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Occurrence and Charactrisation of Superoxide Dismutases in the Female Reproductive Structures of Petunia

A thesis

submitted in partial fulfillment

of the requirements for the Degree

of

Master of Science in Plant Biotechnology

in the

University of Canterbury

by

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2006

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ABBREVIATIONS

ABA	Abscisis acid
ANOVA	Analysis of variance
APS	Ammonium persulphate
APX	Ascorbate peroxidase
Bis	N, N'- Methlene-bis-acrylamide
BSA	Bovine Serum Albumin
^{o}C	Degree Celcius
CAT	Catalase
CO_2	Carbon dioxide
Cu/Zn-SOD	Copper-zinc containing superoxide dismutase
DHA	Dehydroascorbate
DHAR	DHA reductase
DTT	DL-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
Fe-SOD	Iron-containing superoxide dismutase
Fd	Ferredoxin
GR	Glutathione reductase
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
JA	Jasmonic acid
K buffer	Potassium dihydrogen phosphate buffer
KCN	Potassium cyanide
Mn-SOD	Manganese-containing superoxide dismutase
MDA	Monodehydroascorbate
MDAR	MDA reductase
NaCl	Sodium chloride
NADPH	Nicotinamine adenine dinucleotide phosphate
NBT	Nitroblue trtrazolium

$(NH_4)_2SO_4$	Ammonium sulphate
Ni-SOD	Nickel-containing SOD
NO	Nitric oxide
NOX	Nicotinamine adenine dinucleotide phosphate oxidase
O_2	Dioxygen
O_2^-	Superoxide
OH	Hydroxyl radical
ONOO	Peroxinitrite
PAGE	Polyacrylamide Gel Electrophoresis
PVCV	Petunia vein clearing virus
PPVP	Polyvinylpyrrolidone
PSI	Photosystem I
PSII	Photosystem II
ROS	Reactive oxygen species
SA	Salicylic acid
SDH	Glacial acetic acid
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
tAPX	Thylakoid-bound APX.
TEMED	N-N-N’-N’-Tetra Methylethlenediamine
Tris	Trizma base
µ	Micro
UV	Ultraviolet

ABSTRACT

Superoxide Dismutase (SOD) activity in cell-free extracts prepared from healthy mature flowers of *Petunia hybrida* (variety 'Hurrah') was studied. The SOD activity in the crude extracts was stable for more than one month when stored at -20 ^{o}C. It was found that pH 7.8 is optimal for SOD activity.

Different flower tissues of petunia (stigma, style and ovary) at various stages of development were extracted and analysed for SOD activity. SOD activity was found to be significantly highest in the ovary tissue of dehiscent petunia flowers.

Three SOD isozymes were detected after crude extracts of the different female reproductive tissues of petunia flowers were analysed on a non-denaturing polyacrylamide gel electrophoresis system. Based on a difference in the sensitivity of the SOD isoforms to H_2O_2 and KCN, it is suggested that Mn-SOD, Fe-SOD and Cu/Zn-SOD were present in the crude extracts of the female reproductive tissues of petunia flowers.

The response of the female reproductive parts of petunia flowers was also tested under water deficiency and high temperature (35 ^{o}C) stress. The SOD activity seemed to increase more in response to the high temperature than the water deficiency stress.

Intense blue staining was observed from developing younger buds, and much lower formazan deposition was detected at the later stage. This indicates the lower O_2^- produced during later stages mainly due to increasing SOD synthesis.

DEAE cellulose chromatography was successfully used to partially purify SOD from the ovaries of petunia flowers. The characteristics of the partially purified enzyme fraction were found to be very similar to those of the crude extracts.

ACKNOWLEDGMENTS

I would not have been able to complete my research without help I needed from many people in different way. Firstly, my sincere thanks to my supervisor, Dr. David Leung, for giving me an opportunity to carry out this research under his supervision. He has been an encourager and cheerful supervisor to me, his enthusiasm, and help with all aspects of my thesis. I would also like to thank my associate supervisor, Dr Matthew Turnbull, for his constructive criticism of this work, also giving me valuable suggestions.

I would also like to thank Ms. Nicole Lauren, Mr. Matt Walters, and Mr. Dave Conder and Mr. Bruce Boon, for their technical assistance, photography and taking care of my plants during my study. Special mention goes to my co-workers Lily, Shugai, Ali, Greta, Moon, and everyone else in the Plant Biotechnology laboratory for their friendship and making the laboratory a pleasant place to work in. I had many precious memories, countless long list of kind helping hands and loving cares that I would treasure all my life.

Most of all, I am indebted to my family members and my friends. I am really pleased for their support, love, care and help. Without them, I would not have accomplished what I have done so far.

CHAPTER 1
INTRODUCTION

1.1 General Introduction

Oxidative stress is induced by a wide range of environmental factors including pathogen invasion (hypersensitive reactions), herbicide action, radiation, ozone, temperature fluctuations and oxygen shortage (Lee and Bennett 1982; Bowler et al. 1992; Scandalios 1993). There are three physiologically different states, which are transient hypoxia, anoxia and reoxygenation. All these can be distinguished by oxygen deprivation stress in plant cells. Generation of reactive oxygen species (ROS) is characteristic of hypoxia and especially of reoxygenation (Blokhina et al. 2003). In plants, the ROS (superoxide free radical and singlet oxygen) are commonly produced in illuminated chloroplasts by the occasional transfer of an electron from an excited chlorophyll molecule or photosystem I (PSI) components under conditions of high NADPH/NADP ratios to molecular O_2 (Scandalios 1993).

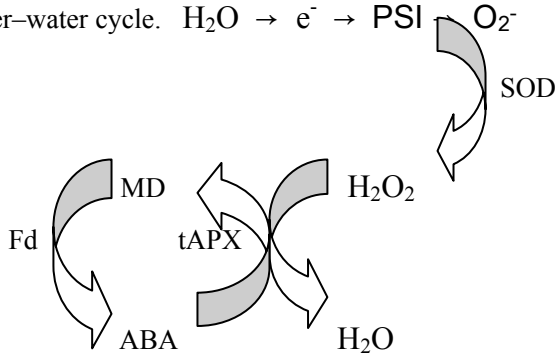
Aerobic organisms cope with ROS that are generated as products of enzymatic reactions or as accidental side products of cellular redox reactions. Traditionally, ROS were considered to be toxic by-products of aerobic metabolism. The toxic products of ROS such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) are produced in a number of cellular reactions, including the iron-catalysed Fenton reaction, and inhibition of enzymes such as lipoxygenases, peroxidases, NADPH oxidase and xanthine oxidase (Blokhina et al. 2003). ROS can cause oxidative damage to many cellular components, which are lipids (for example, peroxidation of unsaturated fatty acid in

membranes), proteins (denaturation and oxidation), carbohydrates and nucleic acids. Oxidative damage may result from the alteration of the balance between the production of ROS and their detoxification by the antioxidative systems. According to the properties of ROS, plant cells require at least two different mechanisms to regulate their intracellular ROS concentrations by scavenging of ROS: one that will enable the fine modulation of low levels of ROS for signalling purposes, and one that will enable the detoxification of excess ROS, especially during stress conditions (Mittler 2002).

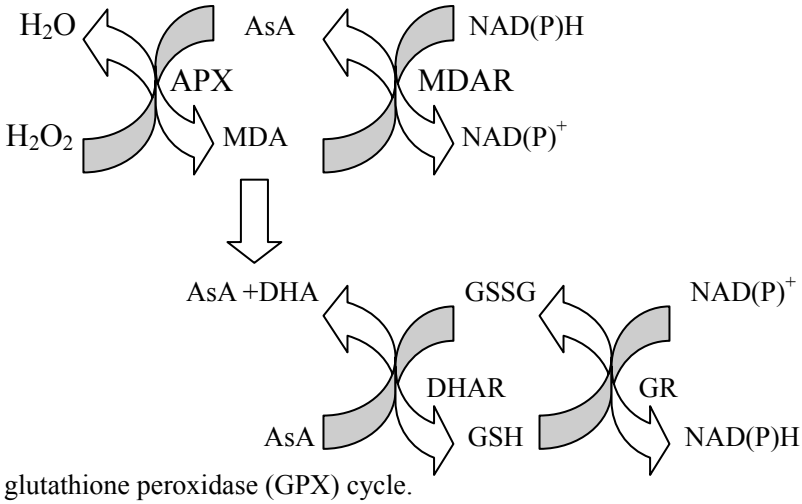
To prevent oxidation of cellular components, cells maintain low steady state levels of ROS by a variety of enzymatic and nonenzymatic antioxidant mechanisms (Larson 1988; Asada and Takahashi 1987). Major ROS-scavenging in plants by a protective system of enzymes includes superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) (Asada and Takahashi 1987; Bowler et al. 1992). The pathways of ROS-scavenging in plants including SOD are found in almost all cellular compartments, the water-water cycle in chloroplasts, the ascorbate-glutathione cycle in chloroplasts, cytosol, mitochondria, apoplast and peroxisomes, glutathione peroxidase, and CAT in peroxisomes (Fig.1). This cycle uses electrons directly from the photosynthetic apparatus (a) or NAD(P)H (b,c) as reducing power (Mittler 2002). The upstream enzyme SOD acts as the first line of defense system converting superoxide (O_2^-) into hydrogen (H_2O_2) and dioxygen (O_2), to protect cells against the oxidative damages. Since superoxide radicals are found to be toxic to living cells, oxidizing and degrading biologically important molecules, the observations on SOD activity in different plant species suggest that different mechanisms may be involved in oxidative stress injury. The biological importance of SOD has been examined. This has mainly been done in relation

Fig 1.1Pathways for reactive oxygen species (ROI) scavenging in plants.(Mittler 2002) Superoxide dismutase (SOD) acts as the first line of defense converting O_2^- into H_2O_2. Ascorbate peroxidases (APX), GPX and CAT then detoxify H_2O_2. In contrast to CAT (d), APX and GPX require an ascorbate (AsA) or a glutathione (GSH) regenerating cycle (a–c).
Abbreviations: DHA, dehydroascorbate; DHAR, DHA reductase; Fd, ferredoxin; GR, glutathione reductase; GSSG, oxidized glutathione; MDA, monodehydroascorbate; MDAR, MDA reductase; PSI, photosystem I; tAPX, thylakoid-bound APX.

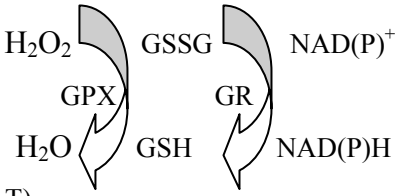
(a) The water–water cycle. $H_2O \rightarrow e^- \rightarrow PSI \rightarrow O_2^-$



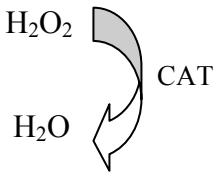
(b) The ascorbate–glutathione cycle.



(c) The glutathione peroxidase (GPX) cycle.



(d) Catalase (CAT).



to response to stress and in the field of biotechnology. It was investigated as a potential therapeutic agent in diseases related to oxidative stress and for its role in moderating the aging process.

1.2 Oxidative Stress in Living Organisms

1.2.1 ROS Formation

Oxidative stress is a general term used to describe the steady state level of oxidative damage that can affect a specific molecule in a cell, tissue, or organ, caused by the ROS. The production of ROS, such as O_2^- and H_2O_2, is an unavoidable consequence of aerobic metabolism. ROS are free radicals, which are atoms or groups of atoms that have at least one unpaired electron. They are highly reactive and promote oxidation that is beneficial because it produces energy and kills bacterial invaders (Frei 1994). However, this is an extremely unstable configuration, and radicals quickly react with other molecules to create more free radicals, because electrons like to pair up to form stable two-electron bonds (a landmark discovery made many decades ago by Linus Pauling, culminating in his Nobel Prize in Chemistry in 1954). ROS may play two different roles: exacerbating damage and signalling the activation of defense responses (Dat et al. 2000; Vranova et al. 2002).

ROS are formed by several different mechanisms. Firstly, biological interaction of ionising radiation can occur with molecules of all aerobic system. Secondly, they can be toxic by-products of cellular respiration, for example, when some electrons are passing through the respiratory chain, some might leak away from the main path and go directly to reduce dioxygen to superoxide anion. The dismutation of two superoxide anions produces H_2O_2, which is also a product of the microbody-associated β-oxidation of fatty acids and peroxisomal

photorespiration reactions (Tolbert 1982). Another type of formation is synthesised by dedicated enzymes in phagocytic cells, like neutrophils and macrophages, which kill some types of bacteria. For example, xanthine oxdiase, aldehyde oxidase, and other flavin dehydrogenases are capable of generating superoxide as catalytic by-products (Fridovich 1998; Tolbert 1982). ROS arise normally during metabolism and all living organisms can handle them under some circumstances. However, if the ROS production becomes excessive, damage can occur.

The oxygen taken by living organisms is converted to O_2^-, H_2O_2, and hydroxyl radical (OH^-) and other molecules by various enzymatic metabolism systems. OH^- and O_2^- have the shortest life spans. OH^- has the highest reactivity and it reacts with metal ions and nitric oxide (NO) to quench the physiological activities such as vascular relaxation. Meanwhile, O_2^- generates peroxinitrite (ONOO) that causes oxidative damage, and it is transformed into OH^- to react indirectly with lipids, proteins and nucleotides.

Besides normal conditions of metabolic mechanisms for generating ROS production, various environmental perturbations (e.g. intense light, drought, and temperature stress) can cause excess ROS, overwhelming the system and necessitating additional defenses (Scandalios 1993). For instance, stomatal closure resulting from drought conditions limits CO_2 availability for photosynthetic carbon assimilation, therefore the excessive superoxide production in the chloroplast can cause photoinhibition and photooxidation damage. Plants produce ROS via oxidase enzymes when attacked by pathogens (the oxidative burst). ROS also initiate signal transduction cascades that activate defense-related gene expression along with varied input from other signalling molecules, such as salicylic acid (SA), nitric oxide (NO) and

jasmonic acid (JA) (Smirnoff 2005). Plasma membrane localised nicotinamine adenine dinucleotide phosphate oxidase (NOX) enzymes that generate extracellular superoxide are implicated in many mechanisms.

1.2.2 Sources of ROS

ROS are produced in significant quantities in various subcellular compartments or organelles (Table 1). There are many different sources from which the ROS are generated. Most of them come from endogenous sources as by-products of normal and essential metabolic reactions: energy generation from mitochondria, photosynthesis and respiration are considered to be the main sources of ROS (Mittler 2002). Other sources of ROS have been discovered in recent years that belong to pathways enhanced during abiotic stresses, such as glycolate oxidases, amino oxidases and cell-cell-bound peroxidases, which are tightly regulated and participate in the production of ROS during processes such as programmed cell death and pathogen defense (Hammond-Kosack and Johns 1996; Grant and Loake 2000).

Under normal growth conditions, the production of ROS in cells is low (240 μM S^{-1} O_2^- and a steady-state level of 0.5 μM H_2O_2 in chloroplasts) (Polle 2001). However, under environmental stress situations, the cellular homeostasis of cells can be disrupted in response to an elevated production of ROS (240-720 μM S^{-1} O_2^- and a steady-state level of 5-15 μM H_2O_2 in chloroplasts) (Polle 2001). The production of ROS by a plant during stress results from pathways such as photorespiration, from the photosynthetic apparatus and from mitochondrial respiration. In addition, pathogens and wounding or environmental stresses (e.g. drought and osmotic stress) have

Table 1.1 Subcellular localisation of ROS and antioxidant enzyme system in plants.

Subcellular localisation	Primary ROS	Sources of ROS	Enzymatic Scavenging Systems	Products	References
Chloroplast	O_2^- H_2O_2	PSII Enzymic Excited chlorophyll	SOD Ascorbate peroxidase	H_2O_2 Dihydroa-scorbate GSH $NADP^+$	Asada and Takahashi (1987) Hammond-Kosack and Johns (1996) Grant and Loake (2000) Munné-Bosh and Alegre (2002)
Mitochondria	O_2^- H_2O_2	Respiration electron transport Enzymic	Peroxidase SOD CAT	H_2O H_2O_2 H_2O,O_2	Mittler (2001) Dat et al. (2000)
Cytosol	O_2^- H_2O_2	Enzymic	Peroxidase SOD CAT	H_2O H_2O_2 H_2O, O_2	Asada and Takahashi (1987) Noctor and Fpyer (1998)
Glyoxysome and peroxisome	H_2O_2	Fatty acid β-oxidation Photorespiration Enzymic	CAT	H_2O, O_2	Corpas et al. (2001) Willekens et al. (1997)
Plasma membrane	O_2^-	Enzymic	SOD	H_2O_2	Hammond-Kosack and Johns (1996) Grant and Loake (2000)
Cell wall	O_2^- H_2O_2	NADH and Mn^{2+} Enzymic	SOD CAT	H_2O_2 H_2O, O_2	Hammond-Kosack and Johns (1996) Grant and Loake (2000)
Apoplast	H_2O_2	Enzymic	CAT	H_2O, O_2	Corpas et al. (2001)

been shown to trigger the active production of ROS by NADPH oxidases (Hammond-Kosack and Johns 1996; Orozco-Gardenas and Ryan 1999; Pei et al. 2000). Therefore, ROS can be considered as cellular indicators of stresses and as secondary messengers involved in the stress-response signal transduction pathways. The determination of the steady levels of ROS can be changed drastically depending upon the physiological condition of the plant and the integration of different environmental, developmental and biochemical stimuli (Mittler 2002).

Besides many different endogenous sources inside cells, tissues and organs, there are also some exogenous sources of ROS that can cause damage to living organisms. They include exposure to environmental pollutants such as emission from automobiles and industries, exposure to ionizing radiation, and bacterial, fungal or viral infections.

1.2.3 Aging and Oxidative Stress

Of particular importance is that ROS damage accumulates with aging for all organisms. Aging is an inevitable biological process that affects most living organisms. For the past two decades, many investigations have been carried out directly toward the basic understanding of biological aging mechanisms and possible aging interventions (Byung 1996). These have led to new insights into the molecular bases and the biological events that contribute to age-related deterioration.

The first step to understanding the basic mechanisms involved in biological aging is of differentiating the biological aging process from age-related disease

processes. In general, aging of human and animals is characteristically described as a time-dependent functional decline, which leads to incapacity of cells to withstand internal and external challenges (Byung 1996). The factors that cause aging process have not been well defined yet. However, two interdependent biological processes were considered as consequences of aging: one is the loss of functionality and the loss of resistance or adaptability to stress (Lim 2000).

Scientists have discovered that free radical actions can damage molecules they react with and sometimes cause the cell's damage. Oxidative stress has been linked to premature aging and cited as a performance-limiting factor in physical activity (Byung 1996). Some evidence indicates people with mental illness may be under oxidative stress, which may lead to a loss of some types of fatty acid from the brain, leading to problems in brain function and ultimately disturbances in the person's behavior (Lim 2000). The antioxidant defense systems in the human body are extensive and consist of multiple layers, which protect at different sites and against different types of ROS.

1.2.4 Plant Aging Increases with Oxidative Stress

Aging has been classically defined as the accumulation of changes in plant development responsible for slow, progressive, and sequential alterations that accompany the plants as they age (Harman, 1991). However, little is known about the relationship between oxidative stress and plant aging, and the regulatory mechanisms responsible for the aging not associated with senescence in plants.

Plants maintain the capability to develop new leaves and grow throughout their lives until they become a developmentally controlled senescing process that inevitably leads to plant death (Munné-Bosch and Alegre 2002). Mitochondria are regarded as the target organelles for oxidative stress in age-related changes observed in animals and plants, whereas the role of chloroplasts can also be as targets of the oxidative stress associated with aging in plants. Some relevant plant-specific features of the mitochondrial electron transport chain are described as main sites of ROS production (Möller 2001). The age-dependent increase in oxidative stress in plants also possibly occurs in chloroplasts, because chloroplasts are the organelles most exposed to oxygen toxicity according to their function under high oxygen and in the light. The consequence of plant aging is a slow progressive process, which does not immediately lead to death, either in cells, leaves and whole plant, but oxidative stresses can cause plant aging before normal senescence occurred (Munné-Bosch and Alegre 2002).

1.2.5 ROS at the Interface between Biotic and Abiotic Stresses

ROS play a central role in the defense of plants against pathogen attack. Therefore, they are produced by plant cells via enhanced enzymatic activity (e.g. NADPH oxidase, amine oxidase and glycolate oxidase) and resulted in the over-accumulation of ROS and the activation of programmed cell death. H_2O_2 produced as a result of SOD can diffuse into cells to activate many of the plant defenses. The over-accumulation of ROS diminishes the capacity to scavenge H_2O_2 and activates cell death in plants. The activity of enzymes of plant defense systems is suppressed by the plant hormones salicylic acid and nitric oxide (Klessig et al. 2000).

The differences in the functions of ROS between abiotic and biotic stresses might result from the action of molecules such as salicylic acid and nitric oxide, and from cross-talk between different signalling pathways or from differences in the steady-state level of ROS produced during the different stresses. The question is how plant manipulates its rate of ROS production and ROS scavenging when it comes under biotic attack during an abiotic stress. Tobacco plants that were subjected to oxidative stress had a reduced rate of programmed cell death compared with unstressed control plants (Mittler et al. 2001).

1.2.6 ROS-scavenging System-Defense Mechanism of ROS

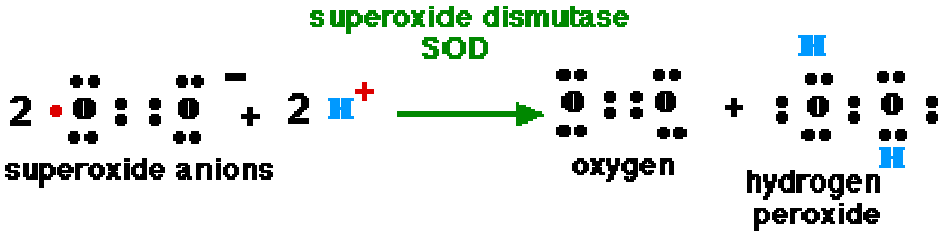
Both enzymic and nonenzymic defense mechanisms have emerged to protect cells against oxidative injury. The protective system of enzymes such as SOD, APX and CAT is a major ROS-scavenging mechanism of plants (Asada et al.1987; Bowler et al. 1992; Willekens et al. 1997). Those enzymes are capable of removing, neutralising, or scavenging free radicals and ROS. The balance between SOD and APX or CAT activities in cells becomes crucial for determining the steady-state level of ROS (Bowler et al. 1991). SOD acts as first line of defense to catalyse the dismutation of superoxide radicals into molecular oxygen and hydrogen peroxide. Then, APX and CAT remove H_2O_2 very efficiently. SODs and CATs are most important, efficient antioxidant enzymes in cells. Their combined action converts the potentially dangerous O_2^- and H_2O_2 to water and molecular oxygen, thus averting cellular damage. Both the mitochondrion and the chloroplast contain ROS-scavenginng mechanisms. The oxidative stresses cause the proliferation of peroxisomes, which might be highly efficient in scavenging of ROS, especially H_2O_2 (Mittler 2002).

Antioxidants such as ascorbic acid and glutathione, which are found at high concentrations in chloroplasts and other cellular compartments, are crucial for plant defense against oxidative stresses (Noctor et al. 1998). Maintaining a high reduced per oxidised ratio of ascorbic acid and glutathione is essential for the proper scavenging of ROS in cells (Conklin et al. 1996). In addition, the overall balance between different antioxidants has to be tightly controlled as they prevent oxidative damage from spreading, e.g. by interrupting the radical chain reaction of lipid peroxidation. Other antioxidants include GSH, cysteine, hydroquinones, mannitol, vitamins C and E, flavonoids, some alkaloids, and β-carotene (Larson 1988).

1.3 Superoxide Dismutase

1.3.1 Introduction

The enzyme superoxide dismutase (SOD) catalyses the breakdown of superoxide anion (O_2^-) and provides the first line of defense against oxygen toxicity. It is a type of metalloprotein catalysing dismutation of O_2^- to molecular oxygen and H_2O_2 (Giannopolitis and Ries 1977). The following figure shows one common series of reactions.



SOD is one of the most important enzymes used against oxidative stress in defense system, and an endogenously produced intracellular enzyme present in essentially every cell of living organisms. It has the distinct capability to

neutralise superoxide anions.

The first isolated SOD (EC 1.15.1.1) was from bovine blood. It is a green copper protein, and its biological function was believed to be copper storage (Scandalios 1993). The catalytic function of the enzyme was discovered by McCord and Fridovich in 1969. It is also a factor that controls an organism's life-span and is known as a stable enzyme because of its β-barrel structure with a low content of œ-helix structure (Khanna-Chopra and Sabarinath 2004). Therefore, research on SOD activity and properties will be essential for understanding of various mechanisms of a living organism.

1.3.2 Classification (Isozymes)

All SODs, irrespective of source, are mutlimeric metalloproteins that are very efficient at scavenging superoxide radicals. Several types of SODs (isoforms) have been reported, based on the metal species present at the active site.

