Free Radical Mediated Mechanisms in Stress and Development in *In Vitro* Crop Plant Systems

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I certify that this thesis is the true and accurate version of the thesis approved by the examiners.

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Abstract

Aldehydic lipid peroxidation products, free radical mediated oxidative stress and antioxidant status were investigated in three *in vitro* plant systems with the objective of exploring the involvement of oxidative stress in plant tissue cultures. *Daucus carota* was used as a model system for the study of somatic embryogenesis and for the development of an enzyme linked immunosorbant assay (ELISA) technique, for the detection of hydroxynonenal-protein adducts in plant extracts. This study shows for the first time, in plants, that exogenously applied lipid peroxidation products hydroxynonenal (HNE) and malondialdehyde (MDA) inhibit callus proliferation and development (as somatic embryogenesis). Removal of the aldehydes led to a reversal of inhibitory effects.

For the first time, HNE and MDA were measured in extracts of callus generated from different explants of *Ipomoea batatas* (an economically significant crop species). Using ELISA, HNE-protein adducts have been detected, in extracts from all three cultivars of *I. batatas* tested. Increases in the activities of the antioxidants, catalase and peroxidase were also observed in nodes of *I. batatas* after transfer to callus induction medium.

Three callus cultures of *Glycine max*, one of which was habituated against benzylamino purine, the second contained chlorophyll (green) and the third, devoid of chlorophyll (white) were profiled for lipid peroxidation products and antioxidant activity. This is first report of the detection free HNE and MDA in

G. max and were detected in all three callus types, with the highest concentrations for both aldehydes in the habituated callus line. HNE-protein adducts were only detected in the white callus line of *G. max*. The level of hydroxyl radical activity was found to be increased in aged callus compared to callus taken from the mid-point of the subculture cycle. Low activities of catalase, peroxidase and other antioxidants were found in the habituated callus. It is proposed that increased amounts of hydroxyl radicals, high levels of HNE and MDA and the low activity of antioxidants leads to oxidative stress in extreme conditions such as habituation in *in vitro* plant systems. This study has implications regarding the understanding of *in vitro* plant recalcitrance.

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Declaration

This thesis records the results of experiments carried out by myself in the School of Science and Engineering, University of Abertay Dundee and the Scottish Crop Research Institute, under the supervision of Dr. D. H. Bremner, Dr. E. E. Benson, Dr. H. J. Staines, and Dr. N. Deighton between October 1996 and October 1999. It is of my own composition and has not been previously submitted in part or whole for a higher degree.

Signed. Linda K Adams.

Linda Kay Adams

Abbreviations

A	
Abscisic acid	ABA
Analysis of Variance	ANOVA
B	
Benzylamino purine	BAP
Bovine Serum Albumin	BSA
C	
Centimetre	cm
International Potato Center, Lima, Peru	CIP
D	
Degrees Celsius	°C
De-ionised water	dH_2O
Deoxyribonucleic acid	DNA
2,4-dichlorophenoxyacetic acid	2,4-D
Dinitrophenylhydrazone	DNP
5,5'-dithiobis-(2-nitrobenzoic acid)	DTNB
Dimethylsulphoxide	DMSO
E	
Enzyme linked immunosorbant assay	ELISA
Electron Spin Resonance	EPR
Ethylenediaminetetraacetic acid	EDTA
Exotic Collection (Indian Accessioning)	EC
F	
G	
Gas Chromatography	GC
Glutathione (reduced)	GSH
Glutathione (oxidised)	GSSG
Glutathhione S-transferase	GST
Glutathione reductase	GR
Gram	g
H	
Hour	hr
Horse Radish Peroxidase	HRP
Hydrogen peroxide	H_2O_2
Hydroxyl free radical	$HO^{\bullet}$

I

International Plant Genetic Resources Institute	IPGRI
Indole acetic acid	IAA

J, K

Kilo Pascal (pressure)	kPa

L

Liquid Chromatography Mass Spectrometry	LC-MS
Liquid Nitrogen	LN_2
Litre	L

M

Malondialdehyde	MDA
Methanol	MeOH
Microgram	µg
Milligram	mg
Millimolar	mM
Millimoles per litre	mmol/L
Minute	min
Moles per litre	mol/L
Murashige & Skoog	M&S

N

Nanometre	nm
Nanomole	nmol
Nanomolar	nM
Napthaleneacetic acid	NAA
National Bureau of Plant Genetic Resources	NBPGR
Negative Ion Chemical Ionisation	NICI
Nicotinamide-adenine dinucleotide phosphate	NADP
Nicotinamide-adenine dinucleotide phosphate (reduced NADP)	NADPH
Nitrotetrazolium blue	NBT
Number	No.

O

Oxidised glutathione	GSSG

P

Parts per million	ppm
Peroxide ion	O_2^{2-}
Phosphate buffered saline	PBS
Polyunsaturated fatty acids	PUFA

Q,R

Revolutions per minute	RPM
Room Temperature	RT

S

Scottish Antibody Production Unit	SAPU

Second	s
Singlet oxygen	$^1O_2$
Sodium hydroxide	NaOH
Sulphydryl groups	SH
Sulphosalicylic acid	SSA
Superoxide dismutase	SOD
Superoxide radical	$O_2^{\cdot -}$
T	
Thiobarbituric acid	TBA
Thiobarbituric acid Reactive Substances	TBARS
Trichloroacetic acid	TCA
2,4,5-Trichorophenoxyacetic acid	2,4,5-T
Trimethylbenzidine	TMB
Triplet oxygen	$^3O_2$
U	
Ultra violet	UV
Units of Enzyme Activity	U
V	
Volume/volume	v/v
2-vinyl pyridine	2-VP
W	
Watt	W
Weight/volume	w/v
Weight/weight	w/w
X,Y,Z	

Chapter 1 Introduction

Contents

1.1 Aims and Objectives of Project

1.1.1 Research Aim

The overall aim of this project is to investigate the possible role of oxidative stress, free radical damage and antioxidant status in tissue culture stress, recalcitrance, habituation and the morphogenetic decline in *in vitro* plants. A specific aim is to investigate the role of the aldehydic lipid peroxidation products hydroxynonenal (HNE) and malondialdehyde (MDA) within plant tissue culture regimes, as these compounds have been implicated in the control and decline of development and growth in *in vitro* mammalian systems.

1.1.2 Experimental Aims

1. Profile and evaluate the different morphologies of embryogenic and non-embryogenic lines of callus from a range of cultivars of *Daucus carota,* and *Ipomoea batatas* (L.) Lam. and habituated and non-habituated lines of *Glycine max*.
2. Profile and evaluate embryogenic and non-embryogenic callus lines generated by different methods from a range of micropropagated plantlets of *I. batatas*.
3. To assess the potential of using antioxidants and detoxification systems for free radical and secondary oxidation products as potential markers of morphogenesis and habituation in *I. batatas* cultures and *G. max* callus cultures.

4. Profile the $HO^{\bullet}$ radical status of different morphogenetic and habituated lines of *G. max* callus cultures and *I. batatas*.
5. Profile the HNE and MDA content, in different morphogenetic and habituated lines, of tissues of *D. carota, I. batatas* and *G. max.*
6. Development of an enzyme linked immunosorbent assay (ELISA) using an anti-HNE specific antibody for the detection of HNE-protein adducts in protein extracts of tissue from cultures of *D. carota, I. batatas* and *G. max.*

1.1.3 Systems of Investigation

In vitro plant tissue culture systems were the subject of investigation for assessing growth, morphogenesis and development in relation to *in vitro* oxidative stress. Profiles of different culture morphologies and habituated types and the rate of callus initiation and somatic embryo production were also used to compare and contrast the different capabilities of cultures within different culture regimes. Culture systems were chosen as representatives of the most important *in vitro* morphogenetic processes, which have a potential for morphogenetic decline and recalcitrance. A model system, *D. carota* was also investigated.

Daucus carota

D. carota was used as the model system of study due to its amenability in tissue culture for the production of somatic embryos (Zimmerman, 1993), and the fact that the species has been extensively studied physiologically *in vitro*

(Ammirato, 1986). Somatic embryogenesis has been used as the model system for the study of development and the initiation and development of somatic embryos within *D. carota* forms an ideal system for the study of morphogenic pathways and the associated processes connected with development and oxidative stress.

Ipomoea batatas (L.) Lam

I. batatas was selected as the second species for study as it is an economically significant crop species and it remains one of the most nutritionally important root crops within the developing world. It has been shown by a number of research groups to have a wide range of genotype responses relating to the initiation of somatic embryos and the manipulation of micropropagated plantlets in tissue culture (Zheng, *et. al.*, 1996, Jarrett, *et. al.* 1984, Al-Mazrooei, *et. al.* 1997).

Glycine Max

Long-term aged cultures that display different types of hormone-habituation and pigmentation of *G. max* were selected for study. Three different lines of habituated and non-habituated *G. max* callus formed the basis of studies in callus systems for the comparison of biochemical profiles of antioxidants and other species associated with oxidative stress. The habituated culture is a "plant cancer" and may help elucidate the biochemical status of this type of system and indicate the role of oxidative stress in habituation (Gaspar, *et. al.* 1991, Franck, *et. al.* 1998).

1.1.4 Tools of Investigation

Tissue Cultures

Different cultivars and culture types of each species were profiled, compared and contrasted for growth and response to *in vitro* culture. Comparisons of the growth and morphogenetic behaviour and variability between cultivars and culture regimes formed the basis of the study for a range of cultivars of *D. carota*, *I. batatas*, and *G. max*. Aspects considered included: the height of the plantlet, rate of callus growth, health, and the rate and production of somatic embryos under established and new regimes.

A range of different biochemical techniques were used to profile different aspects of *in vitro* biochemical status for the three crop species and to correlate this with growth, morphological development, secondary oxidative stress, and antioxidant status.

Biochemical assays

1. Spectrophotometric assays of enzymes and antioxidants associated with oxidative stress were monitored using a range of spectrophotometric assays (e.g. for catalase, peroxidase, superoxide dismutase, glutathione reductase, sulphydryl groups, oxidised and reduced glutathione and glutathione S-transferase). The assays selected give an indication of the protective status of the cells and the mechanisms employed by different species to cope with oxidative stress within the tissue culture environment.

2. Enzyme linked immunosorbant assay (ELISA) developed and adapted from mammalian methods and used to measure the content of HNE-protein adducts within soluble protein extracts of the three plant species. The anti-HNE antibody was developed for use in mammalian systems and this study sees the first application of this immunological technique within plant tissue culture systems for the detection of HNE-protein adducts.
3. Liquid chromatography mass spectrometry (LC-MS) analysis of the lipid peroxidation products hydroxynonenal and malondialdehyde as derivatives extracted from the tissue of the three plant species was used to definitively detect the presence of toxic aldehydic products in *in vitro* plant cultures.
4. The analysis and measurement of volatile hydrocarbons released from plant tissue exposed to the free radical trap dimethyl sulphoxide by gas chromatography (GC) was used to monitor the hydroxyl radical status within plant tissue.

1.2 Project Introduction: Concepts of Plant Tissue Culture and Its Limitations

1.2.1 History of Plant Tissue Culture

Biotechnology, and in particular plant tissue culture, has been the main techniques employed in the improvement of a wide range of important crop species in recent years. Many studies have been performed on the diverse range of species, from which domestic crops are derived. The totipotent potential of plants (the ability to regenerate whole plants from single cells) has been widely exploited in plant tissue manipulations and underpins the *in vitro*

improvement initiatives for plant conservation, reproductive technologies and genetic modifications (Benson, *et. al.* 1997).

The first person to postulate that isolating plant cells in culture may give interesting insights into the potentialities of the cell and the inter-relationships within multicelluar organisms was Gottlieb Haberlandt (1854-1945) (see Bhojwani & Razdan, 1983). Haberlandt cultured isolated differentiated cells from leaves of *Lamium purpureum* as early as 1898. It was not until the 1930's that major discoveries were made that pushed the advancement of plant tissue culture with the identification of auxins and the recognition of the significance of the B group of vitamins (Bhojwani & Razdan, 1983). The complete nutritional requirements for maintaining different types of plant cells in culture were established in the 1960's and include a wide range of substances such as sucrose, basic salts, vitamins and plant growth regulators (Murashige & Skoog, 1962). These constituents now form the basis for the majority of modern plant tissue culture media. The advances in plant tissue culture, since then have been vast. However, there are still many problems associated with growing plants in an *in vitro* environment.

1.2.2 Tissue Culture Systems

Callus Cultures and Somatic Embryogenesis

The totipotent potential of plants to regenerate whole plants from single cells is unique to the plant kingdom and this means that the number of techniques used in plant tissue culture is wide and varied. Manipulations for the

generation of cultures are based on the production of dedifferentiated tissue by subjecting explants, taken from micropropagated plantlets or glass house plants grown from seed, to high concentrations of plant growth regulators or hormones. The tissue responds to the hormones producing dedifferentiated tissue called callus, usually at the cut ends. This tissue can then be sub-cultured onto fresh medium containing the plant growth regulator, and maintained in this way. Callus can be initiated in a large number of species and forms the main tissue system of study for *D. carota,* and *G. max* used in this investigation. Callus cells can be stimulated into development by manipulation of the levels of hormones within the culture media to give rise to somatic embryos. In *D. carota* the onset of somatic embryogenesis can be induced by the removal of the auxin 2,4-dichorophenoxyacetic acid (Zimmerman, 1993), and was first reported in 1958 by Reinert and Steward (Bhojwani & Razdan, 1983). These embryos will eventually form plantlets, after progressing through the sequence of cellular developmental stages from globular to torpedo, and if allowed to grow to maturity the plantlets can then be used as the basis for maintaining micropropagated plants in *in vitro* culture (Zimmerman, 1993).

The progression of differentiation through the various stages in embryo development, offers the possibility for the study of physiological and biochemical aspects of development and differentiation of plant cells that can be stimulated in an *in vitro* environment. Almost any type of tissue from the plant can be used for the initiation of somatic embryogenesis and by the

1980's over 80 species from 33 different families had been induced to produce somatic embryos (Bhojwani & Razdan, 1983).

Somatic embryogenesis has been initiated in a large number of systems but one of the most widely studied is *Daucus carota*. *D. carota* has been found to be very amenable to the generation of somatic embryos, and hence has been used as a model systems for the study of development and has been well documented by a number of researchers (Zimmerman, 1993, de Vries, *et. al.* 1994). Somatic embryogenesis has also been widely studied in *I. batatas* (Jarrett, *et. al.* 1984, Al-Mazoorei, *et. al.* 1997) and is particularly crucial for the production of somatic embryos for artificial seeds (Chee & Cantliffe, 1992). The International Potato Center (CIP) in Peru is continually striving for the breeding and development of new cultivars with stress tolerance, drought resistance, disease and pest resistance, and increased productivity. The improvement of existing cultivars through *in vitro* manipulations could achieve the desired characteristics for increased production in the developing and third worlds. However, one of the problems associated with somatic embryogenesis is the gradual loss of this embryogenic potential over time, eventually making previously embryogenic cultures non-embryogenic. This loss of morphogenic capacity can severely inhibit developmental programmes and restrict the application of somatic embryogenesis to plant regeneration. It is for this reason that somatic embryogenesis has been investigated in this work with respect to oxidative stress. Furthermore, this study examines somatic embryogenesis in a model species (*D. carota*), and an important crop species (*I. batatas*).

Micropropagated Plants

Somatic embryogenesis can be considered as a route for micropropagation. The proliferation of roots, shoots, and nodes form a second important *in vitro* system. Micropropagation can be used as a method for the rapid multiplication of plants *in vitro* without the need for the full life cycle of the plant, thus avoiding lengthy seed production stages or vegetative reproduction in the field. Using this method a large number of plants can be established from one parent plant, producing populations of clonal plants in a relatively short period of time (Bhojwani & Razdan, 1983). One advantage of this method, is the ability to produce large quantities of plants from species that generate little viable or poor quality seed, such as some cultivars of banana, grape, fig and petunias (Bhojwani & Razdan, 1983). This process has been adopted for the production of commercial plants in the horticulture industry for species such as african violets, primulas, and begonias.

Micropropagated plants can be generated from either apical shoots or stem nodal cuttings taken from seed or tuber grown plants. These plants, once established in a suitable tissue culture routine, can be used for the study of growth and development and the initiation of callus and somatic embryos from a range of explants. Many culture systems have been established by this method and it is a useful way of maintaining healthy plants in a disease and virus free environment (Bhojwani & Razdan, 1983). International organisations and culture collections, such as International Potato Center (CIP) in Peru (Golmirzaie, *et. al.* 1999) and the National Bureau of Plant Genetic

Resources (NBPGR) in India, screen all stocks to ensure they are virus and disease free and are exported for research throughout the world. Five of the *I. batatas* cultivars used in this project were imported from NBPGR, some of which had originally originated from CIP (for full details see 2.1).

The production of micropropagated plants depends on the use of minerals, organic compounds, and hormones for the culture media and a range of different environmental conditions. The role of auxins and cytokinins is important and the balance between the two will determine whether the formation of roots or shoots is favoured. However, there are a number of other factors that have only recently been considered (Gaspar, 1990, Lowe, *et. al.* 1996). For example, ethylene is produced by plants, and affects growth and development. Allowing ethylene to accumulate in the first part of the subculture cycle and allowing it to escape in the second half, has been shown to improve the growth of rose cultures (Gaspar, 1990). Studies of this nature will help to further the understanding of the growth and development of plants in *in vitro* culture.

1.2.3 Recalcitrance and Problematic Plants

One of the main problems still affecting the extent to which different species can be manipulated within plant tissue culture is recalcitrance; the inability to successfully manipulate some types of plants in culture (Benson, *et. al.* 1997). The recalcitrance of some species *in vitro* has become an increasingly important area of research and to ascertain the underlying causes and possible solutions are a major concern for tissue culturists. So much so, that the

Society for In Vitro Biology (SIVB), at its international conference in New Orleans in June 1999 dedicated an entire symposium to the subject, to highlight this difficult problem (for review see Benson, 2000a). Interest is now focusing on the possible involvement of free radicals in recalcitrance, where some of the changes induced by *in vitro* culture may predispose the tissue to increased levels of production of secondary oxidation products associated with lipid peroxidation and oxidative stress (Benson, 2000b).

Some of the most recalcitrant groups of species include for example woody plants, cereals and legumes. These groups of plants can be extremely difficult to establish in culture and manipulate within *in vitro* systems (Burdon, *et. al.* 1990). Woody plants, in particular, produce high levels of phenolics that promote oxidation during explant initiation and culture (Benson & Roubelakis-Angelakis, 1992, 1994) and subsequently this group are particularly problematic and many are recalcitrant. However, there is some evidence to suggest that phenolics may also provide antioxidant properties to the plant and may even account for the anti-cancer properties attributed to certain types of fruit and vegetables (Rice-Evans, *et. al.* 1997). A woody species that is particularly recalcitrant to manipulation in tissue culture is *Vitis vinifera* (Benson & Roubelakis-Angelakis, 1992, 1994). This recalcitrance has been particularly associated with oxidative stress and lipid peroxidation, the production of fluorescent oxidation products and fluctuations in associated enzymes such as catalase and superoxide dismutase (Benson & Roubelakis-Angelakis, 1994).

Many of the problems associated with the difficulty of manipulating monocotyledonous species is their complicated life cycle which has many stages including reproduction, senescence, dormancy, and rejuvenation (Benson, 2000a). These different stages mean that there are predictable components within the life cycle of the plant but there are only certain stages where the most responsive explants can be used for the initiation of new cultures. This restricts the time available for the gathering of fresh explants and, in the case of species with long life cycles such as trees, makes the manipulation of these species within tissue culture extremely problematic. The associated "in built" problems with many of these stages, makes the recalcitrance within *in vitro* culture difficult to overcome. The responses, generated by different species to *in vitro* tissue culture, are dictated by the genetic content of the plants. This phenomenon is known as genetic pre-determinism (McCown, 2000) and means that some groups of species are particularly difficult to manipulate in an *in vitro* environment (for more details see section 1.2.5.1). The more information gained from the study of the life cycles of problematic species will help in overcoming the associated recalcitrance.

Developing tissue culture systems for cereals, in particular, has been troublesome and is important due to our dependence on a few selected species of cereals. Many cereal crops are extremely difficult to manipulate and are generally recalcitrant towards normal culture techniques, which makes any improvement of existing cultivars difficult. The mechanisms of recalcitrance are still unclear although in recent years the focus of techniques within cereal

systems for genetic manipulation has been the use of protoplast culture (Vasil, 1987). This type of culture has been particularly successfully applied to rice (*Oryza satixa* L.) by using callus generated from embryos to form protoplast cultures. Removing the cell wall in this species seems to dramatically aid its manipulation for the insertion of foreign DNA and makes regeneration of transformed plants possible (Thomson, *et. al.* 1986). The contents of the media have been an important consideration, and the inclusion of amino acids promotes protoplast division in a wide range of cultivars (Thompson, *et. al.* 1986). The gelling agent used may also have a significant effect in the establishment and maintenance of cells in culture (Thompson, *et. al.* 1986).

Many attempts have been made to try and overcome recalcitrance (Benson, 1990, 2000a), by manipulation of conditions and the application of growth regulators, antioxidants and other chemical aids but with varying degrees of success (Benson, *et. al.* 1997). Progress has been made with careful application and manipulation of natural and synthetic analogues of plant growth regulators, particularly auxins and cytokinins (Bhojwani & Razdan, 1983).

1.2.4 Habitutation

Recalcitrance is the inability to successfully manipulate a species within tissue culture whereas habituation is the ability of tissue to grow without the requirement for hormones, these cells are able to continually divide and they appear to behave the same way as a plant tumour cell (Hagège, 1996). The phenomenon can arise spontaneously and generate a neoplastic state (Hagège,

1996). Eventually the culture completely losses the ability to respond *in vitro* and will grow continually without the aid of plant growth regulators (Kevers, *et. al.* 1996, Gaspar, 1995). This phenomenon is problematic to the manipulation of plants within plant tissue culture and makes the continued application of biotechnology to a wide range of species within horticulture and agriculture difficult.

Gaspar and colleagues have reported extensively on the phenomenon (Gaspar, 1995, Kevers, *et. al.* 1996, Bisbis, *et. al.* 1998, Gaspar, *et. al.* 1991, Gaspar, 1998) and Gaspar postulates that because habituated plant cells carry many of the characteristics animal metastases including hormone independence, complete loss of cell-to-cell adhesion, permanent oxidative stress, and accumulation of polyamines (Gaspar, 1998), they can be considered as cancer cells. Normal plant cells have the capacity to organise themselves into organogenic or regenerating meristems, and therefore the typical plant cancer has been defined as the irreversible loss of organogenic totipotency (Gaspar, 1998).

Habituation has been observed as a gradual process but in some cases it has been spontaneous (Gaspar, 1995). Habituation appears similar to tumour transformation in crown gall disease where tissue grows independently of exogenous hormones. There are many possible explanations as to why the cells are able to continue dividing without the requirement of hormones such as autonomous production of auxins and cytokinins, altered sensitivity to endogenous hormones, accumulation of metabolites that may replace

cytokinins, altered metabolism of ethylene, or altered gene expression. However, currently the actual mechanism of habituation and its progression remains unclear. Habituation has been shown to be initiated in *G. max* by exposing tissue to 2,4-dinitrophenol or phenoxyisobutyric acid for a short period of time (Christou, 1988). In this study, ageing cultures of *G. max* have been chosen that demonstrate different types of hormone requirements.

A "permanent stress" hypothesis has been proposed to explain the onset of habituation in *Beat vuglaris* (Le Dily, *et. al.* 1993). This hypothesis assumed that the stress caused by the accumulation of peroxides and low catalase and peroxidase activities would result in the accumulation of hydrogen peroxide that would be responsible for the oxidative stress (Hagège, 1996). Another hypothesis is the "antioxidant adaptive strategy", where the cells have adapted by developing a higher level of protection from activated oxygen species and free radicals (Hagège, 1996).

Ethylene is an important hormone in the growth and development of plants, and its level within growing habituated callus cultures appears to be lower than that of auxin-dependant cultures. Although, the habituated callus will still produce ethylene without the application of auxin (Köves & Szabó, 1987). The investigation into the role of ethylene within habituated callus may help to elucidate the mechanisms involved. However the true mechanisms and implications of habituation still remain unclear. As part of this study, profiles of the ethylene production of different lines of *G. max*

exhibiting habituation were performed using gas chromatography, and this may help to elucidate some of the mechanisms involved in habituation.

1.2.5 Genetic Factors Affecting Plant Tissue Culture

1.2.5.1 Genetic Predeterminism

The genetic makeup, natural habitat and life cycle of the plants under investigation all have a significant influence on the ability of the species to respond under *in vitro* culture conditions. The life cycle is particularly significant in affecting the way in which species can be manipulated within *in vitro* culture for woody plants and perennial plants, and determines the stages of the life cycle from which explants will respond in culture (McCown, 2000). Some species are pre-determined to be difficult to establish in tissue culture due to the type of growth and reproduction methods, for example determinant herbaceous species (*Panix, Paeonia*) or episodic woody organisms (*Quercus, Pinus*) have proven predetermined to be difficult to *in vitro* manipulation (McCown & McCown, 1999). Overcoming the pre-set patterns and seasonal variability within plant species for regeneration within plant tissue culture has proven a difficult part of establishing plants within culture. However, even when certain regimes work for particular varieties and cultivars of a species they may not necessarily be suitable or the most efficient for other cultivars. Much of the problem with establishing commercial programmes is the large variability between the genotypes of different cultivars. These large genetic differences make establishing favourable programmes for the production of large numbers of healthy and responsive plants costly and difficult.

1.2.5.2 DNA Methylation

DNA in eukaryotic organisms is routinely modified by methylation of cytosine residues (Lambe, *et. al.* 1997). In higher plants up to 30% of cytosine residues are methylated and the level of methylation is changed during development (Lambe, *et. al.* 1997). The amount of methylation of cytosine has been shown to be less for genes which are being expressed, suggesting that methylation is being used as a method of controlling DNA replication and translation into protein. Lambe, postulates that hypermethylation of DNA during tissue culture could explain the appearance of habituated cells and non-differentiating callus (Lambe, 1997). The continued methylation of genes essential for cell differentiation during cell division and multiplication and the continuous removal of cells capable of differentiation are suggested to be responsible for the gradual progressive loss of totipotency during plant tissue culture.

DNA methylation has also been a focus of attention within mammalian cells and raises considerable interest in the quest to find the true mechanism of cellular ageing. Methylation appears to play an important part in gene regulation and any interference of this process could have significant effects on the expression and control of the genes involved in differentiation (Catania & Fairweather, 1991). DNA methylation patterns have been investigated in hazel cultures for the connection to reinvigoration induced by tissue culture. The morphogenic potential of these cultures may be dependant of the level of DNA methylation and may even be one of the mechanisms connected to habituation and somaclonal variation (Diaz-Sala, *et. al.* 1995). The levels of

methylation and the genes involved may play a role in ageing in woody plant such as *Vitis vinifera* (Harding, *et. al.* 1996). DNA methylation has therefore been of considerable interest in cellular ageing and habituation and the search for the mechanisms involved remains an interesting topic of research.

1.2.5.3 Somaclonal Variation

Somaclonal variation is the heritable change in the genetic material induced by plant tissue culture manipulations (Larkin & Scowcroft, 1981). The definition of a clone dictates that all the plants produced from a single parent plant will be identical (Mantell, 1986). However, the clones may actually differ slightly in their genetic composition due to mutations generated during culture manipulation (Karp, 1993). The incidence of the number and mutations produced will vary according to the culture conditions and may produce phenotype differences between clones that are meant to be identical (Mantell, 1986). The incidence of somaclonal variation appears to be greatest in cultures that are made to pass through a callus stage of development (Mantell, 1986). The influence of plant growth regulators and the age of the callus appear to have a significant effect on the rates of somaclonal variation in strawberry cultures and many regenerants from 16 or 24 week old callus exhibited deformed leaf shape and yellow leaf variants (Nehra, *et. al.* 1992). However, many of the mutated regenerants could be used as an additional source of variability and may help in the improvement of asexually propagated crop plants (Nehra, *et. al.* 1992). Maintaining callus in this dedifferentiated state for long periods of time increases the likelihood of mutation and variations appearing in the regenerated plants. Therefore, the level of plant

growth regulators used and the length of time cells are maintained in the dedifferentiated state increases the likelihood of generation of somaclonal variants and investigation into the causal mechanisms of this phenomenon will help to minimise the losses through mutation.

After 14 months of culture of *Triticum aestivum* up to 80 % of the regenerants showed altered chromosome complements and shows that even when somatic embryogenesis occurs it does not mean that the DNA is complete (Henry, *et. al*. 1996). The effect of such changes can be devastating and can severely inhibit the manipulation of different species *in vitro*.

1.3 Oxygen, Oxidative Stress, and the *In Vitro* Environment

1.3.1 Oxygen and Free Radicals

Aerobic organisms are all dependent on oxygen and redox reactions involving the transfer of single electrons and are involved in many essential life processes including oxidative respiration, photosynthesis, lipid metabolism and cell signalling (Salin, 1987). The fact that plants cannot escape the stresses of the environment in which they grow, they have acquired a number of responses and defences to manage the different conditions and stresses within that environment. Thus, plants have integrated a wide range of environmental signals into their developmental pathways as a response to stress (Scandalios, 1990). The aerobic environment therefore presents a number of problems to all organisms that live within it and one of the most troublesome is oxidative stress.

"Oxidative Stress" is used to describe cells which have an increase in the concentration of reactive oxygen species (Scandalios, 1990). Oxidative stress occurs when the balance between mechanisms triggering oxidative stress and cellular antioxidant defences is disrupted or disturbed (Scandalios, 1990). Causal mechanisms of oxidative stress in plants include: radiation, Fenton reactions, impairment of the normal functioning cytochrome electron-transport chains, xenobiotic reagents and pollutants (such as ozone and sulphur dioxide), pathological stress due to disease, infectious agents, pathogens (Scandalios, 1990), light, experimental manipulations and ageing (Bartosz, 1997). Defence systems present within living cells include enzymes (such as superoxide dismutase, catalase and peroxidase), low molecular weight compounds (such as glutathione) as well as vitamins E, C and A, the relationships between many of these antioxidants are shown in Figure 1.3.1.

The reactive oxygen species generated during oxidative stress are formed from stepwise single electron additions to molecular oxygen generating a unique spectrum of reactive species which includes superoxide ($O_2^{\bullet -}$) and hydroxyl radicals ($HO^{\bullet}$) (Punchard & Kelly, 1996). Many of these intermediates are reactive free radicals - compounds that have one unpaired electron. This reactivity is driven by the requirement to find an electron to make a pair and it achieves this by extracting an electron from a neighbouring molecule (Benson, 1990). Superoxide and hydrogen peroxide can lead to the generation of hydroxyl radicals through the Fenton Reaction with iron as a catalyst and therefore generation of reactive oxygen compounds can lead to others

Figure 1.3.1 Relationships between Reactive Oxygen Species, Lipid Peroxidation and Antioxidants

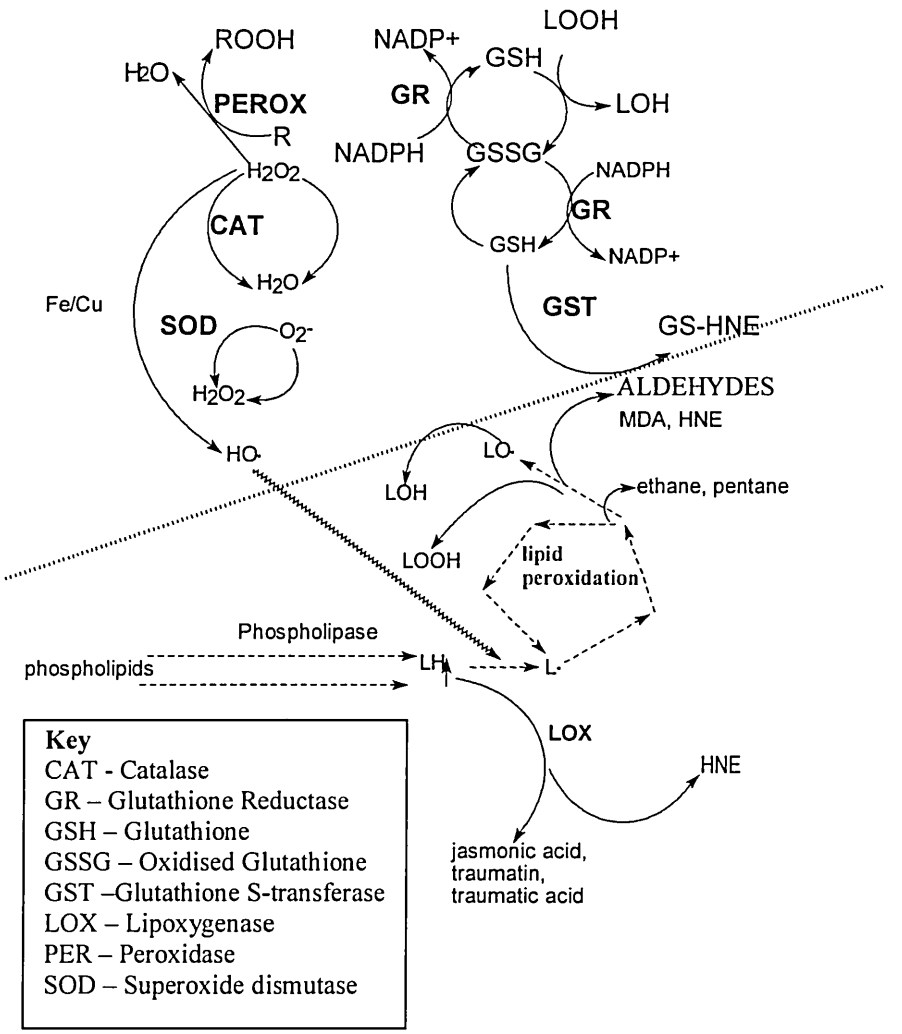


Figure 1.3.1: Relationships between reactive oxygen species, antioxidants and lipid peroxidation in plant cells (adapted from N. Deighton, personal communication).

(Benson, 1990). Oxygen free radicals have been implicated in cellular differentiation of many different organisms including fruit flies (*Ceratitis capitata*), slime mould (*Didymimium iridis*), and the nematode, (*Caenorhabditis elegans*) (Sohal, 1986).

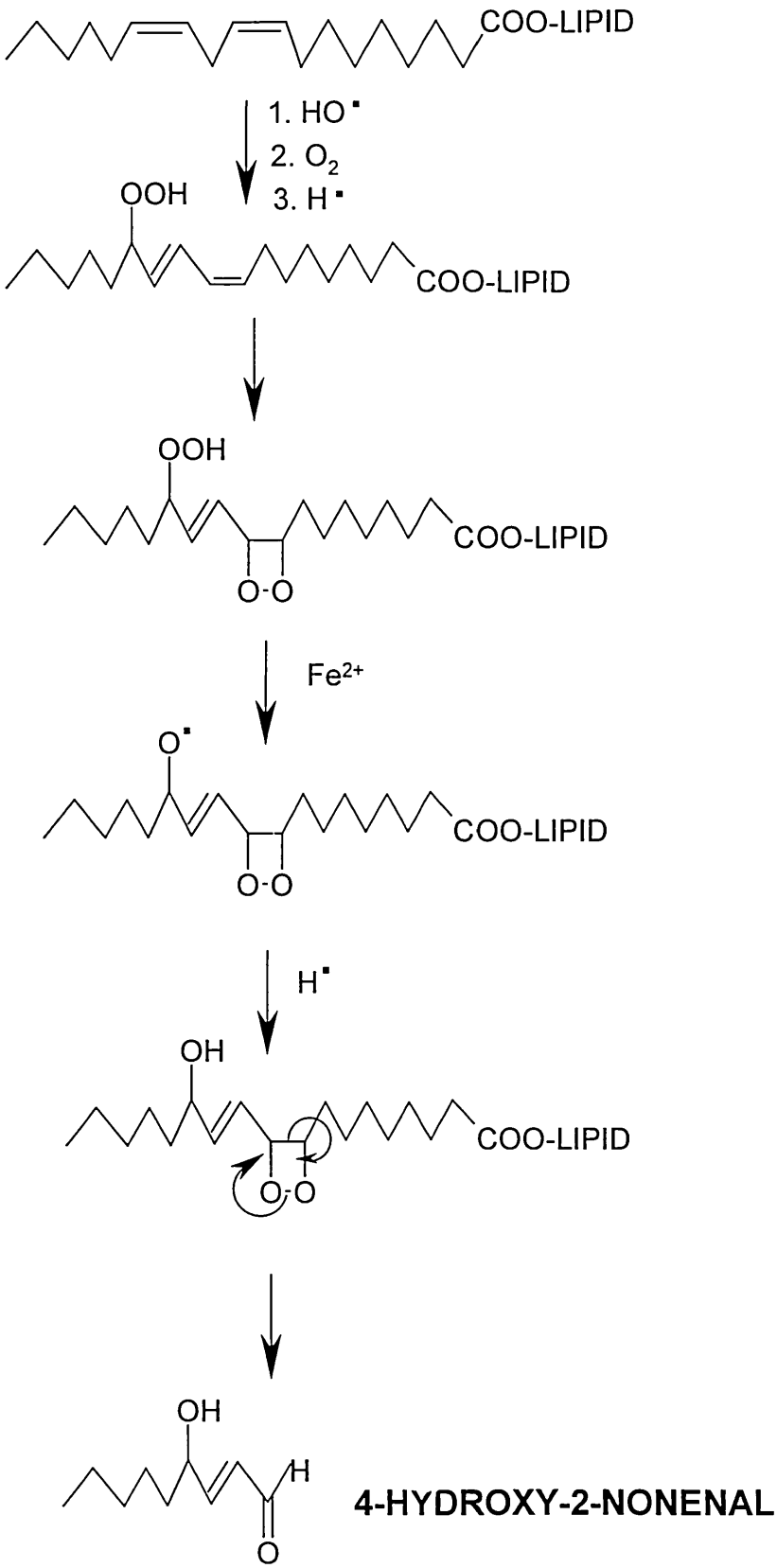
In plants, active oxygen species, in particular superoxide radicals and singlet oxygen, are produced in chloroplasts by photo-reduction of oxygen and energy transfer from triplet excited chlorophyll to oxygen (Smirnoff, 1993, Scandalios, 1990, Salin, 1987). Some of these free radicals and intermediate species escape from these processes and can lead to deleterious effects and oxidative stress.

Oxidative stress involves the direct attack of free radicals on macromolecules and the secondary damage caused by free radical reaction products (Zollner, *et. al.* 1991, Yang & Schaich, 1996). Macromolecules affected include the fatty acids within membranes, functional proteins and enzymes and DNA molecules, and detrimental effects range from enzyme inhibition or inactivation to the ultimate disruption of essential cellular membranes leading to cell lysis and death. To combat these effects, species have developed many complex systems to control and remove these damaging free radical species. Most of these systems involve enzymes such as catalase, superoxide dismutase and glutathione reductase. Other defence mechanisms include low molecular weight antioxidants such as glutathione and ascorbate (Esterbauer, 1996). There is evidence to suggest that some of the polyphenolic compounds present in woody species have antioxidant properties and may be partly responsible for the anti-cancer properties of fruit, vegetables, and cereals (Rice-Evans, *et. al.* 1997).

1.3.2 Oxygen Radicals and Lipid Peroxidation

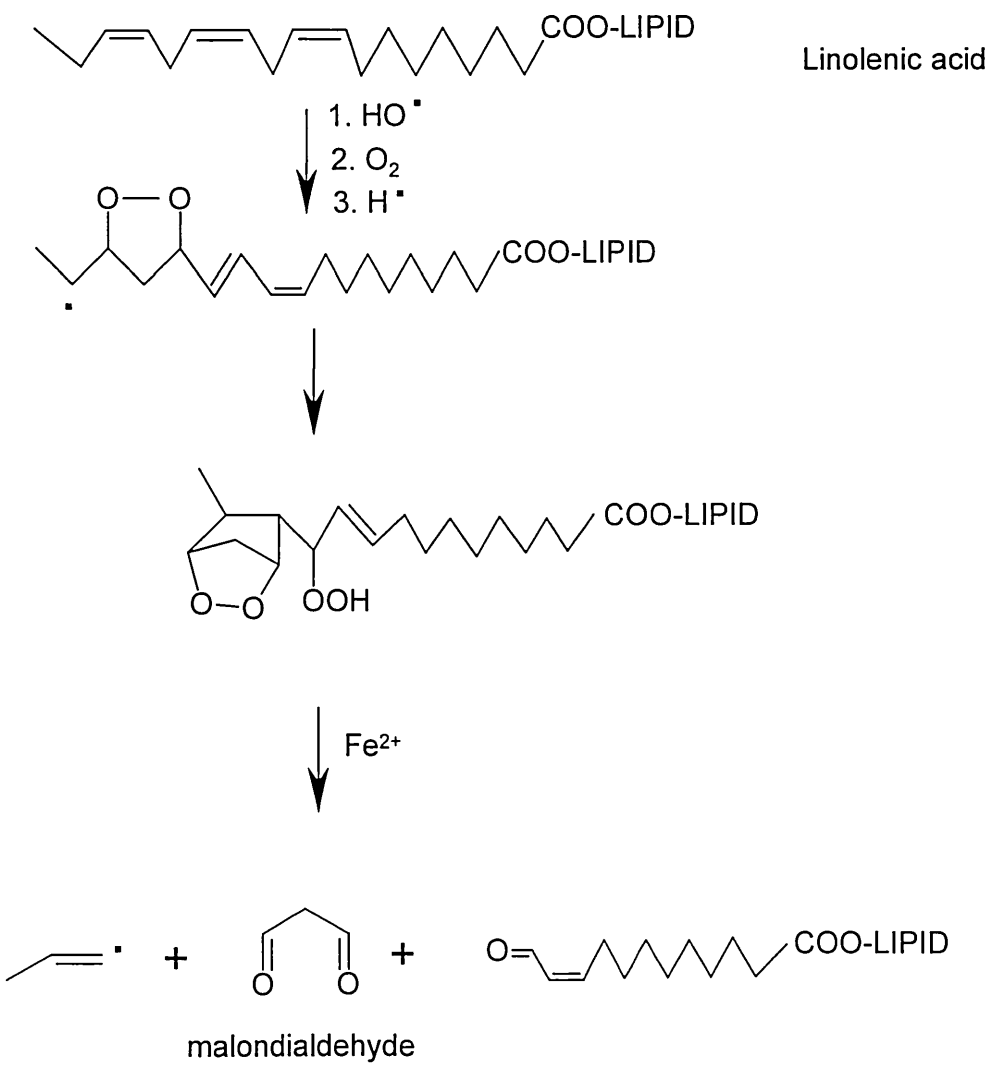
Hydroxyl radicals are the most potent naturally occurring oxidant known, and intense reactivity can initiate the damaging process of lipid peroxidation and is one of the most important mechanisms involved in free radical damage to cells (Cheeseman, 1994). Injury to cells can be direct by destroying the integrity of the membranes of intracellular organelles or the plasmalemma which can lead to leakage of cellular contents, rapid desiccation and ultimately cell death (Scandalios, 1990), or indirectly, by producing damaging secondary products which can affect macromolecules and other cellular contents. One important group of these secondary products is the hydroxyalkenals, and perhaps the most significant of these is 4-hydroxynonenal (HNE) (see Figure 1.3.2.1) (Cheeseman, 1994, Zollner, *et. al.* 1991). The mechanism of production of HNE is from lipid hydroperoxides by homolytic scission (β-cleavage) of the C-C bonds on either side of the hydroperoxy group, accelerated by traces of reduced forms of transition metals such as Fe^{2+}, or Cu^{+}. HNE is generated from ω-6 poly unsaturated fatty acids (PUFAs) and the principal PUFA in plant cells, that can be oxidised to aldehydes, is linoleic acid (18:2), (Esterbauer, *et. al.* 1990) (see Figure 1.3.2.1). HNE has a high biological activity and has been widely investigated within mammalian systems and exhibits cytotoxic, mutagenic and genotoxic properties by linking with DNA, proteins and enzymes (Burdon, *et. al.* 1990, Esterbauer, *et. al.* 1988, Esterbauer, *et. al.* 1990 Zollner, *et. al.* 1991). HNE-protein adducts are formed when HNE reacts with histidine residues forming a Michael-adduct with a hemiacetal structure of the aldehyde by a nucleophilic addition reaction (Waeg, *et. al.* 1996). *In vivo*, significantly increased levels of HNE and other

Figure 1.3.2.1 Formation of Hydroxynonenal from Linoleic Acid



lipid peroxidation products are found in plasma and various organs under conditions of oxidative stress (for review see Esterbauer, *et. al.* 1990). Lipid peroxidation has been investigated within a number of different plant systems but HNE production and its effects within *in vitro* plant tissue cultures have not been considered.

Figure 1.3.2.2 Formation of Malondialdehyde from Linolenic Acid



Analytical techniques developed by the University of Abertay free radical research group were used in this study to investigate HNE within plant systems (Deighton, *et. al.* 1997, Bremner, *et. al.* 1997). These techniques were used in conjunction with the application of existing techniques to new systems to investigate the effects of the production HNE within *in vitro* plant tissue (Adams, *et. al.* 1999).

Another important product of lipid peroxidation is malondialdehyde (MDA) and this has been widely studied in different organisms, including animals (Esterbauer, *et. al.* 1990, Frankel, 1987), plants (Franck, *et. al.* 1998), and micro-organisms (Turton, *et. al.* 1997). MDA in many systems is the most abundant aldehyde arising from lipid peroxidation and consequently its production has been used as a marker of lipid peroxidation (see Figure 1.3.2.2) (Brown & Kelly, 1996). MDA production has been examined in a number of plant systems including senescing maize leaves (Hung & Kao, 1997), *Prunus avium* L. (Frank, *et. al.* 1998), and *Lycopersicon esculentum* (subjected to a challenge by pathogens) (May, *et. al.* 1996).

MDA has been quantified through its reaction with thiobarbituric acid (TBA) (Esterbauer & Cheeseman, 1990). However, this reaction has inherent problems when applied to tissue and body fluids for the measurement of MDA. TBA will also react with a number of other substances that are present within biological samples, including carbohydrates, nucleic acid bases and haemoglobin, which produce coloured products which overlap the absorbance spectrum of MDA-TBA (Brown & Kelly, 1996). Therefore, another

technique for the measurement for MDA needed to be found (Robertson, *et. al.* 1995). This became one of the priorities of the University of Abertay free radical research team and lead to the development of the LC-MS assay for the determination of MDA and HNE based on the derivitisation reaction with 2,4-dinitrophenylhydrazine (Deighton, *et. al.* 1997). This assay is one of the main biochemical tools used for the investigation of HNE and MDA within this project (Bremner, *et. al.* 1997, Adams, *et. al.* 1999).

1.3.3 Lipid Peroxidation and Mammalian Disease

The majority of the research into lipid peroxidation to date has focused on mammalian systems and diseases including arthritis, cancer, atherosclerosis, inflammation and liver injury (Esterbauer & Ramos, 1995, Zollner, *et. al.* 1991, Spiteller, 1996). Lipid peroxidation has been linked with disease and clinical conditions in all parts of the body. The liver can be particularly targeted and can be affected by iron overload causing liver dysfunction or liver cirrhosis initiated by alcoholism (Cheeseman, 1994).

In atherosclerosis one of the lipid peroxidation products HNE, reacts with amino side chains of low-density lipoprotein. The modified protein is then recognised by the "scavenger receptor" of a macrophage cell which ingests the modified protein and changes into a foam cell (Cheeseman, 1994). The formation of these foam cells is one of the first steps in the process of the creation of an atherosclerotic lesion (Cheeseman, 1994). The toxicity of HNE within mammalian cells varies greatly depending on the type of cell involved,

but in many cases it is cytostasis, causing the cells to stop growing but not killing them (Cheeseman, 1994).

Lipid peroxidation has been associated with an increasing list of diseases and conditions including: diabetes, respiratory disease syndrome and shock, Parkinson's disease, inflammatory conditions, ischaemia, and reperfusion injury to the heart, brain and intestine (Cheeseman, 1994). HNE, as a product of lipid peroxidation, has been shown to inhibit a large number of enzymes and this fact may help to account for its toxic effects (for review see Zollner, *et. al.* 1991). Enzymes affected include, DNA polymerase, glyceraldehyde phosphate dehydrogenase (Uchida & Stadtman, 1993), lactate dehydrogenase and protein kinase C.

1.3.4 Oxidative Stress in Plant Tissue Culture

Plants have acquired unique sets of responses to deal with difficult environment and environmental changes (Scandalios, 1990). A number of stress response genes have been shown to be selectively increased or decreased under stressful situations; in particular many of the antioxidant enzymes have been shown to be increased after the onslaught of a number of environmental stresses and have been associated with developmental changes (Scandalios, 1990).

However, *in vitro* culture imposes a different set of environmental conditions and induce an alternative set of stress responses than a natural environment. There is mounting evidence to support the hypothesis that oxidative stress is

prevalent in *in vitro* culture and that it plays a significant role in the responses of the tissue to culture conditions (Ishii, 1987, Cutler, *et. al.* 1989, Bailey, *et. al.* 1994, Benson & Roubelakis-Angelakis, 1992, 1994, Marco & Roubelakis-Angelakis, 1996, Bremner, *et. al.* 1997, Benson, *et. al.* 1997). Furthermore, it may be a causal factor in *in vitro* plant recalcitrance and in the production of active oxygen species has been associated within the *in vitro* manipulation of plants (Benson & Roubelakis-Angelakis, 1992, 1994, Marco & Roubelakis-Angelakis, 1996). Studies of lipid peroxidation in plant tissue cultures demonstrate that aldehydes are produced during culture initiation and throughout routine sub-culturing (Benson & Roubelakis-Angelakis, 1994, Robertson, *et. al.* 1995). They also accumulate in cultures which have lost their totipotency, compared to those which have maintained their regeneration potential (Benson, *et. al.* 1992a). Hung and Kao (1997) have studied lipid peroxidation and MDA production pathways in senescing maize leaves and Franck, *et. al.* (1998), have monitored lipid peroxidation pathways and markers of physiological disorders in plant tissue culture.

Cereals appear to be particularly prone to oxidative stress and the process of removing the cell walls to produce protoplasts generates increased levels of superoxide resulting in oxidative stress (Ishii, 1987, 1988). In cultures of wheat and maize there is evidence to suggest that oxidative stress plays a role in the recalcitrance of establishing these species in culture (Culter, *et. al.* 1989). Raised levels of lipoxygenase activity and lower activity of antioxidants were associated with the increased production of oxidative species and cause damage (Culter, *et. al.* 1989). During the initiation of callus

in *Vitis vinifera* L. there was increased levels of fluorescent products and thiobarbituric acid reactive substances, both of which were associated with oxidative stress (Benson & Roubelakis-Angelakis, 1994). All these studies indicate the potential for oxidative stress to have a significant effect on the growth and responses of plant tissue in *in vitro*.

1.3.5 Measurement of Free Radical Species and Secondary Oxidation Products

Electron spin resonance (EPR) can be used to directly measure the amount of free radicals within plant tissue and has been sued to detect free radicals within potato tissue place on callus induction medium (Bailey, *et. al.* 1994). EPR has also shown that wounding produces the most detectable free radical signatures and that light is associated with the most oxidative stress (van Doorslaer, *et. al.* 1999). However, EPR is expensive and requires careful interpretation and therefore other assays are more commonly used. Assays monitoring the activity of free radicals measure secondary reaction products and associated compounds have given much needed insight into the activity of free radicals within active tissues. The range of assays now employed to profile the extent of oxygen-derived free radicals within tissues and the secondary products produced when they react with cellular components.

Hydroxyl radicals generated within tissue are measured by profiling the amount of methane released by dimethylsulphoxide (DMSO) as it reacts with the hydroxyl radical using gas chromatography (Benson & Withers, 1987). Chemiluminescence techniques employ the use of compounds such as luminol to indicate the presence of superoxide radicals (Weber, 1990). UV-

spectrophotometry (see section 1.3.6) has been used to measure the activity of superoxide radicals by the reduction of compounds such as nitrotetrazolium blue (Weber, 1990). Spectrophotometry techniques have also been used to measure antioxidant activity such as catalase and peroxidase (Aebi, 1983, Castillo, *et. al.* 1984).

A range of techniques for profiling the extent of production of lipid peroxidation products and associated species have been developed for mammalian systems and include: loss of PUFAs, rate of oxygen consumption, increase of MDA (Esterbauer, 1996, Zollner, *et. al.*, 1991, Deighton, *et. al.*, 1997), formation of fluorescent reaction products of lipids and proteins (Benson & Roubelakis-Angelakis, 1992, Fleck, *et. al.* 1999), and detection of lipid peroxidation products conjugated to proteins with ELISAs (Waeg, *et. al.* 1996). Many of these techniques have been employed in *in vitro* cultures of plant tissues.

