MOLECULAR BASIS OF ANTHOCYANIN PRODUCTION IN CALLUS AND CELL CULTURES OF *OXALIS RECLINATA*

BY

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DECLARATION

The experimental work described in this thesis was conducted in the Department of Botany, University of Natal, Pietermaritzburg, under the supervision of **Professor J. van Staden** and co-supervision of **Doctor W. A. Cress.**

These studies were the result of my own investigations, except where the work of others is acknowledged.

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ABSTRACT

Oxalis reclinata Jacq., is a dicotyledonous plant. *O. reclinata* belongs to the family Oxalidaceae. This plant produced callus which accumulated red coloured anthocyanin pigments when cultured *in vitro*. The levels of anthocyanin accumulated by *O. reclinata* callus were higher than in the intact plant. The major pigment was isolated and identified as cyanidin-3-glucoside (CROUCH, VAN STADEN, VAN STADEN, DREWES & MEYER, 1993). In nature, anthocyanins are responsible for orange, red, purple and blue colouration of certain tissues of higher plants. Due to the toxicity of many synthetic red colouring agents, anthocyanins are regarded as potential substitutes for synthetic food colourants. This research was aimed at investigating mechanisms which induce pigment production as well as to optimize anthocyanin yield from callus cultures of *O. reclinata*, once anthocyanin production was stimulated.

Pigmented and non-pigmented callus lines were generated from *O. reclinata* (CROUCH & VAN STADEN, 1994) and maintained on MURASHIGE & SKOOG (1962) agar medium (0.8% [w/v], pH 5.7) supplemented with 0.5 mgℓ^{-1} BA, 5 mgℓ^{-1} NAA, 30 gℓ^{-1} sucrose and 0.1 gℓ^{-1} myo-inositol. Plant tissue culture studies were conducted on red and white lines of *O. reclinata* to optimize callus yield and anthocyanin production *in vitro*. This involved manipulating contributory factors of the culture environment (carbohydrates, nitrates, phosphates, phytohormones, light and temperature).

In vitro studies showed that, light played an inductive role in anthocyanin production in callus cultures of *O. reclinata*. The auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) reduced pigment production but increased callus biomass. This hormone probably exerted its effect by reducing the pool of anthocyanin precursors, such as phenylalanine, resulting in increased primary metabolic activity. Suspension cultures were shown to be a viable means of propagating pigmented callus cells of *O. reclinata*. The growth curves for red and white callus cells were determined using the settled cell volume (SCV)

method. Pigmented cell cultures grew for longer periods compared to non-pigmented cells of *O. reclinata*. White callus cells reached the stationary phase after 18 days. Red callus cells continued growing exponentially for an extra three days compared to white callus cells. The vacuole was identified as the organelle where anthocyanins accumulate using the light microscope.

The molecular techniques of two-dimensional electrophoresis and *in vitro* translation were utilized to analyze differences in gene expression between white and red callus cultures of *O. reclinata.* Thus far, two-dimensional electrophoresis has shown that the red callus of *O. reclinata* had more polypeptides compared to the white callus. The level of gene expression was higher in the red callus compared to white callus, as revealed by non-radioactive *in vitro* translation. With optimization of radioactive *in vitro* translation, identification of specific structural anthocyanin genes which are under regulatory control should be possible.

Future research should aim at acquiring a better understanding about the genetic control of anthocyanin biosynthesis in order to manipulate this pathway effectively.

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ABBREVIATIONS

APS	Ammonia persulfate
AS	Anthocyanin synthase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
β	Beta
BA	Benzyladenine
bis	N'N'methylene bisacryalamide
Bq	Becquerel
°C	Degrees celsius
cDNA	Copy deoxyribonucleic acid
CHI	Chalcone isomerase
C4H	Cinnamate 4-hydroxylase
CHS	Chalcone synthase
Ci	Curie
4CL	4-Coumaryl CoA ligase
CoA	Co-enzyme A
cpm	Counts per minute
2D	Two-dimensional
2D-PAGE	Two-dimensional polyacrylamide electrophoresis
2,4-D	2,4-Dichlorophenoxyacetic acid
DFR	Dihydroflavonol-4 reductase
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DR	Dark-grown red callus
DW	Dark-grown white callus
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
EtBr	Ethidium bromide
FAB-MS	Fast atom bombardment mass spectrophotometry
F3H	Flavonone hydroxylase

FW	Fresh weight
g	Gram(s)
g	Standard acceleration of gravity
μg	Microgram
GA	Gibberellin
g ℓ^{-1}	Grams per litre
GTP	Guanosine triphosphate
h	Hour(s)
HPLC	High-performance liquid chromatography
IAA	Indole acetic acid
IBA	Indole butyric acid
IEF	Isoelectric focusing
KDa	Kilodalton
KIN	Kinetin
ℓ	Litre
ℓ^{-1}	Per litre
LS	Leucoanthocyanidin synthase
LDR	Red callus exposed to a light-dark cycle
LDW	White callus exposed to a light dark cycle
LLR	Red callus grown at low-light intensity
LLW	White callus grown at low-light intensity
LR	Red callus grown at high light intensity
LW	White callus grown at high light intensity
M	Molar
μM	Micromolar
mA	Milliampere
mM	Millimolar
mg	Milligrams
mg ℓ^{-1}	Milligrams per litre
Mg^{2+}	Magnesium ion
$MgSO_4$	Magnesium sulfate
μmol photons m^{-2} s^{-1}	Micromole photons per square meter per second

mRNA	Messenger ribonucleic acid
MEC	Molecular exclusion chromatography
MS	Murashige and Skoog medium
Met	Methionine
N	Nitrate
nm	Nanometer(s)
NAA	Naphthalene acetic acid
NAD	Nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance
NP-40	Nonidet P-40
P	Phosphate
pI	Iso-electric point
pKa	Acid dissociation constant
PMSF	Phenylmethylsulfonyl fluoride
PAL	Phenylalanine ammonia lyase
PCR	Polymerase chain reaction
PVP	Polyvinylpyrollidone
PVPP	Polyvinylpolypyrollidone
pp.	Page(s)
R	Red callus
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SCV	Settled cell volume
[^{35}S] Methionine	Radioactive methionine
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
Temed	NNN'N'tetramethylethylene diamine
TLC	Thin layer chromatography

™	Registered trademark
tRNA	Transfer ribonucleic acid
Tris	Tris(hydroxymethyl) amino methane
UF3GT	UDP glucose: flavonoid 3-O-Glucosyl transferase
UV	Ultraviolet
UV/VIS	Ultraviolet/visible spectrophotometry
v	Volume
V	Volts
Vh	Volt hours
w	Weight
W	White callus
X	Times

CHAPTER 1

GENERAL INTRODUCTION

Oxalis reclinata Jacq., is a dicotyledonous plant. It belongs to the family Oxalidaceae. This family consists of approximately 800 species worldwide (HEYWOOD, 1978). This family has major diversity centres in South America and South Africa. There are about 150 temperate species native to South Africa and these bear a wide range of inflorescence structures and herbaceous forms (SALTER, 1944). These geophytic South African species have a wide variety of perennating organs, such as tubers, stolons, bulbili, aerial bulbili and bulbs. Formation of trifoliar leaves and flowers occurs during the winter months when climatic conditions are wet and cold. Flowers of *Oxalis* open under well-lit conditions and the petals may exhibit a wide range of hues across the genus, ranging from white, yellow, orange to scarlet. *Oxalis reclinata* Jacq. has lightly coloured pink corollas and green vegetative organs (HEYWOOD, 1978). The South African genera of Oxalidaceae were taxonomically described last by SALTER in 1944, and no taxonomical reviews have been published since.

In vitro, propagation of *Oxalis* species was reported by CROUCH & VAN STADEN (1994). Generation of plantlets was achieved on modified MURASHIGE & SKOOG (1962) (MS) medium supplemented with either 5 mg ℓ^{-1} naphthalene acetic acid (NAA) and 0.5 mg ℓ^{-1} benzyladenine (BA) or 2 mg ℓ^{-1} NAA and 0.1 mg ℓ^{-1} kinetin. Production of heterogenous mixtures of white, yellow, green and red callus was initially noted for *O. reclinata*. Transfer of routinely subcultured callus types from a 25°C growth room to a 10 to 12°C cold room resulted in extensive organogenesis on both media types (CROUCH & VAN STADEN, 1994). This lower culture temperature closely parallels the natural conditions where *Oxalis* species are found. Maintenance of white and red callus lines at 25°C promoted dedifferentiation and resulted in proliferation of callus which requires a three to four week growth period before

subculturing. The red pigmented callus generated accumulated anthocyanins. The major pigment was identified as cyanidin-3-glucoside (CROUCH, VAN STADEN, VAN STADEN, DREWES & MEYER, 1993).

Conclusion

Studies on the biosynthesis and accumulation of anthocyanins are relevant as anthocyanins are the main pigments of higher plants (CONE, COCCIOLONE, BURR & BURR, 1993). These pigments have been consumed by man without apparent ill effects for years as they are the main pigments of fruits and flowers. Anthocyanins are presently being studied with a renewed interest as they are highly desirable substitutes for synthetic food colourants (BROUILLARD, 1982; BROUILLARD, 1988; CROUCH, VAN STADEN, VAN STADEN, DREWES & MEYER, 1993; MEYER & VAN STADEN, 1995). In recent years banning of especially red colourants for use in food products has occurred. This is due to the toxicity of many synthetic food colouring agents (MORI, SAKURAI, SHIGETA, YOSHIDA & KONDO, 1993). Alternative natural sources of these pigments are currently being investigated by research companies. The small biomass produced by members of the Oxalidaceae does not make it economically feasible to produce pigments on a large scale from these plants. CROUCH, VAN STADEN, VAN STADEN, DREWES & MEYER (1993) showed that callus and suspension cultures of *O. reclinata* may produce pigments, characteristic of anthocyanins, to levels that exceed those in the intact plant. Optimisation of anthocyanin accumulation in *O. reclinata* using *in vitro* culture techniques was seen as one option or direction for studying secondary metabolism involved in anthocyanin production.

Many studies involving the use of plant biotechnology have aimed at manipulating plant cells in culture to increase metabolic flux into specific pathways to increase secondary metabolite product formation (DIXON & BOLWELL, 1986). This involves alteration of biotic components (carbohydrates, nitrates, phosphates and plant growth regulators) and abiotic factors (light and temperature) which contribute to the culture environment. Molecular analyses resulting from such changes have generally been limited to measurements of

end-product accumulation only. This approach has undoubted value for preliminary optimisation of culture conditions for production of secondary metabolites such as anthocyanins. However, it has disadvantages as it may fail to identify positive or negative endogenous biochemical regulatory mechanisms which may act to control the flux through pathways under study, and which, if able to be triggered or circumvented, may result in increased metabolite yield. Therefore, studies based on end-product accumulation only, may not necessarily indicate the total attainable capacity for production of the anthocyanin by that particular species. Assessment of the operation of endogenous regulatory mechanisms controlling secondary product accumulation requires knowledge of the enzymology of the biosynthetic pathways under consideration and of the factors which might control enzymic capacity both *in vitro* and *in vivo*. Positive and negative effectors may be investigated to assess the *in vitro* situation, whereas the effects of transcription, translation and post-translation modification, including enzyme inactivation and/or degradation, are areas which require investigation to assess *in vivo* control (DIXON & BOLWELL, 1986).

Most studies conducted on anthocyanins have dealt with the chemistry of anthocyanins. This involves the elucidation of chemical structures of the pigments and quantification of anthocyanins in accumulating tissues. Anthocyanins and other flavonoids have also formed a basis of chemotaxonomical studies in plants (SPARVOLI, MARTIN, SCIENZA, GAVAZZI & TONELLI, 1994). To date, biosynthesis of flavonoids at a molecular level has been extensively studied in only three plant species, namely, *Petunia hybrida* (VAN TUNEN & MOL, 1991); *Zea mays* (PAZ-ARES, WIENAND, PETERSON & SAEDLER, 1987) and *Antirrhinum majus* (SPARVOLI, MARTIN, SCIENZA, GAVAZZI & TONELLI, 1994). These molecular studies have disclosed the existence of both structural and regulatory classes of genes involved in anthocyanin biosynthesis. While structural genes encode enzymes involved in the biosynthetic pathway, the regulatory genes are involved in control of the activity of the biosynthetic genes, thereby conditioning temporal and spatial

accumulation of the pigments in higher plants (KOES, QUATTROCHIO & MOL, 1994). Synthesis of enzymes encoded by the structural genes is highly regulated in the intact plant. It is usually flower specific and under developmental control. Synthesis in otherwise non-expressing tissues can be induced by environmental stress factors, such as, ultraviolet (UV) light, deficiency of nutrients, high-light intensity, low temperatures, drought, hormonal changes and phytopathogens (OZEKI & KOMAMINE, 1983; SPARVOLI, MARTIN, SCIENZA, GAVAZZI & TONELLI, 1994).

This study was undertaken with the objective of identifying the environmental control of anthocyanin production in cell and callus cultures of *O. reclinata*. It deals with the physiological effects of exogenous plant growth regulators and nutrients on callus growth and anthocyanin yield. It reports on the effect of light and temperature on *O. reclinata* callus cultures. At a molecular level, differences between anthocyanin-rich and anthocyanin-poor callus lines were investigated using the techniques of two-dimensional electrophoresis and *in vitro* translation.

The use of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) in studying proteins allows for the identification of proteins whose expression is changed by an external stimulus or stimuli; or which are developmentally regulated. The use of two-dimensional electrophoresis coupled with the technique of *in vitro* translation provides information about mechanisms involved in protein synthesis. These molecular techniques allow for the identification of specific messenger ribonucleic acid (mRNA) molecules and the study of properties for which they code (BROWN, 1990). These two techniques were chosen in order to identify the factor(s) that induces anthocyanin biosynthesis and to make comparative analysis of the enzyme composition between the red and white callus of *O. reclinata*.

CHAPTER 2

LITERATURE REVIEW

The anthocyanins (Greek *anthos*, flower, and *kyanos*, blue) belong to a subclass of secondary metabolites collectively known as the flavonoids. Flavonoids represent a class of plant constituents that are synthesized in almost every vascular plant examined. Therefore, the distribution of anthocyanins within the Plant Kingdom is widespread. In fact, anthocyanins are responsible for blue, purple and red pigments of higher plants. In flowers and fruits, anthocyanins are thought to be essential for fertilisation and seed dispersal (MAZZA & MANIATI, 1993).

2.1 CHEMICAL STRUCTURE OF ANTHOCYANINS

The basic chemical structure of flavonoids is relatively simple and it is composed of two aromatic C_6 rings held together by a C_3 unit. The degree of oxidation of the carbon (C) ring, other additions and rearrangements determines the subclass formed, such as chalcones, flavonones, flavonols, isoflavonoids, flavones, and anthocyanins (VAN TUNEN & MOL, 1991; VAN DER MEER, STUITJIE & MOL, 1993). Anthocyanins are glycosides of polyhydroxy and polymethoxy derivatives of two phenylbenopyrilium of flavylium salts (Figure 2.1). Differences between individual anthocyanins are the number of hydroxyl groups in the molecule, the degree of methylation of these hydroxyl groups, the nature and the number of sugars attached to the molecule, the position of attachment, and the number of aliphatic or aromatic acids attached to the sugars in the molecule. The types of naturally occurring anthocyanidins which are frequently found in plants are pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvinidin (Figure 2.2). Since each anthocyanidin may be glycosylated and acylated by different sugars and acids, at different positions, the number of anthocyanins is approximately 15 to 20 times greater than the

number of anthocyanidins. The sugars commonly associated with anthocyanidins are glucose, galactose, rhamnose, and arabinose (MAZZA & MANIATI, 1993).

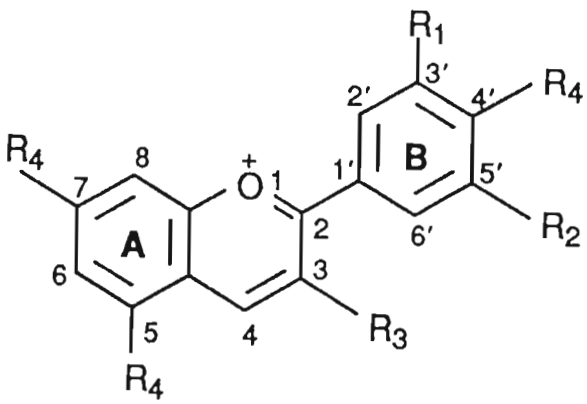
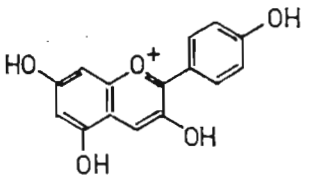
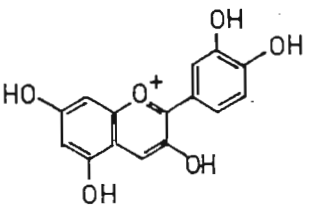


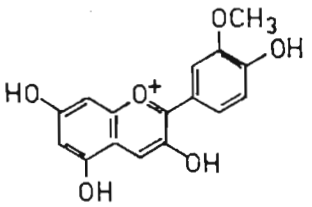
Figure 2.1: The flavylium cation. R_1 and R_2 are H, OH, OCH_3; R_3 is a glycosyl or H; and R_4 is a glycosyl or OH (MAZZA & MANIATI, 1993).



