blackamber' and 'Amber Jewel' and then declined, which is in contrast to 'Angeleno' which showed a progressive increase over the entire ripening period (Fig. 4.5B). The highest CAT activity in 'Angeleno' on the 8^{th} day of fruit ripening points towards the functioning

of a highly efficient system of H_2O_2 removal, whereas high POD activities in 'Blackamber' and 'Amber Jewel' cultivars during the same period showed that H_2O_2 removal is occurring at the expense of cellular reductants (Fig. 4.5C).

Significant changes in the AA–GSH cycle were also observed during fruit ripening of Japanese plums (Figs. 4.6 to 4.9). The differences among three cultivars with regard to changes in concentrations of AA, DHA, GSH and GSSG did not show a clear trend to completely support the hypothesis. In general, a continuous increase in concentrations of DHA and GSSG, and decease in AA:DHA and GSH:GSSG ratios signified the increased oxidative state of the tissue in all cultivars. However, the maintenance of the higher concentration of total glutathione (GSH + GSSG), GSH and GSH:GSSG ratio was observed during most of the period of fruit ripening in 'Angeleno' compared to other two cultivars (Fig. 4.8). This could be explained on the basis of higher GR and lower GT activity in this cultivar (Fig. 4.9). The lower GT activity, which is responsible for detoxification of lipid hydroperoxides, in 'Angeleno' is another evidence of lower levels of oxidative stress in this cultivar.

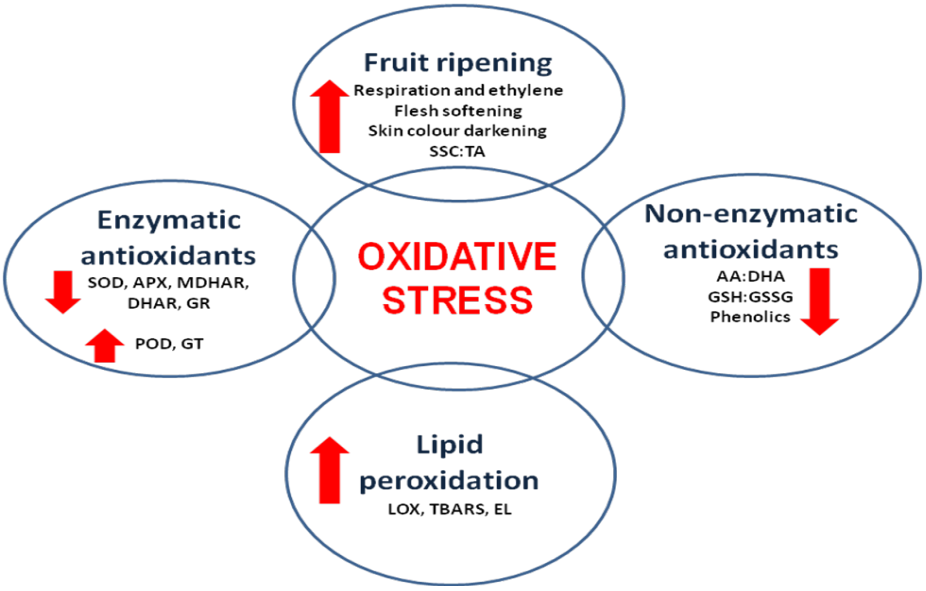


Fig. 10.1. Postharvest oxidative stress during fruit ripening in Japanese plums.

In conclusion, the postharvest oxidative stress develops during fruit ripening of Japanese plums (Fig. 10.1), but the rate was dependent on the climacteric behaviour of fruit. The degree of lipid peroxidation and activities of enzymatic antioxidants, particularly CAT and POD, were closely related to the climacteric

levels of cultivars of Japanese plums. The progress in fruit ripening as marked by fruit softening and skin colour changes were accompanied by the increased oxidative state of the flesh tissue as demonstrated by the redox potential of the two important buffers, AA and GSH. Japanese plum cultivars experiencing oxidative injury at the faster rate show enhanced membrane disintegration resulting in accelerated rates of ripening and senescence, thus limiting their postharvest life. Therefore, oxidative behaviour of Japanese plums during fruit ripening is determined by their climacteric levels.

10.3 Harvest maturity and antioxidant metabolism during cold storage of Japanese plums

Harvest maturity is an important determinant of fruit quality and potential storage/SL of Japanese plums. The storage/shipping life of Japanese plums is limited by their susceptibility to CI symptoms which mainly appear in the form of flesh browning, mealiness and flesh translucency (Crisosto et al., 1999). Harvest maturity has been considered to be one of the important factors that determine the incidence, severity and nature of CI symptoms. Harvesting before maturity leads to CI symptoms in the form of flesh browning, mealiness and leathery texture (Crisosto et al., 2003; Crisosto et al., 1999). The delayed harvesting has been reported to increase the susceptibility of fruit to flesh translucency (Abdi et al., 1997a; Taylor et al., 1993; Taylor et al., 1995). Moreover, CI has been described as an oxidative phenomenon in which the activation of the antioxidant defense system is required to encounter increasing pro–oxidant levels during the chilling stress. However, no information is currently available on the oxidative behaviour of Japanese plums during prolonged cold storage in relation to harvest maturity. This research gap has been addressed by the information presented in the Chapter 5.

CI symptoms appeared after 3 weeks of cold storage at 0°C in ‘Amber Jewel’ fruit harvested at both maturities and the differences were not significant in two maturity groups for the first 5 weeks of storage (Fig. 5.3). The incidence and severity of CI in delayed–harvested fruit surpassed the commercial harvest during the last 2 weeks of storage. Both maturity groups showed similar rates of lipid peroxidation during cold storage with a continuous increase in the accumulation of TBARS (Fig.

5.4). The average LOX activity and EL remained slightly higher in the fruit from delayed harvest compared to commercial harvest which demonstrates greater extent of membrane disintegration in the former.

The higher activities of primary antioxidant enzymes are vital to impart tolerance against chilling stress in fruits (Imahori et al., 2008; Rivera et al., 2007; Wang et al., 2004; Zhao et al., 2009). Fruit harvested at commercial maturity had better response to chilling conditions with their SOD and CAT activities remained at higher levels during most of the cold storage period than the delayed harvest (Fig. 5.5). A significant decline in the activities of SOD, CAT, and POD beyond 5 weeks of storage indicates the reduction in the capability of antioxidant system to cope with increasing stress from prolonged storage duration.