1.3.6 Defence Mechanisms and Antioxidants

Aerobic organisms have developed numerous detoxifying mechanisms to combat the 5- 10% of oxygen that escapes from respiration and can be reduced to form reactive oxygen species (Esterbauer, 1996). Plants, particularly, have to cope with free radical formation as a result of photosynthesis and antioxidants are present within chloroplasts to deal with the reactive oxygen radicals produced, by "mopping-up" these compounds and protecting the surrounding cell (Salin, 1987). These antioxidants include a large group of detoxifying enzymes (e.g. catalase, superoxide dismutase, glutathione

peroxidase, glutathione reductase, and glutathione S-transferase) and low molecular weight antioxidants such as ascorbate and glutathione (Esterbauer, 1996). Different organelles within plant cells have alternative enzymes or isoforms of the enzymes to cope with the products generated under oxidative conditions and many are associated with membranes (Bartosz, 1997). The range of defences against reactive oxygen species is wide and includes ascorbate, phytochelatins, carotenoids, flavanoids, thioredoxin (Bartosz, 1997). Ferritin indirectly prevents lipid peroxidation resulting from reactive oxygen species by sequestering iron preventing it from catalysing the Haber-Weiss reaction (Bartosz, 1997). There is increasing evidence to support the fact that some of these systems, under certain conditions, may be overwhelmed by the onslaught of reactive oxygen species and lipid peroxidation products (Esterbauer, 1996).

1.3.6.1 Catalase and Peroxidase

Hydrogen peroxide (H_2O_2) is constantly produced in biological tissues as an essential component of oxidative metabolism and electron transport, such as the action of glycollate oxidase in the choroplast (Foyer, *et. al.* 1997). H_2O_2 is a major product of photorespiration and its generation is increased during environmental stresses involving water deficits (Foyer, *et. al.* 1997) and is accumulated in stressful situations such as exposure to ultra violet light, low temperatures, and heat shock (Foyer, *et. al.* 1997). H_2O_2 is also formed as a result of the dismutation of superoxide by superoxide dismutase (see section 1.3.6.2). Under normal conditions these reactions producing H_2O_2 are tightly controlled, however during stress, ageing, and disease these controls may not

be sufficient to overcome the amount of free radicals produced (Benson, *et. al.* 1992a). H_2O_2 can be broken down by a number of different mechanisms, and includes the action of the enzyme catalase. The role of catalase is important, as some of the mechanisms that remove other dangerous oxygen species produce hydrogen peroxide. The level of catalase within plant tissue can have significant implications as to the morphological status of the tissue and low levels of catalase have been implicated in habituation (Le Dily, *et. al.* 1993). This low activity can allow H_2O_2 to accumulate, and can therefore lead to the generation of hydroxyl radicals through the Fenton reaction, which can in turn lead to the initiation of lipid peroxidation (Le Dily, *et. al.* 1993). H_2O_2 can easily penetrate cellular membranes and therefore can react at locations away from the original site of generation (Bartosz, 1997), which is in contrast to other free radical products which have a very short half-life and therefore react with molecules immediately adjacent to the site of generation.

Catalase

Catalase activity can be easily measured using spectrophotometric assays (Cohen, *et. al.* 1970) and has been investigated in a large number of plant systems *in vitro* including *Vitis viniferia* (Benson & Roubelakis-Angelakis, 1992), *Oryza sativa* (Benson, *et. al.*1992a), and *Zea mays* (Prasad, 1997). In rice, decreased levels of catalase are associated with tissues that have lost embryogenic capacity and have increased levels of lipid peroxidation products (Benson, *et. al.*1992a). Monitoring the level of catalase in tissues under

stressful situations may give an indication as to the oxidative status of the tissue.

Peroxidases

Peroxidases are another group of enzymes that are integrally involved in growth and development but also play a role as detoxification enzymes, in the defence of oxidative stress and the control of reactive oxygen compounds within plant cells. Peroxidase activity has been the focus of a great deal of attention and is now considered as a developmental marker (Krsnik-Rasol, 1991). Cell wall peroxidases have been linked with lignification processes (Kevers & Gaspar, 1985) and changes in levels of activity have been associated with vitrification (Kevers & Gaspar, 1985), habituation (Kevers & Gaspar, 1985) and general declines in organogenic capacity (Gaspar, 1995; Kevers, *et. al.* 1996; Le Dily, *et. al.* 1993). There are many different types of peroxidase enzymes, including ascorbate peroxidase, which removes H_2O_2 by reduction of ascorbate (Alscher, *et. al.* 1997). The different peroxidases are important in the regulation of H_2O_2 and preventing excess H_2O_2 accumulating, which can initiate the production of hydroxyl radicals and subsequently lipid peroxidation. There are also a number of non-specific peroxidases present within the chloroplasts (Bartosz, 1997).

Increased peroxidase activity has been shown to occur when plants are exposed to a variety of stresses including viral, microbial or fungal infection, high salt concentration, wounding, or air pollution (Castillo, *et. al.* 1984). In

wounded tissue, damaged cell walls must be quickly repaired to prevent cell lysis and an isozyme of peroxidase has been associated with lignin biosynthesis (Wakamatsu & Takahama, 1993). Peroxidase enzymes, involved in the process of lignification, are decreased in *Dianthus caryophyllus* cells that have undergone vitrification (Kevers & Gaspar, 1985) indicating that a detrimental change in the activity of peroxidase enzymes can affect the morphogenic and health status of tissue within an *in vitro* environment. Changes in the levels of peroxidase within plant cells have been linked with stress (Wakamatsu & Takahama, 1993) and, particularly, on exposure to ozone (Castillo, *et. al.* 1984). The level of peroxidase present in tissues of *Sedum album* rapidly rises within 24 hr after exposure to ozone and may form part of the basis of the tolerance of this species to ozone (Castillo, *et. al.* 1984).

During the initiation of callus within *Vitis viniferia* from mature embryos the activity level of catalase and guaiacol peroxidase are markedly reduced and are much lower than in mature tissues (Dey & Kar, 1995). This indicates that lower antioxidant enzyme activities during changes in morphogenic status and that callus tissues are less able to remove free radicals and active oxygen species within *in vitro* culture. Changes in peroxidase activity were also noted when lupin shoots were transferred onto media containing auxin for the induction of root formation (Jouve, *et. al.* 1994). Peroxidase activity rapidly decreased to a minimum level after 9 hours on the auxin-containing media indicating the level of peroxidase activity may have a role within developmental changes (Jouve, *et. al.* 1994). Peroxidase activity has been

monitored within a large number of different *in vitro* plant tissue systems and has been particularly profiled in tissue with different degrees of habituation and vitrification (Kevers & Gaspar, 1985, Le Dily, *et. al.* 1993).

Peroxidase activity has therefore been used as a developmental marker due to its importance in the regulation of H_2O_2 during different stages of morphological development. Monitoring levels of peroxidase within different cultures under varying conditions will give an indication as to the morphogenic potential of the tissue.

1.3.6.2 Superoxide Dismutase

Superoxide dismutase (SOD) is a general term for a group of metalloenzymes which contain manganese, iron, copper or zinc as their metals (Benson, 1990). In plants, the highest concentration of SOD activity is located within the chloroplasts and is generally associated with membranes, although Cu/ZnSOD, the major form of SOD, can also be found in the cytosol. SOD catalyses the conversion of $O_2^{\bullet -}$ to H_2O_2 and the resultant H_2O_2 is metabolised to H_2O by glutathione peroxidase. The action of SOD is very important to combat oxidative stress under a number of different environmental conditions.

In varieties of *Sorghum bicolor* (L.) the activity of SOD and catalase are both increased under temperature and light stress in drought tolerant species compared to susceptible varieties, indicating that toleration is partly mediated by manipulation of the levels of antioxidants (Jagtap & Bhargava, 1995).

Increased SOD levels have also been shown to play a role in the tolerance of *Zea mays* seedlings to chilling stress in combination with raised levels of ascorbate peroxidase (Pinhero, *et. al.* 1997). Therefore, SOD activity is known to play an important role in the defence of tissues against oxidative and environmental stresses and monitoring activity levels of SOD (Beauchamp & Fridovich, 1971) within tissues will give an indication of the ability of the tissues to remove dangerous superoxide from its cells.

1.3.6.3 Sulphydryl Groups

Compounds containing sulfydryl groups (SH groups) can be divided into two groups, low molecular weight compounds such as glutathione (see 1.3.5.4) and high molecular weight, protein bound SH groups (Faure & Lafond, 1995). Protein SH groups have been considered as important agents in protecting cells against NO_2 and associated lipid peroxidation (Halliwell, *et. al.* 1982). The oxidation of protein SH groups has been proposed as the primary reaction leading to membrane damage in plant cells affected by ozone (Chevrier, *et. al.* 1988). The oxidation of SH groups is likely to lead to alterations in membrane properties, ionic imbalance and the disturbance of metabolic processes and subsequently growth (Chevrier, *et. al.* 1988). Therefore, monitoring the levels of both protein-bound and non-protein SH groups will give an indication as to the role of SH groups in oxidative stress.

1.3.6.4 Oxidised and Reduced Glutathione and its Associated Enzymes

Glutathione is a low molecular weight antioxidant consisting of a tripeptide with a thiol group (S-H) on the cysteine residue and was discovered in the 1920s (Cotgreave & Gerdes, 1998). Increasingly, glutathione has been associated with apoptosis and may have significant reactions with proteins, although its role and effects are not currently fully understood (Cotgreave & Gerdes, 1998).

Glutathione metabolism in plants has been considered for many years and has been shown to be the same series of reactions noted in animal cells (Rennenberg, 1982). Since then a large number of groups have shown glutathione and its associated enzymes to have important roles within stress tolerance, senescence and defence (Foyer, *et. al.* 1997, May, *et. al.* 1996, Xiang & Oliver, 1998, Jiménez, *et. al.* 1998, and Stajner, *et. al.* 1993).

Glutathione is very important for the protection of membranes sensitive to reactive oxygen species and acts as a competitor to oxidative damage by being preferentially oxidised over membrane lipids. Glutathione in its reduced form can be recycled by associated enzymes such as glutathione reductase, which links it to the oxidation of NADPH in the Halliwell-Asada cycle (Bartosz, 1997). Glutathione peroxidase uses glutathione to reduce H_2O_2 (Bartosz, 1997) and, more importantly, organic peroxides which are the first products of lipid peroxidation.

Glutathione plays a central role in the detoxification of reactive oxygen species within green plant cells (Alscher, *et. al.* 1997). Without glutathione the integrity of membranes under attack by oxidative stress would be severely threatened and its action is vital in maintaining structural integrity of cells under stress. Glutathione is particularly associated with lipid peroxidation and the role of its associated enzymes are shown to have increased activity in *Phaseolus vulgaris* tissue under attack from herbicides which activate lipid peroxidation (Schmidt & Kunert, 1986). Increased expression of glutathione S-transferase (which conjugates glutathione to a variety of compounds to reduce their toxicity and aid their detoxification) and glutathione peroxidase appears to enhance the growth of tobacco seedlings during chilling and salt stress (Roxas, *et. al.* 1997). It appears that the enzymes involved in glutathione conjugation and recycling are more significant than the levels of the glutathione synthetase enzyme, particularly for the resistance to photo-inhibition in poplar trees (Foyer, *et. al.* 1995). The evidence suggests that the involvement of glutathione is essential in combating environmental stresses.

1.3.6.5 Other Antioxidants

Vitamins C and E play important roles in the protection of cells from oxidative stress. Vitamin C (ascorbic acid) and its associated enzyme ascorbate peroxidase are important in the removal of H_2O_2 and the ascorbate is recycled by dehydroascorbate reductase in conjugation with glutathione (Bartosz, 1997). Vitamin C is also important in the control of superoxide and hydroxyl radicals (Kitts, 1997) both of which cause significant damage within plant cells. Vitamin C is important in the regeneration of vitamin E, where it

mediates the reduction of α-tocopherol quinone back to α-tocopherol (Packer, *et. al.* 1979).

Vitamin E is particularly important in the prevention of lipid peroxidation since it is an effective free radical scavenger due in part to its lipophilic nature and is associated with membranes (reviewed by Benson, 1990). Vitamin E is often partly imbedded within the lipid layers of membranes and therefore it "mops-up" hydroxyl radicals that may initiate lipid peroxidation. Vitamin E has been particularly associated with chronic neurological syndrome, and the lipid peroxidation level is increased in rats that are deficient in vitamin E (MacEvilly & Muller, 1996). Therefore, vitamin E seems to be important in mediating oxidative stress and in the propagation of free radical species within mammalian disease.

Carotenoids play an important role in the defence against oxidative stress in plants. In choroplasts, carotenoid pigments are the first line of defence and serve to prevent the propagation of free radicals by preventing excited chlorophyll molecules passing on their energy to oxygen, thus preventing reactive oxygen species from being produced (Benson, 1990, Smirnoff, 1993). Carotenoids can also quench singlet oxygen, which provides an important protective role in non-photosynthetic tissues (Benson, 1990).

The number of low molecular weight antioxidants is vast. One of the most significant is glutathione but the list includes cysteine, phytic acid, phytochelatins, flavanoids, cinnamic acids (e.g. caffeic acid) and polyphenols

which are all present in aqueous phases (Bartosz, 1997). Other antioxidants are lipophilic and are present within or associated with membranes and these include tocopherols (e.g. vitamin E), tocotrienols, xanthophylls, ubiquitinol and vitamin D and these are all involved in the protection of a wide range of processes throughout the cell (Bartosz, 1997).

1.3.7 Plant Membranes, Lipids and Lipoxygenase

Cellular membranes play a vital role in how the cell senses and reacts to the environment around it. Membranes are also critical for controlling the flow of substances in and out of the cell. Therefore, attack on membrane integrity and damage to its components can cause cell necrosis and death. The study of plant membranes has been greatly aided by investigations on animal and microbial cells, but unique organelles such as chloroplasts make plant models unique. The ranges of fatty acids present in mammalian and plant membranes are similar but have some important differences. A common component of mammalian membranes is arachidonic acid (20:4) but in plant cells it is replaced by linoleic acid (18:2) or linolenic acid (18:3) (Hildebrand, 1989). Lipid peroxidation has been recognised in plants since the 1930's (Zimmerman & Vick, 1988), however, progress since the 1970's has been limited and the true role of many of the aldehydic products still remains unclear (Zimmerman & Vick, 1988).

Lipoxygenase (linoleate: oxygen oxidoreductase EC 1.13.11.2) enzymes catalyse the incorporation of molecular oxygen into linoleic and linolenic acids (Zimmerman & Vick, 1988). The products are fatty acid hydroperoxides

and serve as substrates for enzymes producing a wide range of products. Many of the products derived from this pathway have been associated with foodstuffs and the problems linked with long term storage (Siedow, 1991). A significant product formed from lipoxygenase initiated pathway is jasmonic acid and its derivative methyl jasmonate (Zimmernam & Vick, 1988).

Jasmonates have been implicated as plant hormones (Staswick, 1992) and have been particularly associated with the wound response and are initiators of plant defence genes in reaction to various environmental stresses (Koda, 1997). Jasmonates appear to regulate cell division by inhibiting cell elongation and promoting cell expansion, leading to defined shapes of organs and tissues (Koda, 1997). Jasmonates are also known to promote senescence and, in tomatoes, methyl jasmonate was shown to inhibit the synthesis of lycopene and to stimulate the production of ethylene (Zimmerman & Vick, 1988). The addition of jasmonic acid to stem node cultures of *Solanum tuberosum* L. cv. *Vesna* at low concentrations (0.1-1 μM) significantly increased the length of the developed plants, with increased root development, however concentrations added above 10 μM lead to the plants having a stunted appearance (Ravnikar, *et. al.* 1992).

Other products that can be produced through similar lipoxygenase mediated pathways and include γ-ketols which are generated from linolenic acid and are structurally similar to HNE and may have similar effects such as inhibition of enzymes and DNA synthesis (Zimmerman & Vick, 1988). Secondary products of lipid peroxidation such as jasmonates and HNE, could act as

signal regulators and secondary messengers and may have significant implications in the control and manipulation of plant cells *in vitro* and in the field.

1.3.8 Antioxidant Strategies to Overcome *In Vitro* Recalcitrance

Antioxidant enzymes and mechanisms associated with the removal of dangerous active oxygen species play a vital role in the ability of tissue to cope with stressful conditions in or out of culture. Tissue culture regimes, which minimise the amount of oxidative stress will help to maximise the responses and growth of healthy plants *in vitro*. Therefore, the study of oxidative stress and free radical processes and the accompanying antioxidants is becoming increasingly important in tissue culture maximisation and applied plant biology (Pryor, 1997, Scandalios, 1997) as phytopathological and environmentally mediated free radical damage can lead to serious reductions in plant responses and crop productivity. Investigations into the role of antioxidant systems within *in vitro* cultured cells may also provide a needed insight into the potential of overcoming oxidative stress in all environments.

The addition of antioxidants has been used as a method for the improvement of the responses of certain species *in vitro*. Different strategies have been used to try and overcome the problem of recalcitrance. In some species of microorganisms media supplemented with antioxidants increased oxygen tolerance and therefore aided manipulations within *in vitro* culture (Hoffman, *et. al.* 1979). With increasing research on the study of lipid peroxidation in plant tissue culture systems the addition of antioxidants or associated

compounds may help to increase viability, as illustrated by addition of antioxidants to rice cells during isolation of protoplasts (Ishii, 1988). Enhancement of organogenesis in tobacco callus was achieved by the addition of ascorbic acid (Joy, *et. al.* 1988). These studies indicate that using antioxidants within culture systems may aid responses and that oxidative stress is playing a significant role in the ability of tissue to respond in *in vitro* culture.

Chapter 2 Materials and Methods

Contents

2.1 Cultures

Three different crop plant species were selected for investigation, *Daucus carota* (carrot), *Ipomoea batatas* (L.) Lam (sweet potato) and *Glycine max* (soyabean). *Daucus carota* (carrot) has been studied by a large number of research groups for many years and is now considered to be one of the model systems for the investigation of plant growth and development (Zimmerman, 1993). The easy manipulation of this species within the plant tissue culture environment makes it an ideal basis for the study of characteristics and responses of oxidative stress *in vitro* (Benson, *et. al.* 1997).

The second species selected was *Ipomoea batatas* (L.) Lam (sweet potato) as it is one of the most important root crops within the developing world. It has been shown by a number of research groups to have a wide range of responses by different cultivars to manipulation within the tissue culture environment (Al-Mazrooei, *et. al.* 1997, Chee & Cantliffe, 1992). Oxidative stress has been implicated as one of the causes of this variability and further investigation into this phenomenon may gain insights into the manipulation of this and other species within tissue culture (Benson, *et. al.* 1997, Benson, 1990). The *I. batatas* cultures were micro-propagated plant-lets established from plants obtained from the National Bureau of Plant Genetic Resources, India (NBPGR) and the University of Bath.

The final species selected was *Glycine max* (soyabean), a crop which has been widely studied and remains an economically and agronomically important species. One of the cultures selected was habituated and would give an

indication of the differences between the cancer like growth and normal dedifferentiated callus growth (Gaspar, 1995, Kevers, *et. al.* 1996). The three types of *G. max* callus cultures were obtained from the John Innes Centre, Norwich (Wang, 1979).

2.1.1 Sources of Cultures

Daucus carota Cultures

D. carota cultures used were callus lines previously established by the group, and new cultures were initiated from seed stocks, (see section 2.4) bought from commercial suppliers as detailed in Table 2.1.1.

Table 2.1.1 *D. carota* Seed Stocks from Commercial Suppliers

CULTIVAR	COMMERCIAL SUPPLIER
Chantenay Red Cored	Basically Seeds, Bury St. Edmonds; Johnson's Seeds, Boston
Regulus	Basically Seeds, Bury St. Edmonds
Early Nantes	Johnson's Seeds, Boston
Autumn King 2	Unwinds Seeds Ltd, Cambridge
Gold King	Basically Seeds, Bury St. Edmonds
Organza	Basically Seeds, Bury St. Edmonds
New Red Intermediate	Sutton's Seeds, Torquay
Early Scarlet Horn	Basically Seeds, Bury St. Edmonds

Ipomoea batatas (L.) Lam Cultures

I. batatas micro-propagated plantlets were obtained from National Bureau of Plant Genetic Resources, India and are detailed in Table 2.1.2. The other micro-propagated plant-let cultivars obtained from the University of Bath are detailed in Table 2.1.3.

Table 2.1.2 *I. batatas* Cultivars Obtained from NBPGR, India

CULTIVAR NAME	INDIAN/CIP REFERENCE	NAME	ORIGIN	YEAR OF INTRODUCTION TO INDIA
S-66	EC 17762	Nauso local	Fiji Island	1960
S-67	EC 17763	Papava	Fiji Island	1960
IC-21	CIP 420024	Unknown	Peru	1990
IC-14	CIP 420014	Jonathan	Peru	1990
S-256	-	IB-14	India	1966

EC = Exotic Collection (Indian Accessioning)
CIP= International Potato Center, Lima, Peru.

Table 2.1.3 *I. batatas* Cultivars obtained from the University of Bath

CULTIVAR NAME	COUNTRY OF ORIGIN	ORGANISATION
Nemanete	Peru	International Potato Center, Lima, Peru
TIB 10	Nigeria	International Institute of Tropical Agriculture
1023M	China	P. Lepoivre, Gembloux, Belgium

Glycine max. Cultures

Three types *G. max* callus cultures were obtained from the John Innes Centre, Norwich. All three types require naphthaleneacetic acid (NAA) for continued

dedifferentiated growth. Two of the types, SOY + green and SOY + white also require benzyl amino purine (BAP) to maintain healthy growth. SOY-white line is habituated against BAP and only requires NAA in the culture media and is detailed in Table 2.2 (Wang, 1979). Both of the white callus lines are maintained at 25 °C in the dark and the green callus in a 16 hour photo-period in a climate controlled growth room at a temperature of 25 °C, with cool white fluorescent tubes at a photon flux density of 50 μmol m^2 s^{-1}.

2.2 Routine Culture Media

Media for routine sub-culturing were made-up in advance from stock solutions (Murashige & Skoog, 1962). Stock solutions were prepared from de-ionised water and stored at 4 °C (Appendix I, Table 1). Stock solutions of hormone additives were also made from de-ionised water and stored at –20 °C (Appendix 1, Table 2).

Media were prepared by adding measured volumes (10 mL / litre) of x100 stock solutions to de-ionised water containing the carbon source, sucrose and the pH was adjusted to 5.6 using 1M NaOH. The volume was then made up to the final value and then decanted into bottles containing the appropriate weight of agar (7g / litre) and the mixture autoclaved at 121 °C (101 kPa) for 20 minutes. Growth regulators and hormones were either added prior to autoclaving or post-autoclave using a 0.2 μm filter under sterile conditions (Table 2.4). All chemicals were of tissue culture grade and obtained from either Sigma Chemical Co. (USA), or Duchefa (Netherlands). The details of

the media and routine sub-culture for the three species are shown in Table 2.1. Further details of media components are shown in Appendix 1, Tables 1 and 2.

All media except for *G. max* were prepared from the basic Murashige and Skoog recipe with the addition of the appropriate hormones (Murashige & Skoog, 1962) (see Appendix 1, Tables 1 & 2). The methods for preparing the hormone solutions are shown in Appendix 1, Table 2. The details of the *Glycine max* media are in Appendix 1, Table 3 (Wang, 1979). SOY + media contains both NAA and BAP and SOY – media contains only NAA. All media preparations were sterilised by autoclaving at 121 °C for 20mins.

Table 2.2 Media and Sub-culture Cycle for Cultures of *Daucus carota*, *Ipomoea batatas* (L.) Lam and *Glycine Max*

CULTURE	MEDIA	ADDITIVES	SUB-CULTURE CYCLE
DAUCUS CAROTA			
Carrot callus initiation and maintenance	2,4-D media	1 mg/L 2,4-dichlorophenoxy-acetic acid	2 weeks
Carrot callus under embryo initiation	M&S media	None	1 - 3 weeks
Germinating carrot seeds	M&S media	None	Approx. 3 weeks
IPOMOEA BATATAS			
Stock plants (for Indian cultures)	M1 media	0.2 mg/L kinetin, 0.1 mg/L indole acetic acid	6-8 weeks
Stock plants (for Bath cultures)	M&S media (with 20g/l sucrose)	None	6-8 weeks
Callus & Embryo initiation	SP1	2 mg/L naphthalene-acetic acid, 0.1 mg/L benzylamino purine	3 weeks
Callus & Embryo initiation	SP2	0.5 mg/L 2,4-dichlorophenoxyacetic acid; 0.1 mg/L benzyl amino purine	3 weeks
Callus & Embryo initiation (Zheng, *et al*, 1996)	S1	2.5 mg/L 2,4-dichloro-phenoxyacetic acid; 0.25 mg/L benzylamino purine	3 weeks
Callus & Embryo initiation (Zheng, *et al*, 1996)	S2	2.5 mg/L abscisic acid	3 weeks
Callus & embryo initiation (Al-Mazrooei *et al*, 1997)	SP 2.5 (2,4,5-T)	2.5 mg/L 2,4,5-trichloro-phenoxyacetic acid	3 weeks
GLYCINE MAX			
SOY callus green +	Soy +	2 mg/L naphthalene-acetic acid; 1mg/L benzylamino purine	3 weeks
SOY callus white +	Soy +	2 mg/L naphthalene-acetic acid; 1mg/L benzylamino purine	3 weeks
SOY callus white -	Soy -	2 mg/L naphthalene-acetic acid	3 weeks

2.3 Culture Maintenance

Sub-culture Techniques

All cultures were sub-cultured using asceptic techniques in a laminar flow cabinet using an alcohol dip and burner to sterilise instruments. Callus cultures were sub-cultured onto fresh media in Petri dishes at the intervals specified in Table 2.2, making sure that only the new growth free from browning or decaying callus was transferred. Petri dishes were then sealed with Parafilm© and maintained in the dark at 25 °C. *G. max* callus was maintained in honey jars with solid lids.

I. batatas micro-propagated plantlets were sub-cultured using nodal cuttings into honey jars containing media, which were then sealed using Sun-caps. Sun-caps are plastic films with a central hole covered by a 0.2 μm filter to allow gaseous exchange without allowing microbial organisms into the culture vessel (Sigma).

I. batatas plants and the green *G. max* callus were maintained under an 18 hour photo-period in a climate controlled growth room at a temperature of 25°C, with cool white fluorescent tubes at a photon flux density of 50 $\mu molm^2 s^{-1}$.

Sampling Techniques

Samples of callus are taken and weighed under sterile conditions and transferred to 1.5 mL screw capped Eppendorf tubes (Eppendorf, Germany) and stored in liquid nitrogen storage Dewar at –196 °C prior to extraction for biochemical analysis.

2.4 Callus and Somatic Embryogenesis Initiation of *Daucus carota*

Seed Sterilisation

D. carota seeds were sterilised by being immersed in a 10 % of solution of Domestos© bleach (active ingredient: sodium hypochlorite) using a sterilised Wilson sieve. The seeds were incubated in this solution for 10 minutes and then removed from the solution within the sieve and then rinsed three times in sterile de-ionised water. Seeds were then placed on Petri dishes containing M&S media and sealed with Parafilm©. The Petri dishes were then incubated in the light until the seedlings were approximately 3 cm long and mature enough for callus culture initiation.

Initiation of *D. carota* Callus

Once the seedlings, grown from sterilised seeds, had reached approximately 3 cm long, the hypocotyls (the stem) was cut into 0.5 cm long sections and placed on 2,4-D media. The Petri dishes were then sealed with Parafilm© and

placed in the dark at 25 °C. After 3-6 weeks the new callus growth was sub-cultured onto fresh 2,4-D media and subsequently maintained in a two-week sub-culture cycle.

Stimulation of Somatic Embryogenesis in *D. carota*

Stimulation of somatic embryos in *D. carota* is a simple procedure compared to many species. Callus initiated and maintained on 2,4-D can be stimulated into progressing on the somatic embryogenesis pathway by the simple removal of the 2,4-D from the media. Callus on 2,4-D medium was sub-cultured onto M&S media without 2,4-D and the cultures placed in the light. After one week the callus is transferred onto fresh M&S medium, which aids the removal of any remaining 2,4-D that may have been clinging to the callus cells. After 2-8 weeks the different stages of embryo development should be visible. There are a number of different cell morphologies displayed during embryo development, although most stages are visible under a dissecting microscope, the latter stages such as the torpedo are visible to the naked eye (Zimmerman, 1993). Most embryos will progress to produce a plantlet, which appears identical to that of one grown from a seed.

2.5 Biochemical Analysis Methodology

Production of free radicals is intrinsic within aerobic metabolism and consequently they attack key macromolecules and systems within the cell (Frankel, 1987). Aerobic organisms have therefore developed a number of antioxidant systems and enzymes to combat the free radical damage and therefore oxidative stress (Hendry, *et. al.* 1996). Some of these enzymes have also been linked to different developmental stages within plant systems. The activity of these enzymes can be measured using different spectrophotometric techniques on protein and other extracts made from plant tissue.

2.5.1 Protein Extraction from Tissue Samples

Soluble proteins are extracted from tissue samples by a method employing liquid nitrogen (LN_2) and a homogenisation technique. Small samples of tissue are weighed into screw cap 1.5 mL Eppendorfs, frozen in LN_2 and stored until required. Mini-pestles (Sigma) are then used with a minor drill from Drill, France to homogenise the tissue while still frozen. Larger samples are taken fresh and frozen in a ceramic mortar using LN_2 and carefully mashed into a pulp with an appropriate pestle. Once a suitable pulp has been achieved, phosphate buffer (pH 7.0) was then added. For 200 mg of tissue, phosphate buffer (1mL) is added, but for the bulk extractions a higher ratio of tissue to buffer was employed for a more concentrated protein solution. The homogenate was allowed to incubate for 20 minutes on ice, with intermittent vortex mixing. The homogenate solutions were then centrifuged in a micro-centrifuge at 13000 rpm in a cold room at approximately 5 °C. The

supernatant was then transferred into fresh 1.5 mL Eppendorfs using an automatic pipette and stored in LN_2 until needed.

Phosphate buffer pH 7.0 was made up as follows:

A	KH_2PO_4	3.40g in 500 mL of dH_2O
B	K_2HPO_4	4.36g in 500 mL of dH_2O

A is added to B with stirring until the pH is equal to 7.0.

The following salts were then added:

1 mM $CaCl_2$	74.5 mg/L
1 mM KCl	37.5 mg/L
1 mM EDTA (Na_2)	186 mg/L

2.5.2 Protein Assay

Protein concentration was measured using the Coomassie blue dye method (Bradford, 1976).

The reagent was prepared as follows:

Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 mL of 95% ethanol, 100 mL phosphoric acid (85%) was added and the total volume made to 1 litre with dH_2O. The reagent was then decanted into foil wrapped bottles and stored at 5 °C until required.

The protein calibration curve was prepared from ampoules containing 2mg/mL bovine serum albumin (BSA) in the range of 50 μg to 2000 μg/mL (Pierce). The protein standard solution (100 μL) was transferred to a 10 mL test-tube, 5 mL of the Coomassie reagent was added and the solution was mixed using a vortex mixer. The samples are then allowed to stand for two

minutes at room temperature before measuring the absorbance at 595 nm using a spectrophotometer.

2.5.3 Catalase Assay

Catalase activity was determined using an UV spectrophotometric assay, monitoring the decrease of absorbance of hydrogen peroxide (H_2O_2) at 240 nm (Aebi, 1983). Assays are performed on protein extracts (as described in section 2.5.1) at 25 °C. The reaction buffer of 50 mM phosphate buffer (pH 7.0) was prepared as described in section 2.5.1 with the addition of 250 µL of H_2O_2 to 100 mL buffer immediately before use. The reaction buffer (950 µL) was added to a quartz cuvette and then 50 µl of the protein extract were added and the decrease in absorbance at 240 nm was measured every 10 seconds over the first 40 seconds. Samples were measured against a buffer blank. The standard curve was prepared from stock enzyme (Sigma) in the range of 30-300 U/mL and enzyme activity is expressed as specific activity on the basis of the soluble protein content.

2.5.4 Peroxidase Assay

Peroxidase activity in protein extracts was measured, spectrophotometrically, by monitoring the formation of oxidation products of guaiacol by guaiacol specific peroxidase at 470 nm (Castillo, *et. al.* 1984).

The phosphate buffer was made by mixing KH_2PO_4 (3.4g in 500 mL dH_2O) and K_2HPO_4 (4.36g in 500 mL dH_2O) until pH 6.1. The reagent buffer was

prepared immediately prior to use contains 16 mM guaiacol (179 µL per 100 ml buffer) and 2 mM hydrogen peroxide (20.4 µL per 100 ml buffer).

Reagent buffer (950 µL) was added to a plastic cuvette and 50 µL of protein extract added. The absorbance at 470 nm was measured every 15 s and the absorbance change per minute was calculated. A standard curve was prepared in the range of 0.1 – 1.2 U/mL and enzyme activity was expressed as A/min per mg protein.

2.5.5 Superoxide Dismutase Assay

Superoxide dismutase (SOD) activity was determined spectrophotometrically by monitoring the inhibition of the reduction of nitrotetrazolium blue (NBT) at 25 °C by superoxide radicals (produced by light mediated generation from riboflavin and methionine) (Beauchamp & Fridovich, 1971). Four stock solutions (A-D) were prepared in advance and A-C were protected from the light and stored at 4 °C (Table 2.5.5).

Table 2.5.5 SOD Stock Solutions

STOCK	COMPOUND	QUANTITY
A	Riboflavin	4 mg / 100 mL dH_2O
B	NBT	40.9 mg / 50 mL dH2O
C	Methionine	0.746g / 50 mL dH2O
D	"Wing buffer" pH 7.8	3.4 g KH_2PO_4 / 500 mL dH_2O
	Containing 1 x 10^{-4} M EDTA*	4.36 g K_2HPO_4 / 500 mL dH_2O

* 6.72 mg EDTA / 200 mL "Wing buffer"

A reaction mixture of the stock solutions was prepared in sufficient quantity to permit all assays to be performed from the same batch of buffer. The

proportions of the different stocks are as follows: 0.3 A, 0.1 B, 0.15 C and 1.0 D (as 30 mL, 10 mL, 15 mL and 100 mL respectively). Assay reagents (2.98 mL) were added to 20 µL of protein extract in a 10 mL test-tube. Control tubes and standards were distributed randomly within the rack and all tubes were exposed to the light of four 100 W bulbs for 20 min. The absorbance was measured at 570 nm. A standard curve was prepared from purified enzyme (Sigma) in the range of 3-30 U/mL.

2.5.6 Sulphydryl Groups Assays

Sulphydryl groups were determined for total and non-protein bound groups as described by Chevrier, *et. al.* (1988) using the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB).

Table 2.5.6 Stock solutions for SH group assays

STOCK	CONCENTRATION	COMPOUND	QUANTITY	SOLVENT / VOL
A	10 mM	DTNB	0.3963g	MeOH / 100 mL
B	1.0 M	Tris-HCl pH 8.2	36.33g	dH_2O / 300 mL
C	0.2 M	Tris-HCl pH 8.5	2.422g	dH_2O / 100 mL
D	5 % (w/v)	SDS	5g	100 mL dH_2O
E	5 % (w/v)	TCA	5g	100 mL dH_2O

SDS = Sodium dodecyl sulphate; TCA = Trichoroacetic acid

Total SH Groups:

Tissue sample was homogenised as in section 2.5.1 and then 2 mL of stock solution "B" was added and mixed. Solutions "D" (0.5 mL) and "A" (0.1 mL) were added to this suspension and the mixture was intermittently vortexed to ensure complete mixing during the 20 min incubation at room temperature. Ice cold methanol (3 mL) was added and the mixture centrifuged at 10000 rpm. The supernatant was decanted and the absorbance at 412 nm was measured, and compared to a standard curve prepared from glutathione (Sigma) at 10, 50, 100, 200, and 500 nmol levels.

Non-protein Bound SH groups

Tissue sample was homogenised as in 2.5.1 and 1.5 mL of stock "E" was added, the mixture was vortexed and then incubated on ice for 10 min, and finally centrifuged at 13000 rpm for 10 min.

Plastic cuvettes were prepared with 3 ml of stock "C" and 0.1 mL of stock "A". A sample (1 mL) of the supernatant from the extraction was added to the cuvette and was gently mixed and allowed to incubate at room temperature for 2 min. The absorbance was then measured at 412 nm in a spectrophotometer. A standard curve was prepared from glutathione (Sigma) at 10, 50, 100, 200, and 500 nmol levels.

2.5.7 Glutathione Reductase Assay

Glutathione Reductase (GR) activity was determined spectrophotometrically using the method described by Goldberg and Spooner (1983) by monitoring the decrease in absorbance due to the oxidation of nicotinamide-adenine dinucleotide diphosphate (NADPH) at 334 nm. Glutathione reductase catalyses the splitting of oxidised glutathione (GSSG) back to the reduced form GSH with the co-oxidation of NADPH to $NADP^+$. Stock solutions are detailed in table 2.5.7.

Cuvettes were prepared with 2.6 mL of stock "A", 0.1 mL of stock "B", and 0.1 mL of stock "C". Protein extract (0.1 mL) was added and allowed to stand for 5 min, and then 0.05 mL of stock "D" was added. The contents of the cuvette were mixed and the absorbance was measured at 334 nm every 15s to establish the absorbance change per min. A standard curve was prepared from purified enzyme (Sigma) in the range 3-30 U/mL.

Table 2.5.7 Stock Solutions for Glutathione Reductase Assay

STOCK	CONCENTRATION	COMPOUND	QUANTITY	SOLVENT / VOL.
A	0.12 mol/L	KH_2PO_4	16.33g	1L dH_2O*
B	15 mmol/L	EDTA-$Na_2H_2.H2O$	0.56g	100 mL dH_2O
C	65.3 mmol/L	GSSG	40mg	1 mL dH_2O**
D	9.6 mmol/L	β-NADPH	8mg	1 mL 1% $NaHCO_3$ (in dH_2O)**

* Phosphate buffer prepared by dissolving 16.33 g KH_2PO_4 in 800 ml of dH_2O, adjusting the pH to 7.2 with 1 M NaOH and then diluting to a final volume of 1L of dH_2O

** Prepared on day of assay

2.5.8 Assays for Glutathione - Oxidised (GSH) and Reduced (GSSG)

Glutathione in oxidised and reduced forms is measured using the DTNB-GSSG reductase recycling assay method of Anderson (1985).

SSA extraction method for GSH / GSSG

Samples were prepared in the same manner as described in 2.5.1 but 50mg samples were extracted into 500 µl of 5 % sulphosalicylic acid (SSA) by vortex mixing and were allowed to stand incubating on ice for 10 min. The samples were centrifuged at 13000 rpm and the supernatant transferred into fresh vials and stored in LN_2, until assayed.

Assays for the two forms were performed on the same extract with the addition of the GSH inhibitor, 2-vinylpyridine, for the GSSG assay.

GSH assay

Cuvettes (1mL) were prepared with 700µL of β-NADPH, 100µL of DNTB, and 180µl of H_2O and mixed (see table 2.5.8). A sample (10µL) was then added and the cuvette placed in the spectrophotometer. To start the assay, 10 µL of GSSG-Reductase was added and measurements, at 412 nm, were taken every 5s to establish the absorbance change per min. A standard curve was prepared in the range 0-100 µM GSH (Sigma).

Table 2.5.8 Stock solutions for GSH / GSSG assays

COMPOUND	CONCENTRATION	QUANTITY	SOLVENT / VOL
Stock buffer-NaH_2PO_4	0.143 M	22.3 g	1 L dH_2O*
DTNB	6 mM	0.238 g	In 100 mL Stock buffer
β-NADPH	0.248 mg / ml	24.8 mg	100 mL buffer
GSSG Reductase	138.4 U / ml	100 µl of stock	In 900 µL buffer

* Phosphate buffer prepared by dissolving 22.3 g NaH_2PO_4 in 800 mL of dH_2O, adjusting the pH to 7.5 with 1 M NaOH and then diluting to a final volume of 1 L of dH_2O.

GSSG Assay

The samples were pre-treated with 2-vinylpyridine (2-VP) to bind GSH and then triethanolamine to prevent oxidation. 2-VP (2µL) was added to the side of an 1.5 mL Eppendorf containing 100 µL of the SSA extract and then 6 µL of triethanolamine was added. The solution was then vigorously mixed using a vortex mixer and the samples were allowed to incubate for 60 min at room temperature.

The assay for GSSG was performed in the same manner as for GSH, with a standard curve in the range 0-100 µM GSSG (Sigma).

2.5.9 Glutathione S-Transferase Assay

Glutathione S-transferase (GST) was measured using the method of Simons and Vander Jagt (1977) with the artificial substrate 1-choro-2,4-dinitrobenzene and glutathione as co-enzyme (Table 2.5.9).

Plastic cuvettes were prepared with 3 mL of substrate in phosphate buffer and 0.5 mL of glutathione solution and the mixture was allowed to equilibrate for 2 min and then 10 μL of the sample was added. The absorbance at 340 nm was measured every 15 s to calculate the change of absorbance per min. A standard curve was prepared from stock GST enzyme (Sigma) in the range of 14-140 U / mL in phosphate buffer.

Table 2.5.9 Stock solutions for GST assay

STOCK	CONCENTRATION	QUANTITY	SOLVENT / VOL
K_2PO_4	0.2 M	2.72 g	100 mL dH_2O*
$NaHPO_4$	0.1 M	3.12 g	200 mL dH_2O*
Glutathione	17.5 mM	0.1076g	20 mL phosphate buffer
1-choro-2,4-dinitrobenzene	0.1 mM	0.0203 g	100 mL 100% EtOH**

*Phosphate buffer made up of 8 mL 0.2 M K_2PO_4, 28 mL 0.1 M $NaHPO_4$, and 64 mL of dH_2O and adjusted to pH 6.5 with 1 M NaOH, made fresh before use.
**Substrate solution diluted - 4 mL up to 100 mL in phosphate buffer for final working solution for assay, fresh before use.

2.6 LC-MS Determination of Hydroxynonenal and Malondialdehyde

Liquid Chromatography-Mass Spectrometry (LC-MS) has been developed to detect aldehydic products of lipid peroxidation. HNE and MDA are identified by derivatisation with dinitrophenyl hydrazine to form dinitrophenyl hydrazones that can be separated and identified by LC-MS (Deighton, *et. al.* 1997).

Preparation of DNPH derivatives of HNE and MDA

Callus tissues (200mg) were weighed into 1.5ml screw cap Eppendorfs and the samples were frozen in liquid nitrogen (to aid cell disruption). The tissue was then homogenised while partly frozen using micro-pestles and a minor drill from Drill, France. Methanol (1mL) was then added to the homogenised tissue and mixed with a vortex mixer. Following centrifugation (at 13000 rpm, 10 min), 2,4-dinitrophenylhydrazine (DNPH) (1 mL of 0.035% (w/v) in 1M HCl) was added to the supernatant in 10 mL bijou bottles. The extracts were then mixed and left standing in the dark at room temperature for 2 hours and then at 4 °C for a further hour as described by Esterbauer and Cheeseman (1990). The solvent was removed by rotary evaporation (≤ 35 °C) and the residue was re-dissolved in acetonitrile (250 μL) containing the standard, 4-fluorobenzaldehyde 2,4-dinitrophenylhydrazone (DNP-FBz 1 μM) as internal standard to assist in the quantification of the DNPH aldehyde derivatives.

Analysis of DNPH derivatives by LC-MS

Duplicate 5 μL injections were carried out using a Finnigan MAT SSQ710C (Hemel Hempstead, UK) single quadrupole instrument with APCI interface, with a Hypersil C_{18} column (250 x 2mm) from Phenomenex (Macclesfield, UK) using a gradient elution (40:60 to 25:75 water:acetonitrile over 5 minutes) was used throughout. Solvent flow was maintained at 0.25mL/min and UV detection was at 360nm. The mass spectrometer was configured for negative ion chemical ionisation (NICI) with coronal discharge at ~4.5kV.

Selected ion monitoring was used to monitor the ions with the m/z ratios: 234 (MDA 2,4-dinitrophenylhydrazone; DNP-MDA); 251 (butanal 2,4-dinitrophenyl-hydrazone; DNP-butanal); 303 (4-fluorobenzaldehyde 2,4-dinitrophenyl-hydrazone; DNP-FBz), 335 (hydroxynonenal 2,4-dinitrophenylhydrazone; DNP-HNE) (Esterbauer & Cheeseman, 1990, Deighton, *et. al.* 1997, Bremner, *et. al.* 1997). Standards were run approximately every 8 injections and contained 40μM DNP-MDA, 2μM DNP-HNE in acetonitrile.

High purity solvents and reagents were used throughout the analysis and methanol, acetonitrile and dichloromethane were of HPLC grade obtained from either SIGMA or Fisher. Potential contamination of reagents used in the study was under surveillance throughout the study using LC-MS but no solvent or aldehyde contamination was observed. The 2,4-dinitrophenylhydrazine (Aldrich with 30% water) was purified by recrystallisation from butanol (Esterbauer & Cheeseman, 1990). The aldehyde-DNPH standards were prepared using MDA derived from hydrolysed 1,1,3,3 tetramethoxypropane and HNE from hydrolysed 4-hydroxynonenal diethylacetal (Vogel, 1989). To circumvent degradation, aldehyde standards were hydrolysed just prior to the preparation of their DNPH derivatives.

2.7 ELISA – Detection of HNE-Protein adducts using antibodies

Enzyme linked immunosorbant assays (ELISAs) employ antibodies and antibodies coupled to enzymes to detect a specific epitope on a protein. This method employs an indirect detection by binding protein onto a PVC 96 well plate, then binding a specific mouse antibody onto the HNE molecules that had covalently attached themselves onto a protein molecule. These antibodies were raised in female Balb-c mice using 25 μg HNE-keyhole limpet haemocyanin and Freund's aduvant and were 1g4 subtype IgG1κ that were specific for the histidine adduct with less than 5% cross-reactivity with lysine and cysteine adduct (Waeg, *et. al.* 1996). These antibodies are then detected using an anti-mouse antibody linked to the enzyme horseradish peroxidase attached (HRP antibody). A chromogenic reagent is finally added that is cleaved by the peroxidase enzyme that releases a coloured product that can be measured spectrophotometrically in an automatic plate reader.

Preparation of solutions and plates

Two humid chambers were prepared 24 hours in advance of an enzyme linked immunosorbant assay (ELISA). The humid atmosphere was created using an airtight plastic box with a roll of tissue that had been wetted with distilled water and spread around the inside edge of the base and then tightly sealed. One box was incubated at 4 °C and the other at 37 °C.

The solutions used in the preparation and development of the ELISA are detailed in Table 2.7.1. Phosphate buffered saline (PBS) was prepared in advance by adding 95 mL of stock A to 405 mL of stock B and diluting to 1

litre with dH_2O to pH 7.4. NaCl (8.7g) was then added to give a concentration of 0.15 M. The antibody dilutions were only prepared immediately before use by dilution with 1% BSA in PBS. Sodium acetate / citrate buffer was made from 0.1 M sodium acetate solution which had been pH corrected to 6.0 by adding crystalline citric acid. The colour developing solution was prepared immediately before use by dissolving 10 mg of tetramethylbenzidine (TMB) in 1 mL of dimethylsulphoxide (DMSO) and diluting to 100 mL with sodium acetate / citrate buffer and then adding 72.8 µL of 30 % hydrogen peroxide solution.

Table 2.7.1 Stock solutions for ELISA

STOCK	CONCENTRATION	QUANTITY	SOLVENT / VOL
A ($NaH_2PO_4.2H_2O$)	0.02 M	0.312 g	100 mL dH_2O
B ($Na_2HPO_4.12H_2O$)	0.02 M	1.42 g	500 mL dH_2O
1 % BSA in PBS	1 % BSA	1 g BSA	100 mL PBS
Sodium acetate	0.1 M	8.204 g	1000 mL dH_2O
Sulphuric acid	2.5 M	66.5 mL	Diluted to 500mL with dH_2O

96 well plates (with U-shaped bottoms) were filled with tissue protein extracts in a general layout followed for all assays which incorporates bovine serum albumin conjugated to HNE (BSA-HNE) and blank wells and is detailed in Table 2.7.2. Protein extracts (prepared as described in section 2.5) were filled into wells marked 1-6. Blank wells contained phosphate buffered saline (PBS) to monitor background levels of antibody binding. BSA-HNE wells contain 0.2 mg/mL BSA-HNE (in PBS) which act as a standard and a positive control. The first row was filled with 125 µL of either PBS, protein extract or BSA-HNE and the rest of the wells were filled with 100 µL of PBS. Protein

extracts were diluted to ensure an even loading level of protein per well. A multichannel pipette was then used to transfer 25 µL from the top row into the second row and the contents flushed through the pipette tip several times to mix, to form a 1/5 dilution. The serial dilutions are continued down the plate by removing 25 µL from the second row, into the third row, mixed and in this manner until the seventh row. The dilutions are detailed in the first column of Table 2.7.2. The eighth row is left containing PBS. The plate was then sealed and incubated in a humid box overnight at 4 °C.

Table 2.7.2 Layout of samples for 96 well plates

	B	1	2	3	HNE	B	B	4	5	6	HNE	B
neat												
1/5												
1/25												
1/125												
1/625												
1/3125												
1/15625												
B	B	B	B	B	B	B	B	B	B	B	B	B

B= Blank (containing PBS); HNE= BSA-HNE; 1-6 are sample wells.

Development of ELISA

After the overnight incubation at 4°C, the contents of the wells were then removed and the wells washed four times with PBS and lightly blotted on tissue for a few seconds. The free sites on the plate were then blocked by adding 100 µL of 1 % bovine serum albumin (BSA) in PBS to each well using a multichannel pipette. The plate was then sealed and incubated in a humid box at room temperature for 30 minutes.

The wells were then washed four times with PBS and lightly blotted. 100 µL of a solution of the HNE antibody (a 1/200 dilution in 1% BSA in PBS) was

then added to each well and the plate sealed, placed in a humid chamber and incubated for 2 hours at 37°C.

The cells were then washed four times with PBS. The anti-mouse HRP antibody (100 µL of 1/500 dilution on 1% BSA in PBS) was then added to each well, the plate was sealed and then placed in a humid box and incubated at room temperature for 30 min (Scottish Antibody Preparation Unit, Carluke, Scotland). The wells were then washed four times in PBS and 100 µL freshly prepared TMB in sodium acetate / citrate buffer (with H_2O_2 added immediately before use) and the blue colour was allowed to develop for 10 min. The reaction was then stopped by adding 100 µL of 2.5 M sulphuric acid to each well, turning the positive wells yellow. The plate was then placed in the automatic plate reader and read against air at 450 nm. Positive wells were those absorbance value was deemed to be 3 times the value of the average blank.

2.8 Volatile Hydrocarbon Analysis by Gas Chromatography

Various volatile compounds such as ethane are evolved when plant and animal cultures undergo stress and damage. When enclosed within sealed vessels the headspace can be analysed by gas chromatography (GC). Hydroxyl free radicals ($HO^{\bullet}$) can be generated during stress and dimethyl sulphoxide (DMSO) can be used as a free radical scavenger to mop up this compound, and subsequently releases methane (Hebbel, *et. al.* 1982). Methane can be quantified by GC and used as a marker of the level of stress within the tissue.

Ethylene has proven a useful marker for stress and can also be measured in this way (Benson & Withers, 1987).

Vial Preparation

The volume of glass vials with an approximate volume of 15 mL with open lids sealed with silicon-teflon septa (Supelco, UK) was determined as 16.68 ± 0.06 mL. Vials were then sterilised by autoclaving (121 °C for 20 min) with the septa in place but lids loose within foil topped jars to prevent water intrusion. Immediately after sterilisation the vials were transferred to a sterile laminar flow bench, lids removed and the vials allowed to vent for 30 minutes, ensuring that any volatiles were removed.

Media for the time trial experiments were prepared in advance and was decanted into 100 mL bottles before sterilisation (see section 2.1 for cultures and section 2.2 for media recipes). Media (2 mL) was transferred into a sterilised vial with an automatic pipette and the vial tilted to an angle of 45° to create a large surface area of medium. The media was allowed to set and the vial was left open for a further 15 minutes to prevent condensation within the vial. All vials used for one trial were prepared from the same bottle of media.

Paper wicks of 1.5 cm x 1cm were cut from student grade filter paper (Whatman, UK), filled into vials which were then sterilised by autoclaving (121 °C for 20 min). Immediately before use, 1 % dimethyl sulphoxide was prepared with dH_2O and was filter sterilised with a 0.2 μm sterile filter into a

sterile bottle. Sterile water was also prepared in the same manner for the controls.