There are at least three forms of SOD in nature. The prevalent one is copper-zinc containing superoxide dismutase (Cu/Zn-SOD), which is found in chloroplast, cytoplasm and probably in the extracellular space. It is a very stable dimeric protein with a molecular mass about 32,000Da and the two subunits are joined by a disulfide bond. The amino acid sequences of Cu/Zn-SODs have been determined showing that the metal-binding sites are highly conserved (Scandalios 1993). The thermostability of this SOD isozyme from *Chenopodium murale in vitro* has also been demonstrated. Constitutive chloroplastic Cu/Zn SOD present in the leaf protein extract was more resistant to heat treatment at 70ºC (Khanna-Chopra and Sabarinath 2004). The minor

class of SOD has manganese at the catalytic site which is principally destined for the mitochondrial and peroxisomes. These two classes of SOD are widely distributed in the plant world. Another is iron-containing superoxide dismutase (Fe-SOD) that is abundant in the plastids. It has so far been identified in only a few species of plants, but not been found in animals or fungi (Asada et al. 1980; Van Camp et al. 1990). Generally, eukaryotes contain a Mn-SOD in the mitochondria and a Cu/Zn containing enzyme in the cytosol. Prokaryotes possess the manganese form and/or the iron form. The Fe- and Mn-SOD are very similar in their primary, secondary and tertiary structure, whereas the Cu/Zn-SOD is unrelated to these structures (Bowler et al. 1989).

In addition, a novel nickel-containing SOD (Ni-SOD) has recently been found in *Streptomyces griseus* and *S. coelicolor* (Hernández et al. 1996). This type of SOD is composed of four identical subunits of 13.4kDa, stable at pH 4.0-8.0 and up to 70ºC (Youn et al. 1996).

The three main types of SOD are distinguished based on a difference in their sensitivity to inhibitors, KCN and H_2O_2. Cyanide reversibly inhibits the enzyme, whereas H_2O_2 causes irreversible inhibition (Scandalios 1993). Cu/Zn-SOD is sensitive to KCN, while Fe-SOD and Mn-SOD are insensitive. Fe-SOD is sensitive to H_2O_2, but Mn-SOD is not. The activity of Mn-SOD is not inhibited either by H_2O_2 or KCN. Therefore, the sensitivity of Cu/Zn-SOD to cyanide has been used as a diagnostic tool to distinguish Cu/Zn-SOD from Fe- and Mn-SOD that are unaffected by cyanide. As for the new discovery of Ni-SOD, it was found to be inhibited by cyanide and H_2O_2, but slightly inhibited by azide (Youn et al. 1996).

1.3.3 SOD and Relevant Research Fields

SOD activity is of interest in all research fields related with living organisms, particularly the involvement of ROS and SOD as far as plants, human and food are concerned.

1.3.3.1 Plants

Plants have highly sophisticated defense mechanisms toward environmental alterations because they cannot move around freely. The activity of SOD of a plant is increased by drought, the use of herbicides and exposure to high concentrations of metals, which cause ROS to be generated in the plant (Möller 2001; Bowler et al. 1992; Scandalios 1993). The induction of SOD in response to the diverse environmental conditions indicates that it plays an important role in the defense mechanism of plants. The reduction in the toxicity of O_2^- is a very important defense system against oxidative stresses. Therefore, the role of SOD assay techniques is very crucial in the study of plant physiology.

1.3.3.2 Human and Animals

In order to protect the body from highly toxic ROS, the anti-oxidative stress mechanisms including SOD are localised in tissues and cells of the human body. If the amount of ROS exceeds the limit of the defense mechanism of the body for any reason, serious disease such as cancer may be induced. Furthermore, cell damage by ROS is considered to be one of the main causes of various aging-related diseases and the use of radical trapping agents as a possible cure for some diseases is being studied (Noor et al. 2002).

Additionally, SOD activity determination will be utilsed not only for the research of the mechanisms that cause diseases but also for the diagnosis and indication of health condition.

1.3.3.3 Food

The findings suggest that the risk of ROS-related diseases is decreased by reinforcement of the defense mechanism against oxidative stress. Many researchers are interested in the anti-oxidation qualities of plants and their products, for example, red wine and tea. It is thought that there are antioxidants in plant materials that can protect heart disease and cancer as they can induce O_2^- quenching activities, similar to those of SOD (Barrett 2003). It is also suggested the best way to ensure adequate intake of the antioxidant-type nutrients is a balanced diet consisting of 5-8 servings of fruits and vegetables per day.

1.3.4 SOD and Stress Tolerance

In plants, adaptation to environmental changes is crucial for plant growth and survival; environmental diversity can lead to the increased generation of ROS. Consequently, SOD has been proposed to be important in plant stress tolerance. Observations on SOD activity in different plant species under several stress conditions (drought, salinity, heavy metals and high/low temperature) suggest that different mechanisms may be involved in oxidative stress injury (Yu et al. 1999a).

1.3.4.1 Photoinhibition

It has been shown that overexpression of SOD can protect plants from oxidative stress (Sen Gupta et al. 1993; Van Camp et al. 1996). The disturbances of free radicals can be caused by conditions of photoinhibition, in which the absorbed light energy exceeds the capacity of the photosystems to direct it through photosynthetic electron transport. The high light intensities lead to a reduction of photosynthetic capability of plants. For instance, SOD had greater resistance to photooxidative damage and to methyl viologen-mediated oxidative stress in the leaves of transgenic tobacco than controlled plants (Sen Gupta et al. 1993). The overexpression of tomato Cu/Zn-SOD in transgenic potato plants has been shown to enhance protection from methyl viologen toxicity (Perl et al. 1993). A biotype of *Conyza bonariensis* possessing elevated levels of chloroplastic SOD, glutathione reductase, and ascorbate peroxidase was reported to be resistant to photoinhibitory light (Jansen et al. 1989). Injury resulting from the combination of light with temperatures (chilling and heat shock) has different patterns of SOD induction, thus implying the mechanisms of photoinhibition are different for each case.

1.3.4.2 Waterlogging and Drought

Water stress not only induces abscisis acid (ABA) accumulation as a stress signal, but also causes an enhancement in the generation of ROS and antioxidant defense enzyme activities (Smirnoff 1993; Bartoli et al. 1999). Waterlogging imposes an oxygen shortage on submerged plant organs and plants respond by altering its pattern of protein synthesis. Drought stress is a complex phenomenon that appears to involve the synthesis of polyamines and

a new set of proteins whose function is unknown (Caplan et al. 1990). Increasing ABA stimulates stomatal guard cells to close, reducing water loss and creating ROS. Chloroplastic and cytosolic Cu/Zn SOD activity increased in pea subjected to water deficit, while osmotic stress enhanced Mn-SOD transcript abundance in maize (Iturbe-Ormaetxe et al. 1998; Zhu and Scandalios 199). Activation of oxygen may proceed through different mechanisms, not necessarily producing a substrate for SOD. For example, waterlogging stress did not affect SOD activity, while a significant increase was detected under drought conditions in leaves of three contrasting wheat genotypes (Sairam et al. 1998).

1.3.4.3 Herbicides

Paraquat and other herbicides that directly affect chloroplast activity can stimulate processes that induce ROS and block photosynthetic electron transport. They can also act by inhibiting synthesis of carotenoids such as aminotriazole and fluridone (Halliwell 1987). Paraquat is a redox-active compound that is photoreduced by PSI and subsequently reoxidised by transfer of its electrons to oxygen to form ROS (Asada and Takahashi 1987). The effects of paraquat on the endogenous SOD enzymes in illuminated plants have been studied in several cases. For example, treatment of *Phaseolus vulgaris* and lemna leaves caused a general increase in SOD activity (Clare et al. 1984; Srivastave and Tel-Or 1991). In *Nicotiana plumbaginifolia*, chloroplastic, cytosolic, and mitochondrial SOD expression was anyalysed at mRNA level, all three were strongly induced by paraquat, but the cytosolic Cu/Zn-SOD was the least affected (Tsang et al. 1991). Increases in SOD activity have been observed in response to treatment with herbicides that serve as terminal

electron acceptors to block electron transportation (Matters and Scandalios 1986).

1.3.4.4 Temperature

Temperature stress is a typical stress plants receive from their surroundings. The range of temperatures varies experienced by plants at different scales both spatially and temporally. Each plants species has its own optimal temperature for growth by the temperature zone in which it can be survived. Global warming that result from atmospheric concentrations of CO_2 and other greenhouse gasses have been considered to affect agriculture and forests. Therefore, understanding of adaptation of plants under temperature stress conditions is very beneficial for future prospects.

The amount of ROS in plants increases when they are exposed to low temperature or high temperature, and other stresses (Asada 1997). SOD activity in the leaves and roots under stress conditions combined with cold acclimation of cold-resistant and control wheat cultivars was unaffected by the low temperature treatment but plants exhibited higher guaiacol peroxidase activity (Scebba et al. 1998). When tobacco plants are chilled under normal lighting condition, Fe-SOD was induced, followed by Cu/Zn-SOD induction (Tsang et al. 1991). Chloroplastic Cu/Zn-SOD in transgenic tobacco has been linked to the improvement of resistance to intense light and low temperature (Ashima et al. 1993), whereas transgenic alfalfa expressing Mn-SOD and Fe-SOD showed improved resistance to low temperature (Mckersie et al. 1999).

1.3.4.5 Atmospheric Pollutants

Industrialisation has considerably increased the concentrations of harmful chemicals such as ozone and sulfur dioxide in recent decades. The sensitivity of plants varies greatly between species and with environmental conditions. The main consequence of these pollutants is reduction of photosynthetic activity, whereas respiration is often increased. Lipid peroxidation similar that occurring during senescence has been found in plants following ozone fumigation (Pauls and Thompson 1984). The participation of SOD has been examined in different plant species. Ozone treatment of spinach leaves induced SOD, peroxidase, and CAT, whereas enhanced SOD and CAT activities in snap beans were correlated with ozone resistance (Lee and Bennett 1982). Evidence for the involvement of oxygen radicals in SO_2 toxicity is more substantial, which its damage to biological systems is probably a result of radicals generated during its oxidation to sulphate (Asada and Takahashi 1987). Plants varieties with differing sensitivities to atmospheric pollutants provide a valuable tool for identifying the mechanism of phytotoxicity.

Consequently, the ability of plants to overcome oxidative stress only partly relies on the induction of SOD activity and other factors can regulate the availability of the substrate for SOD.

1.3.5 Mechanism of SOD Regulation

It is clear that SOD plays an important role in ROS-scavenging system in plant kingdom. SOD activity is induced by diverse environmental stress conditions, so that these stresses can be mediators of SOD gene regulation. The expression

of different forms of SOD is based on the metal present at the active site within different subcellular compartments. Then, the effects of a particular stress on SOD gene expression is likely to be governed by the subcellular sites at which oxidative stress is generated. For instance, Mn-SOD responses to increased free radical formation in mitochondia while Fe-SOD responses to such an event occurring in the chloroplast in *N. plumbaginifolia* (Bowler et al. 1989; Tsang et al. 1991). If the signalling factor regulating each class of SOD were generated in specific compartments, specific regulation could be achieved (Bowler et al. 1992).

1.3.6 Function of SOD in the Development of a Plant

The biological role and importance of SOD as protective enzymes against ROS toxicity have been studied in different plant species for a long period of time. ROS are thought to be key players in cell death that accompanies senescence in plants (Pastorl et al. 1997; Jimenez et al. 1998). The appearance of SODs was triggered by the proliferation of photosynthetic organisms that began to produce oxygen.

Under physiological conditions, the production of ROS is regularly destroyed by biological mechanisms in the cell. Micronutrients such as zinc, Mn and Cu have been correlated with numerous growth and developmental processes of plants. Deficiencies of micronutrients are recognised as the most common and widespread deficiencies in agricultural zones of Australia (Donald and Prescott 1975). Another experiment has shown that the activity of SOD was altered depending on the kind and severity of the micronutrient deficiencies stress in narrow-leafed lupins (Yu et al. 1999b). Therefore, SOD can be involved in the

developmental growth of plants. The process of development is expression of selective genes, and operating on a constant pool of genetic information to produce a complex adult organism from a single fertilised cell. Tissue differentiation is essential to the diverse and integrated functions of an adult organism from developmental regulation by functional enzymes. The enlargement of organs (e.g. ovary) appears to result from cell enlargement rather than new cell division during the later period of growth of plants (Slocum and Galston 1985). Furthermore, senescence takes place at every stage of natural plant organ development and leads to cell death in the final stage of organ development. The death of cells is preceded by a loss of membrane permeability, due in part to an increase in the levels of ROS that are in turn related to upregulation of antioxidative enzymes such as SOD and CAT (Kim et al. 2005).

The ROS level increases during senescence in many plants. To a great extent, the differences in SOD activity were shown to be related to subcellular localisation of SOD isoforms and to the cellular decompartmentalisation that results from membrane deterioration during oxidative burst (Droillard and Paulin 1990). It is likely that oxidative enzymes play an important role during plant development and senescence, but there are only few studies carried out on this subject. For instance, leaf senescence is strongly associated with increased oxidative damage to macromolecules by ROS. These changes are due to a sharp decline in the level of certain antioxidant enzymes such as APX and CAT, but an increase in SOD level has been shown in the chloroplasts of cucumber (Kim 2004). The abundance of SOD isoforms is highly variable and is regulated by environmental and developmental stimuli. Fath et al. (2001) have shown a direct correlation between the amounts of ROS-scavenging

enzymes and hormonal regulation of cell death in aleurone cells. The sensitivity of response to photooxidative stress decreases when the leaf becomes old. However, it is not clear if the decrease in antioxidant activities during senescence is due to a low response to oxidative stress or if oxidative stress is low due to the decreased metabolic activity as tissues age.

1.3.7 SOD in Flowers

1.3.7.1 Structure of Flowers

Typically, a flower is made of four kinds of organs: sepals, petals, stamens and ovary. For example, the flower of a buttercup has five green sepals, which enclose and protect the developing bud, and five glossy yellow petals. It has a large number of stamens, each consisting of a filament bearing an anther which will dehisce to release yellow pollen powder. Then, in the centre of the flower there is a cluster of carpels, each one with a receptive stigma at the tip, and each containing an ovule, which after fertilisation, can develop into a seed (Proctor et al. 1996). A similar basic structure can be recognised in most flowers, but there is an enormous variation in the number of different floral parts.

1.3.7.2 Pollination and Structure of Reproductive Parts of Flowers

Some flowers have both stamens and carpels in the same flower, in which the flowers are hermaphrodite or bisexual. This is the usual condition in the flowering plants. Others have them separately, which are referred to as “male” and “female” flowers.

Sexual reproduction in flowering plants is dependent on the correct delivery of pollen grains to conspecific stigmata. In the young bud, the stamen first appears as a projection on the developing receptacle, and the filament and anther are soon recognisable (Baker et al. 1973). When the pollen grains become mature, they are transferred to the surface of stigma by different ways of pollination. Then they start to imbibe water and germinate to become a pollen-tube. The pollen tube penetrates the stigma surface to grow between the cell tissues of the style, eventually down through the style to the ovary. The ovary is closely bound up with growth of the receptacle. The remaining living contents of the pollen tube after entering the ovule are discharged into the embryo-sac, where one of the two male gametes fuses with the egg to create a fertilised egg (Proctor et al. 1996). This fertilised egg can develop to form the embryo of the future seed.

1.3.7.3 Indentification and Characterisation of Surface Components within Stigma

Female reproductive function, that is the ability of a flowering plant to set viable seed, depends on both stigma receptivity and ovule viability (Dumas et al. 1984). The stigma is the first site where the recognition events lead to the acceptance of compatible pollen or the rejection of incompatible pollen (Cynthia et al. 1990). Stigma receptivity is defined as the period of effective pollination, and has its basis in temporal changes that occur in the pollen-receiving cells of the pistil. Morphological changes may also be associated with the onset of receptivity. The stigma is made up of elongate papillae cells, each of which has a single, fingerlike projection that is receptive to pollen binding. Wet stigma such as those found in the Solanaceae, Leguminosae and

Orchideceae are coated with sticky secretions like exudate, and dry stigmas such as those from Brassicaceae, Gramineae and Compositae are covered with a protein-containing pellicle (Heslop-Harrison 1992). For instance, the stigma of *Petunia Lindl*. (Solanaceae) is bilobed, with the middle part of each lobe raised upwards, and the surface of the stigma has a large number of papillae (Konar and Linskens 1966). The stigmatic exudate of *Nicotiana* consists of lipids, phenols proteins and polysaccharides, and the proteins are present in the layer covering the stigmatic papillae or dissolved in the exudates upon the stigma (Cresti et al. 1986). In the wet types of stigma, carbohydrates and proteins are normally detected in the stigma and exudates of stigmatic surface. The carbohydrate-containing components presented in the stigma and contained monosaccharides galactose, arabinose, mannose, and rhamnose. The surface of stigma also contained an arabinogalactan or arabinogalactan protein as a major component (Clarke et al. 1979). Predominant monosaccharides were glucose and fructose in the stigma, and some enzymes were found to be associated with stigmatic tissues such as β-1,3-glucanse (Price 1996). The polysaccharide component of the *Lilium longiflorium* stigmatic exudates has been shown to contain an arabino-3,6-galactan component but differs from the Gladious stigma material in having a higher arabinose content as well as rhamnose and glucuronic acid as major components (Aspinall and Rosell 1978). Chitinase enzyme activity is present in the extracts of healthy and non-senescent petunia flower tissues localised in the stigma and its activity increases about five-fold therein-following anther dehiscence (Leung 1992). The complexity of biochemical structure of stigma is of potential interest for further investigation.

1.3.7.4 SOD and Flowers

The biological role and significance of SODs as protective enzymes against O_2^- toxicity have been studied in different species. Plants have multiple enzymatic forms of SOD (isozymes) unlike most other organisms. The existence and localisation of SODs within cells, tissues, or organelles and any changes undergoing during development may imply separate metabolic roles for each of the SOD isozymes. A promoter of cytosolic Cu/Zn-SOD from *Nicotiana plumbaginifolia* was cloned in transgenic tobacco to induce expression of SODc-gusA in protoplasts in response to various environmental stress conditions (Herouart et al. 1994). However, the role and characteristic of SOD in flower development and senescence have been studied to a limited extent. Thus, this topic is of particular interest and should be an area for further investigation.

1.3.7.5 Petunia as a Potential Model

1.3.7.5.1 General Introduction

The genus Petunia can be referred to as one potential model in different research fields, because there is a considerable body of genetic information and molecular tools for studying this plant. It is calssified in the division Magnoliophyta, class Magnoliopsida, order Polemoniales. It is established by Jussieu in 1803, comprises at least 30 (sub) species, which belong to the family of the Solanaceae (Gerats and Vandenbussche 2005). The main distribution of petunia is from Argentina to Uruguay, in the Southern part of Brazil and in the Andean foothills (Wijsman 1990; Ando 1996). Petunia remains as one of the favorite genera for developing new varieties.

Petunia has a typical flower comprising sepals, petals, stamens and carpels, it can be referred to as a model system for different research fields (Plate 1.1). It has many optimal qualities as a role model system for plant biology studies. Firstly, it has an easy growth habit and a relatively short lifecycle of roughly four months from seed to seed. The second important quality is that asexual propagation of it can be achieved from cuttings, callus or protoplasts. Thirdly, it is easy to produce a new set of varieties by transformation. Then, it has a large and expanding set of functionally and molecularly well characterised genes. Last but not the least, the availabilities of large sets of mutants of petunia are mainly caused by insertion of endogenous transposable elements (Gerats and Vandenbussche 2005). Therefore, the capacity of combination between petunia system and many potential technical features with a wide range of research possibilities is a major advantage as a model.

1.3.7.5.2 Structure of Reproductive Parts of Petunia

Petunia has different species of self-incompatible, self-compatible pollination and cross-pollination types. Pollination begins when pollen and stigma cells from productive contacts that lead to pollen tube germination. Pollen-stigma adhesion is highly species-specific. Petunia has wet stigmas on which a sticky exudate is secreted. The exudates are carbohydrate and lipid- rich secretions, which are a complex mixture of proteins, lipids and saccharides that can hydrate pollen indiscriminately (Cresti et al. 1986; Konar and Linskens 1966). When mature pollen land on a mature stigma, they first come into contact with the emerging pollen tubes, which grow further through the exudates in the intercellular spaces toward the transmitting tissue (Verhoeven et al. 2005). The exudation takes place in two stages. In the first stage, the epidermal and

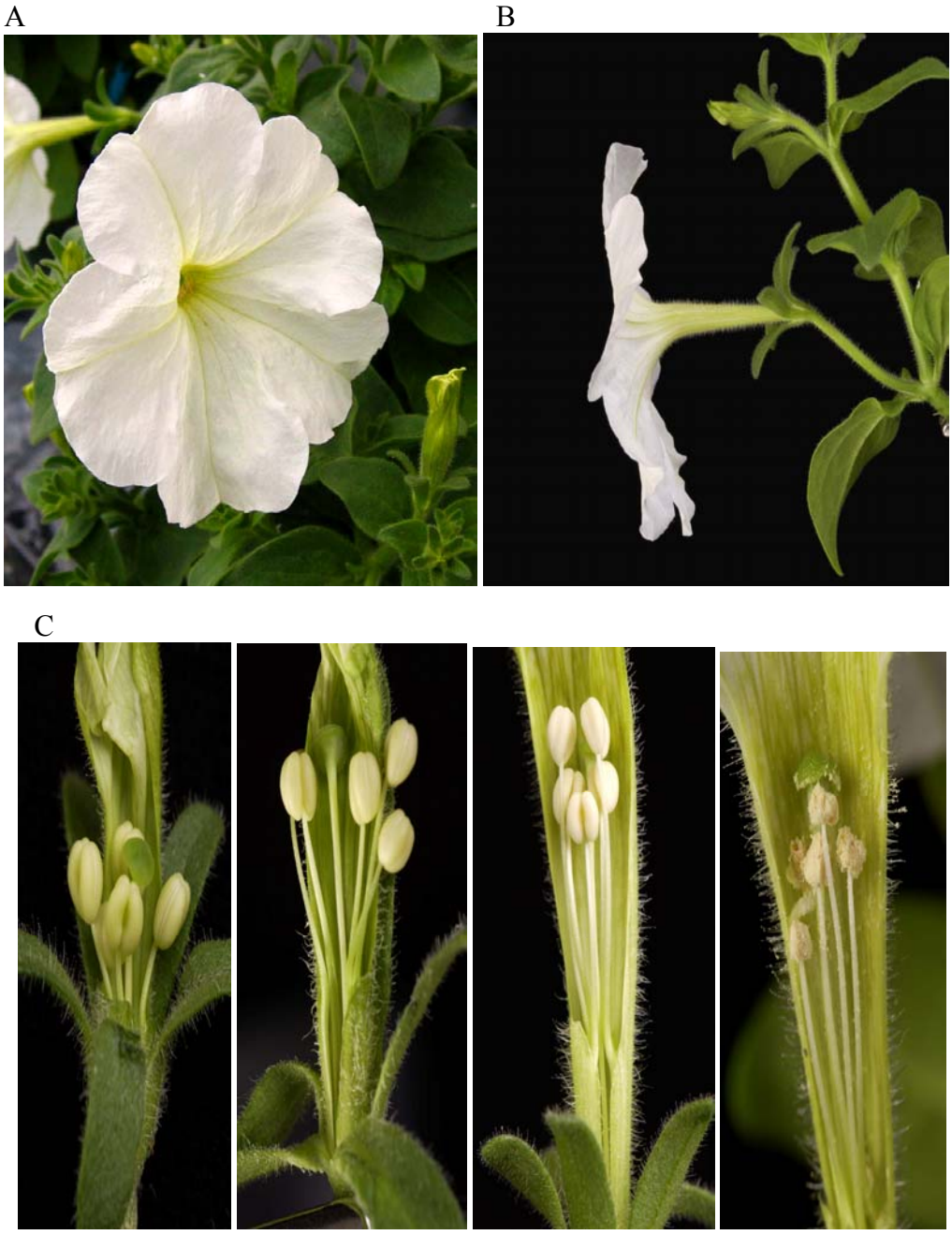


Plate 1.1 *Petunia hybrida* (‘Hurrah’) species accessions. Flower in front view (A), side view (B), and internal views from young buds to opening flowers(C).

papillae cells release out the oily exduate upon of the cuticle. The second phase of exudation begins with anthesis. The lipids present in the exduate are sufficient and essential for pollen tube initiation and can be functionally replaced both in exduate and the stigmatoid tissue (Wolters-arts et al. 1998). The lipid composition influences water uptake by pollen grains and enables successful pollen-pistil interactions (Wolters-Arts et al. 2002). Once the pollen tube has moved through the style and targeted a particular ovule in the ovary, a fertilised egg can be eventually created.

1.3.7.5.3 Relevant Research Fields

Petunia has been used to research a diverse set of interesting questions. Petunia research proliferated further with progressively more work on different aspects of plant and flower development since late 1980s. Normally, large mutant collections are maintained availability for different purposes such as taxonomy, meristem activity, genetic maps, self-incompatibility, senescence, so on. Specifically, the petunia vein clearing virus (PVCV), which combines features of both viral and non-viral retroelements has been studied for the particular interest of retroelement activity in plant breeding programmes (Galliano et al. 1995; Richert-Poggeler et al. 2003). Special attention has been paid to male sterility cloning a mitochondrial gene from petunia (Bentolila et al. 2002). Furthermore, petunia has also been studied for a special expansin to reduce the amount of crystalline cellulose in cell walls and lead to phenotypic changes in petal limbs. Expansins are involved in disruption of the noncovalent bonds between cellulose microfibrils and cross-linking glycans, thereby promoting wall creep (Zenoni et al. 2004). Since the early 1990s, extensive molecular studies of plant floral mutants in a range of species have gathered evidence for

the MADS-box gene transcription factor family in floral development and plant architecture including petunia (Gerats and Vandenbussche 2005). Researching the function of these genes can help better understand the evolution of flower and plant shape. Therefore, petunias become a broad sampling genus for study on diverse topics of interest like branching patterns, volatile production, pollination syndromes and mycorrhiza-plant interactions.