At harvest, AA and GSH were slightly higher in fruit from delayed harvest compared to commercial harvest, but both showed a significant decrease with the progression of storage (Figs. 5.6 & 5.7). The levels of DHA and GSSG showed an interesting pattern of a sudden increase after 4 weeks of storage in fruit from both maturity groups, except for DHA increase which was after 3 weeks in delayed–harvested fruit. The activities of various enzymes responsible for oxidation and recycling of AA and GSH also support these observations. APX activity showed an upsurge after 2 and 3 weeks in delayed and commercial harvests, respectively, whereas the activities of MDHAR and DHAR showed a significant decrease beyond 3 weeks (Fig. 5.8). The fall in GR and rise in GT activities may also explain the decrease in GSH and increase in accumulation of GSSG after 4 weeks of storage (Fig. 5.9). These observations suggest that the storage period between 3 and 4 weeks is a critical point from where onwards the antioxidant system continues to collapse as shown by various components of AA–GSH cycle.

The concentration of total phenolics increased during the initial 2 weeks of storage (Fig. 5.10A) which is an indication of the activation of defence mechanism against chilling stress (Galli et al., 2009). This increase was significantly higher in the fruit from commercial harvest than in the delayed harvest. However, a decrease followed as the storage duration increased which could be due to their utilization as substrate of PPO in the browning reactions as the severity of flesh browning increased with storage. Another possible explanation for decrease in phenols is that these may be oxidized by peroxidases in the presence of H_2O_2 because of the co–

existence of phenolics and peroxidases in vacuoles (Takahama, 2004). The pattern of changes in DPPH scavenging activity (Fig. 5.10B) was quite similar to that of the total phenolics because the latter is the primary contributor to the antioxidant capacity of the flesh tissue (Gil et al., 2002).

The comparison of the two maturity groups suggest that fruit from commercial harvest had overall better retention of the antioxidant system compared to those from delayed harvest. The acclimatization response to chilling stress in Japanese plums as indicated by the activities of various antioxidant enzymes, levels of AA and GSH, and AA–GSH cycle enzymes was too weak and slow to compensate for the presumed increased production of ROS during prolonged cold storage. It is also evident from the data that the changes in antioxidant components during cold storage of Japanese plums seem to be more important in providing protection against oxidative injury than their at–harvest antioxidant status. Based on the response of antioxidant system to chilling stress, it may be concluded that the cold storage (0°C) of 'Amber Jewel' plums harvested at commercial and delayed maturity is safe for 2 weeks, but may be extended to 3 weeks. CI symptoms appeared after 3 weeks of storage in both maturity groups, but the level of CI incidence (~10%) was below the benchmark of 25% which is generally considered for termination of storage/shipping life of Japanese plums.

10.4 Storage temperature and antioxidant metabolism in Japanese plums

The maintenance of optimum storage temperature is the first requirement in the postharvest handling and storage of Japanese plums, like other fruits. Japanese plums can be stored at 0°C for 3–5 weeks depending upon the cultivar's susceptibility to CI. The storage temperatures between 2.2°C and 7.6°C have been considered to be lethal for development of CI in Japanese plums. However, the sensitivity to CI is strictly controlled by genetic factors in Japanese plums as some cultivars are susceptible or tolerant to CI at both temperatures 0°C and 5°C, and a few cultivars show CI at one temperature and not at the other (Table 2.6). The response of antioxidant system to these storage temperatures in Japanese plums, which could possibly develop a relationship between the susceptibility to CI and oxidative

behaviour of fruit, has not been studied. Therefore, storage of Japanese plums at two different temperatures (0°C and 5°C) was carried out with the objective of elucidating the effect of storage temperature and storage duration on the changes in enzymatic and non–enzymatic antioxidants in relation to CI and fruit quality.

The potential storage/shipping life of Japanese plums is reduced to almost half by storage at 5°C compared to at 0°C (Crisosto et al., 2003; Crisosto et al., 1999). The data presented in Chapters 5 and 6 also show that storage life of 'Amber Jewel' plums was reduced to 2 weeks at 5°C compared to 3–4 weeks at 0°C. The accumulation of TBARS, a marker for lipid peroxidation, after 4 and 6 weeks of storage was significantly greater in fruit kept at 5°C compared with those at 0°C (Fig. 6.2B). The peak in LOX activity also appeared about 2 weeks earlier in fruit at 5°C than at 0°C (Fig. 6.2A). The response of SOD, CAT, and POD was significantly higher during the initial 2–4 weeks of storage at 5°C than at 0°C (Table 6.2). The fruit metabolism at 0°C could be slower than at 5°C as evident from the changes in fruit softening and skin colour (Table 6.1). Therefore, the production of ROS might be lower due to slower metabolism at 0°C than at 5°C, resulting in a weaker response of these enzymes at 0°C. The storage at 5°C might have resulted in increased ROS production than at 0°C which might have caused strong antioxidant enzymatic response at 5°C. The concentration of TBARS clearly shows that oxidative stress level after the first 4 weeks of storage was significantly greater at 5°C than at 0°C, and supports the viewpoint that more ROS production was occurring at 5°C. It appears that the up–regulation of SOD, CAT, and POD in response to fruit storage at 5°C compared with 0°C appeared not to be sufficient to encounter the increased ROS production and could not sustain for longer storage duration of 6 weeks.

Similar to primary antioxidant enzymes, the concentrations of AA and GSH were also retained better at 5°C than at 0°C (Tables 6.3 & 6.4). AA:DHA ratio suggested a more reductive cellular environment in the fruit at 5°C than at 0°C. The activities of various enzymes involved in recycling of AA and GSH were also favourable during storage at 5°C, indicating the successful operation of AA–GSH cycle at 5°C than at 0°C. However, the increased activity of GT at 5°C (Table 6.4) points towards the need for removal of lipid hydroperoxides produced as a result of greater lipid peroxidation (Fig. 6.2B). The higher levels of GT could be attributed to

the increased accumulation of GSSG and a lower GSH:GSSG ratio at 5°C than at 0°C.

The method of modified dual temperature (DT) storage, which involves the storage of fruit at –0.5°C for a maximum of 10 days followed by 18 days at 7.2 °C and then 14 days at 10°C, had been successful to reduce the incidence of CI in Japanese plums, but the fruit ripened and softened to a greater extent than single temperature (ST) (Taylor et al., 1994). The greater antioxidant response might be associated with DT storage, but it has never been investigated. Similar to storage at 5°C, DT storage also promotes flesh softening as a consequence of triggering of fruit ripening process at higher temperatures.