A paper wick is carefully placed on the medium surface and either 500 μL of 1% DMSO or water was carefully added to the wick. Callus or nodes were then weighed onto a sterile Perti dish within the laminar flow bench before being transferred to the vial. The vial was then sealed and the time noted. Vials containing *Glycine max* green callus or sweet potato nodes were then incubated at 25 °C for a 16-hour photoperiod until sampled. All other vials were incubated at 25 °C in the dark. Controls were empty vials, vials with only media (all types used), vials with media, a wick and DMSO and vials with media, a wick and water. The laboratory air was also injected into the GC to account for the background air methane.

Gas Chromatography of Headspace Samples

Headspace samples (1 mL) were drawn from the vials using a 5mL gas-tight syringe. In order to ensure complete mixing of the headspace, the syringe was flushed several times before removal. The vials were aerated in the laminar flow bench after sampling and the time noted when re-sealed and incubated.

Samples were injected into a Perkin Elmer 8310 Gas Chromatography (Perkin Elmer, UK) fitted with a 2m Propak Q column (Supelco, UK) and a hydrogen/air flame ionization detector. Volatiles were separated using a temperature programme, with an oven temperature of 35 °C and an isothermal

time of 6 minutes. The injector and detector temperatures were 200 °C and 225 °C respectively. Nitrogen was used as the carrier gas at a flow rate of 20 $mL.min^{-1}$. Gas chromatographic peaks were identified using standard gas mixtures containing methane and ethylene at known ppm and a computerised data handling facility.

2.9 Statistical Analysis

Data for statistical analysis were entered and manipulated in Excel (Version 5 and Version 97, Microsoft). Data were organised into columns containing factors and responses. Factors are variables such as the media used or morphogenic status of the cultivar that may have had an effect on the response. Response variables were those variable that were measured or evaluated data such as growth rate, embryogenic development, or protein content. Raw data was then imported into Minitab version 11.22. To ensure that the data had a normal distribution, a normality plot was performed and only data that formed a normal distribution within a 95 % confidence interval was accepted ($p>0.05$).

To test whether a factor had a significant effect on the response, Analysis of Variance (ANOVA) was performed on general linear models. This determined the significance of the main effects and interactions. Significant, highly significant, and very highly significant effects were designated by p values <0.05, <0.01, <0.001, respectively. Outliers from the model were removed if they a standard residual that was >2 or <-2, to remove their large influence on the model, and standard diagnostic tests were performed to

ensure that the normal distribution remained intact. Dunnett's test was used to compare controls and samples. Scheffe's analysis was used to determine the effect of the significant trends (Sokal & Rohlf, 1995).

Chapter 3 *Daucus carota* – A Model Species

Contents

Daucus carota, commonly known as carrot, has been used as a model species for the study of plant growth and development for many years. In fact, it has been successfully manipulated to produce embryos since the first reports of somatic embryogenesis in 1958 by Reinert and Stewart (see section 1.2.2). The onset of somatic embryogenesis in *D. carota* can be induced by the removal of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) (Zimmerman, 1993). The study of somatic embryogenesis is particularly interesting as it can be monitored on a single cell basis, independently from the parent plant. This observation is in direct contrast to the early events of the development of the zygotic regeneration system which is minute and surrounded by maternal and endosperm tissues and therefore very difficult to observe during early developmental stages (Zimmerman, 1993). However, even in the case of a model system such as *D. carota* there are still reports of embryogenic and non-embryogenic tissues and the loss of the embryogenic capacity with time (Bhojwani & Razdan, 1983, Krul, 1993). The comparison of non-embryogenic and embryogenic lines forms the basis of many studies of molecular and biochemical markers of morphogenesis within *in vitro* systems.

Somatic embryogenesis in *D. carota* has been used as a model system for a range of biochemical and molecular studies (Wakamatsu & Takahama, 1993, Sung & Okimoto, 1981, Choi, *et. al.* 1987, Borkird, *et. al.* 1986, Michalczuk, *et. al.* 1992). Some of these studies focus on the change in gene expression during the onset of somatic embryogenesis and show that there are a number of proteins which are preferentially expressed in the early stages of embryogenesis (Sung & Okimoto, 1981, Choi, *et. al.* 1987, Borkird, *et. al.*

1986). Some of these proteins have also been shown to be present in a number of other species including cassava, peach and maize (Choi, *et. al.* 1987). One of the proteins investigated has been shown to code for a lipid transfer protein that is expressed during embryogenesis (Sterk, *et. al.* 1991). The study of these proteins and other markers of morphogenesis within *D. carota* can give indications of the mechanisms of control of such developmental pathways and lead to methods of increasing the response of recalcitrant species and cultivars to *in vitro* manipulation.

In addition to fundamental biological studies, somatic embryogenesis has the potential to regenerate large numbers of young plants from a single parent without the need for going through the full life cycle of the plant, in certain species. One major application of this type of regeneration is in forestry. Tree species typically have very long life cycles that can last many decades, and can include long periods of dormancy. These long life cycles could be by-passed with the development of suitable somatic embryogenesis systems. A small number of tree species, such as *Prunus avium* (wild cherry), have been successfully regenerated in this manner (Reidoboym-Talleux & Grenier-De March, 1999). However, many important crop and domestic species are recalcitrant or respond poorly to such *in vitro* treatments and suitable regeneration systems on a large scale using somatic embryogenesis have not been established (see section 1.2.2). Progress has been made with species such as sweet potato through the efforts of organisations such as the International Potato Center (CIP). However, despite significant biotechnological impact in recent years, the horticultural and forestry

industries are still largely relying on traditional breeding and propagation methods.

In view of the problems associated with somatic embryogenesis, even in model systems, this chapter will explore the possibility that recalcitrance and lack of response to *in vitro* manipulation may be linked to oxidative stress and lipid peroxidation. The more information that can be gained *in vitro* on the responses of *D. carota* tissue under oxidative and *in vitro* stress, the greater the possibilities for the improvement of culture regimes. Since *D. carota* has been studied for so many years, a great deal of information has been documented on the physiological and biochemical properties of the species in culture and this makes it an ideal model plant for preliminary investigations.

The Free Radical Research group at the University of Abertay Dundee has used *D. carota* for many of its preliminary studies into oxidative stress and recalcitrance. In particular, *D. carota* callus cultures have been previously used as the basis for the development of new techniques for the analysis of lipid peroxidation products generated in *in vitro* culture (Bremner, *et. al.* 1997, Deighton, *et. al.* 1997, Benson, *et. al.* 1997, Adams, *et. al.* 1999, Robertson, *et. al.* 1995).

Aims and Objectives of the Study of *D. carota*

The main aims of the study of *D. carota* tissue are:

1 Establishment of the basic techniques and validation of the assays for use throughout the project.

2 To adapt techniques previously used within mammalian systems for use in plant systems, with the development of an enzyme linked immunosorbant assay for the detection of HNE-protein adducts from tissue extracts.

3 To establish profiles of the differences in the HNE and MDA content in non-embryogenic and embryogenic callus for a range of cultivars.

4 To study of the effects of the exogenous application of the lipid peroxidation products HNE and MDA on the growth and development of callus tissue and somatic embryo development.

3.1 Results - Characterisation of *D. carota* Cultivars

3.1.1 Seed Germination Studies

A number of studies were carried out to ascertain any differences in the germination rates of a range of commercially available *D. carota* cultivars. Seeds were sterilised in 10% Domestos (for full method see section 2.4) prior to germination under *in vitro* conditions. Seedlings generated were later used for the initiation of callus on 2,4-D media (see section 2.4 for details) using hypocotyl sections as explants.

Seeds from a range of cultivars were sterilised in batches of 12 seeds and the germination rates are detailed in Fig 3.1.1. There was a significant difference between the cultivars, as shown by a Chi-Squared test (χ^2 (4)=16.22, $p<0.01$), with cultivars New Red Intermediate and Early Nantes having germination rates of over 60%.

Figure 3.1.1 Germination Rates of *D. carota* Cultivars

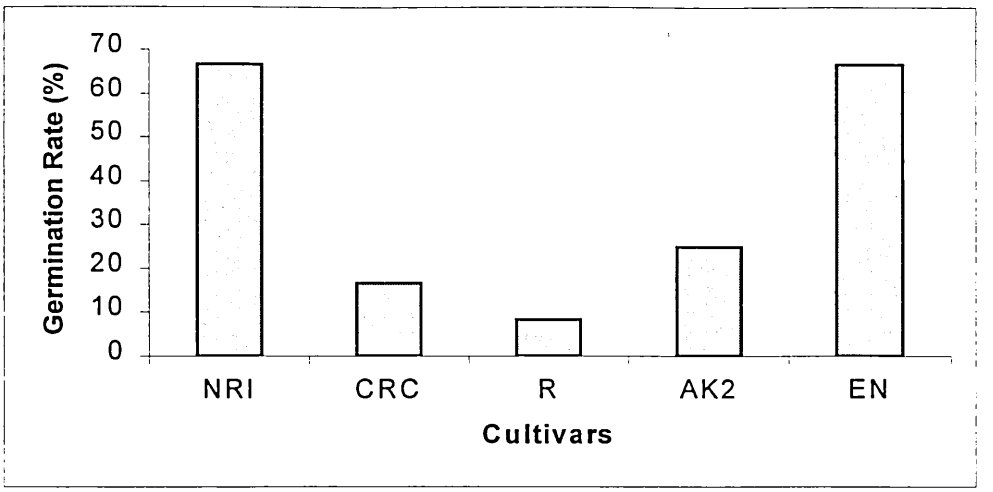


Fig 3.1.1: Germination of seeds of 5 cultivars of *Daucus carota*, New Red Intermediate (NRI), Chantenay Red Cored (CRC), Regulus (R), Autumn King 2 (AK2), and Early Nantes (EN) (12 seeds per cultivar), data points are expressed as a % of total sterilised seeds that germinated after 28 days.

3.1.2 Somatic Embryogenesis in Newly Established Callus

Newly established callus lines generated from *D. carota* seedlings were compared to profile the differences in embryogenic potential between lines and cultivars after the removal of the hormone 2,4-dichlorophenoxyacteic acid. Seedlings of Early Scarlet Horn (for method see section 2.4) were germinated and hypocotyl sections were placed onto 2,4-D media in order to initiate the production of callus. Six lines of callus were successfully established and maintained in a two-week sub-culture cycle; these six lines were then sub-cultured onto MS media that was hormone free in order to initiate somatic embryogenesis. The different responses are detailed in Figure 3.1.2.1 ($\chi^2(5)=31.10$, $p<0.001$).

Figure 3.1.2.1 Somatic Embryogenesis in Six Lines of the *D. carota* Cultivar Early Scarlet Horn

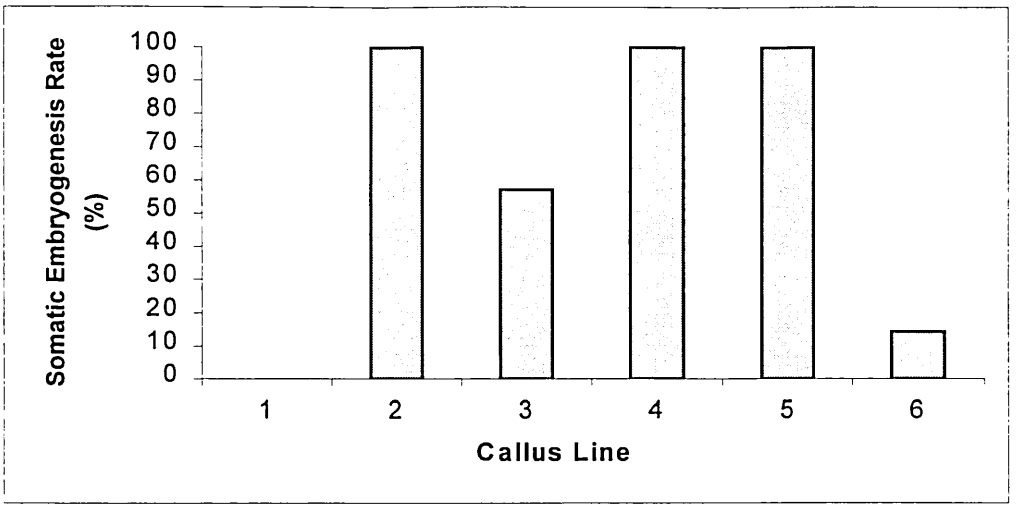


Figure 3.1.2.1: Somatic embryogenesis rates of 6 lines of Early Scarlet Horn. Data points are expressed as a percentage of the total number of colonies (average 7 colonies per plate) that have developed somatic embryos to the plantlet stage after 28 days.

Further callus cultures were transferred onto MS medium for initiation of somatic embryogenesis. The responses are detailed in Figure 3.1.2.2 and show the variation in somatic embryogenesis rates of different cultivars, although there were no significant differences between the lines (χ^2 (4)=2.72, p>0.05).

Figure 3.1.2.2 Somatic Embryogenesis Rates of Lines of *D. carota*

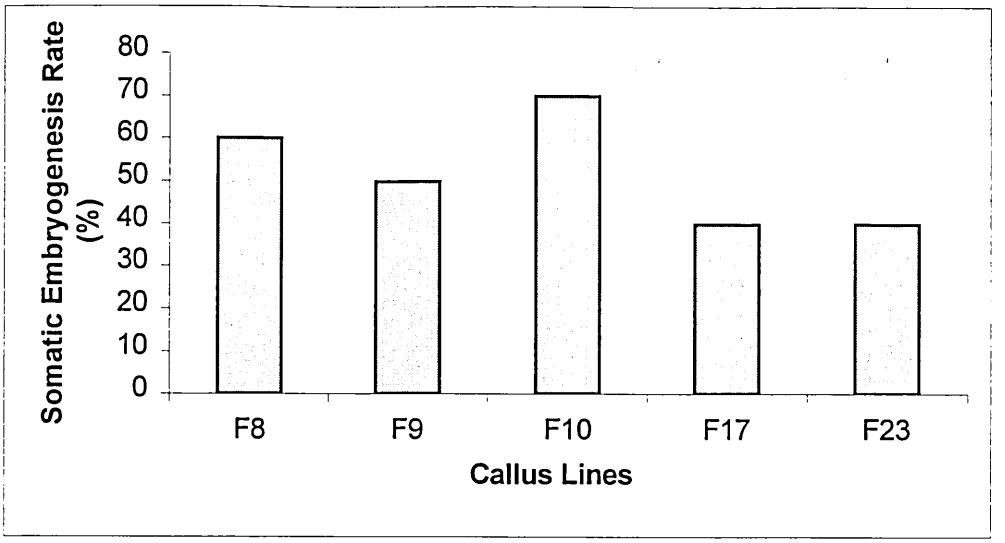


Figure 3.1.2.2: Somatic embryogenesis rates of new established callus lines F8 (Early Nantes), F9 (Gold King), F10 (Gold King), F17 (Chantenay Red Cored), F23 (Autumn King 2). Data points are expressed as a percentage of the total number of colonies (average 10 colonies per plate) that have developed somatic embryos to the plantlet stage after 28 days.

3.1.3 Somatic Embryogenesis Initiation from Established Cultures

Established callus lines from a range of cultivars were profiled for the ability of the callus to respond to the removal of 2,4-D which thus initiated the somatic embryogenesis developmental pathway. Three lines of callus of the cultivar Autumn King 2, were transferred but only one of the three lines produced embryos. The other two failed to respond and were therefore classed non-embryogenic. The embryos produced developed to the plantlet stage. Two further lines of Autumn King 2 were sub-cultured onto MS media for the initiation of somatic embryogenesis; the first line (6) produced no embryos and the responses of the second line (B50) are detailed in Figure 3.1.3.1 ($\chi^2(4)=5.83$, $p>0.05$). These two lines were later used for HNE and MDA studies.

Figure 3.1.3.1 Somatic Embryogenesis Rates in five plates of the *D. carota* cultivar Autumn King 2 line B50

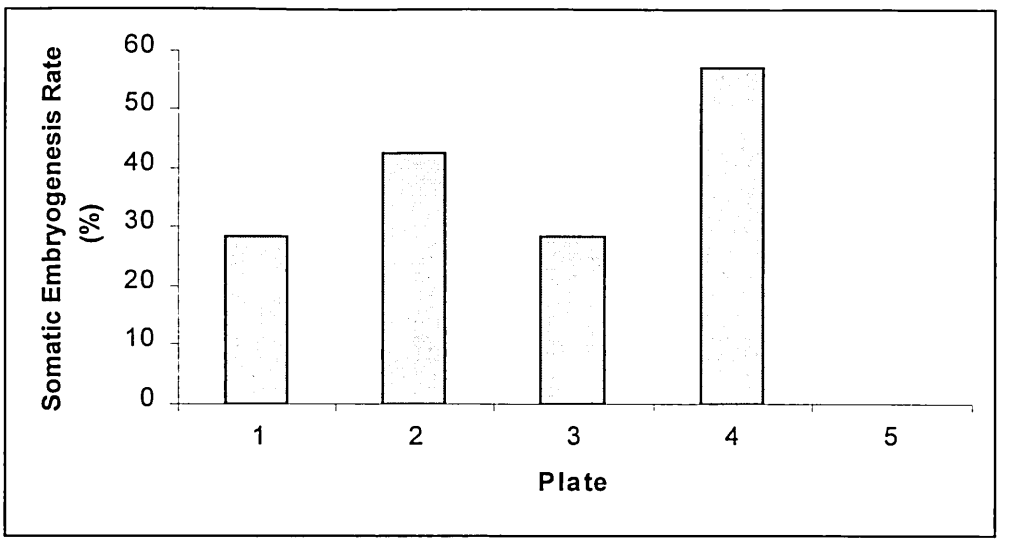


Figure 3.1.3.1: Somatic embryogenesis rates for 5 plates of the line B50 from the cultivar Autumn King 2. Data points are expressed as a percentage of the total number of colonies (average 7 colonies per plate) which developed somatic embryos to the plantlet stage after four weeks.

Another group of callus lines stimulated to produce embryos were from the cultivar New Red Intermediate and the results are detailed in Figure 3.1.3.2 ($\chi^2(4)=20.03$, $p<0.001$). Two of the lines failed to produce any embryos, and therefore shows that there is variability in the level of response of different callus lines.

Figure 3.1.3.2 Somatic Embryogenesis Rates of Callus Lines of the *D. carota* cultivar New Red Intermediate

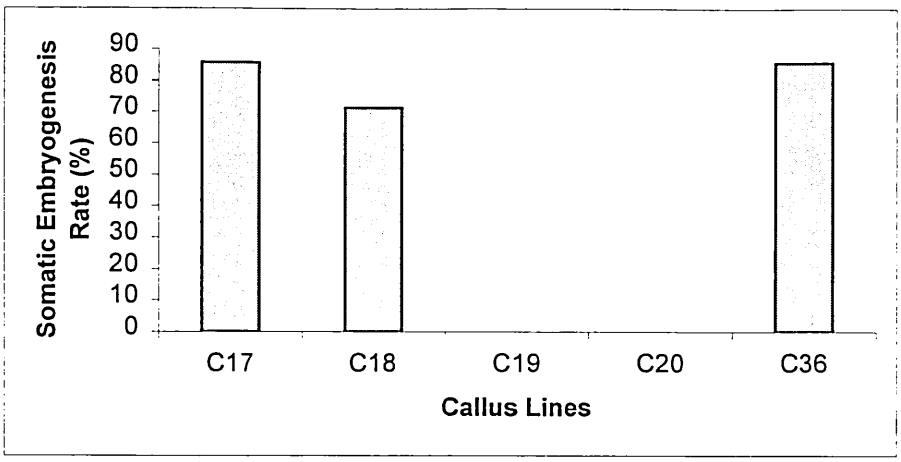


Figure 3.1.3.2: Somatic embryogenesis rates of 5 lines of New Red Intermediate. Data points are expressed as a percentage of the total number of colonies (average 7 colonies per plate) which have developed somatic embryos to the plantlet stage after four weeks.

Other lines that were profiled for their ability to produce embryos were two lines of Oranza which both failed to produce embryos, one line of Chantenay Red Cored 57% of which produced embryos, one line of Chantenay Royal that produced 71% and one line of Autumn King that produced 100%. Lines selected for further study were mainly from the cultivars Autumn King 2, Chantenay Red Cored, Early Nantes, and New Red Intermediate.

3.2 Preliminary Biochemical Studies – Protein

Early studies of the protein content of *D. carota* tissues were used to establish routine assay and sampling methods. Soluble protein extracts contain a number of active enzymes and can be used for the analysis of activity of a number of antioxidants. Antioxidant enzymes such as catalase, peroxidase, and superoxide dismutase can be measured with spectrophotometric assays

using soluble protein extracts. These enzymes are important in the control of reactive oxygen species that are generated in metabolically active cells and particularly under conditions of oxidative stress (see sections 1.3.4 and 1.3.5). The levels of activity of these and other related species such as glutathione can give important indications as to the level of oxidative stress experienced by the cells under *in vitro* conditions.

D. carota tissues were used for the establishment of the biochemical extraction techniques and analysis of protein and enzyme activity using spectrophotometric analysis. The protein content of tissue may vary depending on the embryogenic status of cells and a range of cultivars were profiled for protein content from non-embryogenic and embryogenic callus lines. Soluble protein extracts from *D. carota* cultivars were also utilised for the development of the enzyme linked immunosorbant assay for the detection of HNE-protein adducts (see section 3.5).

3.2.1 Comparing Embryogenic and Non-embryogenic *D. carota* lines

Protein was extracted from a range of established cultivars of *D. carota* (see section 2.3 for sampling techniques and section 2.5.1 for protein extraction method). Protein assays were performed on the soluble protein extracts (for method see section 2.5.2) based on the assay of Lowry modified by Bradford (1976). Pairs of embryogenic and non-embryogenic callus lines of a range of cultivars were used to compare routine small-scale extractions with large-scale extractions that used several grams of tissue. Results of the small-scale extractions are shown in Figure 3.2.1.1 and one-way analysis of variance

shows (see section 2.9) that there is a significant difference in protein content between the different lines ($F_{(7,16)}=13.17$ $p<0.001$). There is also a significant difference in the protein content between the embryogenic lines and the non-embryogenic lines, with the former having a higher value ($F_{(1,22)}=31.65$ $p<0.001$).

The extracts, prepared using large scale extraction using approximately one gram of tissue was macerated using liquid nitrogen and a ceramic mortar and pestle, and final centrifugation and sample storage were as previously described (section 2.4), with the results shown in Figure 3.2.1.2. There was a significant difference in protein content between the callus lines ($F_{(7,16)}=78.20$ $p<0.001$) and there was also a significant difference between the embryogenic and non-embryogenic lines ($F_{(1,22)}=32.74$ $p<0.001$).

Figure 3.2.1.1 Protein Content of Embryogenic and Non-Embryogenic Callus Lines of *D. carota* Prepared using Small-Scale Extractions

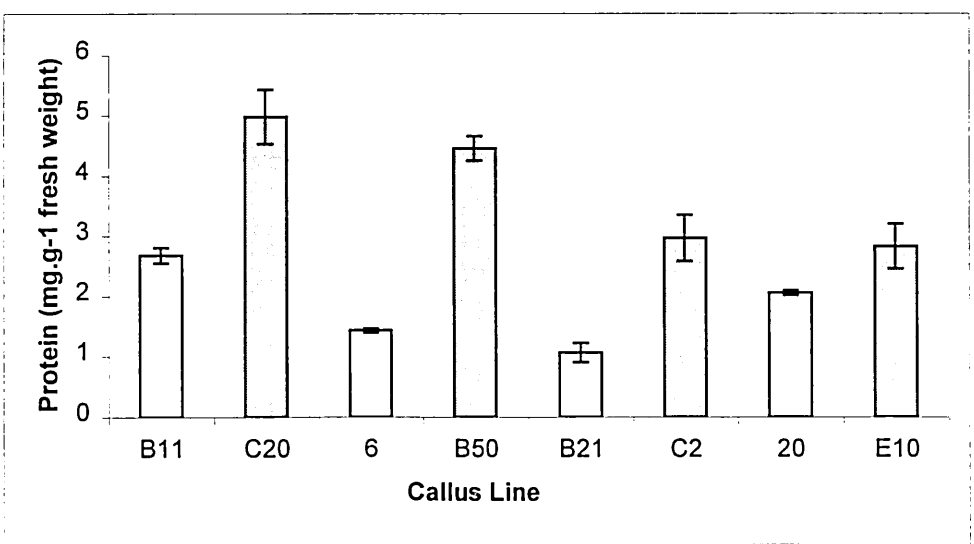


Figure 3.2.1.1: Protein concentrations in embryogenic callus lines (C20, B50, F9, E10, grey bars) and non-embryogenic (B11, 6, B21, 20, white bars) expressed as $mg.^{-1}$gram fresh weight of tissue on 2,4-D media. Cultivars were New Red Intermediate (B11 and C20), Autumn King 2 (6 and B50), Gold King (B21 and C2) and Chantenay Red Cored (20 and E10). Data points are means of 3 replicates and the error bars are expressed as standard deviations.

Figure 3.2.1.2 Protein Content of Embryogenic and Non-Embryogenic Callus Lines of *D. carota* Prepared from Bulk Extracts

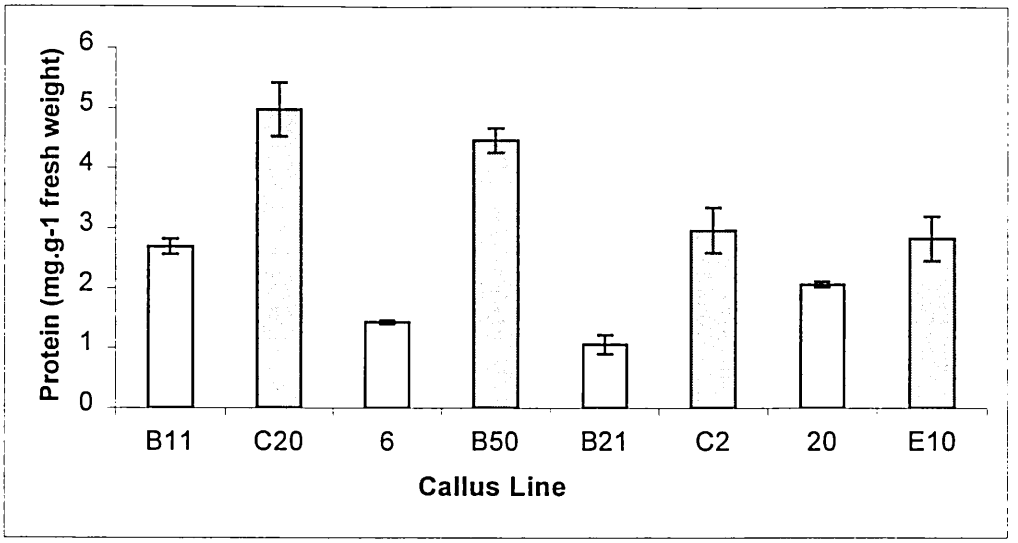


Figure 3.2.1.2: Protein concentrations in embryogenic callus lines (C20, B50, C2 E10, grey bars) and non-embryogenic (B11, 6, B21, 20, white bars) expressed as mg.$^{-1}$gram fresh weight of tissue on 2,4-D media. Cultivars were New Red Intermediate (B11 and C20), Autumn King 2 (6 and B50), Gold King (B21 and C2) and Chantenay Red Cored (20 and E10). Data points are means of 3 replicate extractions from one sample of tissue and the error bars are expressed as standard deviations.

3.3 Initial Studies–Extraction of Hydroxynonenal and Malondialdehyde

Since, the lipid peroxidation products HNE and MDA have been implicated in oxidative stress, profiling the concentration of these two products will give an indication of the oxidative status of the cells *in vitro*. Initial studies of the MDA and HNE content of a range of *D. carota* callus cultures were prepared using the extraction and analysis technique that is detailed in section 2.6. The MDA content of four lines of *D. carota* was determined and the results are represented in Figure 3.3.1 and show that E10 had significantly lower MDA concentration than the other three lines ($F(3,8)=6.88$ $p<0.05$). The HNE content of the same samples is represented in Figure 3.3.2 and shows that there was a significant difference between the lines ($F(3,7)=30.89$ $p<0.001$).

Figure 3.3.1 MDA Content of Embryogenic and Non-embryogenic Lines of *D. carota*

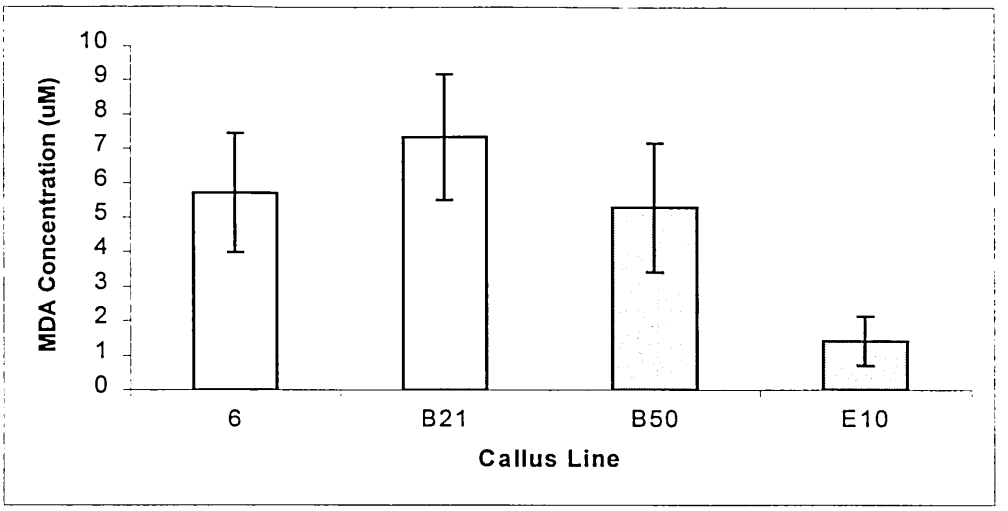


Figure 3.3.1: MDA content of embryogenic lines (grey bars): B50 (Autumn King 2) and E10 (Chantenay Red Cored) and non-embryogenic lines (white bars) 6 (Autumn King 2) and B21 (Chantenay Red Cored). Data points are expressed as means of three replicate samples that were run in duplicate, with errors expressed as standard deviations.

Figure 3.3.2 HNE Content of Embryogenic and Non-embryogenic Lines of *D. carota*

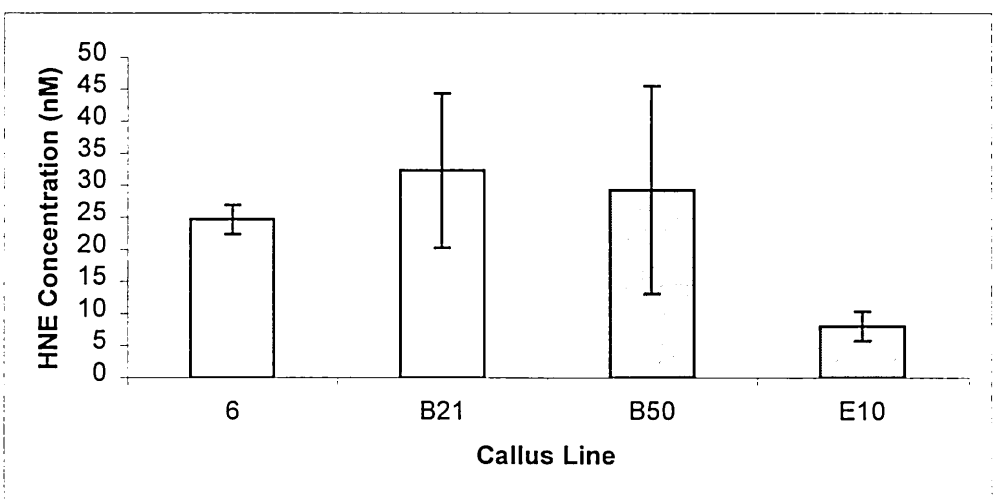


Figure 3.3.2: HNE content of embryogenic lines (grey bars): B50 (Autumn King 2) and E10 (Chantenay Red Cored) and non-embryogenic lines (white bars) 6 (Autumn King 2) and B21 (Chantenay Red Cored). Data points are expressed as means of three replicate samples that were run in duplicate, with errors expressed as standard deviations.

Three lines of Chantenay Red Cored of varying embryogenic capability were sub-cultured on MS media for the initiation of somatic embryogenesis. Callus

samples were taken immediately upon sub-culture (day 0) and after 24 and 48 hours (day 1 and day 2) and stored in liquid nitrogen. The HNE and MDA were extracted from the frozen samples and the concentration determined. There was no significant difference between the days but there was a significant difference between the lines for both MDA ($F(2,24)=14.80$, $p<0.05$) and HNE ($F(2,24)=8.39$ $p<0.01$).

The same set of Chantenay Red Cored lines were used in the next trial with the addition of another callus line (line 20), that had never shown any embryogenic potential. The MDA content of the lines is shown in Figure 3.3.3 and there was no significant difference between the different lines ($F(3,8)=3.36$ $p>0.05$). The HNE content of the lines is shown in Figure 3.3.4 and, in this case, there was a significant difference between the lines ($F(3,7)=11.32$ $p<0.01$).

Figure 3.3.3 Malondialdehyde Content of four lines of *D. carota* with different embryogenic capacities (First Set)

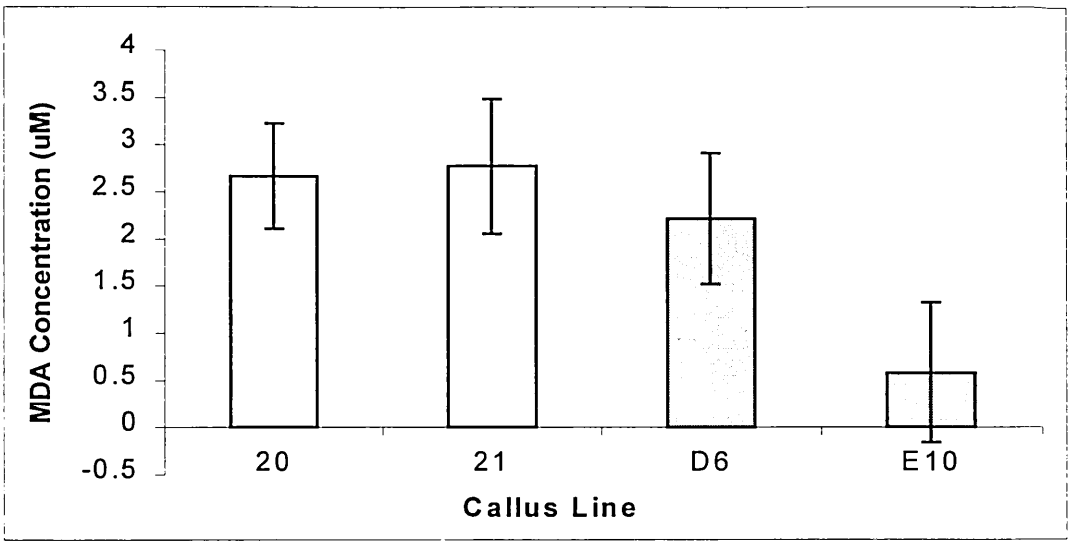


Figure 3.3.3: MDA content of Chantenay Red Cored, non-embryogenic lines 20 and 21 (white bars), poorly embryogenic line D6 and embryogenic line E10 (grey bars) on 2,4-D media. Data points are expressed as means of three replicate samples that were run in duplicate, with errors expressed as standard deviations.

Figure 3.3.4 Hydroxynonenal Content of four lines of *D. carota* with different embryogenic capacities (First Set)

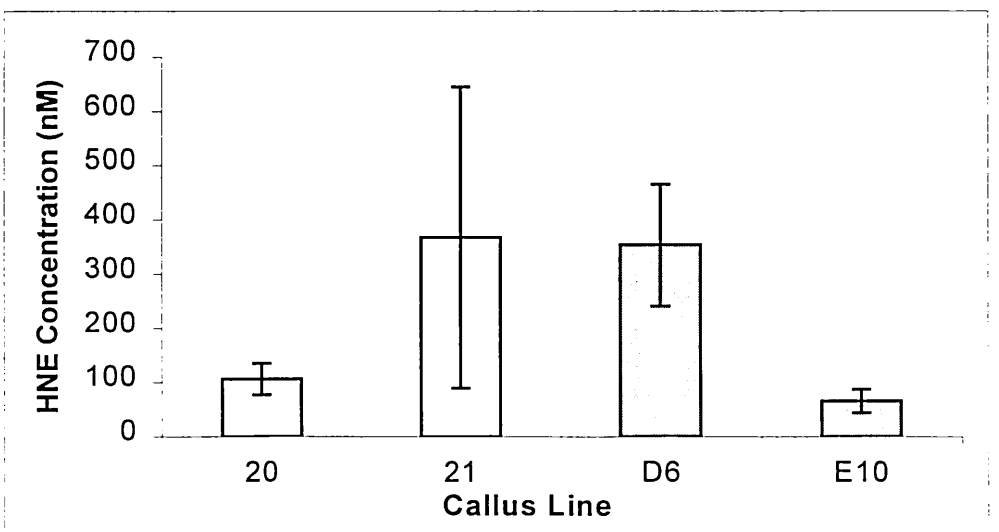


Figure 3.3.4: HNE content of Chantenay Red Cored, non-embryogenic lines 20 and 21 (white bars), poorly embryogenic line D6 and embryogenic line E10 (grey bars) on 2,4-D media. Data points are expressed as means of three replicate samples that were run in duplicate, with errors expressed as standard deviations

A replicate of the previous trial was performed from the Chantenay Red Cored group. The MDA content is detailed in Figure 3.3.5, and shows a significant difference between the lines, with E10 having a much lower value than the other lines ($F(3,7)=24.89$ $p<0.000$). The HNE content is detailed in Figure 3.3.6 and shows a significance difference between the lines, with D6 having a significantly higher HNE content than the other lines ($F(3,7)=13.30$ $p<0.01$). Although the MDA and HNE content of the blanks was higher that some of the samples giving negative results.

Figure 3.3.5 Malondialdehyde Content of four lines of *D. carota* with different embryogenic capacities (Second Set)

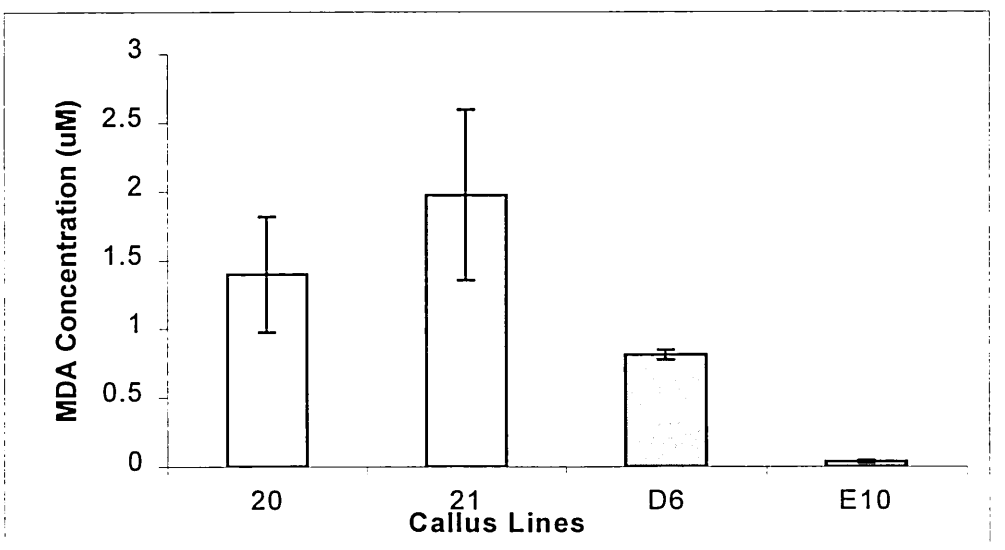


Figure 3.3.5 MDA content of Chantenay Red Cored, non-embryogenic lines 20 and 21 (white bars), poorly embryogenic line D6 and embryogenic line E10 (grey bars) on 2,4-D media. Data points are expressed as means of three replicate samples that were run in duplicate, with errors expressed as standard deviations.

Figure 3.3.6 Hydroxynonenal Content of four lines of *D. carota* with different embryogenic capacities (Second Set)

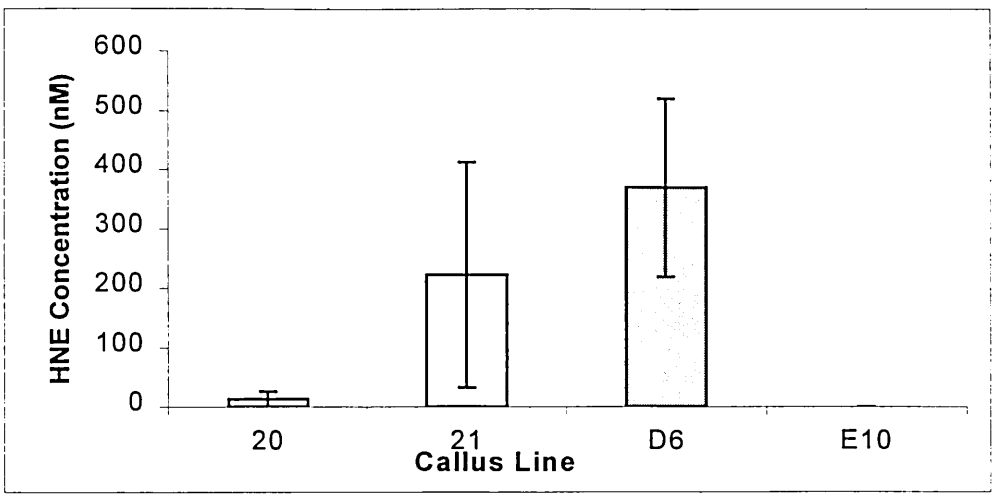


Figure 3.3.6: HNE content of Chantenay Red Cored, non-embryogenic line 20, non-embryogenic line 21, poorly embryogenic line D6 and embryogenic line E10 on 2,4-D media. Data points are expressed as means of three replicate samples that were run in duplicate, with errors expressed as standard deviations.

The previous samples of the Chantenay Red Cored lines of *D. carota* were re-analysed, but the order of analysis was non-random as had been for previous trials. Non-random order was used to monitor any drift in analyses through the long run of injections. The MDA results are detailed in Figure 3.3.7 and there is a significant difference between the lines, with E10 having a much lower MDA content than the other lines ($F(3,7)=26.52$ $p<0.001$). The HNE content is detailed in Figure 3.3.8 and it can be seen that the levels in lines 20 and E10 are much lower than in the other two lines ($F(3,7)=56.94$ $p<0.001$). The results were very similar to those represented in Figures 3.3.5 and 3.3.6, showing the consistency of analysis of the samples injected in a different order on a different day, and that there was no difference between random and non-random order of analysis.

Figure 3.3.7 Malondialdehyde Content of four lines of *D. carota* with different embryogenic capacities (Non-random Analysis)

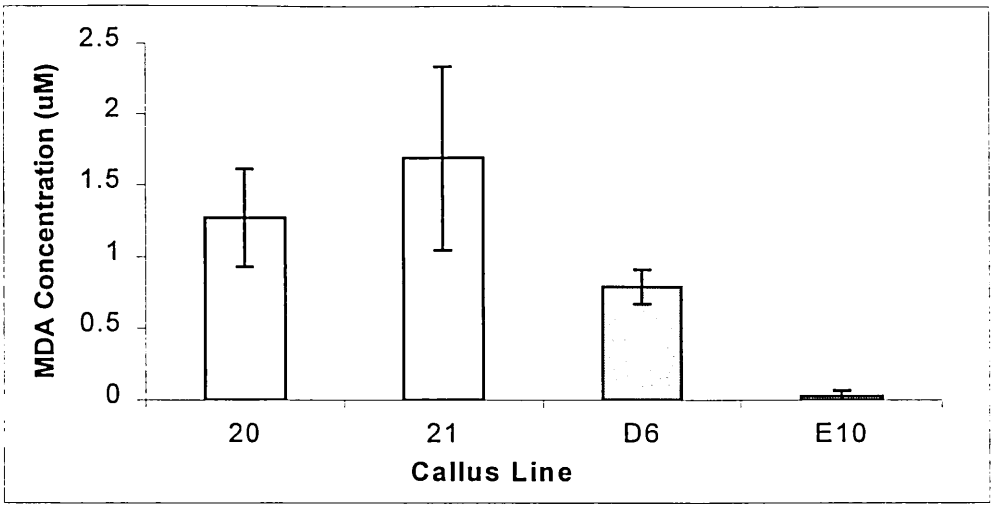


Figure 3.3.7: MDA content of lines of Chantenay Red Cored, non-embryogenic 20, non-embryogenic line 21 (white bars), poorly embryogenic line D6 and embryogenic line E10 (grey bars) on 2,4-D media. Data points are expressed as means of three replicate samples that were run in duplicate, with errors expressed as standard deviations.

Figure 3.3.8 Hydroxynonenal Content of four lines of *D. carota* with different embryogenic capacities (Non-random Analysis)

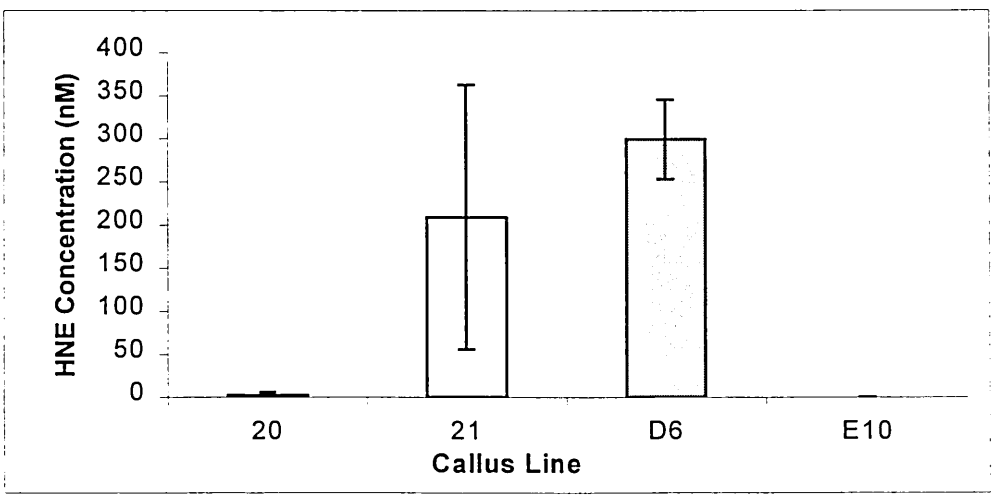


Figure 3.3.8: HNE content of lines of Chantenay Red Cored, non-embryogenic lines 20 and 21 (white bars), poorly embryogenic line D6 and embryogenic line E10 (grey bars) on 2,4-D media. Data points are expressed as means of three replicate samples that were run in duplicate, with errors expressed as standard deviations.

3.4 Effects of the Exogenous Application of HNE and MDA on the Growth and Development of Rapidly Differentiating *D. carota* Callus

Lipid peroxidation has been associated with many different diseases and HNE and MDA in particular have been linked with conditions such as arthritis, cancer, atherosclerosis (heart disease), inflammation and liver injury (Esterbauer & Ramos, 1995, Zollner *et. al.* 1991, Spiteller, 1996). HNE and MDA have been shown to have a wide range of effects on different tissue and cells types. Although results vary greatly between experimental conditions, many of these effects are caused by concentrations as low as 1 nM, and HNE in particular has been shown to be exceptionally cytotoxic at very low concentrations. In plants, hormones have stimulatory or inhibitory effects on tissues and, in particular, jasmonic acid, now considered a plant hormone has an active range up to 50 μM (Creelman, *et. al.* 1992). The purpose of this study was to try and evaluate the effects on callus growth and development of adding HNE and MDA to the culture media. Concentrations were chosen in the active range of lipid peroxidation and lipoxygenase derived products at the lower end of the scale, with the highest concentration of 420 nM. The study incorporates a number of other factors and includes two callus lines of different embryogenic potentials and two different types of media.

3.4.1 Experimental Design

The experiment was designed to compare a number of different factors:

1. The two media: hormone free (MS) used for inducing embryogenesis and hormone media containing 2,4-dichlorophenoxyacetic acid (2,4-D), for maintaining callus.

2. Two lines of *D. carota* that have different embryogenic capabilities. Two lines of Autumn King 2 were chosen, line 6 (non-embryogenic) and line B50 (embryogenic).
3. The effect of two different lipid peroxidation products HNE and MDA, at four different concentrations.

Stocks of both cultures were maintained on 2,4-D media until the samples were taken for the initial experimental set up. The experimental design is shown in Table 3.4.1.1 and incorporates five levels of concentration of both HNE and MDA, including a control. The numbers give each plate its unique identification number and each treatment has three identical replicates. All media was prepared as detailed in section 2.2 and autoclaved in 120 ml aliquots and the aldehydes were added after filter sterilisation immediately before dispensing, the aliquot being poured into 6 Petri dishes and three of the plates used for each of the two callus lines. Controls were prepared at the same time without the addition of any aldehydes. During the course of the experiment the callus was transferred onto new media approximately every two weeks. At this stage the weight of the callus could be evaluated.

Table 3.4.1.1 Summary of Experiment Layout

	MEDIA MS		MEDIA 2,4-D	
Concentration (n M)	Line 6	Line B50	Line 6	Line B50
HNE 0.42	1-3	25-27	49-51	73-75
HNE 4.2	4-6	28-30	52-54	76-78
HNE 42.0	7-9	31-33	55-57	79-81
HNE 420.0	10-12	34-36	58-60	82-84
MDA 0.42	13-15	37-39	61-63	85-87
MDA 4.2	16-18	40-42	64-66	88-90
MDA 42.0	19-21	43-45	67-69	91-93
MDA 420.0	22-24	46-48	70-72	94-96
Control	MS A-C	MS D-F	2,4D A-C	2,4-D D-F

Table 3.4.1.1: Layout of experimental plates for two media types (MS – hormone free and 2,4-D), for two lines of Autumn King 2 (embryogenic line B50, and non-embryogenic line 6) on media containing 4 concentration levels of HNE and MDA (0.42 – 420 nM), with controls. Each plate keeps same identification number throughout the experiment.

At the third transfer it was decided to split the current callus into two, the first half was continued on the treatment media and the second half being transferred onto fresh media without the added aldehydes. This would give an indication as to the effect post-treatment and the recovery potential of the stressed callus and to monitor which treatments were fatal without any invasive assays. At each transfer the callus was evaluated for growth and the weight on each plate recorded. The schedule of transfers is detailed in Table 3.4.1.2.

Table 3.4.1.2 - Transfer days

	TREATMENT	POST TREATMENT
Set up	Day 0	-
Transfer 1	Day 19	-
Transfer 2 & post-treat set up	Day 33	Day 0
Transfer 3	Day 52	Day 19
Final weight and samples	Day 66	Day 33

At the end of the experiment samples were taken from each plate and one of each set of replicates was randomly allocated for protein extraction, for analysis of HNE and MDA using LC-MS and for analysis of dry weight. The major results of this experiment were reported at an international conference in 1998 and are fully discussed in an article published in 1999 (Adams, *et. al.* 1999) and includes images of the callus under treatment.

3.4.2 Growth Profiles after Exogenous Treatment with HNE and MDA

The effects of HNE and MDA on the rate growth of the *D. carota* callus tissue are shown in figures 3.4.2.1 and 3.4.2.2 respectively and show the significant difference between the controls and the callus under treatment, ($F(4,101)=26.26$, $p<0.001$). Similar results were noted for both HNE and MDA, and demonstrated the fact that the tissue was still dividing but at a much reduced rate compared to the controls.

Figure 3.4.2.1 Effect of applied HNE on the growth rate after 19 days

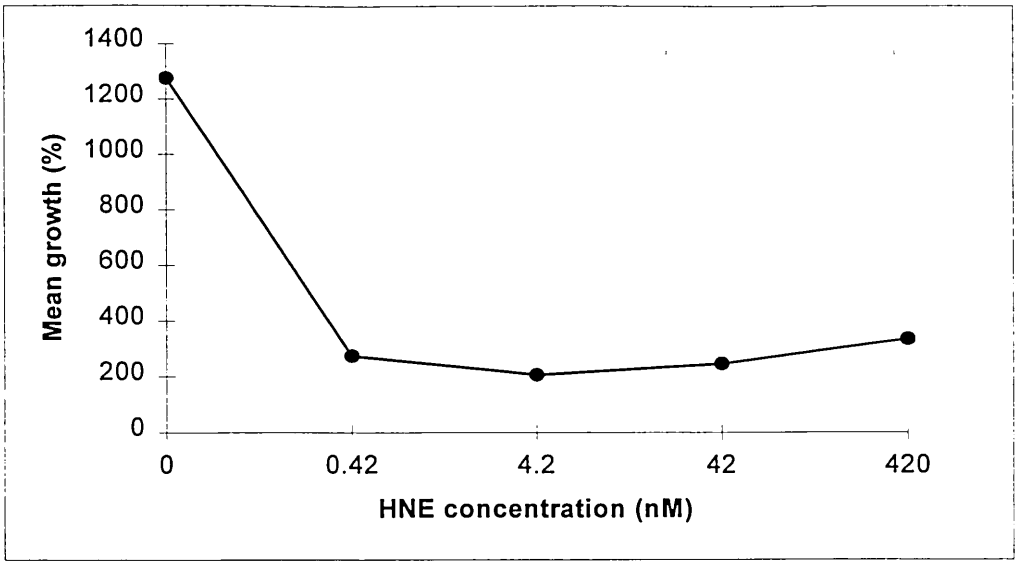


Figure 3.4.2.1: Effect of exogenously applied HNE on the growth rate after 19 days of treatment. Growth rate is expressed as a percentage of the initial callus fresh weight, before treatment application. Means comprised an average of the total replicated treatments (n=3) for both the embryogenic and non-embryogenic callus lines cultured on MS and 2,4-D media.