1.4 Aims and Objectives

It is clear from a literature review regarding the role of SOD in living organisms, but the understanding of its function within flowers is lacking. The identification and characterisation of factors and elements in SOD regulation and expression in flowers will provide in-depth understanding of the entire signal transduction pathway during oxidative stress. Consequently, further investigation into how to engineer more stress tolerant plants should concentrate on over-expressing or under-expressing SOD levels and on maintaining the optimal endogenous levels of all isozymes of SOD when plants are under oxidative stress conditions. In addition, the question should be resolved with keeping a well-balanced and coordinated expression of all essential antioxidative enzymes in the various cell compartments.

Petunia has become popular for different research fields because it is a typical flower that can be referred as a model system of flower kingdom. It also can be a potential source for the study of characteristics and localisation of SOD. It is possible that oxidative burst is prevalent at different stages of flower development and senescence. SOD might play a role in controlling the pollen-pistil system following compatible and self-incompatible pollination in the

stigma, style and ovary.

The initial part of this study was to determine the occurrence of SOD activity in the female reproductive parts of petunia and the profile of different SOD isoforms, and their characteristics. Once this was established, the tissues selected were examined for developmental regulation of SOD activity. Then, SOD enzyme was to be purified from the ovary. The characteristics of original SOD and partially purified SOD were compared as another objective of this study.

CHAPTER 2
MATERIALS AND METHODS

2.1 Plant Material

2.1.1 Growth of Petunia Plants

The plant selected for this study was *Petunia hybrida* ('Hurrah'). Initially, eight or more small branches (with three or four leaves) from seedlings of petunia were grown in a tray of potting mix containing 60% bark, 20% peat, and 10% sterilized soil for a period until root system was growing well enough to be transferred individually into pots for each cutting. Plants were maintained in the glasshouse at 25 -30^{o}C/15-20 ^{o}C day/night during summer time and 20-25^{o}C/10-15 ^{o}C day/night, and under 16 hr photoperiod (sodium light, 400w) during winter in the glasshouse at the University of Canterbury (Plate 2.1). Flowering normally began 1-2 months after potting but took longer during winter. Collection of floral tissues for this study was then possible. Petunia plants over 5 months of age were not included in this study as the floral morphology of these plants became inconsistent.

2.1.2 Harvest of Floral Tissues

The superoxide dismutase activity was analysed using several types of floral tissue at different developmental stages (Plate 2.2). Forceps were used to grasp the style of the flower and pull the pistil out of the flower, usually breaking off at the junction of the style and the ovary. The stigmata and style were subsequently separated using a scalpel in some experiments. The ovary was collected using a scalpel to cut off the connection between the ovary and sepal.

Plate 2.1 Flowers of petunia.

The flowers of petunia cultivars were grown in the glasshouse of the University of Canterbury for this study.

The tissues collected for different experiments were used immediately for preparing enzyme extracts. If there was more plant material left, they would be frozen and stored at -80 ^{o}C until use.

2.2 Methods

2.2.1 Preparation of Enzyme Extracts

Usually, the sooner the raw material is used, the better and the more physiologically relevant the preparation will be. Initially, the plant tissues were used for different types of experiments. The plant tissues were thoroughly ground with a pestle and a mortar on crushed ice to disrupt cells and release proteins into solution. The relative amounts of 0.1 M potassium phosphate buffer (the extraction buffer, see Appendix 1.1) added to plant tissues varied depending on different types of experiments. For instance, for SOD activity determination in crude extracts the ratio was 20 flower tissues to 1.5 mL of extraction buffer, whereas for enzyme purification the ratio was 1000 tissues to 30 mL of extraction buffer. The homogenate was centrifuged at 10000g for 10 minutes in a refrigerated centrifuge at 4^{o}C. The supernatants as crude extracts were transferred to a measuring cylinder to determine the volume, and then placed in Eppendorf tubes for the appropriate experiments. They were stored for a period of time until needed at -20 ^{o}C. For long term storage purpose such as purification, freezers were operated at temperature down to -80 ^{o}C.

The effect of polyvinylpyrrolidone (PVPP) in the extraction buffer on the level of extractable SOD activity in the floral tissues was also tested by adding 0.05 mg powered PPVP to the extraction buffer. In another experiment, 100 ul of 1 mM DL-Dithiothreitol (DTT) and 1 mM ethylenediamineteraacetic acid

(EDTA) were also added to the extraction buffer.

2.2.2 Establishment of SOD Assay

SOD activity was determined and modified according to Giannopolitis and Ries (1977) using fresh extracts. The crude extracts were diluted with 0.1 M K-phosphate buffer as required, and then aliquots were boiled in a boiling bath for 5 minutes as control. The boiled enzyme extracts were centrifuged for 5 minutes. The reaction mixture was composed of 0.1 M K-phosphate extraction buffer (pH 7.8), 10 mM EDTA, 63 mM NBT as an indicator, 13 mM riboflavin as a source of O_2^- and an appropriate volume of enzyme extract in a test tube as one replicate. After preparation of three replicates, the reaction mixture was vortexed at room temperature and illuminated at 22-25 ^oC for 15 minutes in a plant growth room. Immediately after this the absorbance was read at 560 nm in a spectrophotomemter. After numerous preliminary experiments, a reliable assay for measuring SOD activity in crude extracts of petunia was established. One unit of SOD activity is expressed as the amount of enzyme that inhibited the NBT photoreduction by 50% and the enzyme was quantitated on the basis of the percent inhibition of NBT photoreduction it caused (Giannopolitis and Ries 1977).

2.2.3 Effect of Storing Crude Enzyme Extracts at Different Temperatures on the Stability of SOD

The enzyme extracts from different parts of fully opened petunia flowers, collected on the day of opening, were stored in three groups, each in Eppendorf tubes as 1.3 mL aliquots at room temperature (25 ^oC), 4 ^oC (in a fridge), and

-20 ^{o}C (in a freezer) for up to one month or longer before SOD assays were performed. The SOD activity of the fresh extracts was considered as the control (100% enzyme activity).

2.2.4 Effects of pH on SOD Activity and Stability

2.2.4.1 Effect of Incubation of Crude Extracts at Different pH before SOD Assay

The crude enzyme extracts from the pistil and ovary of fully open flowers collected on the day of opening were diluted with either 1 mM citrate phosphate buffer or 2 mM Tris-HCl buffer (see Appendix 1.1). The reaction mixture with 0.1 M phosphate buffer, as in a routine standard SOD assay protocol, was assayed immediately for 25 minutes and absorbance was read at 560 nm. The diluted extracts were also incubated for 2 hr and overnight at 20-25 ^{o}C before SOD assay as standard SOD assay protocol (see 2.2.2).

2.2.4.2 Dependence of SOD Activity on pH of the Assay Reaction Mixture

In order to obtain the desired pH, the reaction buffer (0.1 M K buffer) in the standard SOD assay (see 2.2.2) was replaced by either 1 mM citrate phosphate buffer for SOD assay at pHs 3.0, 4.0, 5.0, 6.0, 7.0, or 2 mM Tris-HCl buffer for SOD assay at pHs 8.5, 9.0 and 10.0 (see Appendix 1.1). The reaction mixture with citrate phosphate buffer and with Tris-HCl buffer was then assayed for 15 minutes. Then, absorbance was read at 560 nm.

2.2.5 Determination of SOD Activity during Developmental Stages

The reproductive parts of mature petunia flowers were divided into four different tissues (pistil, ovary, stigma and style), and then tested for SOD activity as described in 2.2.2. One unit of SOD activity is defined as the amount of enzymes that inhibits NBT photoreduction by 50% (Beauchamp and Fridovich 1971). Dehiscent pistils were also collected for detecting SOD activity in the exudates of stigmatic surface. Protein concentration of each extract from different parts of flower was also determined (Bradford 1976). At least three replicates were completed for each flower part and over three treatments. Data was analysed by ANOVA and the Tukey test for determination of activity significance. Plant tissues (pistil, stigma, style and ovary) were collected at different stages of flower development of the petunia from small bud (referred to as the first stage, see Plates. 2.2 and 2.3) to dehiscent withered flower (referred to as the sixth stage, see Plates. 2.2 and 2.3). The crude extracts were prepared from these materials and SOD assays as described in 2.2.2 were performed for each stage. At least three groups of flowers at different stages were collected for making extractions to get reliable data for analysis.

Plate 2.2 Developmental stages of petunia flowers

Stage 1. Day 0-1, young petunia flowers – tip of petals is beginning to extend past the sepals (12-14mm).

Stage 2. Day 2, corolla extending – half of the full length (30-35mm).

Stage 3. Day 3, corolla fully extending – petals are beginning to open and full length (60-70mm).

Stage 4. Day 4, petals are opening at an angle of 45º to floral stem axis, anthers are not dehiscent.

Stage 5. Day 5, petals are fully opening at an angle of 90º to floral stem axis, anthers are predehiscent.

Stage 6. Day 6, petals are fully opening at an angle of 90º to floral stem axis, anthers are dehiscent.

Plate 2.3 Developmental stages of the female reproductive parts of petunia.

Stage 1. Day 0-1, young petunia flowers – tip of petals is beginning to extend past the sepals (12-14mm).

Stage 2. Day 2, corolla extending – half of the full length (30-35mm).

Stage 3. Day 3, corolla fully extending – petals are beginning to open and full length (60-70mm).

Stage 4. Day 4, petals are opening at an angle of 45º to floral stem axis, anthers are not dehiscent.

Stage 5. Day 5, petals are fully opening at an angle of 90º to floral stem axis, anthers are predehiscent.

Stage 6. Day 6, petals are fully opening at an angle of 90º to floral stem axis, anthers are dehiscent.

2.2.6 Stress-Induced Response on SOD Activity

2.2.6.1 Water Deficiency

Twelve petunia cuttings were potted in the glasshouse for a month until flowering began, then six pots were watered normally to act as control and the other six were subjected to water stress treatment. The water potential was measured in randomly selected flower branches with a Scholander pressure chamber everyday from the first day after watering to six days without watering. Water potential was measured by placing the basal portion of a flower branch into the chamber and applying sufficient pressure from a dry air tank to return the meniscus of the xylem sap to the cut surface of the midrib. In several petunia cultivars, this balancing pressure gives a reliable estimate of the water potential measured. The weight of each pot was measured using scales for water loss every day, and then buds and fully opened flowers were collected and assayed for SOD activity as described in 2.2.2 (Plate 2.4).

2.2.6.2 High Temperature

Nine Seedlings were potted in the glasshouse for a month until flowering began. Six pots were stressed inside a temperature controlled cabinet kept at around 30-35 ^{o}C, 720 uE light intensity, and 16 hr photoperiod. The buds and fully opened flowers were assayed according to section 2.2.2 for SOD activity after a period of stress (Plate 2.5).

Plate 2.4 **Comparison of petunia flowers under water deficiency stress (left) and normal conditions as control (right).**

Plate 2.5 **Comparison of petunia flowers under normal conditions as control (left) and high temperature stress conditions (right).**

2.2.7 Gel Electrophoresis

2.2.7.1 Non-denaturing PAGE for Determination of SOD Isozymes

Enzyme extracts from several tissues (including stigma, style, pistil and ovary) of different developmental stages of petunia flowers were used for this experiment. Gels were cast and run using the Mini-PROTEAN II Dual Slab Cell System (Bio-rad, USA). Electrophoresis of crude extracts with compatible dilutions of different types of tissue was performed on 12% separating and 4% stacking gels at 200 volts for 42 minutes at room temperature. Separating gels contained 30% (w/v) polyacrylamide, 1.5M Tris-HCl (pH 8.8), 0.05% (w/v) ammonium persulphate (APS) and 0.1% (w/v) TEMED. Stacking gel was comprised of 30% (w/v) acrylamide monomers, 0.5 M Tris HCl buffer (pH 6.8), 0.05% (w/v) APS and 0.1% (w/v) TEMED. The running buffer for electrophoresis contained 37.5 mM Tris and 0.29 M glycine. The acrylamide concentration was 12% and 60% sucrose was added to the samples to increase the density of extracts. Ten µL of mixed samples for loading were prepared by mixing two volumes of enzyme extract with one volume of 60% (w/v) sucrose in an Eppendorf tube. The appropriate dilution of enzyme extract was also required before mixing with sucrose.

2.2.7.2 SDS PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis)

Enzyme extracts from different tissues (including stigma, style, pistil and ovary) of fully opened flowers were prepared using an extraction buffer at pH 7.8 as described in 2.2.1.1. The enzyme extract was mixed with SDS sample

buffer in a 4:1 ratio and boiled for 5 min before loading into the wells. The SDS sample buffer consisted of 20% (v/v) glycerol, 10% (w/v) SDS, 5% β-mercaptoethanol and 0.1% (w/v) bromophenol blue (M & B, UK) in 0.125 M Tris-HCl (pH 6.8).

Enzyme samples were loaded based on calculations of mg protein determination, and gels were cast and run using the Mini-PROTEAN II Dual Slab Cell System (Bio-rad, USA). SDS PAGE was performed with 12% separating and 4% stacking gels at 200 volts for 42 minutes at room temperature. The separating gels were made up of 12% polyacrylamide, 10% SDS, 1.5M Tris-HCl (pH 8.8), 0.05% (w/v) ammonium persulphate and 0.1% (w/v) TEMED. Stacking gels consisted of 30% (w/v) polyacrylamide, 10% SDS, 0.5 M Tris-HCl buffer (pH 6.8), 0.05% (w/v) ammonium persulphate and 0.1% (w/v) TEMED. The electrophoresis running buffer included 37.5 mM Trizma base (Tris) (Sigma), 0.29 M glycine and 0.15% SDS.

The SDS PAGE molecular weight markers (Bio-rad standard) used for comparison were phosphorylase B (108,000 Da), bovine serum albumin (90,000 Da), ovalbumin (50,700 Da), carbonic anhydrase (35,500 Da), soybean trypsin inhibitor (28,600 Da) and lysozyme (21,200 Da). After taking photos of SDS PAGE gels, the distance traveled by each marker protein was measured using a ruler in order to establish a standard curve (molecular weight versus distance migrated) to estimate the molecular weights of the denatured proteins in the enzyme extracts.

2.2.7.3 Staining for SOD Activity in Non-denaturing Gels

After running electrophoresis, the non-denaturing gels were stained at room temperature for 20 minutes with 0.01 mM NBT solution, and then incubated at 22-25 ^{o}C in 12 mL mixture consisting of 11 mL of extraction buffer (pH 7.8), 200 µL EDTA (10 mM) and 800 µL riboflavin (13 mM) in a growth room overnight until clear bands appeared on a dark purple background.

2.2.7.4 Staining for SOD Isozymes

To distinguish different forms of SOD isozymes, the appropriate non-denaturing gels were incubated after electrophoresis in 5 mM hydrogen peroxide solution for 30 min and then soaked in distilled water for 5 min. Then the same staining procedure for SOD activity as described before was used. Also a non-denaturing gel was incubated after electrophoresis in 10 mL of 4 mM potassium cyanide instead of H_2O_2 before staining for SOD isozyme activities as described before to distiginguish isozymes of SOD according to sensitivity to those two inhibitors (Scandalios 1993).

2.2.7.5 Coomassie Blue Staining Procedure for SDS PAGE Gels

After SDS PAGE electrophoresis, the gels were stained for 30 min at room temperature by shaking at 20 rpm, in about 100 mL of Coomassie blue stain containing 50% absolute methanol, 10% glacial acetic acid (BDH) and 0.1% (w/v) Coomassie Briilliant Blue R 250 (Sigma). The staining solution was discarded and gels were destained (200 mL) with 40% (w/v) methanol and 10% (w/v) glacial acetic acid at room temperature overnight until the bands

were clear, and then washed with several changes of distilled water.

2.2.8 Imaging of Superoxide Responses in Tissues

Petunia tissues were excised at the conjunction of the pistil and ovary with a razor blade. Pistils from each developmental stage of flowers were immersed in 0.01mM NBT solution at room temperature, and ovaries were split apart. Styles were stained two different ways: whole styles and styles split longitudinally before being immersed in the NBT solution.

As a control, the pistils, ovaries and styles of fully opened petunia flowers were stained in 0.01 mM NBT with or without 0.2 mM $MnCl_2$ solution.

2.2.9 Purification

2.2.9.1 Ammonium Sulphate Precipitation

Initially, crude enzyme extracts were prepared from the pistils and ovaries of 100 fully blooming flowers, collected on the day of opening, in the extraction buffer as described in 2.2.1. One-step and stepwise additions of ammonium sulphate were both performed to choose which percentage of the salt would be the best for SOD purification.

For the stepwise method, 176 g/L $(NH_4)_2SO_4$ (ammonium sulphate) was added, a little at a time, to the crude enzyme extract in a cold room at 4 ^oC, stirring to get to 30% (w/v) saturation, then this mixture was centrifuged for 15 min at 10,000 rpm at 4 ^oC to obtain the supernatant and a pellet which was dissolved in 3 mL of 0.1 M potassium phosphate buffer (pH 7.8). Then, 1 mL of the

supernatant or the dissolved pellet was assayed as described in 2.2.2 after dialysis overnight with agitation at 4 ^{o}C in one L of the extraction buffer (pH 7.8).

Then, the supernatant was brought to 60% saturation with 198 g/L $(NH_4)_2SO_4$ added little by little at a time at 4 ^{o}C, and was centrifuged at 10,000 g for 15 min at 4 ^{o}C. Next, the supernatant obtained from the 60% saturation was brought to 90% saturation with 227 g/L $(NH_4)_2SO_4$ and centrifuged as before.

In addition, the one-step method of adding $(NH_4)_2SO_4$ to crude enzyme extracts was evaluated: 0 to 30%, 0 to 60% or 0 to 90% of $(NH_4)_2SO_4$. There was exactly same process as stepwise method to make certain percentage of addition of $(NH_4)_2SO_4$.

The dialysed extract after the appropriate percentage addition of $(NH_4)_2SO_4$ was centrifuged at 10,000 g for 15 min at 4 ^{o}C, and the supernatant was placed inside dialysis tubing and covered with 20,000-PEG powder in a cold room at 4 ^{o}C to reduce the volume to about 3 mL.

2.2.9.2 Determination of Total Protein Concentration

Total protein concentrations in the extracts were determined according to Bradford (1976). One hundred μL of protein extract were mixed with 1 mL of Coomassie Brilliant Blue G (Sigma) dye reagent and vortexed immediately. The samples were incubated for at least 5 minutes at room temperature before the absorbance was measured at 595 nm. A standard curve was prepared with 0-10 μg/100 μL bovine serum albumin (BSA). The extraction buffer was used

as the blank and the diluant if dilution of the protein extract was needed.

The protein concentration of the enzyme extracts from stepwise and one-step $(NH_4)_2SO_4$ precipitation were determined and the absorbance was read at 595 nm in a spectrophotomemter (Bio-Rad, USA).

2.2.9.3 Purification by DEAE Cellulose Column

A DEAE cellulose (Sigma) column (24×1.5 cm) was attached to a peristaltic pump (Masterflex, Cloe Parmer Instrument Co, USA, model 7014.20) and a fraction collector (Bio-Rad, model 2110) in this step. DEAE cellulose (5 g) was soaked with 100 mL washing buffer (0.01 M potassium phosphate buffer, pH 7.8) and changed frequently with the same amount of buffer for three days, then degassed overnight. The column was packed with the degassed DEAE cellulose and equilibrated with 300 mL of 0.01 M potassium phosphate buffer (pH 7.8). The supernatant from 1000 pistils or ovaries was dilyised overnight against 2 L of the extraction buffer. Then, another important application of salting out procedures was to concentrate samples to 3 mL using PEG-20000 (see 2.2.9.1) before loading onto a DEAE cellulose column equilibrated with 0.01 M potassium phosphate buffer. 100 fractions (2 mL each) were collected at a flow rate of 80 drops per min and assayed for SOD activity. The first 50 fractions were eluted with 80 mL of 0.01M potassium phosphate buffer (pH 7.8) and SOD isozymes in those fractions did not bind to the column for this stage. Then separation of SOD isozymes in other 50 fractions was achieved by anion-exchange chromatography when salt gradient (from 0.05 to 0.4 M sodium chloride (NaCl)) was performed. The absorbance of all fractions was read at 280 nm and SOD activity in all fractions was assayed as described in

2.2.2.

2.2.10 Characteristics of Partially Purified Enzyme

The peak fractions with the highest SOD activity were collected for further purposes. Three fractions of each peak SOD activity were pooled and concentrated against 20,000-PEG powder to reduce volume to 2 mL. This was used for the determination of the total protein content, SOD activity, non-denaturing PAGE and SDS-PAGE gel analysis of the partially purified enzyme extracts to check the progress of purification. Each peak fraction was divided into different categories for different analyses.

The following analyses were also carried out using the same procedure as described as earlier:

1. Effect of storage at different temperatures on the activity and stability of partially purified SOD
2. Effect of pH on the activity and stability of partially purified SOD
3. Non-denaturing PAGE of partially purified SOD
4. SDS PAGE of partially purified SOD

2.3 Data Analysis

The Microsoft Office XP, Excel XP and statistics versions were used for the analysis of all the data. One way analysis of variance ANOVA and Tukey tests for comparison of the means were performed ($P<0.05$) using Microsoft Excel Data Analysis tools.

CHAPTER 3
RESULTS

3.1 Occurrence of SOD in Female Reproductive Parts of Petunia Flowers

Many methods of cellular disintegration have been used for enzyme extraction from different cell types. Addition of substances such as insoluble PVPP, DTT and EDTA to extraction buffer is often beneficial improving enzyme extraction. SOD activity extractable from petunia female reproductive parts was also found to be approximately doubled in the presence of these substances when compared with using only the reaction buffer (0.1 M K buffer). A common SOD detection method involving spectrophotometric detection was used to measure SOD activity. After numerous preliminary experiments, a reliable assay for measuring SOD activity in crude extracts of fully open, healthy petunia flowers was established.

SOD activity was found in all the female reproductive parts of a petunia flower examined. The highest SOD activity was found in the ovary tissue of petunia. Activity within the ovary was significantly higher than that of the pistil. Analysis of the data by multiple comparisons of means showed no significant difference ($P<0.05$) between the activity of SOD in stigma of mature fully bloom flower and that of the style.

3.2 Freshness and Storage

The availability of fresh material does not always coincide with one's ability to

use it. Frozen storage, either of the material as received or of an extract from it, should be considered. The aim of freezing is reaching a temperature below -20 ^{o}C quickly, and storage should be at even lower temperatures if possible. The effect of storing extracts at a low temperature was determined by storing them at 4ºC in a fridge and by freezing at -20ºC in a freezer. SOD activity was measured when extracts were fresh, and then after two days, one week, two weeks, three and four weeks. There was no significant difference in SOD activity between fresh extracts and those that had been stored at -20ºC, however, a dramatic decrease after storage at 4ºC was detected as only 16% of activity remained after four weeks (Fig. 3.1). Extracts from the pistil and the ovary of fully open petunia flowers stored at room temperature (25ºC) lost their activities to undetectable levels after 16 days (Fig. 3.2). The SOD activity of crude extracts could be stable for more than one month when stored at -20 ^{o}C. Therefore, -20ºC was selected as the temperature to store extracts for short-term purpose whereas -80ºC was chosen for long term storage.

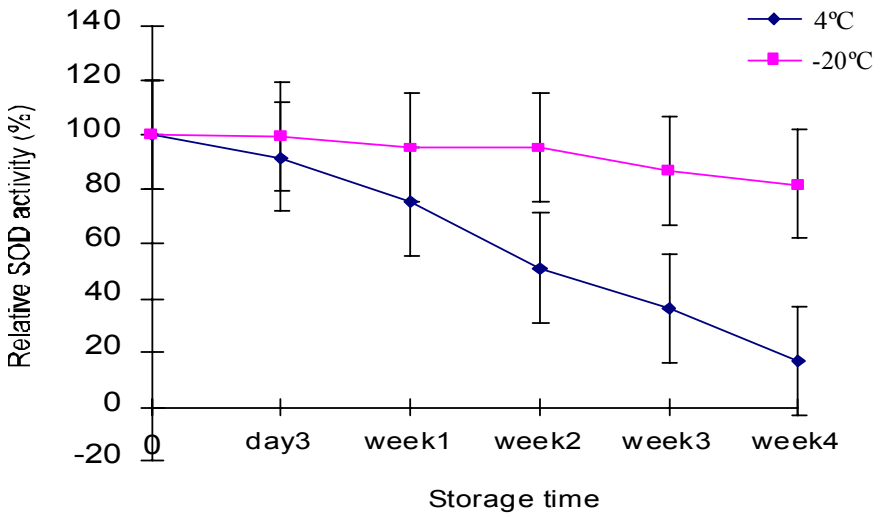


Fig 3.1 Effect of storage time at 4ºC in a fridge and at -20ºC in a freezer on SOD activity. Enzyme extracts were prepared from pistils of fully open petunia flowers. Fresh extracts used as 100% activity. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with extract of pistils.