The data presented in chapter 6 suggest that the responses of enzymatic and non–enzymatic antioxidants to two storage temperatures were remarkably different in 'Amber Jewel' plums. The antioxidant response is generally triggered by the onset of stress conditions which result into initiation of cellular responses such as enhancement of antioxidant protection system through the regulation of genes affecting metabolic changes which may ameliorate the stress response (Toivonen, 2004). But the duration of stress is very important because if the stress continues, the antioxidant system may not be able to compensate for oxidative processes and damage may occur at cellular and subcellular levels. There is a possibility that storage at 5°C might have been perceived by the fruit as a stress condition which triggered the stronger antioxidant response to encounter the increased ROS production. However, the extended storage to 4 and 6 weeks might have resulted in accumulation of ROS to the toxic levels despite the higher activities of antioxidant enzymes causing oxidative stress.

It appears that the antioxidant response of 'Amber Jewel' fruit was not strong enough to prevent lipid peroxidation as the degree of lipid peroxidation was significantly higher at 5°C than at 0°C, particularly after 4 and 6 weeks of storage. The process of fruit ripening as evident from changes in skin colour, flesh firmness and loss of titratable acidity, was significantly faster at 5°C than at 0°C. These observations suggest that the storage temperature should be sufficiently low to control fruit ripening. It is not always necessary that postharvest conditions which promote the antioxidant system in fruit will yield the better storage life and fruit quality. The greater antioxidant response may sometimes not be strong enough to

quench the increased load of ROS due to prolonged stress conditions. The effects of other physiological processes such as fruit ripening may override the antioxidant system to limit the storage life of fruit. Further investigations are required to test the possibility of DT storage regime comprising of 7–10 days at 5°C followed by storage at 0°C.

10.5 Antioxidative response of Japanese plums to cold storage duration and shelf life

Chilling stress has been known to trigger the antioxidant response in fruits (Hariyadi and Parkin, 1991; Sala, 1998; Zhao et al., 2009). Chapters 5 and 6 have dealt with the development of CI during cold storage of 'Amber Jewel' cultivar of the Japanese plums, with regard to harvest maturity and storage temperature. However, studies have shown that the CI symptoms intensify during the SL period after cold storage in Japanese plums (Candan et al., 2008; Crisosto et al., 1999, 2004). Oxidative processes involved in the development of CI in the plant system are highly dynamic in nature. To better understand the development of postharvest oxidative stress in fruits, a multiple–point time course analysis of the antioxidant system has been suggested (Toivonen, 2004). Many researchers have undermined the importance of this factor in other fruits. The need to address this research gap in Japanese plums was realized and an experiment on 'Blackamber' plums was conducted. The results are presented in Chapter 7 providing information on the influences of storage period and subsequent SL on the development of CI and fruit quality in relation to lipid peroxidation, enzymatic and non–enzymatic antioxidants. The major objective was to expand the knowledge of development of oxidative stress during SL after each week of storage for 5 weeks so as to determine the critical point beyond which the antioxidant system collapses. The data from this experiment provided an insight into the relationship between the duration of storage, the build up of oxidative stress and the manifestation of CI in Japanese plums.

Post–cold storage physiological activity of 'Blackamber' plums was accelerated with the prolonged storage duration and is evident from the rates of respiration and ethylene production after 4 and 5 weeks (Fig. 7.1). Due to this physiological activity, the accelerated rates of fruit ripening were observed as shown

by faster rates of fruit softening (Fig. 7.2), skin colour changes (Table 7.1), and reduction in titratable acidity (Table 7.2). These observations provide evidence that a significant reduction in SL can be expected with the increase in cold storage duration beyond 3 weeks for 'Blackamber' plums. Symptoms of CI appeared after 3 weeks of storage with very low severity and incidence, and intensified with the increase in storage duration and SL. Even after 5 weeks of storage, the severity and incidence of these symptoms were rated low (20%–40% flesh area affected) and 35%, respectively. Therefore, based on the criterion of CI, the potential storage/market life of 'Blackamber' plums, under this experiment, has to be considered for 4 weeks.

The increases in activity of LOX and concentration of TBRAS were significantly greater during the last 2 weeks of cold storage and SL compared to the first 3 weeks (Fig. 7.4A & B). The higher levels of EL during the same period also indicate the increased membrane disintegration (Fig. 7.4C). Lipid peroxidation indicated by TBARS concentration and membrane degradation as marked by increase of EL could possibly be due to both chilling injury and fruit senescence (Du and Bramlage, 1995). The stimulation of SOD, CAT, and POD activities was observed immediately after 3 weeks of cold storage which might be to scavenge the increasing levels of ROS (Fig. 7.5). These activities could not sustain for the next 2 weeks. Furthermore, the activities of SOD, CAT and POD did not sustain at higher levels during the SL after 4 and 5 weeks as compared to previous weeks and the decreased activities were also coincident with the higher incidence of CI, particle after 5 weeks of storage. The correlation analysis also showed that the severity of CI was negatively correlated with the activities of SOD, CAT, and POD (Table 7.3). These observations were indicative of the declined capability of the primary antioxidant enzymes due to prolonged exposure to cold stress resulting in the expression of CI symptoms.

Contrary to 'Amber Jewel' cultivar (Fig. 5.6A), AA concentration did not decrease significantly during cold storage of 'Blackamber', rather a substantial increase in AA was observed in response to chilling stress (Fig. 7.6B). Post–cold storage APX activity sustained at higher levels (Fig. 7.7A), while the activities of MDHAR (Fig. 7.7B) and DHAR (Fig. 7.7C) declined significantly after 6 and 8 days of SL. A significant increase in AA and DHA also occurred during SL period after cold storage which illustrates the simultaneous occurrence of biosynthesis and

degradation of AA in 'Blackamber' plums (Fig. 7.6B & C). The increase in AA during fruit ripening and chilling stress has also been reported in tomatoes (Ioannidi et al., 2009; Jiménez et al., 2002a; Stevens et al., 2008). Further investigations are required to explore the variations among cultivars of Japanese plums with regard to AA metabolism.