Figure 3.4.2.2 Effect of applied MDA on the growth rate after 19 days

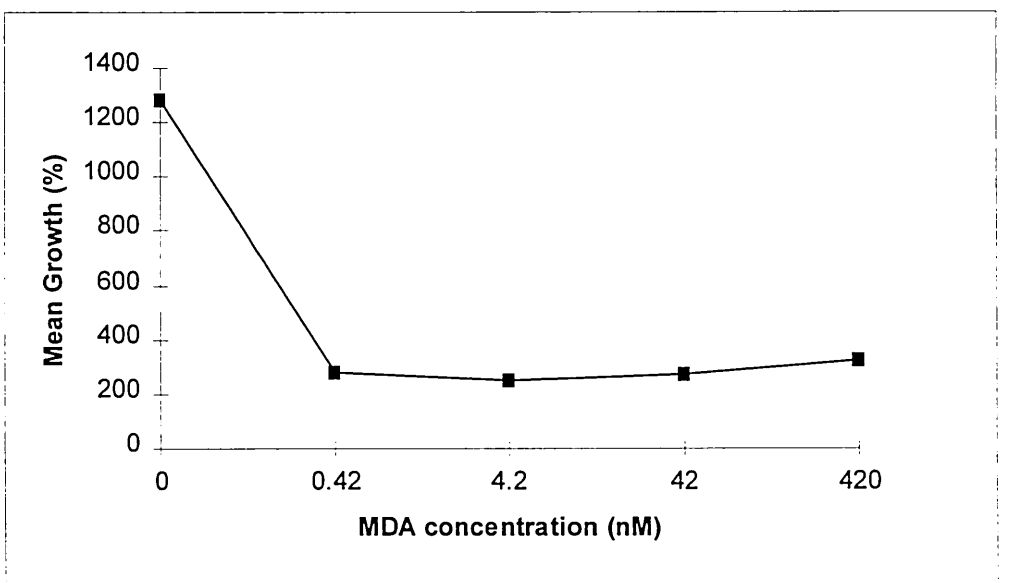


Figure 3.4.2.2: Effect of exogenously applied MDA on the growth rate after 19 days of treatment. Growth rate is expressed as a percentage of the initial callus fresh weight, before treatment application. Means comprised an average of the total replicated treatments (n=3) for both the embryogenic and non-embryogenic callus lines cultured on MS and 2,4-D media.

In order to evaluate the effects of the different factors within the aldehyde and post-aldehyde treatments the control data were removed from the data sets.

After removal of the highly significant control results, interaction plots of the overall effects of line and media are shown in Figure 3.4.2.3 for HNE treatments and post-treatments and in Figure 3.4.2.4 for MDA treatments and post-treatments. The interaction between line and media is significant for HNE treatments ($F(1,43)=9.79$ $p<0.01$), and HNE post-treatments ($F(1,44)=28.30$ $p<0.001$), MDA treatments ($F(1,44)= 37.13$ $p<0.001$) and MDA post-treatments ($F(1,44)= 58.65$ $p<0.001$).

Figure 3.4.2.3 Interaction plot for HNE Treatments and Post-treatments

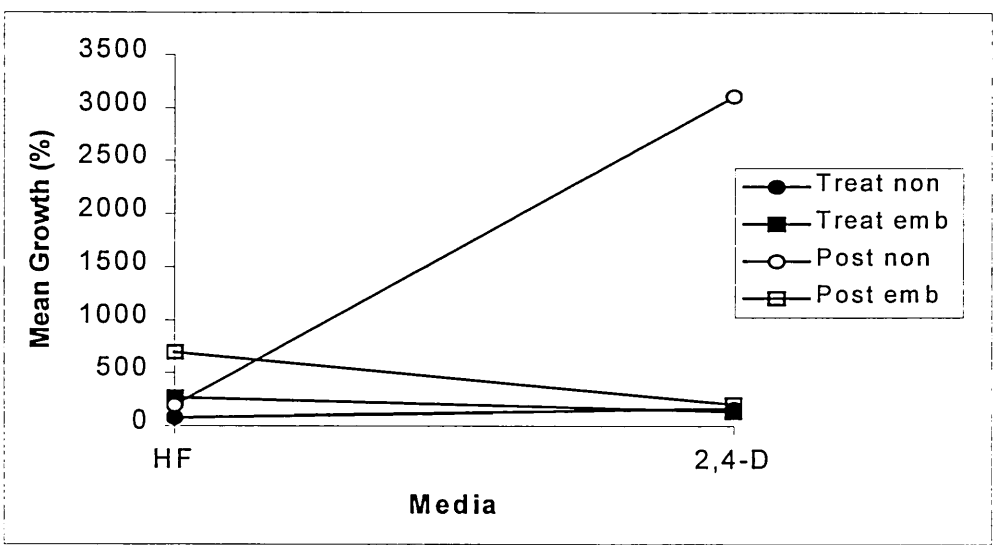


Figure 3.4.2.3: Callus proliferation assessments were performed after 66 days of treatment with HNE (designated as treatments (treat) –•– non-embryogenic and –■– embryogenic callus growth) and after 33 days treatment followed by 33 days post-aldehyde removal (designated as post treatment (post) for : –o– non-embryogenic and –□– embryogenic callus growth). Growth rate is expressed as a % of the initial weight before treatment application (for post-treatment as a % from the half of the fresh weight transferred from the treatment at day 33 onto the post-treatment aldehyde- free media, the other half being continued on the treatment). Means comprise an average of the growth responses for the total replicated (n=3) treatments for all concentrations (n=8), of HNE applied in embryo induction medium (HF) and callus proliferation medium (2,4-D). Statistical analyses were performed on treatment data only (without controls).

Figure 3.4.2.4 Interaction plot for MDA Treatments and Post-treatments

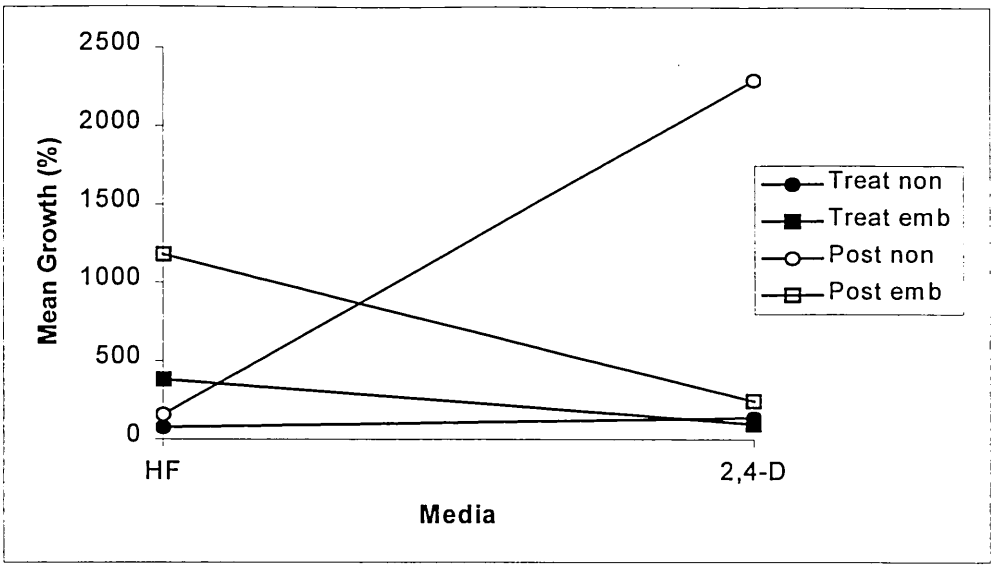


Figure 3.4.2.4: Callus proliferation assessments were performed after 66 days of treatment with MDA (designated as treatments (treat) –•– non-embryogenic and –■– embryogenic callus growth) and after 33 days treatment followed by 33 days post-aldehyde removal (designated as post treatment (post) for : –o– non-embryogenic and –□– embryogenic callus growth). Growth rate is expressed as a % of the initial weight before treatment application (for post-treatment as a % from the half of the fresh weight transferred from the treatment at day 33 onto the post-treatment aldehyde- free media, the other half being continued on the treatment). Means comprise an average of the growth responses for the total replicated (n=3) treatments for all concentrations (n=8), of MDA applied in embryo induction medium (HF) and callus proliferation medium (2,4-D). Statistical analyses were performed on treatment data only (without controls).

3.4.3 Somatic Embryogenesis During and Post Hydroxynonenal and Malondialdehyde Treatment

During the course of the experiment the tissue was evaluated for the production of embryos and the amount produced by each treatment are shown in Figures 3.4.3.1 (HNE) and in Figure 3.4.3.2 (MDA). These figures show that there are more embryos produced by tissue under MDA treatment than under HNE treatment, indicating that MDA may be less toxic to the cells.

Figure 3.4.3.1 Embryo production of HNE treated *D. carota* tissue

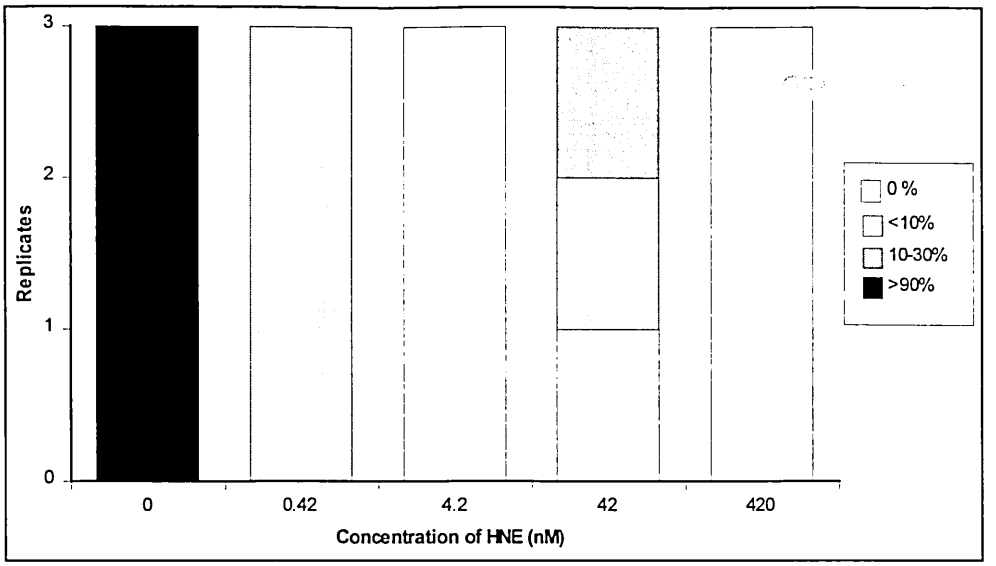


Figure 3.4.3.1:Embryo production assessments were performed 66 days after treatment with HNE. Embryo production is expressed as a percentage of the number (n=3) of the callus colonies producing advanced stage embryos (torpedo) in three replicate plates (designated as replicate plates 1,2, and 3 on the Y axis).

Figure 3.4.3.2 Embryo Production of MDA treated *D. carota* tissue

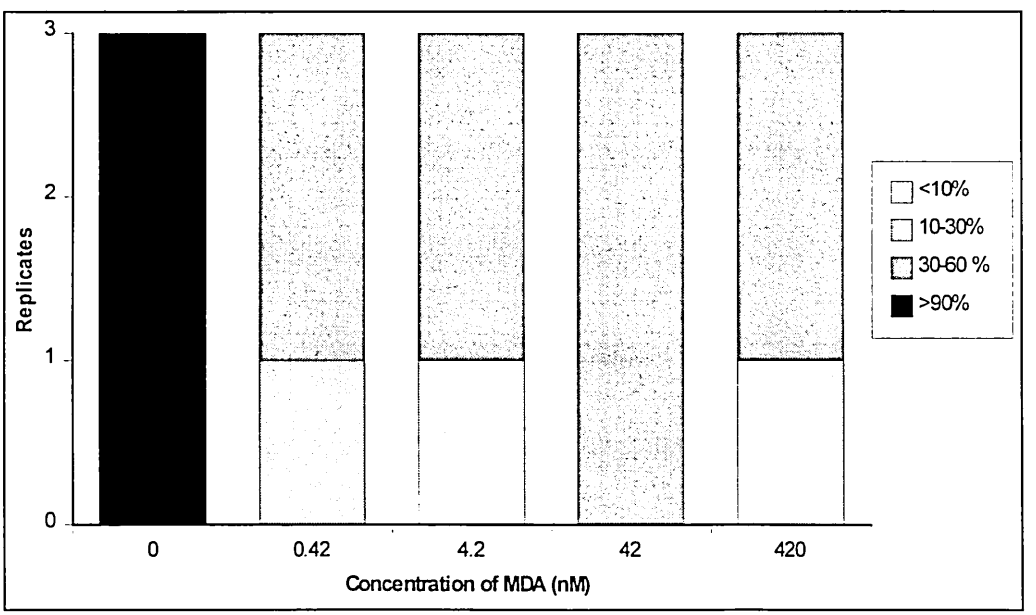


Figure 3.4.3.2:Embryo production assessments were performed 66 days after treatment with MDA. Embryo production is expressed as a percentage of the number (n=3) of the callus colonies producing advanced stage embryos (torpedo) in three replicate plates (designated as replicate plates 1,2, and 3 on the Y axis).

The level of production of embryos in the post-treatment set was also evaluated after previous treatment with HNE and MDA and the results are shown in Figures 3.4.3.3 and 3.4.3.4 respectively. The number of embryos produced compared to the treatment set had increased, particularly for the two highest concentrations of HNE treatment and all of the concentrations for the post-treated MDA, with levels of production of embryos recovering almost to that of the controls. This indicates the potential for the cells to overcome the aldehyde treatment and initiate somatic embryogenesis. However, the aldehyde treatment did not stimulate embryo production in non-embryogenic cells since only the line classed as embryogenic produced any embryos.

Figure 3.4.3.3 Embryo Production of *D. carota* HNE Treated and Post Treated

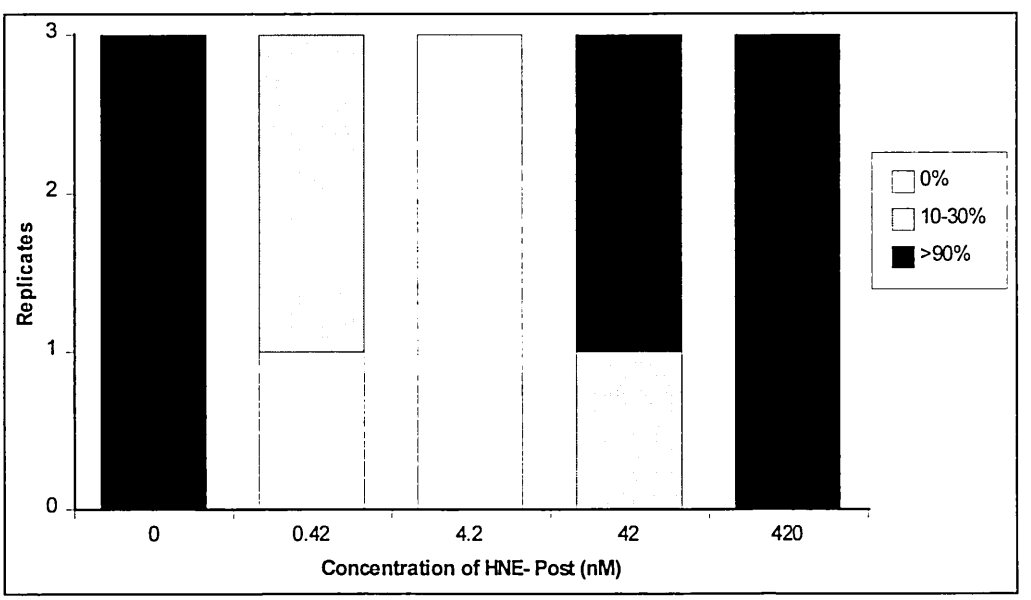


Figure 3.4.3.3: Embryo production assessments were performed after a 66 day, two-stage, phased treatment comprising: (stage 1) 33 days growth in the presence of HNE and (stage 2) 33 days growth following the removal HNE. Embryo production is expressed as a percentage of the number (n=3) of the callus colonies producing advanced stage embryos (torpedo) in three replicate plates (designated as replicate plates 1,2, and 3 on the Y axis).

Figure 3.4.3.4 Embryo Production of MDA Treated and Post Treated

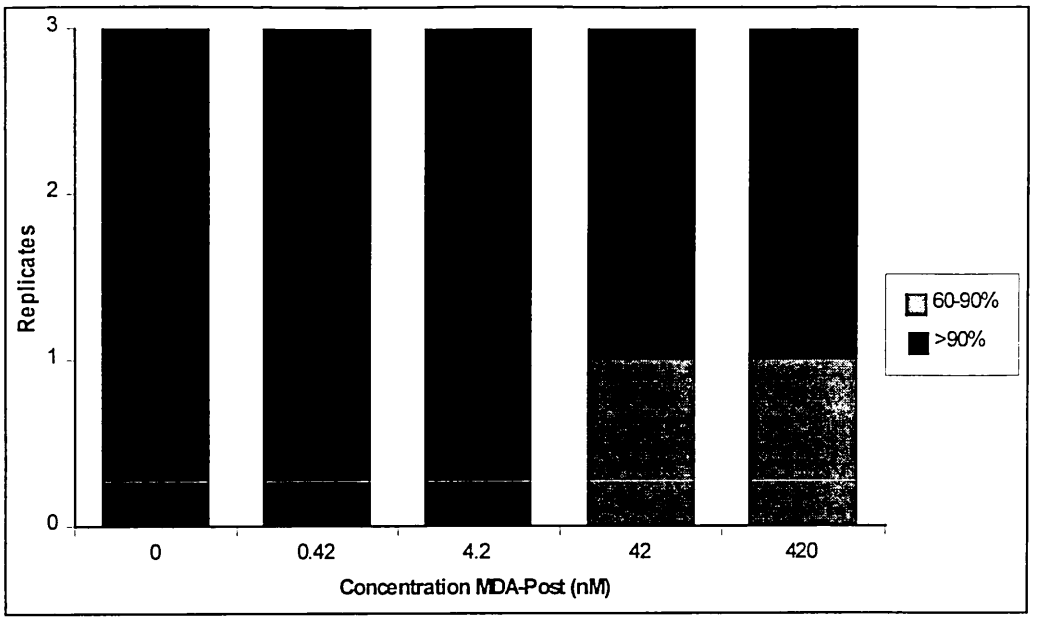


Figure 3.4.3.4: Embryo production assessments were performed after a 66 day, two-stage, phased treatment comprising: (stage 1) 33 days growth in the presence of MDA and (stage 2) 33 days growth following the removal of MDA. Embryo production is expressed as a percentage of the number (n=3) of the callus colonies producing advanced stage embryos (torpedo) in three replicate plates (designated as replicate plates 1,2, and 3 on the Y axis).

3.4.4 Hydroxynonenal and Malondialdehyde Content of Hydroxynonenal Post-treated *D. carota* Callus

Samples taken at the end of the experiment were profiled for HNE and MDA content as measured by LC-MS (see section 2.6 for full details of extraction and analysis procedures). There was a marked difference between the embryogenic and non-embryogenic callus on 2,4-D media after treatment with HNE followed by post treatment. Both the HNE and the MDA content of this group of samples were similar and showed higher levels of both aldehydes in the embryogenic callus. The results are shown in Figures 3.4.4.1 (HNE content) and 3.4.4.2 (MDA content).

Figure 3.4.4.1 HNE content of *D. carota* tissue after HNE treatment and post-treatment

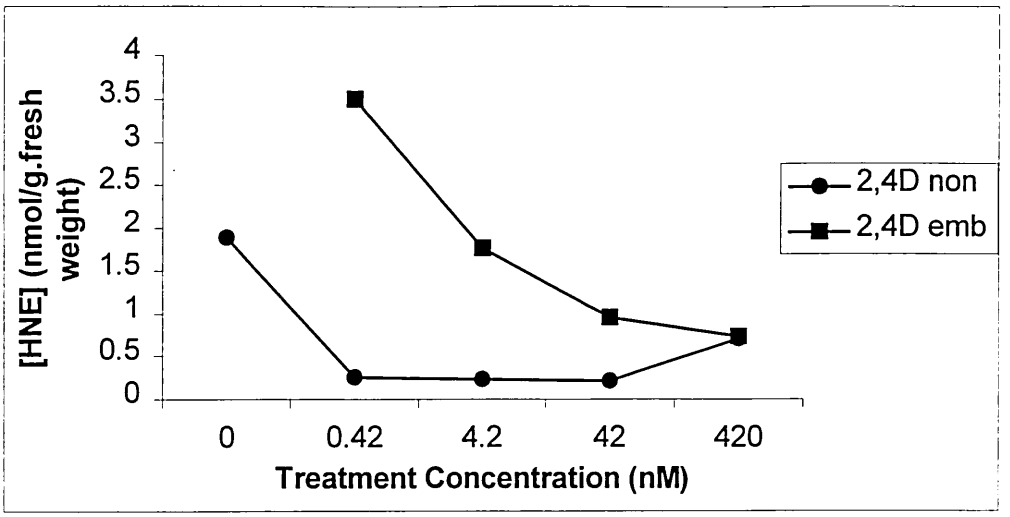


Figure 3.4.4.1: LC-MS was used to assess endogenous HNE levels in non-embryogenic (non -•-) and embryogenic (emb -■-) callus cultures of *D. carota* (maintained on 2,4-D medium) after a 66 day, two stage, phased treatment comprising: (stage 1) 33 days growth in the presence of different concentrations of HNE followed by (stage 2) 33 days of growth following HNE removal.

Figure 3.4.4.2 MDA Content of *D. carota* Tissue after HNE Post-treatment

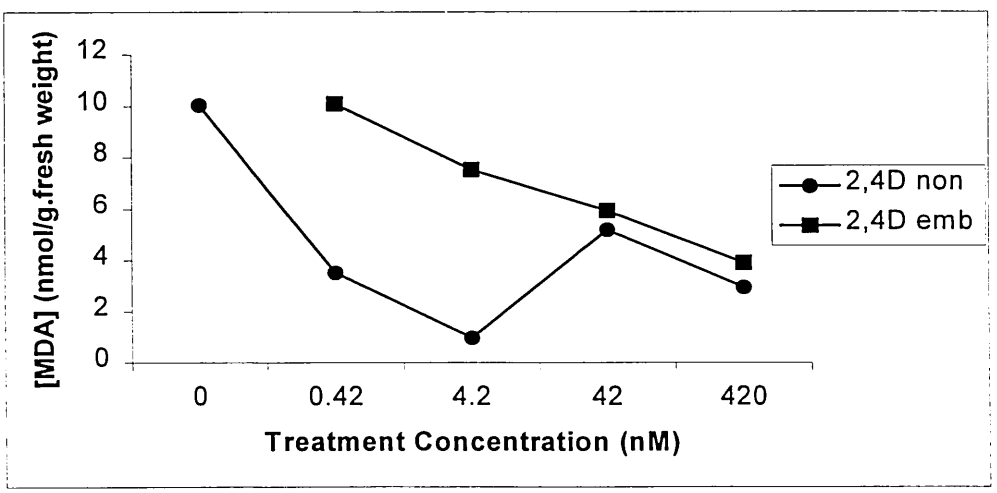


Figure 3.4.4.2: LC-MS was used to assess endogenous MDA levels in non-embryogenic (non -•-) and embryogenic (emb -■-) callus cultures of *D. carota* (maintained on 2,4-D medium) after a 66 day, two stage, phased treatment comprising: (stage 1) 33 days growth in the presence of different concentrations of HNE followed by (stage 2) 33 days of growth following HNE removal.

Other groups of samples taken from the end of the experiment and analysed for HNE and MDA content showed no clear patterns of HNE and MDA content.

3.4.5 Catalase and Peroxidase Activity of HNE and MDA Treated and Post-Treated *D. carota*

Analysis of the catalase and peroxidase antioxidant activity in samples from the end of the experiment showed that catalase activity was in the range of 0-0.2 A/min/mg protein, but showed no clear patterns between the HNE and MDA treated or post-treated sets. Peroxidase activity showed a clearer pattern, with higher activity in the non-embryogenic callus compared to the embryogenic callus for HNE and MDA treatments and post-treatments on 2,4-D media and is demonstrated as an interaction plot in Figure 3.4.5.1.

Figure 3.4.5.1 Peroxidase Activity of Callus Exposed to HNE and MDA on 2,4-D media

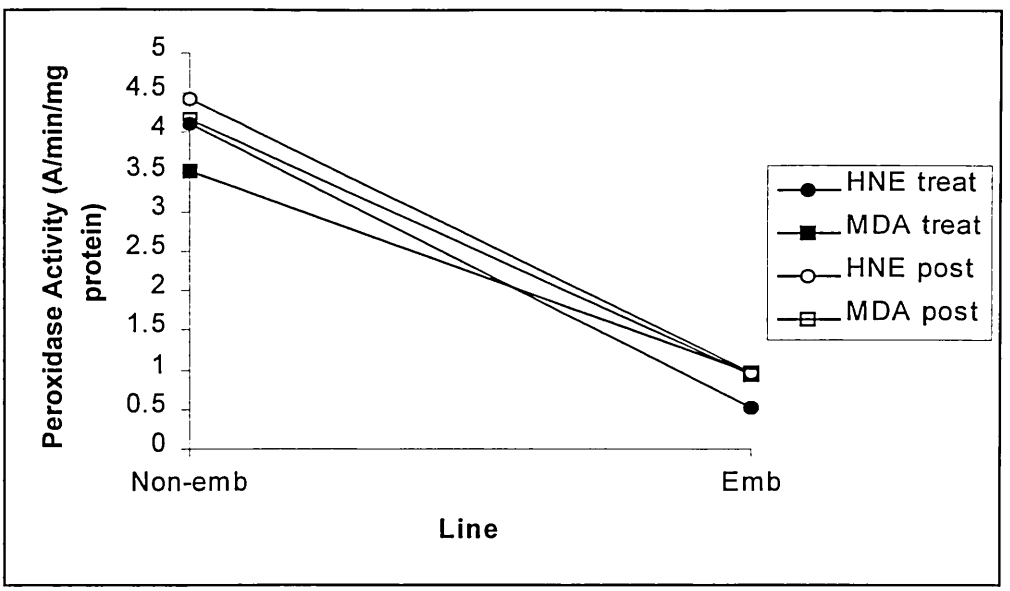


Figure 3.4.5.1: Peroxidase activities were measured in (1) embryogenic and non-embryogenic proliferating callus (maintained on 2,4-D medium) after 66 days of culture in the presence of HNE (HNE treat -•-) and MDA (MDA treat -■-) and (2) embryogenic and non-embryogenic callus cultures following 33 days of treatment followed by 33 days of culture after aldehyde removal (HNE post -o-, MDA post -□-). Means comprise of an average of the treatments (n=2 for duplicated samples from one of the three randomly selected treatment replicates in duplicate) for each HNE and MDA treatment and post-treated callus lines, cultured on 2,4-D containing media (n=10 for each aldehyde line and treatment).

3.5 Development of ELISA for the Detection Hydroxynonenal Protein Adducts for Plant Tissue Using *D. carota* as a Model

HNE-protein adducts have been detected in mammalian tissues and have been associated with tissue that is undergoing apoptosis or oxidative stress (Kirichenko, *et. al.* 1996, Cohn, *et. al.* 1996) but there has been little such work reported in plant systems. These HNE-adducts have been investigated and HNE has been shown to conjugate to a number of different amino acid side chains, but mainly histidine (Uchida & Stadtman, 1992 a, b, Uchida & Stadtman, 1993, Uchida, *et. al.* 1994). The presence of HNE-adducts in plant tissues has until now not been investigated and may give a true indication of the "bound" amount of HNE present within the cells. The amount of these

adducts can be estimated with the use of enzyme linked immunosorbant assays (ELISA). The enzyme linked immunosorbant assay (ELISA) has been widely used as an analytical tool in mammalian systems to study activities of a range of biochemical and immunological compounds (Waeg, *et. al.* 1996). These assays take many forms and can be used to detect the presence of antibodies or antigens or even to study the structure of these antigens by changing certain key conditions and can detect very low concentrations, often down to 10^{-12} M (Harlow & Lane, 1988). The investigation of lipid peroxidation has benefited from the application of ELISA techniques and has been used by research groups to detect the presence of HNE-protein conjugates. A number of antibodies have been generated for the detection of HNE-protein adducts (Uchida, *et. al.* 1995, Kim, *et. al.* 1997, Waeg, *et. al.* 1996) and MDA adducts (Craig, *et. al.* 1994) and have been used in ELISAs to characterise the epitopes to which the lipid peroxidation products conjugate. Auto-antibodies of MDA-modified proteins have been found in tissue that is diseased and in particular has been associated with systemic lupus erythematosus (Amara, *et. al.* 1995). ELISAs have become an increasing useful tool in the study lipid peroxidation products and their association with proteins. ELISAs have also been increasingly used in plant systems but until now only for the investigation of plant viruses and pathogens (Sutula, *et. al.* 1986). Therefore the use of ELISAs for the study of HNE and its conjugation with proteins forms a unique tool for the investigation of lipid peroxidation products within different plant species.

3.5.1 Preliminary ELISA Trials

Trials using *D. carota* protein extracts were used to develop the methodology for the ELISA for plant tissue extracts. The method is fully detailed in section 2.7, although for the preliminary trials BSA-HNE was not used. Four *D. carota* callus lines were extracted for protein as detailed in section 2.5.1, with two samples of each being used to fill the wells in the 96 well plate. The results are detailed in Table 3.5.1.1 and are scored in the range of – to +++, with +/- showing only a little colour indicating that some wells may show the presence of HNE-adducts but results were not conclusive. To account for background levels of signal for the subsequent trials blanks or negative controls were included, which contained phosphate buffered saline instead of protein extract.

Table 3.5.1.1 ELISA of Embryogenic and Non-embryogenic lines of *D.carota*

	Un-dil	Un-dil	1/4	1/4	1/16	1/16	1/64	1/64	1/256	1/256	1/1024	1/1024
6	+	+	+	+	-	-	-	-	-	-	-	+
6	+	-	-	-	-	-	-	+	-	-	-	-
B21	-	-	++	++	-	-	-	-	-	-	-	+
B21	-	-	-	-	-	-	-	+	-	-	-	-
B50	+/-	-	-	-	-	-	-	-	-	-	-	+
B50	-	-	-	-	-	-	-	-	-	-	-	-
E10	+/-	-	-	-	+	-	-	-	-	-	-	-
E10	-	-	+	-	-	-	-	-	-	-	-	-

Table 3.5.1.1: ELISA of four callus lines: 6 (non-embryogenic, Chantenay Red Cored), B21 (non-embryogenic, Gold King), B50 (embryogenic, Autumn King 2) and E10 (embryogenic, Chantenay Red Cored), with serial dilutions (1/4 to 1/1024) made across the plate from the undiluted extract (UN-DIL).

At this stage the methodology for the development of the ELISA was in place and a checkerboard assay was then set up to ascertain the levels of the first and second antibodies needed to achieve a consistent positive result. The

checkerboard assay uses the 1st antibody at three dilutions, 1/10, 1/100, and 1/200 and the 2nd antibody at two dilutions, 1/100 and 1/500. The samples were diluted down the plate and the results are shown in Table 3.5.1.2, for the non-embryogenic samples and Table 3.5.1.3 for the embryogenic line. Table 3.5.1.2 shows a strong signal in the top left hand corner of the plate at the highest possible concentration of the two antibodies. This combination has a concentration far higher than is routinely used in a standard ELISA and may indicate a low concentration of the antigen present or low antibody specificity. Table 3.5.1.3 also shows the highest signal at the top concentrations but to a lesser degree than the non-embryogenic samples.

Table 3.5.1.2 Checkerboard Assay of a Non-embryogenic line of *D. carota*

	|2nd Antibody 1/100						|2nd Antibody 1/500					
	1/10	1/10	1/100	1/100	1/200	1/200	1/10	1/10	1/100	1/100	1/200	1/200
Un-dil	+++	+++	+/-	+/-	+	+	+	+	-	-	-	+
1/4	+++	+++	+/-	+	+	+	-	-	-	-	-	-
1/16	++	+	+/-	+/-	+/-	+/-	-	-	-	-	-	-
1/64	+	+/-	+/-	+/-	+/-	+/-	-	-	-	-	-	-
1/256	+	+/-	+/-	+/-	+/-	+/-	-	-	-	-	-	-
1/1024	-	+/-	+/-	+/-	+/-	+/-	-	-	-	-	-	-
1/4096	-	+/-	+/-	+/-	-	-	-	-	-	-	-	-
Blank	+/-	+/-	+/-	-	+/-	-	-	-	-	-	-	-

Table 3.5.1.2: Checkerboard Assay of Non-embryogenic Line B21 (Gold King) in serial dilutions (un-diluted to 1/4096) with 1st antibody dilutions at 1/10, 1/100, 1/200 and 2nd antibody dilutions at 1/100 and 1/500.

Table 3.5.1.3 Checkerboard Assay of an Embryogenic line of *D. carota*

	|2nd Antibody 1/100						|2nd Antibody 1/500					
	1/10	1/10	1/100	1/100	1/200	1/200	1/10	1/10	1/100	1/100	1/200	1/200
Un-dil	++	++	+	+	+	+	+/-	+	+	-	-	+
1/4	+	+	+/-	+/-	+/-	-	+/-	+/-	-	-	-	-
1/16	+/-	+/-	+/-	+/-	+	+/-	+	-	+	-	-	-
1/64	+/-	+/-	+/-	+/-	+/-	+/-	-	+	-	-	-	-
1/256	-	-	+/-	+/-	-	-	-	-	-	-	-	-
1/1024	-	-	-	-	-	-	-	-	-	-	-	-
1/4096	-	-	-	-	-	-	-	-	-	-	-	-
Blank	+/-	-	-	-	-	-	-	-	-	-	-	-

Table 3.5.1.3: Checkerboard Assay of Embryogenic Line B50 (Autumn King 2) in serial dilutions (un-diluted to 1/4096) with 1st antibody dilutions at 1/10, 1/100, 1/200 and 2nd antibody dilutions at 1/100 and 1/500.

A micro-titre plate reader was used for all subsequent assays, giving absorbance reading to three decimal places. Positive wells were considered as three times the average blank. Four lines of the *D. carota* cultivar Chantenay Red Cored of different embryogenic potentials were extracted for protein. Standardised samples were then used in an ELISA with the 1st antibody at 1/10 dilution, and the 2nd antibody at a 1/100 dilution. At the final stage, on development of the blue colouration after a 10 min development, 100 µl of 2.5M sulphuric acid was added to stop the reaction and turning the colour yellow, with the absorbance measured at 450 nm. Results for triplicate samples of the four callus lines are shown in Table 3.5.1.4.

Table 3.5.1.4 ELISA of four lines of *D. carota* of different embryogenic potentials

	20	20	20	21	21	21	D6	D6	D6	E10	E10	E10
Un-dil	.158	.163	.174	.198	.175	.299	.304	.155	.133	.125	.142	.112
1/ 4	.125	.092	.198	.090	.121	.077	.087	.114	.156	.100	.106	.162
1/ 16	.150	.183	.079	.102	.098	.089	.127	.108	.130	.120	.088	.282
1/ 64	.184	.183	.139	.254	.294	.096	.401	.093	.087	.102	.093	.094
1/ 256	.128	.096	.310	.306	.264	.089	.110	.148	.102	.079	.100	.126
1/ 1024	.088	.098	.209	.105	.114	.083	.083	.083	.126	.103	.106	.115
1/ 4096	.104	.339	.099	.088	.081	.094	.099	.093	.094	.104	.115	.138
Blank	.223	.275	.307	.247	.147	.123	.156	.162	.190	.270	.148	.299

Table 3.5.1.4: Four lines of Chantenay Red Cored, non-embryogenic line 20, non-embryogenic line 21, poorly embryogenic line D6 and embryogenic line E10 in triplicate samples serial diluted up to 1/4096. Data are absorbance values at 450 nm. 1st antibody at 1/10 dilution and 2nd antibody at 1/100 dilution.

A number of assays were performed on similar *D. carota* extracts, but no clear difference was found between the samples and blank wells. Three similar assays were performed but there was little difference between samples and blanks. At this stage a fresh batch of the second antibody was received and with it a sample of HNE conjugated to bovine serum albumin protein (BSA-HNE) which could be used as a positive control.

3.5.2 Quantification of ELISAs with HNE-BSA

A checkerboard assay was performed with a combination of both the first and second antibodies at 1/200 dilution and 1/500 dilution, with only BSA-HNE or blank wells containing PBS, to give positive and negative controls. The results are shown in Table 3.5.2.1. Positive signals were achieved with all combinations, and the absorbance values for the blanks were in average under 0.100. The positive wells are represented in Table 3.5.2.2.

Table 3.5.2.1 Checkerboard assay of BSA-HNE

	Ab 2 1/200			Ab 2 1/500			Ab 2 1/200			Ab 2 1/500		
	Ab 1 1/200						Ab 1 1/500					
	HNE	HNE	B	HNE	HNE	B	HNE	HNE	B	HNE	HNE	B
Un-dil	.841	.884	.133	.471	.430	.072	.597	.605	.080	.338	.27	.056
1/ 5	.95	.889	.056	.467	.485	.085	.629	.609	.058	.330	.303	.055
1/ 25	.883	.941	.054	.429	.457	.063	.650	.630	.053	.345	.281	.055
1/125	.924	.909	.067	.462	.479	.065	.691	.645	.052	.347	.337	.062
1/625	.747	.705	.110	.359	.357	.078	.500	.485	.056	.258	.242	.069
1/ 1325	.383	.427	.061	.210	.228	.069	.235	.237	.050	.154	.162	.054
1/ 6625	.186	.149	.073	.116	.165	.090	.134	.141	.068	.107	.174	.065
B	.123	.101	.082	.062	.075	.078	.081	.079	.078	.066	.076	.064

Table 3.5.2.1: Checkerboard assay of BSA-HNE (HNE) with 1st and 2nd antibody dilutions at 1/200 and 1/500, and blanks containing PBS (B). Data are absorbance at 450 nm.

Table 3.5.2.2 Checkerboard assay of BSA-HNE

	HNE	HNE	B	HNE	HNE	B	HNE	HNE	B	HNE	HNE	B
Un-dil	+	+	-	+	+	-	+	+	-	+	+	-
1/ 5	+	+	-	+	+	-	+	+	-	+	+	-
1/ 25	+	+	-	+	+	-	+	+	-	+	+	-
1/125	+	+	-	+	+	-	+	+	-	+	+	-
1/625	+	+	-	+	+	-	+	+	-	+	+	-
1/ 1325	+	+	-	-	+	-	+	+	-	-	-	-
1/ 6625	-	-	-	-	-	-	-	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-	-	-	-

Table 3.5.2.1: Checkerboard assay of BSA-HNE (HNE) with 1st and 2nd antibody dilutions at 1/200 and 1/500, and blanks containing PBS (B). Positive wells (+) have absorbance over 0.216 and negative wells (-) have absorbance less than 0.216, absorbance at 450 nm.

The two highest concentrations of antibody used in the last assay gave the highest absorbance values for BSA-HNE and were adopted as the concentrations for subsequent assays. The two samples detected by these dilutions of antibody are shown in Figure 3.5.2.1 and show the drop off in signal after the 1/125 dilution. The highest concentration of BSA-HNE was 0.2 mg/mL, therefore detection was in the range of 160 ng to 0.02 mg, giving a maximal signal and the detection limit was down to 15 ng.

Figure 3.5.2.1 Absorbance Profile of BSA-HNE

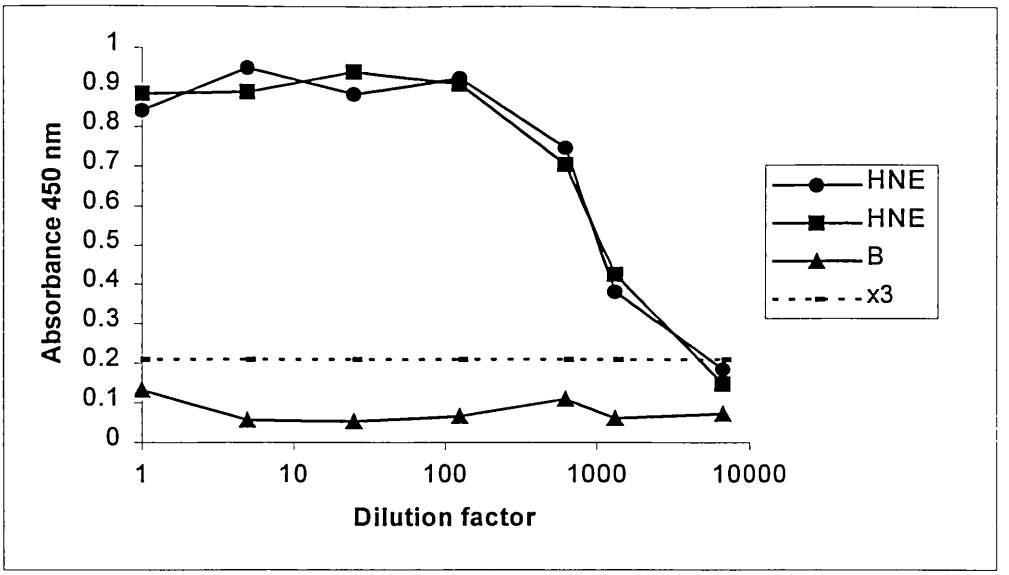


Figure 3.5.2.1: Absorbance at 450 nm of duplicate samples of BSA-HNE (HNE), blank containing only PBS (B) and the positive-negative threshold 3 times the average blank (x3) that have been serially diluted, depicted in a log scale (Dilution factor). Primary and secondary antibodies were used at a 1/200 dilution.

Pairs of embryogenic and non-embryogenic lines of a range of cultivars were assayed for the presence of HNE-adducts. Autumn King 2 lines B50 (embryogenic) and 6 (non-embryogenic), Gold King lines B21 (embryogenic) and F9 (non-embryogenic) and a range of other lines were assayed but only the BSA-HNE gave a positive signal. Lines C20 (Chantenay Red Cored), B50 (Autumn King 2) and F8 (Early Nantes), gave high levels of extracted protein, increasing the loading level of protein by three times but no positive samples were achieved, only the BSA-HNE gave a maximal absorbance down to 1/125 dilution.

In general there seemed to be little indication of the presence of HNE-protein adducts in soluble protein extracted from samples of callus of a range of cultivars of *D. carota*. The technique has been proven to be fairly consistent with duplicate samples of BSA-HNE giving similar results within each plate,

although there was some variation in the level of the signal between plates. However this remains one of the first reports of applying such technology to a plant system and thus provides valuable indications as to future directions. For further work in this area see chapters 4 and 5.

3.6 Discussion

3.6.1 *Daucus carota* as a Model System

Somatic embryogenesis forms an ideal model for the study of early regulatory and morphogenetic events in plant embryogenesis (Zimmerman, 1993). *D. carota* has been used as a model species for the study of somatic embryogenesis for many years, and is very amenable and easily manipulated with the exogenous control of one auxin (Zimmerman, 1993, de Vries, *et. al.* 1994, Borkird, *et. al.* 1986, Ammirato, 1986). Somatic embryogenesis in *D. carota* has been very well characterised and a great deal of information is available as to the role of hormones and the genes expressed during this developmental pathway (Krul, 1993, Michalczuk, *et. al.* 1992, Kawahara, *et. al.* 1992, Sung & Okimoto, 1981, Choi, *et. al.* 1987). However, there are limitations to even this well characterised system. Some callus lines generated with the application and removal of 2,4-D do not produce embryos and other lines that did produce embryos initially, lose the ability over time. This indicates that although *D. carota* is considered a responsive species, some cultivars can be partly recalcitrant and that factors, such as the age of the culture can influence its ability to produce embryos. Therefore, conclusions drawn from the study of a narrow range of cultivars may not fully represent

the responses throughout that species, particularly, as most commercial species are drawn from a narrow genetic base that accounts for only a small portion of the gene pool of the wild species. Rice and grapevine have proven particularly difficult to establish and manipulate within *in vitro* culture and have special problems (Benson & Roubelakis-Angelakis, 1992, 1994, Benson, *et. al.* 1992a & b). Careful considerations need to be made when applying techniques developed within a model species to these and other recalcitrant systems that have a different range of responses.

3.6.2 Characterisation of Different Cultivars

One of the problems of establishing cultures *in vitro* for the study of developmental pathways is the genetic and physiological variability between cultivars and sources of seed stocks. Different cultivars have a range of discrete differences in their genetic make-up which reflect in their response to *in vitro* stimulus therefore forming a genetic pre-determinism that can give each a unique response (McCown, 2000). Many of the cultivars used within this investigation have been previously used for studies and illustrate the variability observed between the response of different cultivars (Robertson, *et. al.* 1995, Benson, *et. al.* 1997, Bremner, *et. al.* 1997). For example, early Nantes was previously shown to respond well to the induction of somatic embryogenesis for most of the lines tested (Benson, *et. al.* 1997). Variability has contributed to the fact that certain of the seed cultivars are poorly represented in the callus lines established from seedlings grown in culture. Cultivars Regolus, and Oranza were particularly poor performers and therefore these cultivars are not used in later protein and ELISA studies due to a lack of

suitable callus lines. Cultivars selected for further study were Autumn King 2, Chantenay Red Cored, New Red Intermediate and Early Nantes as they had generated the greatest number of embryogenic and non-embryogenic established callus lines. Seeds that have been kept in storage for long periods of time may exhibit reduced germination rates. The variability of different cultivars and the gradual loss of responsiveness over time within a species form a problematic area of establishing significant commercial, endangered and domestic species within *in vitro* systems and gene banks to maintain a wide genetic base.

3.6.3 Somatic Embryo Initiation in a Range of Callus Cultures

The ability of newly established callus cultures to generate somatic embryos varies greatly, with some cultures failing to produce any and therefore classed as non-embryogenic (Figures 3.1.2.1 and 3.1.2.2). The three main types of non-embryogenic culture are those which never produced embryos from the point of initiation, those genetically pre-disposed to produce embryos and those that were embryogenic but have gradually or suddenly lost the ability, showing time related decline in embryogenic capacity. This decline in embryogenic capacity is also demonstrated within more established cultures, many of which exhibit reduced powers of development with lines that were previously embryogenic becoming non-embryogenic (Figures 3.1.3.1, 3.1.3.2). The decline in the embryogenic capacity can be linked to time-related ageing. In whole organisms, the exact mechanism of ageing is unknown and is indeed the subject of controversy. However, ageing has continued to be linked with oxidative stress and the generation of free radical species within *in vitro*

culture (Benson, *et. al.* 1992 a, b, Benson & Roubelakis-Angelakis 1992, 1994, Benson, *et. al.* 1997, Bremner, *et. al.* 1997, Robertson, *et. al.* 1995). The decline in *in vitro* morphogenetic capacity with time is a major difficulty in the manipulation of plants within *in vitro* cultures and means that new embryogenic cultures of each species have to be continually initiated to maintain a supply of embryogenic cultures. This is time consuming and costly.

3.6.4 Protein Content of Embryogenic and Non-Embryogenic Cultures

3.6.4.1 Method Development and Validation

In order to validate fully the procedures used for the extraction of proteins from callus cultures, comparisons were made between established small-scale and large-scale extractions. Small-scale extractions used weights of callus under one gram, typically 200 mg with 1.5 mL Eppendorfs while large scale extractions used several grams of tissue cooled with liquid nitrogen in a ceramic mortar and pestle. There was no difference between the extraction procedures (Figures 3.2.1.1 & 3.2.1.2) and that both methods could be used and were valid and directly comparable. The large-scale extractions provided samples containing a higher concentration of protein and they were used to maximise the protein loaded into 96 well plates for the analysis of protein adducts using an ELISA technique (see section 3.5). The small-scale extractions were used for studies of protein content and peroxidase activity.

3.6.4.2 Differences in Protein Content of Embryogenic and Non-Embryogenic Cultures

Analysis of the protein content of embryogenic and non-embryogenic callus lines shows that the former generally have a higher protein content than the latter (Figure 3.2.1.1, 3.2.1.2). In all pairs of embryogenic and non-embryogenic callus lines, for a range of cultivars, the embryogenic had a higher protein content than the non-embryogenic line and the highest protein content was observed in the New Red Intermediate and Autumn King 2 cultivars. The least difference noted in protein content was between the Chantenay Red Cored lines. The increased protein content may be due to the fact that the embryogenic cells have a number of transcriptionally active genes associated with somatic embryogenesis and that protein synthesis from these genes may not have been completely inhibited by the presence of 2,4-D (Choi, *et. al.* 1987, Kawahara, *et. al.* 1992). Several studies have focused on the genes expressed during the initiation of somatic embryogenesis within *D. carota*. A number of proteins are also produced during the early stages of morphogenesis and many are linked to the change in cell status and the initiation of new developmental pathways (Zimmerman, 1993) (see sections 1.2 and 3.1). The data discussed above was presented as protein content per gram fresh weight. These data should be interpreted cautiously because the observed variations could be affected by fluctuations of the water content of the samples.

3.6.5 Implications of the Presence of Hydroxynonenal and Malondialdehyde Content of *D. carota*

The concentration of hydroxynonenal and malondialdehyde present within cells gives an indication of the level of lipid peroxidation experienced by the cell (Zollner, *et. al.* 1991, Esterbauer, *et. al.* 1996, Esterbauer, *et. al.* 1990, Esterbauer, *et. al.* 1988). Many studies have measured the content of HNE within different types of mammalian cells and show a range from 2 μM in bronchial fibroblasts to 200 μM in peritoneal Ehrlich ascite (Zollner, *et. al.* 1991). The HNE and MDA content of *D. carota* quantified by LC-MS (Deighton, *et. al.* 1997) showed that the most embryogenic line had the lowest HNE and MDA content (Figures 3.3.1 and 3.3.2). The levels of HNE observed in *D. carota* extracts are much lower that any of those measured within mammalian systems (Deighton, *et. al.* 1997, Zollner, *et. al.* 1991). The low MDA content of the most embryogenic line is consistent with early analysis of *D. carota* tissue when the method was developed (Figure 3.3.1) (Bremner, *et. al.* 1997). This shows that the more embryogenic callus may be better able to remove the harmful lipid peroxidation product HNE under standard *in vitro* conditions.

3.6.6 Effects of Hydroxynonenal and Malondialdehyde on Growth and Somatic Embryo Development

The exogenous addition of HNE and MDA has been shown to have significant effects on the growth rate of rapidly proliferating *D. carota* callus, with both HNE and MDA having a highly significant effect on the rate of growth of callus even for the lowest concentration of the aldehyde added (Figures 3.4.2.1

and 3.4.2.2). Interestingly, the effect on the growth rate could be partly overcome by the removal of the aldehydes and the continued culture of the affected callus on aldehyde free media. The best recovery, showing growth rates up to 30 times that of the control, was observed in the non-embryogenic line which had been cultured on 2,4-D media. This remarkable growth rate recovery is unlike most of the similar studies in mammalian systems that generally either killed the cells or irreversibly inhibited growth rate (Zollner, *et. al.* 1991).

The effects of the exogenous application of HNE and MDA was also dramatic on the ability of the embryogenic line to produce embryos under the addition of aldehyde on hormone free media, with MDA showing higher rates of embryo production (Figures 3.4.3.1, 3.4.3.2, 3.4.3.3, 3.4.3.4). However, the post-treatment culturing showed increased embryo production with the restoration, almost to controls levels, for the previously MDA treated callus (Figure 3.4.3.4). Some of the post-treatment concentration from HNE addition also showed increased embryo production after HNE removal but one set of callus previously exposed to 4.2 nM HNE showed a complete inhibition of embryo production. This indicates an irreversible inhibition of somatic embryogenesis was possible without killing the cells under treatment (Figure 3.4.3.3) and shows that plant cells are able to cope with exogenous application of aldehydes and are even still able to commence somatic embryogenesis. This ability to cope with the application of HNE and MDA may be due to the activity of detoxification mechanisms that are stimulated by the presence of

the aldehydes and may involve glutathione and its associated mechanisms (Fukuda, *et. al.* 1997).