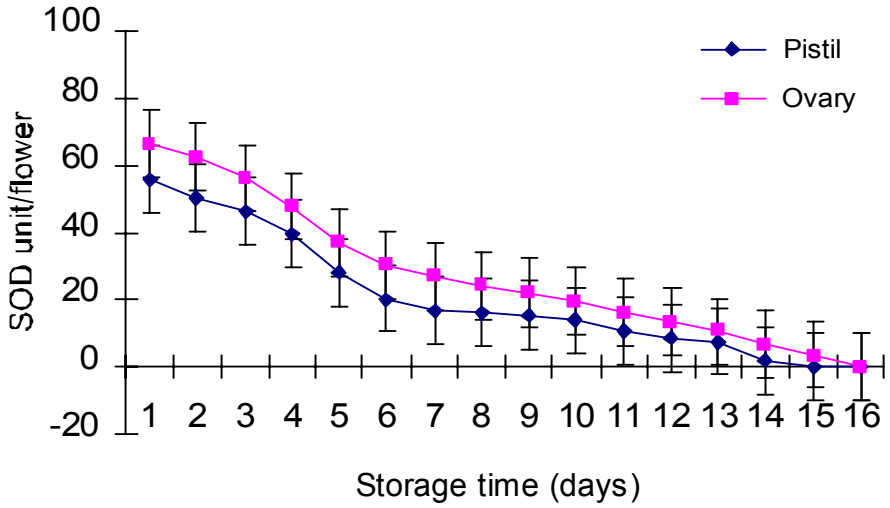


Fig 3.2 Effect of storage time at room temperature (25ºC) on SOD activity. Enzyme extracts were prepared from pistils and ovaries of fully open petunia flowers. One unit of SOD activity is expressed as the amount of enzyme that inhibited the NBT photoreduction by 50%. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with each extract.

3.3 Optimisation of Enzyme Reactions and Effect of Different pHs on SOD Activity

For optimisation of SOD assays, several aspects were examined including enzyme kinetics with respect to reaction time and volume of the enzyme extract in the reaction mixture, and optimum pH. The pH curve of SOD activity in extracts of pistils and ovaries of petunia showed an optimum at pH 7.8 (Figs. 3.3 and 3.4).

Incubating diluted enzyme extracts with 1 mM citrate phosphate buffer or 2 mM Tris HCl buffer had a similar effect on the stability of SOD activity. Similar results were obtained with extracts of both pistils and ovaries of petunia (Figs. 3.5 and 3.6).

3.4 Development of Petunia Flowers

3.4.1 Morphological Changes

Six major developmental stages of petunia flowers were included in this study (Plates. 2.2 and 2.3). Developmental stages of flowers were as follows: stage 1, very young bud, tip of petals just longer than sepals, day 0-1; Stage 2, corolla extending half of full length, 30-35 mm, day 2; Stage 3, corolla fully extending to full length and petals are beginning to open, 60-70mm, day 3; Stage 4, petals are opening at an angle of 45º to floral stem axis, day 4; Stage 5, petals are fully opening at an angle of 90º to floral stem axis, anthers are predehiscent, day 5; Stage 6, petals are fully opening at an angle of 90º to floral stem axis, anthers are dehiscent, day 6.

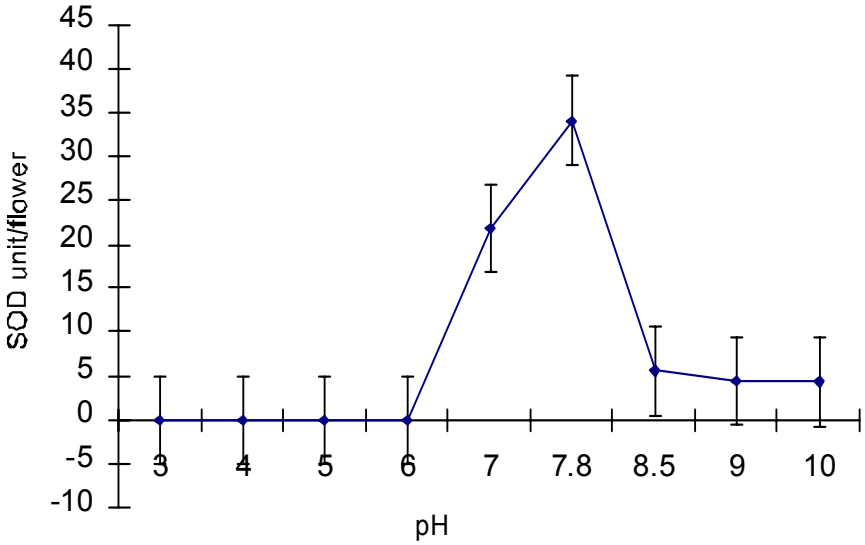


Fig 3.3 Effect of pH on SOD activity in the pistils of fully open healthy petunia flowers. One unit of SOD activity is expressed as the amount of enzyme that inhibited the NBT photoreduction by 50%. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with extract of pistils.

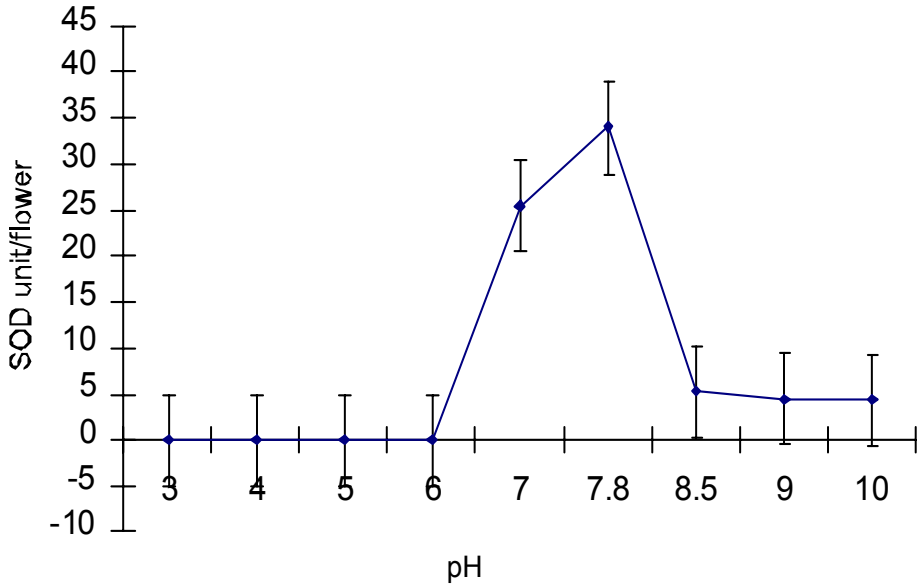


Fig 3.4 Effect of pH on SOD activity in the ovaries of fully open healthy petunia flowers. One unit of SOD activity is expressed as the amount of enzyme that inhibited the NBT photoreduction by 50%. Error bars represent standard deviation for three flower extracts. Three replicate activity determinations were carried out with extract of ovaries.

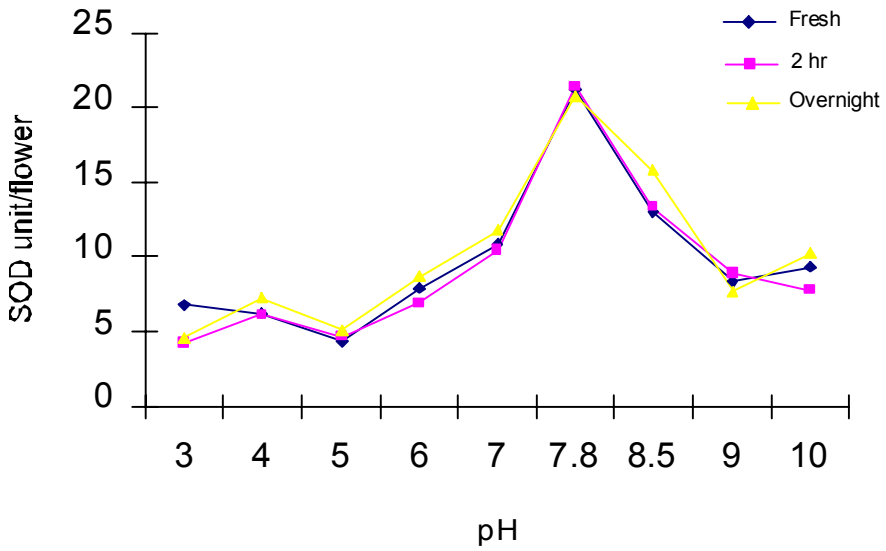


Fig 3.5 Effect of pH on SOD stability in the pistils of fully open healthy petunia flowers. One unit of SOD activity is expressed as the amount of enzyme that inhibited the NBT photoreduction by 50%. Three replicate activity determinations were carried out with extracts.

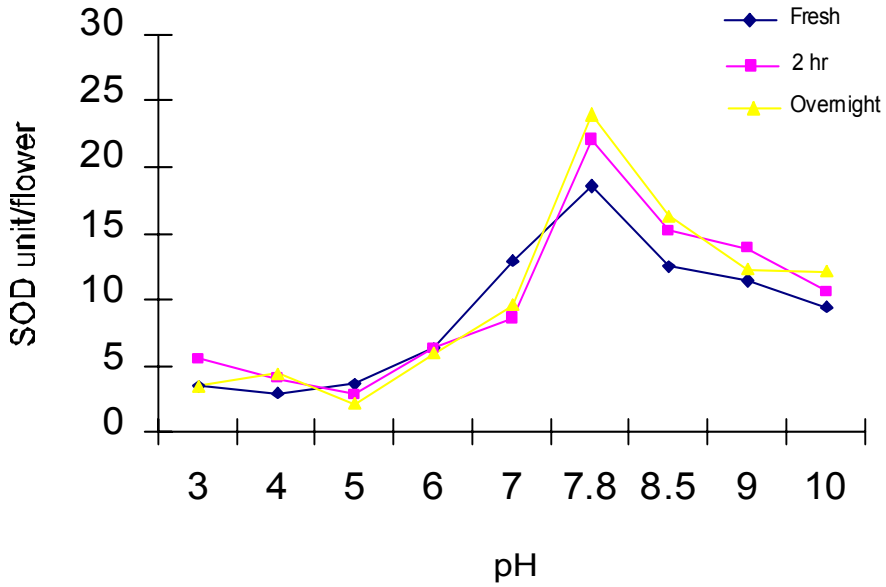


Fig 3.6 Effect of pH on SOD stability in the ovaries of fully open healthy petunia flowers. One unit of SOD activity is expressed as the amount of enzyme that inhibited the NBT photoreduction by 50%. Three replicate activity determinations were carried out with extracts.

While the flower buds enlarged in size, their colour began to change from green to white. As the buds started to enlarge, the stigma was of the same height as the sepals from the first day of flowering, and then they were growing to half the full length of mature flowers the following day. The buds were almost of maximum size and had tightly closed or slightly open sepals, and the colour of the tip of petals began to change the third day. The pistils were extending to full size at this stage and ovaries started to grow faster. After that, flowers were half open at an angle of 45 º to floral stem axis in the morning of the next day and fully open in the form of typical shape of petunia flowers in the same day. With time, anthers began to become dehiscent to release pollen grains. The symptoms of senescence started to appear after dehiscent anthers were released pollen grains that landed on the surface of stigmata for pollination. The stigmata of petunia changed colour from green to yellow-green and ovaries became mature. By this time, flowers had begun to close. When petals became completely dry, the flowers were fully wilted. If the ovules were fertilised, the remaining ovary had begun to develop further.

3.4.2 Changes in Fresh Weight during Flower Development

Fresh weight changes appear to follow a pattern during petunia flower development and senescence. Therefore, the change in fresh weight during the developmental stages and senescence of petunia flowers was determined by weighing buds and flowers collected at different stages. Generally, the fresh weight of petunia flowers reached a maximum at the full bloom stage as the buds developed, and then dropped as the flower wilted (Table 3.1). Fresh weights of different reproductive parts of petunia flowers were also selected to get a general pattern of changes during different developmental stages and

senescence. An increasing trend was recorded from all the reproductive parts collected in this experiment (Table 3.1). The fresh weight of pistil and ovary increased dramatically to reach a maximum after the flower wilted. The fresh weight of the style increased sharply from stage 1 to stage 3, and then remained the same in following four stages. The fresh weight of the stigma increased slightly between young buds and fully open flowers.

3.4.3 Changes on SOD Activity during Flower Development

Changes in SOD activity during flower development (see Plate 2.2) was studied using reproductive tissues of petunia flowers (stigma, style, pistil and ovary) collected at different stages of development. The results show increasing trends of SOD activity in crude extracts from the four different reproductive parts of petunia. Generally, the highest SOD activity was found at the fifth or sixth stage, while the first stage had the lowest activity of SOD (Figs. 3.7, 3.8, 3.9 and 3.10). Activity of SOD in the ovary increased as the flower grew and developed to the stage when the anthers were dehiscent (Fig 3.8, stage 6), whereas the pistil of petunia reached a peak of SOD activity when the anthers were predehiscent (Fig 3.7). Although the results show a clear trend, the activity in stigma was not significantly different at different developmental stages except stage 1, according to a multiple comparison of means using Tukey test (Fig 3.9). SOD activity in the last four developmental stages of the style were not significantly different from each other, but that of the first two was different compared to that of the last four stages (Fig 3.10).

Table 3.1 Changes of physiological pattern of petunia during flower development.

	Fresh weight (g/flower)				
Stages of Flower Development	Whole Flower	Pistil	Ovary	Stigma	Style
D1 stage 1	0.118	0.065	0.067	0.040	0.010
D2 stage 2	0.2	0.129	0.103	0.064	0.051
D3 stage 3	0.354	0.165	0.131	0.073	0.089
D4 stage 4	0.406	0.172	0.137	0.076	0.092
D4 stage 5	0.608	0.189	0.143	0.079	0.092
D5 stage 6	0.56	0.191	0.155	0.081	0.095

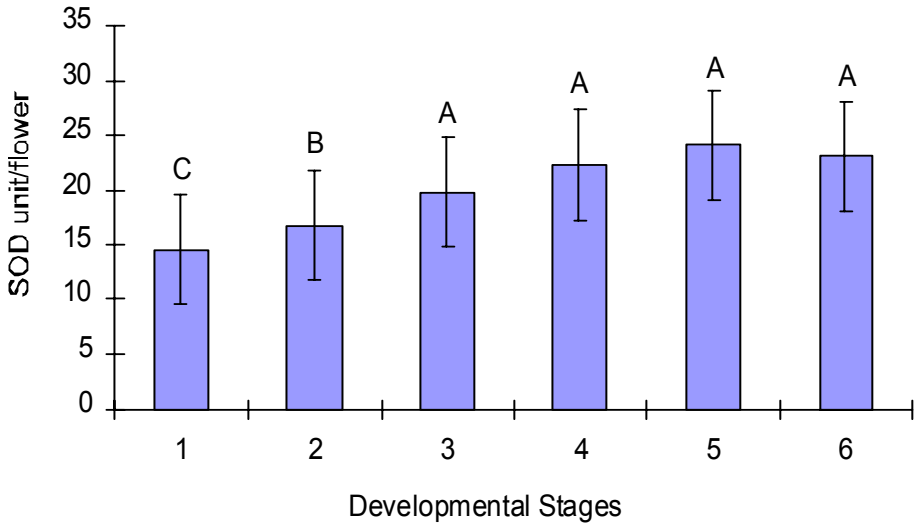


Fig 3.7 Activity of SOD in the pistils of healthy petunia flowers during different developmental stages. One unit of SOD activity is expressed as the amount of enzyme that inhibited the NBT photoreduction by 50%. The developmental stages are illustrated in Plate 2.2 and 2.3. Error bars indicate standard error of the mean. Letters above each value represent results from the Tukey test. Values with the same letter were not significantly different. 1-6 refers to the developmental stages. (For details, see Plate 2.3).

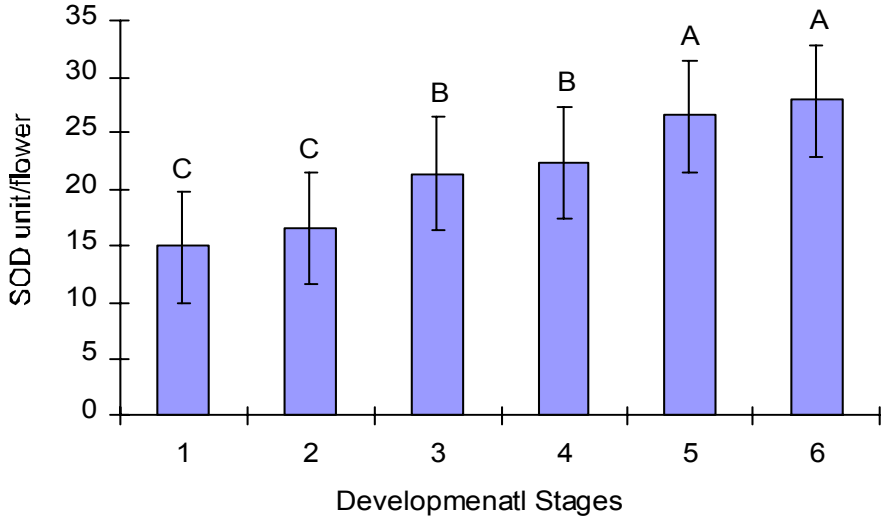


Fig 3.8 Activity of SOD in the ovaries of healthy petunia flowers during different developmental stages. One unit of SOD activity is expressed as the amount of enzyme that inhibited the NBT photoreduction by 50%. The developmental stages are illustrated in Plate 2.2 and 2.3. Error bars indicate standard error of the mean. Letters above each value represent results from the Tukey test. Values with the same letter were not significantly different. 1-6 refers to the developmental stages (For details, see Plate 2.3).

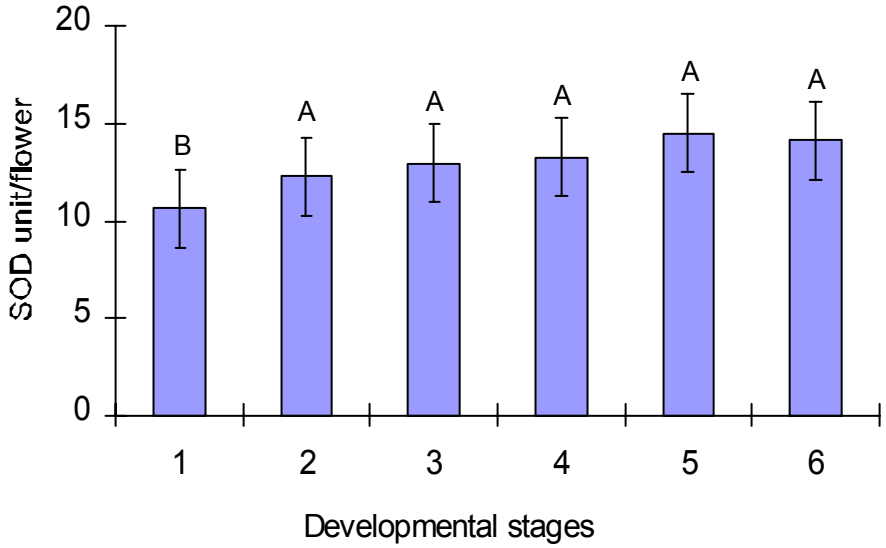


Fig 3.9 Activity of SOD in the stigmas of healthy petunia flowers during different developmental stages. One unit of SOD activity is expressed as the amount of enzyme that inhibited the NBT photoreduction by 50%. The developmental stages are illustrated in Plate 2.2 and 2.3. Error bars indicate standard error of the mean. Letters above each value represent results from the Tukey test. Values with the same letter were not significantly different. 1-6 refers to the developmental stages (For details, see Plate 2.3).

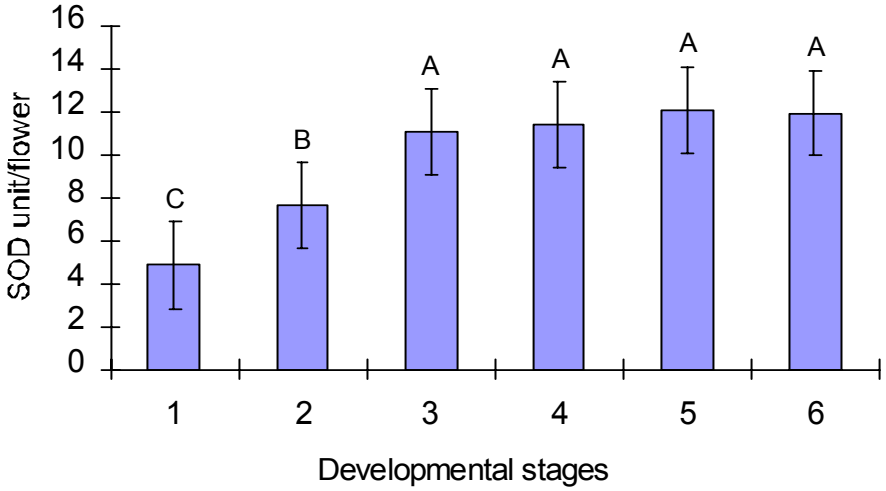


Fig 3.10 Activity of SOD in the styles of healthy petunia flowers during different developmental stages. One unit of SOD activity is expressed as the amount of enzyme that inhibited the NBT photoreduction by 50%. The developmental stages are illustrated in Plate 2.2 and 2.3. Error bars indicate standard error of the mean. Letters above each value represent results from the Tukey test. Values with the same letter were not significantly different. 1-6 refers to the developmental stages (For details, see Plate 2.3).

3.5 Stress-induced Response on SOD Activity

It has been suggested that different types of stress might induce the expression of SOD in different ways. Two different stress conditions were studied using only buds and fully open flowers. The shape and size of flowers was smaller and unhealthier when petunia was under stress conditions compared to the healthy control flowers (Plate 3.1). However, the development of the female reproductive structures of the flowers appeared to be unaltered under stress conditions (Plate 3.2). During water deficiency stress, there was a significant increase in SOD activity in each female reproductive tissue of young buds except the ovary compared to the control (Fig 3.11). As for fully open flowers, a significant increase was found in all the tissues collected. The pistil exhibited the highest increase of SOD activity in fully open flowers under water dificiency, approximately 31% more than that of the control, whereas the lowest increase was found in the stigma, which was only 12% more. By contrast, the highest increase in SOD activity in young buds was found in the stigma (a 29% increase) under water deficiency, whereas the increase (16%) in the ovary was the lowest. There was no significant difference in the percentage increase in SOD activity between young buds and fully open flowers under water deficiency.

SOD activity was found to increase in the female reproductive parts of both young buds and fully open flowers (except the stigma) under high temperature stress (Fig 3.12). In response to the high temperature stress, the styles of young buds exhibited the highest increase in SOD activity, which was approximately double that of the control. As for fully open flowers, the ovary exhibited the highest increase (54%) in SOD activity, while the lowest increase (18%) was

Plate 3.1 The pictures above show the shape and size of petunia flowers under stress conditions and normal conditions as control. In each picture, buds and fully open flowers on the left side are under stress conditions, whereas normal condition on the right side.

Plate 3.2 The picture above shows the shape and size of the female reproductive parts of petunia flowers under stress conditions and normal conditions as control. In the picture, buds and fully open flowers on the left side are under stress conditions, whereas normal condition on the right side.

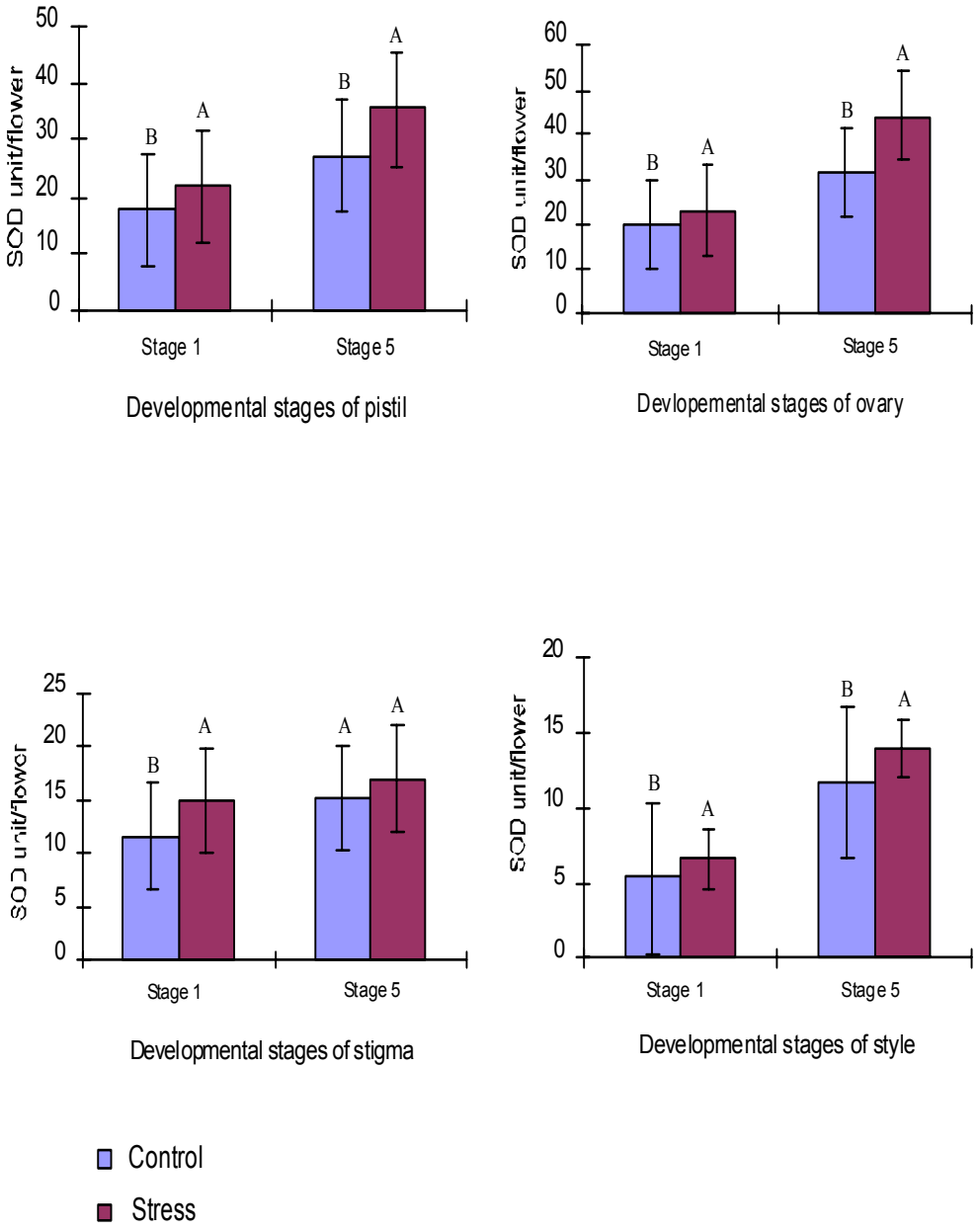


Fig 3.11 Activity of SOD in the pistils, ovaries, stigmas and styles of healthy petunia flowers in response to water deficiency stress. One unit of SOD activity is expressed as the amount of enzyme that inhibited the NBT photoreduction by 50%. The stage 1 and 5 were chosen for this experiment (For details, see Plate 2.3). The blue and red bars indicate control, and the stress treatment, respectively. The treatment was repeated three times with similar results of comparison within same stage. Error bars indicate standard error of the mean.