The concentrations of GSH showed a significant decrease during SL after each week of cold storage and the level of decrease was significantly greater after 4 and 5 weeks of storage (Fig. 7.8B). The accumulation of GSSG continued during cold storage, but was aggravated after 4 days of SL following cold storage (Fig. 7.8C) which resulted in decreased GSH:GSSG ratio (Fig. 7.8D). The lower and higher activities of GR and GT, respectively, could be responsible for the decline in the redox potential of the glutathione pool (Fig. 7.9). The data indicate that the ascorbate pool in 'Blackamber' fruit was affected to lesser extent compared to the glutathione pool by the chilling stress. As the duration of storage and SL progressed, the redox status of the cell, based on the AA:DHA and GSH:GSSG ratios, was relatively more oxidized. It is also clear that the concentrations of total ascorbate and total glutathione, which are sometimes measured as the indicators of oxidative stress, may not present the actual redox potential of the cell.

The concentrations of total phenolics and DPPH scavenging activity generally showed a slight increase during the initial phase of SL after cold storage up to 3 weeks (Fig. 7.10). Such increases after 4 and 5 weeks were marginal and declined significantly towards the end of SL. The increase in concentration of total phenolics during storage or SL period could be due to activation of phenylpropanoid metabolism in response to chilling stress (Díaz–Mula et al., 2009; Galli et al., 2009; Sanchez–Ballesta et al., 2007). The phenolic compounds in co–operation with other antioxidants might have contributed to maintaining ROS at low levels, and thus protected the tissue from oxidative injury. The increase in storage duration accompanied by the decrease in total phenols and reduced efficiency of other antioxidative systems might have contributed to the build up of oxidative stress to potentially damaging levels resulting in increased severity of CI symptoms.

The data on the effects of storage duration and SL on lipid peroxidation, enzymatic and non–enzymatic antioxidants in Japanese plums showed that a strong relationship exists among three factors: storage duration, SL and CI. It is also

expected that the oxidative stress developed during SL might be the collective result of CI and fruit ripening as both involve oxidative processes. The multiple–point time course analysis was beneficial in determining the critical point of 3 weeks after which the capability of antioxidant system to cope with the increasing oxidative stress from CI and fruit ripening began to decline in 'Blackamber' plums.

10.6 Antioxidative response of 1–MCP–treated and –untreated Japanese plums to cold storage under controlled/modified atmospheres

Oxidative stress develops during postharvest fruit ripening, storage and SL of Japanese plums (Chapters 4, 5, 6, and 7). The experiments presented in the Chapter 8 were conducted with the objective of investigating the antioxidant metabolism in the fruit in response to 1–MCP treatment and storage atmospheres. Storage under CA/MA, 1–MCP treatment and a combination of both have been reported to exert several beneficial effects on fruit quality and alleviation of physiological disorders. CA/MA can limit the abundance of O_2 required for normal physiological activities of the fruit and can possibly also reduce the development of oxidative stress which was one of the bases of hypothesis in these experiments. Storage atmospheres differing mainly in O_2 concentrations (1.0%, 2.5%, and 10%) along with 3%–4% CO_2 (balance N_2) were achieved either by CA system or MAP and the regular air (O_2 = 20.8%) served as the control (Table 8.1).

The fruit ripening was greatly retarded in fruit stored under CA containing either 1.0% or 2.5% O_2 compared to those under MAP and regular air (Fig. 8.1). CA storage was effective in reducing the incidence and severity of CI after 5 weeks of storage, but its efficacy declined with the extension in storage duration to 8 weeks (Fig. 8.2). The pre–storage treatment with 1–MCP enhanced the beneficial effects of CA in 'Blackamber' plums and vice–versa. The retardation of fruit softening and loss of titratable acidity and reduction in incidence and severity of CI were among the greatest benefits derived from the combination of CA and 1–MCP (Fig. 8.9 & 8.10). 1–MCP–treated fruit had relatively lower incidence and severity of CI than the untreated fruit, irrespective of storage atmosphere. After 6 days of SL, the fruit quality in terms of flesh firmness and CI symptoms was much better in 1–MCP–

treated fruit kept under CA either for 5 or 8 weeks. The beneficial effects of the combination of CA and 1–MCP could be related to the better control of ethylene action in fruit.

The role of ethylene in development of CI in Japanese plums has been reported (Candan et al., 2008), but needs further investigations and evidence. The results are in agreement with previous findings on the effectiveness of 1–MCP in alleviating CI in climacteric type 'Larry Ann' and suppressed–climacteric type 'Angeleno' plums (Candan et al., 2008; Menniti et al., 2006). The inhibition of fruit softening by 1–MCP might have been achieved through the reduced activities of cell–wall degrading enzymes (Khan and Singh, 2008). The reduced rates of respiration and ethylene production in response to CA during cold storage and the residual effects of CA during SL might have contributed to the retention of fruit quality in 'Blackamber' plums (Maré et al., 2005). The data show synergistic effects of 1–MCP and CA in reducing the incidence and severity of CI and maintaining fruit quality in 'Blackamber' plums during 8 weeks of cold storage.

LOX activity was suppressed to some extent during cold storage under CA conditions, but the activity was up–regulated in response to fruit ripening during SL (Table 8.2). The suppression of LOX could be considered as an indication that the storage atmosphere was not significantly stressful. It has been demonstrated that LOX activity during postharvest storage can be triggered by chilling stress, storage atmospheres and a combination of both, which may enhance lipid peroxidation leading to initiation of certain physiological disorders and senescence–related processes (Ding et al., 2006; Larrigaudiére et al., 2001a; Larrigaudiére et al., 2001b; Mao et al., 2007; Rivera et al., 2007). 1–MCP treatment also had an inhibitory effect on the activity of LOX in Japanese plums (Table 8.6) as reported in loquat and pears (Cao et al., 2009b; Li and Wang, 2009). The regulation of ethylene production and action could be attributed to the 1–MCP treatment, resulting in suppression of LOX. It appears that LOX is also strictly regulated by ethylene in the Japanese plums as reported in other fruits such as apple and kiwifruit (Schaffer et al., 2007; Zhang et al., 2009).

The accumulation of lipid peroxidation product, TBARS, was also limited in 1–MCP–treated and untreated fruit held under CA conditions (Tables 8.3 and 8.6). The inhibition of lipid peroxidation could be attributed to reduced LOX activity and

oxidative stress under low O_2 atmospheres. It has been reported that lipid peroxidation can be inhibited by storage under CA (Hodges and Forney, 2000; Larrigaudiére et al., 2001b) and postharvest treatment with 1–MCP (Li and Wang, 2009; Singh and Dwivedi, 2008; Vilaplana et al., 2006). As a result of lower LOX activity and lower levels of TBARS, the membrane integrity was also maintained as indicated by the lower EL (Tables 8.3 and 8.6). These observations support the fact that the expression of CI symptoms was significantly lower in CA–stored fruit, especially those pre–treated with 1–MCP, compared to those kept under MAP or regular air.