3.6.7 Measurement of HNE- Protein adducts using an ELISA

HNE has been shown to form adducts with protein and lipoprotein and has been characterised within mammalian systems (Uchida, *et. al.* 1995, Craig, *et. al.* 1994, Cohn, *et. al.* 1996) and these adducts have been implicated in apoptosis (Kirichenko, *et. al.* 1996). Antibodies against these adducts have been used for quantification within tissue samples and extracts (Waeg, *et. al.* 1996) and these antibodies have now been used in attempts to quantify HNE adducts within plant cells. ELISA assays of HNE-protein adducts shows that this technique can be used as a tool for the detection of HNE-adducts, however the concentration appears to be too low for successful detection in samples of *D. carota* (section 3.5). Although, later studies indicated that other plant species, such as *Ipomoea batatas* and *Glycine max* contain HNE-protein adducts that can be detected in this manner (see chapters 4 and 5). ELISA has been used for the detection of pathogens within plant cells (Sutula, *et. al.* 1986) and may on refinement prove an extremely useful tool for the detection of HNE adducts in order to further current knowledge on HNE and its activity within plant cells.

3.6.8 Conclusions

Overall, the studies of *D. carota* have shown that there is great variability between different cultivars and lines of callus generated from these cultivars,

with a wide range of responses. *D. carota* has also been shown to be sensitive to the exogenous application of HNE and MDA, with reduced growth rates and rates of somatic embryogenesis. However, these inhibitions can in general be reversed, with the cells able to recover growth rates and the number of somatic embryos produced. The development of new techniques such as the ELISA of HNE-protein adducts has shown that many of the methods developed for mammalian systems can be successfully converted for use within plant systems. Many of these studies have brought further insight into the role of lipid peroxidation products in oxidative stress within plant tissue cultures.

D. carota Main Findings

- Reversible inhibition of callus proliferation and somatic embryo development by the exogenous application and removal of hydroxynonenal and malondialdehyde.
- Development of an ELISA for the detection of HNE-protein adducts in plant extracts, although they were not detected within *D. carota.*
- Routine measurement of hydroxynonenal and malondialdehyde extracted from tissues of *D. carota*

Chapter 4 *Ipomoea batatas* (L.) Lam – An Economically Important Species

Contents

D. carota was used as a model system to establish a range of biochemical techniques (see chapter 3), but though *D. carota* is a valuable vegetable crop in certain areas, it is not one of the major global food sources. It was decided, therefore, to direct studies towards somatic embryogenesis in the globally significant crop *I. batatas*. *Ipomoea batatas,* or sweet potato, is an important root crop of the developing world, forming a staple of subsistence farmers (Henderson, *et. al.* 1986, Golmirzaie, *et. al.* 1999). Furthermore, it has been an important crop of focus for the International Potato Center (CIP) in Peru. *I. batatas* is the seventh most important food crop after wheat, rice, maize, potato, barley and cassava. *I. batatas* has a higher edible energy content than many other crops and will produce abundant yields even in poorer soils with limited water supplies. The improvement of existing species and cultivars forms one of the main goals of research at CIP, to maximise crop yields by producing cultivars that are resistant to diseases and insect pests such as weevils. Routine propagation in the field is by vine cuttings and this approach makes it difficult to maintain virus and pathogen free stocks. Maintaining culture collections in the field is also very costly, and cultivars are at risk from a major disease outbreak or natural disaster resulting in a loss of the entire crop. *In vitro* propagation forms one of the best methods for maintaining the greatest range of viable cultivars that are pathogen and virus free (Henderson, *et. al.* 1986). However, even this can be expensive, and some cultivars are difficult to establish in a productive propagation routine (Henderson, *et. al.* 1986).

One method of improving this species is applying *in vitro* manipulation of cell lines that are able to produce somatic embryos. A range of species and cultivars have been established in gene banks and culture collections and form the basis of many *in vitro* studies (Golmirzaie, *et. al.* 1999). A number of studies have attempted to generate somatic embryos from *I. batatas* species derived from a wide geographical distribution (Al-Mazrooei, *et. al.* 1997, Zheng, *et. al.* 1996, Jarrett, *et. al.* 1984, Bieniek, *et. al.* 1995). Some studies have been successful and have generated somatic embryos from a range of cultivars (Zheng, *et. al.* 1996), however there are a small number that fail to produce embryos (Jarret, *et. al.* 1984, Al-Mazrooei, *et. al.* 1997). However, variability among different cultivars makes finding a suitable regime to maximise responses from the majority very difficult and some cultivars may not respond sufficiently to produce adequate numbers of embryos. It has been shown by a number of research groups that there are a wide range of responses by different cultivars to the initiation of somatic embryos and the manipulation of micro-propagated plantlets in tissue culture (Zheng, *et. al.* 1996, Jarrett, *et. al.* 1984). The concentration of the hormones can be crucial in forming embryogenic callus, for example for the cultivar TIB 10, a concentration of 10 μM 2,4-D was needed to achieve a embryogenic response of over 60%, lower concentrations produced little or no response (Al-Mazrooei, *et. al.* 1997).

Oxidative stress has been implicated in such variability in other species and may be influential in dictating the responsiveness of different cultivars of *I. batatas* under *in vitro* conditions. Under some circumstances certain cultivars may be completely recalcitrant (Benson, *et. al.* 1997, Benson & Roubelakis-

Angelakis, 1992 & 1994). Therefore, investigations into causal mechanisms affecting oxidative stress, antioxidants and their associated enzymes, within the cells that combat reactive oxygen species responsible for the stress may give indications into the best regimes to minimise stress and maximise the responses of the widest range of cultivars possible.

Aims and Objectives of the Study of *I. batatas*

The objectives of the study of *I. batatas* are, using the tools developed within *D. carota*, were to investigate the role of oxidative stress and its implications in the responses of this economically significant crop.

The main aims of the study of *I. batatas* tissue are:

1 To compare two different culture regimes for the generation of callus and somatic embryos.

2 To profile the activity of a range of antioxidants over the early stages of callus initiation.

3 To determine level of HNE-protein adducts within extracts of *I. batatas* using the enzyme linked immunosorbant assay developed within *D. carota* systems.

4 To establish profiles of the HNE and MDA content of callus generated from a range of cultivars and explants.

5 To study the generation of hydroxyl radicals using gas chromatography during the early stages of callus initiation.

4.1 Results - Characterisation and Profile of Cultivars of *I. batatas*

4.1.1 Micropropagation of Different Cultivars

Using the method of Al-Mazoorei *et. al.* (1997) which employs the hormone 2,4,5-trichorophenoxy acetic acid (2,4,5-T), 5 different clones of *I. batatas* were observed over a four-week period using the parameters, total height of the plantlets and the number of nodes of the plantlets. Figure 4.1.1.1 and 4.1.1.2, show a significant difference between the clones for both height and the number of nodes ($F(4,12)=3.28$, $p<0.05$, and $F(4,12)=5.26$, $p<0.05$, respectively).

Figure 4.1.1.1 Time Course of Growth (plant height) of *I. batatas* Plantlets during Micropropagation on 2,4,5-T Media over 6 weeks

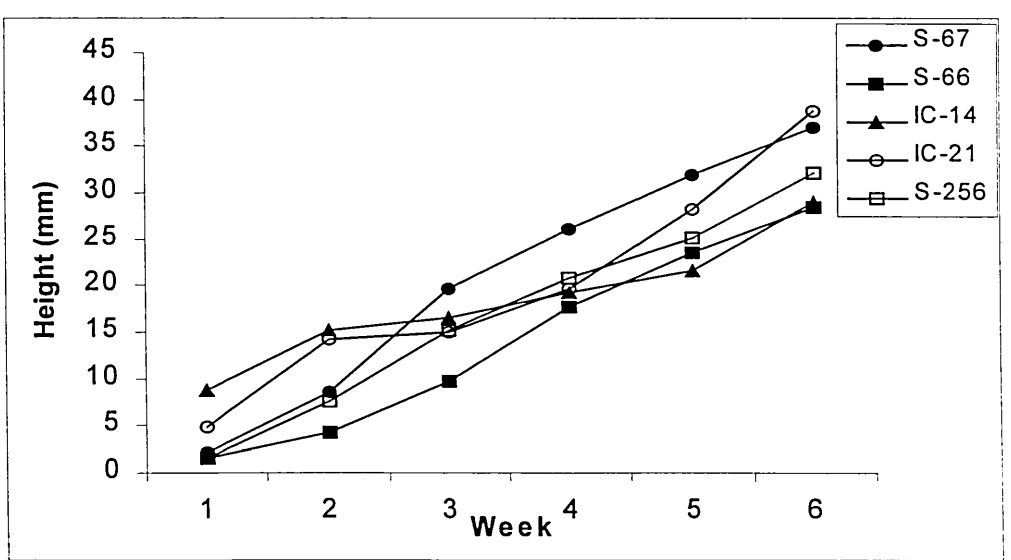


Figure 4.1.1.1: Time course of growth (plant height) of *I. batatas* plantlets during micropropagation on 2,4,5-T Media over 6 weeks for 5 cultivars. Data points are means of three replicates.

Figure 4.1.1.2 Time Course of Growth (number of nodes) of *I. batatas* Plantlets during Micropropagation on 2,4,5-T Media over 6 weeks

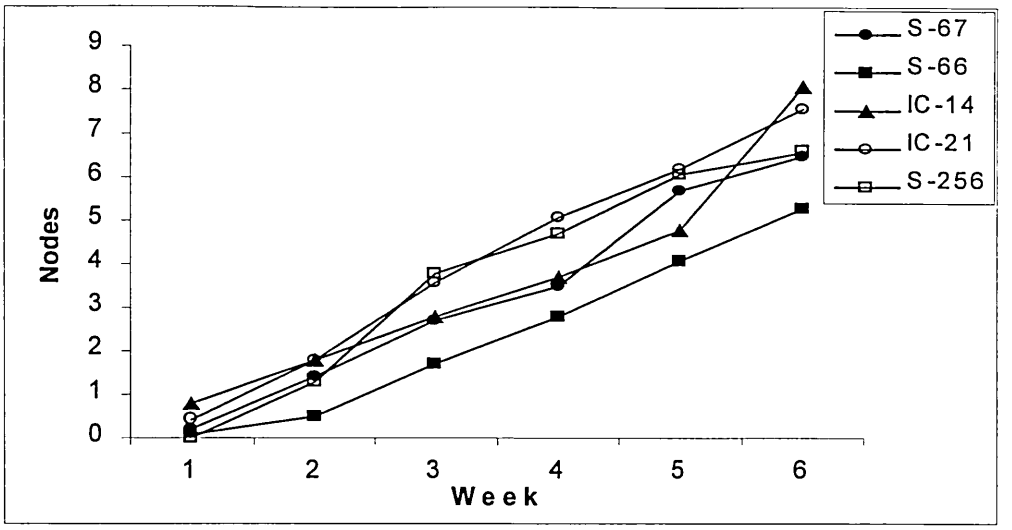


Figure 4.1.1.2: Time course of growth (number of nodes) of *I. batatas* plantlets during micropropagation on 2,4,5-T Media over 6 weeks for 5 cultivars. Data points are means of three replicates.

4.1.2 Induction of Callus in Different Cultivars of *I. batatas* using 2,4,5-T Medium

2,4,5-Trichorophenoxyacetic acid (2,4,5-T) is a related derivative of 2,4-dichorophenoxyacetic acid (2,4-D) and both have been used as hormones for the induction of callus from a range of species. Al-Mazrooei, *et. al.* (1997) have used 2,4,5-T and 2,4-D to generate embryogenic callus from a range of different *I. batatas* cultivars. Various concentrations of the compounds have been used to generate embryogenic callus from cultivars from countries such as Peru, Nigeria and South Africa (Al-Mazrooei, *et. al.* 1997). The concentration within the medium that gave the best response from the widest range of the cultivars was used for the induction of callus from leaves of three cultivars of *I. batatas*, IC-14, IC-21 and S-256, and was designated as 2,4,5-T medium. The results are shown in Figure 4.1.2.1 and show the significant

difference between the cultivars ($F(2,54)=41.87$, $p<0.001$) and over the course of the 7 weeks ($F(6,54)=13.97$, $p<0.001$).

Figure 4.1.2.1 Time Course of Proliferation (as % callus coverage) of Leaves of 3 Cultivars of *I. batatas* on 2,4,5-T Medium

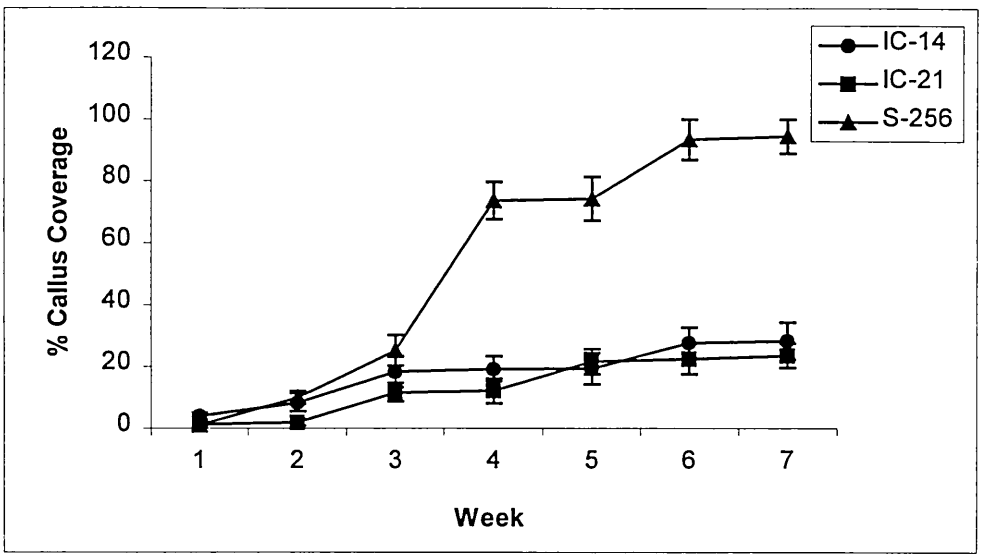


Figure 4.1.2.1: Time course of proliferation as % of leaves covered by callus of three cultivars of *I. batatas* after 7 weeks on 2,4,5-T medium. Data points are means of 3 tissue replicates, with errors expressed as standard deviations.

4.1.3 Induction of Callus in Different Cultivars of *I. batatas* using Medium Containing Abscisic acid

The method of Zheng, *et. al.* 1996, using medium containing abscisic acid (S2 medium, see section 2.2) was used to compare the rate of callus growth on three cultivars of *I. batatas*, in comparison to the rates grown on 2,4,5-T medium (see section 2.2). Figure 4.1.3.1 shows that there are no significant differences between the three cultivars ($F(2,36)=3.14$, $p>0.05$), but there is a significant difference over the course of the 5 week period ($F(4,36)=103.01$, $p<0.001$). This is in contrast to the results for callus induction on 2,4,5-T medium, which did show a difference between the cultivars. It was also noted

that during the early weeks of callus induction some of the callus contained some anthocyanin pigmentation. However, despite the production of a large number of different callus lines, no line produced was ever capable of producing any somatic embryos, and only rhizogenesis was noted in a number of the callus lines.

Figure 4.1.3.1 Time Course of Proliferation (as % callus coverage) of 3 Cultivars of *I. batatas* on Abscisic acid Medium Over 5 Weeks

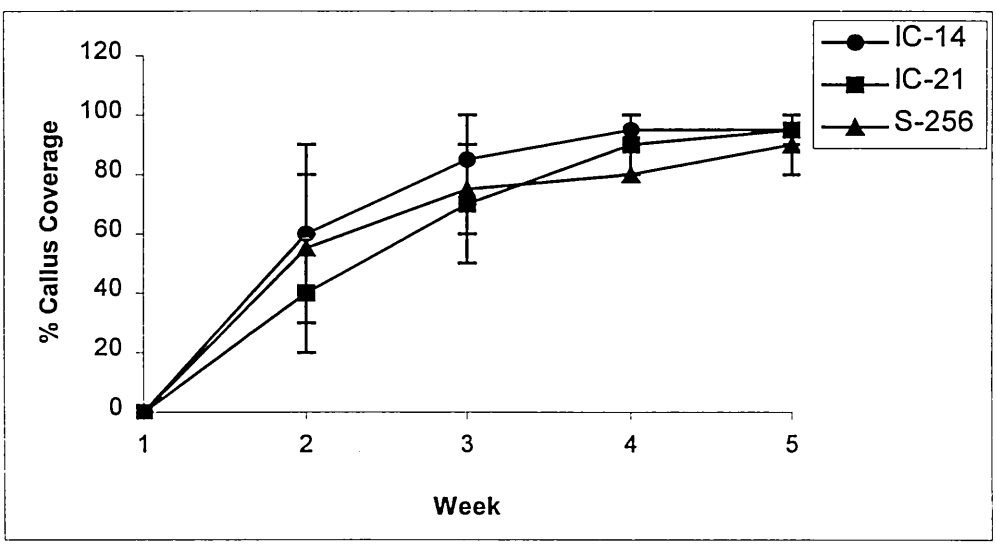


Figure 4.1.3.1: % of Leaves covered by callus of three cultivars of *I. batatas* after 5 weeks on S2 (abscisic acid) medium. Data points are means of 3 tissue replicates, with errors expressed as standard deviations.

4.2 Biochemical Studies - Protein and Antioxidants

Antioxidants are thought to play an important role in mechanisms of defence within plant cells and their activity can give an indication of the level of oxidative stress being experienced in the cells (Bartosz, 1997). Monitoring changes in levels of activity during the early stages of callus initiation may give an indication as to the level of oxidative stress within the tissues (Benson & Roubelakis-Angelakis, 1994). Studies in *I. batatas* followed those in *D. carota* and extended the range of enzymes investigated. Glutathione and the ratio between its reduced and oxidised forms have been particularly implicated in somatic embryogenesis (Wann, *et. al.* 1989) and therefore emphasis was placed on these and associated enzymes during studies of the early stages of callus initiation.

Peroxidase activity has been the focus of a great deal of attention and is now considered as a developmental marker (Krsnik-Rasol, 1991). Therefore a rise in activity may give an indication of a change in developmental status. Monitoring the level of catalase in tissues under stressful situations may also give an indication as to the oxidative status of the tissue and to whether dangerous levels of H_2O_2 are being allowed to accumulate and damage cells.

Compounds containing sulfhydryl groups (SH groups) can be divided into two types, low molecular weight compounds such as glutathione (see 1.3.5.4) and high molecular weight thiols (protein bound SH groups) (Faure & Lafond, 1995). Protein SH groups have been considered as important agents in

protecting cells against NO_2 and lipid peroxidation (Halliwell, *et. al.* 1982) (see section 1.3.5.3). The oxidation of SH groups can lead to membrane damage and altered metabolism (Chevrier, *et. al.* 1988) and therefore monitoring the degree of SH groups within protein and non-protein could give an indication of the role of SH groups within oxidative stress.

Glutathione and associated enzymes have important roles in stress tolerance, senescence, defence and tissue culture (Foyer, *et. al.* 1997, May, *et. al.* 1996, Xiang & Oliver, 1998, Jiménez, *et. al.* 1998, Stajner, *et. al.* 1993) (see section 1.3.5.4). Glutathione is very important for the protection of membranes sensitive to reactive oxygen species and acts as a competitor to oxidative damage by being preferentially oxidised over membrane lipids. The tripeptide form has to be recycled after it has been sacrificed and oxidised by reacting with reactive oxygen species. The enzymes involved in this recycling also play an important role in the supply of adequate glutathione for the protection of growing cells. Profiling the oxidised and reduced forms could give an indication as to the role of glutathione in cells of different morphological status.

Glutathione in its reduced form can be recycled by associated enzymes such as glutathione reductase which link it to the oxidation of NAPDH in the Halliwell-Asada cycle (Bartosz, 1997). Glutathione can also be used for the conjugation and removal of reactive compounds such as herbicides by the action of glutathione S-transferase and the increased expression of this enzyme appears to enhance the growth of tobacco seedlings during chilling and salt

stress (Roxas, *et. al.* 1997). It appears that the enzymes involved in glutathione conjugation and recycling are more significant than the levels of the glutathione synthetase enzyme particularly for the resistance to photo-inhibition in poplar trees (Foyer, *et. al.* 1995). Therefore monitoring the activity of such enzymes would indicate the extent that glutathione can be recycled and utilised within plant cells.

Glutathione S-transferase (GST) mediates the conjugation of glutathione to a variety of potentially toxic species and is particularly important in the detoxification of herbicides and appears to enhance the growth of tobacco seedlings during chilling and salt stress (Roxas, *et. al.* 1997). GST activity within mammalian systems has been linked to the metabolism of hydroxynonenal (Ansari, *et. al.* 1996).

To assess some of the effects attributable, nodes of *I. batatas* were transferred onto Petri dishes containing 2,4,5-T medium as in section 2.2. Samples were taken over approximately two weeks in order to determine the changes in antioxidants that the explants experience under the influence of the high hormone concentrations used to induce the production of dedifferentiated callus tissue. The activity of a range of antioxidants was profiled and the emphasis was placed on glutathione and associated enzymes.

4.2.1 Changes in Protein Content of *I. batatas* Nodes Undergoing Callus Induction

Two cultivars of *I. batatas* were profiled for protein content over a time-course, 15 days after transfer to medium containing 2,4,5-trichoro-phenoxyacetic acid (2,4,5-T). Figure 4.2.1 indicates significant differences between the cultivars ($F(1,32)=22.46$, $p>0.001$) and for the days after transfer onto 2,4,5-T media ($F(6,32)=59.05$, $p<0.001$) and indicate an increase in protein content over the course of two weeks after transfer.

Figure 4.2.1 Time Course of Changes in Protein Content of Two Cultivars of *I. batatas* after Transfer to 2,4,5-T Medium

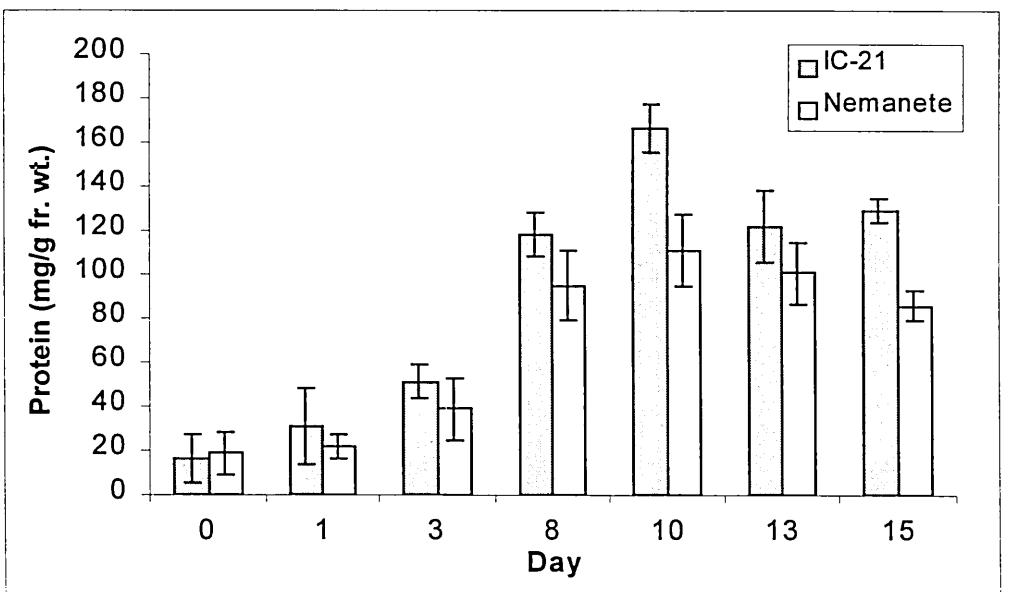


Figure 4.2.1: Protein concentrations in cultivars IC-21 (grey bar) and Nemanete (white bar) of *I. batatas* after transfer to 2,4,5-T media and expressed as $mg.^{-1}$gram fresh weight of tissue. Data points are means of 3 tissue replicates and the error bars are expressed as standard deviations.

4.2.2 Peroxidase and Catalase Activity of *I. batatas* Nodes Undergoing Callus Induction

Peroxidase activity was determined for nodes of *I. batatas* for two cultivars undergoing callus initiation on 2,4,5-T medium over a two-week period. The results are shown in Figure 4.2.2 and show significant differences over the time course period ($F(6,33)=10.35$, $p<0.001$), although there was no difference between the two cultivars ($F(1,33)=0.00$, $p>0.05$). Catalase activity was measured from the same samples and showed very low levels for almost all the samples, only the final days results showed any activity and the average for IC-21 was 0.034±0.021 Absorbance/min/g fresh weight.

Figure 4.2.2 Time Course of Changes in Peroxidase Activity of Two Cultivars of *I. batatas* after Transfer to 2,4,5-T Medium

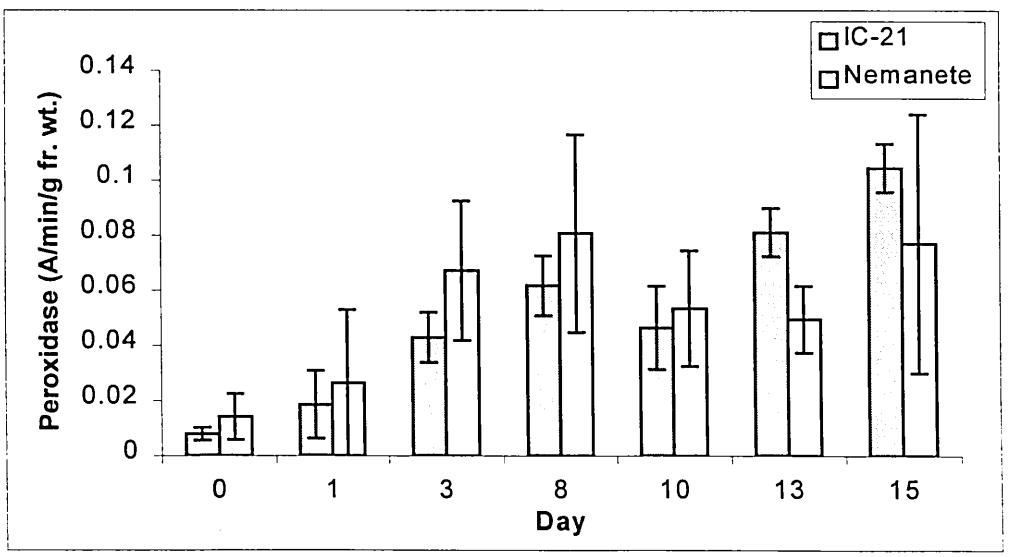


Fig 4.2.2: Peroxidase activity in cultivars IC-21 (grey bar) and Nemanete (white bar) of *I. batatas* after transfer to 2,4,5-T media and expressed as Absorbance/min/mg protein. Data points are means of 3 tissue replicates and the error bars are expressed as standard deviations.

4.2.3 Sulphydryl Group Content of Nodes of *I. batatas* after transfer to 2,4,5-T Medium

4.2.3.1 Total SH Group Content of Nodes of *I. batatas* after transfer to 2,4,5-T Medium

The total SH group content and non-protein SH group content (see 4.2.4.2) were profiled for nodes of two cultivars of *I. batatas* over a two-week period after being transferred to 2,4,5-T medium in order to monitor changes stimulated by the induction of the formation of callus.

Figure 4.2.3.1 shows significant differences in SH group content between the days after transfer and that there was an interaction between the cultivar and day, although there was no significant difference between the two cultivars ($F(5,22)=3.61$, $p<0.05$, $F(5,22)=2.74$, $p<0.05$, and $F(1,22)=1.72$, $p>0.05$ respectively).

Figure 4.2.3.1 Total SH Groups Content of Two Cultivars of *I. batatas* after Transfer to 2,4,5-T Medium

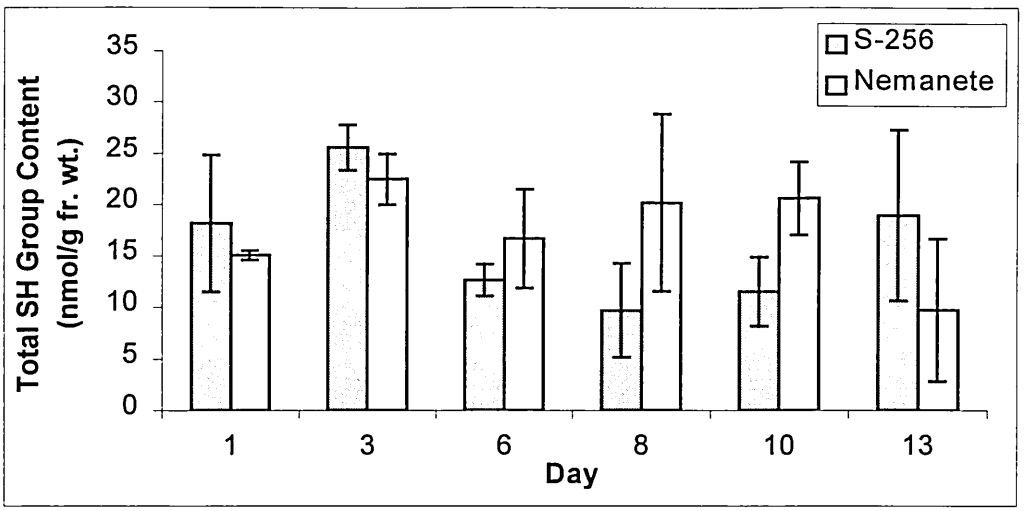


Figure 4.2.3.1 Total SH group content of cultivars S-256 (grey bar) and Nemanete (white bar) of *I. batatas* after transfer to 2,4,5-T media and expressed as nmol/g fresh weight. Data points are means of 3 tissue replicates and the error bars are expressed as standard deviations.

4.2.3.2 Non-protein SH Group Content of Nodes of *I. batatas* after transfer to 2,4,5-T Medium

The non-protein SH groups were measured from similar samples and the results ranged from 3.3 ± 2.5 to 11.3 ± 5.9 nmol/gram fresh weight, although there were no significant differences between either the cultivars or over the days ($F(1,27)=0.03$, $p>0.05$, $F(6,27)=1.60$, $p>0.05$).

4.2.4 Glutathione Content of Nodes of *I. batatas* after transfer to 2,4,5-T Medium

Glutathione is both its reduced and oxidised forms was profiled in nodes of two cultivars of *I. batatas* after transfer to 2,4,5-T medium for the induction of callus over a two-week period.

4.2.4.1 Reduced Glutathione Content of Two Cultivars of *I. batatas* after Transfer to 2,4,5-T Medium

The reduced glutathione content was measured in nodes of cultivars Nemanete and S-256, after transfer to 2,4,5-T medium over a period of two weeks. Figure 4.2.4.1 shows that there were no significant differences between the two different cultivars, however there were significant differences between the days and an interaction between the cultivars and the days ($F(1,24)=0.09$, $p>0.05$, $F(6,24)=21.59$, $p<0.001$, and $F(6,24)=12.79$, $p<0.001$).

Figure 4.2.4.1 Time Course of Reduced Glutathione Content of Two Cultivars of *I. batatas* after Transfer to 2,4,5-T Medium

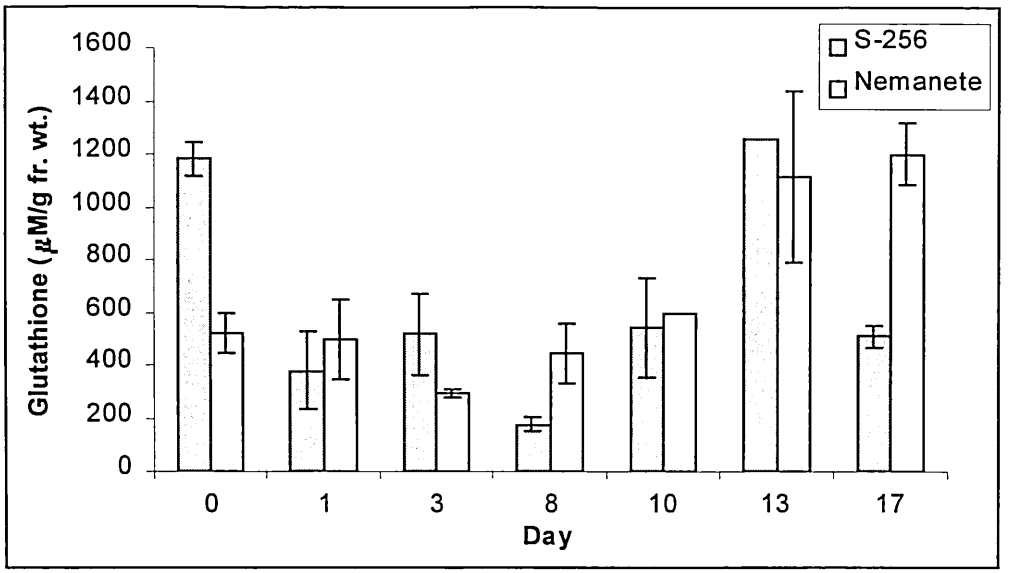


Figure 4.2.4.1: Reduced glutathione (GSH) content of cultivars S-256 (grey bar) and Nemanete (white bar) of *I. batatas* after transfer to 2,4,5-T media and expressed as µM/g fresh weight. Data points are means of 3 tissue replicates and the error bars are expressed as standard deviations.

4.2.4.2 Oxidised Glutathione Content of Two Cultivars of *I. batatas* after Transfer to 2,4,5-T Medium

The oxidised glutathione content was measured in nodes of cultivars Nemanete and S-256, after transfer to 2,4,5-T medium over a period of two weeks. Figure 4.2.4.2 shows that there were no significant differences between the two different cultivars, however there were significant differences between the days and an interaction between the cultivars and the days ($F(1,23)=2.58$, $p>0.05$, $F(5,23)=4.16$, $p<0.001$, and $F(5,23)=4.68$, $p<0.001$).

Figure 4.2.4.2 Time Course of Oxidised Glutathione Content of Two Cultivars of *I. batatas* after Transfer to 2,4,5-T Medium

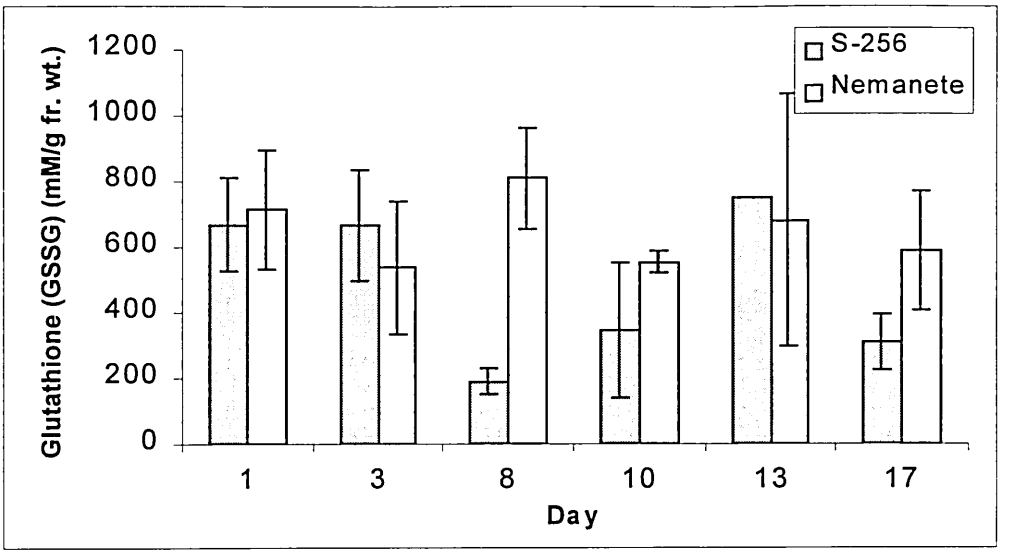


Figure 4.2.4.2: Oxidised glutathione (GSSG) content of cultivars S-256 (grey bar) and Nemanete (white bar) of *I. batatas* after transfer to 2,4,5-T media and expressed as μM/g fresh weight. Data points are means of 3 tissue replicates and the error bars are expressed as standard deviations.

4.2.5 Glutathione Reductase Activity of Two Cultivars of *I. batatas* after Transfer to 2,4,5-T Medium

Glutathione reductase activity was measured in nodes of *I. batatas* during the first two weeks after being transferred onto 2,4,5-T media, for the induction of callus. Activity was noted in all samples and Figure 4.2.5 shows the significant differences between the cultivars and over the time-course of two weeks ($F(1,29)=15.40$, $p>0.001$, and $F(6,29)=7.15$, $p>0.001$) and generally indicates a decrease in activity over time.

Figure 4.2.5 Time Course of Glutathione Reductase Activity of Two Cultivars of *I. batatas* after Transfer to 2,4,5-T Medium

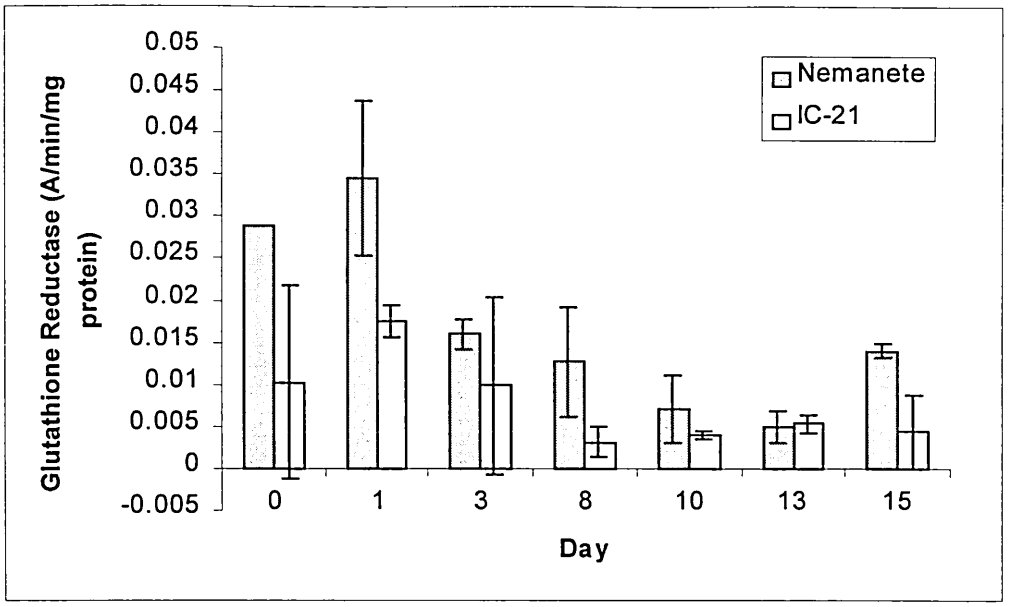


Figure 4.2.5: Glutathione reductase activity in cultivars IC-21 (grey bar) and Nemanete (white bar) of *I. batatas*, after transfer to 2,4,5-T media and expressed on A/min/mg protein. Data points are means of 3 tissue replicates and the error bars are expressed as standard deviations.

4.2.6 Glutathione S-transferase Activity in Two Cultivars of *I. batatas* after Transfer to 2,4,5-T Medium

Glutathione S-transferase was measured in nodes of two cultivars of *I. batatas* during the first two weeks after being transferred onto 2,4,5-T media (for the induction of callus). Very low activities were measured in all samples, although there were no significant differences between the two cultivars, there were significant differences over the two week period and the results are shown in Figure 4.2.6 ($F(1,24)=0.63$, $p>0.05$, $F(6,24)=3.15$, $p<0.05$).

Figure 4.2.6 Time Course of Glutathione S-transferase Activity of Two Cultivars of *I. batatas* after Transfer to 2,4,5-T Medium

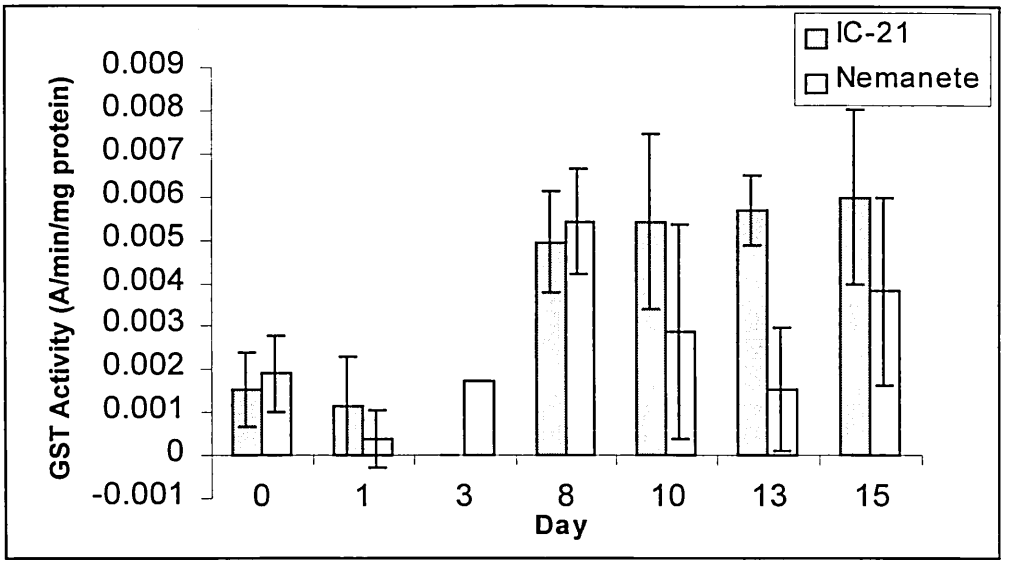


Figure 4.2.6: Glutathione S-transferase activity in cultivars IC-21 (grey bar) and Nemanete (white bar) of *I. batatas*, after transfer to 2,4,5-T media and expressed as Absorbance/min/mg protein. Data points are means of 3 tissue replicates and the error bars are expressed as standard deviations.

4.3 Hydroxynonenal and Malondialdehyde Content of *I. batatas*

Oxidative stress and lipid peroxidation has been documented within a number of different plants species and has shown the presence of various oxidative products (Benson & Roubelakis-Angelakis, 1992, Chaoui, *et. al.* 1997, Culter, *et. al.* 1989, Robertson, *et. al.* 1995). Initial studies investigating hydroxynonenal (HNE) were performed in cultures of *D. carota* and showed the presence of HNE in a range of cultures with and without embryogenic capacity (Roberston, *et. al.* 1995, Deighton, *et. al.* 1997). Further studies were performed on *D. carota* (see section 3.3) and compared the HNE and malondialdehyde (MDA) content within non-embryogenic and embryogenic tissue. However, the presence of HNE has been little documented within other species of plant tissue and not before within *I. batatas.* Furthering

investigations into lipid peroxidation and the associated secondary products has lead to the detection of HNE and MDA using LC-MS, in tissue generated from of range of explants of several different cultivars of *I. batatas* originally collected in different countries (see Table 2.1.2, for further details). The comparison of HNE and MDA content of callus generated from a range of explants may hold important implications in practical tissue culture on the explants chosen for conservation and germplasm collections. High levels of HNE and MDA may indicate a higher level of oxidative stress and therefore callus initiated from explants that show high levels of both compounds is likely to be less responsive.

Callus initiated from a range of explants was compared for their HNE and MDA content. Callus initiated from leaves on two different media regimes were also compared for their HNE and MDA content.

4.3.1 Comparison of Hydroxynonenal and Malondialdehyde Content of Callus Generated from Different *I. batatas* Explants

Three different explants were used to generate callus on S1 medium (that contained 2,4-dichorophenoxy acetic acid (2.5 mg/L) and benzylamino purine (0.25 mg/L)) from three different cultivars, IC-14, IC-21, and S-256. The explants were leaves, petioles and internodal sections (stem sections cut between leaf nodes). HNE and MDA (after extraction and derivatisation) were measured by LC-MS in callus samples that had been maintained on S1 medium (see methods in section 2.6). Figure 4.3.1.1 shows the MDA content and that there were significant differences between the cultivars and explants ($F(2,79)=44.15$, $p<0.001$, $F(2,79)=20.77$, $p<0.001$) and there was also a

significant interaction between the cultivars and explants ($F(4,79)=10.41$, $p>0.001$).

Figure 4.3.1.1 Malondialdehyde Content of Callus Generated from Different Explants of three Cultivars of *I. batatas*

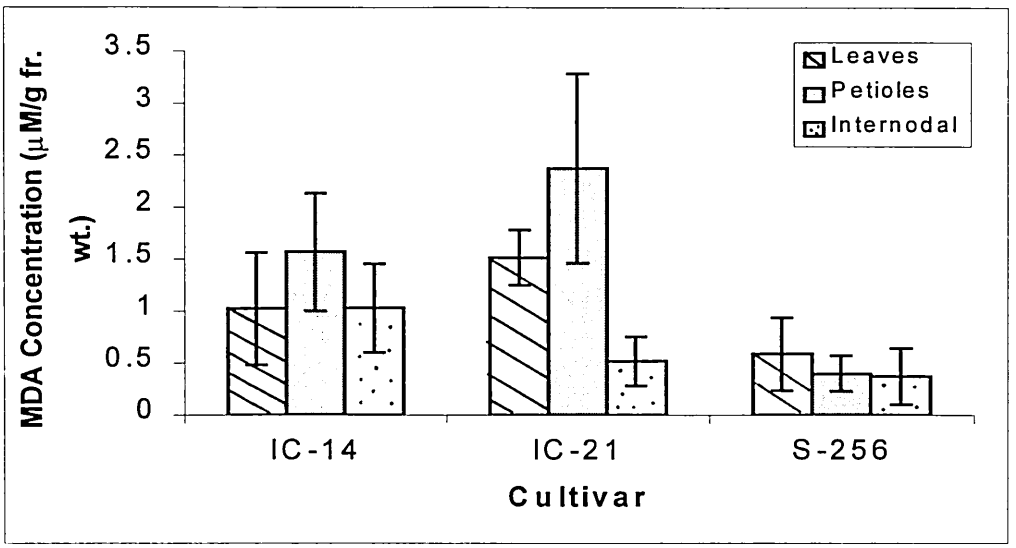


Figure 4.3.1.1 Malondialdehyde content of lines of *I. batatas* cultivars IC-14 (striped bars), IC-21 (solid grey bars), S-256 (spotted bars), on S1 media. Data points are expressed as means of nine tissue replicate samples that were run in duplicate, with errors expressed as standard deviations.

Figure 4.3.1.2 shows the HNE content and that there were significant differences between both the cultivars and the explants ($F(2,82)=25.71$, $p<0.001$, $F(2,82)=14.11$, $p<0.001$, respectively). There was also a significant interaction between the cultivars and explants ($F(4,82)=5.38$, $p<0.001$).

Figure 4.3.1.2 Hydroxynonenal Content of Callus Generated from Different Explants of three Cultivars of *I. batatas*

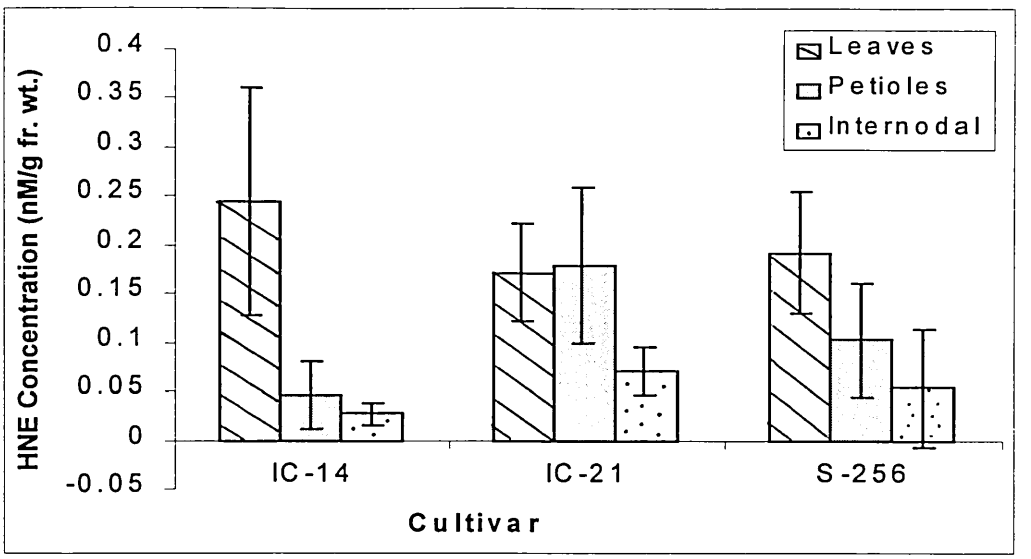


Figure 4.3.1.2 Hydroxynonenal content of lines of *I. batatas* cultivars IC-14 (striped bars), IC-21 (solid grey bars), S-256 (spotted bars), on S1 media. Data points are expressed as means of nine tissue replicate samples that were run in duplicate, with errors expressed as standard deviations.

4.3.2 Hydroxynonenal and Malondialdehyde Content of Callus Generated From Leaves of *I. batatas* Exposed to Different Media Regimes

Callus was generated from leaf sections of three cultivars of *I. batatas* using two different media regimes using 2,4,5-T, and S1 media that contained 2,4-dichorophenoxy acetic acid (2.5 mg/L)and benzylamino purine (0.25 mg/L) (Zheng, *et. al.* 1996). Samples were then compared for hydroxynonenal and malondialdehyde content using LC-MS. MDA and HNE were measured in samples from leaves of *I. batatas.* HNE was measured in the range of 0.03 – 0.37 nM /g fresh weight, however there was no significant trends in the HNE content between cultivars or media regimes. The MDA content of samples from leaves, are shown in Figure 4.3.2.1, and show significant trends for both cultivar and media and an interaction between cultivar and media

($F(2,11)=161.68$, $p<0.001$, $F(1,11)=242.02$, $p<0.001$, and $F(2,11)=150.40$, $p<0.001$).

Figure 4.3.2.1 Malondialdehyde Content of Callus Generated from Leaves from three Cultivars of *I. batatas* on Two Different Media Types

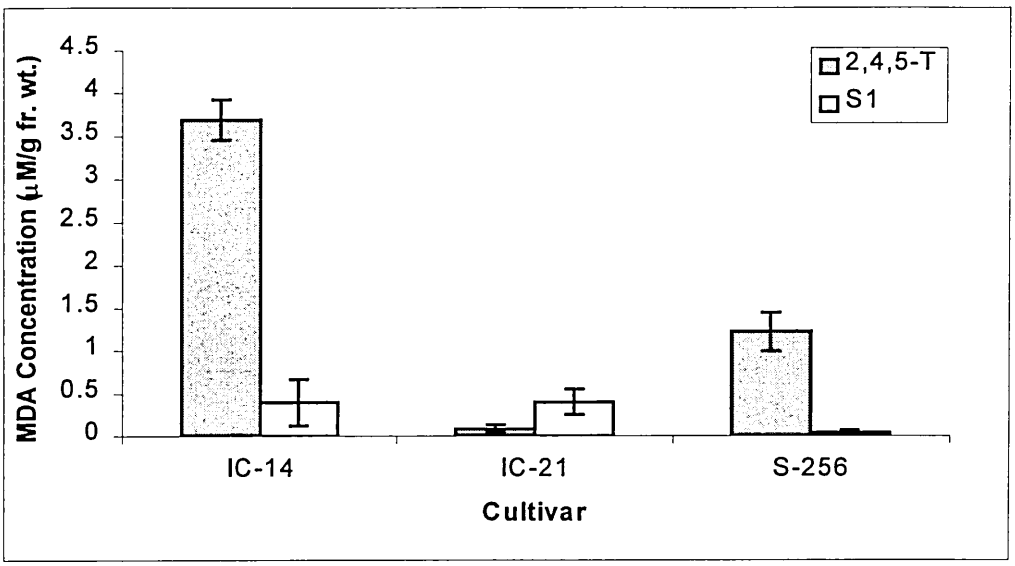


Figure 4.3.2.1 Malondialdehyde content of callus lines generated from leaves of *I. batatas* cultivars IC-14, IC-21, and S-256 on 2,4,5-T media (grey bars), and S1 media (white bars). Data points are expressed as means of three tissue replicate samples that were run in duplicate, with errors expressed as standard deviations.

4.4 Quantification of HNE-Protein Adducts by ELISA in *I. batatas*

Following the establishment of an enzyme linked immunosorbant assay (ELISA) using protein extracts of *D. carota,* extracts of a range of samples of *I. batatas* were profiled for the presence of HNE-protein adducts.