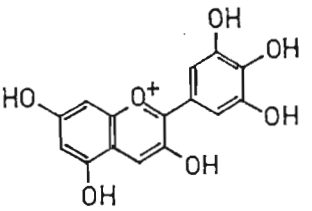
Perlagonidin



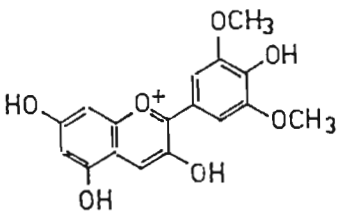
Cyanidin



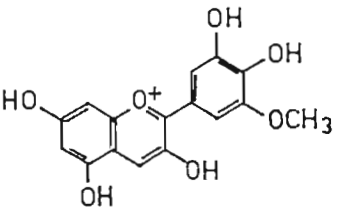
Peonidin



Delphinidin



Malvinidin



Petunidin

Figure 2.2: Most frequently found anthocyanidins in plants (BROUILLARD, 1982)

2.2 HISTORICAL BACKGROUND

The biosynthetic pathway of flavonoids has been subject to extensive study for more than a century. The pathway has been looked at using various techniques at multiple levels. By the seventeenth century, extraction of flavonoids from flowers, and knowledge about how to change extract colour by addition of salts and acids was known. The nineteenth century brought studies that were involved in the biochemistry of flavonoids. A breakthrough was accomplished in the 1960's with the development of techniques involving chromatography and nuclear magnetic resonance (NMR) spectroscopy (VAN TUNEN & MOL, 1991). The analysis of anthocyanins is said to be complicated as they undergo structural transformations and complexation reactions. Identification of anthocyanins was initially carried out using paper and/or thin layer chromatography (TLC), UV/VIS spectroscopy, and controlled hydrolysis and oxidation tests. High-performance liquid chromatography (HPLC) is a technique which is frequently used for both preparative and quantitative work of flavonoids. This is a powerful tool for separating anthocyanin mixtures. Structural elucidation of anthocyanins involves NMR and fast-atom bombardment-mass spectrometry (FAB-MS) (MAZZA & MANIATI, 1993). In the late 1980's more than 3 500 different flavonoids from all kinds of plant species had been identified and characterised (HARBORNE, 1988) and at present new structures are still being reported (KOES, QUATTROCHIO & MOL, 1994).

The physiology and biochemistry, and especially the enzymology of anthocyanin biosynthesis (Figure 2.3 and Figure 2.4) have been studied extensively (VAN DER MEER, STUITJIE & MOL, 1993). However, the last steps of the pathway are unclear (JENDE-STRID, 1993). Genetic studies of flavonoid metabolism were initiated around 1900, when pigments of flowers were used to study Mendelian inheritance. The formation of end products of the flavonoid biosynthetic pathway involves a number of different steps and sequential action of many enzymes. Mutations that are visible but not lethal to the plant have provided a genetic model system. The use of mutants has led to the

elucidation of the biochemistry of flavonoid biosynthesis (DOONER, ROBBINS & JORGENSEN, 1991). Flavonoid biosynthesis is regarded as being one of the best systems available for the study of regulation of plant gene expression. At the genetic level, three plant species have been mainly utilized to study flavonoid biosynthesis; namely, *Petunia hybrida* (petunia), *Antirrhinum majus* (snapdragon) and *Zea mays* (maize) (VAN TUNEN & MOL, 1991; VAN DER MEER, STUITJIE & MOL, 1993). Approximately 35 genes are involved in flavonoid synthesis in *Petunia* (VAN DER MEER, STUITJIE & MOL, 1993). Twelve genes influence the pathway in *Antirrhinum* (DOONER, ROBBINS & JORGENSEN, 1991). In *Z. mays*, at least 18 loci are implicated (DOONER, ROBBINS & JORGENSEN, 1991). Many of the loci contain structural genes coding for biosynthetic genes, but genes coding for regulatory mechanisms that control several steps have also been identified. Synthesis of the enzymes of the flavonoid pathway is co-ordinately and developmentally regulated in a tissue-specific manner. Several genes encoding enzymes and regulatory proteins involved in flavonoid biosynthesis have been cloned from a number of plant species (VAN TUNEN & MOL, 1991; VAN DER MEER, STUITJIE & MOL, 1993).

Molecular isolation of structural genes has been established by means of biochemical, genetic and molecular strategies. These strategies may often be used in combination. Biochemical strategies involve the use of antibodies prepared against purified gene products, and are most useful for structural genes that encode enzymes that can be assayed *in vitro* and that are sufficiently stable during purification. Genetic strategies involve the use of transposable elements in the induction of a mutation in an anthocyanin gene, which can be subsequently isolated by the use of a physical probe for the transposon. Molecular means utilize the differential regulation of transcripts of anthocyanin genes by different alleles of regulatory genes. Screening of copy deoxyribonucleic acid (cDNA) libraries prepared from tissues expressing anthocyanin genes by ribonucleic acid (RNA) probes from the same genotypic tissues that do not express these genes may be performed. Use of

heterologous hybridisation to isolate a homologous gene from another species may be used depending on the evolutionary distance between species. The polymerase chain reaction (PCR) may be used to isolate genes that are from distantly related species (DOONER, ROBBINS & JORGENSEN, 1991).

2.3 ANTHOCYANIN BIOSYNTHESIS

Anthocyanins are synthesized through the flavonoid biosynthetic pathway which is one of the side branches of the more general phenylpropanoid pathway (Figure 2.3) which branches off primary metabolism (OZEKI & KOMAMINE, 1985a; KOES, SPELT & MOL, 1989). The general phenylpropanoid pathway refers to a three step mechanism which involves phenylpropane based structures. L-phenylalanine is channelled into the formation of hydroxy-cinnamoyl co-enzyme-A (CoA) thiol esters. These esters and other intermediates of this pathway lead to the formation of a number of compounds, such as lignins, coumarins, stilbenes and flavonoids (HARBORNE, 1988).

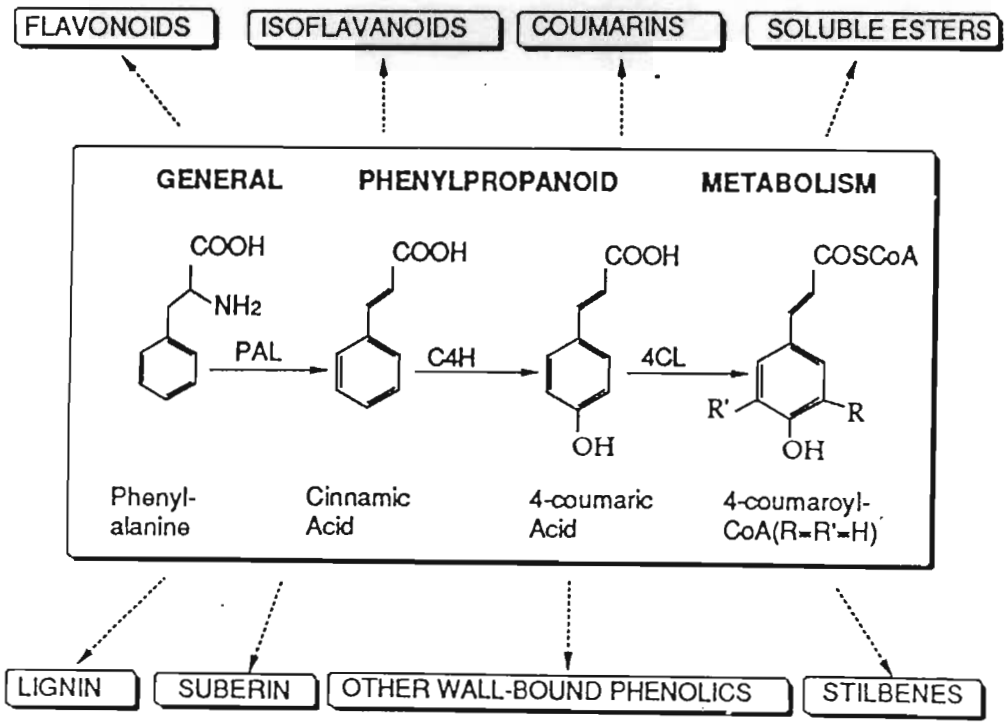


Figure 2.3: The general phenylpropanoid pathway (DIXON & BOLWELL, 1986)

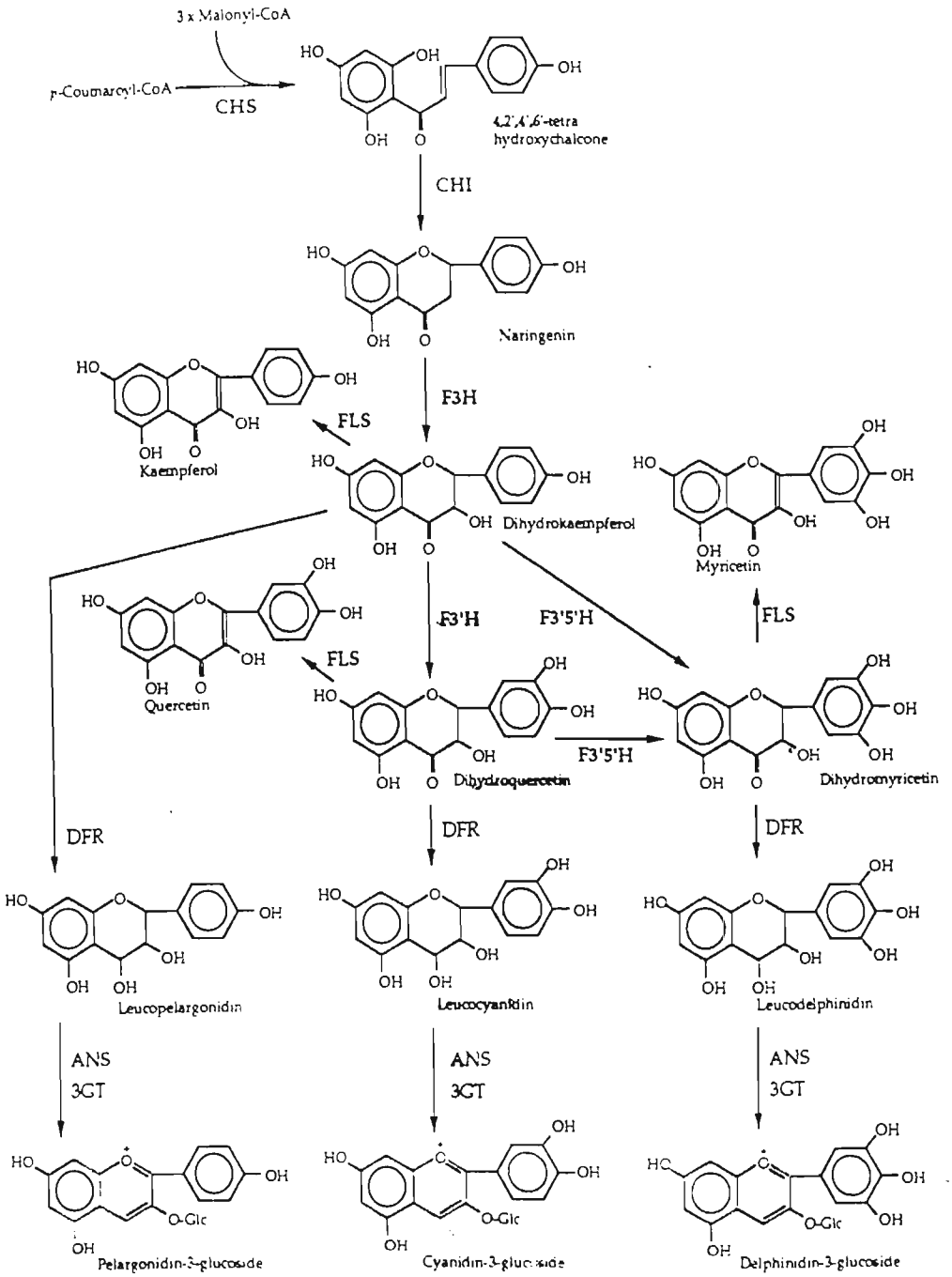


Figure 2.4: Anthocyanin biosynthetic pathway (HOLTON & CORNISH, 1995)

Production of the hydroxylcinnamoyl CoA thiol esters occurs via activation of transcinnamic acids produced from phenylalanine (OZEKI & KOMAMINE, 1985a). The first key step of this pathway is catalysed by phenylalanine ammonia lyase (PAL) which converts the aromatic amino acid L-phenylalanine into cinnamic acid. Cinnamate 4-hydroxylase (C4L) is involved in producing 4-coumaric acid and 4-coumaroyl-CoA ligase (4CL) is responsible for production of 4-coumaroyl CoA. The activity of PAL is said to control the entry of L-phenylalanine pools into the phenylpropanoid pathway and 4CL activity is responsible for the removal of CoA esters into end product specific metabolic branches. These two enzymes have been shown to be co-ordinately induced in UV-irradiated parsley cell cultures (VAN TUNEN & MOL, 1991).

Malonyl-CoA, which is the other precursor for flavonoid biosynthesis, is synthesized from the glycolysis intermediate acetyl-CoA and CO_2. This carboxylation reaction is catalysed by the enzyme Acetyl-CoA carboxylase. Acetyl-CoA is a central intermediate in the Krebs cycle of primary metabolism while production of 4-coumaroyl-CoA via PAL links the phenylpropanoid pathway to primary metabolism as phenylalanine is produced from the shikimate/arogenate pathway (HARBONE, 1988). Production of flavonoids is initiated with the stepwise condensation of three malonyl-CoA molecules and a molecule of coumaroyl-CoA (or related cinnamic esters) by chalcone synthase (CHS) yielding a C_{15} chalcone intermediate, 42'1'4'1'6'1-tetrahydroxy chalcone. This is said to be the first committed step in flavonoid metabolism and CHS is regarded as the key enzyme of flavonoid biosynthesis, as the C_{15} chalcone intermediate forms the basic or fundamental structure from which all flavonoids originate. Derivation of aurones and other diphenylpropanoids is also dependent on this intermediate. Transformation of the yellow-coloured tetrahydroxy chalcone by stereospecific action of chalcone flavonone isomerase (CHI), where intramolecular closure of the carbon ring occurs, produces a naringenin. This compound is a colourless flavonone. This isomerisation proceeds spontaneously at a low rate, but the activity of CHI increases the rate of reaction. Virtually all flavonoid classes are derived from

a flavonone. The enzyme 2-oxoglutarate-dependent dioxygenase: flavonone 3β-hydroxylase (F3H) is responsible for the production of dihydrokaempferol. This reaction involves the β-hydroxylation of flavonones at the 3-position of the C-ring. The enzyme requires Fe^{2+} and ascorbate as co-factors. Dihydrokaempferol may be converted to dihydroflavonols which are direct precursors for anthocyanin biosynthesis.

According to OZEKI & KOMAMINE, 1983; DOONER, ROBBINS & JORGENSEN, 1991; VAN DER MEER, STUITJIE & MOL, 1993; KOES, QUATTROCHIO & MOL, 1994; HOLTON & CORNISH, 1995, the type of anthocyanin ultimately produced is determined by the type of dihydroflavonol precursor synthesized. Dihydrokaempferol can be hydroxylated by F3H to produce dihydroquercetin or by flavonone 3',5'-hydroxylase (F35H) to produce dihydromyricetin. The activity of F3H results in hydroxylation of the B-ring taking place at the 3' position only and the resultant production of dihydroquercetin may lead to red-coloured cyanidins being produced. Dihydromyricetin production involves the hydroxylation of the B-ring to completion. Dihydromyricetin is a direct precursor of blue or purple coloured delphnidins. In the absence of both 3' and 3'5' hydroxylases, dihydrokaempferol acts as a precursor of the orange-coloured pelargonidins. The conversion of colourless dihydroflavonols into anthocyanins is highly complex and requires the action of a different number of enzymes, some of which have been identified. The dihydroflavonols (dihydromyricetin, dihydroquercetin and dihydrokaempferol) are reduced to flavan-3,4-*cis*-diols (unstable proanthocyanidins) by dihydroflavonol-4-reductase (DFR). The next two steps in the pathway are not clearly understood and defined, but it is thought that leucoanthocyanidin dioxygenase and a dehydratase enzyme may be responsible for converting proanthocyanidins into anthocyanidins. Further oxidation, dehydration, and glycosylation of the different proanthocyanidins produce corresponding brick-red perlargonidin, red cyanidin and blue delphinidin pigments. Production of the first stable anthocyanin is due to the activity of UDP glucose : flavonoid 3-O-glucosyl transferase (UFGT). The step, catalysed by this enzyme, is an obligatory glycosylation reaction, usually a glycosylation

in the 3′ position of the anthocyanidin or a suitable intermediate. Anthocyanidin-3-glucosides may be further modified in many species by glycosylation, methylation and acylation.