Generally, CA was found effective to some extent in maintaining SOD activity slightly higher than in 1–MCP–treated and untreated fruit compared to those held in MAP and regular air during 5 and 8 weeks of cold storage and subsequent SL of 6 days (Figs. 8.3A & 8.11A). The higher SOD activities indicate the efficient removal of the O_2^- from the system and thus converting them into H_2O_2. The sustainable levels of CAT and POD activities were observed during storage under all atmospheres with a few exceptions and might have contributed to the prevention of accumulation of H_2O_2 to toxic levels (Figs. 8.3 & 8.11). The higher levels of activities of SOD and CAT have been reported during CA storage of pears and peaches compared to storage in normal air (Larrigaudiére et al., 2001b; Wang et al., 2005). The beneficial effects of 1–MCP in stimulating the primary antioxidant mechanism in some fruits such as apricot, mangoes, and pears have also been demonstrated (Larrigaudiére et al., 2004; Martino et al., 2006; Singh and Dwivedi, 2008). The effects of the combination of low O_2 atmospheres and 1–MCP appeared to be responsible for the efficient removal of ROS and thus reducing the levels of oxidative stress.

The ascorbate component of the AA–GSH cycle appears to be less affected by the storage atmosphere and more affected by the storage duration (Figs. 8.4 & 8.12). The influence of 1–MCP treatment also had no remarkable effect on the ascorbate component to bring forward conspicuous results (Fig. 8.12). The increase in storage duration from 5 to 8 weeks significantly decreased the concentrations of AA, irrespective of storage atmosphere and 1–MCP treatment. On the other hand, DHA accumulation occurred during the first 5 weeks of storage and continued during the SL period as well (Figs. 8.4C & 8.12C). However, a significant decrease in DHA

was noticed during the last 3 weeks of storage. The decrease in AA in response to cold storage under normal air and CA had been attributed to the development of internal browning disorders in apples and pears (De Castro et al., 2008; Franck et al., 2003; Galvis–Sánchez et al., 2006; Larrigaudiére et al., 2001b; Veltman et al., 2000; Veltman et al., 1999). The decrease in AA in CA–stored fruit might have been compensated by other components of the antioxidant defence to provide protection against the oxidative stress. In general, the retention of AA for maintaining fruit quality, nutritional value and preventing storage disorders is therefore of paramount importance.

The higher APX activity and lower activities of recycling enzymes, MDHAR and DHAR, could be primarily attributed to the losses of AA and increase in concentration of DHA (Figs. 8.6 & 8.14). The activities of MDHAR and DHAR either remained stable or increased in response to storage under different atmospheres. The higher activity of MDHAR was observed during SL period in CA–stored fruit which is in agreement with an earlier study on CA storage of spinach (Hodges and Forney, 2000). Ironically, the concentrations of AA and DHA can't be completely explained by the activities of enzymes involved in oxidation and recycling process. The role of AA as a single important antioxidant to provide protection against chilling and gaseous stresses is therefore questionable in Japanese plums.

The response of glutathione system to CA storage was relatively better than the ascorbate. The concentration of GSH decreased during cold storage with the increase in concentration of O_2 in the storage atmosphere, resulting in retention of higher levels of GSH in CA–stored fruit (Fig. 8.5). 1–MCP treatment was also effective in retaining the concentrations of GSH in fruit. The higher level of total glutathione in 'Conference' pears was also reported during the first 22 days of storage in CA (Larrigaudiére et al., 2001b), while another report indicated a decline in the concentration of glutathione in spinach during CA storage as compared to storage in normal air (Hodges and Forney, 2000). The data on 'Blackamber' plums suggest that CA storage was not helpful in maintaining AA components, whereas the GSH component was preserved to a greater extent under similar storage atmospheres. The response of these components to CA storage in Japanese plums is opposite to that of spinach (Hodges and Forney, 2000). The antioxidant potential has

been found to be enhanced by the 1–MCP treatment in loquat (Cao et al., 2009b), pears (Larrigaudiére et al., 2004; 2009), and mangoes (Singh and Dwivedi, 2008). The higher levels of GSH (Fig. 5.13) could be another effect contributing to some benefits to the non–enzymatic antioxidants in 'Blackamber' plums. An increase in GR (Fig. 8.15A) and slight reduction in GT (Fig. 8.15B) activities in response to 1–MCP treatment could be one of the factors contributing to the higher GSH levels.

The concentration of total phenolics was maintained at slightly higher level in CA–stored fruit compared to normal air and MAP for the first 5 weeks of storage, but showed a slight decrease during the last 3 weeks of storage (Fig. 8.8A). CA–stored fruit also showed a minor increase during the SL after 5 weeks of cold storage. 1–MCP treatment also had a positive effect on the retention of total phenols in the flesh tissue of 'Blackamber' plums (Fig. 8.16A). A significant positive correlation between the total phenolics and DPPH scavenging activity ($r = 0.90$) explains the similarities between the pattern of their changes during storage and SL. The combination of 1–MCP with any of the storage atmospheres did not yield much favourable results in terms of antioxidant activity (Fig. 8.16B). Previously, the total antioxidant activity in the skin tissues of 1–MCP treated 'Empire' and 'Delicious' apples was significantly higher than the untreated fruit (McLean et al., 2003). Recently, 1–MCP treatment and CA–storage have been shown to affect the phenols and antioxidant capacity of fruit inconsistently (Fawbush et al., 2009). It is clear from the data that CA storage for 5 weeks helps maintain the concentration of phenolics and DPPH scavenging activity in the flesh tissue of 'Blackamber' plums (Figs. 8.8 & 8.16). This is an evidence of better antioxidative system under low O_2 atmospheres to provide protection against the ROS. Further research is required to understand the effects of 1–MCP and CA storage on the concentration of phenolics and antioxidant capacity in various fruits in relation to development of storage disorders.