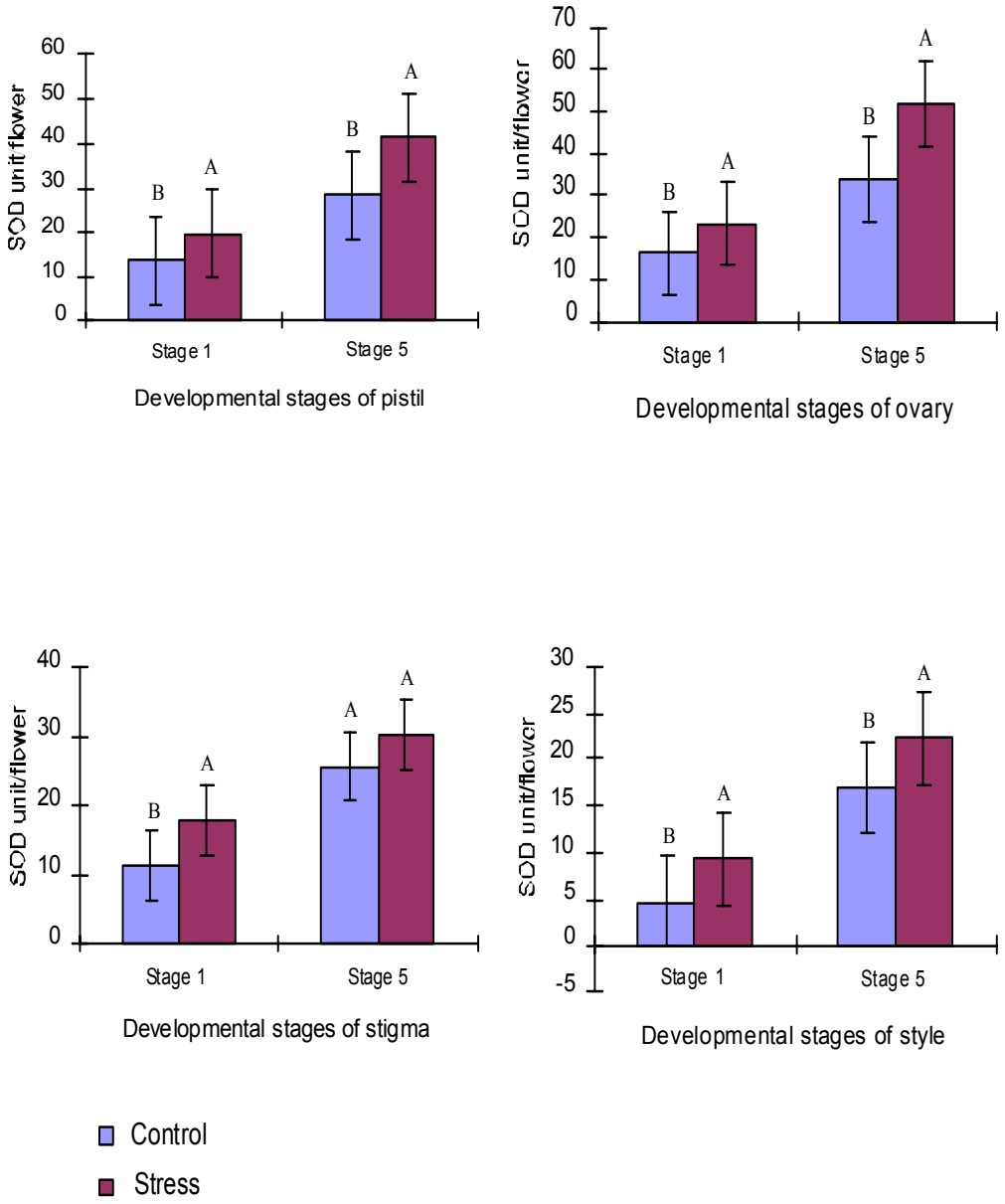


Fig 3.12 Activity of SOD in the pistils, ovaries, stigmas and styles of healthy petunia flowers in response to high temperature stress. One unit of SOD activity is expressed as the amount of enzyme that inhibited the NBT photoreduction by 50%. The stage 1 and 5 were chosen for this experiment (For details, see Plate 2.3). The blue and red bars indicate control, and the stress treatment, respectively. The treatment was repeated three times with similar results of comparison within same stage. Error bars indicate standard error of the mean.

found in the stigma in response to high temperature stress. Therefore, the stigmas and styles of young buds were more sensitive to high temperature stress than those of fully open flowers. In contrast, the ovaries of fully open flowers appeared to be more sensitive to high temperature stress than those of young buds.

3.6 Gel Electrophoresis

3.6.1 Isozyme Analysis of SOD

The SOD isozymes present within developmental stages of reproductive parts of petunia were analysed using a non-denaturing PAGE gel system. Samples of reproductive parts of flowers (pistil, ovary, stigma and style) at different developmental stages were initially homogenised in extraction buffer and diluted before loading onto the gel. Three clear distinct bands of SOD activity were detected in each 12% acrylamide gel (Plates 3.3, 3.4, 3.5 and 3.6). There was a clear trend of increase in SOD activity of each reproductive part of petunia flowers selected for this experiment. Manipulation of the amount of extracts loaded and incubation time in the stain were performed to try to obtain optimal band intensity during flower development. Sometimes an extra band might be visible at the bottom of a gel (Plate 3.7), but it was no longer detectable when the extract was boiled 5 min before gel electrophoresis. All of the gels tried had the same number of bands with varying intensity and size.

3.6.2 Classification of SOD Isozymes

In order to distinguish different types of SOD isozymes, the method used is based on a difference in the sensitivity of different SOD isoforms to inhibitors

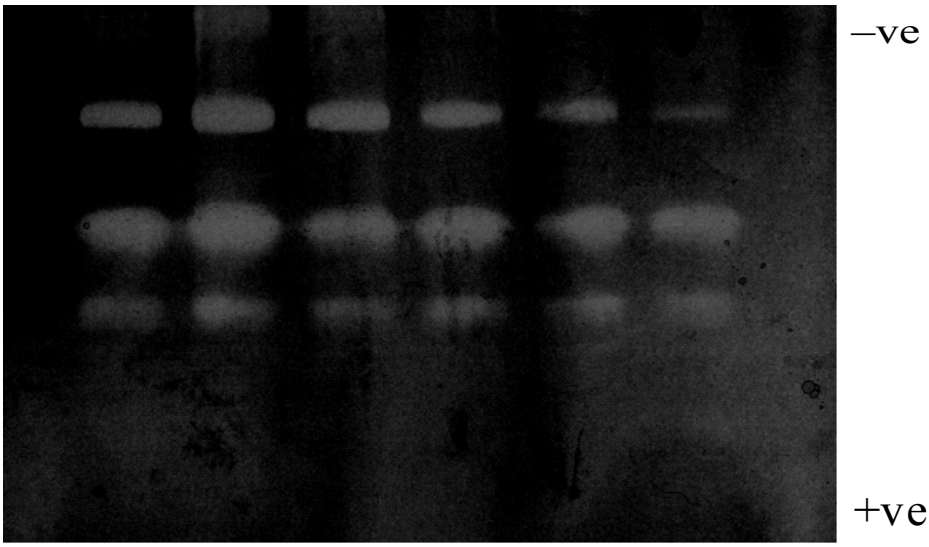


Plate 3.3 Non-denaturing PAGE gel with SOD activity in the pistils at six different developmental stages of petunia flowers. Each lane was loaded with 1:10 dilution of extract. Three distinct clear bands were presented on a dark purple background. There was an increase on SOD activity as the flower developed and grew.

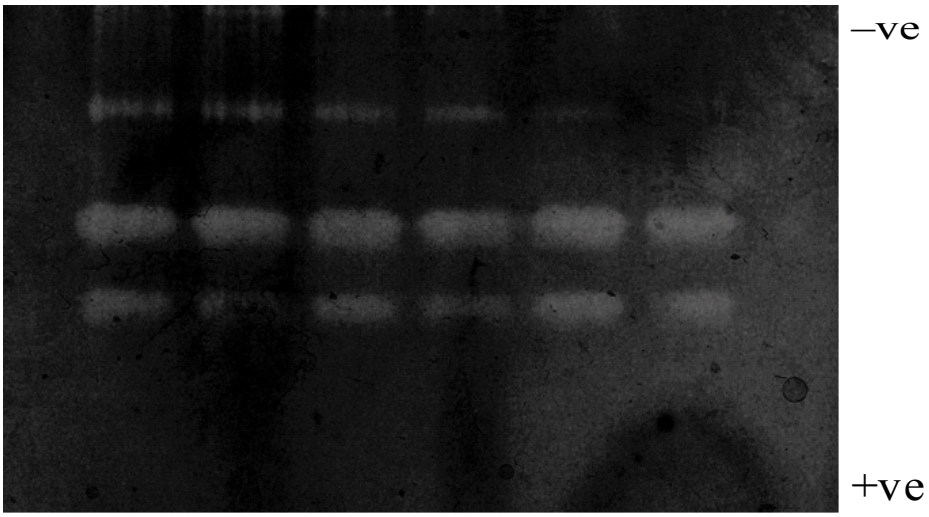


Plate 3.4 Non-denaturing PAGE gel with SOD activity in the ovaries at six different developmental stages of petunia flowers. Each lane was loaded with 1:10 dilution of extract. Three distinct clear bands were presented on a dark purple background. There was an increase on SOD activity as the flower developed and grew.

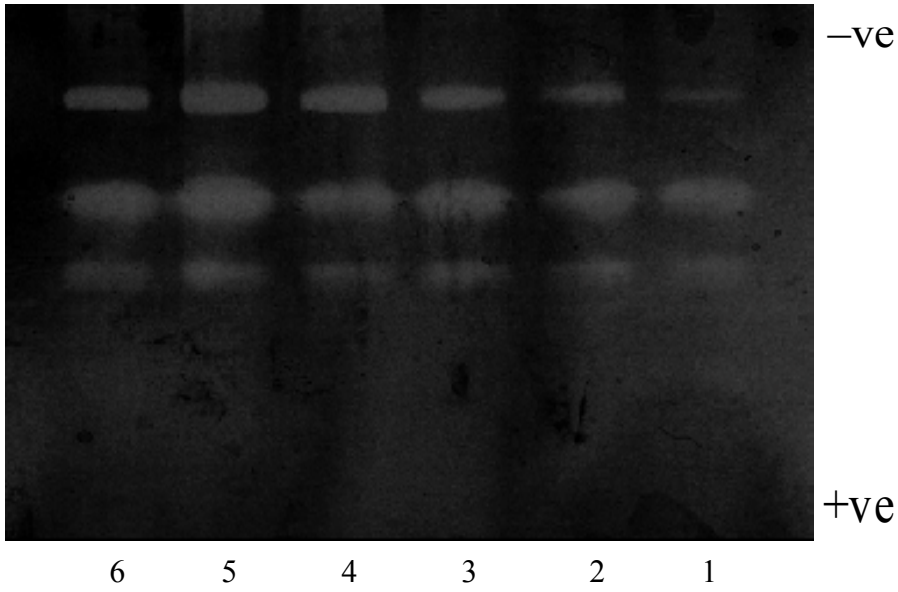


Plate 3.5 Non-denaturing PAGE gel with SOD activity in the stigmas at six different developmental stages of petunia flowers. Each lane was loaded with 1:2 dilution of extract. Three distinct clear bands were presented on a dark purple background. There was an increase on SOD activity as the flower developed and grew.

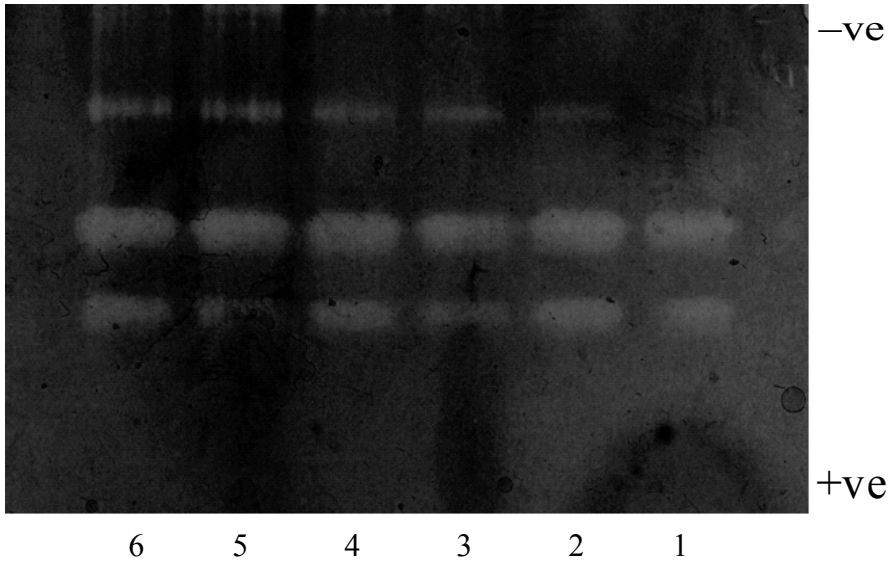


Plate 3.6 Non-denaturing PAGE gel with SOD activity in the styles at six different developmental stages of petunia flowers. Each lane was loaded with 1:2 dilution of extract. Three distinct clear bands were presented on a dark purple background. There was an increase on SOD activity as the flower developed and grew.

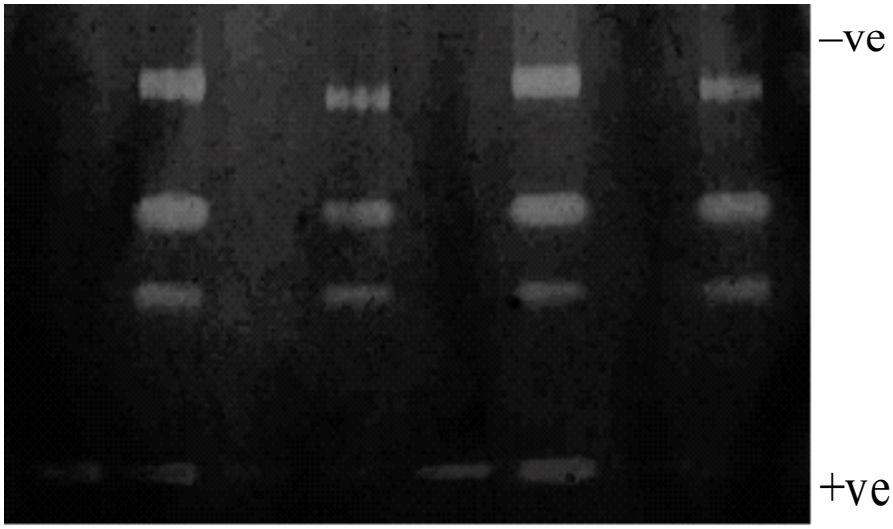


Plate 3.7 The comparison of Non-denaturing PAGE gel with SOD activity and boiling diluted extracts after 5 min in the female reproductive parts of petunia flowers. Lane 1 and 2: boiling and without boiling extracts of the ovary. Lane 3 and 4: boiling and without boiling extracts of the pistil. Lane 5 and 6: boiling and without boiling extracts of the stigma. Lane 7 and 8: boiling and without boiling extracts of the style.

Plate 3.8 Non-denaturing polyacrylamide gel electrophoresis of SOD within the ovaries of petunia after H_2O_2 treatment. The result shows that only first band left after SOD isozymes were treated to test a difference in sensitivity to H_2O_2.

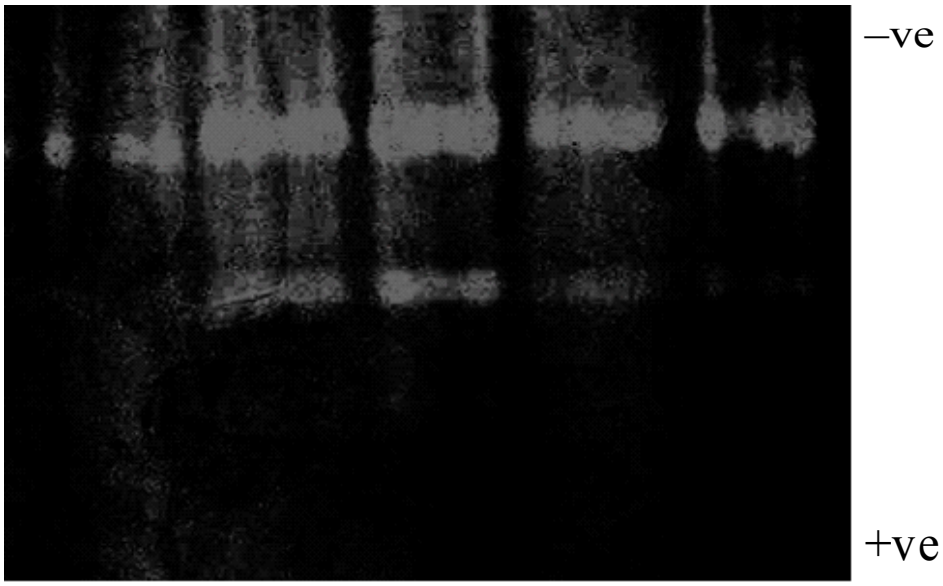


Plate 3.9 Non-denaturing polyacrylamide gel electrophoresis of SOD within the ovaries of petunia after KCN treatment. The result shows that the first two bands were left after SOD isozymes were treated to test a difference in sensitivity to KCN.

such as H_2O_2 and KCN. The result in Plate 3.8 show that only the first band was left after treatment with H_2O_2, whereas the third band disappeared completely when the gel was treated with KCN (Plate 3.9). Consequently, the first band left in the gel is Mn-SOD because the activity of Mn-SOD is not inhibited either by H_2O_2 or KCN (Plate 3.8). The second band is Fe-SOD as Fe-SOD is sensitive to H_2O_2 but not KCN (Plate 3.9). The sensitivity of Cu/Zn-SOD to cyanide suggested that the third band is a Cu/Zn-SOD (Plate 3.9).

3.7 Detection of Superoxide in Female Reproductive Tissues

Generally, the young bud had the fastest staining response among the tissues from the six developmental stages when immersed in a NBT solution. Tissues from the sixth stage had the lowest response with NBT, except for styles (Plate 3.10 A, C, D). The style is quite a difficult type of tissue for uptake of NBT solution compared to others (Plate 3.10 B, D). It had to be split apart to let solution penetrate into tissues over certain period of time, pictures show that small amount of solution could enter through cutting points until it was immersed in NBT solution for 8 hr (Plate 3.10 B). However, a style split from the longitudinal side was stained blue after immersing in NBT solution for only 1 hr (Plate 3.11 D).

To determine the specificity of the NBT reaction for superoxide determination, different types of tissues were incubated in the NBT solution with or without $MnCl_2$ over a certain time period. $MnCl_2$ is a superoxide dismutation agent which functions just like SOD. The stigma and ovary were stained blue after immersing in NBT solution for 2 hr, whereas there was no change in colour

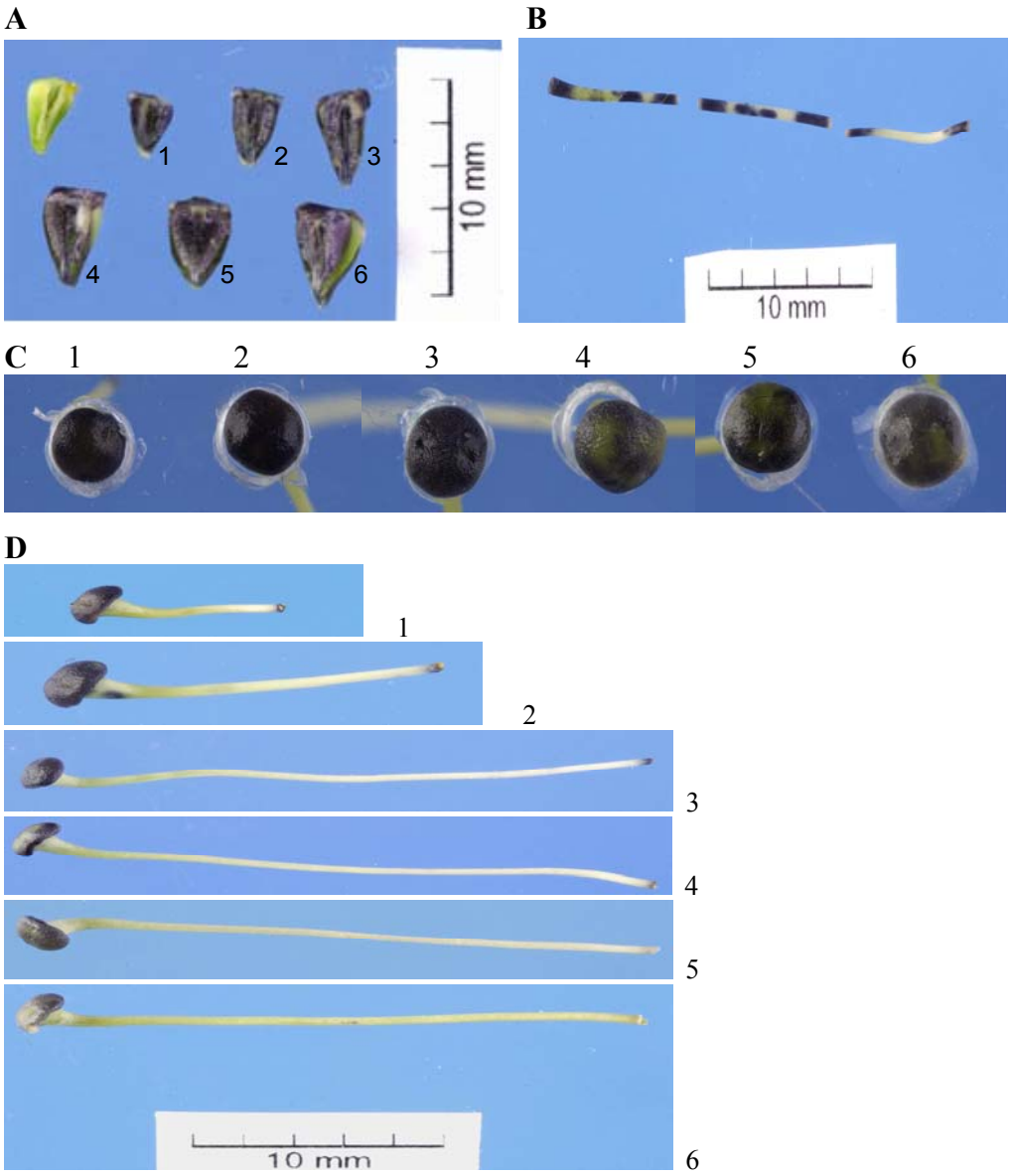


Plate 3.10 Response of superoxide in the female reproductive parts of petunia at different developmental stages. A. The ovaries split apart longitudinally at developmental stages after immersing in NBT solution for 2 hr. B. The style splits apart after immersing in NBT solution for 8 hr. C. Front view of the stigmas from developmental stages after immersing in NBT solution for 2 hr. D. Side view of the pistils from developmental stages after immersing in NBT solution for 2 hr. 1-6 refers to the developmental stages (For details, see Plate 2.3).