2.3.1 Structural genes involved in anthocyanin biosynthesis

Studies using mutants with a block in anthocyanin pigmentation have disclosed the existence of two classes of genes which affect anthocyanin biosynthesis. One class composed of the structural or effector genes of the pathway (Figure 2.3 and 2.4.) common to different species (FOSKET, 1994; SPARVOLI, MARTIN, SCIENZA, GAVAZZI & TONELLI, 1994).

Genes involved in the biosynthetic pathway of flavonoids have been characterized and cloned by differential and antibody screening of cDNA libraries or by using transposable elements. Table 2.1, summarizes the structural genes isolated from each of the species listed.

The second class consists of regulatory genes that control the activity of the biosynthetic genes. These genes regulate the spatial and temporal accumulation of anthocyanin pigments. The intensity of the pigment is also influenced by these genes. Evidence for the regulatory control of anthocyanin biosynthesis was obtained by enzyme assays or mRNA assays of structural gene activity (HOLTON & CORNISH, 1995). The regulatory genes described in the best studied plant systems, namely, maize, snapdragon and petunia are summarized in Table 2.2.

Table 2.1: Cloned structural genes of the anthocyanin biosynthetic pathway

Gene product	Source	Gene number	Comments	References
PAL	*Arabidopsis thaliana*	3-4	Differential expression of PAL genes in plant tissues	Wanner *et al.* (1995)
	Cucumis melo		Wound-induced synthesis of PAL genes in melon fruit	Diallinis and Kanellis (1994)
	Ipomea batatas			Tanaka *et al.* (1989)
	Orzya sativa	3-4	Genes are regulated by light	Minami *et al.* (1989)
	Phaseolus vulgaris	3-4	Differential expression of genes	Cramer *et al.* (1989)
	Petroselinum crispum	4-5		Lois *et al.* (1989)
	Solanum tuberosum		Genes isolated from elicitor-induced cell suspension cultures	Fritzemeier *et al.* (1987)
	Vitis vinifera	15-20	Snapdragon and maize heterologous probes were used to screen a cDNA library obtained from light grown seedlings	Sparvoli *et al.* (1994)
4CL	*P. crispum*	2	Both genes induced by UV light and phytopathogens	Douglas *et al.* (1987)
	S. tuberosum	2	cDNA library constructed form mRNA isolated from elicitor-treated cell suspension cultures	Fritzemeier *et al.* (1987)
CHS	*Antirrhinum majus*	1	Multiple alleles as a result of transposon insertions	Sommer and Saedler (1986)
	A. thaliana	1	Gene induced by high-light intensity	Feinbaum and Ausebel (1988)
	Glycine max	6	Only *CHS*1 gene is induced by UV light and phytopathogens	Wingender *et al.* (1989)
	Hordeum vulgare		Phylogenetic study	Niesbach-Klogen *et al.* (1987)
	Magnofolia liliflora		Phylogenetic study	Niesbach-Klogen *et al.* (1987
	Matthiola incana		Sequencing of cDNA	Epping *et al.* (1990)

Table 2.1. continued

Gene product	Source	Gene number	Comments	References
	P. vulgaris	6-8	Genes differentially expressed	Ryder *et al.* (1987)
	Ranonculus acer		Phylogenetic study	Niesbach-Klogen *et al.* (1987)
CHI	*Petunia hybrida*	2	Differential expression of genes	van Tunen *et al.* (1988)
	P. vulgaris	1	Inducible by wounding and fungal infection	Mehdy and Lamb (1987)
	A. majus		Multiple alleles as a result of transposon insertions	Martin *et al.* (1985)
	Z. mays		Multiple alleles as a result of transposon insertions	O'Reilly *et al.* (1985)
F3H	*A. majus*		Differential screening and genetic mapping was used to isolate the cDNA corresponding to the *incolorata* locus which is known to encode F3H	Martin *et al.* (1991)
	P. hybrida		High sequence homology exists between the snapdragon and petunia genes	Britsch *et al.* (1993)
DFR	*A. majus*		Transposon tagging was used to isolate the gene	O'Reilly *et al.* (1985)
	P. hybrida		A snapdragon clone was used to isolate a homologous gene from petunia	Beld *et al.* (1989)
	V. vinifera	1	Expression induced by light	Sparvoli *et al.* (1994)
UFGT	*A. majus*		A putative UFGT clone was isolated from snapdragon using the maize gene as a probe	Martin *et al.* (1991)
	V. vinifera	1	A snapdragon clone was used to isolate a partial clone from grape	Sparvoli *et al.* (1994)
	Z. mays		maize *Bz1* gene encoding UFGT was isolated by transposon tagging	Dooner *et al.* (1985)

Table 2.2: Cloned regulatory genes of flavonoid metabolism

Species	Locus	Cloned	Structural genes regulated	Gene cloning reference
Zea mays	*R*	+	*chs,dfr,ufgt*	Dellaporta *et al.* (1988)
	R(S)	+	*chs,dfr,ufgt*	Perrot & Cone (1989)
	R(Sn)	+	*chs,dfr*	Holton & Cornish (1995)
	R(Lc)	+	*chs,dfr*	Ludwig *et al.* (1989)
	B	+	*dfr,ufgt*	Chandler *et al.* (1989)
	C1	+	*chs,dfr,ufgt*	Cone *et al.* (1986)
	Pl	+	*chs,dfr,ufgt*	Cone and Burr (1989)
	Vp1	+	*C1*	McCarty *et al.* (1989)
Antirrhinum majus	*Delila*	+	*F3H,DFR,AS,UFGT*	Goodrich *et al.* (1992)
	Eluta	-	*F3H,DFR,AS,UFGT*	
	Rosea	-	*F3H,DFR,AS,UFGT*	
Petunia hybrida	*An1*	-	*chsJ,F3'5'H,DFR,AS,UFGT*	
	An2	+	*chsJ,DFR,AS,UFGT*	Holton & Cornish (1995)
	An4	+	*chsJ,DFR,AS,UFGT*	Holton & Cornish (1995)
	An11	-	*chsJ,DFR,AS,UFGT*	

Anthocyanin biosynthesis is regulated primarily at the transcriptional level. Regulatory genes involved in controlling anthocyanin biosynthesis in *Z. mays* appear to control the whole pathway as a single unit as pigmentation of the aleurone cell layer involves the simultaneous induction of the structural genes. This multiple transcriptional activation is due to the *R* and *C1* transcription factors which act to induce all the committed steps of the pathway (DOONER, ROBBINS & JORGENSEN, 1991; MARTIN & GERATS, 1993). The *R* family is encoded by functionally duplicate, unlinked *R* (which includes *S*, *Lc* and *Lw*) and *B* loci. The R proteins belong the class of helix-loop-helix type transcription factors (BODEAU & WALBOT, 1995). The *R* family comprises of a set of regulatory genes consisting of the *R* locus (which includes *S*, *Lc* and *Lw*), and the *B* locus. The C1 proteins are encoded by *C1* and *Pl* loci.

This family resembles the Myb proto-oncogene type transcriptional activators. The properties of individual alleles is responsible for the tissue specificity of anthocyanin synthesis as each gene determines pigmentation of different parts of the maize plant (DOONER, ROBBINS & JORGENSEN, 1991; BODEAU & WALBOT, 1995; HOLTON & CORNISH, 1995). BODEAU & WALBOT (1995) showed that the same biosynthetic pathway and regulatory mechanisms were operative in maize callus as in the intact plant. At least one *R* gene-family member is required for production of anthocyanin and the *Pl* locus acts with the *R(S)* locus to control pigmentation in the dark. Genotypes expressing *Pl* gene showed increased anthocyanin production in the presence of light.

In dicotyledonous plants, anthocyanin biosynthesis does not depend on a single induction mechanism for all the biosynthetic genes as in the maize system. It seems that different biosynthetic genes are regulated separately. In snapdragon, three regulatory genes have been identified: *delila*, *eluta* and *rosea* (HOLTON & CORNISH, 1995). Cloning and sequencing of *delila* have shown that this gene is highly homologous to the maize *R* family. The first two steps of the pathway, CHS and CHI, show minimal regulation, but subsequent steps (F3H, DFR, AS, UF3GT) have an absolute requirement for the *delila* (Del) gene

product (KOES, QUATTROCHIO & MOL, 1994; MARTIN & GERATS, 1993; HOLTON & CORNISH, 1995). *Petunia* has been shown to have the largest collection of loci that influence anthocyanin production. The genes that control anthocyanin production have been divided into two groups. One set of loci controls the activity of a single enzyme from the biosynthetic pathway and they appear to contain the structural gene encoding the enzyme. The second class of loci controls the activity of multiple enzyme steps. These loci are said to encode regulatory factors. The first and second parts of anthocyanin biosynthesis are under different transcriptional control. The late steps of the anthocyanin biosynthetic pathway are controlled by *An1*, *An2*, *An10* and *An11*. These loci control the activity of DFR, UF3GT and AS. Mutations at these loci result in unpigmented tissues but accumulation of dihydroflavonols is maintained, indicating the activity of early biosynthetic enzymes encoded by *CHS, CHI* and *F3H* genes (VAN TUNEN & MOL, 1991; KROON, SOUER, DE GRAAFF, XUE, MOL & KOES, 1994; HOLTON & CORNISH, 1995).

2.4 EVOLUTION AND FUNCTIONS OF ANTHOCYANINS AND OTHER FLAVONOIDS

2.4.1 Evolution of flavonoids

Different classes of flavonoids are distributed in a manner which suggests that their appearance occurred sequentially during evolution. The chalcones, flavonones and flavonols appeared with the ancestors of a class of *Bryophytes* (*musci*). Proanthocyanidins appeared with the first vascular plants (*Pteridophyta*), and anthocyanins only appeared with the emergence of flowering plants (*Angiospermae*). The genes encoding these compounds are thought to have also evolved sequentially. Many of the structural genes which have been sequenced, as well as their gene products, have shown homology with enzymes from primary metabolism (KOES, QUATTROCHIO & MOL, 1994). The initial reaction leading to the first C_{15} compound is catalysed by chalcone synthase. This condensation reaction utilises phenylpropanoid and malonyl-CoA

pathway products. This reaction shows high homology to reaction mechanisms found in primary metabolism. It is thought that enzymes of fatty acid metabolism, such as β-ketoacyl carrier protein of fatty acid synthases may be 'parent' enzymes of chalcone synthase (STAFFORD, 1991).

The function of flavonoids is thought to have appeared at different points during evolution in correspondence with the appearance of different flavonoids. KOES, QUATTROCHIO & MOL (1994), consider their function as UV protectors to have been the first to be established. STAFFORD (1991) argued that a function as internal physiological regulators or signal molecules was the first to have been established, as the first enzymes capable of synthesising flavonoids were not as plentiful, nor as efficient, as modern day forms. Therefore large amounts of flavonoids did not accumulate initially, and a relatively large concentration would have been required for their function as UV filters. Anthocyanins which appeared relatively late, are thought to have evolved with the appearance of flowers, and the function of flavonoids in the attraction of pollinators would have been acquired at this stage (KOES, QUATTROCHIO & MOL, 1994). According to SWAIN (1986) anthocyanins do occur sporadically in lower plants, but their role is unclear. The full range of their orange to blue colours is not expressed until flowering plants coupled with the advent of specialized animal pollinators and animal fruit dispersal agents. SWAIN (1986) presumes that the biosynthetic steps involved in anthocyanin production may have arisen early in the evolution of plants, but were not utilized until required. The yellow chalcones are the first products of anthocyanin biosynthesis and they occur on the outside of fern fronds, yet this situation does not exploit their colour; instead they function to deter a host of potential pathogens. KOES, QUATTROCHIO & MOL (1994) consider the acquisition of function by the flavonoids in the interaction with microbes (rhizobia or pathogens) to be more recent, as this function is found mainly in a single family of plants, namely *Leguminoseae*.

KOES, QUATTROCHIO & MOL (1994) presented a model which attempted to describe the evolution of mechanisms involved in the regulation of flavonoid biosynthesis. Structural genes for proanthocyanidin and anthocyanidin synthesis (the late acting genes of the pathway) are thought to be under a linked or related set of regulatory genes, i.e. ancestors of *Lc* and *C1*. This set of genes ensures co-ordinated expression in the flower. It is thought that the expression of early genes of the pathway were linked to the same ancestral *C1* and *R* regulatory genes as that of late genes. This would have allowed flavonones, flavonol, proanthocyanidin and anthocyanidin synthesis to occur independently. Co-ordination between late and early genes is thought to have been achieved in two ways during evolution. Firstly, addition of new appropriate modules in promoters of ancient genes would have occurred, thereby giving them broad specificity. Alternatively duplication of some structural genes, followed by coupling of one set of genes to newly acquired regulators occurred. The specific *cis*-elements are presumed to have been lost during later stages of evolution from multi-purpose genes, or specialised gene copies may have been lost or inactivated (KOES, QUATTROCHIO & MOL, 1994). The regulation of *chs*, *chi* and *f3h* genes in primitive ferns and mosses has not yet been elucidated. KOES, QUATTROCHIO & MOL (1994) suggested that if the model presented holds true, then no activation of *chs*, *chi* and *f3h* genes in primitive ferns and mosses would occur by present day *R* and *C1* regulatory gene families.

2.4.2 Functions of anthocyanins and other flavonoids in nature

Plants that are insect-pollinated have a tendency to have large, often brightly coloured petals; whereas wind-pollinated plants generally have small, dull coloured petals, or no petals. This is clearly demonstrated by petunia and maize (KOES, QUATTROCHIO & MOL, 1994). Red or purple coloured anthocyanins or aurones and chalcones (yellow coloured flavonoids) are mostly responsible for flower pigmentation (MARTIN & GERATS, 1993). Besides anthocyanins, accumulation of flavonols or flavonones in petals of many plant species has

also been observed. These colourless compounds alter flower colour through co-pigmentation by forming complexes with anthocyanins and metal ions. Strong blue colours of flowers are a result of co-pigmentation and metal chelation (JACKMAN, YADA, TUNG & SPEERS, 1987; MAZZA & MANIATI, 1993). Intermolecular co-pigmentation involves the association of anthocyanins with other flavonoids to form weak complexes through presumably hydrogen bonding. Intramolecular co-pigmentation has been regarded as a more efficient mechanism in the stabilisation of anthocyanins as opposed to intermolecular co-pigmentation. It may occur in conjunction with metal complexing (JACKMAN, YADA, TUNG & SPEERS, 1987).

Flower pigments act as visual signals to attract pollinators (insects or birds). Anthocyanin biosynthesis is usually under spatial and temporal control, and this is consistent with a role as a visual signal. Anthocyanins accumulate mainly in the inner epidermis of petals. Transcriptional activity of structural genes and the rate of anthocyanin biosynthesis reach a maximum prior to opening of the flower bud (VAN TUNEN, KOES, SPELT, VAN DER KROL, STUITJIE & MOL, 1988; BELD, MARTIN, HUITS, STUITJIE & GERATS, 1989; KOES, VAN BLOKLAND, QUATTROCHIO, VAN TUNEN & MOL, 1990; VAN TUNEN, MUIR, BROUNS, RIENSTRA, KOES & MOL, 1990; MARTIN & GERATS, 1993). It has been demonstrated that removal of petals from flowers results in a decrease in the number of insects that visit flowers. Removal of petals does not completely eliminate visitation of pollinators, as other factors, such as fragrance, are involved in the attraction of pollinators (KOES, QUATTROCHIO & MOL, 1994). Wind-pollinated and self-fertile plant species, such as maize, accumulate anthocyanins in several plant parts (e.g. anthers, leaves and stems). The function of this pigmentation is unclear. In some cases, accumulation of anthocyanins might be to attract fruit-eating animals and as a result contributes in dispersal of seeds.

Accumulation of anthocyanins and other flavonoids in the anthers and the pistil of many plant species has been reported. Anthocyanins, as well as flavonols and chalcones, are the most commonly found flavonoids in anthers. The structural genes responsible for their biosynthesis, and the enzymes encoded by those genes, are active in the tapetum and the connectivum. These are tissues which are important for the nourishment of developing pollen grains (KOES, VAN BLOKLAND, QUATTROCHIO, VAN TUNEN & MOL, 1990). The use of spontaneous and engineered mutants in flavonoid research has shown that flavonoids play an essential role in pollen development. Maize plants with mutations in *chs* genes produce unpigmented white pollen that is sterile (COE, McCORMICK & MODENA, 1981). In petunia plants, blockage of *chs* gene expression through antisense RNA or sense RNA (TAYLOR & JORGENSEN, 1992) resulted in white pollen that failed to produce a functional pollen tube. In the pistil of petunia flowers, flavonoid biosynthetic genes like *chs* and *chi* are highly active in the ovary and they result in flavonol accumulation (KOES, VAN BLOKLAND, QUATTROCHIO, VAN TUNEN & MOL, 1990; VAN TUNEN, MUIR, BROUNS, RIENSTRA, KOES & MOL, 1990). It has been suggested that flavonols form a gradient along which growing pollen tubes are guided to the ovule, as ovules are the primary sites for *chs* and *chi* expression (KOES, QUATTROCHIO & MOL, 1994).