In conclusion, the postharvest oxidative stress in 'Blackamber' plums could be mitigated by storage under CA containing 1.0% O_2 + 3% CO_2 for 5 weeks. The prolonged cold storage for 8 weeks required the pre–storage treatment with 1–MCP to have synergistic effects of both CA (1.0% or 2.5% O_2 + 3% CO_2) and 1–MCP on the alleviation of oxidative stress. 1–MCP treatment alone was also effective in inhibiting lipid peroxidation and alleviating oxidative stress during cold storage for 5

weeks under regular air. MAP of 1–MCP–treated fruit did not provide significant protection against lipid peroxidation, resulting in unacceptable levels of CI symptoms. The beneficial effects derived from either CA or the combination of CA and 1–MCP resulted in retention of fruit quality and alleviation of CI in 'Blackamber' during cold storage for 5 and 8 weeks, respectively.

10.7 Antioxidative response of Japanese plums to postharvest fumigation with nitric oxide gas

The multifunctional roles of NO have been elucidated in plants, especially during biotic and abiotic stresses (Arasimowicz and Floryszak–Wieczorek, 2007). The signalling processes during stress conditions involve NO's cross–link with other plant hormones and signalling molecules (Arasimowicz and Floryszak–Wieczorek, 2007; Lamattina et al., 2003; Wendehenne et al., 2004). Earlier reports have shown the effects of NO in delaying fruit ripening and/or senescence processes in horticultural commodities (Eum et al., 2009; Flores et al., 2008; Leshem et al., 1998; Wills and Bowyer, 2003; Wills et al., 2000; Zhu et al., 2006; Zhu and Zhou, 2007). But there are a few reports on its effects on pro– and anti–oxidant components in fruits treated with NO (Duan et al., 2007a; Duan et al., 2007b; Fan et al., 2008; Flores et al., 2008; Li–Qin et al., 2009; Zhu et al., 2008). The use of NO has also been proposed among the postharvest procedures to alleviate oxidative stress in fruits (Toivonen, 2004). Therefore, the effects of postharvest NO fumigation on fruit quality, CI and oxidative behviour of Japanese plums during storage at ambient and low temperature were investigated. The data presented in Chapter 9 demonstrated the effects of NO in alleviation of oxidative stress in Japanese plums, resulting in enhanced SL and storability with reduced CI symptoms.

NO fumigation was beneficial in delaying fruit softening during storage at both ambient and low temperature conditions. The response of 'Amber Jewel' cultivar to NO fumigation in terms of retention of fruit quality was better than 'Blackamber'. The differential responses of these two cultivars might be due to differences in the rates of respiration and ethylene production; 'Amber Jewel' is moderately–climacteric and 'Blackamber' is highly–climacteric (Fig. 4.1). The lower doses of NO fumigation (5, 10 and 20 μL L^{-1}) might not have been sufficient to

inhibit the high rates of respiration and ethylene production in 'Blackamber' compared to 'Amber Jewel'. Further testing of higher concentrations of NO in 'Blackamber' cultivar may be required, provided the cytotoxic effects of higher NO concentrations should not reverse the protective effects gained with lower concentrations (Zhu et al., 2008).

The alleviation of CI symptoms in NO–fumigated Japanese plums could be attributed to the inhibition of ethylene production during cold storage of NO–fumigated fruit as reported in kiwifruit and peaches (Zhu et al., 2010; Zhu et al., 2006; Zhu et al., 2008). The inhibition of ethylene production and action during cold storage of Japanese plums has been shown to reduce CI symptoms (Candan et al., 2008). The cross–link of NO with other plant hormones including ethylene and abscisic acid has been reported to be complex and not fully understood (Arasimowicz and Floryszak–Wieczorek, 2007). The inhibition of ethylene production by NO fumigation could be related to one of the factors that are responsible for enhancing the chilling tolerance in Japanese plums.

Both cultivars of Japanese plums fumigated with 10μL L^{-1} NO showed a decrease in the rate of accumulation of TBARS during ambient and low temperature conditions (Figs. 9.3 & 9.12). However, in most cases, the differences in concentrations of TBARS among fumigated and non–fumigated fruit were non–significant after either 12 days at ambient or 7 weeks at 0°C plus 5 days at ambient. The data suggest that NO fumigation only reduced the rate of lipid peroxidation and might have enhanced the capacity of fruit to counteract the ROS in delaying the onset of oxidative stress. Therefore, the beneficial effects of NO on fruit quality diminished with the increased duration of storage/SL.

A clear trend in the activities of primary antioxidant enzymes, SOD, CAT and POD, did not emerge in response to NO fumigation in both cultivars of Japanese plums at both storage conditions. Only some general conclusions can be drawn from these results presented in Figure 9.4 and Tables 9.4, 9.5 and 9.6. The maintenance of higher activities of SOD and CAT in NO–fumigated fruit between 3 and 9 days of storage at ambient conditions could also be related to delayed fruit ripening and senescence in 'Amber Jewel' cultivar than in 'Blackamber' which showed a decreasing trend. The higher levels of CAT activity in fruit fumigated with 20 μL L^{-1} were observed after 5 days of SL following 5 and 6 weeks of cold storage. The

efficient removal of H_2O_2 by the action of CAT has been found to improve chilling tolerance in fruits (Sala, 1998; Sala and Lafuente, 2000).

The concentrations of total ascorbate and AA decreased during 12 days of storage at 21±1°C and were significantly influenced by NO fumigation , but a consistent trend was not observed (Fig. 9.5). On the other hand, fruit subjected to cold storage for 5, 6 and 7 weeks showed an increase in concentrations of AA and DHA during SL of 5 days at 21±1°C (Tables 9.8 & 9.9). The stimulation of AA biosynthesis as an acclimatization response may be expected to counteract the increasing oxidative stress occurring during the post–cold storage ripening. Fruit fumigated with 10 or 20μL L^{-1} NO showed higher AA:DHA ratio during some stages of storage at ambient conditions (Fig. 9.5D) and also after cold storage (Table 9.10) in both cultivars. These observations indicate the role of NO in the maintenance of redox potential of AA buffer in the fruit tissue.

NO fumigation did not significantly influence the changes in concentrations of GSH, GSSG and GSH:GSSG ratio during storage at 21±1°C for 12 days (Fig. 9.7). However, these results were significant in fruit kept in cold storage for 5, 6, and 7 weeks and subsequently held for 5 days at 21±1°C (Tables 9.15 to 9.17). The retention of higher concentration of GSH and higher GSH:GSSG ratio during cold storage in fruit fumigated with 10 or 20 μL L^{-1} NO also reflect the positive role of NO in maintaining the antioxidant potential of fruit. GR activity was not influenced by NO fumigation which is important in maintaining the concentration of GSH in the glutathione pool for efficient operation of AA–GSH cycle (Tables 9.18). Contrary to GR, the activity of GT was found to be significantly influenced by NO fumigation during cold storage (Tables 9.19) as the fruit fumigated with 20 μL L^{-1} NO showed significantly lower GT activity compared to other treatments. This could be related to the higher concentration of GSH in these fruit during cold storage because GT utilizes GSH as a substrate to detoxify lipid hydroperoxides accumulated as a result of increased lipid peroxidation (Rogiers et al., 1998).