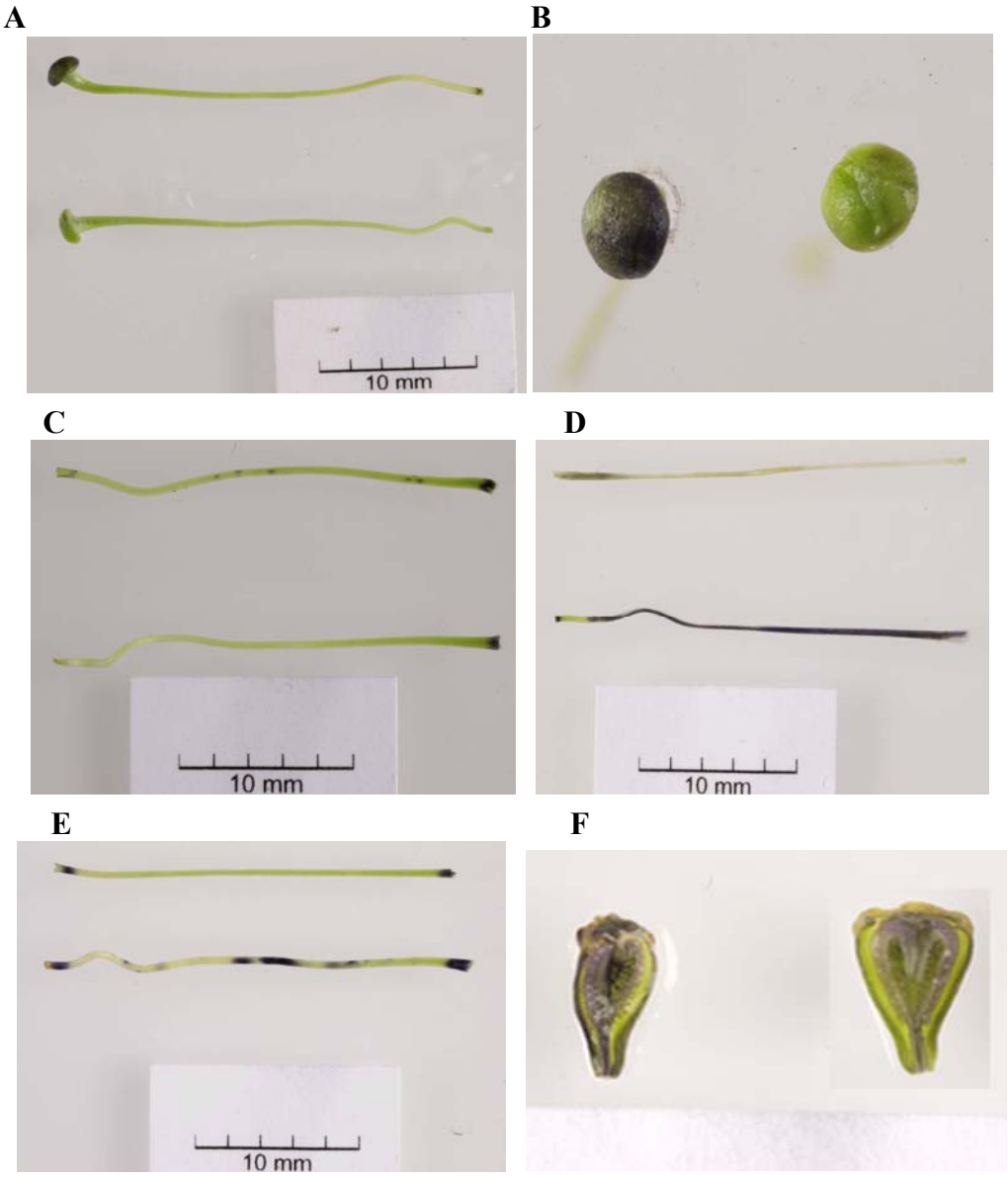


Plate 3.11 The comparison of the female reproductive parts of petunia immersed in the 0.01mM NBT solution and NBT + 0.2 mM $MnCl_2$ solution. A. The pistils immersed in NBT + $MnCl_2$ solution (bottom) and in NBT solution (top) after 2 hr. B. The stigmas immersed in NBT + $MnCl_2$ solution (right) and in NBT solution (left) after 2 hr. C. Whole styles immersed in NBT + $MnCl_2$ solution (bottom) and in NBT solution (top) after 4 hr. D. The split style immersed in NBT + $MnCl_2$ solution (top) and in NBT solution (bottom) after 1 hr. E. Whole style immersed in NBT + $MnCl_2$ solution (top) and in NBT solution (bottom) after 1 day. F. The ovaries immersed in NBT + $MnCl_2$ solution (right) and in NBT solution (left) after 2 hr.

with $MnCl_2$ during same period of time (Plates 3.11 A, B and F). The whole style exhibited no significant change in detection of O_2^- after immersing in both NBT solutions with or without $MnCl_2$ for 3 hr except cutting points (Plate 3.11 C), but some parts stained blue after approximately one day compared to the one in NBT solution with $MnCl_2$ (Plate 3.11 E). The split style had the fastest rate of reaction with NBT solution after only 1 hr immersion, while there was no change when incubating in NBT solution with $MnCl_2$ during the same time period (Plate 3.11 D).

3.8 Purification of SOD from the Pistils and Ovaries

Partial purification of SOD enzymes had been performed using the pistils and ovaries of fully open flowers of petunia. In the early stages of purification, attempts were made to precipitate the enzyme by ammonium sulphate ($(NH_4)_2SO_4$). The crude extracts of the pistils and ovaries from 100 flowers collected on the day of flower opening were subjected to $(NH_4)_2SO_4$ precipitation to test the appropriate saturation of $(NH_4)_2SO_4$ and which part of fractionation could be retained for further purification. After several treatments of this precipitation experiment, it was found that the highest SOD activity was obtained with 60% $(NH_4)_2SO_4$ precipitation. For the pistils, from 0 to 30% of $(NH_4)_2SO_4$ saturation was also examined and the supernatant from this step was chosen for further purification after several runs.

After preliminary experiments, the precipitate formed at 60% $(NH_4)_2SO_4$ saturation of the crude extracts from 1000 ovaries of petunia flowers previously stored at -80ºC and the supernatant from 30% saturation with $(NH_4)_2SO_4$ of the extracts from 1000 pistils were processed further. Dilysed

and concentrated extracts of the pistils and ovaries from the ammonium sulphate step were passed through a DEAE cellulose column.

The elution profiles with the ovary extract from the DEAE cellulose column are shown in Figs. 3.13 and 3.14. There was a major peak of SOD activity in the fractions from 7 to 13, indicating that the enzyme had been eluted out without binding to the column with 0.01M K buffer (pH 7.8). Then after applying a salt gradient (NaCl, from 0.05 M to 0.4 M), fractions from 68 to 94 had four different peaks of SOD activity (Fig 3.13). Similarly, with the pistil extract, there was also a major peak of SOD activity in the fractions from 11 to 23 and four peaks of SOD activity in the fractions from 53 to 84 that were eluted after applying a salt gradient (Fig 3.14). All fractions were examined for enzyme activity and protein concentration. The peak fractions were pooled for enzyme characterisation. The percentage of yield and the purity factors at each step of purification within both cases of the pistils and ovaries are summarised in Tables 3.1 and 3.2. The enzyme yields obtained, however, were low. SOD of the ovary extracts was purified approximately 15-fold at the final step to a specific activity of 530 units mg^{-1} protein (Table 3.1), whereas there was no significant changes of specific activity between $(NH_4)_2SO_4$ precipitation and anion-exchange chromatography from the pistils (Table 3.2). Therefore, the present methods are not appropriate for SOD enzyme purification from the pistils.

Table 3.2 Purification steps of SOD from the ovaries of petunia flowers.

Procedures	Volumn(mL)	Protein (mg)	Activity (units)	Sp activity (unit/mg)	Recovery (%)	Purification
Crude extract	31	72.80	2569.88	35.3	100	1
0-60% $NH_4(SO_4)_2$	34	9.92	865.67	87.28	33.69	2.47
Fraction No.11	2	1.29	84.67	65.47	3.29	1.85
Fraction No. 69	2	0.03	17.67	530	0.69	15.01
Fraction No. 76	2	0.09	16	181.56	0.62	5.14
Fraction No. 80	2	0.12	35	281.53	1.36	7.98
Fraction No. 83	2	0.05	20.67	440.43	0.80	12.48

Table 3.3 Purification steps of SOD from the pistils of petunia flowers.

Procedures	Volumn(mL)	Protein (mg)	Activity (units)	Sp activity (unit/mg)	Recovery (%)	Purification
Crude extract	29.5	64.82	2497.67	38.53	100	1
0-30% $NH_4(SO_4)_2$	31	41.48	1539.67	37.12	61.64	0.96
Fraction No.15	2	13.73	77	5.61	3.08	0.15
Fraction No. 60	2	0.67	18	27	0.72	0.70
Fraction No. 64	2	1.11	22.67	20.42	0.91	0.53
Fraction No. 68	2	1.37	19.33	14.08	0.77	0.37
Fraction No. 73	2	0.85	22.33	26.28	0.89	0.68

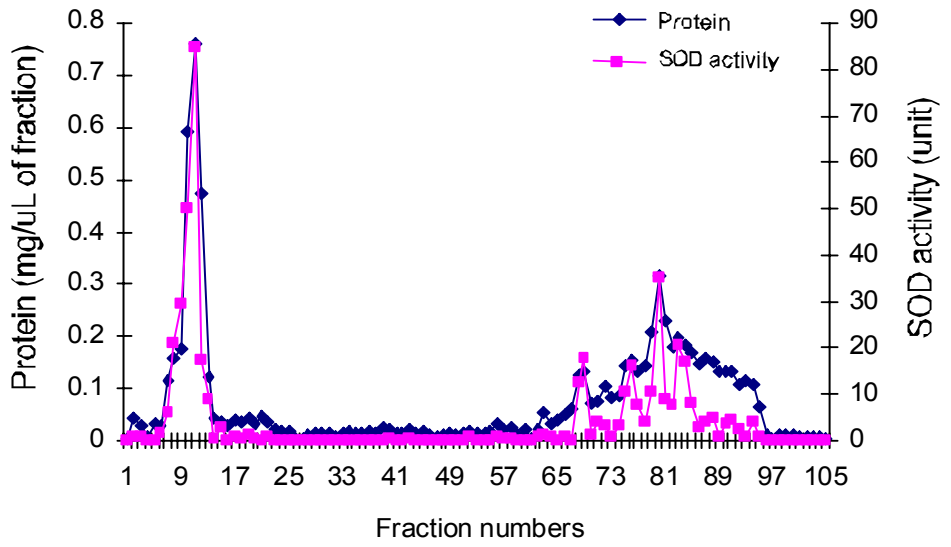


Fig 3.13 Elution profile of total protein and SOD activity from a DEAE-Cellulose column within the ovaries of petunia. The first 50 fractions were eluted with 0.01 M phosphate buffer (pH 7.8), while the next 50 fractions were eluted using 0.01 M phosphate buffer (pH 7.8) containing salt gradient from 0.05 to 0.4 M NaCl. Protein content was measured as absorbance at 280 nm. One unit of enzyme activity is defined as a change in one unit of absorbance at 560 nm per μL of fraction..

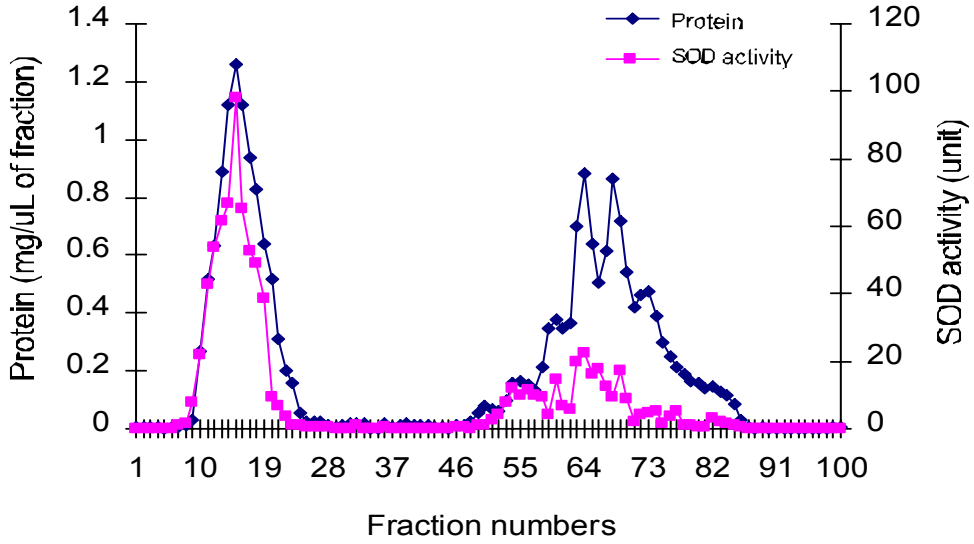


Fig 3.14 Elution profile of total protein and SOD activity from a DEAE-Cellulose column within the pistils of petunia. The first 50 fractions were eluted with 0.01 M phosphate buffer (pH 7.8), while the next 50 fractions were eluted using 0.01 M phosphate buffer (pH 7.8) containing salt gradient from 0.05 to 0.4 M NaCl. Protein content was measured as absorbance at 280 nm. One unit of enzyme activity is defined as a change in one unit of absorbance at 560 nm per μL of fraction.

3.9 Characteristics of Purified Enzyme

3.9.1 Effect of Storage at Different Temperatures on the Activity and Stability of Partially Purified SOD

Fractions corresponding to peak protein and activity were examined for effects of storage at different temperatures. The thermostability of the enzyme was examined at pH 7.8. The effect of storing purified fractions at low temperature was determined by storing at 4ºC in a fridge and at -20ºC in a freezer. Room temperature was also included in an attempt to compare the difference between crude extracts and partially purified SOD fractions.

For the ovaries, storage of the partially purified extracts at -20ºC resulted in approximately 30% loss in SOD activity after 4 weeks, whereas there was a dramatic decrease in SOD activity when the partially purified extracts were stored at 4ºC as only 20% of activity remained for both unbound and bound fractions during same time period (Fig 3.15.A & B). Generally, the SOD activity in the bound fractions from the DEAE-cellulose column was more stable than that in unbound ones when stored at -20ºC, whereas both unbound and bound fractions became undetectable when they had been stored at 4ºC after 3 weeks. There was a dramatic drop in SOD activity when the unbound fractions were stored at room temperature (25ºC), and that in the bound fractions became undetectable only after 6 days of storage at this temperature (Fig 3.16). The partially purified extracts from the pistils of fully open flowers were also examined to attempt comparison with earlier results from crude extracts. There was a similarity to that of the ovaries (data not shown).

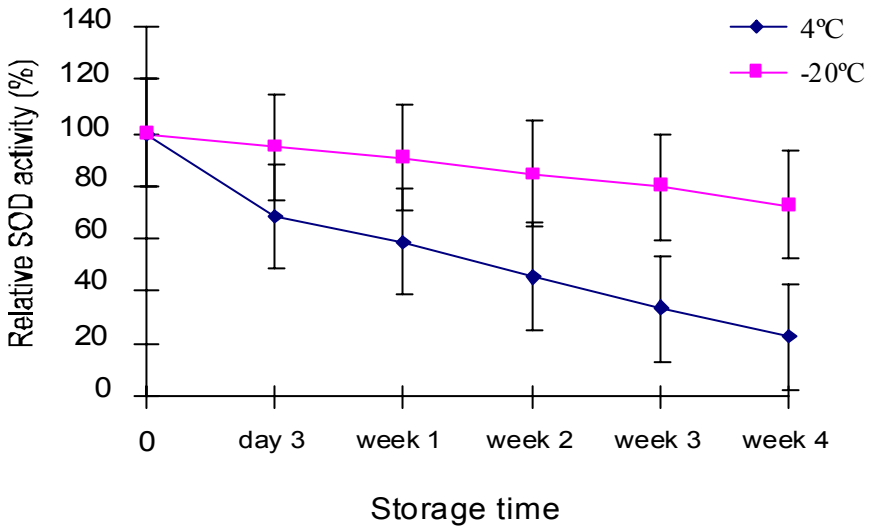


Fig 3.15.A Effect of storage time at 4ºC in a fridge and at -20ºC in a freezer on activity of partially purified SOD. Unbound peak fraction (No. 11) from normal wash of 0.01 M phosphate buffer (pH 7.8). Fractions were purified from the ovaries of fully open petunia flowers. One unit of enzyme activity is defined as change in absorbance at 560 nm per µL of fraction. Error bars represent standard deviation.

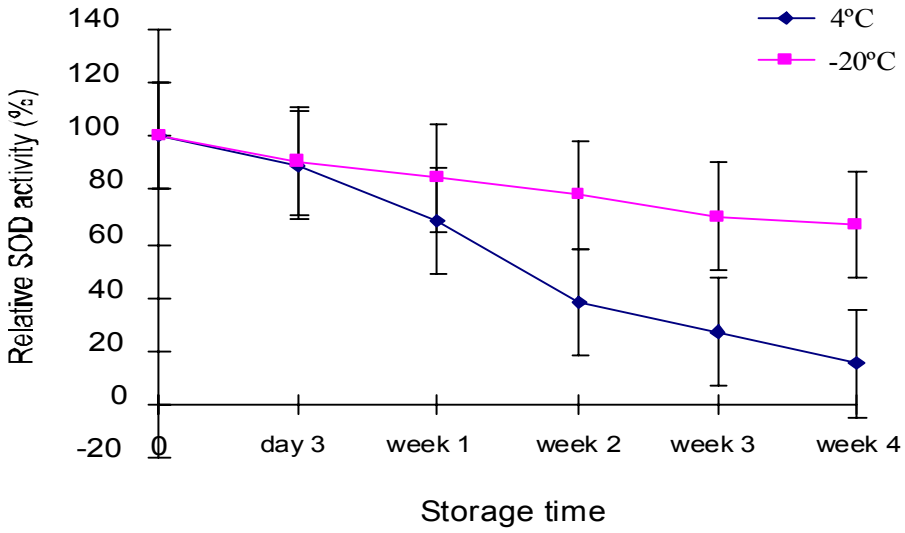


Fig 3.15.B ffect of storage time at 4ºC in a fridge and at -20ºC in a freezer on activity of partially purified SOD. Bound peak fraction (No. 76) from salt gradient. Fractions were purified from the ovaries of fully open petunia flowers. One unit of enzyme activity is defined as change in absorbance at 560 nm per µL of fraction. Error bars represent standard deviation.

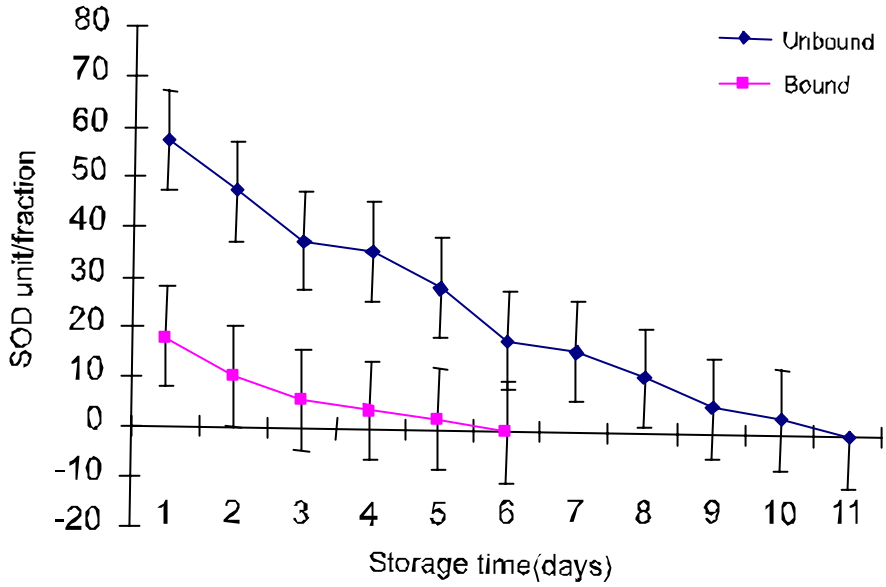


Fig 3.16 Effect of storage time at room temperature (25ºC) on activity of partially purified SOD. Fractions were purified from the ovaries of fully open petunia flowers. One unit of enzyme activity is defined as change in absorbance at 560 nm per µL of fraction. Error bars represent standard deviation.

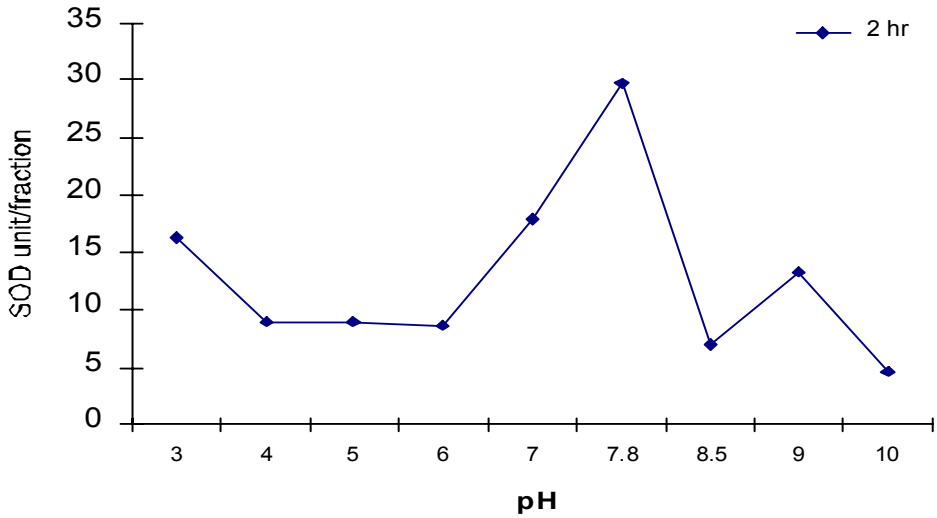


Fig 3.17 Effect of pH on SOD stability in the ovaries of partially purified SOD. Fraction No. 69 was purified from the ovaries of fully open petunia flowers. One unit of enzyme activity is defined as change in absorbance at 560 nm per µL of fraction.

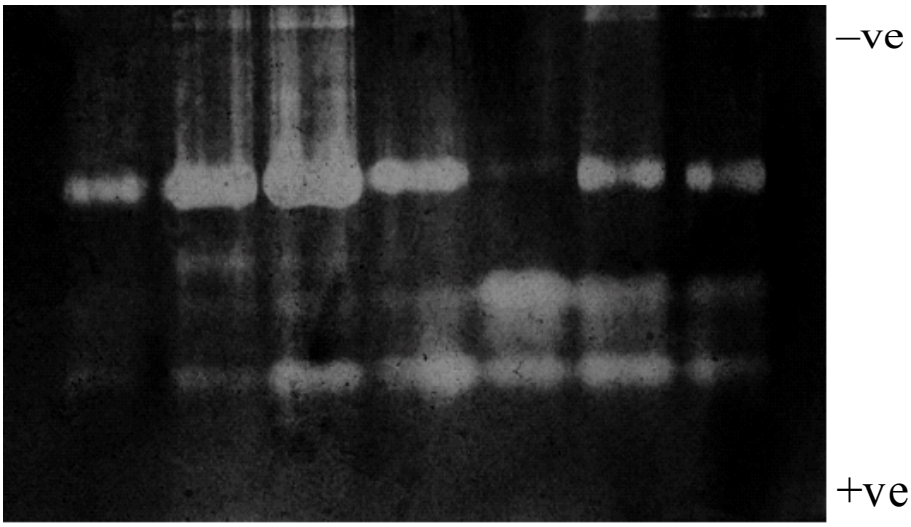
3.9.2 Effect of pH on the Activity and Stability of Partially Purified SOD

The SOD activity in partially purified extracts from the ovaries of petunia was optimal at pH 7.8 (data not shown) which is the same as the crude extracts (Fig 3.4). Based on experience from the earlier results (Figs 3.5 and 3.6), an attempt to study the effect of pH stability on the SOD in partially purified extracts was performed after only 2 hr incubation. The results obtained (Fig 3.17) were similar to those obtained with crude extracts except some difference showed up at some pH levels in a particular fraction. For instance with fraction No. 69 showed a minor peak at pH 10 besides the peak at pH 7.8.

3.9.3 Non-denaturing PAGE of Partially Purified SOD

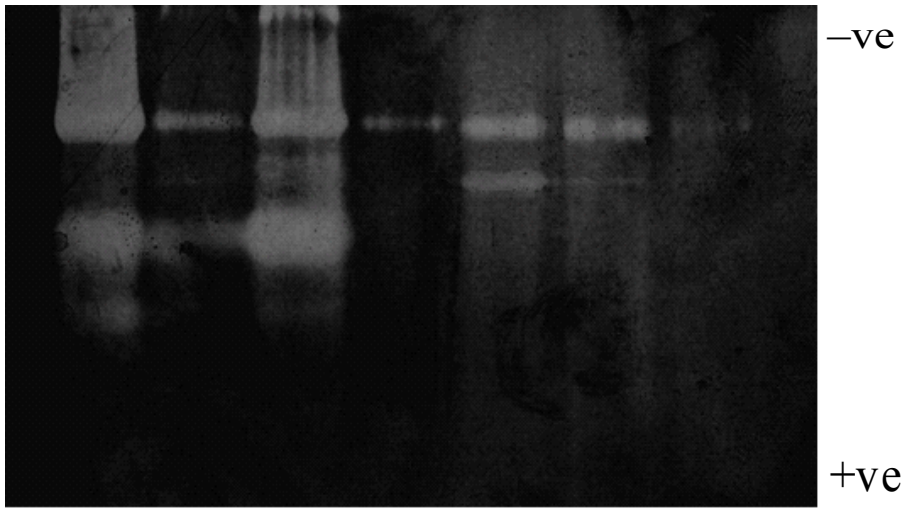
Non-denaturing PAGE of the partially purified extracts from the pistils and ovaries were performed. The location of the purified enzymes in the gel was identical to the activity bands from the crude extracts. There was approximately no difference shown in the gel from the crude extract, ammonium sulphate fraction, the unbound fraction and bound fractions from the DEAE-cellulose column of the pistils of petunia flowers (Plate 3.12). The bound fractions from the DEAE-cellulose column of the ovaries of flowers had a major SOD isozyme band in the non-denaturing PAGE gel, which corresponded to the location of one of the three isozymes bands in the crude extracts (Plate 3.13). There was also another band visible in some fractions obtained from purification of the ovaries (Plate 3.13).