Sunlight is required by plants for photosynthesis, and the UV component of light is a potential hazard as it can damage DNA and impair certain physiological processes. Flavonoids are thought to act as ultra-violet protectants as they strongly absorb UV light. They also accumulate mainly in epidermal cells after expression of structural biosynthetic genes of flavonoid metabolism due to UV-induction. Therefore, they have been regarded as a protective shield against UV light. With the accumulation of flavonoids, most cells become shielded and biosynthesis then ceases. This is thought to be the reason for transient expression of flavonoid genes under continuous UV light

conditions. Flavonoids have been reported to prevent UV-induced damage and plants with decreased levels of flavonoids show increased sensitivity to damage by UV irradiation (LI, OU-LEE, RABA, AMUNDSON & LAST, 1993; KOOSTRA, 1994). Ultra-violet irradiation or white light containing UV leads to a massive increase in transcriptional activity of CHS in *Petroselinum crispum* cell suspension cultures (CHAPPELL & HAHLBROCK, 1984). Recently, the analysis of UV light on *Arabidopsis* flavonoid mutants for the *tt4* and *tt5* genes (encoding CHS and CHI, respectively) demonstrated the role of flavonoids in the protection against UV light. These mutants lacked flavonols in all tissues due to the synthesis of flavonol derivatives being blocked. When placed under short wavelength UV light, growth of the mutants became strongly retarded (LI, OU-LEE, RABA, AMUNDSON & LAST, 1993).

2.5 MANIPULATION OF CULTURED CELLS TO SYNTHESIZE ANTHOCYANINS *IN VITRO*

2.5.1 Importance of accumulation of anthocyanins in cultured cells - possible role as food colourants

Researchers have long recognized the importance of cell cultures in the production of secondary metabolites, even though many advances have been made in organic chemistry to synthesize these metabolites that are of industrial and medicinal importance. Many secondary metabolites have industrial applications as pharmaceuticals and as agents in food flavouring and perfumery (DODDS & ROBERTS, 1985). Initial proposals for using plant tissue culture techniques in synthesising secondary metabolites were made by KLEIN (1960). The basic technology involved in suspension cell cultures on a large scale was described by NICKELL (1962). Plant tissue culture systems have allowed the identification of previously undescribed secondary compounds. Cultures of higher plants are seen as an important source of new and economically important compounds (DODDS & ROBERTS, 1985). With the recent advancement in plant biotechnology, many reports have been made regarding the accumulation of anthocyanins in plant cell and callus cultures from a wide

variety of plant species (MORI, SAKURAI, SHIGETA, YOSHIDA & KONDO, 1993). The production of naturally occurring anthocyanin pigments in cell cultures is a potential alternative to synthetic food colourants that have been banned due to their toxicity (TIMBERLAKE & HENRY, 1986; MORI, SAKURAI, SHIGETA, YOSHIDA & KONDO, 1993). Due to the low toxicity of anthocyanins which have been consumed by man without any apparent ill-effects for thousands of years, anthocyanins are now seen as a new source of food colourants. Intensive research is being conducted by many research institutes and food manufacturing companies into producing anthocyanin pigments in cultured cells.

2.5.2 Effects of carbohydrate manipulation

In the intact plant, carbon dioxide is assimilated into sucrose which is the main translocatable carbon source (CRESSWELL, FOWLER, STAFFORD & STEPAN-SARKISSIAN, 1989). *In vitro* cell cultures require carbon and an energy source as well, and it has been suggested that sucrose-fed cell cultures are probably similar in terms of primary metabolic pathways. Feeding of nutrients should be at concentrations similar to that of the whole plant. Sucrose and D-glucose are generally added to culture media in concentrations of 20 g ℓ^{-1} to 30 g ℓ^{-1} (DODDS & ROBERTS, 1985) and these sugars may be found present in phloem and cell sap of cultured cells at levels of around 10% to 25%, considerably higher than conventional concentrations (CRESSWELL, FOWLER, STAFFORD & STEPAN-SARKISSIAN, 1989). In plant tissue culture systems, the favoured source of carbon appears to be sucrose, as nearly all cultures appear to respond optimally to its presence (DODDS & ROBERTS, 1985). It has been demonstrated that sucrose added *in vitro* to cell suspensions is rapidly hydrolysed to glucose and fructose, which are then taken up either passively or actively, depending on the plant species. It is thought that invertases, which are responsible for sucrose hydrolysis, may reside in the external medium, the cell wall or the plasmalemma. Uptake of glucose is more rapid than that of fructose from culture medium. Sucrose may therefore be viewed as an

alternative means of supplying glucose (CRESSWELL, FOWLER, STAFFORD & STEPAN-SARKISSIAN, 1989). Reports on the effects of sucrose and glucose upon culture growth and secondary metabolism have indicated that their mode of uptake and utilization is regulated in a rather different manner. Other glucose-containing disaccharides, such as maltose and lactose, may be used as energy sources by certain cell lines, but they are generally less effective than sucrose. According to CRESSWELL, FOWLER, STAFFORD & STEPAN-SARKISSIAN (1989) the effect of these alternative carbon sources on secondary metabolism requires further investigation as they may have detrimental effects on secondary metabolite production.

Carbohydrates are known to function in the regulation of external osmotic potential, which governs the uptake of water by plant cells between vacuolar sap and the external medium. Water availability to cultured cells is influenced by the concentration of agar and other non-metabolites, as well as the carbohydrate source (DODDS & ROBERTS, 1985). It is important to consider the effect of various carbohydrates on cell growth and productivity of secondary metabolites such as, anthocyanins.

SAKAMOTO, IIDA, SAWAMURA, HAJIRO, ASADA, YOSHIKAWA & FURUYA (1993) reported the isolation of an anthocyanin producing cell strain of *Aralia cordata*, which had a high and stable production capacity in conditions of darkness or light. The cell line was produced from both *A. cordata* leaves and stems, and was obtained by continuous cell-aggregate cloning. Investigations dealing with the effects of several sucrose concentrations on cell growth and anthocyanin yield suggested that sucrose concentrations higher than 5% reduced cell growth and anthocyanin accumulation by affecting the osmotic strength. High concentrations of sucrose results in higher osmotic strength of the media and the higher osmotic strength negatively affects the water content of the vacuole. Anthocyanins have been shown to accumulate in the vacuole in *A. cordata* cells. *Aralia reptans* callus also tended to show reduced growth and production at higher sucrose concentrations. The best conditions for

anthocyanin production in dark or light conditions were observed on LINSMAIER & SKOOG (1965) basal medium, supplemented with 2% sucrose (anthocyanin yield: 9.0%; growth index: 7.5%) and 4% sucrose (anthocyanin yield: 7.5%; growth index: 8.0%) respectively (SAKAMOTO, IIDA, SAWAMURA, HAJIRO, ASADA, YOSHIKAWA & FURUYA, 1993).

The effects of different carbon sources other than sucrose have been investigated on *A. cordata* cells. Under a light-dark cycle, glucose, sucrose and fructose yielded the best results with respect to callus growth as compared to xylose, cellobiose and maltose. The best anthocyanin yields were observed with glucose and fructose at 5.9% and 7.9% in the light and dark, respectively. In callus cultures of *Hibiscus sabdariffa* L., fructose was also shown to be most effective toward anthocyanin production in the dark but the growth rate was low in comparison with sucrose. Xylose was shown to be inhibitory to growth and production of anthocyanin, whereas galactose, cellobiose and maltose did not support growth. Sucrose, therefore, appears to yield the best levels of anthocyanin, and it is capable of maintaining callus growth (SAKAMOTO, IIDA, SAWAMURA, HAJIRO, ASADA, YOSHIKAWA & FURUYA, 1993).

The effect of sucrose on cell growth and production of callus that does not necessarily accumulate anthocyanins, was similar in species that do accumulate anthocyanins. YAMAMOTO, YAN, IEDA, TANAKA, IINUMA & MIZUNO (1993) showed that cell growth in *Vancouveria hexandra* cells accumulation of flavonol glycoside paralleled an increase of sucrose up to 7% on LINSMAIER & SKOOG (LS) (1965) gellan gum medium, and then became independent of the sucrose increase. These authors observed that the most suitable sucrose concentration for flavonol glycoside production was 7%.

In *in vivo* propagated plant cells, application of sugars affected anthocyanin production at the gene level as the expression of a particular CHS gene (*CHS-A*) from petunia in transgenic leaves of *Arabidopsis* was induced. Organ-

specific and sugar-responsive expression of CHS cDNA have been isolated from *Camellia sinensis*, which accumulates the flavonoid, catechin. Leaves of *C. sinensis* were treated with different carbohydrates. Fructose, sucrose or maltose resulted in increased levels of CHS-transcripts. The other sugars, i.e. glucose, galactose, sorbitol and mannitol had no effect on chalcone synthase transcript levels. Transcript levels were observed to be lower in the darkness than in continuous light. These researchers suggest that sugar effects are mediated through balances of sugars and/or changes in energy metabolism. They suggest that levels of sugars increase due to photosynthetic activity of cells under continuous light, and balances and energy metabolism is changed under conditions of light, causing differences with respect to sugar-responsiveness of CHS transcripts under different light conditions. Sugars are suggested to act as inducers of CHS transcripts *in vivo* and the induction of CHS gene expression by sucrose is thought to reflect the requirement for a large amount of a carbon source for catechin accumulation (TAKEUCHI, MATSUMOTO & HAYATSU, 1994).

2.5.3 Effects of manipulating inorganic salts on anthocyanin biosynthesis

A continuous supply of macronutrient elements, such as nitrogen, phosphorous, potassium, calcium, magnesium, and sulphur, is required by cultured plant tissues. Nitrogen may be added in the largest amount as either a nitrate or ammonium ion, or a combination of these ions. Magnesium sulfate ($MgSO_4.7H_2O$) generally satisfies the magnesium and sulphur requirements and phosphorus can be provided by $NaH_2PO_4.H_2O$ or KH_2PO_4. Potassium, the cation found in the largest amount, is given as either KCl, KH_2PO_4 or KNO_3. The calcium requirement may be provided by $CaCl_2.2H_2O$, $Ca(NO_3)_2.4H_2O$, or an anhydrous form of either salt may be added (DODDS & ROBERTS, 1985).

A high energy demand on nitrogen assimilation is imposed on cultured plant cells when nitrogen is supplied as nitrate. The supply of nitrogen as ammonia

is energetically more efficient than supplying the more oxidised nitrate form. Removal of NH_3 from culture medium results in a pH decrease, especially with the weak buffering capacity usually present in tissue culture media. It appears that NH_3 present in a culture medium, with other more oxidised forms (NO_3^-) is generally preferentially removed and utilized.

Nitrogen effects on cultured *A. cordata* cells were investigated by varying the nitrogen concentration in different types of basal media (LS and MS medium with 3% (w/w) sucrose, 1 mg ℓ^{-1} 2,4-D and 1 mg ℓ^{-1} kinetin). One of the cell lines was grown in the dark for 21 days at 25°C, whilst the other line was exposed to a light-dark regime. Cell growth in the dark was better promoted by 1/5 total nitrogen of the standard MS medium, and the highest anthocyanin yield under light conditions was obtained by 1/5 total nitrogen of the standard MS medium. Increasing the nitrogen concentration resulted in decreasing anthocyanin yield. In the dark, the NO_3^- ion was speculated to be solely responsible for activation of anthocyanin biosynthesis. Higher ratios of NO_3^-/ NH_4^+ have been demonstrated to be more effective for anthocyanin production. In the presence of light, 15 mM and 30 mM total nitrogen were preferable for callus growth (SAKAMOTO, IIDA, SAWAMURA, HAJIRO, ASADA, YOSHIKAWA & FURUYA, 1993).

2.5.4 Effects of manipulating plant growth regulators on anthocyanin biosynthesis

For most callus cultures, the growth regulator requirements are generally auxin and cytokinin. Auxins are compounds that stimulate shoot cell elongation. They resemble indole acetic acid (IAA) in their spectrum of activity. Cytokinins are known to promote cell division in plant tissues, and regulate cellular growth and development. Auxin-cytokinin supplements are instrumental in the regulation of cell-division, cell elongation, cell differentiation and organogenesis (DODDS & ROBERTS, 1985).

The effects of 2,4-D on secondary metabolic processes have been examined by several researchers (OUELHAZI, FILALI, CRECHE, CHÉNIEUX & RIDEAU, 1993: *Catharanthus roseus* cell cultures; OZEKI, DAVIES & TAKEDA, 1993: *Daucus carota* suspension cultures; OZEKI, KOMAMINE & TANAKA, 1990: *Daucus carota* suspension cultures; MEYER & VAN STADEN, 1995: *Oxalis reclinata* cultured cells). Increasing concentrations of 2,4-D resulted in increased callus production in *O. reclinata* callus (MEYER & VAN STADEN, 1995). The repression of anthocyanin production in *Oxalis* cultures by auxins was said to be in agreement with reports made on *Haplopappus gracilis* (CONSTABLE, SHYLUK & GAMBORG, 1971); *Helianthus tuberosus* (IBRAHIM, THAKUR & PERMANAND, 1971) and *Daucus carota* L. cv Korudagosun (OZEKI & KOMAMINE, 1986).

OZEKI & KOMAMINE (1985a) found that transfer of *D. carota* cells from a medium containing 2,4-D to a 2,4-D lacking medium, cell division continued for four days after the transfer. This was suggested to be due to 2,4-D carry-over, as cell division ceases after four or five days and accumulation of anthocyanin occurs in the vacuole after five days. Addition of 2,4-D to anthocyanin synthesising cells six days after the transfer resulted in a gradual disappearance of anthocyanin and an initiation of cell division in the same cells when anthocyanin had almost vanished. This observation confirmed that a cell in which cell division had stopped and anthocyanin synthesis was taking place in a medium lacking 2,4-D, had the ability to regain cell division activity when it was transferred again to a 2,4-D containing medium. Anthocyanin biosynthesis induction and cell division appeared to be a reciprocal phenomena. The use of DNA synthesis inhibitors indicated that anthocyanin synthesis is regulated by 2,4-D, irrespective of cell division, and that anthocyanin synthesis and cell division may be regulated by different mechanisms.

Investigations conducted to elucidate regulatory mechanisms involved in the expression and suppression of secondary metabolism by auxins at the enzymatic level have revealed that enzymes involved in the more general phenylpropanoid pathway, mainly PAL and 4-CL have increased catalytic activity during the first two days of transfer from media lacking or containing 2,4-D. This effect was assigned to a transfer response. Maintenance of cultured cells on 2,4-D containing medium resulted in a decrease of enzymatic activities of these enzymes. Low levels of the enzymes were maintained on 2,4-D containing medium. Chalcone synthase and chalcone isomerase were detected at low levels at all times in the presence of 2,4-D. In particular, chalcone synthase activity was below detectable levels throughout the culture period. Transfer of cells to 2,4-D lacking medium resulted in the induction of anthocyanin, which was reflected by increased levels of all enzymes. Chalcone synthase induction was particularly noted (OZEKI & KOMAMINE, 1985a). Ultraviolet irradiation or elicitor treatment of cultured cells resulted in rapid induction of flavonoid enzymes. Induction of PAL and CHS in 2,4-D lacking medium is said to be slow, as three or four days are taken for enzyme induction. The rapid induction of flavonoid metabolism by UV light and elicitors relates to the fact that defense responses need to be rapid in order to protect plant cells against environmental changes. In general, the induction of enzymes related to flavonoid metabolism is regulated at the transcriptional level and the induction of mRNA synthesis occurs prior to the induction of the synthesis of enzyme proteins (LAWTON, DIXON, HAHLBROCK & LAMB, 1983a and b; EDWARDS, CRAMER, BOLWELL, DIXON, SCHUCH & LAMB, 1985).

Cytokinins are important regulators of many aspects of plant development, including cell division, nutrient mobilisation, senescence, chloroplast development and apical dominance (DEIKMANN & HAMMER, 1995). For *in vitro* culture purposes, the most widely used cytokinins in growth media are kinetin, benzyladenine (BA) and zeatin. Kinetin is typically added to media at a concentration of 0.1 mg ℓ^{-1} for the induction of callus (DODDS & ROBERTS, 1985).

Cytokinins affect the expression of specific genes by both increasing and decreasing particular protein or mRNA abundance. Cytokinins have also been shown to affect accumulation of anthocyanins in plants. Increases in anthocyanin accumulation have been noted for tissues in culture as well as in parts of intact plants. Anthocyanin accumulation in response to cytokinins has been shown in *D. carota* suspension culture cells (OZEKI & KOMAMINE, 1981). Recently induction of accumulation of anthocyanins by cytokinins has been shown in *Arabidopsis thaliana* seedlings (DEIKMANN & HAMMER, 1995).

Induction of anthocyanin accumulation by cytokinins is said to be reminiscent of the classical cytokinin bioassay of betacyanin induction in *Amaranthus* seedlings. Even though betacyanins are chemically unrelated to anthocyanins, and they accumulate in plants that do not produce anthocyanins, they play a similar physiological role to anthocyanins. Betacyanins have the same set of signals as anthocyanins, mainly light, wounding and development (DEIKMANN & HAMMER, 1995).

In *Arabidopsis*, a large increase in anthocyanin accumulation is thought to result from increased accumulation of mRNA's encoded by four genes in the anthocyanin biosynthetic pathway. Two of the genes, namely, *CHI* and *DFR*, are regulated at the transcriptional level and the other two genes, *PAL1* and *CHS*, are post-transcriptionally regulated (DEIKMANN & HAMMER, 1995).