The possibility of reaction of NO with GSH to form S–nitrosoglutathione can serve as a mobile reservoir of NO bioactivity or a S–nitrosylating agent (Feechan et al., 2005). The binding of a NO group to the thiol side chain of a cystein residue is involved in a large part of the almost ubiquitous influence of NO on the cellular signal transduction. The characterization of mechanisms regulating *S*–

nitrosylation/de–nitrosylation is still not completely understood in plants in relation to NO (Lindermayr et al., 2005). Studies on the role of NO in such mechanisms in fleshy fruits are lacking and warrant investigations.

In conclusion, postharvest application of NO (10 or 20 μL L^{-1}) has potential in delaying the fruit ripening and maintaining quality in Japanese plums for 9–12 days at 21±1°C. The effectiveness of NO at 21±1°C was significantly higher in 'Amber Jewel' cultivar than in 'Blackamber'. The alleviation of oxidative stress by NO fumigation was evident from the reduced rates of lipid peroxidation in fruit. NO fumigation with 20 μL L^{-1} was found useful in improving the storage potential of Japanese plums at 0°C through alleviation of CI symptoms for 5–6 weeks. The positive effects of NO fumigation on enzymatic and non–enzymatic antioxidants in addition to reduced lipid peroxidation might have resulted in enhancing the chilling tolerance in Japanese plums. More research is required to understand the mechanisms of action of NO gas in the climacteric and suppressed–climacteric type plums.

10.8 General conclusions

The factors affecting the development of oxidative stress in Japanese plums and strategies to combat it are shown in a generalised model (Fig. 10.2). The fruit ripening, delayed harvest maturity, storage at 5°C and duration of cold storage (0°C) for more than 2–3 weeks enhanced the rate of development of oxidative stress. The storage under CA, postharvest treatment with 1-MCP, a combination of CA and 1–MCP, and fumigation with NO can potentially mitigate the oxidative stress leading to better postharvest storability and retention of fruit quality with lower incidence and severity of CI.

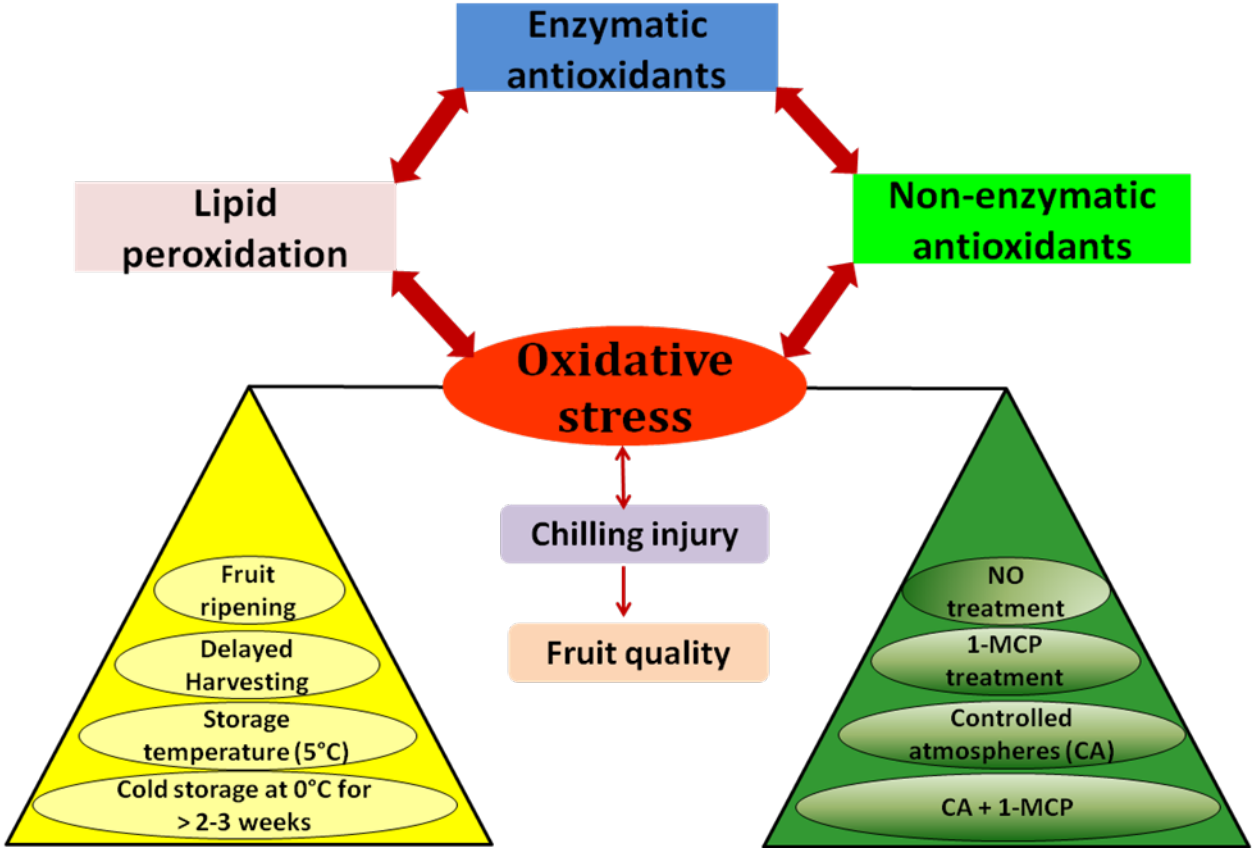


Fig. 10.2. A generalised model depicting the development of postharvest oxidative stress in Japanese plums. The cellular homeostasis in the fruit flesh tissue was disturbed by the postharvest factors in the yellow triangle and maintained by the treatments in the green triangle.