4.4.1 ELISA of Callus from Leaves of Three Cultivars of *I. batatas* Exposed to Two Media Regimes

Callus was established from leaves of three cultivars of *I. batatas* using two different media regimes. The first set of samples was from callus generated on medium containing 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (see section 2.2 for full details of media and routines). The second set of samples was from callus established on S1 medium (containing 2.5 mg/L 2,4-D and 0.25 mg/L BAP). Soluble protein was extracted from all samples using the routine method detailed in section 2.5.1. Extracts were prepared from samples of IC-14, IC-21, and S-256 for both media regimes. Results of the first group are shown in figure 4.4.1.1 and indicate that there are HNE-protein adducts within all three cultivars on 2,4,5-T medium. A replicate assay showed similar results (results not shown).

Figure 4.4.1.1 ELISA of Callus lines of Leaves of Three Cultivars of *I. batatas* on 2,4,5-T medium

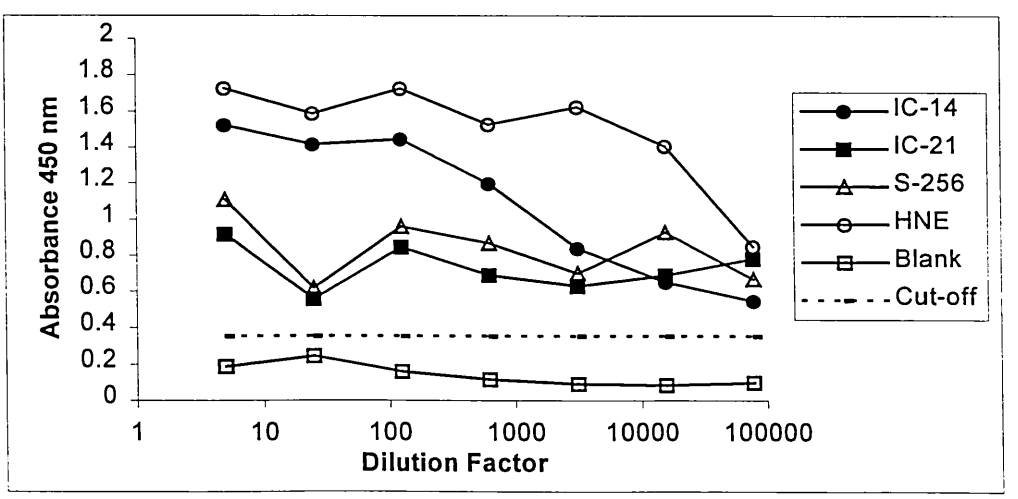


Figure 4.4.1.1 Absorbance at 450 nm of soluble protein samples of callus lines generated from leaves of IC-14, IC-21, S-256 cultivars of *I. batatas* on 2,4,5-T medium, with standard of HNE-BSA (HNE), and controls containing only phosphate buffered saline (Blank). The positive-negative threshold is three times the average blank (Cut off). Primary and secondary antibodies were used at a 1/200 dilution.

The second set of samples of callus maintained on S1 medium was assayed for HNE-protein adducts and the results in Figure 4.4.1.2 show that there are HNE-adducts present within all of the cultivars assayed. Although, from this medium only IC-21 and IC-14 showed positive samples, in contrast to the samples on 2,4,5-T medium that showed that all three cultivars had HNE-protein adducts.

Figure 4.4.1.2 ELISA of Callus lines of Leaves of Three Cultivars of *I. batatas* on S1 Medium

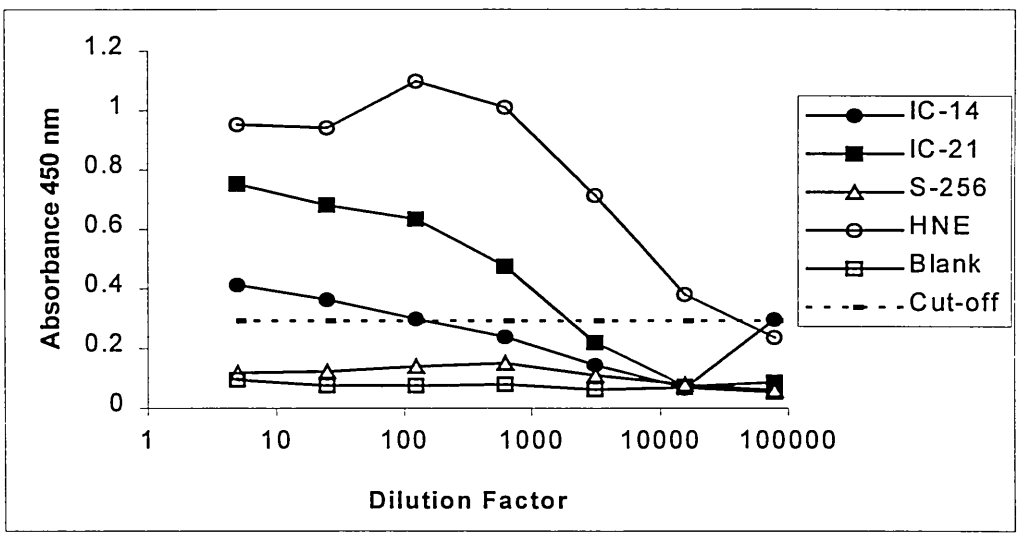


Figure 4.4.1.2 Absorbance at 450 nm of soluble protein samples of callus lines generated from leaves of IC-14, IC-21, S-256 cultivars of *I. batatas* on S1 medium, with standard of BSA-HNE (HNE), and controls containing only phosphate buffered saline (Blank). The positive-negative threshold is three times the average blank (Cut off). Primary and secondary antibodies were used at a 1/200 dilution.

4.4.2 ELISA of Callus from Internodal Sections of Three Cultivars of *I. batatas*

Protein extracts of callus generated on S1 media (containing 2.5 mg/L 2,4-dichorophenoxyacetic acid, 0.25 mg/L benzylamino purine) from internodal sections of cultivars IC-12, IC-21 and S-256 were assayed for HNE-protein adducts. The results are given in Figure 4.4.2.1 and show that all three

cultivars had HNE-protein adducts and two identical assays showed similar results, although IC-21 showed only a marginally positive result.

Figure 4.4.2.1 ELISA of Callus lines of Internodal Sections of Three Cultivars of *I. batatas* on S1 Medium

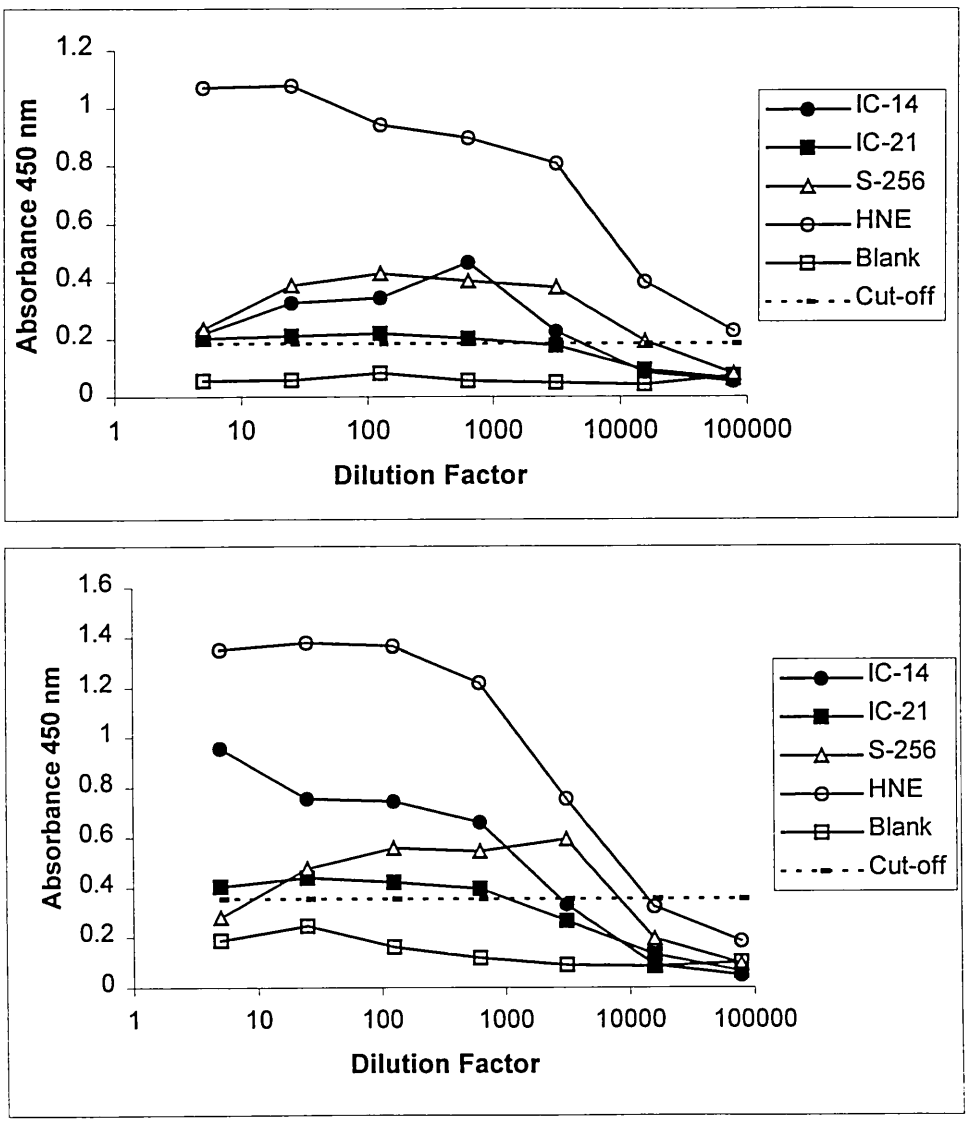


Figure 4.4.2.1 Absorbance at 450 nm of soluble protein samples of callus lines generated from internodal sections of IC-14, IC-21, S-256 cultivars of *I. batatas* on S1 medium, with standard of HNE-BSA (HNE), and controls containing only phosphate buffered saline (Blank). The positive-negative threshold is three times the average blank (Cut off). Primary and secondary antibodies were used at a 1/200 dilution.

4.5 Determination of Volatiles as Measurement of Hydroxyl Radical Activity and Ethylene by Gas Chromatography in Nodes of *I. batatas*

Hydroxyl radicals are generated when superoxide reacts with hydrogen peroxide, which are routinely generated within the cell (see section 1.3.2). If catalase activity is low and the hydrogen peroxide concentration is allowed to build up it will lead to the increased generation of hydroxyl radicals. Therefore, the level of activity of catalase can directly effect the level of generation of hydroxyl radicals. The hydroxyl radical is an extremely reactive free radical species and has been implicated in the initiation of lipid peroxidation. Therefore monitoring the activity of hydroxyl radicals within plant tissue cultures can give an indication as to the extent of free radical mediated stress within *in vitro* culture. Measurement of the activity of hydroxyl radicals directly is difficult due to the highly reactive nature of the species, techniques such as Electron Spin Resonance (EPR) can be used, but are expensive and the results are often difficult to interpret. An alternative way to measure hydroxyl radical activity is to measure the activity indirectly. This can be achieved by the use of dimethyl sulphoxide (DMSO), which reacts with hydroxyl radicals producing methane (Babbs & Steiner, 1990). The evolution of this methane can be measured using gas chromatography (GC) (Dillard & Tappel, 1979, Benson & Withers, 1987, Fleck, *et. al.* 2000).

Gas chromatography can also be used to detect the presence of ethylene and both compounds can be detected from the samples simultaneously. Ethylene is an important signal molecule within plant systems. Its generation has been linked to a number of different metabolic processes and stresses such as

senescence, mechanical trauma, and pathogen infection (Ecker, 1995). Therefore peaks of ethylene generated within plant cultures may be indicative of stress being experienced by the culture.

To monitor changes in the production of hydroxyl radicals during the initiation of callus, nodes of two cultivars of *I. batatas* were profiled for hydroxyl radical activity over three weeks after transfer to callus induction medium (2,4,5-T medium). The evolution of ethylene was also monitored and would give an indication of stress.

4.5.1 Hydroxyl Radical Activity of Nodes of *I. batatas* under callus initiation

Nodes from two cultivars of *I. batatas*, IC-14 and Nemanete were excised and placed onto 2,4,5-T medium in the presence of DMSO (full details are in section 2.8). Over a period of three weeks, headspace analysis was used to quantify the amount of methane and ethylene produced by the nodes on 2,4,5-T medium. Analysis was performed approximately every 4 days with venting every two days. Methane was measured for all samples of *I. batatas*, in the range of 0.2-8 ppm/hr/gram fresh weight and indicates the presence of hydroxyl radicals. However, there was no significant differences between the cultivars for all the sample days except day 2, where there was a significant difference between the two cultivars ($F(1,7)=19.22$, $p<0.01$) and the results are shown in figure 4.5.1.

Figure 4.5.1 Hydroxyl Radical Activity of *I. batatas* nodes on 2,4,5-T medium after 3 days

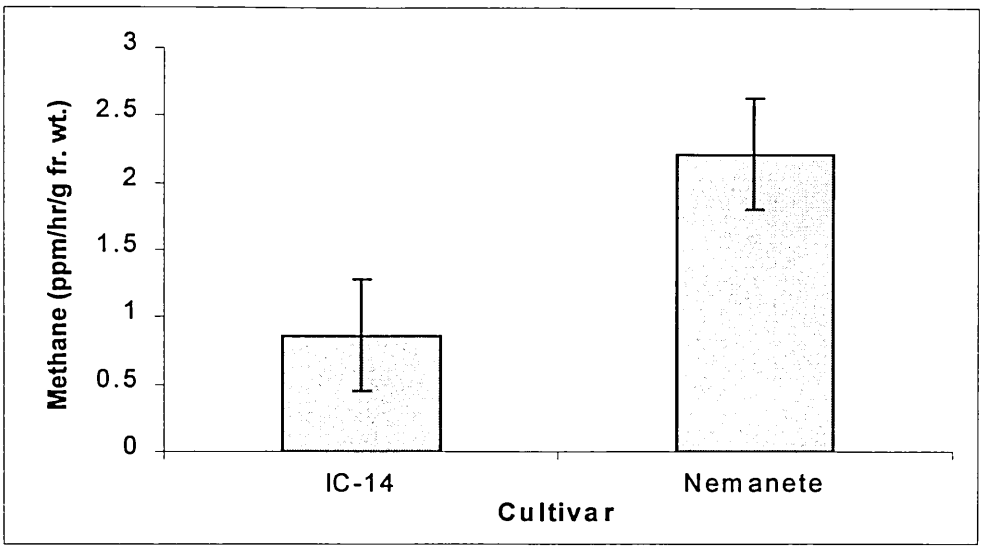


Figure 4.5.1. Hydroxyl radical activity of cultivars of *I. batatas,* Nemanete and IC-14 on 2,4,5-T media. Data points are expressed as means of five tissue replicates, with errors expressed as standard deviations.

Ethylene was also produced by many of the samples particularly for days 3 and 7 where the rates were as high as 30 ppm/hr/gram fresh weight. The results in Figure 4.5.2 show no ethylene content in IC-14 after the 7th day, and no ethylene was measured for either cultivar by the 21st day.

Figure 4.5.2 Ethylene Emission from Nodes of *I. batatas* on Callus Induction Medium after 21 days

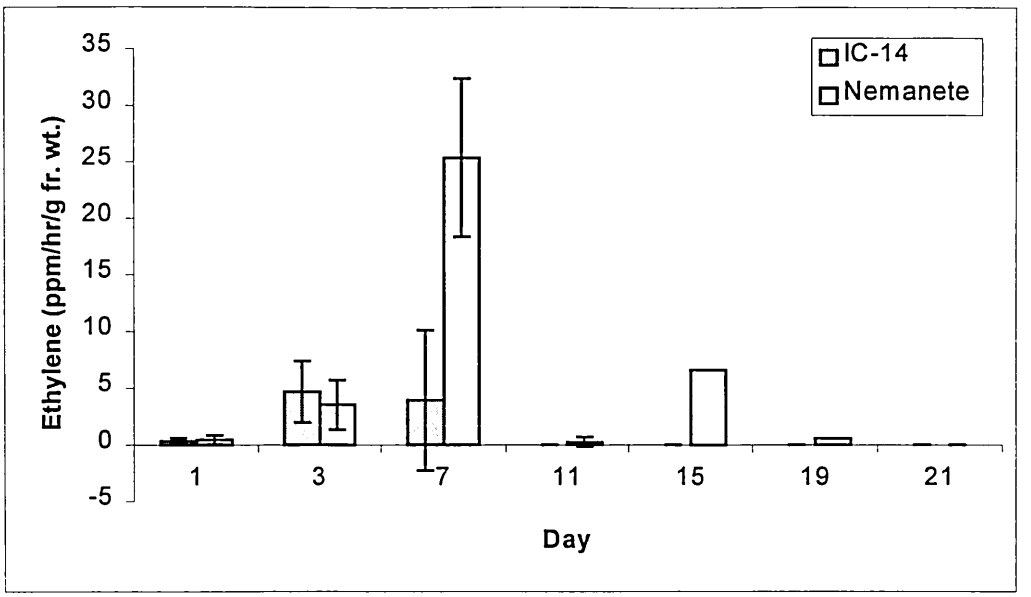


Figure 4.5.2. Hydroxyl radical activity of cultivars of *I. batatas,* IC-14 (grey bars) and Nemanete (white bars) on 2,4,5-T media. Data points are expressed as means of five tissue replicates, with errors expressed as standard deviations.

4.6 Discussion

4.6.1 *In vitro* Micropropagation and Callus Induction

D. carota has been used as a model system and as a basis for the study of oxidative stress and lipid peroxidation in plant systems within this project. However, despite range of studies on somatic embryogenesis in this species, it is a temperate species and therefore systems devised for this species may not be applicable to species from other climatic regions. Therefore, other species need to be studied to fully benefit the greatest number of conservation and germplasm collections and improvement of field cultivars. *I. batatas* was chosen in this study as it is an economically important third world crop for which the main route of field propagation is by vine cuttings, this method

makes keeping stocks virus and pathogen free difficult (Jarret, *et. al.* 1984). One method of trying to enhance the response and yield within the field is an improvement of the range of cultivars through *in vitro* micropropagation and somatic embryogenesis. Micropropagation methods can be used to rapidly multiply clonal stocks of suitable cultivars that are pathogen, virus and disease free. Various methods have been established that ensure rapid rates of producing plantlets from a wide range of cultivars from all areas of the world. Two of these methods have been employed within this project and show that a range of cultivars can be grown successfully within an *in vitro* environment by micropropagation methods using nodes from healthy (see section 4.1). However, methods developed for the induction of embryogenic callus are more problematic. Of the two methods used within this project, neither was able to successfully produce callus with the ability to produce somatic embryos (sections 4.1.2 and 4.1.3), although rhizogenesis was achieved in a number of cases. The first method was based on that of the study by Al-Mazrooei, *et. al.* 1997 and showed that the responses were cultivar dependant and some only responded on either 2,4-D or 2,4,5-T medium, and some of the cultivars they tested did not respond on either hormone. 2,4,5-T medium was chosen as it showed the greatest number of cultivars that were recalcitrant on 2,4-D to be responsive under 2,4,5-T treatment. Leaves were used as explants to compare with the other method (Zheng, *et. al.* 1996) and increased regeneration would likely to have been achieved using axillary buds as explants, of which the size of bud has been found to be crucial to achieve somatic embryogenesis (Al-Mazrooei, *et. al.* 1997). The callus generated was primarily of a yellow colour with some green lines and not the deep red /

purple colour exhibited by many of the most embryogenic lines (Al-Mazrooei, *et. al.* 1997). Cultivar S-256 showed the greatest callus production under 2,4,5-T treatment and may indicate that the cultivars originating from India are predetermined to respond more than those of the Peruvian cultivars obtained from CIP (McCown, 2000). The cultivars used within this study have not been profiled previously and may not be responsive using this method.

Previous studies performed on *I. batatas* for the induction of somatic embryogenesis focused on one cultivar "White Star", cultivars from other countries have not been reported (Bieniel, *et. al.* 1995, Chee & Cantliffe, 1992). Leaf and petiole explants had been chosen, based on the study of Zheng, *et. al.* 1996, and had been successfully induced to produce embryos in one selected cultivar. However, only 3 out of a further 10 cultivars tested in this published study showed any responsiveness, therefore only a small number of the cultivars were responsive and embryogenic. This method was based on a two stage process with variable lengths of time on each stage (Zheng, *et. al.* 1996) therefore finding the optimum time of exposure for each stage was difficult with some cultivars needed much longer times than others. A range of different hormone concentrations was also used and other concentrations than those chosen may prove to have been more suitable.

4.6.2 Protein Levels in Tissue Undergoing Callus Initiation

The response of different cultivars of *I. batatas* to the initiation of callus and somatic embryogenesis is dictated by many factors. In studies of *D. carota*, embryogenic lines contained more protein than non-embryogenic lines for all cultivars tested (see section 3.2). A number of proteins have been shown to be produced in *D. carota* during the change in cell status and the initiation of new developmental pathways (Zimmerman, 1993, Choi, *et. al.* 1987, Kawahara, *et. al.* 1992, Sung & Okimoto, 1981, Sterk, *et. al.* 1991). The protein content of the tissue is therefore indicative of the extent of metabolic activity, and an increase in activity indicates a change in cell status and the initiation of new pathways. This reflects the formation of new dedifferentiated callus cells, formed as a result of the influence of the hormones. An increase in protein content was shown over the first ten days in nodes of *I. batatas* transferred to 2,4,5-T medium (see Figure 4.2.1).

Oxidative stress is one of the factors influencing the responsiveness of cultivars *in vitro*. Studies have monitored the changes in antioxidant enzymes and play a vital role in the control of reactive oxygen species and changes in developmental status. Part of the increase in protein expression may be due to increases in antioxidant activity and in other studies increases in antioxidants have been associated with the initiation of callus (Benson & Roubelakis-Angelakis, 1992 & 1994). Oxidative stress also forms one of the areas of greatest interest in research into recalcitrance and may be one of the best routes for the improvement of existing culture programs (Benson, 2000a & b).

4.6.3 Antioxidants in Tissue Undergoing Callus Initiation

Oxidative stress can be generated by a number of different mechanisms, and one of these is the generation of reactive oxygen species. Some of these species are routinely generated in normal metabolism, but antioxidant enzymes that remove or transform them into less dangerous species control activity and prevent damage within the cells. Peroxidase is an antioxidant and has been established as a developmental marker (Krsnik-Rasol, 1991).

Increased levels of peroxidase activity have been associated with changes in developmental pathways and in particular the initiation of somatic embryogenesis (Krsnik-Rasol, 1991). Increased levels of peroxidase were also shown in lupin shoots after transfer to rooting medium (Jouve, *et. al.* 1994). Such an increase in peroxidase activity was noted in nodes of *I. batatas* after transfer to 2,4,5-T medium (see Figure 4.2.2), and showed an increase of the course of two weeks, with similar patterns for both cultivars tested. An increase in the level of peroxidase was shown in cultures of flax after two weeks on callus induction medium and indicates the preferential expression of specific peroxidases (McDougall, *et. al.* 1992).

Catalase is an important antioxidant that mediates the removal of hydrogen peroxide and cells undergoing oxidative stress can generate increased levels of H_2O_2 (Bartosz, 1997, Rao, *et. al.* 1997, Prasad, 1997). Catalase is also significant in the prevention of the generation of hydroxyl radicals which are formed by the reaction of hydrogen peroxide with iron ions (reviewed Benson, 1990). Therefore catalase activity is significant in removing hydrogen

peroxide and preventing the initiation of lipid peroxidation by hydroxyl radicals. Catalase activity in nodes of *I. batatas* was not detectable until two weeks after the transfer onto 2,4,5-T callus initiation medium, and only at a low level of activity. This is consistent with findings of Benson and Roubelakis-Angelakis, (1992 & 1994), in studies of grapevine under similar conditions where increased catalase activity was noted 1-2 weeks after explant excision.

Non-enzymatic antioxidant species containing sulphydryl groups (SH groups), can be split into two groups. The first group is high molecular weight protein bound thiols. The oxidation of protein SH groups has been proposed as the primary reaction leading to membrane damage in plant cells affected by ozone (Chevrier, *et. al.* 1988). The other groups of SH groups containing species are low molecular weight compounds such as glutathione, which play significant roles in stress tolerance, senescence and defence (Foyer, *et. al.* 1997, May, *et. al.* 1996, Xiang & Oliver, 1998, Jiménez, *et. al.* 1998, Stajner *et. al.* 1993).

The sulphydryl group status of plant cells can be measured for both the total SH groups content and the non-protein content. Both were measured in nodes of *I. batatas* after transfer to 2,4,5-T medium over a course of two weeks. A peak in activity was noted for the total SH group content at the third day after transfer, and was similar in trend for both the cultivars tested, with no significant difference between the two (see Figure 4.2.3.1). The increase in activity is similar to that experienced by grapevine tissue undergoing callus initiation, although the increase was less pronounced (Benson & Roubelakis-

Angelakis, 1994) and may reflect increases in oxidative stress experienced by tissue undergoing dedifferentiation.

The non-protein sulphydryl group content was also measured in the same samples and showed no significant differences in activity between the cultivars or over the course of the two-week period. This indicates that there seems to be little change in the non-protein SH group content over the course of the early initiation of callus under 2,4,5-T treatment. Further information on particular species in these groups of compounds, such as glutathione, will give more indication as to the responses of low molecular weight SH groups containing species during callus initiation.

Glutathione is very important for the protection of membranes sensitive to reactive oxygen species, and acts as a competitor to oxidative damage by being preferentially oxidised over membrane lipids. Glutathione plays a central role in the detoxification of reactive oxygen species within green plant cells and protects the integrity of membranes under attack by oxidative stress (Alscher, *et. al.* 1997). Glutathione has been associated particularly with lipid peroxidation and the role of its associated enzymes has been shown to have increased activity in *Phaseolus vulgaris* tissue under attack from herbicides which activate lipid peroxidation (Schmidt & Kunert, 1986). Glutathione in its reduced form can be recycled by associated enzymes such as glutathione reductase which links it to the oxidation of NAPDH in the Halliwell-Asada cycle (Bartosz, 1997).

Many of the compounds and enzymes associated with glutathione can be measured in extracts of plant cells and were profiled in nodes of *I. batatas* undergoing callus initiation on 2,4,5-T medium. The two forms of glutathione, reduced and oxidised were both measured in the nodes over a two-week period after transfer to the 2,4,5-T medium. The reduced form increased in concentration towards then end of the two-week period after the cultivar S-256 showed a high initial value after transfer to the 2,4,5-T medium (see Figure 4.2.4.1). The oxidised form of glutathione (GSSG) for the cultivar S-256, exhibited a general decrease in concentration with a peak of concentration at day 13, the pattern was less clear for Nemanete (see Figure 4.2.4.2), showing differences in the two cultivars.

A decrease was noted in glutathione reductase activity during the course of the first two weeks of nodes of *I. batatas* undergoing callus initiation (See Figure 4.2.5). Glutathione reductase mediates the recycling of oxidised glutathione back to reduced glutathione, and is vital for maintaining a pool of reduced glutathione within the cell (Alscher, *et. al.* 1997). Alternative antioxidant systems such as superoxide dismutase which shows a continued increased over the complete incubation period are likely to be active in the dedifferentiated tissue induced by the action of 2,4,5-T.

The final glutathione enzyme measured in the nodes of *I. batatas* under callus induction was glutathione S-transferase, which mediates the conjugation of glutathione to various substrates including hydroxynonenal (McGonigle, *et. al.* 1998, Fukuda, *et. al.* 1997). Activity of this enzyme was very low, although

an increase was shown after day 3, particularly for cultivar IC-21. This may indicate a rise in lipid peroxidation products stimulated by the high concentration of hormones used to initiate callus.

4.6.4 Malondialdehyde and Hydroxynonenal Content in Callus of *I. batatas*

The presence of lipid peroxidation products malondialdehyde (MDA) and hydroxynonenal (HNE) gives an indication of the extent to which lipid peroxidation is being experienced by cells in culture. Both compounds were detected in embryogenic and non-embryogenic culture of *D. carota* (see section 3.3) and were previously profiled in a range of cultivars (Deighton, *et. al.* 1997). Both compounds were detected in extracts of *I. batatas* from callus generated from a range of explants including petioles and internodal stem sections. This is the first time they have been detected simultaneously in *I. batatas*. Of the three cultivars tested, S-256 showed the lowest MDA content for callus from the three explants tested leaves, petioles and internodal stem sections (Figure 4.3.1.1). For both MDA and HNE content (Figures 4.3.1.1. and 4.3.1.2), the callus from internodal sections showed the lowest values for all three cultivars. This may indicate that this callus has mechanisms in place to remove either the hydroxyl radicals that initiate lipid peroxidation or the secondary products such as HNE and MDA.

Malondialdehyde and hydroxynonenal were also profiled in callus generated from leaves of three cultivars on 2,4,5-T medium and S2 medium. For HNE there was no significant differences, but the cultivar IC-14 on 2,4,5-T medium showed a much higher MDA content than all other cultivars on 2,4,5-T or S2

medium (see Figure 4.3.2.1). This result was the highest measured MDA content for all samples tested of *I. batatas*, and may indicate that this culture is under more oxidative stress than the other cultivars on either media type. The levels of oxidative stress within *in vitro* culture are significant in the recalcitrance of many species and cultivars and methods employed to reduce the effects of oxidative stress may have implications for the improvement of conservation programmes.

4.6.5 ELISA of Callus Generated from Leaves and Internodal Sections of *I. batatas*

Enzyme linked immunosorbant assays (ELISA) were used to determine the presence of HNE-protein adducts in protein extracts of *I. batatas*. Callus generated from leaves on 2,4,5-T medium and S1 medium were profiled for HNE-protein adducts, and on both media cultivars IC-21 and IC-14 showed the presence of the adducts. S-256 only showed the presence of HNE-protein adducts on 2,4,5-T medium. Callus generated from internodal sections of *I. batatas* on S1 medium, was also profiled for the presence of HNE-adducts, and all three cultivars tested showed positive signals.

HNE-protein adducts have never been detected in extracts of protein from plant cultures before and were undetectable in extracts from *D. carota* (see section 3.5). Interestingly, HNE-protein adducts have been detected in *I. batatas* and show that the lipid peroxidation product HNE is not only extractable and detectable in its free form by LC-MS (see section 4.3) but conjugates to proteins. The significance of these adducts within plant systems

has yet to be investigated although is likely to be a factor in damage accumulated as a result of oxidative stress in the form of lipid peroxidation.

4.6.6 Hydroxyl Radical Activity in Nodes of *I. batatas* under Callus Initiation on 2,4,5-T Medium

Nodes of from two cultivars of *I. batatas*, IC-14 and Nemanete were excised and placed onto 2,4,5-T medium in the presence of DMSO, and over a period of three weeks headspace analysis was used to quantify the amount of methane and ethylene produced by the nodes. Over the time course, only at day two results showed a significant difference in the OH radical activity between the two cultivars with Nemanete having a higher activity than IC-14. However, more interestingly, there was a large peak of ethylene release at day seven (Figure 4.5.2), which decreased until little was produced by the end of the three-week period. Ethylene biosynthesis is known to be stimulated by a number of different environmental stresses such as mechanical trauma and pathogen infection and its production plays a role in a varied group of physiological and developmental processes including ripening of fruit and root elongation (Ecker, 1995). Ethylene production can be monitored as an indicator of plant stress and the peak in production suggests that the nodes are experiencing stress as a result of the application of 2,4,5-T. Similar trends were noted in normal auxin requiring callus of *Nicotiana tabacum* after subculture with a peak of ethylene production at 6 days, suggesting that even routine culture on auxin medium can cause stress (Koves & Szabo, 1987).

4.6.7 Conclusions

Studies of *Ipomoea batatas* have shown that a range of cultivars micropropagated onto 2,4,5-T medium can have similar growth responses, although responses for other growth regulator treatments can vary greatly. *I. batatas* has been shown, by a number of different groups, to be a difficult species to initiate somatic embryogenesis in, and even when achieved, rates tend to be low, with many cultivars only producing non-embryogenic callus (Jarret, *et. al.* 1984, Al-Mazrooei, *et. al.* 1997). The application of antioxidants may increase the responsiveness of cultures by removing damaging free radicals (Zhang & Kirkham, 1996, Earnshaw & Johnson, 1987).

The activity of protein and antioxidants have been shown to have activity within explants undergoing callus initiation and in particular, peroxidase levels were increased over the first two weeks of initiation. Evidence of the effect of lipid peroxidation was the presence of MDA, HNE and HNE-protein adducts. The report of HNE-protein adducts is the first in plant cells and further investigation into the role of these adducts may give important clues within cells undergoing oxidative stress. HNE has been shown to be conjugated to enzymes within mammalian systems (Uchida & Stadtman, 1993) and therefore may have a role within the regulation of enzyme activity. HNE adducts could be used as markers for oxidative stress and their presence has been associated with apoptosis (Kirichenko, *et. al.* 1996).

Chapter 5 *Glycine max* – A Study of Habituation

Contents

Glycine max, commonly known as soya, has been an important legume crop for many years and consequently has been used as basis for the study of physiology and biotechnology since the early establishment of tissue culture (Brar & Carter, 1993). Many of the most recent developments have been utilised within this species, with *G. max* and tomato being two of the first species to have been commercially grown from genetically modified seeds which has brought the use of biotechnology to the forefront of public perception. *In vitro* studies provide fundamental information with wider applications for improving the performance of the crop within the field environment. One of the problems of manipulating plant tissues in an *in vitro* environment is the gradual loss of regenerative potential in cultures that are maintained long term. Eventually this gradual loss can result in cell lines that had been previously embryogenic or organogenic become non-regenerative. An important phenomenon associated with the decline in responsiveness is habituation, which is exhibited by a number of species (Jackson & Lyndon, 1990, Meins, 1989, Christou, 1988, Gaspar, *et. al.* 1999, Gaspar, *et. al.* 1983, Bennici & Bruschi, 1999, Arbillot, *et. al.* 1991).

Habituation as a phenomenon has been documented in plant systems since the 1940's, and has been studied within a number of different species (Jackson & Lyndon, 1990, Meins, 1989, Gaspar, *et. al.* 1999, Gaspar, 1999, Hoffman & Hoffman-Tsay, 1994, Bennici & Bruschi, 1999). Habituated growth is described as the acquired and hereditary capacity for autonomous growth, in the absence of exogenously applied auxins and/or cytokinins in tissue culture (Gaspar, 1999, Christou, 1988, Jackson & Lyndon, 1990). Habituation has

been observed in some cases as a gradual process while in other cases it has occurred spontaneously generating a neoplastic stage (Gaspar, 1995, 1999). It has been suggested that this ability to continually divide makes the tissue appear to behave in the same way as a plant tumour cell (Hagège, 1996). It has been postulated that habituated cells are analogous to cancer cells as they have many common characteristics shared with animal metastases, including hormone independence, complete loss of cell-to-cell adhesion, permanent oxidative stress and accumulation of polyamines (Gaspar, 1998).

The change in status of the habituated cells has been linked to the altered metabolism of ethylene. Decreased levels of ethylene production by habituated tissues may indicate a link between auxins and ethylene formation (Köves & Szabó, 1987). A number of other processes within the metabolism of habituated and non-habituated tissues linked to oxidative stress have been investigated (Le Dily, *et. al.* 1993, Hagège, 1996). The presence and role of a range of antioxidant compounds and enzymes has formed the basis of one of the theories regarding habituation as an antioxidant adoptive strategy (Hagège, 1996). The enzymes removing reactive oxygen species such as catalase and superoxide dismutase have been deemed particularly important. Lipid peroxidation has also been investigated within habituated cultures (Cherif, *et. al.* 1996), although there is evidence to suggest that the MDA may not be linked to lipid peroxidation in these cultures (Cherif, *et. al.* 1996). This could be due the methods by which MDA has been measured. Further investigation into the presence of this and other lipid peroxidation products, using a broader range of analytical methods within a range of *G. max* cultures may provide

further evidence for the role of lipid peroxidation. The role of antioxidants and lipid peroxidation products within habituated tissues could give important information as to differences in metabolism of habituated tissues compared to responsive cultures.

Aims and Objectives of the Study of *Glycine max*

The objectives of the study of *G. max*, using the tools developed within *D. carota*, were to investigate the role lipid peroxidation and oxidative stress in *in vitro* culture and their implications in habituation.

The main aims of the study of *G. max* tissue are:

1 To profile the activity of a range of antioxidants in three types of *G. max* callus with different hormone requirements.

2 To establish profiles of the HNE and MDA content of three types of *G. max* callus

3 To determine level of HNE-protein adducts within extracts of *G. max* using the enzyme linked immunosorbant assay developed within *D. carota* systems.

4 To study the generation of hydroxyl radicals using gas chromatography of three types of *G. max* callus

5.1 Culture System

Many different callus types have been generated in the species *G. max* for use within physiological studies using a range of auxins and cytokinins, including indole acetic acid, benzyl amino purine and napthaleneacetic acid (NAA) (Wang, 1979). Three types of *G. max* tissues were received from the John

Innes Centre, Norwich, which had been generated on different hormone containing media. These were designated SOY – white, SOY + white and SOY+ green (see section 2.1) (Wang, 1979). All the lines were generated on NAA (2 mg/L) and benzylamino purine (BAP, 1 mg/L) containing medium but the SOY – white line had been subsequently grown on medium containing only NAA (2 mg/L) had showed continued growth and was considered to be habituated. Only the SOY + green callus under light for growth (photon flux density of 50 μmol m^2 s^{-1}) and grew at a much reduced rate compared to both the white callus lines which grew rapidly under dark conditions.

Table 5.1 *G. max* Callus Types

	Media	Hormones	Light/Dark
G.max callus Green +	Soy +	NAA (2 mg/L), BAP (1 mg/L)	Light
G.max callus White +	Soy +	NAA, (2 mg/L), BAP (1 mg/L)	Dark
G.max callus White -	Soy -	NAA, (2 mg/L)	Dark

5.2 Biochemical Studies – Protein and Antioxidants

5.2.1 Protein Content of *G. max* Callus

Preliminary studies were performed to determine the difference between the different types of callus regarding their soluble protein content. Triplicate assessments were made on five callus replicate samples of each callus type. Analysis of variance showed that there was a significant difference between the different types and is shown in Figure 5.2.1 a) ($F(2,12)=78.42$ $p<0.001$), b) ($F(2,12)=36.21$ $p<0.001$) and c) ($F(2,11)=14.33$ $p<0.001$). Scheffe's

Analysis of Contrasts showed that the green hormone dependant line had a significantly higher protein content than the two white lines ($p<0.05$).

Figure 5.2.1: Comparison of Protein Content of Three Types of *G. max* Callus

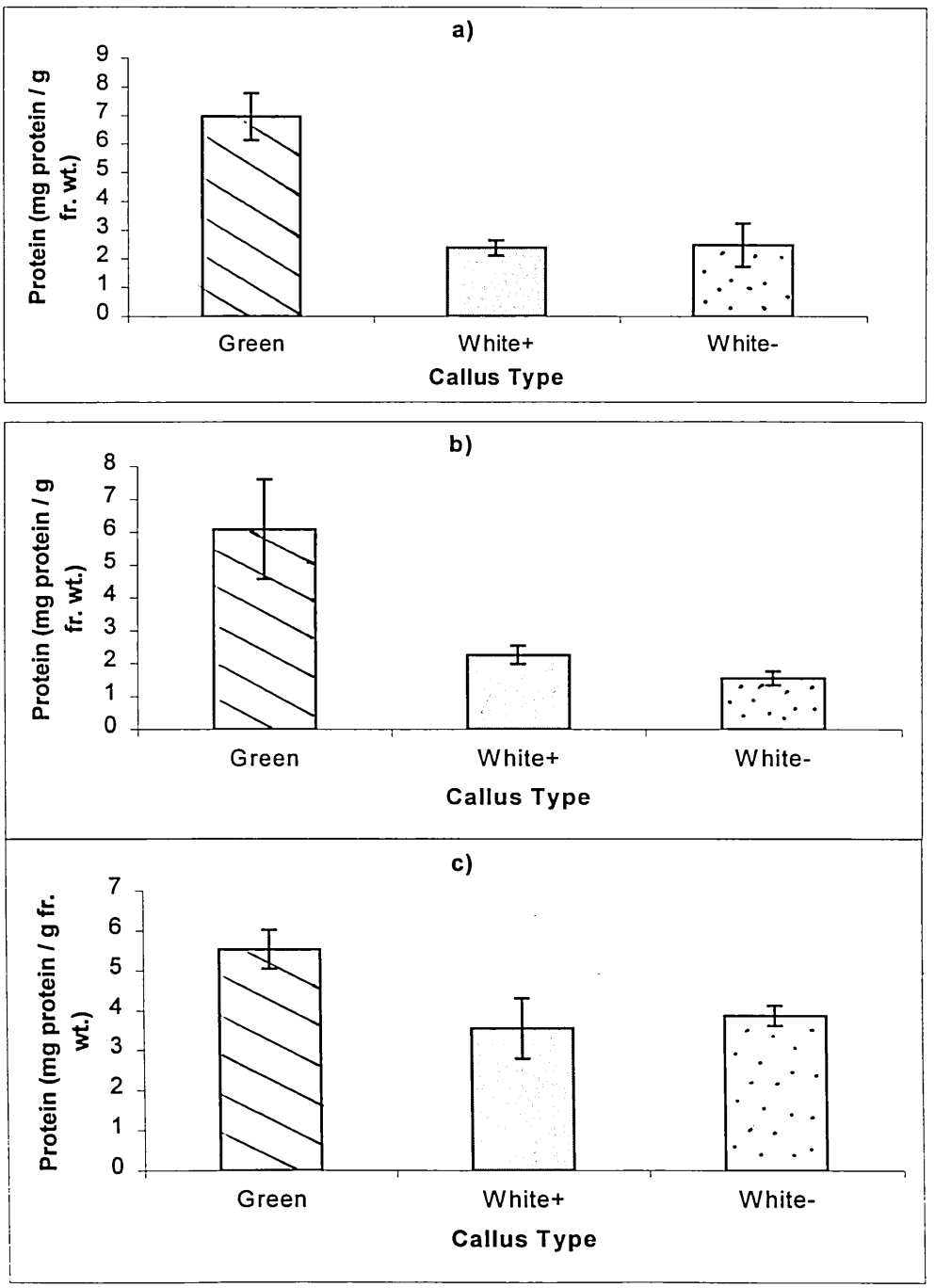


Figure 5.2.1: Protein concentrations in *G. max* callus: green (striped bar), white + (solid grey bar) and white – (spotted bar) expressed as mg.$^{-1}$gram fresh weight of tissue. Data points are means of 5 tissue replicates and the error bars are expressed as standard deviations.

5.2.2 Catalase Activity of *G. max*

The level of catalase within plant tissue can have significant implications as to the morphological status of the tissue (Benson & Roubelakis-Angelakis, 1992, 1994, Dey & Kar, 1995, Scandalios, 1990) and low levels of catalase have been implicated in habituation (Le Dily, *et. al.* 1993). Monitoring the level of catalase in tissues under stressful situations may give an indication as to the oxidative status of the tissue and to whether dangerous levels of H_2O_2 are being allowed to accumulate and damage cells. The catalase activity in the three types of *G. max* callus were established to determine whether the habituated callus line had a lower level of catalase activity than the two other callus lines.

The catalase activity was profiled for three replicate sets of protein extracts from the three types of *G. max* tissue. Analysis of variance shows a highly significant difference between the callus lines and the results are shown in Figure 5.2.2 [a) $F(2,11)=20.20$, $p<0.001$, b) $F(2,11)=60.13$, $p<0.001$, c) $F(2,11)= 70.01$, $p<0.001$]. Scheffe's analysis of contrasts showed that the green callus had a much higher catalase activity than the other two lines ($p<0.05$). Overall it is clearly shown that there is a highly significant difference between the catalase activity of the chlorophyll containing green line as compared to the two white lines. This indicates that the green line is better able to control the levels of hydrogen peroxide under *in vitro* conditions.

Figure 5.2.2. Catalase Activity of Three Types of *G. max* Callus

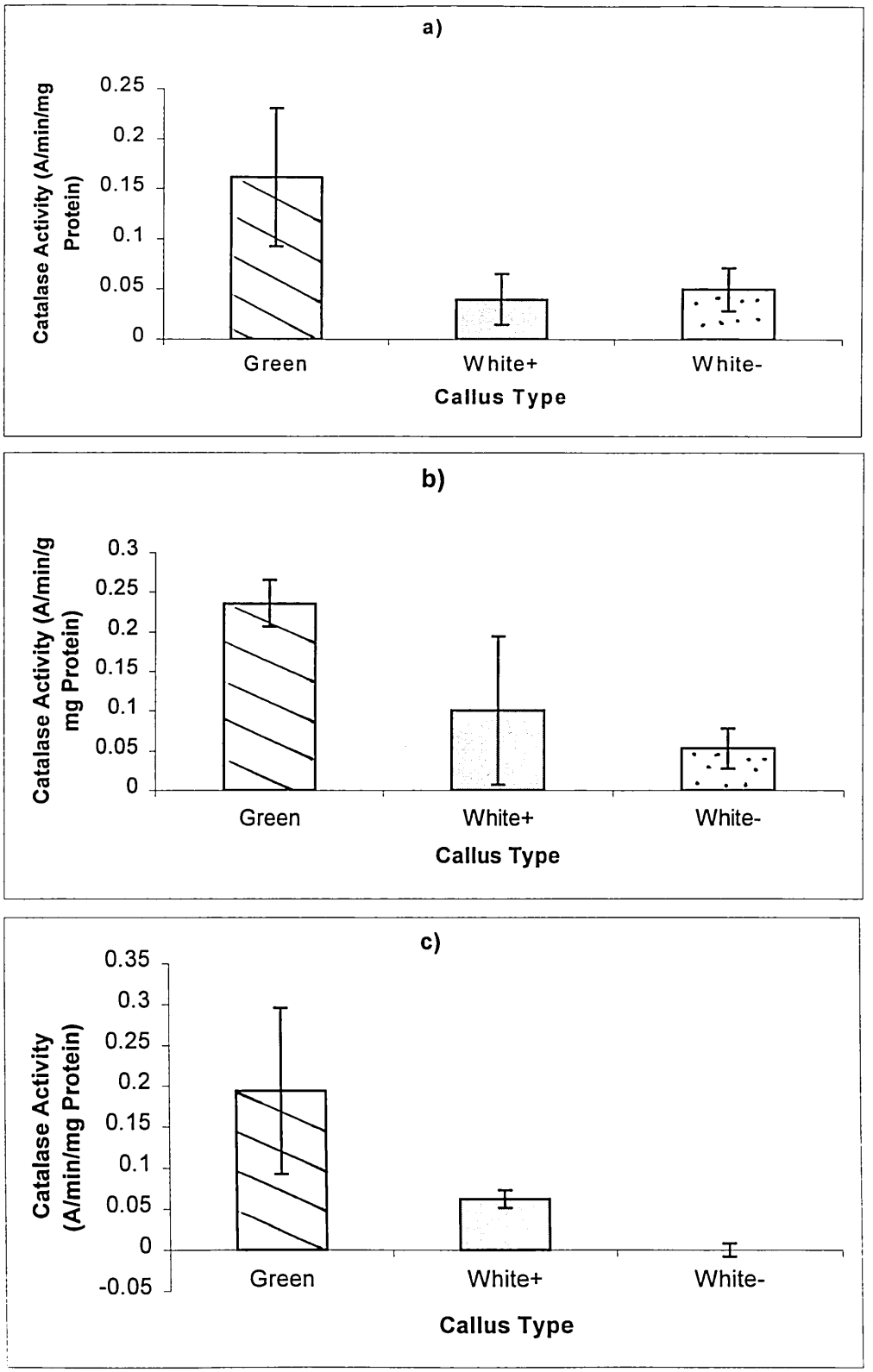


Figure 5.2.2: Catalase Activities in *G. max* callus : green (striped bar), white + (solid grey bar) and white – spotted bar) callus expressed as A/min/g fresh weight of tissue. Data points are means of 5 tissue replicates and the error bars are expressed as standard deviations.

5.2.3 Peroxidase Activity of *G. max*

Peroxidase activity was measured within protein extracts of the three types of *G. max* callus tissue in three replicate sets with tow of the sets showing significant differences and the data is shown in Figure 5.2.3. Analysis of variance showed that there is a significant difference between the three types of callus a) $F(2,12)=33.38$, $p<0.001$ and b) $F(2,12)= 28.12$, $p<0.001$. Scheffe's analysis of contrasts showed that the white + callus had a much higher peroxidase activity than the other two callus lines ($p<0.05$).

Figure 5.2.3 Peroxidase Activities of Three Types of *G. max* Callus

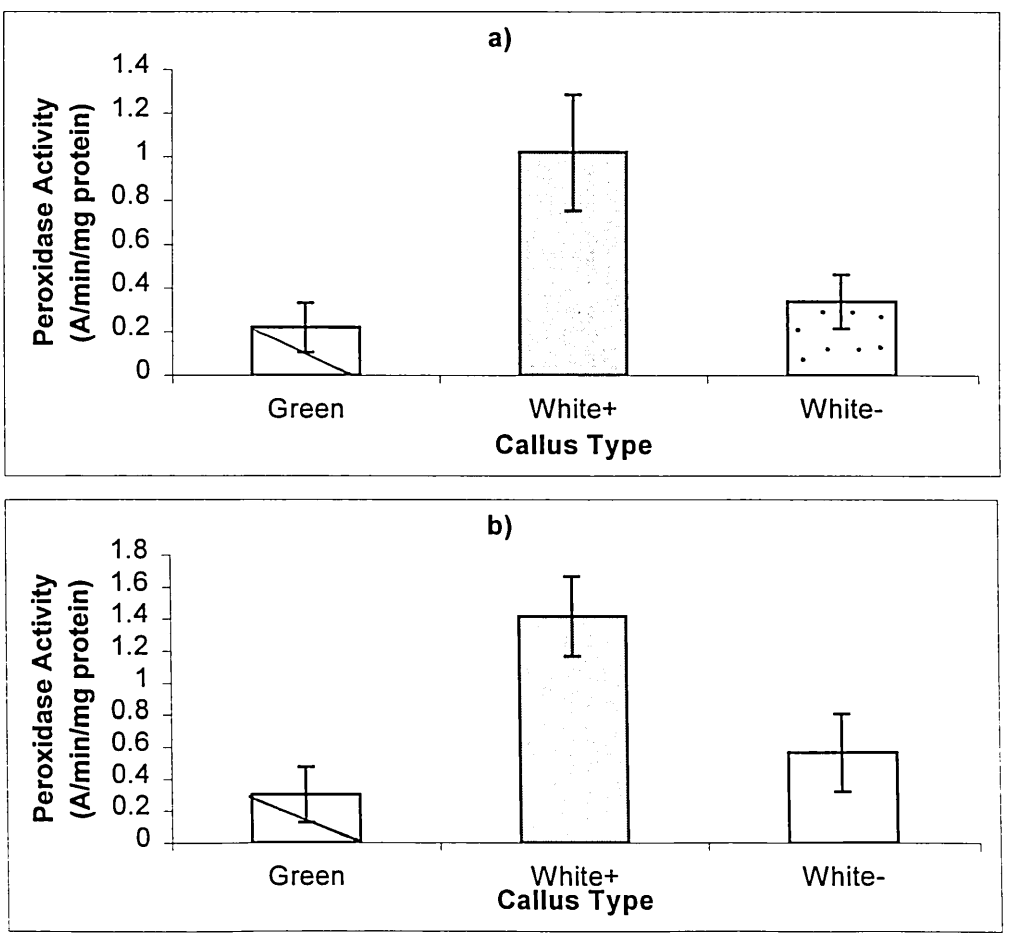


Figure 5.2.3: Peroxidase Activities in: green (striped bar), white + (solid grey bar) and white – (spotted bar) callus expressed as A/min/mg protein. Data points are means of 5 tissue replicates and the error bars are expressed as standard deviations.

5.2.4 Superoxide Dismutase Activity

Superoxide dismutase (SOD) is an important antioxidant enzyme in the control of reactive oxygen species (Cutler, *et. al.* 1989, Scandalios, 1990). SOD catalyses the conversion of $O_2^{\bullet -}$ to H_2O_2 which is then metabolised to H_2O by glutathione peroxidase (see section 1.3.5.2). The action of SOD is very important in combating oxidative stress under a number of different environmental stresses (Scandalios, 1990, Sutherland, 1991, Wingsle, *et. al.* 1992). SOD activity can be measured using a spectrophotometric assay utilising the photo-reduction of the nitro blue tetrazolium dye (Beauchamp & Fridovich, 1971).