In cultured cell systems, there is controversy about the effects of cytokinins on anthocyanin production. Some reports on inhibitory effects of cytokinins have been made. KINNERSLEY & DOUGALL (1980) showed that kinetin decreased the yield of anthocyanin in a suspension culture of *D. carota*. In the system of OZEKI & KOMAMINE (1985b) where anthocyanin synthesis was induced in relation to embryogenesis in suspension cultures of *D. carota*, cytokinins were reported to have promoted synthesis of anthocyanins. In this system, anthocyanin biosynthesis was viewed as 'metabolic differentiation', as anthocyanin biosynthesis is an expression of secondary metabolism. In all the

systems, where cytokinins have a positive effect on anthocyanin production, these promotions are closely associated with illumination (OZEKI & KOMAMINE, 1981; TAKEDA, 1988; DEIKMANN & HAMMER, 1995).

OZEKI & KOMAMINE (1981) suggested that disagreement arising from the controversial effects of growth regulators on anthocyanin biosynthesis may be due to physiological developmental differences of the cells used for experiments, as well as the different levels of endogenous growth regulators used.

Controversy surrounds the effect of gibberellins on anthocyanin production. Gibberellins increased anthocyanin accumulation in the corolla of petunia flowers by increasing flavonoid gene transcription (WEISS, VAN DER LUIT, KNEGT, VERMEER, MOL & KOOTER, 1992). In other systems, they have been found to have inhibitory effects on anthocyanin production. Gibberellins were shown to decrease anthocyanin accumulation in carrot cell suspension cultures (HINDERER, PETERSEN & SEITZ, 1994; OZEKI & KOMAMINE, 1986). The mechanism(s) of gibberellin inhibition have not yet been identified (ILAN, ZANEWICH, ROOD & DOUGALL, 1994).

In intact plants, gibberellins are known to have profound and diverse effects on growth and development. In petunia flowers, stamens contain high levels of gibberellins and the gibberellins promote pigmentation by playing a key role in the regulation of anthocyanin synthesis in corolla tissues of *Petunia hybrida* (WEISS & HALEVY, 1989). Removal of the stamens or anthers at an early stage of corolla development, before the onset of anthocyanin synthesis, inhibits growth and anthocyanin accumulation in the attached corollas. The effect of gibberellins on corolla growth is independent of its effect on anthocyanin biosynthesis (WEISS, VAN TUNEN, HALEVY, MOL & GERATS, 1990).

The expression of anthocyanin genes has been shown to be induced by exogenous application of gibberellins. Immunoblotting using specific antibodies showed that significantly higher levels of the flavonoid enzymes, CHS and CHI, were detected in gibberellin *in vitro* cultured corollas of *P. hybrida* in the presence of sucrose. Examination of the steady-state levels of mRNA for CHS and CHI revealed that gibberellins enhanced CHS and CHI steady-state mRNA levels (WEISS, VAN TUNEN, HALEVY, MOL & GERATS, 1990). Gibberellin has also been shown to increase the production of anthocyanin through increased PAL activity. It is thought that gibberellin may operate at the transcriptional level, or may aid in stabilisation of specific flavonoid mRNAs (WEISS, VAN TUNEN, HALEVY, MOL & GERATS, 1990; HOOLEY, 1994). Gibberellin has also been thought to act on regulatory genes encoding transcription factors of the basic helix-loop-helix and c-myb classes, which, in part, appear to control expression of anthocyanin biosynthetic genes. This particular concept requires testing (HOOLEY, 1994).

2.6 AIMS AND OBJECTIVES

The economic importance of red coloured anthocyanins in the food industry was the main motivation behind this study as cyanidin-3-glucoside which accumulates as the major pigment in *O. reclinata* could potentially be used industrially as an alternative source to synthetic red food colourants. This research was aimed at investigating mechanisms which could be employed to induce pigment production as well as optimising anthocyanin yield from callus cultures of *O. reclinata*. Accumulation of red pigments in callus tissues was hypothesized to be due to external factors as anthocyanins have been shown to accumulate in response to environmental stimuli in usually non-expressing tissues.

The major objectives were to determine the 'switch' for anthocyanin production in *O. reclinata* callus cultures. With the establishment of the inducing factor(s), optimisation of red colourant production from *O. reclinata* was to be achieved. This involved determining the optimal conditions necessary for anthocyanin

yield as well as the maintenance of callus growth. The major objective of increased pigment production would be achieved through tissue culture based studies. The approach involved determination of physical and physiological factors which could switch on the expression of the genes coding for anthocyanin production during culturing of red and white callus lines. The following factors were investigated:

i) different carbohydrate sources;
ii) the effect of inorganic salts (nitrogen and phosphate);
iii) the influence of plant growth regulators; and
iv) the effect of light and temperature on production of anthocyanin and generation of callus biomass.

Tissue culture studies based on the measurement of secondary end product accumulation only were foreseen as limiting as they may not truly reflect the total attainable capacity for accumulation of the secondary metabolite. In order to recognize and ascertain biochemically important mechanisms which affect end product accumulation at the protein and gene level, molecular techniques were employed. Two-dimensional electrophoresis and *in vitro* translation were chosen in order to complement findings from plant tissue culture studies. These molecular techniques were utilized to acquire knowledge about the differences between pigment producing callus and non-pigmented callus of *O. reclinata*. Differences with respect to proteins, especially enzymes associated with the anthocyanin biosynthetic pathway were investigated.

Although, these techniques have been reported to be simple, rapid and possess a high resolution capacity, the establishment and optimisation of these techniques had to be achieved before they could be used successfully. Plant pigments and other secondary products tend to interfere with the isolation of proteins and nucleic acids (WANG & VODKIN, 1994), as well as polyacrylamide gel electrophoresis. Elimination of this problem had to be achieved prior to the

molecular analysis of the differences between the red and white callus types of *O. reclinata*.

CHAPTER 3

IN VITRO CULTURE STUDY

3.1 INTRODUCTION

The use of plant tissue culture systems has allowed for elucidation of protective responses of plant cells to environmental stress. In cultured plant cells, responses to stress occur even in the dedifferentiated state (DIXON & BOLWELL, 1986). Plant tissue culture systems are theoretically ideal for metabolic studies involving synthesis of secondary metabolites. Production of pigment-containing cells in anthocyanin synthesizing cultures is usually small. The cells may be diffusely distributed, or may be localised, thus giving a patchy appearance to the cultures (OZEKI, KOMAMINE & TANAKA, 1990). These heterogenous cultures may be manipulated *in vitro* to produce optimal levels of the desired secondary metabolite. Alteration of physical and chemical factors contributing to the culture environment may result in an increase in the metabolic flux towards secondary metabolite formation (DIXON & BOLWELL, 1986). This would result in reduced callus growth, even though the desired product is synthesized.

3.1.1 Effects of physiological factors on anthocyanin production

Accumulation of anthocyanins in cultured cells may often be restricted to certain cells or to a small region of cells. This results in a heterogenous type of callus being produced. Most plant cell cultures are heterogenous, with some more so than others. The component cells may differ in size, structure, deoxyribonucleic acid (DNA) content and in many other ways, including metabolism. The properties of cell types varies within and between cultures, and changes with time. One may often observe a decline in the ability of a cell

population to accumulate a designated secondary metabolite, but there are times when variation may give rise to cultures with an increased level of biosynthetic activity. Therefore, factors affecting such changes, namely, the heritable variation present in the explant, and the influence of culture conditions must be considered with seriousness in terms of manipulating cultured cells for increased metabolite production (HOLDEN, HOLDEN & YEOMAN, 1988).

Factors affecting increases or decreases in biosynthetic activity of cell cultures are important to researchers that are looking for high yields of secondary metabolites. Exposure of cells to appropriate cultural stimuli may lead to cultures producing a significant and stable yield of product. Such stimuli may be in two forms, that is, coming from a physical environment such as light or temperature, or in a physiological form, from the components of the medium. HOLDEN, HOLDEN & YEOMAN (1988) suggested that the components of the culture medium have the greatest effect on product yield. The concentrations and sources of carbon in the medium have been known to affect secondary metabolism. The levels of inorganic salts are known to affect both primary and secondary metabolic processes. In some cases, cultured cells may produce greater levels of the desired secondary product in medium devoid of these nutrients. Reduced nitrate levels also appear to stimulate biosynthetic activity (HOLDEN, HOLDEN & YEOMAN, 1988).

Plant growth regulators are used primarily to induce or establish callus from an explant and to maintain proliferation (DODDS & ROBERTS, 1985). They can also be effective in stimulating or inhibiting secondary products in cultured cells. Auxins appear to have the greatest influence on biosynthetic activity. The synthetic auxin 2,4-D has been shown to affect inductive activities of anthocyanin biosynthetic enzymes, PAL and CHS (OZEKI & KOMAMINE, 1986). Application of this plant growth regulator appears to inhibit production of the key regulatory enzymes of the anthocyanin biosynthetic pathway. Removal of 2,4-D activates expression of PAL and CHS mRNA's. Other auxins,

such as IAA, have promotory characteristics in production of secondary metabolites (HOLDEN, HOLDEN & YEOMAN, 1988; SATO, NAKAYAMA & SHIGETA, 1996).

The main motivation behind plant tissue culture studies in *O. reclinata* was to establish the factor(s) which induce or repress anthocyanin biosynthesis. The second objective was to manipulate anthocyanin production and to increase callus and anthocyanin yield.

3.2 MATERIALS AND METHODS

3.2.1 Plant material and callus generation

Pigmented and non-pigmented callus of *Oxalis reclinata* was generated by the method described by CROUCH, VAN STADEN, VAN STADEN, DREWES & MEYER (1993). Callus was initiated by surface-sterilization of stem-internodal explants in 1.75% NaOCl for four minutes prior to rinsing. A modified MURASHIGE & SKOOG (1962) medium (without glycine) was supplemented with 30 g ℓ^{-1} sucrose. BA (0.5 mg ℓ^{-1}) and NAA (5 mg ℓ^{-1}) were added to the medium prior to adjusting the pH to 5.7. The medium was solidified with agar (8 g ℓ^{-1}) and sterilized by autoclaving. Explants (3 mm) were transferred to the solid medium and placed with their long axes in contact with the agar. Culture vessels were placed under white cool fluorescent light (44.5 μmol photons m^{-2} s^{-1}) in a growth room with a 16 hour (h) light regime at 25°C. Production of callus occurred and heterogenous mixtures of white, yellow, green and red callus were observed. Red callus formation occurred with the induction of organogenesis. In order to create a homogenous red cell line, red cell aggregates were clustered together and subcultured. A white callus line was generated in a similar manner. The callus lines isolated were maintained on MURASHIGE & SKOOG (1962) medium (0.8% agar [w/v]; pH 5.7) supplemented with 5 mg ℓ^{-1} NAA, 0.5 mg ℓ^{-1} BA, 30 g ℓ^{-1} sucrose and 0.1 g ℓ^{1-} myo-inositol. Stock cultures were sub-cultured at four weekly intervals.

3.2.2 Manipulation of chemical components of culture medium

Unless otherwise stated, all experiments were conducted at constant light (48 μmol photons $m^{-2} s^{-1}$) at 22°C for 28 days.

3.2.2.1 Carbohydrate manipulations

The effect of varying concentrations of sucrose (10 g ℓ^{-1} to 60 g ℓ^{-1}) were investigated using both white and red callus lines. Alternative carbon sources were examined on MS basal medium (0.8% agar [w/v]; pH 5.8). The alternative sources of carbohydrate investigated were glucose, galactose, fructose, xylose, lactose and maltose at 30 g ℓ^{-1}.

3.2.2.2 Nitrate and phosphate manipulations

To investigate the effect of inorganic ions on anthocyanin yield, the target compound concentration was changed and the other nutrient concentrations maintained in the original MS medium (MURASHIGE & SKOOG, 1962) previously described for maintaining *O. reclinata* callus (Section 3.2.1) This medium contains 1.8 mM KNO_3 and 2 mM NH_4NO_3 as the sources of nitrogen; and 1.24 mM KH_2PO_4 as the main phosphate source. The above mentioned concentrations of nitrogen and phosphate sources were quartered, halved or doubled to test the effect of varying nitrogen and phosphate levels.

3.2.2.3 Phytohormone manipulations

The effect of growth regulators on callus growth and anthocyanin production was determined by subjecting dark grown white callus and light grown red callus to basal MS medium (MURASHIGE & SKOOG, 1962) containing different types of plant growth regulators (BA, KIN, NAA, IAA, IBA (indole butyric acid), 2,4-D, GA_3) at 0.1 mg ℓ^{-1}.

3.2.3 Manipulation of physical factors of the culture environment

3.2.3.1 Temperature effects

Cultures were placed at 10°C, 22°C and 35°C. The influence of these temperatures on white and red callus were recorded by weighing them after 28 days and thereafter extracting for anthocyanins (Section 3.2.4).

3.2.3.2 Light effects

The absence of light on anthocyanin accumulation was observed by placing callus lines in the dark for 28 days. Different light regimes were also investigated. Control cultures of both red and white callus were placed in continuous light under white fluorescent tubes (44 μmol photons $m^{-2}s^{-1}$) at 22°C. Experimental cultures were incubated under a light-dark cycle (16 h light, 21.1 μmol photons $m^{-2}s^{-1}$) at 22°C, alternatively placed in a growth room that was dimly illuminated continuously (2 μmol photons m^{-2} s^{-1}) at 22°C.

3.2.4 Measurement of callus growth and determination of anthocyanin content

In all experiments, final growth was measured by weighing callus after 28 days of growth at the various treatments. The anthocyanin content was determined by extracting weighed callus in 0.1% HCl-methanol at 4°C overnight. The absorbance of the clear methanolic supernatant, after centrifugation for five minutes at 10 000*g*, was measured at 535 nm with a spectrophotometer (Beckman DU-65 Spectrophotometer). A standard, cyanidin-3-glucoside (ROTH), was used as a reference solution for quantification. Anthocyanin content (mg g^{-1} FW) was calculated.

3.2.5 Analysis of data

All experiments were done using five replicates for statistical purposes. Every experiment was repeated five times. Data were subjected to a one-way

Analysis of variance (ANOVA) using the Statgraphics statistical programme. When the ANOVA indicated statistical significance, a Tukey's multiple comparison test was used to distinguish differences between treatments.

3.3 RESULTS

Production of anthocyanin containing *O. reclinata* callus was initially achieved on MS medium (MURASHIGE & SKOOG, 1962) supplemented with 30 g ℓ^{-1} sucrose. Application of sucrose at this concentration to basal MS medium resulted in the highest callus growth and anthocyanin yield for red and white callus (Figure 3.1). Concentrations of 40 g ℓ^{-1} or more resulted in decreased accumulation of callus biomass. Therefore, a concentration of 30 g ℓ^{-1} was chosen to test the effect of different carbohydrate sources on anthocyanin accumulating callus cultures of *O. reclinata*. Sucrose was used as the carbohydrate source in subsequent experiments, because it was found to have the greatest effect on callus growth and anthocyanin production. However, both sucrose and glucose had a positive effect on the growth of red callus. Anthocyanin production was positively influenced by sucrose and maltose. Fructose inhibited callus growth of both red and white cultures (Figure 3.2)

The effect of nitrates and phosphates on *O. reclinata* callus cultures are given in Figures 3.3 and 3.4. Cell growth was best promoted by 1.8 mM KNO_3 and 2 mM NH_4NO_3 for the white callus (Figure 3.3A). The other concentrations resulted in far lower generation of callus biomass. Optimal white callus growth was obtained once the phosphate level was halved from 1.24 mM KH_2PO_4 to 0.62 mM KH_2PO_4. For the red callus, increasing the concentration of nitrates had no significant effect on callus growth. Reducing the phosphate concentration to 0.31 mM KH_2PO_4 in the growth medium induced greater accumulation of anthocyanin. However, increasing the total nitrate source promoted anthocyanin biosynthesis for the red callus but resulted in a reduction in growth (Figure 3.4).