10.9 Recommendations to the industry

The experimental data suggest that the postharvest practices can potentially minimize the impact of oxidative stress on fruit quality and development of chilling injury in Japanese plums. Therefore, the following recommendations are made to the Japanese plum industry of Australia:

- The storage of 'Amber Jewel' plums harvested at commercial maturity may be considered safe for 2–3 weeks at 0°C. With the increase in storage duration beyond 2 weeks, the risk of chilling injury development may increase.
- The delay in harvesting of 'Amber Jewel' plums by one week after commercial maturity can potentially increase fruit softening and limit its storability. It may be practiced to supply fruit to the local markets, but the storage and marketing period should not exceed 2 weeks at 0°C.
- 'Blackamber' plums harvested at commercial maturity may be safely stored or transported for 3 weeks at 0°C with a potential SL of 6–8 days at 21±1°C. With the increase in storage duration beyond 3 weeks, the SL may be expected to decline along with the increased risk of chilling injury.
- Pre–storage treatment of commercially mature 'Blackamber' plums with 0.6 μL L^{-1} dose of 1–methylcyclopropene[6] (1–MCP) for 12 hours, as recommended by AgroFresh Inc.[7], has potential in increasing the storability and maintaining fruit quality during 5 weeks of cold storage at 0–1°C plus 6 days of SL at 21±1°C.
- The alleviation of CI and maintenance of fruit quality in 'Blackamber' plums can also be achieved by storage at 0–1°C under controlled atmospheres (CA) containing 1.0% O_2 + 3% CO_2 for 5 weeks, plus 6 days of SL at 21±1°C.
- The storage potential of 'Blackamber' plums can be further enhanced to 8 weeks at 0–1°C plus 6 days of SL at 21±1°C by the combination of pre–storage treatment with 1–MCP (0.6 μL L^{-1} for 12 hours) and CA containing 1.0% or 2.5% O_2 + 3% CO_2.

[6] 1–MCP is registered for postharvest use on Japanese plums in Australia.
[7] Use of company name does not imply endorsement of its products or criticism of one not mentioned.

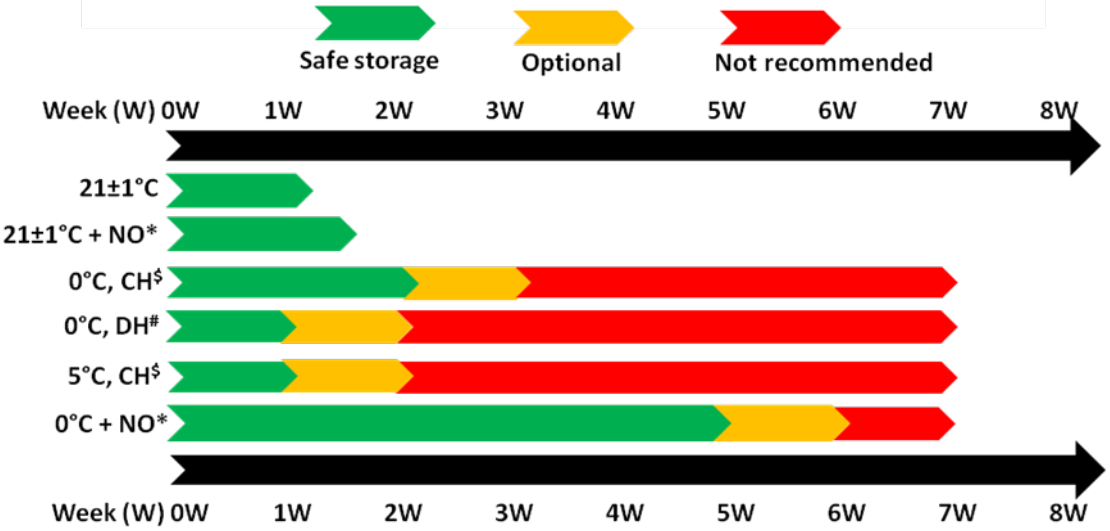
- Postharvest fumigation with nitric oxide (NO) gas[8] at 10 μL L^{-1} for 2 hours in air tight containers has potential in delaying fruit ripening in 'Amber Jewel' and 'Blackamber' plums during storage at ambient conditions (21±1°C). The additional SL of 3 days in fumigated fruit may be expected against the 8–9 days in control.
- NO fumigation (10 or 20 μL L^{-1}) may be recommended for the alleviation of chilling injury and retention of fruit quality in 'Amber Jewel' and 'Blackamber' plums during 5 or 6 weeks of cold storage at 0°C, plus 5 days of SL at 21±1°C.
- ***Disclaimer:*** *These recommendations are purely based on the laboratory experiments conducted under strictly controlled conditions. The discrepancies may arise in the outcomes under commercial situations. The project investigator, Sukhvinder Pal Singh, and Curtin University accept no liability whatsoever reason of negligence or otherwise arising from the reliance or use of these recommendations.*

[8] Nitric oxide is currently not registered for postharvest application in Japanese plums in Australia.

Fig. 10.3. Estimated Storage Potential of 'Blackamber' plums

*Postharvest fumigation with 20 µL L^{-1} nitric oxide (NO) gas
Controlled atmosphere storage (1 or 2.5% O_2 + 3% CO_2)
$ Postharvest fumigation with 1-methylcyclopropene (1-MCP; 0.6 µL L^{-1}) for 12 h

Fig. 10.4. Estimated Storage Potential of 'Amber Jewel' plums



*Postharvest fumigation with nitric oxide 10 or 20 µLL^{-1}
$ CH refers to commercial harvest maturity, as defined by DAFWA
# DH refers to delayed harvest maturity, one week after CH

10.10 Future research

- The comprehensive screening of Japanese plum cultivars for their enzymatic and nonenzymatic antioxidants is required to be conducted. This information may be very useful for developing biochemical markers for determining the potential storage life of various cultivars to be evolved through future breeding programs.
- The effects of preharvest practices such as orchard management, mineral nutrition, irrigation regimes and climatic conditions are required to be investigated for their potential roles in affecting the antioxidative metabolism of Japanese plums.
- The roles of postharvest factors such as heat and cold shock treatments in alleviation or aggravation of oxidative stress in Japanese plums need to be explored.
- The complex interactions among various plant hormones including ethylene and signalling molecules during postharvest oxidative stress are worthy of investigations in the future.
- The climate change effects such as increased surface–air temperature, spring frost, longer and drier summer, and increased risk of bush–fires would likely influence the antioxidative system of fruit and its potential postharvest storage life and quality. The future impacts of climate change on fruit quality of Japanese plums need to be investigated.
- The application of emerging tools of genomics, proteomics, metabolomics and computational biology can be beneficial to improve the understanding of the physiological development of disorders and biological basis of fruit quality in Japanese plums.

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