The activity of superoxide dismutase was measured in soluble protein extracts of the three types of *G. max* callus in three replicate sets. SOD activity measured within the green callus was significantly differences between the callus types and the results are shown in Figure 5.2.4, a) $F(2,12)= 1108.5$, $p<0.001$, b) $F(2,11)=21.23$, $p<0.001$ and c) $F(2,11)= 90.16$, $p<0.001$). Scheffe's Analysis of Contrasts showed that the green hormone dependant line had a significantly higher SOD content than the two white lines ($p<0.05$).

Figure 5.2.4 Superoxide Dismutase Activities of Three Types of *G. max* Callus

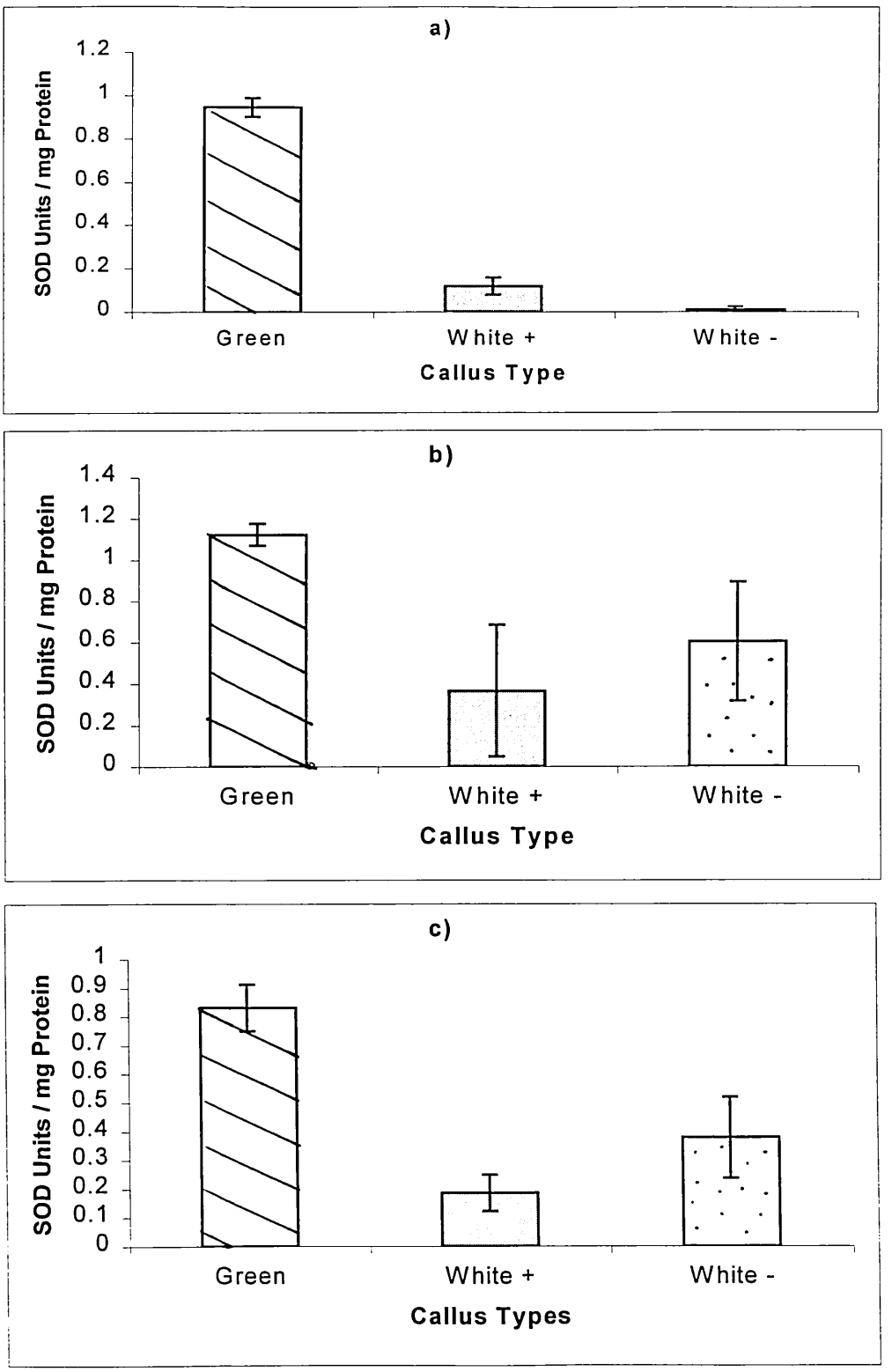


Figure 5.2.4: Superoxide Dismutase Activities in : green (striped bar), white + (solid grey bar) and white – (spotted bar) callus expressed as Units/mg protein. Data points are means of 5 replicates and the error bars are expressed as standard deviations.

5.2.5 Sulphydryl Group Content of *G. max*

Compounds containing sulfydryl groups (SH groups) can be divided into two types, low molecular weight compounds such as glutathione (see 1.3.5.4) and high molecular weight thiols (protein bound SH groups) (Faure & Lafond, 1995). Protein SH groups have been considered as important agents in protecting cells against NO_2 and associated lipid peroxidation (Bartosz, 1997) (see section 1.3.5.3). The oxidation of SH groups can lead to membrane damage and altered metabolism (Chevrier, *et. al.* 1988) and therefore monitoring the degree of SH groups within protein and non-protein could give an indication of the role of SH groups within oxidative stress.

Total SH Groups

The total concentration of the SH groups within three types of *G. max* callus was measured. The level of total SH groups was much higher in the green callus than in both white callus lines and is shown in Figure 5.2.5.1 a) $F(2,12)=28.6$, $p<0.001$, b) $F(2,12)=5.69$, $p<0.05$, c) $F(2,12)=34.26$, $p<0.001$. The levels measured in the white callus lines were extremely low and almost too low to be measured for the majority of samples.

Figure 5.2.5.1 Total SH Group Concentration in three types of *G. max* Callus

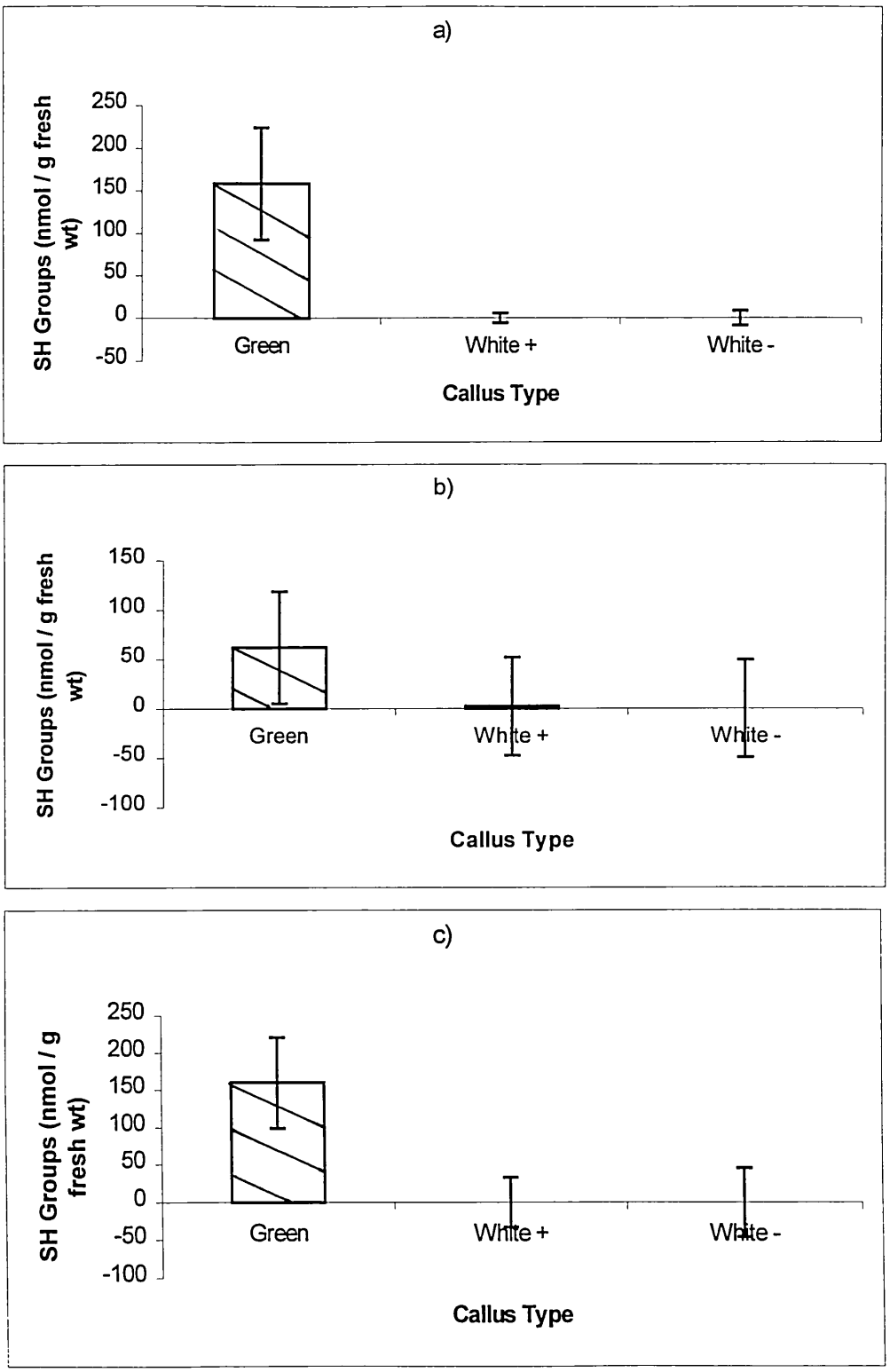


Figure 5.2.5.1 Total Sulphydryl Group content in : green (striped bar), white + (s solid grey bar) and white – (spotted bar) callus expressed as nmol/ g fresh weight. Data points are means of 5 replicates and the error bars are expressed as standard deviations.

SH Groups (Non-Protein)

The SH group content for non-protein based groups was also measured. The three replicate result sets are represented in Figure 5.2.5.2, and shows that the green callus has a higher content than the two white callus lines a) $F(2,11)=5.6$, $p<0.05$, b) $F(2,11)=6.46$, $p<0.05$, c) $F(2,11)=8.8$, $p<0.05$.

Figure 5.2.5.2 Non-Protein SH Group Content of three types of *G. max* Callus

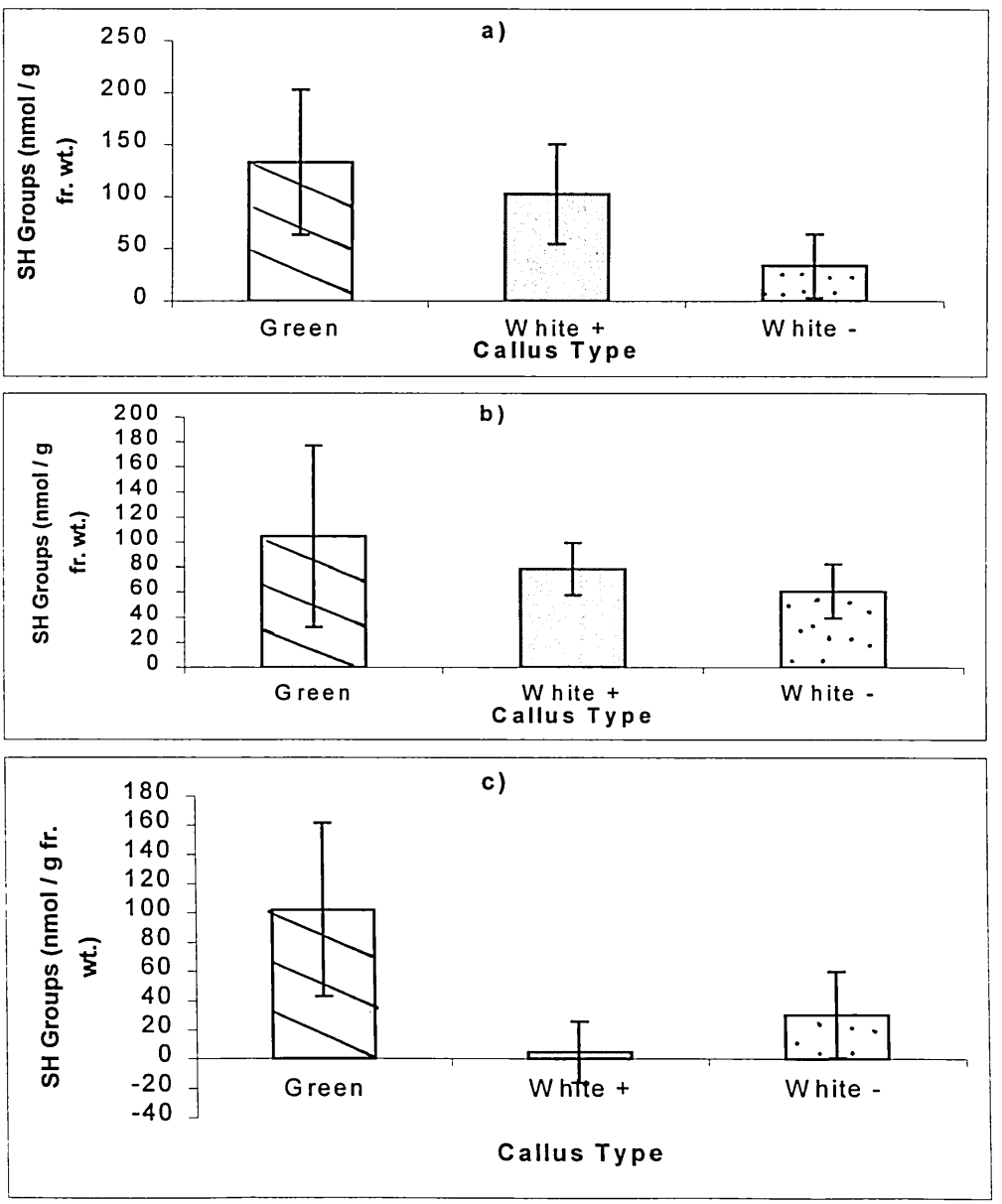


Figure 5.3.5.2: Non-protein Sulphydryl Group content in green (striped bar), white + (solid grey bar) and white – (spotted bar) callus expressed as nmol/ g fresh weight. Data points are means of 5 tissue replicates and the error bars are expressed as standard deviations

5.2.6 Glutathione Activity within *G. max*

Glutathione and its associated enzymes have important roles within stress tolerance, senescence and defence (Foyer, *et. al.* 1997, May, *et. al.* 1996, Xiang & Oliver, 1998, Jiménez, *et. al.*, 1998, Stajner, *et. al.* 1993) (see section 1.3.5.4). Glutathione is very important for the protection of membranes sensitive to reactive oxygen species and acts as a competitor to oxidative damage by being preferentially oxidised before membrane lipids. The tripeptide form has to be recycled after it has been sacrificed and oxidised by reacting with reactive oxygen compounds. The enzymes involved in this recycling also play an important role in the supply of adequate glutathione for the protection of growing cells. Profiling the oxidised and reduced forms can give an indication as to the role of glutathione in cells of different morphological status.

Glutathione in its reduced form can be recycled by associated enzymes such as glutathione reductase which links it to the oxidation of NAPDH in the Halliwell-Asada cycle (Bartosz, 1997). Glutathione can also be used for the conjugation and removal of compounds such as herbicides by the action of glutathione S-transferase and the increased expression of this enzyme appears to enhance the growth of tobacco seedlings during chilling and salt stress (Roxas, *et. al.* 1997). It appears that the enzymes involved in glutathione conjugation and recycling are more significant than the levels of the glutathione synthetase enzyme particularly for the resistance to photo-inhibition in poplar trees (Foyer, *et. al.* 1995). Therefore monitoring the

activity of such enzymes will indicate the level that glutathione can be recycled and utilised within plant cells.

5.2.6.1 Reduced Glutathione Content of *G. max*

The concentration of reduced glutathione was measured in three types of *G. max* callus tissue. Samples were profiled for reduced glutathione content and for one of the sets there was a significant difference between the lines and the results are shown in Figure 5.2.6.1 ($F(2,10)=20.02$, $p<0.001$).

Figure 5.2.6.1 Reduced Glutathione content of three types of *G. max*

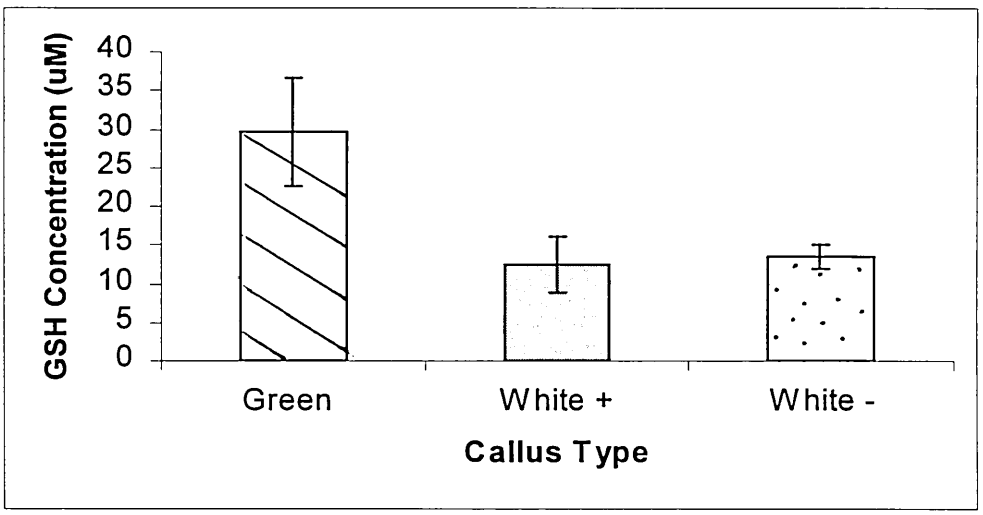


Figure 5.2.6.1: Reduced glutathione content of green (striped bar), white + (solid grey bar) and white – (spotted bar) callus expressed as μM / g fresh weight. Data points are means of 5 replicates and the error bars are expressed as standard deviations

5.2.6.2 Oxidised Glutathione Content of *G. max*

The concentration of the oxidised form (GSSG) of glutathione was also measured. Three sets of samples were profiled for the concentration of GSSG, however, there was no significant difference between the GSSG content within the three types of *G. max* callus ($F(2,12)=0.43$, $p>0.05$, $F(2,11)=0.83$, $p>0.05$, $F(2,11)=3.09$, $p>0.05$).

5.2.6.3 Glutathione Reductase Activity in *G. max*

Glutathione reductase activity was profiled within three sets of samples of the three types of *G. max* callus. For the first set of samples there was a significant difference between the different types of callus, and the results are shown in Figure 5.2.6.3.1 ($F(2,11)=4.41$, $p<0.05$). For the other two sets there was no significant difference between the callus lines ($F(2,11)=3.53$, $p>0.05$, $F(2,11)=0.88$, $p>0.05$).

Figure 5.2.6.3 Glutathione Reductase Activity in *G. max*

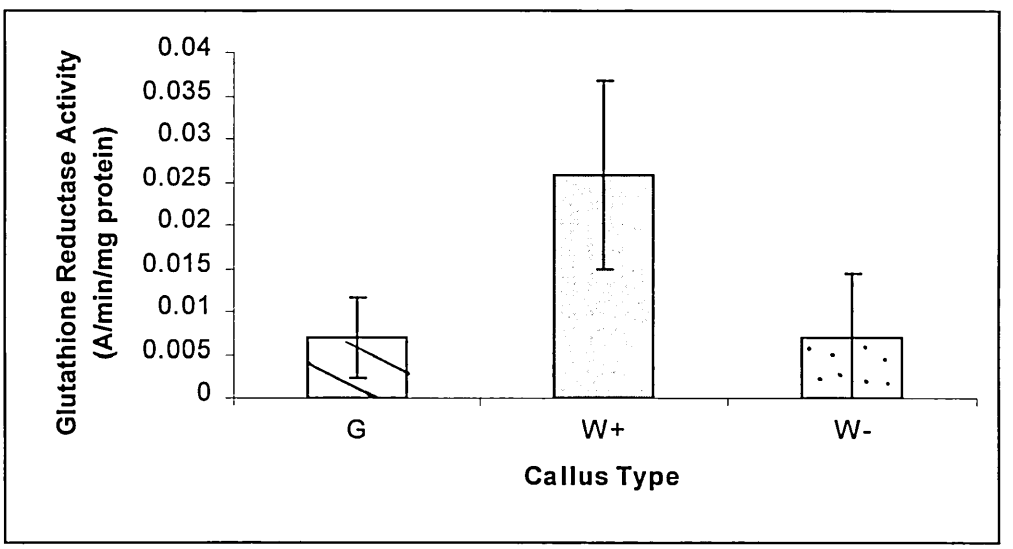


Figure 5.2.6.3 Glutathione reductase activity of green (striped bar), white + (solid grey bar) and white – (spotted bar) callus expressed as A/min/mg protein. Data points are means of 5 replicates and the error bars are expressed as standard deviations

5.2.6.4 Glutathione S-Transferase Activity in *G. max*

Glutathione S-transferase activity was measured within samples of three types of *G. max* callus. Methodology established from mammalian systems was used to establish the presence of glutathione S-transferase within soluble protein extracts of *G. max*. Activity of a range of standard concentrations from 14-140 Units was established, however, there was no detectable activity

measured within any of the three sets of samples. This indicates that there appears to be no measurable activity within soluble protein extracts of *G. max*, although activity may be too low to be detected using this method. GST activity has been shown in *G. max* (McGonigle, *et. al.* 1998), however there is evidence to suggest that this enzyme might not play as important a role in plants as it does in mammalian systems (Dean & Devarenne, 1997).

5.3 Determination of Volatiles as Measurement of Hydroxyl Radical Activity by Gas Chromatography in Three Types of *G. max*

The hydroxyl radical is an extremely reactive free radical species and has been implicated in the initiation of lipid peroxidation (Cheeseman, 1994), therefore monitoring the activity of hydroxyl radicals within plant tissue cultures may give an indication as to the extent of free radical mediated stress within *in vitro* culture. Measurement of the activity of hydroxyl radicals directly is difficult due to the highly reactive nature of the species and is only possible using extremely expensive equipment. An alternative way to measure hydroxyl radicals is to measure the activity indirectly, which can be achieved by the use of dimethyl sulphoxide (DMSO) which reacts with the hydroxyl radical producing gaseous methane (Babbs & Steiner, 1990). The evolution of methane can be measured using gas chromatography (Dillard & Tappel, 1979, Benson & Withers, 1987). A baseline profile of the activity of the hydroxyl radicals present within three types of *G. max* was established from callus in the mid-point of its subculture cycle, 10 days since the last sub-culture. This activity was then compared to callus that was sampled at the very end of the subculture cycle one week after the normal sub-culture date that may show an ageing response.

5.3.1 Hydroxyl Radical Activity of Three Types of *G. max*

Three types of *G. max* callus were profiled for the presence of hydroxyl radicals using DMSO as a trap, and monitoring the release of methane using gas chromatography. Samples of callus were taken at the middle of the sub-culture cycle of green, white + and white – callus lines approximately 10 days after the previous sub-culture. Three replicate runs of samples were profiled for hydroxyl radical activity measured every 24 hours over a three-day period. The full details of the method and instrument set up are in section 2.8. Controls were: empty vials, vials containing each media type, vials with media and DMSO, vials with each media and filtered distilled water and the laboratory air.

Only two sample points showed significant differences between the three callus lines. The methane emissions after one day treatment are shown in Figure 5.3.1.1 and show significant differences between the callus types ($F(2,6)=7.49$, $p<0.05$). After two days treatment the methane emissions of the three types of callus are shown in Figure 5.3.1.2 and show significant differences between the three callus types ($F(2,5)=5.76$, $p<0.05$).

Figure 5.3.1.1 Methane Emission of *G. max* after one days treatment

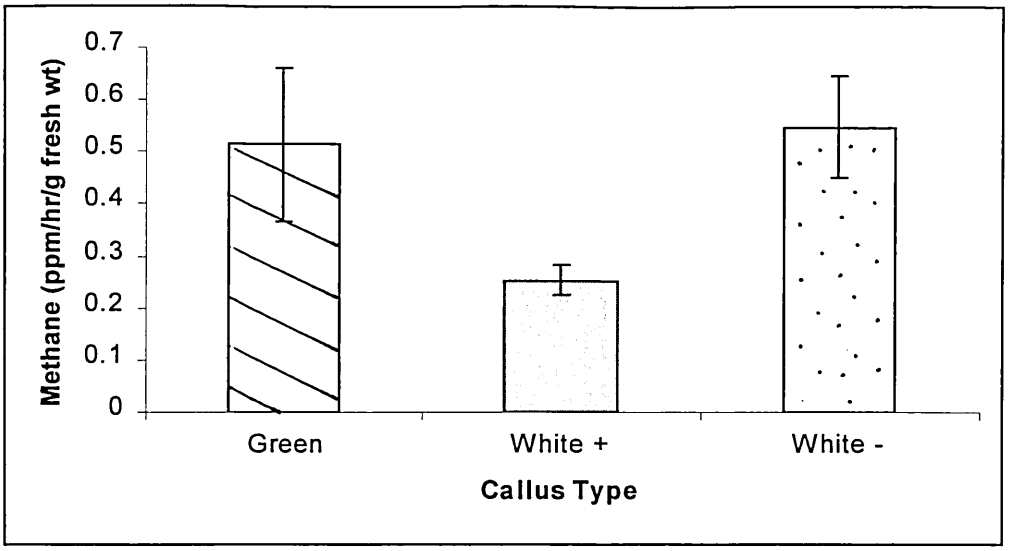


Figure 5.3.1.1 Methane emission of three types of *G. max*, green (striped bar), white (solid grey bar), and white – (spotted bar) callus after treatment with DMSO. Data points are expressed as the mean of three replicates and the error bars are standard deviations.

Figure 5.3.1.2 Methane Emission of *G. max* after two days treatment

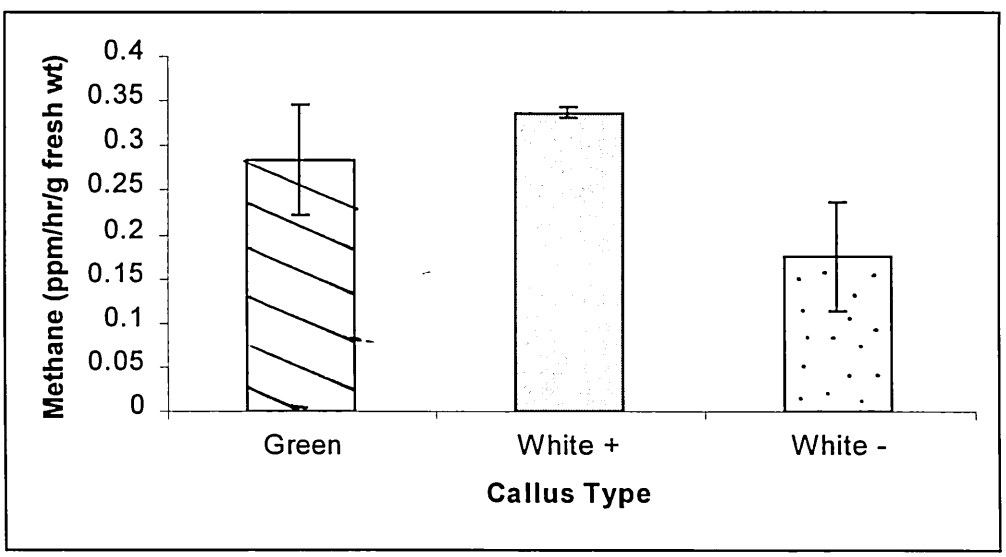


Figure 5.3.1.2 Methane emission of three types of *G. max*, green (striped bar), white (solid grey bar), and white – (spotted bar) callus after treatment with DMSO. Data points are expressed as the mean of three replicates and the error bars are standard deviations.

5.3.2 Hydroxyl Radical Activity of Three Types of Aged *G. max*

G. max callus was also profiled for the presence of hydroxyl radicals using DMSO as a trap for callus aged past the end of a normal sub-culture cycle. Samples of callus were taken from aged cultures after the end of a normal sub-culture cycle of green, white + and white – callus lines approximately one week after the end of the normal sub-culture date. Three replicate runs of samples were profiled for hydroxyl radical activity measured every 24 hours over a three days period.

On the second day of treatment there was a significant difference in the levels of methane produced by the three callus lines ($F(2,6)=4.00$, $p<0.01$). The results are shown in Figure 5.3.2.1. At the third day treatment there was a significant difference between in the levels of methane produced by the three callus lines ($F(2,6)5.37$, $p<0.05$) and the results are shown in Figure 5.3.2.2. After the first day's treatment, some of the samples showed the presence of ethylene, particularly in the green and white + callus types and ranged from 0.09 to 0.3 ppm/hr/gram fresh weight, but there was no significant difference between the types of callus ($F(2,5)=0.66$, $p>0.05$).

Figure 5.3.2.1 Methane Emission of aged *G. max* after two days treatment

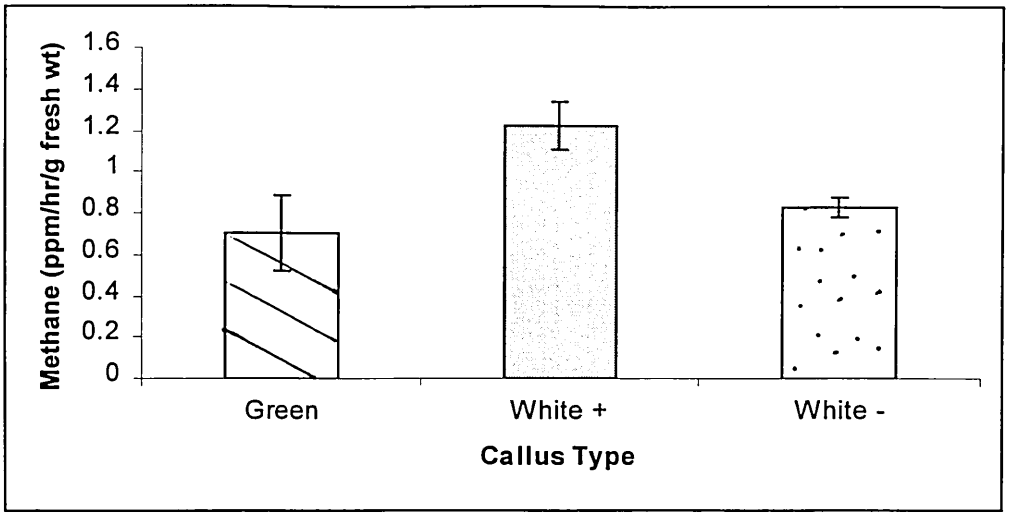


Figure 5.3.2.1 Methane emission of three types of *G. max*, green (striped bar), white (solid grey bar), and white – (spotted bar) callus after treatment with DMSO. Data points are expressed as the mean of three replicates and the error bars are standard deviations.

Figure 5.3.2.2 Methane Emission of aged *G. max* after two days treatment

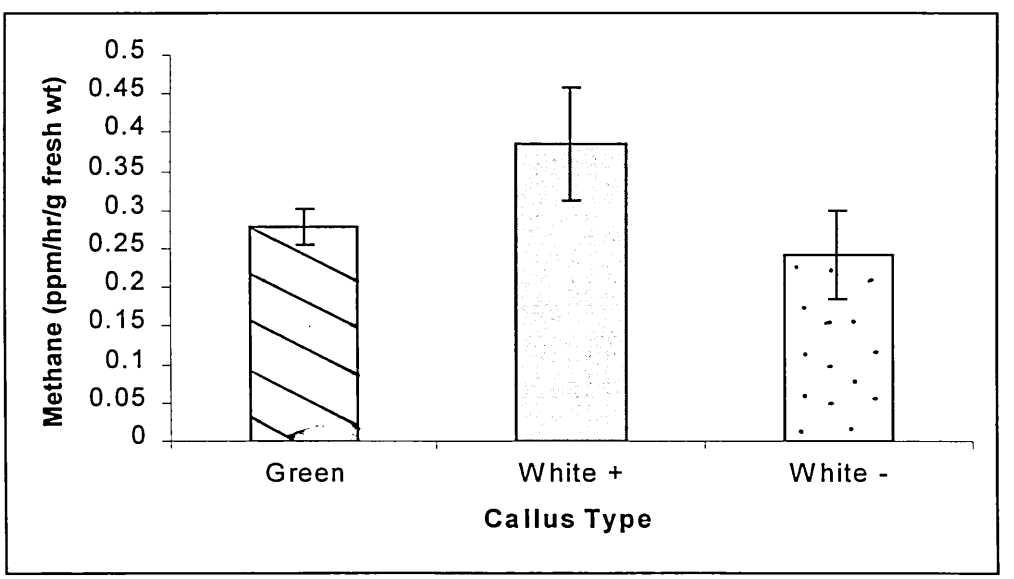


Figure 5.3.2.2 Methane emission of three types of *G. max*, green (striped bar), white (solid grey bar), and white – (spotted bar) callus after treatment with DMSO. Data points are expressed as the mean of three replicates and the error bars are standard deviations.

5.4 Malondialdehyde and Hydroxynonenal Content of *G. max*

Hydroxyl radicals are extremely reactive oxygen species and are responsible for the initiation of lipid peroxidation. The main toxic compounds produced by this reaction include malondialdehyde (MDA) and hydroxynonenal (HNE) and their presence within plant systems can lead to secondary oxidative stress. The concentration of MDA and HNE were measured in three types of *G. max* callus, green, white + and white – at the mid-point of the sub-culture cycle using Liquid Chromatography – Mass Spectroscopy (LC-MS) (Deighton, *et. al.* 1997). There were significant differences between the content within the different callus types for both MDA and HNE and the results are shown in Figures 5.4.1 and 5.4.2 ($F(2,22)=25.86$, $p<0.001$ $F(2,24)=13.14$, $p<0.001$). Lipid peroxidation is apparently higher within the habituated line white -, with higher concentrations of both MDA and HNE.

Figure 5.4.1 Malondialdehyde Content of Three Types of *G. max*

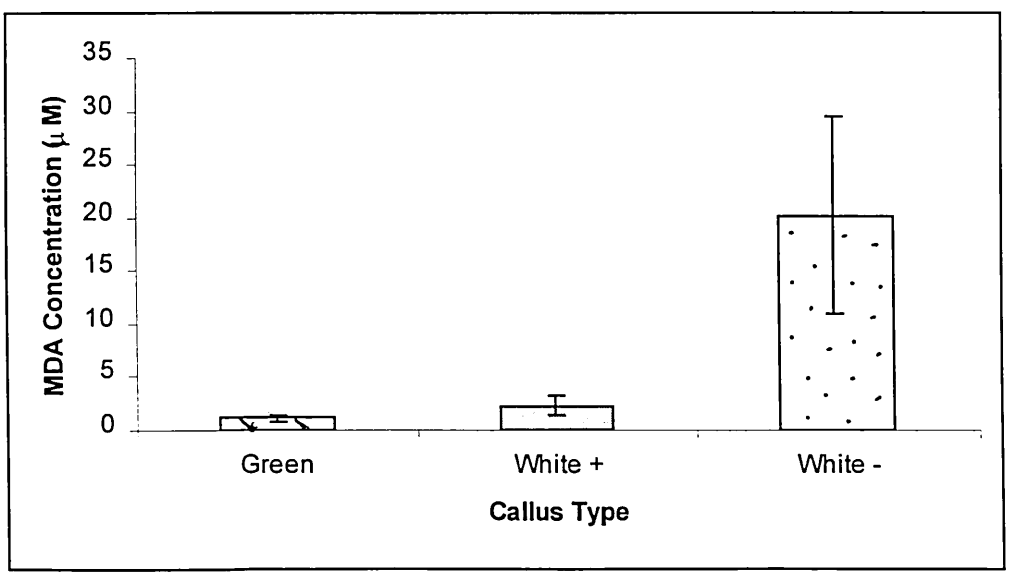


Figure 5.4.1 MDA concentration within green (striped bar), white + (grey bar) and white – (spotted bar) *G. max* callus. Data points are means of 5 replicates, run in duplicate and the error bars are expressed as standard deviations.

Figure 5.4.2 Hydroxynonenal Content of Three Types of *G. max*

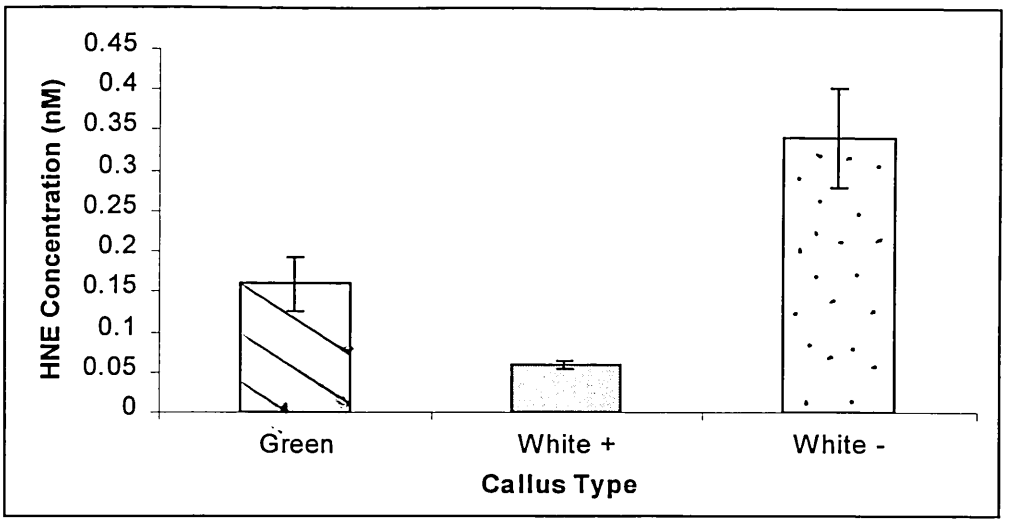


Figure 5.4.2 HNE concentration within green (striped bar), white + (grey bar) and white – (spotted bar) *G. max* callus. Data points are means of 5 replicates, run in duplicate and the error bars are expressed as standard deviations.

5.5 Quantification of HNE-Protein Adducts by ELISA in *G. max*

The development of an enzyme linked immunosorbent assay (ELISA) with *D. carota* extracts enabled the quantification of HNE-protein adducts for other plant extracts. This assay was used to determine the quantity of HNE-protein adducts present within samples of soluble protein extracted from tissue of *G. max*. ELISA was used to compare the three different types of *G. max* used in this study for the presence of HNE-protein adducts. Until recently measurement of HNE has concentrated on the free or extractable HNE content of plant tissue (Deighton, *et. al.* 1997, Adams, *et. al.* 1999), whereas ELISA gives an indication as to the level of HNE that is bound to proteins within the cell. HNE has been shown in mammalian studies to conjugate to a number of different proteins including a number of enzymes such as glyceraldehyde-3-phosphate dehydrogenase (Uchida & Stadtman, 1993). ELISA was used to

determine the quantity of HNE-protein adducts present in samples of soluble protein extracted from three callus types of *G. max*.

The full method for the detection of HNE-protein adducts is given in section 2.7 and was followed for all ELISA assays performed. Several columns of blank wells that contained only phosphate buffered saline were included to account for any signals created by non-specific binding of the antibodies. The "cut-off" line shows the value that is three times the average blank and gives the absorbance value above which wells were considered to have a positive signal. Samples were run in duplicate or triplicate groups of wells. Soluble protein extracts of the three types of *G. max* callus were profiled for HNE-protein adduct content, and the results are shown in Figure 5.5.1. The cut-off line for this ELISA was fairly high level of absorbance, giving only the standard HNE-BSA a positive trend. Although, it is likely that with lower levels of absorbance in the blanks the green and white + line may give positive signals and future ELISA trials focused on these two callus types.

Figure 5.5.1 ELISA of Three types of *G. max* callus

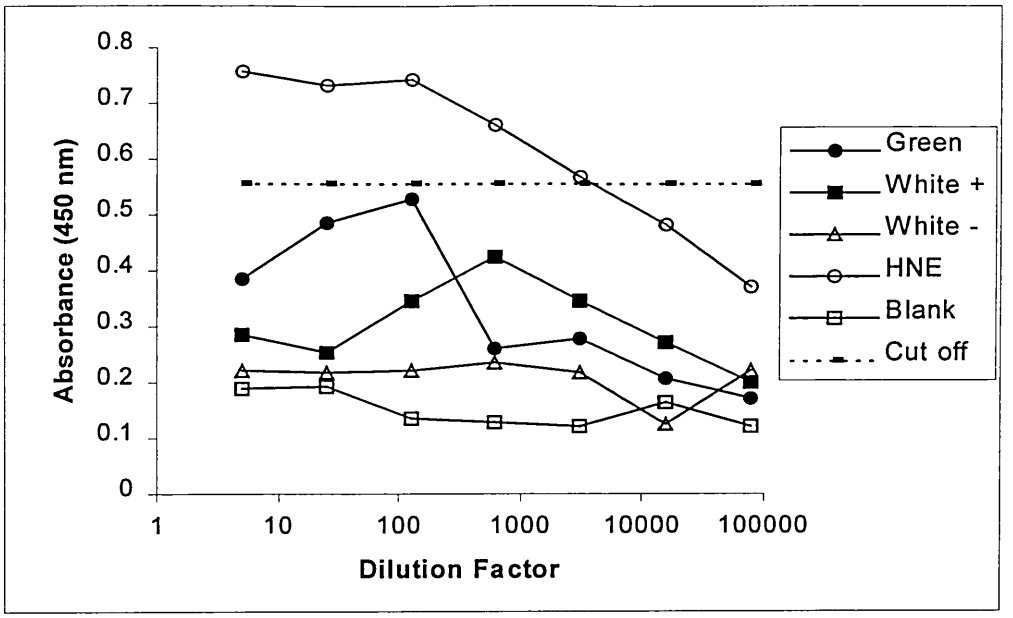


Figure 5.5.1 Absorbance at 450 nm of soluble protein samples of Green, White + and White – *G. max* callus lines, with standard of HNE-BSA (HNE), and controls containing only phosphate buffered saline (Blank). The positive-negative threshold is three times the average blank (Cut off). Primary and secondary antibodies were used at a 1/200 dilution.

Two identical ELISA trials were performed on soluble protein extracts from the green and white + callus lines that had been generated using the large scale extraction method. The results of the first assay are shown in Figure 5.5.2 and show that positive signals were achieved for the white + callus line and the HNE-BSA standard. Similar results were achieved for both assays and give the maximal absorbance for the HNE-BSA from 0.2 mg/mL down to 1.6 μg/mL protein and for the white + callus 1.0 ± 0.034 mg/mL to 1.59 ± 0.05 μg/mL.

Figure 5.5.2 ELISA of Two Types of *G. max*

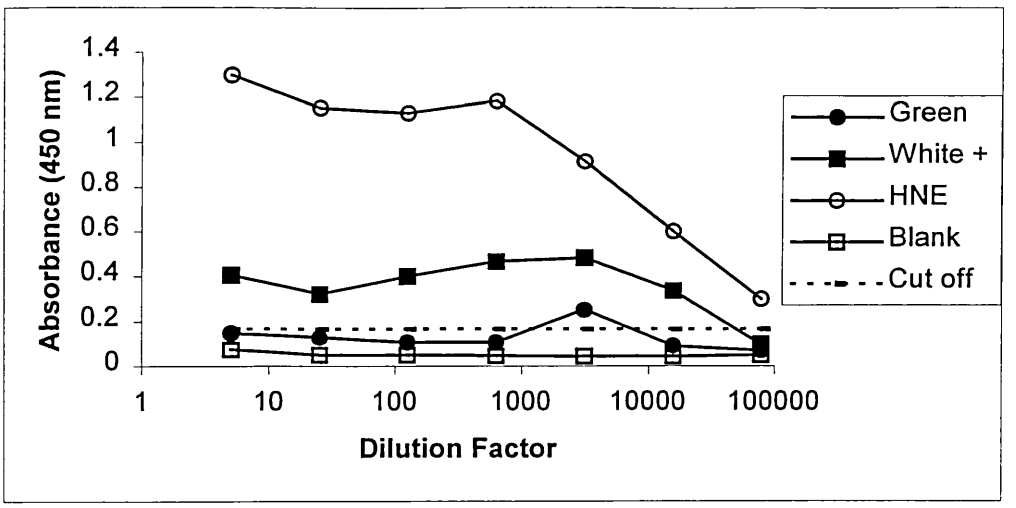


Figure 5.5.2 Absorbance at 450 nm of soluble protein samples of Green, White + *G. max* callus lines, with standard of HNE-BSA (HNE), and controls containing only phosphate buffered saline (Blank). The positive-negative threshold is three times the average blank (Cut off). Primary and secondary antibodies were used at a 1/200 dilution.

Three identical assays were performed for green and white + callus protein generated from small-scale extractions performed on samples taken from the same cultures as the large scale extraction. All three assays showed very similar profiles and the first assay is shown in Figure 5.5.3. Maximal absorbance for the HNE-BSA was from 0.2 mg/mL down to 3.1 μg/mL protein and for the white + callus from 0.79 ± 0.12 mg/mL to 6.33 ± 0.95 μg/mL.

Figure 5.5.3 ELISA of Two Types of *G. max*

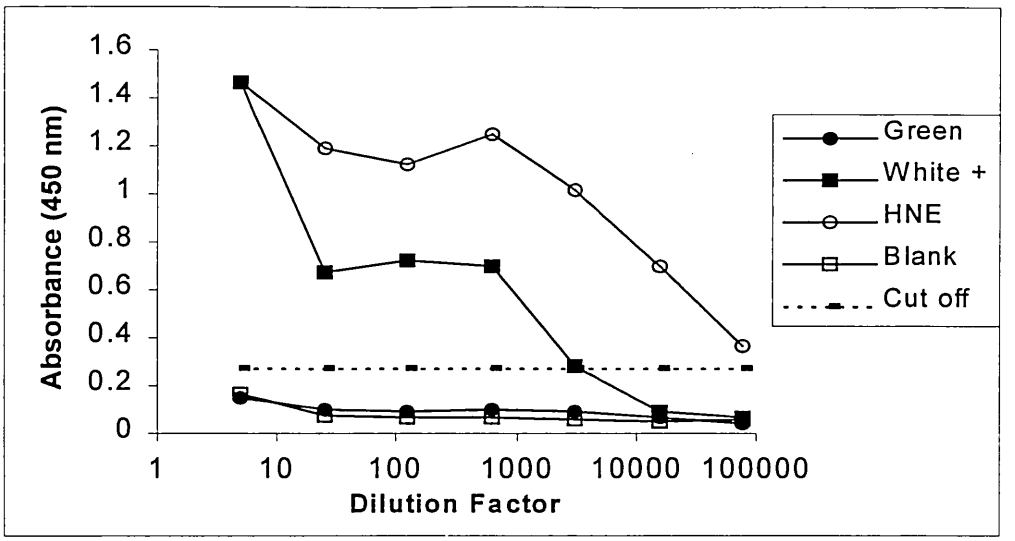


Figure 5.5.3 Absorbance at 450 nm of soluble protein samples of Green, White + *G. max* callus lines, with standard of HNE-BSA (HNE), and controls containing only phosphate buffered saline (Blank). The positive-negative threshold is three times the average blank (Cut off). Primary and secondary antibodies were used at a 1/200 dilution.

Three identical assays were performed for green and white + callus protein generated from large-scale extractions to maximise the protein in each sample. All three assays showed very similar profiles and the first assay is shown in Figure 5.5.4. Maximal absorbance for the HNE-BSA was from 0.2 mg/mL down to 0.32 μg/mL protein and for the white + callus was from 0.71 ± 0.01 mg/mL to 1.14 ± 0.02 μg/mL.

Overall the profile of HNE-protein content of soluble protein extracts of *G. max* indicated that only the white + callus line had any detectable adducts. The maximal absorbance in extracts of white + was from 1mg/mL protein down to a lowest concentration showing maximal absorbance of approximately 1 μg/mL protein for both large scale and small scale extractions.

Figure 5.5.4 ELISA of Two Types of *G. max*

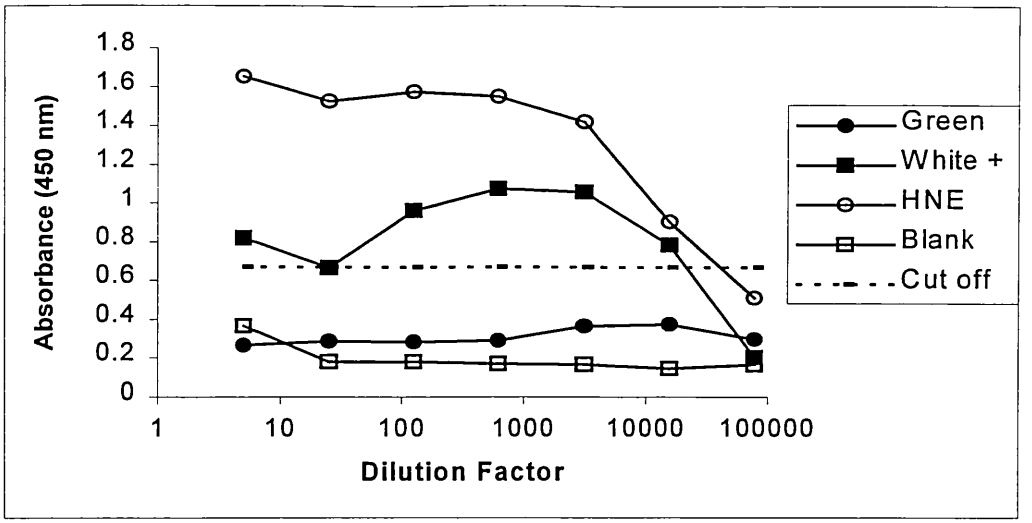


Figure 5.5.4 Absorbance at 450 nm of soluble protein samples of Green, White + *G. max* callus lines, with standard of HNE-BSA (HNE), and controls containing only phosphate buffered saline (Blank). The positive-negative threshold is three times the average blank (Cut off). Primary and secondary antibodies were used at a 1/200 dilution.

5.6 Discussion

5.6.1 Protein Content and Antioxidant Activity in *G. max*

Protein content and a range of antioxidant activities were determined in three types of *G. max* callus (designated green, white + and white -). For the majority of enzymes profiled, the activity was greatest in the chlorophyll containing green line that was hormone and light dependant. This suggests that the green line is likely to be more metabolically active than the two white callus lines and has more efficient antioxidant systems in place to combat the generation of reactive oxygen species and secondary products. More protective systems will be needed to control the tetrapyrrolic compound pathways that produce chlorophyll which are not activated in the white callus lines (Gaspar, *et. al.* 1999). In addition, pigments can generate oxygen and free radicals through photooxidation mechanisms (Salin, 1987). The green

line showed highest content for protein, catalase, superoxide dismutase, reduced glutathione (GSH), and protein and non-protein sulphydryl groups. Catalase activity in the white – line was particularly low and this was in accordance with findings in other species (Benson, *et. al.* 1992b), catalase has been shown to have a very low if not non-existent activity in habituated callus lines of sugarbeet (Hagège, 1996). Low antioxidant activity has been associated with habituation particularly for catalase and peroxidase, where the low activity allows hydrogen peroxide to accumulate, which may trigger hypersensistive cell death (Hagège, 1996, Gaspar, *et. al.* 1991). Peroxidase is intrinsic within many metabolic processes and its activity has been particularly investigated in lignification (Kevers & Gaspar, 1985). Low levels of lignin and cellulose have been shown in habituated cells (Crevecoeur, *et. al.* 1987, Gaspar, 1991). Peroxidase activity was highest in the white + callus line and low in the habituated white – callus tissue protein extracts. Peroxidase is vital in development and low levels may indicate a lower level of potential for development inherent in the cells under *in vitro* treatment (Krisnik-Rasol, 1991), particularly in habituated systems which may eventually lose their totipotent potential (Gaspar, *et. al.* 1991). The superoxide dismutase activity was low for the habituated line, although there was little difference between the levels of activity between the two white lines. Low activity of this enzyme will result in the accumulation of superoxide that can lead to increased oxidative stress.

Imbalance in the activities of the different antioxidants can lead to the accumulation of superoxide and hydrogen peroxide and other compounds such

as secondary oxidation products, that can increase the level of oxidative stress. Low levels of antioxidants present in habituated callus can allow the initiation of other pathways such as lipid peroxidation which can lead to higher concentrations of secondary products such as HNE and MDA, which may in turn cause further changes in the *in vitro* culture of plant cells (Benson, *et. al.* 1997, Knozner, *et. al.* 1996).