The fractions were run on non-denaturing PAGE for analysis based on the



7 6 5 4 3 2 1

Plate 3.12 Non-denaturing polyacrylamide gel electrophoresis of partially purified SOD within the pistils of petunia in the different purification steps. Lane 1, crude extracts of 1000 pistils; Lane 2, 0-30% $(NH_4)_2SO_4$ fractionation; Lane 3, unbound enzyme fraction from a DEAE-cellulose column; Lane 4—7, bound enzyme peak fractions from a DEAE-cellulose column.



1 2 3 4 5 6 7

Plate 3.13 Non-denaturing polyacrylamide gel electrophoresis of partially purified SOD within the ovaries of petunia in the different purification steps. Lane 1, crude extracts of 1000 ovaries; Lane 2, 0-60% $(NH_4)_2SO_4$ fractionation; Lane 3, unbound enzyme fraction from a DEAE-cellulose column; Lane 4—7, bound enzyme peak fractions from a DEAE-cellulose column.

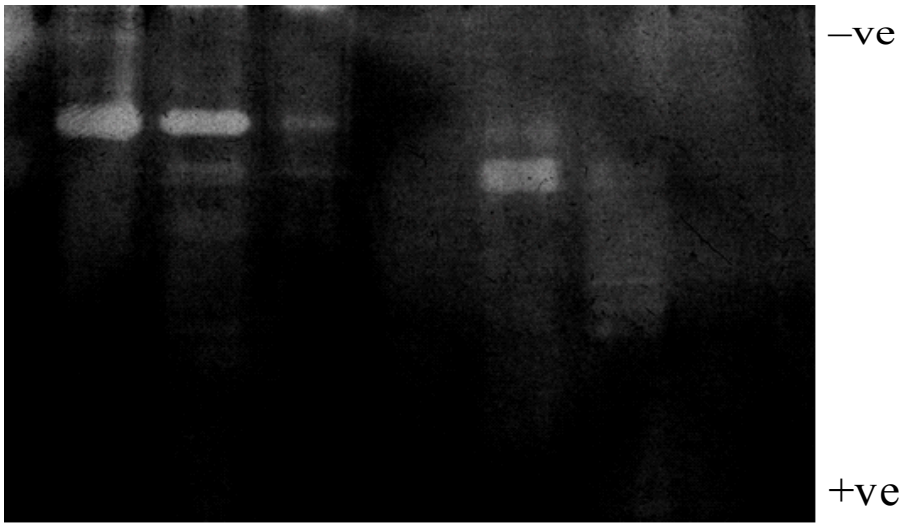


Plate 3.14 Non-denaturing polyacrylamide gel electrophoresis of partially purified SOD within the ovaries of petunia in the different purification steps after H_2O_2 treatment. Lane 1, crude extracts of 1000 pistils; Lane 2, 0-30% $(NH_4)_2SO_4$ fractionation; Lane 3, unbound enzyme fraction from a DEAE-cellulose column; Lane 4—7, bound enzyme peak fractions from a DEAE-cellulose column.

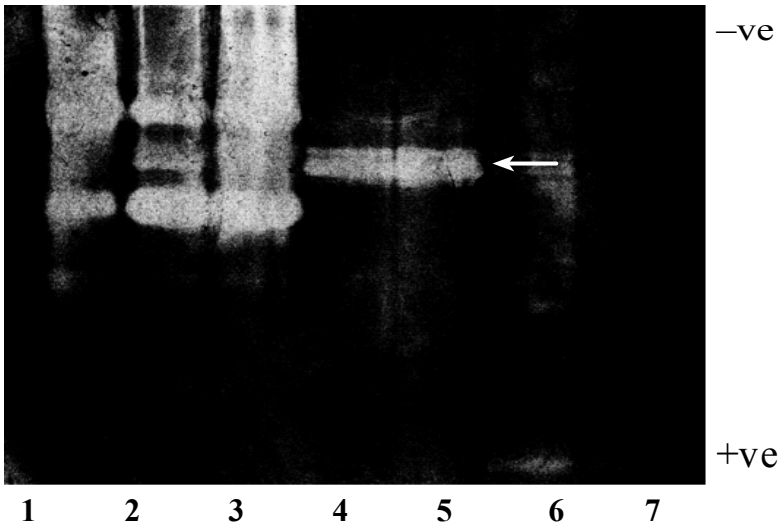


Plate 3.15 Non-denaturing polyacrylamide gel electrophoresis of partially purified SOD within the ovaries of petunia in the different purification steps after KCN treatment. Lane 1, crude extracts of 1000 pistils; Lane 2, 0-60% $(NH_4)_2SO_4$ fractionation; Lane 3, unbound enzyme fraction from a DEAE-cellulose column; Lane 4—7, bound enzyme peak fractions from a DEAE-cellulose column.

differences in sensitivity to H_2O_2 and KCN. In the crude extracts, ammonium sulphate fraction, and the unbound fraction from the DEAE-cellulose column, there was only one clear band left after H_2O_2 treatment (Plate 3.14). There was also second band only present in the $(NH_4)_2SO_4$, unbound and some bound fractions (Plate 3.14). After KCN treatment, two clear bands were present in the crude extract as well as the first two steps of purification, but not in the bound fraction from the DEAE column (Plate 3.15). Except the crude extracts, other fractions from the different purification steps had another band resistant to the KCN treatment (see the arrow lead in Plate 3.15).

3.9.4 SDS PAGE of Partially Purified SOD

SDS-PAGE gel and Coomasie blue staining of petunia from the pistils of each purification step revealed that almost all the bands in the crude extracts were also present in the ammonium sulphate fractionation and the pooled DEAE-cellulose column fractions (Plate 3.16). A different result was obtained with SDS PAGE analysis of protein samples of the ovary extracts from the different steps of purification. In the bound fractions, compared to the crude extracts, very few protein bands were detectable (Plate 3.17). The molecular weight of SOD from each purification step of the pistil and ovary of petunia flowers was estimated by SDS-PAGE gel analysis using a mixture standard protein marker from BioRad (California, USA). They were: phosphorylase b (108,000Da), bovine serum albumin (90,000 Da), ovalbumin (50,700 Da); carbonic anhydrase (35,500 Da); soybean trypsin inhibitor (28,600 Da), and lysozyme (21,200 Da) (Tables 3.4 and 3.5).

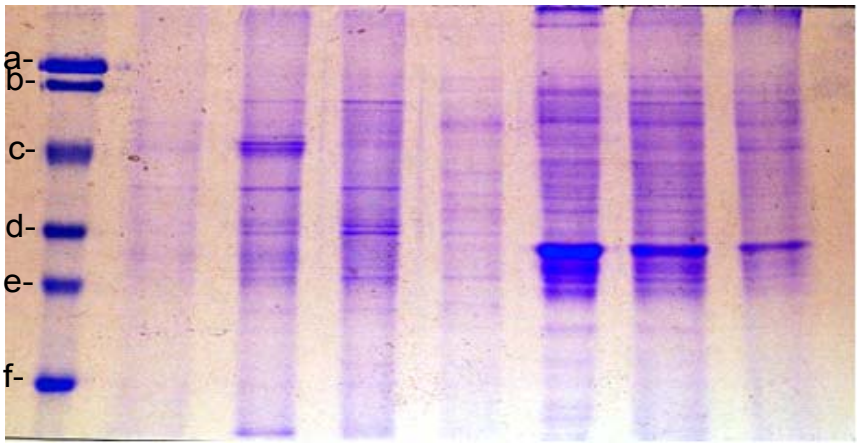


Plate 3.16 SDS-PAGE of partially purified extract from the pistils of petunia. Lane 1, crude extracts of 1000 pistils; Lane 2, 0-30% $(NH_4)_2SO_4$ fractionation; Lane 3, unbound enzyme fraction from a DEAE-cellulose column; Lane 4—7, bound enzyme peak fractions from a DEAE-cellulose column. Lane 8, molecular weight standard marker, from top to bottom: a) phosphorylase (108,000 Da); b) bovine serum albumin (90,000 Da); c) ovalbumin (50,700 Da); d) carbonic anhydrase (35,500 Da); e) soybean trypsin inhibitor (28,600 Da); f) lysozyme (21,200 Da).

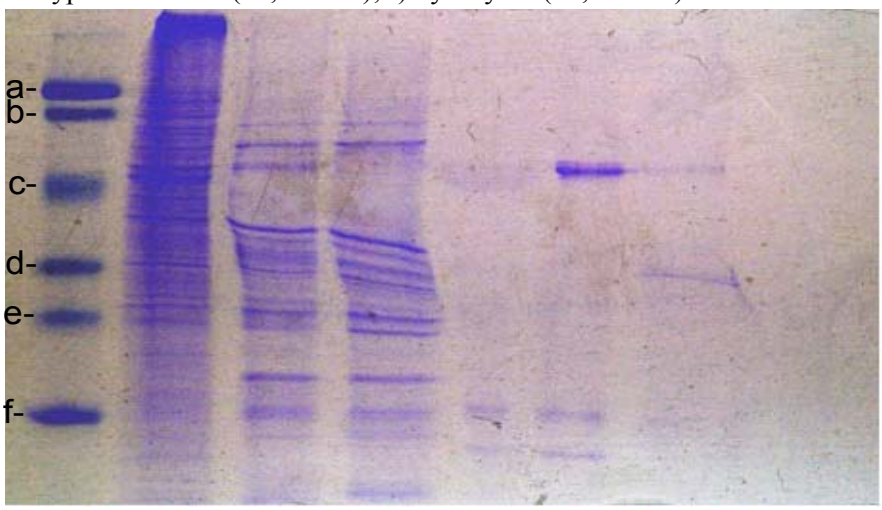


Plate 3.17 SDS-PAGE of partially purified extract from the ovaries of petunia. Lane 1, molecular weight standard marker from top to bottom, a) phosphorylase (108,000 Da); b) bovine serum albumin (90,000 Da); c) ovalbumin (50,700 Da); d) carbonic anhydrase (35,500 Da);e) soybean trypsin inhibitor (28,600 Da); f) lysozyme (21,200 Da). Lane 2, crude extracts of 1000 ovaries; Lane 3, 0-60% $(NH_4)_2SO_4$ fractionation; Lane 4, unbound enzyme fraction from a DEAE-cellulose column; Lane 5—8, bound enzyme peak fractions from a DEAE-cellulose column.

Table 3.4 Molecular weight (in Daltons) estimation from SDS PAGE gel analysis of enzyme extracts from the pistils of petunia flowers.

Distance migrated on the gel (mm)	Crude extract	30% $(NH_4)_2SO_4$ precipitation	Unbound fraction 10-23	Bound fraction 60	Bound fraction 64	Bound Fraction 68	Bound fraction 73
3.4	77370	77370	77370		77370	77370	
4	66328	66328	66328				
4.8						55805	
5	53687	53687	53687			53687	
6.3	43129	43129	43129		43129	43129	43129
8.3	33214	33214	33214	33214			
9.3	29820	29820	29820	29820	29820	29820	
9.7				28653			

Table 3.5 Molecular weight (in Daltons) estimation from SDS PAGE gel analysis of enzyme extracts from the ovaries of petunia flowers.

Distance migrated on the gel (mm)	Crude extract	60% $(NH_4)_2SO_4$ precipitation	Unbound fraction 11	Bound fraction 69	Bound fraction 76	Bound fraction 80	Bound fraction 83
4.5	68165	68165	68165				
5.1	59670	59670					
5.3					57278		
5.5	55065						
6.8	43942	43942	43942				
8.3	35548	35548	35548				
9.5	30793						
9.7		30118	30118				

CHAPTER 4

DISCUSSION

4.1 Importance of New Findings

This is first time the occurrence of SOD activity in female reproductive parts (pistil, ovary, stigma and style) of petunia has been studied. The experiments on general changes associated with developmental stages and stress-induced response of SOD within petunia were also carried out in this research to confirm and extend the findings of previous studies on SOD. SOD activity was found to be significantly highest within the tissues of the ovary of the flower, whereas the style had the lowest. SOD was found to be present on the extracellular surface of the stigma. Activity was also found to be dependent upon the developmental stages of the flower, with the highest enzyme activity within dehiscent flowers. The evidence from this research also shows that SOD activity is associated with plant defense system against stress conditions (water deficiency and high temperature). Some interesting questions have arisen regarding the biological function of SOD activity in these floral tissues of the flower. Does the enzyme play a role in the developmental physiology of the flower? Can this enzyme activity also be associated with plant defense systems or environmental stress induced response? These are worthy of further investigation in the future.

The formation of ROS could cause rapid substrate overload of constitutive SOD, while induction was probably hindered by other factors such as time, activity of downstream enzymes in the ROS-detoxification cascade, inhibition

by the end product (H_2O_2) and consequences of anoxic metabolism. SOD activity was found to be fluctuate in different seasons and under some environmental stresses (e.g. fungi disease, white fly infection and temperature fluctuation).

Characterisation of SOD within the female reproductive parts of petunia was examined regarding to the different forms of isozymes present in different floral tissues of the flower. Three isozymes of SOD were found using a native PAGE gel system. This is the first study of isozymes of SOD in the extracts of the female reproductive parts of a petunia flower. Further study is required to ascertain the biological role of each SOD isozyme in flower development and senescence. These SOD isozymes might have different functions that could be involved in plant defense mechanism and other aspects of physiological importance.

An estimation of superoxide level in the floral tissues was determined using nitro blue tetrazolium (NBT) staining. Intense blue staining was observed from developing younger bud, and at the later stages, much lower formazan deposition was observed (Plates 3.11 A.C.D).

Purification of SOD from the pistils and ovaries of petunia was achieved following $(NH_4)_2SO_4$ (ammonium sulphate) precipitation and ion-exchange chromatography on a DEAE-cellulose column. A considerable amount of time and effort were required to collect sufficient pistil and ovary tissues from 1000 flowers at the same developmental stage for this experiment, which was attempted three times. There was a very low level of SOD activity obtained from the pistils of flowers, and the analysis of SDS PAGE gel also showed that

no progressive process of purification was achieved with the crude extracts from this type of tissue. In contrast, a clearer result was obtained with the crude extracts from the ovaries using the same purification steps.

4.2 Assay of SOD

The activity and the assay techniques are associated with diverse fields such as medicine, biochemistry, plant physiology and food chemistry. Various SOD assay methods and systems have been developed since the discovery of SOD. It is difficult to determine the activity of SOD based on the dynamic concentration of the substrate because O_2^- is unstable. Depending on the product level, it is also not feasible to base activity determination on product formation because hydrogen peroxide is also unstable in the presence of certain metal ions. CAT and peroxidase existing in the crude extracts can also interfere with the result. The methods of SOD assay such as those based on the inhibition of NBT photoreduction (Giannopolitis and Ries, 1977) or on cytochrome C reduction (Beyer and Fridovich, 1987) suffer from interference from oxidases or peroxidases which are also present in plant crude extracts (Beauchamp and Fridovich 1971).

The method modified for this present study presents the composition of a reaction mixture which has been found to be very convenient for measuring SOD activity by the photochemical NBT method. The percent inhibition of NBT reduction is a function of SOD activity in this assay. It is clear that only a portion of the photoreduction of NBT is inhibitable by SOD. Therefore, it is reasonable to define one unit of SOD as that amount which causes a 50% decrease of the SOD-inhibitable NBT.

4.3 SOD Activity in Petunia

4.3.1 SOD Activity in the Female Reproductive Parts of Petunia

The pistils appear to be well designed to encourage pollen-pistil interactions, and a number of mechanisms supporting this general strategy have been described both in the stigma and in the style. SOD forms have been studied to be differentiated in activity, depending not only on their localisation in the plant tissue but also on the stage of development in *Mesembryanthemum crystallinum* L. (Libik et al. 2005). Hérouart et al. (1994) have studied the promoter activity of a cytosolic gene coding for CuZn-SOD in transgenic tobacco. The promoter was fused to the *β*-glucurondase (GUS) reporter gene (*gusA*), and GUS activity was detected in ovules and pollen grains, in pigmented tissues of petals, and in the vascular tissue of ovaries and anthers in the flower, but not in styles. However, based on the results obtained in this study, SOD activity was also found within the style of petunia flowers. Further study of gel electrophoresis also showed evidence of a CuZn-SOD isozyme within the style of petunia flowers.

The developmental anatomy of the stigma of *Petunia hybrida* was studied by Konar and Linskens in 1966. According to their theory, stigma can be classified into two zones: an upper zone (secretory zone) and a lower zone (storage zone). The petunia stigma has a bright green colouration because the cells of the secretory zone are very rich in chloroplasts (Konar and Linskens 1966). They also reported that exudation begins at an early stage and covers stigmas with a layer of exudate after anthesis in petunia. The stigmatic exudate consists of lipids, phenolic compounds, proteins, water and sugars (e.g polysaccharides) (Verhoeven et al. 2005). The lipid composition influences water uptake by

pollen grains and enables successful pollen-pistil interactions (Wolters-Arts at al. 2002), and pollen tube growth is more efficient with unfractionated exudate than when pollen is grown with lipids only (Wolters-Arts at al. 1998). The feature of stigmatic exudate is very complicated, and its roles might vary from the exclusion of fungi, bacteria and foreign pollen from growth on the pistil, to promotion of the growth of native pollen. The stigmatic exudate of dehiscent petunia flowers was tested for SOD activity in a preliminary experiment. SOD activity was found to be present in the stigmatic exudates of dehiscent petunia flowers. This might be SOD of the pollen grain that was loaded on the surface of the stigma. Further study of SOD having a possible role in the process of pollination is interesting. Some constituents of the exudate, such as phenolics, are thought to play a role in repelling insects, or inhibiting germination of spores (Martin and Brewbaker 1971). Therefore, it is possible that SODs present on the stigmatic surface might have a role similar to that proposed for the phenolics.

It is also reported that petunia has a solid style with transmitting tissue which is located in the centre of the style but forked into two vascular strands near the stigma in order to reach the stigmatic lobes (Onus 2000). The nutritive role of the transmitting tissue for the growth of the pollen tubes through the style has been known for some time. Kroh and Helpser (1974) assumed that in pollinated styles the pollen tubes broke down the intercellular material enzymatically and utilsed it for growth within the intercellular substance of the transmitting tissue in petunia. The activity of SOD found in the style of fully open flowers was approximately equal to the stigma at the same developmental stage. According to the antioxidant role of SOD, it might have a role in the development and regulation of elongation of the style or the pollen tube growth

through the style.

In some species, regulation of pollen tube entry into the ovary has been reported (Martínez-Pallé and Herrero 1998). Pollen tube growth in the ovary has been examined to see how changes in the sporophytic tissues of the ovary and ovule relate to pollen tube guidance (Herrero 2000). He reported that once pollen tubes reach the ovary, they have to 'surf' along the placenta, reach the ovule exostome, enter the micropyle, traverse the nucellus and enter the embryo sac via a synergid. Therefore, changes in biosynthesis of protein content are associated with growth and development in the ovary tissues. In the present study, a high level of SOD activity was found in the ovary after anther dehiscence, suggesting that SOD might be associated with regulation of flower development. SOD within the ovary of petunia flower could also be involved in plant defense, and or it might have some as yet underlined developmental functions. Studies are lacking that indicate the functions of SOD in the process of pollination and fertilisation in the ovary.

4.3.2 SOD Activity during Petunia Flower Development

O_2^- was found to be present during, and essential for, morphogenesis of etiolated wheat seedlings. Then, seedlings incubated in antioxidants had reduced O_2^- and distorted development (Shorning et al. 2000). The self-incompatible pollination on stigmas of *L. longiflorum* modulated the activity of stress enzymes, such as superoxide-forming NADH- and NADPH-dependent oxidases, xanthine oxidase, SOD, CAT and ascorbate peroxidase in the pistils (Tezuka et al. 1997). Senescence takes place at every stage of natural plant organ development and is the final developmental stage controlled by planned

gene expression. The loss of the ability to scavenge free radicals during petal senescence is generally attributed to a decrease in activity of antioxidative enzymes such as SOD, CAT and peroxidase (Pastori and Trippi 1993). The expression of two Cu-Zn SOD genes of tomato was followed in different organs and plant developmental stages at the transcript and enzymatic activity levels (Perl-Treves and Galun 1991). The activities of the antioxidative enzymes APX, GR and SOD reached their maximum at the beginning of leaf development, but were reduced in senescing leaves in tobacco (Dertinger et al. 2003).

The female reproductive parts of petunia flower were chosen for the investigation into changes of SOD activity during flower development. The results obtained in the present study are consistent with these studies as SOD activities slightly declined during the later stages of flower development (Figs 3.7 and 3.9). SOD activity in the female reproductive tissues was also found to increase as the petunia flower developed and matured. The highest level of enzyme activity was recorded within the ovary of fully open flowers. A clear, increasing trend of SOD activity in each female reproductive part was found, except the ovary of petunia flower from stage 1 to 5, with a slight decrease at stage 6. SOD activity peaked within each female reproductive part when petunia flowers were fully open and predehiscent, and was significantly higher than stages 1 and 2 except that in the stigmas, but the difference was not big compared to the other three stages. This suggests that SOD activity in the female productive parts of petunia is developmentally regulated. By contrast, the activity found in the ovary tissue where SOD seemed to increase during flower growth and remained highest after anther dehiscence.

More recently, it has become apparent that ROS are required for growth of some cell types. The effects of ROS on growth and development are the result of more direct alterations to cell wall structure (Smirnoff 2005). ROS are involved in the programmed cell death that occurs as part of normal plant development. Plant NADPH oxidases are intrinsic proteins that catalyse the formation of O_2^- from amolecular oxygen using reduced NADPH as an electron donor, and appear to be important for plant cell development (Sagi and Fluhr 2001). Programmed cell death is preceded by a loss of membrane permeability, due in part to an increase in the levels of ROS that are in turn related to upregulation of oxidative enzymes and a decrease in the activity of certain protective enzymes (Kim et al. 2005). Thus, the pattern of SOD activity during petunia flower development might implicate lipid peroxidation by free radicals as a possible mechanism and seems to be the result of the imbalance between lipolytic enzymes and antioxidative enzyme activities.

These results suggest that SODs are not only stress regulated but also that their expression is modulated by the developmental stages of the organ. Thus, young buds could minimise the effects of oxidative stress by increasing levels of transcripts of enzymes, even under conditions of very high rates of oxidative radical formation. It is clear that the pistil has to attain a certain degree of development to support pollen tube growth until flower opening. Several studies have identified roles for ROS in plant development without identifying the source of ROS formation. In particular, the role of ROS as signals that control plant development will be considered. Consequently, SOD plays a special role as a defense protein to scavenge overproduction of ROS that could keep optimal condition for plant growth under oxidative stress conditions, and is involved in regulation of plant development.

4.3.3 Their Role as a Defense Enzyme

In many plant species, several factors including pathogen attack, mechanical damage and environmental stress conditions are known to produce certain proteins and enzymes which are thought have a defensive role in protecting plants (Bowler and Fluhr 2000). Increased SOD activity has been correlated with enhanced stress tolerance in a variety of plants. The critical factor in evaluating the impact of SOD on stress tolerance can be used as a means to quantify the enzymatic activity in plant tissues. It is logical to assume that certain common compounds of environmental stresses are the mediators of SOD gene regulation. Increased production of ROS is associated with the initial phase of the active defense response by the plant. SOD plays a key role in plant defense systems against adverse effects induced by various O_2^- produced under many different kinds of stress conditions and its activity is increased in response to the onset of stress (Strajner et al.1995). Constitutive overproduction of SOD has been shown to confer increased tolerance to stress and has started to reveal subtle biochemical differences between SOD isoforms (Sen Gupta et al. 1993; Van Camp et al. 1996).

As the flower develops and matures, SOD activity of the pistil, stigma and style of petunia flowers decreases. This is consistent with the idea that free radicals play an important role in aging processes (Dertinger et al. 2003). Regulation of SODs in the flower parts also appears to be sensitive to environmental stress conditions, presumably as a consequence of increased oxygen radical formation. The water stress-induced ABA accumulation triggers the increased generation of ROS and up-regulates the activity of antioxidant enzymes in maize leaves (Jiang and Zhang 2002). SOD activity was increased

in all the younger transgenic *Pssu-ipt* tobacco plants rather older ones (Synkova and Valcke 2001). In the present study, water stress cycles started with plants at two contrasting developmental stages, i.e. at the stage of vegetative growth (young buds) and at the full bloom condition. As for younger buds, the highest increase was found in the stigma, implies that it is more sensitive than other tissues under water stress. This suggests that SOD activity might be sensitive to water deficiency stress depending on the types of female reproductive tissues. SOD activity was affected particularly by plant age, but also by growth conditions. Variable responses of SOD to dehydration stress have been reported including decreased activity (Quatracci et al. 1994) or increased activity (Srivalli 2003) depending on plant species, tissue and stage development.