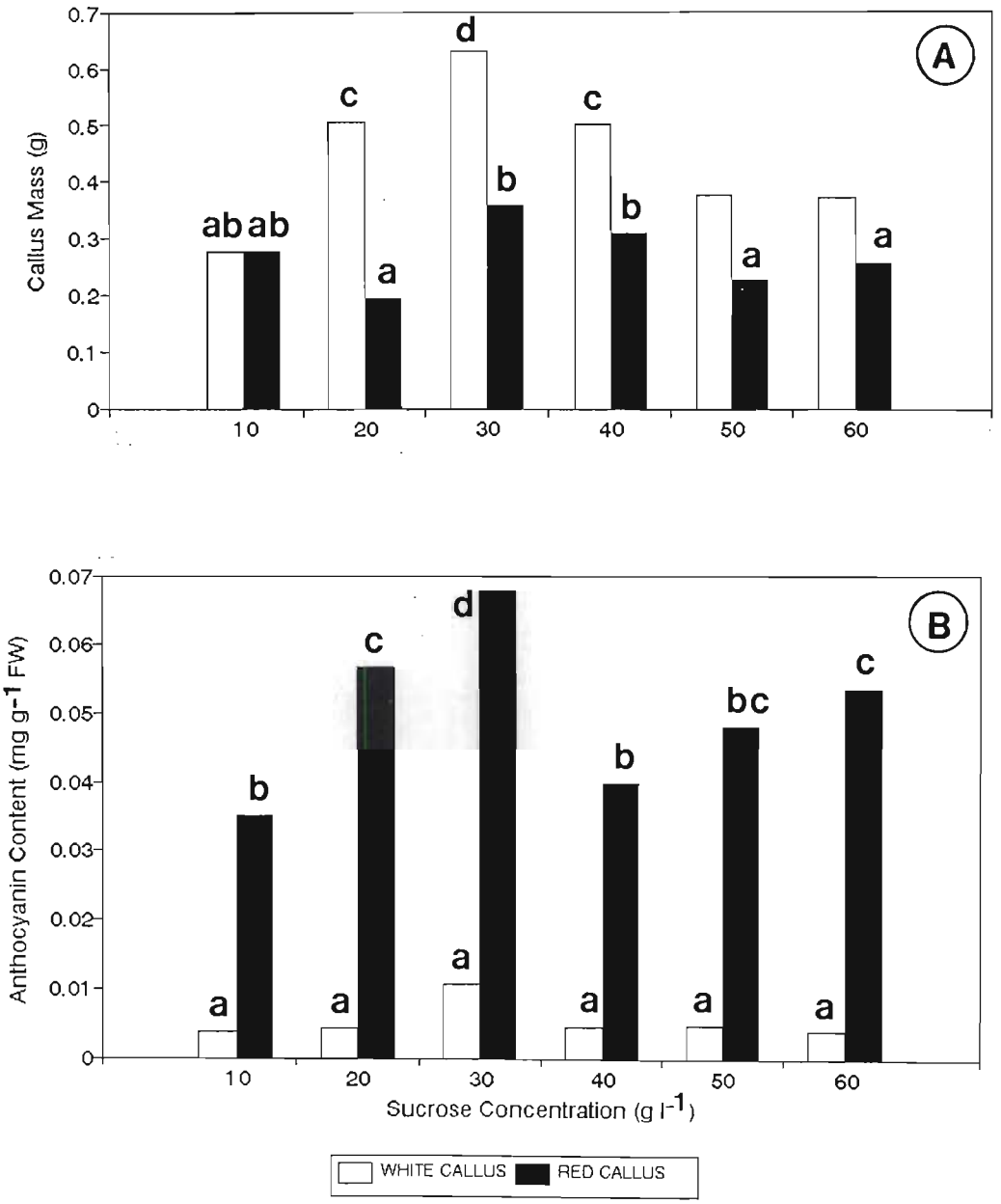


Figure 3.1: Effect of sucrose on callus growth (A) and anthocyanin production (B) in white and red callus cultures of *O. reclinata*. Cultures were maintained on MS basal medium with 5 mg ℓ^{-1} NAA and 0.5 mg ℓ^{-1} BA. Treatments with the same letter were not significantly different, $P < 0.05$

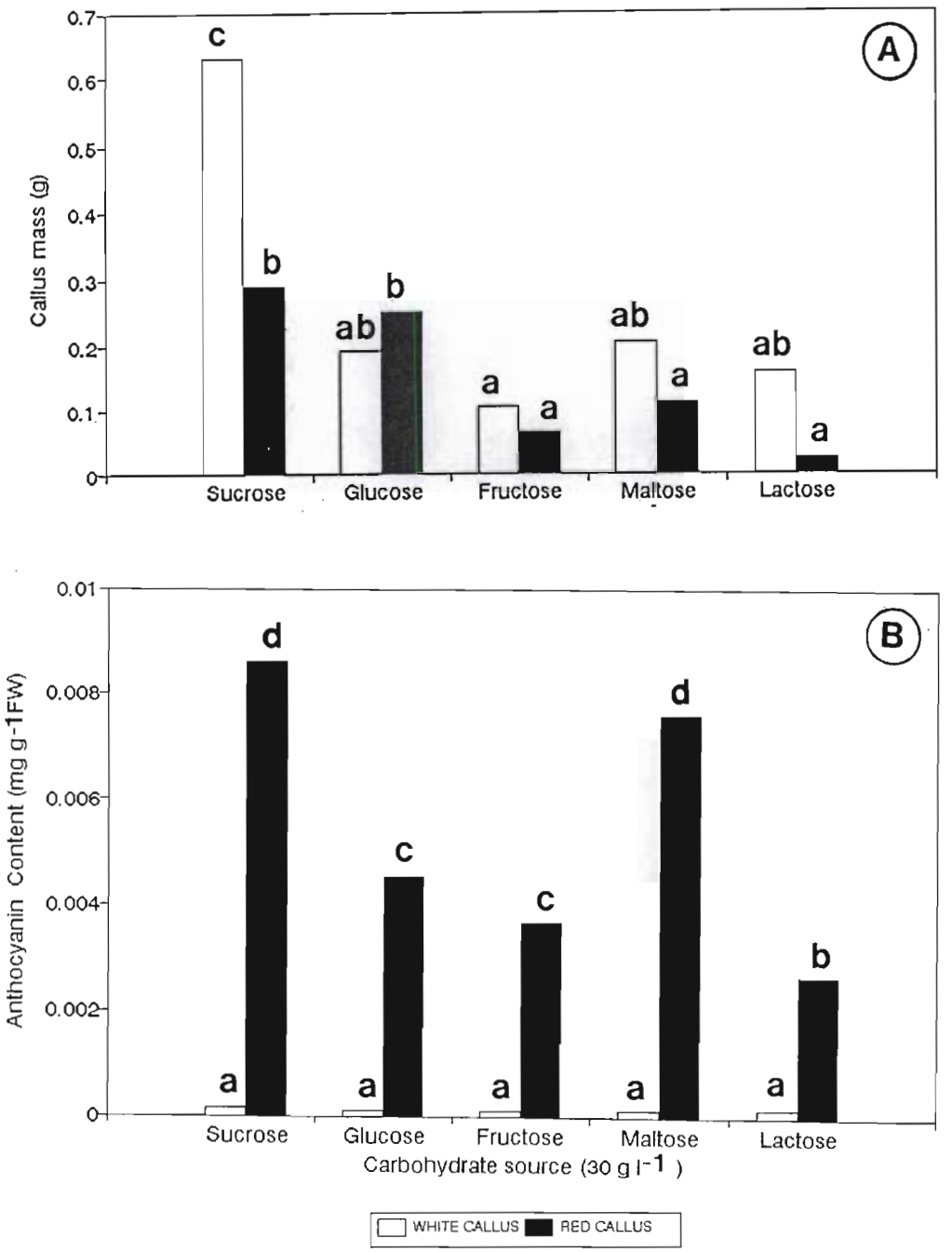


Figure 3.2: Effect of carbohydrate source on callus growth (A) and anthocyanin production (B) in white and red callus cultures of *O. reclinata*. Cultures were maintained in MS basal medium with 5 mg ℓ^{-1} NAA and 0.5 mg ℓ^{-1} BA. Treatments denoted by the same letters were not significantly different, $P < 0.05$

The most effective hormone on cell growth for both red and white callus with an average of 1.4 g and 1.9 g of callus biomass generated, respectively, was 2,4-D (Figure 3.5). The highest anthocyanin yield (0.008 mg g^{-1} FW) was obtained with medium supplemented with 1.0 mg ℓ^{-1} NAA. Kinetin and IBA did not support red callus growth. Instead necrosis of the callus was observed and eventually the cells died.

The requirement of *Oxalis* callus for light in order to produce anthocyanin is shown in Figure 3.6. Dark-grown cultures of the white type did not produce red pigment (Figure 3.6B). To induce anthocyanin production in white cultures, it was necessary to illuminate these cultures with high-light (23,8 μmol photons m^{-2} s^{-1}) for 24 hours daily (Figure 3.6D). This resulted in production of a heterogenous callus which was composed of anthocyanin-accumulating cells and pigment-free cells. Red callus grown in a 24 hour high-light intensity (23,8 μmol photons m^{-2} s^{-1}) growth room was always richly pigmented (Figure 3.6A). Transfer of this callus to the dark resulted in paling of the callus to a pink colour (Figure 3.6C) and eventually complete loss of pigment. This indicated the probable cessation of anthocyanin biosynthesis (Figure 3.7A ii). Shifting this dark grown red callus to the light activated anthocyanin biosynthesis as accumulation the red pigment could be visualized (Figure 3.7B).

Callus exposed to high-light continuously accumulated anthocyanin to the highest quantities (0.008 - 0.01 mg g^{-1} FW) (Figure 3.8). A slight reduction in anthocyanin production was noted for red callus placed under a light-dark illumination cycle (16-8 hours respectively). Cultures placed in the dark showed a significant reduction in anthocyanin synthesis. Red cultures placed under low-light conditions (2 μmol photons m^{-2} s^{-1}) showed moderately lowered anthocyanin levels. On the other hand, white cultures, grown at low-light, hardly accumulated anthocyanins. These cultures accumulated anthocyanin to similar levels as those cultures grown in the dark (Figure 3.8B).

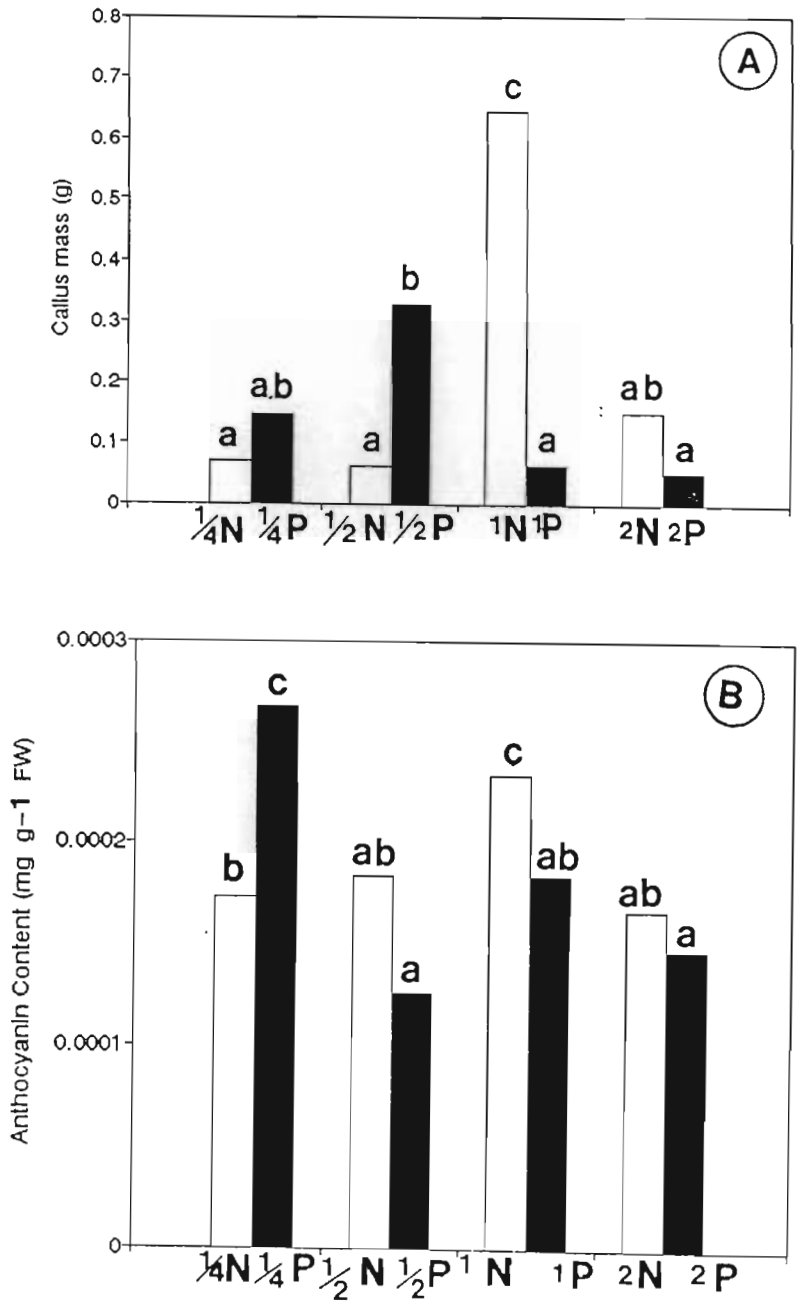


Figure 3.3: Effect of nitrates (□) and phosphates (■) on callus growth (A) and anthocyanin production (B) in white callus cultures of *O. reclinata*. Cultures were maintained in MS basal medium with 5 mg ℓ^{-1} NAA and 0.5 mg ℓ^{-1} BA. Treatments denoted by the same letters were not significantly different, $P < 0.05$

Key to figure: 1/4N, 0.45 mM KNO_3 and 0.6 mM NH_4NO_3; 1/2N, 0.9 mM KNO_3 and 1.2 mM NH_4NO_3; 1N, 1.8 mM KNO_3 and 2.4 mM NH_4NO_3; 2N, 3.6 mM KNO_3 and 4.8 mM NH_4NO_3; 1/4P, 0.312 mM KH_2PO_4; 1/2P, 0.624 mM KH_2PO_4; 1P, 1.24 mM KH_2PO_4 and 2P, 1.24 mM KH_2PO4.

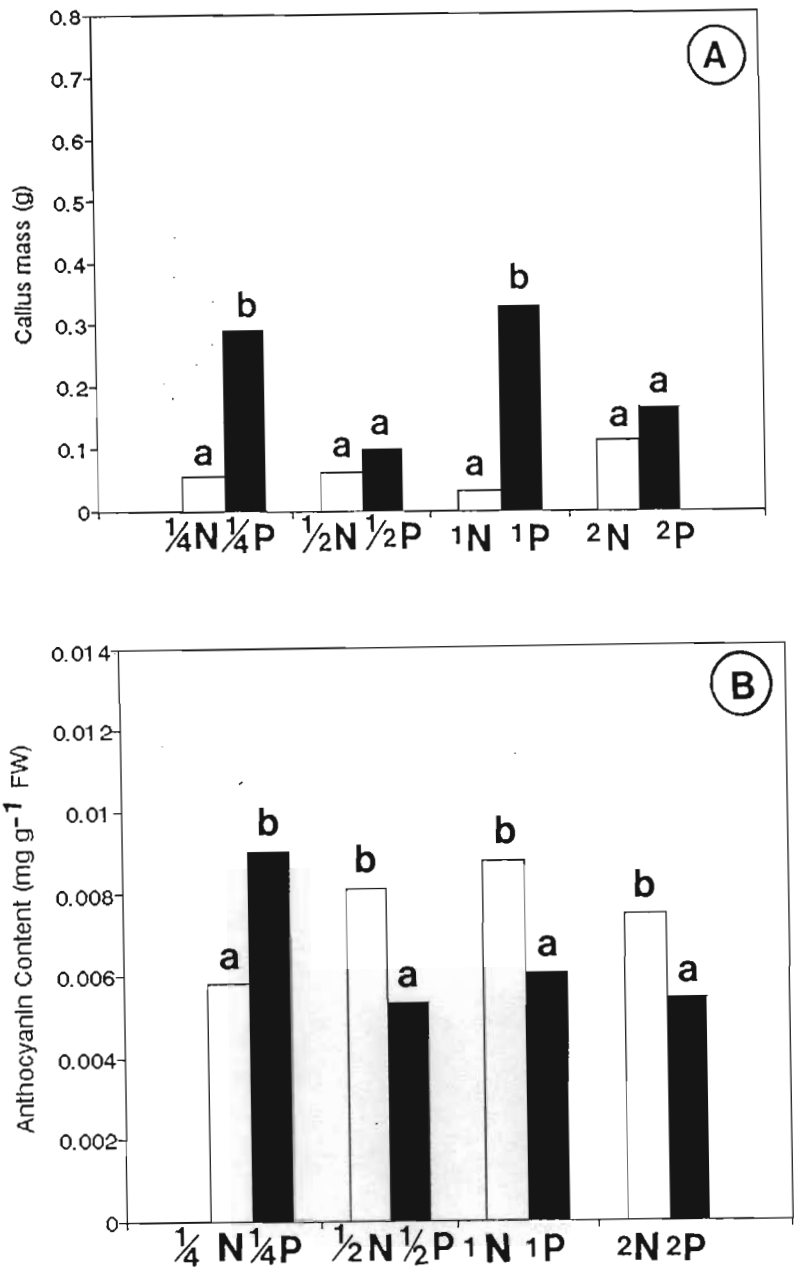


Figure 3.4: Effect of nitrates (□) and phosphates (■) on callus growth (A) and anthocyanin production (B) of red callus cultures of *O. reclinata*. Cultures were maintained in MS basal medium with 5 mg ℓ^{-1} NAA and 0.5 mg ℓ^{-1} BA. Treatments denoted by the same letters were not significantly different, $P < 0.05$

Key to figure: 1/4N, 0.45 mM KNO_3 and 0.6 mM NH_4NO_3; 1/2N, 0.9 mM KNO_3 and 1.2 mM NH_4NO_3; 1N, 1.8 mM KNO_3 and 2.4 mM NH_4NO_3; 2N, 3.6 mM KNO_3 and 4.8 mM NH_4NO_3; 1/4P, 0.312 mM KH_2PO_4; 1/2P, 0.624 mM KH_2PO_4; 1P, 1.24 mM KH_2PO_4 and 2P, 1.24 mM KH_2PO4.