Glutathione and SH group contents were highest in the green callus line and these compounds have been shown to be important in the combat of a range of reactive species. Their high contents mean a greater level of protection for membranes and macromolecules. However, glutathione s-transferase (GST), an enzyme shown in mammalian systems to mediate the conjugation of reduced glutathione with compounds such as HNE (Fukuda, *et. al.* 1997, Simons & Vander Jagt, 1977), was not detected in any of the *G. max* callus. It has been shown to be present within *G. max* (McGonigle, *et. al.* 1998) but there is other evidence to suggest that this type of conjugation may be mediated by peroxidase (Dean & Davarenne, 1997). Glutathione reductase was highest in the white + line (similar to the peroxidase results) and indicates a high recycling potential of this callus to regenerate reduced glutathione.

5.6.2 Hydroxyl Radical Activity in *G. max*

Hydroxyl radicals are one of the most reactive oxygen species and are able to initiate lipid peroxidation, and consequently, profiling the presence of these radicals may give an indication of the potential for the initiation of lipid peroxidation. Hydroxyl radical activity was measured by the use of DMSO as

a trap, generating a $CH_3^{\bullet}$ radical that readily picks up a hydrogen atom to form methane (CH_4) (Babbs & Steiner, 1990), which was measured using gas chromatography (Dillard & Tappel, 1979, Benson & Withers, 1987, Fleck, *et. al.* 2000). The hydroxyl radical activity was determined over three days in callus at the mid-phase of the culture cycle and aged callus that had been sampled one week after the normal sub-culture date (see section 5.3). After two days of treatment the level of methane emission was much higher in the aged callus than in the mid-cycle callus, showing that a higher level of free radical activity occurs in callus that has been aged in culture. This indicates that prolonged sub-culture cycles are likely to induce oxidative stress and, therefore, careful regulation of the sub-culture cycle is crucial in minimising the induction of oxidative stress. Finding the balance between the length of the sub-culture cycle that maximises growth and responsiveness but minimises oxidative stress will form a basis for future improvement in tissue culture regimes. Due to reactive nature of the hydroxyl radicals, the half-life of the compound is very short and they are likely to react with nearby macromolecules such as lipids (Cheeseman, 1994). Therefore, measured levels may not reflect the true significance of this compound within plant systems, and the level of secondary products can also indicate the activity of this type of compound.

5.6.3 Hydroxynonenal and Malondialdehyde Content in *G. max*

The presence of the lipid peroxidation products malondialdehyde (MDA) and hydroxynonenal (HNE) gives an indication of the extent to which lipid peroxidation is being experienced by cells in culture. For the first time HNE and MDA were detected simultaneously within callus cultures of three types of *G. max* callus. MDA has been detected previously within *G. max,* but its activity was determined by TBARS and may not fully represent true MDA content (Vianello, *et. al.* 1986). The LC-MS method employed in this study, (Deighton, *et. al.* 1997), shows the presence of both MDA and HNE within extracts of *G. max.* The habituated callus line showed the highest content for both HNE and MDA compared to the other two callus lines. Hydroxyl radicals have been shown to be present within all types of *G. max* (see 5.3 and 5.6.2) and the habituated callus has a low activity of antioxidants including catalase, peroxidase and glutathione. Hydroxyl radicals are able to initiate lipid peroxidation and are therefore linked to the production of HNE and MDA. The high content of HNE and MDA can therefore be linked to the action of hydroxyl radicals and the low activity of the antioxidants that should be in place to control the propagation of lipid peroxidation. HNE and MDA have been shown to react with a number of macromolecules including proteins (Uchida & Stadtman, 1993), DNA (Chaudray, *et. al.* 1994), they may have significant roles in control and regulation (Parola, *et. al.* 1999) and also influence the progression of conditions in plants such as neoplasia and cancer (Gaspar, 1999). Further investigation is now needed as to the role of lipid peroxidation products within neoplastic plants. Studies of the role of HNE

and MDA in signalling within the cellular *in vitro* environment would be interesting.

5.6.4 ELISA for the detection of HNE-Protein Adducts in *G. max*

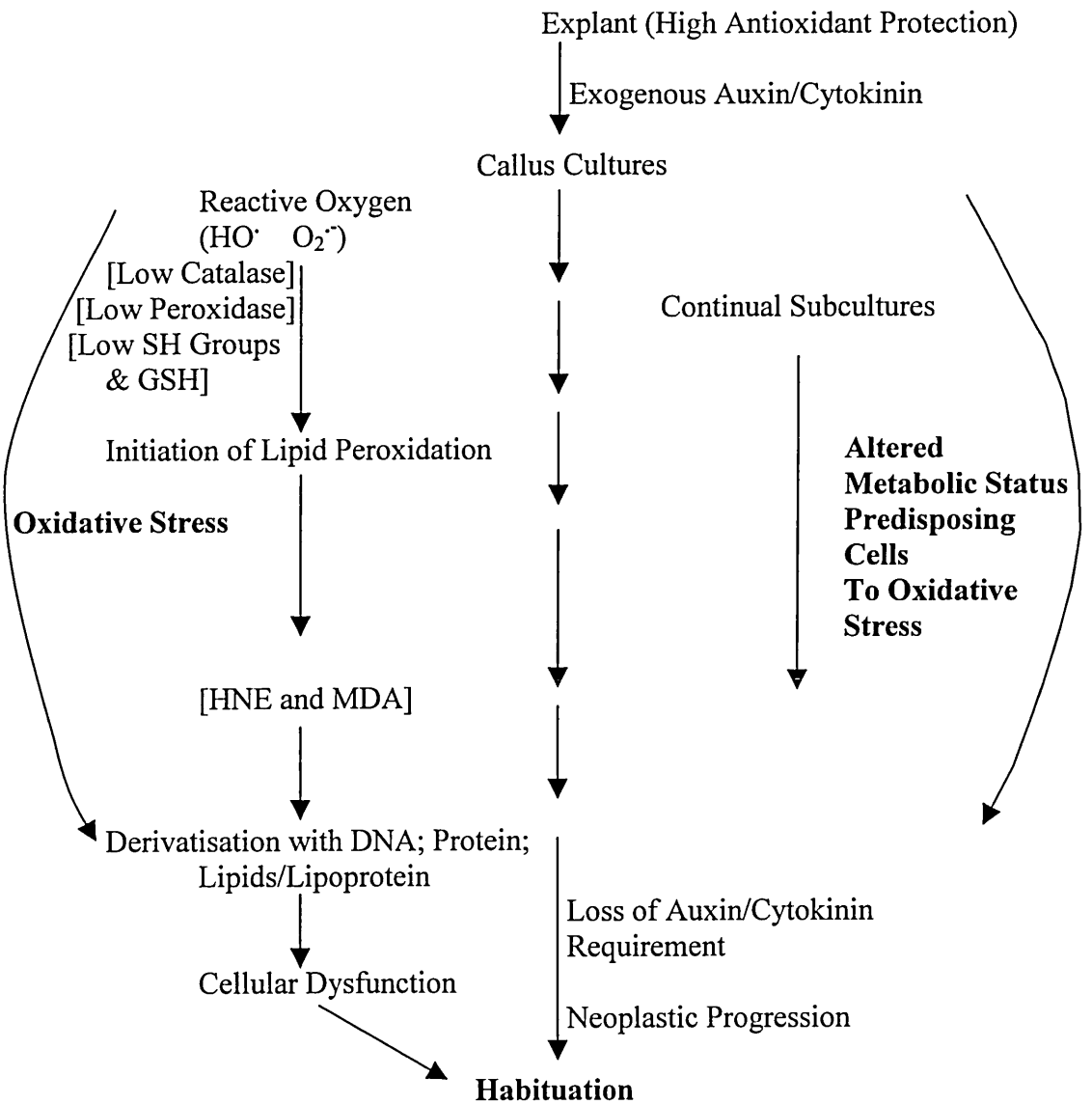
Enzyme linked immunosorbant assays (ELISA) were used to determine the presence of HNE-protein adducts in protein extracts of three callus types of *G. max*. This is the first report that these adducts have been detected in *G. max* cultures, and it is only the second plant species to have been shown to have HNE-protein adducts, after *I. batatas* (see chapter 4.4). HNE-protein adducts were detected in only one of the three callus types tested. Positive reactions were achieved for the white auxin-dependant callus line and not in the other two callus lines. The highest "free" HNE content was detected in the white habituated callus line, and the detection of HNE-protein adducts within the white auxin-dependant callus lines shows that in some plant tissue systems HNE may have more than one role, with the ability to be extractable or bound to proteins. In mammals HNE-lipoprotein adducts have been associated with atherosclerosis (Uchida, *et. al.* 1994) and HNE-protein adducts have been linked to apoptosis after ozone exposure (Kirichenko, *et. al.* 1996). HNE has also been shown to chemically react with glyceraldehyde-3-phosphate dehydrogenase (Uchida & Stadtman, 1993). HNE and HNE-protein adducts have been linked to neoplastic mammalian disease, has shown to be present in a wide range of disease conditions, although the exact role has yet to be established (Parola, *et. al.* 1999). The precise site of reaction of HNE with plant proteins is still unknown and would benefit from further study.

5.6.5 Habituation and Plant Tissue Culture

The links between the activity of antioxidants, and the initiation of pathways such as lipid peroxidation in habituated cells could have important implications in plant tissue culture. The exact relationships between lipid peroxidation, antioxidants and the state of habituation are still unknown and open for debate, however, during the investigation of this system a model can be presented for discussion and detail a number of relationships. This model is detailed in Figure 5.6.5 and illustrates the links between habituation, oxidative stress and the plant systems investigated during this project.

The links between secondary lipid peroxidation products generated as a result of oxidative stress and habituation are still unclear although it has been shown that high levels of the lipid peroxidation products HNE and MDA are found in the habituated callus compared to the auxin-requiring callus (see section 5.4). The low activity of the majority of antioxidants tested suggests that the active oxygen and free radical compounds are allowed to accumulate and therefore cause additional oxidative stress, the implications of these findings will be discussed further in the General Discussion (chapter 6).

Figure 5.6.5 Relationship between Habituation and Oxidative Stress in Plant Tissue Culture



5.6.6 Conclusions

This study is the first report of the presence of both HNE and MDA simultaneously in extracts of three types of *G. max,* including a higher content in the habituated auxin-independent (white-) callus line. It has also shown, for the first time, the presence of HNE-protein adducts within soluble protein extracts from *G. max* callus cultures. Therefore, lipid peroxidation products have been shown to be present in *G. max* in both "free" and bound forms in

extracts from callus cultures. The links between lipid peroxidation, antioxidant activity and the fate of lipid peroxidation products form one area of oxidative stress that requires further investigation to fully understand the responses of *in vitro* cultures. The development of methods that can be used to improve responsiveness and overcome recalcitrance in problematic species will further developments in biotechnology.

G. max Main Findings

- Highest antioxidant activity was in the green hormone-dependant callus line for catalase, superoxide dismutase, SH groups, and oxidised and reduced glutathione and low activity for all antioxidants measured for the habituated white – callus line.
- Hydroxynonenal and malondialdehyde measured simultaneously in callus cultures of *G. max* for the first time.
- HNE-protein adducts detected for the first time in protein extracts from *G. max* callus cultures with the highest level found in the white hormone dependant line.

Chapter 6 General Discussion

Contents

6.1 The Plant Tissue Culture Environment

Plant tissue culture underpins many aspects of modern plant biotechnology and was one of the intrinsic techniques in this study. Though many hundreds of species have been established *in vitro*, many more have been difficult or impossible to establish. Thus recalcitrance forms a significant problem in modern biotechnology and may act as a constraint to the uptake of many conservation programmes due to the problems of establishing endangered species in germplasm collections (Benson, 2000a). However, this is not the only difficulty faced, many species that are established in *in vitro* culture are responsive for some months or even years, but eventually lose their ability to respond to manipulations. This loss of potential means that maintaining responsive cultures indefinitely, within *in vitro* systems is difficult and as a consequence, new cultures have to be continually initiated to maintain adequate supplies of responsive tissue.

In order to understand the mechanisms of recalcitrance and loss of developmental and morphogenetic potential in *in vitro* systems, physiological and molecular studies have been undertaken (Benson, 2000a, McCown, 2000). However, even these tools are limited and to date there is still little understanding of the true mechanisms which cause recalcitrance (Benson, 2000a, Gaspar, *et. al.* 2000). The *in vitro* environment is thought by some researchers to promote stress and in particular, can induce changes in the oxidative/reductive balance within cells (Larson, 1995). The exact composition of the medium used can be crucial in maximising plant culture response, and the addition of particular factors such as hormones, amino acids

or the correct type of carbohydrate source can be used to overcome recalcitrance in some species (Benson, 2000a, Golmirzaie, *et. al.* 1999). Thus, careful manipulation of culture conditions can help to minimise stress and maximise responsiveness.

Animal systems also experience changes in the oxidative/reductive balance and in particular free radicals have become associated with cell signalling and stress (Suzuki, *et. al.* 1997). Exploring the generation of free radicals and the influence they have on cellular responses could give vital clues as to understanding the mechanisms of plant tissue culture stress and eventually recalcitrance (Benson, 2000b). One group of free radicals that have been shown to have a wide range of significant effects in animals systems are the oxygen based radicals and associated compounds such as hydrogen peroxide (Stadtman, 1989, Saran & Bors, 1989). One of the most significant of these are hydroxyl radicals, this species together with superoxide are responsible for the initiation of lipid peroxidation (Esterbauer, *et. al.* 1990). The relationships between the generation of free radicals, the propagation of lipid peroxidation and the antioxidants used by the systems to control them, may have significant implications in tissue culture for both animals and plants. This hypothesis formed the basis of the present study.

6.2 *Daucus carota*

6.2.1 *Daucus carota* - System of Investigation

Three different plant tissue systems were chosen for the investigation of free radical mediated stress. *Daucus carota* was selected as the first of these

species, and has been previously used for the development of techniques for the study of stress induced by lipid peroxidation products. *D. carota* has been studied within *in vitro* culture for many years, and therefore there is a large amount of documentation on its response (Ammirato, 1986). In particular, a great deal of study has focused on somatic embryogenesis (Zimmerman, 1993). This pathway forms an idea system for the study of development, and particularly of the changes induced within cells when undergoing differentiation. Somatic embryogenesis can be induced within *D. carota* with the simple manipulation of 2,4-dichlorophenoxyacetic acid (Zimmerman, 1993). In this study, techniques of the study of oxidative stress used within animal systems were adapted using *D. carota* tissues and extracts for the profiling of plant tissue. An example was the adaptation of an enzyme linked immunosorbant assay (ELISA) for the measurement of HNE-protein adducts (Waeg, *et. al.* 1996). The antibodies used within this assay were generated using mammalian cells (Waeg, *et. al.* 1996) and have never been used before in plant tissue for the detection of HNE-protein adducts. Also, the exogenous application of lipid peroxidation products has been used to test their toxic effects within mammalian cell systems (Zollner, *et. al.* 1991), but to date their effects have not been investigated in plant cells, until this study. This forms a novel approach in plant systems for evaluating the effects of lipid peroxidation products on growth and development in plants.

6.2.2 Summary of Findings for *Daucus carota*

Preliminary profiling of a range of cultivars of *D. carota* showed the variability between cultivars for the rates of germination for seeds and the induction of callus from explants taken from seedlings (section 3.1). Certain

cultivars were poor performers and later studies reflect this when they were not included due to lack of sufficient callus formation. The induction of somatic embryogenesis in *D. carota* is easier than for many other species and embryos can be produced from callus cultures in a few weeks, however this ability can decline with time and eventually almost all cultures will stop producing embryos. A number of these embryogenic and non-embryogenic cultures were compared for protein content and in all cases the protein content for the embryogenic line was higher than that for the non-embryogenic line (section 3.2).

Comparisons were also made between embryogenic lines and non-embryogenic lines for the presence of the lipid peroxidation products malondialdehyde (MDA) and hydroxynonenal (HNE) using the method established by the group using liquid chromatography mass spectrometry (Deighton, *et. al.* 1997). Both lipid peroxidation products were found to be present in extracts from embryogenic and non-embryogenic callus lines. The most embryogenic line E10 showed the lowest levels of both products. This may suggest that levels of lipid peroxidation products free within the cells are lowest in the most embryogenic lines, or that these cultures are more able to control the extent to which lipid peroxidation takes place within the cells. Levels of MDA and HNE are much lower than those measured previously in embryogenic and non-embryogenic lines of *D. carota* (Deighton, *et. al.* 1997), although in the previous study the levels of MDA were lower in the embryogenic than the non-embryogenic lines. The reduced HNE and MDA content may be due to ageing effects being experienced by the callus as some

of the callus lines used were also used in this previous study (Deighton, *et. al.* 1997) and the reduced content may reflect the decline in morphogenetic capacity.

For the first time, this investigation studied the effects of exogenously applied MDA and HNE in plants using embryogenic and non-embryogenic carrot tissue cultures. The effects of MDA and HNE on the growth and development of embryogenic and non-embryogenic callus cultures were evaluated over a range of concentrations. Both lipid peroxidation products were shown to have a significant inhibitory effect on the growth rates of both embryogenic and non-embryogenic callus cultures, with growth rates being reduced by over 4-fold. These effects were shown at all concentrations of HNE and MDA added to the culture medium. After the transfer of the tissue onto medium free of the compounds, growth rates recovered, and for the non-embryogenic line the growth rate was stimulated to over 3000% after the removal of HNE on 2,4-D medium. The growth rates after MDA treatment also recovered, with the greatest growth rate exhibited by the embryogenic line on 2,4-D medium. The exogenous application of both HNE and MDA inhibited the embryogenic callus line on MS medium from forming somatic embryos on hormone-free medium, on two of the HNE concentrations somatic embryogenesis was completely inhibited. On MDA treatment the inhibition was less apparent with all treatment concentrations able to produce some embryos. After the removal of both compounds the rate of somatic embryogenesis was increased in two of the four concentrations, for HNE treatments, and for all the MDA treatments, with the MDA treatments able to produce levels of embryo

production similar to the controls. However, the generation of somatic embryos on 4.2 nM HNE, was irreversibly inhibited and never produced any embryos.

The HNE and MDA concentration within the cells was measured at the end of the experiment. Measured levels were similar to those that had been measured in other embryogenic and non-embryogenic lines of *D. carota,* and that for both compounds showed a decrease in concentration as the level of applied compound increased. Peroxidase was also measured at the end of the experiment and for all treatments and post-treatments showed higher activity in the non-embryogenic tissue compared to the embryogenic tissue. The effect of the exogenous application of HNE and MDA had profound effects on the growth rates and development of somatic embryos in callus cultures of *D. carota.*

Enzyme linked immunosorbant assays (ELISA) have been widely used as an analytical tool in mammalian systems to study activities of a range of biochemical and immunological compounds (Harlow & Lane, 1988). HNE-protein adducts have been detected within mammalian tissues and have been associated with tissue that is undergoing apoptosis or oxidative stress (Kirichenko, *et. al.* 1996, Cohn, *et. al.* 1996) but there has been little such work reported within plant systems. An ELISA was adapted using antibodies generated in mammalian systems for the detection of HNE-protein adducts (Waeg, *et. al.* 1996) in protein extracts from plant cells. Extensive trials using a range of protein extracts from embryogenic and non-embryogenic callus

were used to establish a suitable methodology for the detection of HNE-protein adducts. Positive signals were achieved for the protein-HNE standard, however no positive signals were achieved for any protein extracts from embryogenic or non-embryogenic callus from all the cultivars tested.

D. carota Main Findings

- Reversible inhibition of growth and somatic embryo development by the exogenous application and removal of hydroxynonenal and malondialdehyde
- Development of an ELISA for the detection of HNE-protein adducts in plant extracts, although they were not detected within *D. carota*
- Routine measurement of hydroxynonenal and malondialdehyde extracted from tissues of *D. carota*

6.3 *Ipomoea batatas* (L.) Lam.

6.3.1 *Ipomoea batatas* – System of Investigation

The second crop system selected for study was *Ipomoea batatas* (sweet potato). This crop was chosen as it is a very important crop commercially in the third world and is currently the seventh most important crop in the world. Improvements in existing cultivars related to the ability to be able to resist pests and diseases and survive in very harsh conditions could increase yields in the areas where sweet potato is relied upon for the survival of animals and people alike. Improvement and conservation programmes have been established by the International Potato Center (CIP) based in Peru and the National Bureau of Plant Genetic Resources (NBPGR) in India. One method of improving existing cultivars is through genetic transformation and then mass propagation using somatic embryogenesis (Chee & Cantiliffe, 1992).

Somatic embryogenesis has been achieved in a number of cultivars by different groups, but many cultivars still remain unresponsive or only produce low numbers of embryos (Zheng, *et. al.* 1996, Jarret, *et. al.* 1984, Al-Mazoorei, *et. al.* 1997). The mechanisms of this unresponsiveness or recalcitrance are yet to be established, although oxidative stress has been implicated. Using the tools developed using *D. carota* tissue and extracts, investigations into the role of lipid peroxidation and oxidative stress could have implications for the future treatment of *I. batatas* in *in vitro* systems and treatment in the field.

6.3.2 Summary of Findings for *Ipomoea batatas*

Micropropagation studies showed that all the cultivars tested exhibited similar responses in the height of plantlets and the number of nodes produced. However, for callus induction experiments there were more differences between the cultivars, and the Indian cultivar S-256 showed the highest amount of callus induction on 2,4,5-T medium. On S2 medium (containing abscisic acid), there were little differences between the three cultivars tested. Further callus induction experiments were used to ascertain differences in response between the nodes of two of the cultivars over a two-week period on 2,4,5-T medium. For the protein content and peroxidase activity there was little difference between the two cultivars but there was a significant differences over the two-week period, with both cultivars showing an increased amount of protein, little activity was noted for catalase with only low levels measurable in the final days of culture. The profile for the total sulphydryl group content was less clear, with lower expression during the middle of the two-week period. The reduced glutathione profile showed a

high initial value and then a drop in activity with a slow increase towards the end of the two-week incubation. Glutathione reductase activity steadily decreased over the two-week period, with little difference between the two cultivars. For glutathione S-transferase the first week showed little activity and then a rise in activity for the second week, with little difference between the two cultivars. The HNE and MDA were profiled for a range of callus cultures generated from *I. batatas*. The MDA content of callus generated from leaves, petioles, and internodal sections showed that the cultivar S-256 had the lowest MDA concentration. For the HNE content the callus generated on the internodal sections showed the lowest activity. The HNE and MDA content was also profiled for callus generated from leaves on two different media regimes and a high MDA content was shown in IC-14 on 2,4,5-T medium, but the results for the HNE content showed no significant trends.

The ELISA, developed using *D. carota* extracts, was used for the detection of HNE-protein adducts within extracts of *I. batatas* callus and showed for the first time that HNE-protein adducts could be found within protein extracts of plant tissue cultures. The presence of HNE-protein adducts were profiled in a range of callus cultures generated from different explants and media regimes. Callus generated from leaves using 2,4,5-T medium and S1 medium (containing 2,4-D and BAP) showed higher levels of HNE-protein adducts in the Peruvian cultivars IC-14 on 2,4,5-T and IC-21 on S1 medium. The Indian cultivar S-256 showed the lowest activity for callus generated from leaves. For callus generated from internodal sections on S1 medium there was less

difference between the three cultivars although the Peruvian cultivar IC-21 showed the lowest presence.

The activity of hydroxyl radicals has been shown in nodes of *I. batatas* under callus initiation conditions using a DMSO based assay and the evolution of methane and ethylene measured by gas chromatography. After two days on the callus induction medium the activity was higher in the cultivar Nemanete. A peak on ethylene emission was noted in both cultivars after 7 days of treatment.

I. batatas Main Findings

- Variation in the extent of callus initiation between three cultivars with two methods, but the study was unable to induced somatic embryogenesis.
- The first measurement of HNE and MDA in a range of callus cultures of *I. batatas.*
- First detection of HNE-protein adducts using an ELISA in any plant system.

6.4 *Glycine max*

6.4.1 *Glycine max* –System of Investigation

The final culture system selected in this study was three callus lines of the crop *G. max* that had different auxin and light requirements. Habituation is the acquired and hereditary capacity for autonomous growth, in the absence of exogenously applied auxins and/or cytokinins in tissue culture (Gaspar, 1999, Christou, 1988, Jackson & Lyndon, 1990). Habituation has been observed, in some cases, as a gradual process, while in others it has occurred spontaneously

generating a neoplastic stage (Gaspar, 1995, 1999). There is now increasing evidence to support the proposal that habituated cells are cancer cells as they have many common characteristics shared with animal metastases including hormone independence, complete loss of cell-to-cell adhesion, permanent oxidative stress and accumulation of polyamines (Hagège, 1996, Gaspar, 1998). The investigation of oxidative stress and particularly the influence of lipid peroxidation and its link with antioxidants within habituated systems may give valuable insight into the mechanisms of this inherited condition. Moreover, this system provides the opportunity to study habituation and loss of morphogenetic capacity, which may be considered an extreme recalcitrance condition.

6.4.2 Summary of Findings for *G. max*

The activities of a range of antioxidants and protein were profiled in three callus cultures of *G. max*. The protein content of the callus lines was greatest in the green auxin-dependant line and the two white lines having a similar level. Catalase activity was also highest in the green callus line and low in both the white callus lines. Peroxidase activity however, was highest in the auxin-dependant white line and low in both the green line and white – line. Superoxide dismutase activity was highest in the green auxin-dependant line and low in both the white callus lines. The sulphydryl groups content for the total and the non-protein groups were both highest in the green line and low for both the white lines. Reduced glutathione concentration was highest in the green auxin-dependant line and low in the two white lines and there was little activity in any of the lines for oxidised glutathione. The associated glutathione enzymes, glutathione reductase and glutathione s-transferase were

also measured. The white auxin-dependant line had the highest activity for glutathione reductase and the activity of glutathione s-transferase was undetectable. Generally for most of the antioxidants the highest activity was shown in the green line. Two exceptions were peroxidase and glutathione reductase, for which activities were highest in the white auxin-dependant line. Overall the habituated white – line had low activities for all the antioxidants tested. Low antioxidant activity was previously shown in habituated callus of sugarbeet (Le Dily, *et. al.* 1993).

The hydroxynonenal and malondialdehyde content of the three types of *G. max* callus was profiled in callus at the mid sub-culture cycle phase. The concentration of HNE and MDA was highest in the white auxin-dependant line, with little activity in either of the other two lines. The enzyme-linked immunosorbant assay, established using *D. carota* callus extracts, was used to detect the presence of HNE-protein adducts within *G. max*. The only positive signals were achieved in protein extracts from the white auxin-dependant callus line, with little evident activity within the other two callus lines.

Hydroxyl radical activity was profiled in callus at the mid sub-culture cycle phase of the three cultures of *G. max* and in an equivalent aged set, sampled a week after the end of a normal sub-culture cycle. Increased methane production was noted in the second set for all the callus lines after two days treatment, with the highest activity shown in the white auxin-dependant line.

G. max Main Findings

- Highest antioxidant activity was in the green hormone-dependant callus line for catalase, superoxide dismutase, SH groups, and oxidised and reduced glutathione and low activity for all antioxidants measured for the habituated white – callus line.
- Hydroxynonenal and malondialdehyde measured simultaneously in callus cultures of *G. max* for the first time.
- HNE-protein adducts detected for the first time in protein extracts from *G. max* callus cultures with the highest level found in the white hormone dependant line.

6.5 Implications of the Studies of a Model System on Micropropagation and Somatic Embryogenesis in *In Vitro* Systems

Daucus carota has been used as a model system for many years and a great deal of information is known about its responses within *in vitro* systems. One of the most studied pathways in this species, is somatic embryogenesis and this forms a system of choice of a plant *in vitro* developmental pathway. Studies within this project have emphasised that shown *D. carota* to be a responsive species and easy to generate somatic embryos from a range of cultivars. It was found that embryogenic callus has a higher protein content and is generally more responsive, with higher antioxidant activity and that non-embryogenic callus cultures have a lower protein and peroxidase activity that embryogenic callus. This concurs with previous findings that embryogenic cells of rice have a higher peroxidase activity than previously embryogenic callus (Benson, *et. al.* 1992a).

However, even in a responsive species such as *D. carota*, tissues can lose the ability to generate somatic embryos. Such changes may be due to changes in metabolism as a result of oxidative stress or damage by secondary oxidative products (Benson & Roubelakis-Angelakis, 1992, 1994, Benson, 1990, Robertson, *et. al.* 1995, Benson, *et. al.* 1997, Bremner, *et. al.* 1997). The decline in somatic embryogenic ability within *in vitro* conditions may be due to the generation of damaging free radical species and the inability of antioxidant enzymes to control them. In this study aged *G. max* callus showed a higher rate of evolution of methane generated by the activity of hydroxyl radicals compared to similar callus at the mid-point of the sub culture phase. This may suggest that reactive oxygen species can accumulate throughout the growth phase of a callus culture and can therefore cause damage if not effectively controlled by antioxidant activity and can cause irreversible changes, such as enzymatic dysfunction and genetic damage (Evans & Sharp, 1986, Bartosz, 1997).

Somatic embryogenesis is not always possible in other species and can be difficult in some genotypes of *I. batatas*. A number of different culture regimes have been established and have generated somatic embryos in a number of cultivars (Zheng, *et. al.* 1996, Al-Mazrooei, *et. al.* 1997, Jarret, *et. al.* 1984). However, the regimes employed have not been able to generate somatic embryos in the cultivars tested, in one of the regimes only four out of ten cultivars tested were responsive (Zheng, *et. al.* 1996) and at best fourteen out of sixteen were responsive (Al-Mazooei, *et. al.* 1997). These two methods were employed in this project but failed to produce any embryos (Zheng, *et.*

al. 1996, Al-Mazrooei, *et. al.* 1997) (section 4.1). This may due to the explants chosen may not be the most suitable tissues to use with the hormones involved, using meristematic tissues such as axillary buds may prove more responsive (Al-Mazrooei, *et. al.* 1997). The cultivars used may be recalcitrant with the techniques employed. The use of alternative hormones or different concentrations to those used may initiate responsiveness. The responses of cultivars can be genetically predetermined and for some species cannot be overcome, however different strategies can be used to circumvent this and for instance in woody species the use of juvenile tissues has been beneficial (McCown, 2000). Further investigation into the mechanisms behind recalcitrance may give vital improvements to the culture regimes employed for the generation of somatic embryos in *I. batatas*. Choosing explants that maximise responsiveness and minimise oxidative stress may be crucial in improving *in vitro* regeneration systems. Oxidative stress within the tissue cultures systems may have inhibited the production of somatic embryos and application of additives such as activated charcoal may help to remove toxic species produced by the tissue (Perl, *et. al.* 1996).

6.6 Oxidative Stress and Antioxidants in *In Vitro* Plant Systems

Oxidative stress has been implicated as one of the causes of variability in responses of plant species to culture, and may be influential in plant recalcitrance (Benson, *et. al.* 1997, Benson & Roubelakis-Angelakis, 1992 & 1994, Ishii, 1987, Bailey, *et. al.* 1994, De Marco & Roubelakis-Angelakis, 1996, Benson, 2000b, Gaspar, *et. al.* 2000). Reactive oxygen species are one of the causes of oxidative stress and are controlled by antioxidants. The balance between the generation of reactive oxygen species and the

antioxidants that remove them is crucial in maintaining the oxidative/reductive balance. The relationship between the reactive oxygen species and the initiation of lipid peroxidation is shown in Figure 6.7.1 (adapted from N. Deighton, personal communication). The initiation of lipid peroxidation by reactive oxygen species can be controlled by antioxidants and the removal of these species may prevent this process and the resulting damage. Allowing reactive oxygen species to accumulate can have far reaching consequences, such as through the production of hydrogen peroxide which can cause oxidative stress in its own right, and will also lead to the generation of hydroxyl radicals which can initiate lipid peroxidation. Therefore, any processes within the plant cell that disrupts the removal of oxidative species can initiate damaging processes that can inhibit growth and development within *in vitro* culture.

During changes in morphogenetic status, changes in the activity of many of these antioxidants were noted in *I. batatas* and an increase was shown for peroxidase activity. Increased peroxidase activity was also shown in embryogenic *D. carota* callus compared to non-embryogenic callus. During the early stages of callus initiation, the level of catalase in *I. batatas* was undetectable and activity was only measurable towards the end of the first two weeks (see section 4.2). However, for all SH group containing species and the enzymes associated with glutathione metabolism a decrease in activity was shown, except for glutathione s-transferase in *I. batatas* (see section 4.2). This decrease in activity of a number of antioxidants was similar to that noted in

Figure 6.7.1 Relationships between Reactive Oxygen Species, Lipid Peroxidation and Antioxidants

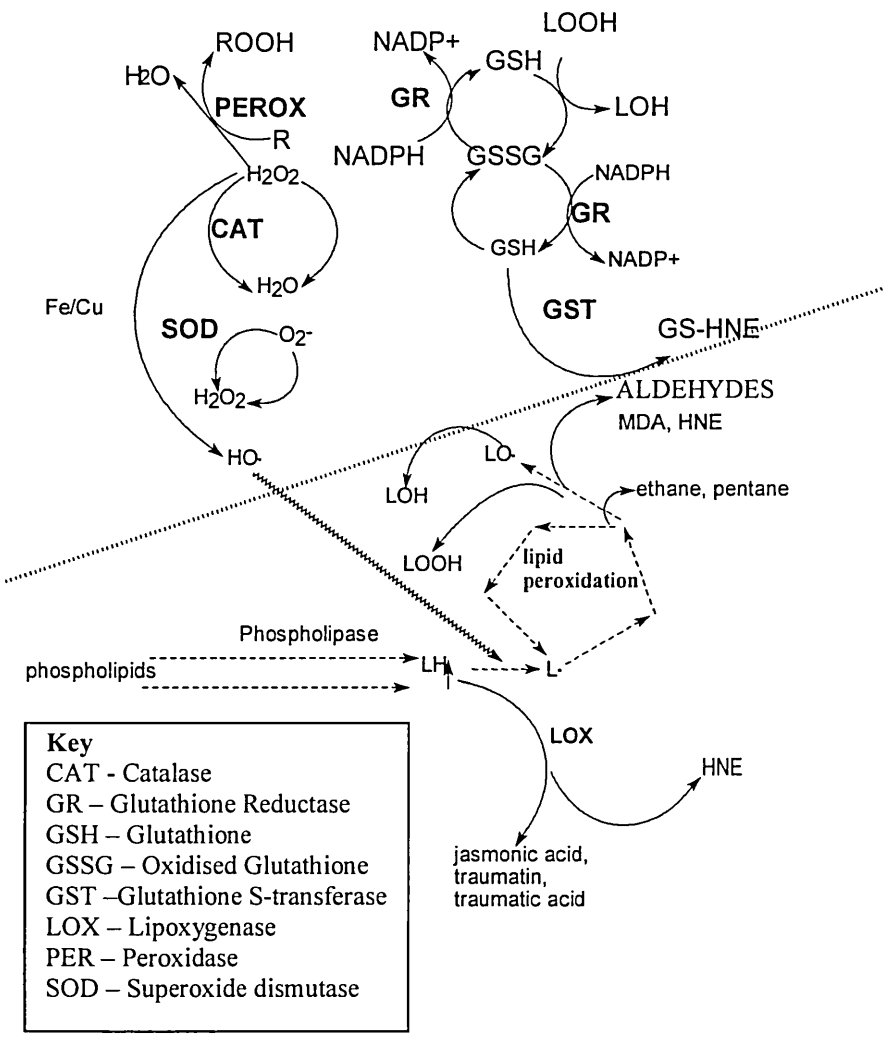


Figure 6.7.1: Relationships between reactive oxygen species, antioxidants and lipid peroxidation in plant cells (adapted from N. Deighton, personal communication).

grapevine callus initiation, and a lowering in antioxidant activity was also necessary to permit somatic embryo development (Benson & Roubelakis-Angelakis, 1994). This suggests that tissues undergoing callus initiation are susceptible to oxidative stress.

A decline in morphogenetic capacity is exemplified by the habituated cultures of *G. max* where all regenerative potential had been lost and cultures are only able to remain in a dedifferentiated state (Kevers, *et. al.* 1996). The progression of hormone-independant tumours that eventually lead to plant cancer and are considered to have accumulated a number of genetic accidents with cumulative effects (Gaspar, *et. al.* 2000). The causes of these changes are unknown, although links have been made with oxidative stress, the generation of malondialdehyde and hydrogen peroxide metabolism (Gaspar, *et. al.* 2000). The activity of antioxidants was generally low in the white auxin-independent habituated callus line and may have allowed reactive oxygen species to accumulate. Such an accumulation of hydrogen peroxide has been associated with habituation in *Beta vulgaris* L. (Le Dily, *et. al.* 1993). The link between reactive oxygen species and lipid peroxidation may give indications towards some of the underlying mechanisms of oxidative stress.

6.7 Lipid Peroxidation and its Implications in Plant Culture

One of the causes of oxidative stress is the generation of free radicals that are responsible for the initiation of lipid peroxidation (see chapter 1). Lipid peroxidation has been well documented within animal systems and has been linked to a wide range of diseases including arthritis, cancer and atherosclerosis (Esterbauer & Ramos, 1995, Zollner, *et. al.* 1991, Spiteller, *et. al.* 1996). Lipid peroxides break down to form a diverse range of aldehydic products which are potentially toxic to cells as, since being potent electrophiles, they are able to form adducts with macromolecules such as DNA, proteins and enzymes (Spiteller, *et. al.* 1996, Yang & Saich, 1996, Grune, *et. al.* 1997).

Two of the most of the most significant lipid peroxidation products are hydroxynonenal (HNE) and malondialdehyde (MDA) and their activity is now thought to contribute to secondary oxidative stress which is thought to be a major factor in free radical mediated damage (Zollner, *et. al.* 1991, Esterbauer, 1996). A wide range of studies in plants has been undertaken to monitor lipid peroxidation under different stressful conditions, but all studies have focused only on MDA (Salata & Tal, 1998, Chaoui, *et. al.* 1997, Barclay & McKersie, 1994, Zhadko, *et. al.* 1994, Hung & Kao, 1997). These studies have used the thiobarbituric acid assay to measure MDA, this assay has a number of drawbacks as many substances can also react and produce mis-leading results (Salata & Tal, 1998, Chaoui, *et. al.* 1997, Barclay & McKersie, 1994, Zhadko, *et. al.* 1994, Hung & Kao, 1997). In order to improve measurement of MDA and HNE, the University of Abertay's Free Radical Research Group together with the Scottish Crop Research Institute, have developed an assay using liquid chromatography mass spectrometry for the measurement of the two aldehydes simultaneously from plant tissue culture extracts (Deighton, *et. al.* 1997). The presence of both compounds has been shown within callus cultures of *D. carota* (Deighton, *et. al.* 1997, Bremner, *et. al.* 1997). However, they have not been measured in any other plant system simultaneously until this project and both were found to be present in *I. batatas* and *G. max*. The presence of these products may be inhibitory to the growth and development of tissues, as the exogenous addition of both compounds was shown to inhibit the growth rate of the *D. carota* callus cultures. The rate of generation of somatic embryos was also inhibited, however, for most concentrations used the effects were reversible when

transferred to medium without aldehydes (Adams, *et. al.* 1999). The effects were experienced by cells with the application of nmol/L concentrations which were much lower than similar studies in animal and microbial cells where μmol/L to mmol/L were required to exhibit inhibitory effects (Poot, *et. al.* 1988, Fazio, *et. al.*1992, Dawes, *et. al.* 1997). This indicates that plant cells are very sensitive to the exogenous application of HNE and MDA, but at nmol/L concentrations the effects are largely reversible, showing that damaging effects are not permanent and do not appear to initiate programmed cell death. However, permanent damage is likely to be caused by the addition of higher concentrations.

In *I. batatas* the measured levels of HNE and MDA were lowest in callus generated from internodal sections of three cultivars (see section 4.3). This indicates that there may be distinct differences in lipid peroxidation in different explants and the callus generated from them, and that the choice of explant may be crucial in maximising the responses of plant species within *in vitro* culture. In generating callus from carnation explants the callus generated from internodal sections was least likely to generate shoots through organogenesis and indicate that certain explants maybe predisposed to poor regeneration (Kallak, *et. al.* 1997). The low levels of HNE and MDA measured within callus generated from internodal sections and the low response of this callus may indicate that the lipid peroxidation products may have a role within signalling (Parola, *et. al.* 1999) and that a certain level may be needed to achieve organogenesis. MDA has been shown to conjugate to

protein (Uchida, *et. al.* 1997) and DNA (Chaudhary, *et. al.* 1994) and therefore it may have a role within regulation.

The measurement of HNE and MDA within callus cultures of *G. max* showed that the habituated white callus line had the highest levels for both aldehydes (see section 5.4). The measured aldehyde levels within this callus line were higher than those measured within callus generated from a range of explants of *I. batatas.* The high levels of aldehydes in the habituated callus compared to the two other callus lines and the associated low levels of antioxidant activity particularly catalase and peroxidase indicate that this systems is likely to be under the most oxidative stress. High levels of MDA were found in habituated callus compared to normal callus in *Beta vulgaris* (Le Dily, *et. al.* 1993). Reduced morphogenetic competence has been correlated with free radical production (Bailey, *et. al.* 1994), and enhanced levels of TBARS (Benson, *et. al.* 1992b) and MDA (Bremner, *et. al.* 1997). Therefore, the high levels of lipid peroxidation products in habituated callus of *G. max* may be associated directly or indirectly with the reduced capacity for development.

ELISA detection of HNE-protein adducts had been established in mammalian cultures (Waeg, *et. al.* 1996) and was adapted for use within plant systems. HNE-protein adducts were detected within a range of extracts from *I. batatas* and *G. max* cultures. This shows that lipid peroxidation products are not only detectable "free" in plant cells but that they have the ability to conjugate to macromolecules in the cell. HNE-protein adducts have been shown to conjugate to important enzymes such as glyceraldehyde -3-phosphatase

dehydrogenase (Uchida & Stadtman, 1993). The significance of HNE-protein adducts within plant systems is yet to be investigated but it has been shown within this project that they are detectable within soluble protein extracts of *I. batatas* and the auxin-dependant white callus line of *G. max*.

6.8 Habituation and Cancer

The ability of plant cultures to continue to divide but become autonomous of growth factors has made habituation an interesting phenomenon within plant tissue culture (Christou, 1988, Jackson & Lyndon, 1990, Droual, *et. al.* 1998, Szabó, *et. al* 1994, Meins, 1989, Gaspar, 1995). Habituation is seen as a neoplastic step towards "plant cancer" as defined by Gaspar (1999) who also states that utilising techniques used within the study of mammalian cancer may give further insight into this condition. The focus of the studies of habituated systems within this project was the role of lipid peroxidation products and their associated antioxidants within different types of callus, one of which was habituated against the hormone benzylamino purine (BAP) but still requiring napthaleneacetic acid (NAA).

Biochemical characteristics of habituated callus associated with plant cancer include low ethylene production, permanent oxidative stress and the irreversible loss of organogenic totipotency (Gaspar, 1999). Protein and the activity of a range of antioxidants were generally highest in the green auxin-dependant line, although for peroxidase and glutathione reductase the activity was highest in the white auxin-dependant line, with the lowest activity noted for the habituated line. A lack of catalase and peroxidase as well as a high level of MDA (as TBARS) have been previously associated with habituation

and may allow hydrogen peroxide to accumulate within habituated cells of sugar beet (Le Dily, *et. al.* 1993). This accumulation of hydrogen peroxide could lead to the increased production of hydroxyl radicals and therefore the initiation of lipid peroxidation. The formation of MDA is attributed to the action of radicals and when the activity was of hydroxyl radicals was measured by their reaction with DMSO and subsequent evolution of methane. The aged habituated callus had a higher hydroxyl radical activity than callus taken from the mid-point of the subculture cycle and indicated that aged callus is likely to be experiencing a greater level of oxidative stress. MDA activity was highest in the habituated callus line and was high compared to those levels measured in callus generated from a range of explants of *I. batatas* or *D. carota* callus after treatment with HNE or MDA. The high activity of hydroxyl radicals, the high levels of MDA and HNE and the low level of activity of antioxidants all indicate that the habituated callus has a higher level of lipid peroxidation that other callus types. The systems in place to remove damaging species appear to be less efficient and allow damaging species to accumulate within *in vitro* culture.

Reactive oxygen species play a role as cell signals (Saran & Bors, 1989, Suzuki, *et. al.* 1997, Degousee, *et. al.* 1997, Alscher, *et. a*l 1997) and are implicated in the redox regulation of cell proliferation, and also in apoptosis occurring frequently in cancer cells (Burdon, *et. al.* 1990). The presence of reactive oxygen species has also been associated with programmed cell death in plants (Pennel & Lamb, 1997). The apparent high activity of such species within habituated callus concurs with previous studies that have shown that

reduced morphogenetic competence has been correlated with free radical production (Bailey, *et. al.* 1994), and enhanced levels of TBARS (Benson, *et. al.* 1992b) and MDA (Bremner, *et. al.* 1997). The accumulation of damage as a result of free radical action may be one of the factors responsible for the decreased ability of the tissue to regenerate within *in vitro* culture.

6.9 Markers of Morphogenesis and Development

Markers of morphogenesis and development form important tools in maximising responses of plant species in *in vitro* culture. Established markers include peroxidase which generally has a higher activity within tissues that are responsive than those that are not and has been shown to be stimulated during development (Krsnik-Rasol, 1991, Wakamatsu & Takahama, 1993, Jouve, *et. al.* 1994). Rises in activity of peroxidase have been shown after explants have been transferred to callus induction medium in *I. batatas* and a higher activity of peroxidase was shown in *D. carota* callus capable of producing somatic embryos. High peroxidase activity is therefore, from the results in this study, associated with responsive tissue and the initiation of developmental pathways. It could be hypothesised that this could be used as a marker to differentiate between embryogenic and non-embryogenic tissue of *D. carota*.

Glutathione has been increasingly associated with apoptosis and may have significant reactions with proteins, although the role and effects are not currently fully understood (Cotgreave & Gerdes, 1998). Glutathione levels have been used to differentiate between embryogenic and non-embryogenic callus, and in loblolly pine glutathione levels are higher in non-embryogenic callus than embryogenic callus (Wann, *et. al.* 1989). Increased levels of GSH

have been associated with ageing in fibroblasts (Poot, *et. al.* 1984); oxidative stress in *G. max* (Knorzer, *et. al.* 1996) and herbicide treatment in bean leaves (Schmidt & Kunert, 1986). The glutathione and the associated enzyme activities decreased over the first two weeks of the callus initiation from nodes of *I. batatas* indicating that lower levels of glutathione may be markers of the onset of developmental pathways (Wann, *et. al.* 1989). However, the control of glutathione is complicated and regulated by a number of different systems (Cotgreave & Gerdes, 1998).

Antioxidants are not the only marker that could be used to differentiate between systems of different morphogenic capability. HNE-protein adducts measured using the ELISA developed during this project have the possibility of being used as an assay to differentiate between different types of tissue. In mammals HNE-lipoprotein adducts have been used as a marker for atherosclerosis (Uchida, *et. al.* 1994). There may be some scope in the further investigation into the levels of HNE-protein adducts within plant systems which prove as useful markers for determining morphogenic capacities within plant tissue culture systems.

6.10 Application of Pro-oxidants and Antioxidants in Tissue Culture

Utilisation of the knowledge gained as to the role of antioxidants and the possibility of the application of them for the increase responsiveness of *in vitro* cultures may form an important area in the improvement of existing culture regimes (Zang & Kirkham, 1996, Earnshaw & Johnson, 1987). The basis for most modern tissue culture regimes has been empirically over many years (Gaspar, 1990), however the advent of new analytical methodologies

may contribute to a better understanding of these procedures. A fuller understanding of the stresses and mechanisms of tissue recalcitrance within *in vitro* conditions will give a better understanding as to the routes for the improvement of these responses. As previously stated, most culture regimes have been developed by trial and error, with profiling of a large range of cultivars on a range of different hormone and culture conditions. However, the responses vary greatly between cultivars and finding a suitable regime to maximise the responsiveness of the greatest number of cultivars can be very difficult in some species (e.g. woody plant species (McCown, 2000)). Using the fundamental knowledge gained within this project further work using ELISA and antioxidant profiles could be used to enhance the responses in culture. Using new approaches such as ELISA and molecular markers will aid the understanding of plant tissue culture systems and their responses *in vitro.*

6.11 Future Work

Further insight into oxidative stress in plant tissue culture, particularly that associated with lipid peroxidation, would benefit from further study into the role of HNE-protein adducts within plant systems. This study has shown the first detection of these adducts within plant systems and establishing that the proteins are derivatised. Their role in development will give an indication as to the significance of these adducts within plant systems. In mammalian systems the conjugation of HNE and MDA onto proteins and lipoproteins has been associated with diseases such as atherosclerosis (see previous). Monitoring changes in the presence of HNE-protein adducts during callus initiation and changes in development status will help to establish if HNE-adducts play a role in development. Furthering the study with the profiling of

MDA-protein adducts, will also help establish if the conjugation of lipid peroxidation to proteins has a role in development. Applying this ELISA technique to a wider range of plant species such as woody plants will help determine the significance of these derivatives. The anti HNE-protein antibodies could be used for Western Blotting, which could isolate the proteins that are derivatised by HNE. The proteins could be identified and proteomics could discover the genes being expressed.

The detection of HNE and MDA in *G. max* and *I. batatas* is the first time these two aldehydes have been measured in these two species. Further studies on changes in HNE and MDA content during the initiation of callus and the onset of somatic embryogenesis may help establish the possible role of lipid peroxidation products within growth and development in plant systems. MDA has been shown to derivatise onto DNA in rat liver (Chaudhray, *et. al.* 1994), although the significance in plants systems has not been investigated and would form a possible role for MDA in the regulation of gene expression. Extending the HNE and MDA detection to further plant species, particularly problematic groups (such as woody species, cereals, and rare species) would help elucidate the significance of these lipid peroxidation products.

Appendices

Contents

Appendix 1 Media Tables

Table 1 Composition of Basic Murashige and Skoog Media

COMPONENT	FINAL CONCENTRATION (mg/L)
$CaCl_2$	440
NH_4NO_3	1650
KNO_3	1900
KI	0.83
$CoCl_2.6H_2O$	0.025
KH_2PO_4	170
H_3BO_3	6.2
$Na_2MoO_4.2H_2O$	0.25
$MgSO_4.7H_2O$	370
$MnSO_4.4H_2O$	22.3
$CuSO_4.5H_2O$	0.025
$ZnSO_4.7H_2O$	8.6
$FeSO_4.7H_2O$	27.85
Na_2EDTA	37.25
Glycine	2.0
Myo-inositol	100.0
Nicotinic acid	0.5*
Pyridoxine HCl	0.5*
Thiamine HCl	0.1*
Sucrose	30 g/L
Agar	0.7% (7 g/L)
PH	Corrected to 5.6 with 1M NaOH

*For S1 and S2: nicotinic acid 2.4 mg/L; pyridoxine HCl 1mg/L; thiamine HCl 1.7 mg/L and the addition of KCl 2.235g/L and phytagel 3g/L instead of agar.

Table 2 Procedures for Preparation of Hormone Solutions

HORMONE	SOLVENT	DILUTENT	AUTOCLAVED/ FILTERED
Abscisic acid (ABA)	1M NaOH	Water	Filtered
Benzyl amino purine (BAP)	1M NaOH	Water	Autoclaved
2,4- dichorophenoxyacetic acid (2,4-D)	1M NaOH / Ethanol	Water	Autoclaved
Indole acetic acid (IAA)	Ethanol / 1M NaOH	Water	Filtered
Kinetin	1M NaOH	Water	Filtered
Napthaleneacetic acid (NAA)	1M NaOH	Water	Autoclaved
2,4,5-Trichorophenoxy-acetic acid (2,4,5-T)	Ethanol	Water	Autoclaved

Table 3 ***Glycine max*** **Media Components**

COMPONENT	FINAL CONCENTRATION (mg/L)
KH_2PO_4	150
KNO_3	500
NH_4NO_3	500
$Ca(NO_3)_2.4H_20$	250
$MgSO_4.7H_20$	36
KCl	32.5
$MnSO_4$	7
Na-Fe EDTA	6.6
$ZnSO_4.\ 7H_20$	1.9
H_3BO_3	0.8
KI	0.4
$Cu(NO_3)_2.3H_20$	0.175
$(NH_4)6Mo_2O_4.4H_20$	0.05
Myo-inositol	0.1
Nicotinic acid	2
Pyridoxine HCl	0.8
Thiamine-HCl	0.8
Agar	7g/L
Sucrose	30g/L
pH	Corrected to 5.8 with 1M NaOH

The contents of Appendix 2, pp. 240-255, have been removed due to copyright restrictions. The content of these pages comprised 2 poster abstracts and 1 journal article. The citations to the published content are given below.

Benson, E.E. et al. (1997) 'Cellular mechanism in vitro: studies of free radical generated lipid peroxidation products in plant tissue culture systems', *Journal of the Society for In Vitro Biology*, 33(3 PART II), pp. 46A

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