High temperature stress was related to oxidative damage to cell membranes by ROS, which results from the dysfunction of the active oxygen-scavenging system under various environmental stresses (Bowler et al. 1992). The effect of temperature stress combined with either light or dark on the level of SOD mRNAs in the mature *N. plumbaginifolia* plants has been examined (Tsang et al. 1991). Heat stress induced oxidative injury and alters the activities of antioxidant enzymes including SOD, CAT, AP and GR in many plant species (Jagtap and Bhargava 1995). The activity of SOD in two cool-season grasses increased transiently after 12 days of heat stress, but was not related to the effects of Ca^{2+} on heat-induced oxidative stress (Jiang and Huang 2001). High temperature can inhibit photosynthesis and accentuate the rate of water loss, plant water deficits, and water stress. Therefore, plants can be manipulated by the evaluation of the combined effects of high temperatures and water stress, the independent effects of high temperature and water stress, and their

interaction. Compared to water deficiency stress, results showed that there were much more significantly increase of each reproductive part of petunia flowers when they were under high temperature stress condition in both cases of buds and fully open flowers. Hence, alleviation of heat injury in the female reproductive parts of petunia flowers could be related to the maintenance of the scavenging ability of antioxidant enzymes at high temperatures. Here the result obtained showed that the styles of young buds exhibited the highest increase in SOD activity, which suggests that it might be more sensitive to high temperature stress than others. It was also found that the female reproductive parts of young buds were much more sensitive to high temperature stress than those of fully open flowers. Thus, the sensitivity to heat treatment of different female reproductive tissues of petunia flower was different, suggesting that the different structure of tissue could be involved in response to the high temperature treatment.

Seasonal temperature fluctuation and unpredictable disease might elevate the amount of superoxide dismutase and also enhance antioxidant scavenging system in the pistils based on the results obtained in this study. Evidence has previously been presented that a modulated expression of antioxidative enzymes is needed for an effective defense against ROS. In this present research, the result obtained under certain stress conditions might indicate that other factors may also be involved in increased resistance to oxidative stress.

4.4 Detection of Superoxide in Female Reproductive Tissues

Specific detection of ROS is of critical importance for the study of O_2^-

production. O_2^- is produced in significant quantities in various tissues, subcellular compartments or organelles. However, the measurement of O_2^- quantity within any cells, are technically difficult and baffled by the presence of SODs. NBT is an artificial electron acceptor which has been suggested to be a useful tool in the study of free radicals (Zhang et al. 1993). Generation of superoxide anions in different types of tissues from female reproductive parts during petunia flower development was detected based on the reduction of nitroblue tetrazolium (NBT) (Radeke et al. 1990). The high production and role of ROS in the expending zone of maize (*Zea mays*) leaf blades were investigated (Rodríguez et al. 2002). Although NBT also reacts with ascorbate, large changes in the ascorbate pool would not be expected in leaf tissues during photo-oxidative stress used. Therefore, imaging of formazan production can be considered to indicate O_2^- accumulation (Fryer et al. 2002). Formazan, produced upon the reduction of NBT by O_2^-, was observed mainly on the surface of the stigma and the inside of ovary tissue. However, this method does not provide information about the number and type of cells producing O_2^-.

Superoxide release was found to be essential for phytoalexin accumulation during defense responses in tobacco cells (Perrone et al. 2003) and has been shown to initiate cell death in soybean (Jads 1999). Hormonal regulation of O_2^- production has been found in maize coleoptiles (Frahry and Schopfer 2001). The production of O_2^- in the cell wall is regulated by the developmental programme and by internal and external stimuli. The cells in the transmitting tissue of the mature style were elongated and more loosely packed than in the immature style (Onus 2000). In an immature pistil, the walls of the cells of the transmitting tissue in the style and the intercellular spaces gave weak NBT-positive reactions, compared to mature pistils. In the late stages of petunia

flower development, the intensity of NBT reaction on the surface of sigma and in the longitudinal section of ovary was less compared to the dark blue staining in the early stages of young buds. This suggests that NBT reduction in female reproductive parts of petunia in this experiment could be due to different quantity of O_2^- produced during different developmental stages. This also indicates the lower O_2^- produced during later stages mainly due to increasing SOD synthesis. In contrast, the highest O_2^- production was found in the longitudinal section of the style after immersing in NBT solution for only one hr. These observations indicate that the structure of style could be the main barrier for uptake of NBT for formazan formation. Deposition of formazan in the present study indicates tissues in which the rate of O_2^- production has become significantly greater than the rate of detoxification. However, it might be possible that some substances other than O_2^- could react with NBT leading to formazan deposition. This possibility was eliminated with the following control.

As a control for the reaction of NBT with superoxide, the staining pattern was examined in normal healthy female reproductive parts of flower tissues immersed in a NBT solution with or without $MnCl_2$. The stigma and the longitudinal sections of ovary incubated in NBT solution alone showed intense formazan staining after two hr, but this was inhibited in NBT with $MnCl_2$, a dismutating catalyst agent (Hernández et al. 2001). Using the same control for NBT reaction, the inhibitory effect of NaCl on formazan staining in the elongation zone of intact maize leaves was shown to be due to salinity-associated changes in O_2^- concentration and not to an artifact associated with formazan generation (Rodriguez et al. 2004).

4.5 Isozyme Analysis of SOD within the Female Reproductive Parts of Petunia

Many studies have reported the occurrence of SOD isozymes in leaves and roots of different plant species. The activity and isozymic composition of SOD were determined in nodules of *Phaseolus vulgaris* L. *Pisum sativum* L. and *Vigna unguiculata* L. This was the first report of a Fe-SOD in the nonphotosynthetic tissue of legumes (Becana et al. 1989). The effects of hypoxia and anoxia on enzymatic activity and expression of SOD isoenzymes have been investigated in wheat (*Triticum aestivum* L.) roots (Biemelt et al. 2000). The cloning of a Fe-SOD gene when using degenerate primers designed for Mn-SODs can be explained by the high structural similarity between Mn-SOD and Fe-SOD (Bowler et al. 1994). A differential regulation of distinct SOD isoenzymes by NaCl and ostotic stress was found in the C_3 halophyte *Suaeda salsa* L. leaf extracts, indicating that this plant possesses an effective antioxidative response system for avoiding oxidative damage (Wang et al. 2004).

The biochemical significance of SODs in plant resistance to environmental stresses and in defense of disease attack necessitate the development of new analytical methods for the quick separation and detection of SODs in plant tissues. In this study, we confirm and provide new information on the occurrence of such metalloenzymic SODs in female reproductive parts of petunia flower. Analysis of isozymes present in crude extracts was achieved after non-denaturing PAGE and a staining method, which directly detects SOD isoforms in the gels. Three classes of SODs were found on the gels after running PAGE using crude extracts of each female reproductive part of petunia

flower tissues. The trace metals such as Zn, Cu and Fe play an important role in many organisms by controlling plant growth and development, and thus in final yield. Zn might be involved in oxidative stress-induced expression of genes encoding antioxidative enzymes such as SOD, H_2O_2-scavenging ascorbate peroxidase and glutathione reductase (Alscher et al. 1997). Several studies have reported that low amounts of Zn in plant cells may enhance production of O_2^- during photosynthetic electron transport (Cakmak and Engels 1999) and induce membrance-bound NADPH oxidase (Pinton et al. 1994). As a cofactor of a variety of enzymes, copper is required in a number of biological reactions involved in cell energy production, oxygen-derived radical detoxification (SOD) and protein cross-linking in connective tissues (Pena et al. 1999). The biological significance of a sufficient supply of iron is known to be essential for all living organisms in order to maintain cellular homeostasis. Van Camp et al. (1996) demonstrated that Fe-SOD and Mn-SOD have different protective properties when targeted to the chloroplast in transgenic plants. This is probably occurred because their biochemical properties differ or their subcellular localisation within the chloroplast is influenced by their affinities for membranes. In order to neutralise the harmful effects of ROS, the cells increase the synthesis of SODs through the metal responsive element within the appropriate SOD genes (Yoo et al. 1999).

The different isozymes of SOD were identified based on the different sensitivity to inhibitors such as H_2O_2 and KCN (Scandalios 1993). The different behaviors of cytosolic and glyoxysomal Cu,Zn-SODs towards cation-exchangers implies differences in their molecular structures, particularly related to the active center area where Zn is bound (Bueno and Del Rio 1992). The different locations of SODs become significant if we accept that the main

role of the SODs is to detoxify superoxide ions, and that the different biological reactions, producers of oxidising agents within the cells, may need various SODs to protect the different macromolecules and organelles from oxidative damage. Based on the limitation of time, the fundamental role of each different isozyme of SOD in the reproductive parts of petunia flower has not been investigated further in this study.

The analysis of SOD isozymes from different stages of growth was also carried out with a native PAGE and SOD activity staining. The result showed three major forms of SOD increased their activity from younger bud stages to later developmental stages in gels. This increasing intensity pattern implies the increase in SOD activity during developmental stages could be due to increased synthesis of SOD isozymes under overproduction of ROS. It is also possible to assume that some changes can occur after pollen grain landed on the surface of stigma to give an increase of SOD activity as pollen has SOD isozymes similar to that in the pistil and ovary found in preliminary experiments.

4.6 Partial Purification of SOD by Chromatography

SOD has been purified in high levels from many plants (Kröniger et al. 1992; Marín et al. 2004; Vyas and Kumar 2005), although the attempt to purify SOD did not result in a high level of purity from the pistil and ovary of fully open petunia flowers in this study. There were several reports on the purification and characterisation of Mn-SOD, for example, from pea (Palma et al. 1998), maize (Baum and Scandalios 1981), watermelon (Pastori et al. 1996), pine (Streller et al. 1994), and other higher plants. The chloroplast-associated form of

superoxide dismutase from maize (*Zea mays* L.) (SOD-1) has been purified 341.6-fold by a stepwise procedure consisting of $(NH_4)_2SO_4$ fractionation, G-100 Sephadex gel filtration, DEAE-Sephacel chromatography, and hydroxylapatite chromatography (Baum et al. 1983). A Cu, Zn SOD was purified 267.2-fold with an activity of 23% recovered, from *Radix lethospermi* seed by ammonium sulphate fractionation and chromatography on DEAE-52, Sephadex G-200 and a second DEAE-52 column (Haddad and Yuan 2005). The isozymes of SOD I were purified approximately 354-fold and SOD II approximately 265-fold from the needles and roots of Norway Spruce (*Picea abies* L.) trees during $(NH_4)_2SO_4$ fractionation, anion chromatography (salt gradient from 0-0.3 M NaCl in 20 mL) and gel filtration (Króniger et al. 1992).

In preliminary experiments, throughout the purification process, SOD activity was steadily lost probably due to the long duration of the process. Eventually, the enzyme was partially purified and analysed and was found to have a comparatively low specific activity (530 units/mg and 27 units/mg, from ovary and pistil, respectively). When the peak fractions that were adsorbed to the DEAE-cellulose column were analysed by nondenaturing PAGE, they were found to contain a single isozyme band that corresponded to the band of enzyme activity from crude ovary extracts, and had similar mobility, indicating the absence of substantial enzyme modification during the purification procedure.

The characterisation of the partially purified SOD fractions was performed as a comparison to the characterisation of SOD in the crude ovary extracts. The results obtained showed that there was no significant difference between the partially purified enzyme and that in the crude extracts. This suggests that

either there were no substances that interfered with SOD activity in crude extracts, or, if there were such substances, they were removed during the purification process. There were also no significant differences between the bound fractions and unbound fractions from the DEAE-cellulose column in the SOD characterisation studied. Due to the time limitation, the present method involved only two purification steps with lower recoveries of the enzymes. Therefore, more purification steps are needed to gain more purified enzyme in the future.

4.7 Conclusions of Physiological Role of SOD within Petunia Flowers

SOD appears to play a role as the first line of defense system which protects plants from oxidative damage. SOD activity is present in each female reproductive part of petunia flowers, suggesting that it might also be associated with flower development. The evidence obtained from gel electrophoresis of SOD isozymes also supports this idea.

As at least three different isozymes of SOD were found within the female reproductive parts of petunia flowers, it is possible that not all of them have the same function. Some may be involved with reproductive functions, while others defend the flower against oxidative stress.

4.8 Future Experiments: Some Suggestions

It is of interest to determine if different isoforms of SOD might play different roles in the regulation of flower development. Further work should also focus

on the cellular localisation of the different SOD isozymes in the female reproductive parts of petunia flowers, and the functions and strategies of the different SOD isoforms in protecting plants from oxidative stress.

In a preliminary study, SOD activity was detectable in petunia stigmatic exudates. A comprehensive study of the SOD isozymes in the stigmatic exudates should be proposed. Does SOD from stigmatic exudates have any effect on pollen germination and tube growth?

SOD activity was significantly induced under water deficiency and high temperature stress. Based on the sensitivity of different tissue types obtained from this study, further works could concentrate on the functions and characterisation of different SOD isozymes within sensitive tissue of female reproductive parts of petunia flower, helping to understand the different roles of SOD isozymes and the mechanisms of adaptive responses of flower plants under environmental stresses.

In biological systems, oxygen-derived free radicals have been demonstrated to play a role in cellular injury through a chain reaction which leads to lipid peroxidation. Several environmental stresses promote the formation of free radicals which mediate in phospholipids degradation, leading to a loss of membrane function (Pastori and Trippi 1995). Analyses of the primary hydroperoxide products are hindered by their instability and detection of oxidative stress has generally relied on measurements of lipid peroxidation end-products (Hodges et al. 1999). Therefore, lipid extraction and analysis could be involved in studying relation between SOD enzymes and lipid peroxidation. It would be of interest to carry out quantitative measurement of

extent of lipid peroxidation that is an essential prerequisite for detailed investigation on peroxidant relative to enzymatic system in plants.

If each of the SOD isoforms can be separated from each other and purified to homogeneity, it should be possible to characterise each isoform fully. The present research has formed a useful basis to start this type of SOD purification project using the ovary extracts of petunia flowers.

Flower development is a complex phenomenon where all the floral parts might be interacting with each other. In this study, only the female reproductive parts of petunia flower have been examined for the occurrence and characterisation of SOD. Therefore, studying the contribution of each floral part, including the male reproductive tissues, towards overall flower development will be necessary to gain a more comprehensive understanding of the role of SOD in flower development. Since SOD appears to be associated with flower development of petunia, it would be fascinating to produce transgenic plants and carry out a similar investigation to further confirm the findings of this research. Further analysis of transgenic plants that may overexpress or down-regulate SOD or isozymes of SOD will gain fundamental information about the effects of oxidative stress on cells and whole plants.

Studies on the genetic manipulation of SOD in plants have been already described. Transgenic tobacco and tomato plants that overproduced a chloroplastic Cu/Zn-SOD derived from petunia were generated (Tepperman and Dunsmuir, 1990). Two genotypes contain a cDNA for *Arabidopsis* Fe-SOD with a chloroplast transit peptide and cauliflower mosaic virus 35S promoter were transferred to alfalfa (*Medicago sativa* L.) plants to overexpress Fe-SOD

for increasing superoxide-scavenging capacity and improving winter survival (Mckersie et al. 2000). If a plant's defense system against oxidative stress could be reinforced with new genes and coordinated to maintain the appropriate physiological balance, stress tolerance would likely be improved because free-radical formation is a ubiquitous component of environmental adversity (Bowler et al. 1992). The reproductive physiology of these transgenic plants could also be studied, in terms of defense function and regulation of flower development. From the knowledge of oxidative stress, it is possible to find an optimised method for improving stress tolerance via manipulation of overproduction and gene expression of SOD.

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APPENDIX

1.1 Buffer Solutions and Reagents

A. 0.1 M Extraction Buffer (Phosphate Buffer) pH 7.8

Solution I. Potassium dihydrogen phosphate, KH_2PO_4 (BDH Laboratory Supplies, Poole England) 13.61 g/100 mL

Solution II. Potassium phosphate, Dibasic, K_2HPO_4 (BDH Laboratory Supplies, Poole England) 87.09 g/500 mL

Solution I (9.2mL) was combined with solution II (90.8mL) at room temperature and made up to 1L using distilled water (dH_2O), and then stored at 4^oC.

B. 10 mM EDTA

Ethylenediaminetetraacetic acid, disodium salt, $C_{10}H_{14}N_2O_6$ $2H_2$ (BDH LaboratorySupplies, PooleEngland 1.8612 g

dH_2O 500 mL

EDTA was dissolved in 400 mL dH_2O, and then brought to 500 mL. The solution was stored at 4^oC.

C. 0.63 mM NBT

NBT 0.1287 g

The chemical was dissolved in 200 mL dH_2O, and then brought to 250 mL.

The solution was stored at 4°C.

D. 0.13 mM Riboflavin

Riboflavin 0.0122 g

The compound was dissolved in 200 mL dH_2O, and then brought to 250 mL. The solution was stored at 4°C.

E. 1 mM Citrate-phosphate buffers

Solution A: 0.1 M Citrate acid (19.21 g / L)

Solution B: 0.2 M Dibasic sodium phosphate (53.65 g / L of $Na_2HPO_4.7H_2O$)

solution A (mL)	solution B (mL)	pH value
39.8	10.2	3.0
30.7	19.3	4.0
24.3	25.7	5.0
17.9	32.1	6.0
6.5	43.6	7.0

Mixed solution A and B as described in the table, diluted to a total volume of 100 mL with dH_2O and stored at 4°C.

G.. 2 mM Tris HCl buffer

Solution A: 25 mM acetic acid (sodium acetate)

Solution B: 25 mM MES

Solution C: 50 mM Tris (Trizma Base) (SIGMA Chemical Company)

solution A (mL)	solution B (mL)	solution C (mL)	pH value
12.5	12.5	25	8.5
12.5	12.5	25	9.0
12.5	12.5	25	10.0

Mixed solution A, B and C as described in the table, diluted to a total volume of 100 mL with dH_2O and stored at 4^oC.

1.2. Protein Determination

Bradford Assay: Estimation of soluble protein concentration (Bradford 1976 with slight modification).

1.2.1 Preparation of Solution

1. Bovine Serum Albumin (BSA) Standard at 1 mg/mL
   Dissolves 100 mg BSA IN 100 mL dH_2O.
   For storage, freeze in 1 mL aliquots.
2. Coomassive blue stock reagent
   Dissolve 100 mg of Coomassie Brilliant Blue G-250 (Sigma) in 50 mL 95% ethanol.
   Add 100 mL of 85% (w/v) phosphoric acid to the solution above.
   Bring to 1000 mL final volume with dH_2O.
   Allow stirring for 3-4 hr or overnight before filtering Coomassie blue stock reagent with Whatman filter paper, and then left to stabilise for three days.

The reagent was stored in a brown bottle.

1.2.2 Preparation of Standard Curve

1. BSA dilution solutions

   Prepare several bovine serum albumin (BSA) solutions ranging from 0 (blank) to 5 and 10 ug/100 μL.

2. Reaction mixture

   In a test tube, mix 100 μL BSA solution and 1 mL Coomassie blue G (Sigma) stock reagent and wait for 5 minutes and read absorbance using spectrophotometer (Pharmacia Biotech, Novaspec II, Sweden) at 595 nm.

3. Standard Curve

   Plot amount of BSA in standard against average absorbance, the equation of the trend line established using Microsoft Excel.

1.2.3 Determination of Protein Concentration in Samples

1. Blank

   Mix 100 μL dH_2O and 1 mL Coomassie blue stock reagent in a test tube as a blank.

2. Reaction mixture

   The protein samples 100 μL were mixed with Bradford reagent 1 mL Coomassie in a test tube, mechanically mixed by vortex, and waited for 5 min and read absorbance using spectrophotometer at 595 nm.

3. Protein concentration estimation

   A standard curve is used to determine protein concentration of the samples.

1.3 Reagents and Gel Preparation for Non-denaturing PAGE Gels (Laemmli Buffer System)

1.3.1 Stock Solution

A. 30% Acrylamide/bis (30%T, 2.6% C) w/v

Acrylamide (BDH Laboratory Supplies, Poole England)	29.2 g/100 mL
Bis (SIGMA Chemical Company)	0.8 g/100 mL

The Acrylamide and Bis were dissolved in dH_2O and made up to 100 mL. The solution was filtered through filter paper and stored at 4^oC.

B. 1.5 M Tris-HCl, pH 8.8 (Separating)

Trizma Base (SIGMA Chemical Company)	18.15 g
dH_2O	100 mL

The Trizma Base was dissolved in half the quantity of dH_2O and adjusted to pH 8.8 with 1M HCl added drop wise. The solution was then made up the final volume and stored at 4^oC.

C. 0.5 M Tris-HCl, pH 6.8 (Stacking)

Trizma Base (SIGMA Chemical Company)	6 g
dH_2O	60 mL

The Trizma Base was dissolved in dH_2O and adjusted to pH 6.8 with 1M HCl added drop wise. The solution was then made up to 100 mL and

stored at 4^{o}C.

D. Native gel running buffer (5X electrons buffer) pH 8.3

Trizma Base (SIGMA Chemical Company)	3 g
Glycine (SIGMA Chemical Company)	14.4 g
dH_2O	200 mL

The compounds were combined and dissolved in distilled water and stored in a plastic bottle at room temperature. During each individual gel runs, 60 mL 5X running buffer was diluted with 240 mL dH_2O.

E. Sucrose 60% (w/v)

Sucrose (Chelsea Refineries)	0.6 g

Sucrose is dissolved in 1 mL dH_2O and stored at -20^{o}C. The solution was mixed with the sample at a ratio of 2:1 (sample: 60%sucrose)

F. Tracking dye

NBT	4.1 mg

The NBT was dissolved in 50 mL dH_2O and then stored at 4^{o}C. 20 Ml NBT solution was used for one gel.

G. 5 mM H_2O_2 (Stocking 30%)

57 μL H_2O_2 was dissolved in 100 mL dH_2O and used for one gel.

H. 4 mM KCN

KCN 13.024 mg

The KCN was dissolved in 50 mL d dH_2O and 10 mL KCN solution was used for one gel.

1.3.2 Neutral pH Gel Preparation

	Separating Gel (12%)	Stacking Gel (4%)
dH_2O	1.74 mL	3.1mL
Tris—HCl pH 8.8	1.25 mL	
Tris—HCl pH 6.8		1.25 mL
Acrylamide/Bis (30%)	2 mL	0.65 mL
*Ammonium Persulphate 10%	25 µL	25 µL
TEMED	2.5 µL	5 µL
Time	45 min--1 hr	30--45 min

* The 10% Ammonium Persulphate solution was made freshly for each gel by dissolved 10 mg APS in 100 µL dH_2O

1.3.3 Running Conditions

The recommended power condition for optimal resolution with minimal thermal band distortion is 200 volts, constant voltage setting. No adjustment of the setting is necessary for spacer thickness or number of gels. The usual run time is approximately 42 minutes.

1.3.4 Staining Procedure

A. The gel is emerged in NBT tracking solution for 20 min

B. Then it is transferred to mixed solution of 11 mL extraction Buffer, 800 µL EDTA and 200 mL Riboflavin, and is left in a growth room overnight.

1.4 Reagents and Gel Preparation for SDS PAGE (Laemmli Buffer System)

1.4.1 Stock Solution

A.30% Acrylamide/bis (30%T, 2.6% C) w/v

Acrylamide (BDH Laboratory Supplies, Poole England)	29.2 g/100 mL
Bis (SIGMA Chemical Company)	0.8 g/100 mL

The Acrylamide and Bis were dissolved in dH_2O and made up to 100 mL. The solution was filtered through filter paper and stored at 4^oC.

B.1.5M Tris-HCl, pH 8.8 (Separating)

Trizma Base (SIGMA Chemical Company)	18.15 g
dH_2O	100 mL

The Trizma Base was dissolved in half the quantity of dH_2O and adjusted to pH 8.8 with 1M HCl added drop wise. The solution was then made up the final volume and stored at 4^oC.

C. 0.5M Tris-HCl, pH 6.8 (Stacking)

Trizma Base (SIGMA Chemical Company)	6 g
dH_2O	60 mL

The Trizma Base was dissolved in dH_2O and adjusted to pH 6.8 with 1N HCl added drop wise. The solution was then made up to 100 mL and stored at 4^oC.

D. 10% SDS (Sodium dodecyl sulphate)

Dissolve 10 g SDS in water with gentle stirring and bring to 100 ml with dH_2O and store at room temperature.

E. Running buffer (5X electrons buffer) pH 8.3

Trizma Base (SIGMA Chemical Company)	9 g
Glycine (SIGMA Chemical Company)	43.2 g
SDS	3 g
dH_2O	600 mL

The compounds were combined and dissolved in dH_2O and stored in a plastic bottle at room temperature. During each individual gel runs, 60 mL 5X running buffer were diluted with 240 mL dH_2O.

F. Sample buffer (SDS reducing buffer)

dH_2O	4.0 mL
0.5 M Tris-HCl, pH 6.8	1.0 mL
Glycerol	0.80 mL

10 % (w/v) SDS	1.6 mL
2-b-mercaptoethanol	0.4 mL
0.05 % (w/v) bromophenol blue	0.2 mL
	8.0 mL

Dilute the sample at least 1:4 with sample buffer, and heat at 95°C for 4 minutes.

1.4.2 Gel Preparation

	Separating Gel (12%)	Stacking Gel (4%)
dH_2O	1.675 mL	3.05 mL
Tris—HCl pH 8.8	1.25 mL	
Tris—HCl pH 6.8		1.25 mL
10% (w/v) SDS stock	50 µl	50µl
Acrylamide/Bis (30%)	2.0 mL	0.65 mL
*Ammonium Persulphate 10%	25 µL	25 µL
TEMED	2.5 µL	5 µL
Time	45 min-1 hr	30-45 min

* The 10% Ammonium Persulphate solution was made freshly for each gel by dissolved 10mg APS in 100 µL dH_2O

1.4.3 Running Conditions

The recommended power condition for optimal resolution with minimal

thermal band distortion is 200 volts, constant voltage setting. No adjustment of the setting is necessary for spacer thickness or number of gels. The usual run time is approximately 42 minutes.

1.4.4 Coomassie Blue Staining Procedure

1. Stain 1/2 hour with 0.1 % Coomassie blue R-250 in fixative (40% MeOH, 10% HOAc).
2. Destain with 40% MeOH/10% HOAc to remove background (usually 1 to 3 hr).