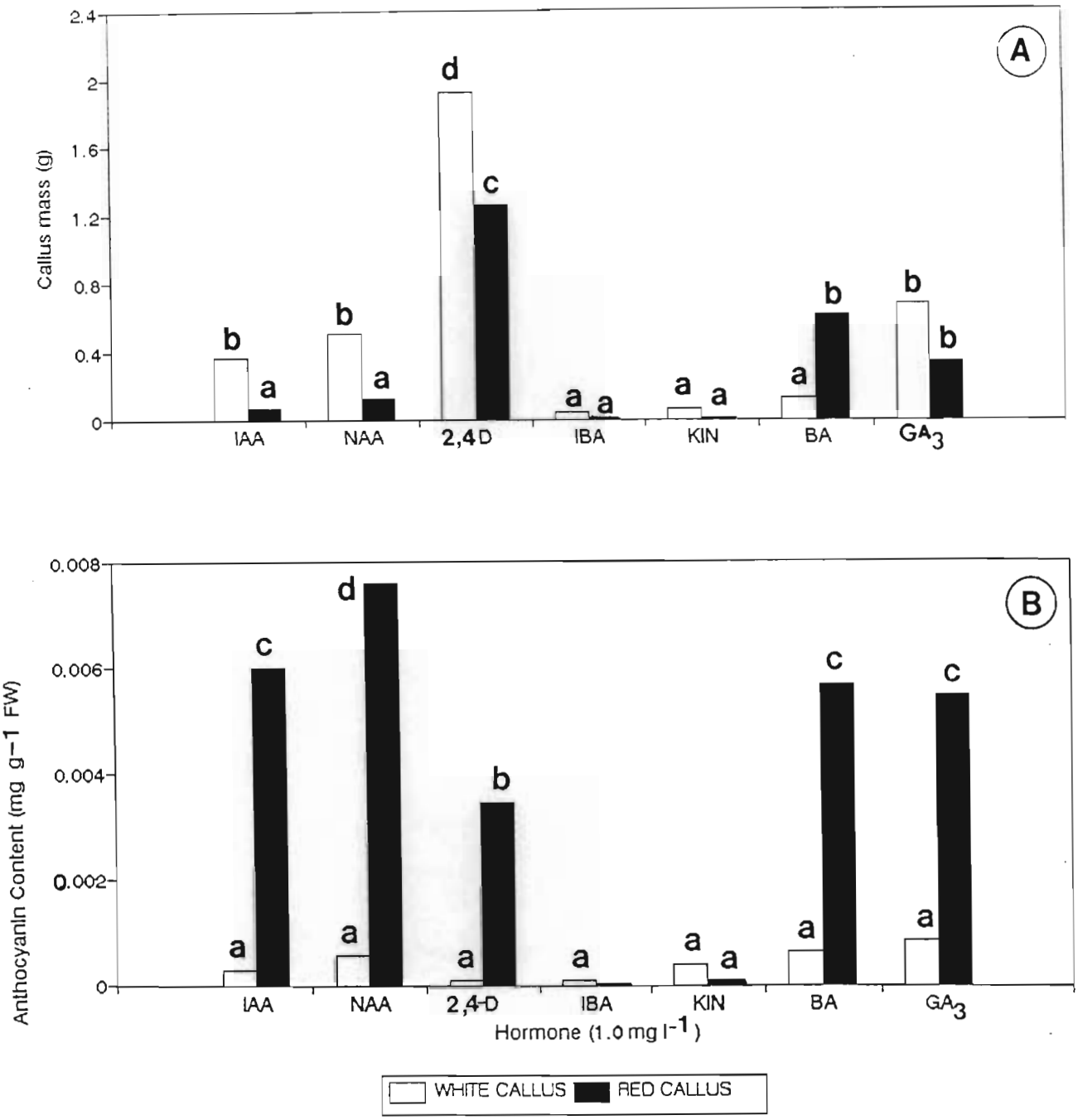


Figure 3.5: Effect of different plant hormones on callus growth (A) and anthocyanin production (B) in white and red callus cultures of *O. reclinata*. Treatments denoted by the same letters were not significantly different, $P < 0.05$

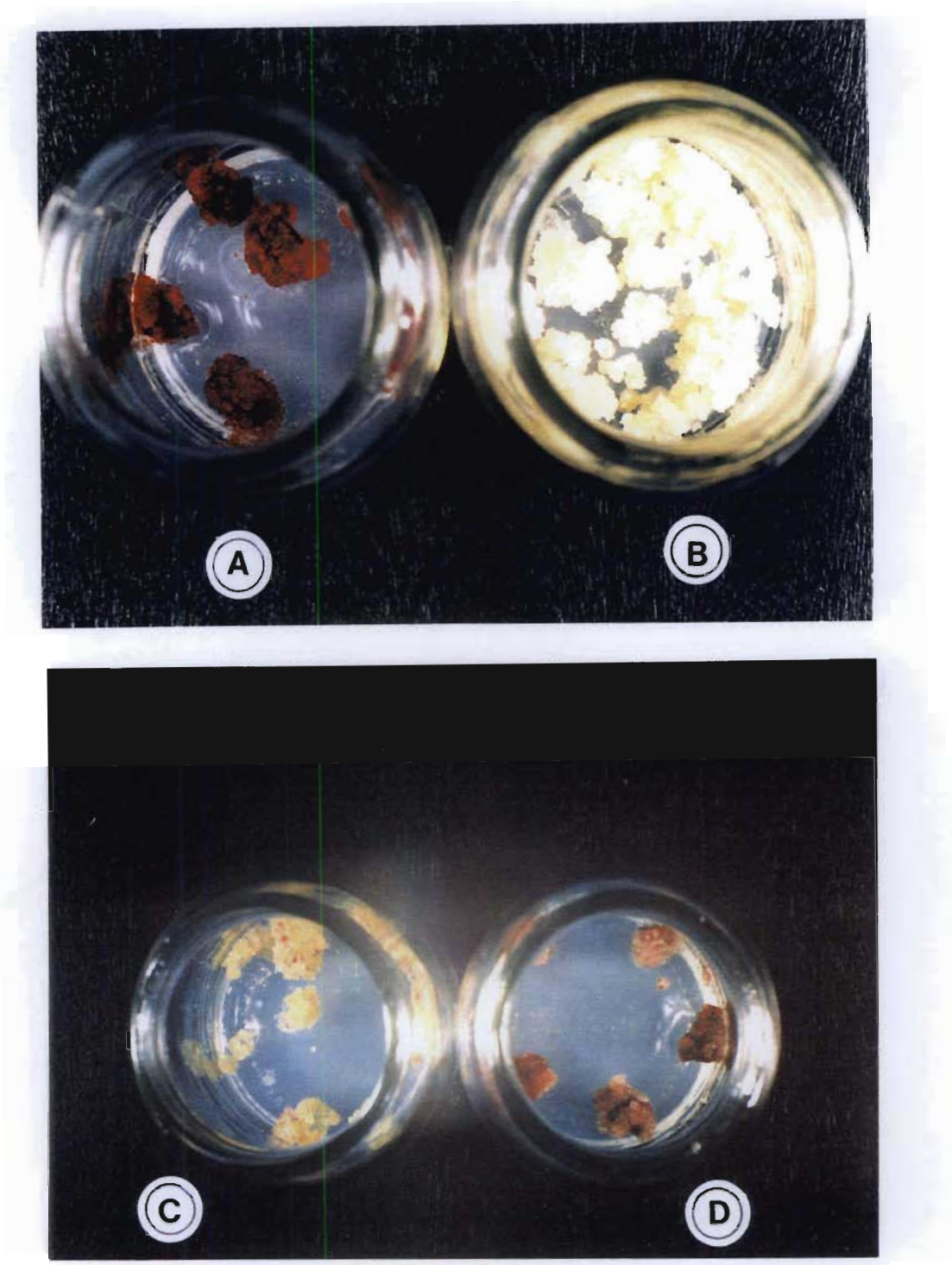


Figure 3.6: Effect of light on *Oxalis* callus grown *in vitro*. Four different callus types were generated. (A) Red callus grown in the light. (B) White callus grown in the dark. (C) A heterogenous red-white line grown in the light and a red callus line which was paling due to absence of light (D)

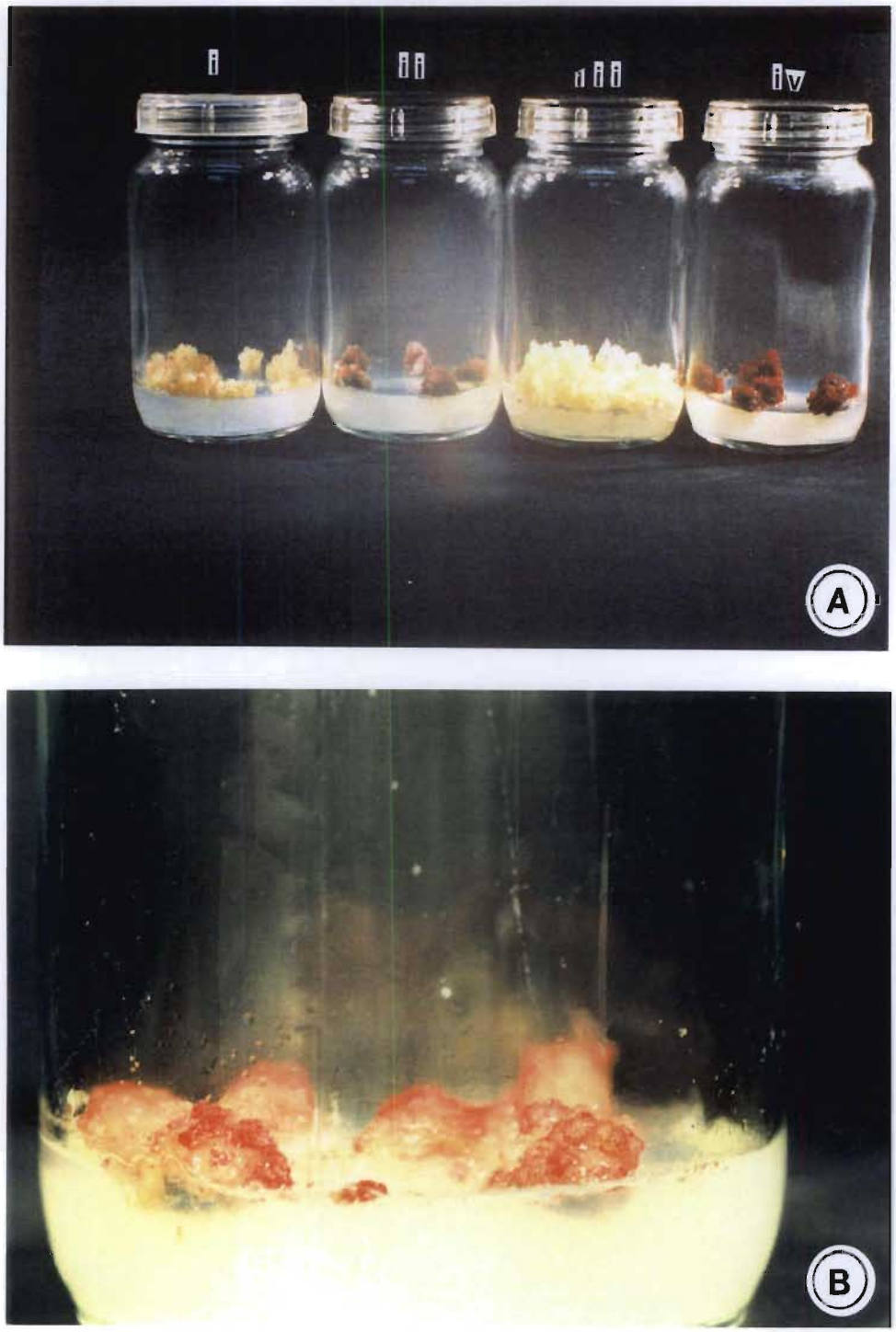


Figure 3.7: (A) Four callus types were generated (i) white callus grown in the light (ii) red callus grown in the dark (iii) white callus grown in the dark (iv) red callus grown in the light. (B) Dark grown callus shows induction of anthocyanin biosynthesis after transfer to the light

Even though light was essential for anthocyanin production, generation of callus biomass appeared to be independent of light. Cultures of the white type grown at high-light intensity and in the dark grew similarly. Continuously illuminated red cultures grew slower than white *Oxalis* cultures exposed to the same light conditions. Placement of red cultures in the absence of light inhibited callus growth (Figure 3.8A).

Production of anthocyanin was independent of temperature treatment as there were no significant differences observed when callus was exposed to temperatures of 10°C and to a 25°C. Red and white callus lines did not survive at 35°C. This temperature was lethal to the callus as it became brown and eventually died. The best temperature was apparently 25°C. At this temperature, callus biomass generated was 0.75 g for the red callus (Figure 3.9).

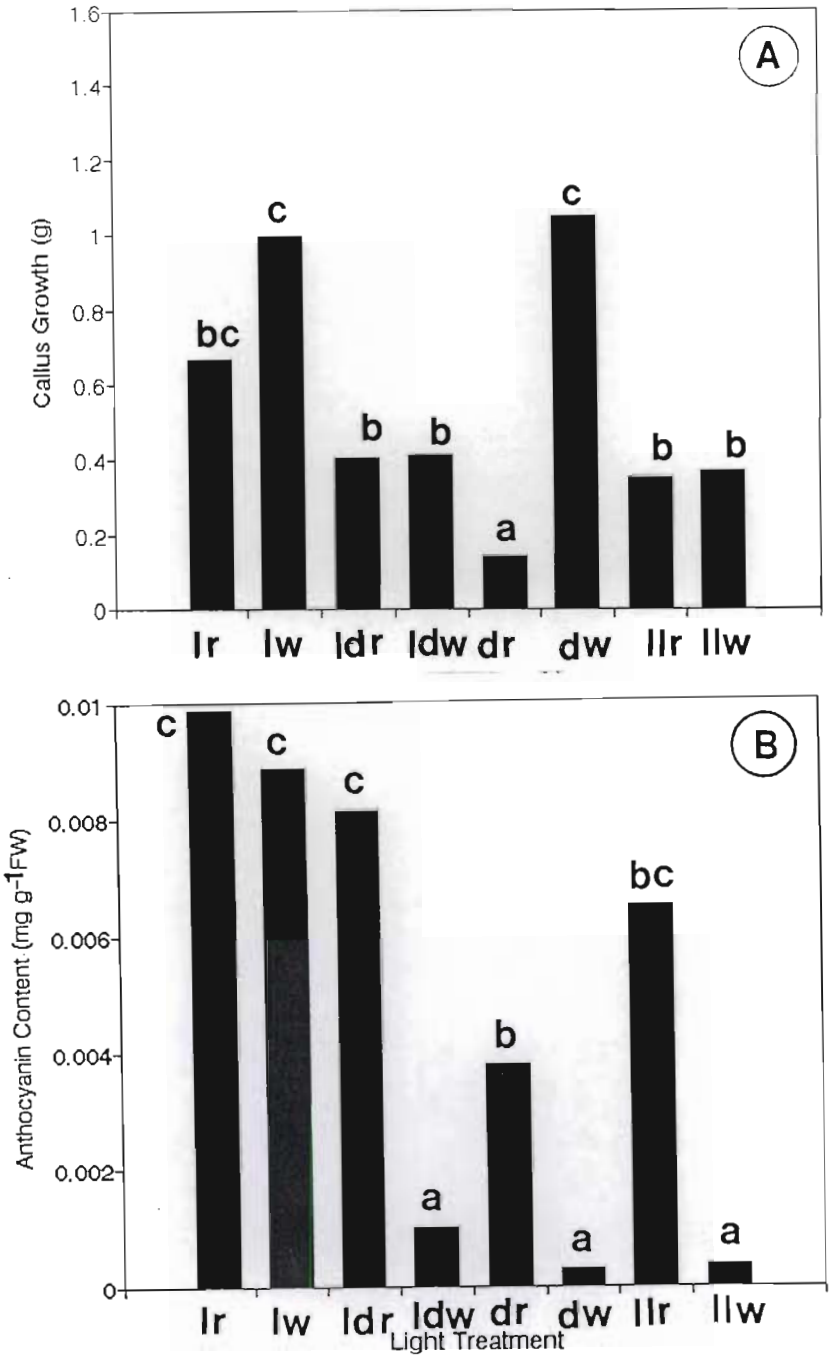


Figure 3.8: Effect of light on callus growth (A) and anthocyanin production (B) in white and red callus cultures of *O. reclinata*. Cultures were maintained in MS basal medium with 5 mg ℓ^{-1} NAA and 0.5 mg ℓ^{-1} BA. Treatments denoted by the same letters were not significantly different, $P < 0.05$

Key to figure: lr, light-grown red callus; lw, light-grown white callus; ldr, red callus exposed to light-dark cycle; ldw, white callus grown exposed to light-dark cycle; dr, dark-grown red callus; dw, dark-grown white callus; llr, red callus exposed to low-light and llw, white callus exposed to low-light

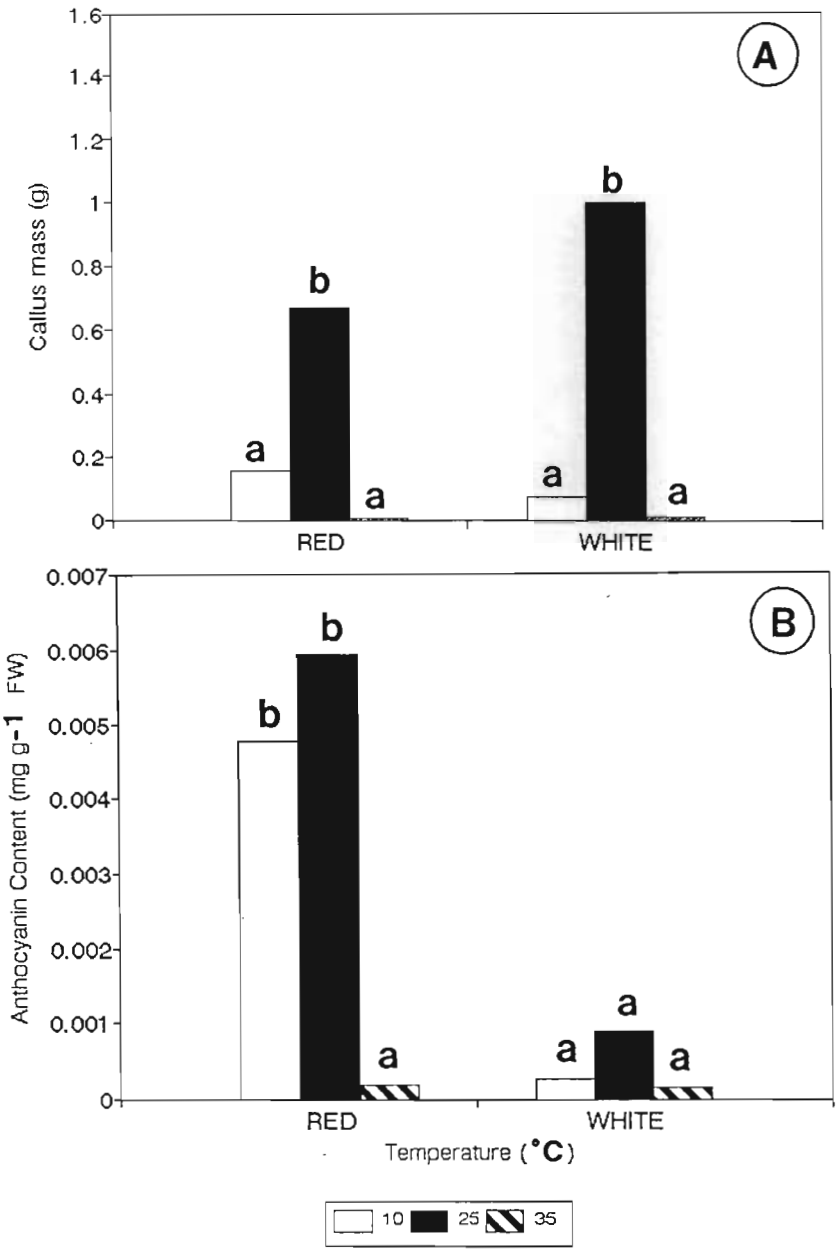


Figure 3.9: Effect of temperature on callus growth (A) and anthocyanin production (B) in white and red callus cultures of *O. reclinata*. Cultures were maintained in MS basal medium with 5 mg ℓ^{-1} NAA and 0.5 mg ℓ^{-1} BA. Treatments denoted by the same letters were not significantly different, $P < 0.05$

3.4 DISCUSSION

The anthocyanin yield is affected by cell growth and anthocyanin content. The anthocyanin content is varied by both the amount of pigmentation in pigmented cells and the number of pigmented callus cells in a culture (SATO, NAKAYAMA & SHIGETA, 1996).

Sucrose concentrations of higher than 50 g ℓ^{-1} reduced cell growth and anthocyanin production of cultured *O. reclinata* (Figure 3.1). It was speculated that this effect is brought about by the higher osmotic strength affecting the water content of the vacuole, negatively and thus, resulting in sucrose limiting anthocyanin accumulation. For *O. reclinata*, the highest growth was obtained with 20 to 40% sucrose for the white callus. This is in accordance with previous findings as *Aralia cordata* cells were found to grow best on medium supplemented with 20 to 40% sucrose. Sucrose acts as an osmotic agent when used at high concentrations. This osmotic effect inhibited cell growth and increased anthocyanin content in *Fragaria ananassa* by increasing the percentage of pigmented cells (SATO, NAKAYAMA & SHIGETA, 1996).

Carbohydrates have been found to have a profound influence on anthocyanin biosynthesis. Different carbohydrates were analyzed for their effect on callus and anthocyanin accumulation in *O. reclinata* cultures. The best carbohydrate source for *Oxalis* callus was found to be sucrose at 30 g ℓ^{-1}. The other carbohydrates tested did not affect anthocyanin production and callus growth significantly. Carbohydrates such as fructose could not support growth of *Oxalis* reclinata callus.

A two-fold increase in the nitrogen source resulted in a decline in anthocyanin production. Reducing the phosphate concentration to a quarter in comparison to the basal medium significantly elevated levels of anthocyanin accumulated by red callus cells (Figures 3.3 and 3.4). Depletion of nutrients is one of the environmental factors which can induce anthocyanin synthesis *in vivo* in

tissues that generally do not accumulate these pigments. In *O. reclinata* callus, decreasing the total phosphate concentration resulted in increased pigment production for the pigmented callus. Increasing the concentration of nitrates for the red callus had no significant effect on growth. In *Oxalis*, nitrates and phosphates appear not to play an inductive role in anthocyanin biosynthesis as no basic trend for accumulation of anthocyanin was identified. Accumulation of anthocyanin due to limiting nutrients, may be due to the cessation of cell division. Through cessation of division, the rate of protein synthesis declines and endogenous accumulation of phenylalanine occurs. Enlargement of this amino acid pool triggers transcription of mRNAs of key anthocyanin biosynthetic enzymes, PAL and CHS. Anthocyanins are then synthesized from phenylalanine (KAKEGAWA, SUDA, SUGIYAMA & KOMAMINE, 1995).

Controversy surrounds the effects of hormones on anthocyanin biosynthesis. KINNERSLEY & DOUGALL (1980) reported that anthocyanin accumulation occurred continuously in culture medium containing the auxin, 2,4-D in carrot cells. In most cases, 2,4-D has been found to have an inhibitory effect on anthocyanin production. OZEKI & KOMAMINE (1981) showed that transfer of anthocyanin-accumulating carrot cells to medium lacking 2,4-D resulted in induction of anthocyanin biosynthesis which was coupled with induction of embryogenesis. Reports on *Daucus carota* L. cv Korudagosun (OZEKI & KOMAMINE, 1986) and *Oxalis reclinata* (MEYER & VAN STADEN, 1995) are consistent with an inhibitory effect this auxin has on anthocyanin production.

This investigation showed that 2,4-D inhibited pigment accumulation but it has a promotive effect on callus growth for red and white callus of *Oxalis*. This auxin probably increases primary metabolism in *O. reclinata* cells and less energy is then spent on secondary metabolic production of anthocyanin pigments.

OZEKI & KOMAMINE (1986) suggested that addition of 2,4-D resulted in greater cell division and removal of this auxin from medium resulted in cell

division ceasing and the ability for cells to accumulate anthocyanin is gained. Enzymatically, continued growth of cells in 2,4-D containing medium results in decreased levels of phenylpropanoid biosynthetic enzymes, PAL and 4CL. Therefore, 2,4-D probably acts by regulating the suppression of genes involved in flavonoid metabolism in most plant species. The other auxins, IAA and NAA had the opposite effect on anthocyanin biosynthesis as they promoted growth in red cultures of *O. reclinata* at 1.0 mg ℓ^{-1}.

The cytokinin, BA, promoted anthocyanin production. It is well-documented that in the intact plant, cytokinins promote secondary processes associated with anthocyanin production (*Arabidopsis thaliana*: DEIKMANN & HAMMER, 1995). In cultured cells, the effect of cytokinins is not clearly defined and presented in the literature. However, in cases where promotions occur with the application of cytokinins, production is closely associated with the inductive effect of light illumination and low temperatures.

Light was found to induce anthocyanin biosynthesis in *O. reclinata* callus cells. Dark grown cultures of the white callus type remained pigment free. Transfer of these cultures resulted in accumulation of pigments (Figure 3.6). High-light intensity induces *de novo* synthesis of enzymes involved in the flavonoid pathway and the spectral sensitivity for anthocyanin induction differs in different plant systems (KOES, SPELT & MOL, 1989). *Oxalis* callus cultures when grown under conditions of high-light intensity accumulated anthocyanins to similar levels. Growth of these cultures under low-light conditions or in the dark did not stimulate anthocyanin biosynthesis. The role played by light with respect to anthocyanin biosynthesis in cultured plant cell systems may be similar to the role played by these compounds in nature. Anthocyanins are known to act as screening pigments in nature and they often accumulate in response to UV-light. Flavonoid pigments strongly absorb UV-light and they are thought to act as a protective shield as the UV-component of sunlight has the potential to damage DNA and impair other physiological processes. Under continuous UV-light transient expression of flavonoid genes occurs (KOES,

SPELT & MOL, 1989). It is thought that, once sufficient flavonoids accumulate most cells will be protected and flavonoid biosynthesis will cease (KOES, QUATTROCCHIO & MOL, 1994).

Regarding callus growth, light does not play a significant role in *O. reclinata*. Light and dark grown cultures of the white type grew to similar levels (Figure 3.8A). However, the red callus maintained under constant high-light grew significantly slower compared to the white callus under the same conditions. This was assigned to secondary metabolic processes which were occurring in red callus cells. Greater energy was being spent on secondary metabolism. Meanwhile, the white callus grew more actively as most energy was utilized for primary metabolic processes. This, resulted in greater biomass generation.

Oxalis reclinata flowers during wet and cold winter months. Organogenesis was achieved by incubation at 10°C using heterogenous callus composed of red, white, yellow and green cells by CROUCH, VAN STADEN, VAN STADEN, DREWES & MEYER (1993). This lowered temperature closely parallels the natural growing conditions of this species. Using the relatively pure red and white lines generated for manipulation studies in this particular investigation, regeneration of plantlets did not occur at lower temperature. Instead, a significant reduction in anthocyanin production was noted for the red callus. This observation was in contrast with other reported data. In most cases, lower temperatures have resulted in accumulation of anthocyanin and steady-state levels of PAL and CHS have been shown to increase in response to lower temperatures. In *Oxalis*, lower *in vitro* temperatures may have reduced primary metabolism but did not necessarily result in increased secondary metabolic activity.

OZEKI & KOMAMINE (1985b) and OZEKI, KOMAMINE & TANAKA (1990) regarded anthocyanin production as a form of 'metabolic differentiation' and they perceived the expression of secondary metabolism to be closely paralleled with morphological differentiation since secondary metabolism is expressed in

differentiated organs and tissues during specific developmental stages *in vivo*. *In vitro*, production of anthocyanin was achieved by these researchers with the induction of embryogenesis in carrot suspension cells. In *Oxalis reclinata*, no morphological differentiation was achieved at either growth temperatures with the pure red and white lines, even though the red callus may be regarded as being differentiated metabolically. To be able to produce organs from this callus, it appears necessary to generate green callus. CROUCH & VAN STADEN (1994) found that incubation of a mixture of red, yellow and green callus of *Oxalis* resulted in organogenesis at 10°C. White or yellow callus has the potential to become red. The yellow callus most probably contains chalcones which are precursors for synthesis of anthocyanins.

CHAPTER 4

SUSPENSION CULTURE

4.1 INTRODUCTION

Suspension cultures of cells is an alternative method of culturing plant tissues. These cultures may be created by transferring fragments of callus tissue into a liquid medium. The system is then agitated during the growth period of the cells (DODDS & ROBERTS, 1985).

This type of *in vitro* culture may have several advantages over conventional solid culture methods. The maintenance of these cultures consumes less time. Sub-culturing can be performed in bulk. Liquid cultures have a potential for greater growth rates and the doubling time of cells may be reduced (DODDS & ROBERTS, 1985). These factors make liquid cultures a desirable means of propagating plant cells and secondary products for commercial purposes.

Suspension cultures have been thought a better alternative compared to solid cultures for the *in vitro* production of pigments and the accumulation of pigment producing cells. Extraction of secondary metabolites from intact plants and *in vitro* cultured hard callus may have some problems associated with it. Extraction of pigments from *in vivo* plant matter may be limited by the season in which they can be collected, age of the plant and other environmental or physiological factors (SATO, NAKAYAMA & SHIGETA, 1996).

Regarding anthocyanin accumulating cells, suspension cultures are useful to study whether changes of anthocyanin accumulation are derived from the increased accumulation of pigment within a cell or the increase of proportion of pigmented cells to total cells (SUZUKI, 1995).

In this study, it was seen necessary to elucidate whether the establishment of suspension cultures for *Oxalis* callus can be achieved and whether the maintenance of pigment production by the red cells of *O. reclinata* can be carried out once the liquid suspension culture had been established. Cells which have the ability to produce anthocyanin may often lose this quality due to a change in environmental and growth conditions. For commercial purposes, it is more economically viable to produce anthocyanin using liquid cultures.

4.2 MATERIALS AND METHODS

4.2.1 Plant material, initiation media and culture conditions

Suspension cultures were initiated from red and white callus stocks of *O. reclinata*. Diced callus (3 g) was used to inoculate MS medium (MURASHIGE & SKOOG, 1962) supplemented with 0.5 mg ℓ^{-1} BA, 5 mg ℓ^{-1} NAA, 30 g sucrose and 0.1 g ℓ^{-1} myo-inositol without the addition of agar at pH 5.7. This medium was similar to that used for solid culture, except that the agar component was omitted. The suspension cultures were initiated in 500 mℓ sterile flasks containing 100 mℓ of autoclaved liquid medium, which were sealed with a cotton wool bung and covered with a tinfoil cap. They were placed on a rotary shaker and shaken at 120 revolutions per minute (rpm). Cultures were grown at 22°C in continuous cool white fluorescent light (24.5 μmol photons $m^{-2} s^{-1}$). Once the cultures were established, subculturing was performed as required by the white lines (3 - 4 weeks) and red lines (1 - 2 weeks).

4.2.2 Data collection for cell growth studies

Cell growth curves were determined for both types of callus lines. The settled cell volume (SCV) was used to measure growth. This value represents the proportion of cell aggregates settled out after 10 minutes in the side-arm of culture vessels. Side-arm flasks (500 mℓ) were suspended so that the angle of the flask arm was perpendicular for a period of five minutes in order to settle

cells at the base of the arm after a ten-minute period. Cell growth was recorded as described on a daily basis at the same time.

4.2.3 Analysis of data

Each experiment was composed of five replicates (flasks) for each callus line and experiments were repeated three times. Data were subjected to a one-way ANOVA using the Statgraphics statistical programme.

4.2.4 Anatomical studies

Once suspension cultures were established and growth curves were determined for both callus lines. Light microscopy (Olympus BH-2 photomicroscope) was used to conduct investigations on the anatomical differences, if any, of the anthocyanin-rich and anthocyanin-free cells. A pasteur pipette volume of the suspension culture was aseptically removed from red and white culture flasks at different stages of the growth cycle and a drop of the suspension viewed microscopically (Olympus BH-2 photomicroscope).

4.3 RESULTS

The establishment of 'single' cell suspension cultures was achieved with ease for the white callus. The friable nature of this callus facilitated fragmentation and dispersion of cellular aggregates throughout the liquid medium. Establishment of 'single' cell suspension of the red type proved to be slightly more difficult as calli tended to grow as hard, globular clumps. Dicing of the red calli resulted in better separation of cells. Microscopic examination of the 'single' cells and cellular aggregates showed that the suspensions were composed of mainly two cell types, elongated and circular cells. The white suspension cultures were composed mainly of circular cells (Figure 4.2A.), whereas the red callus had a high proportion of elongated cells (Figure 4.2B). Red cells had large pigmented vacuoles which extended throughout the cells. This is in accordance with previous findings, where the accumulation of

anthocyanin in vacuoles has been documented (NOZUE, KUBO, NISHIMURA, KATOU, HATTORI, USUDA, NAGATA & YASUDA, 1993). The white cells showed no pigmentation and vacuoles were small.

The settled cell volume (SCV) was utilized to determine cellular growth of red and white callus cells in suspension. This value is a time dependent measurement of growth. It represents the proportion of the culture occupied by single cells and cell aggregates settled out after 10 minutes in side-arm flasks (Figure 4.1). Suspension cultures were established using low amounts of initial inoculum (5-10% SCV). A typical sigmoidal curve was obtained for both red and white callus (Figure 4.3). This growth pattern is representative of most liquid cultures (DODDS & ROBERTS, 1985).

Light microscopic analysis was achieved by removing a pasteur pipette volume from the white and red cell culture at different stages of the growth cycle. This showed that, the white cells were circular or pear-shaped (Figure 4.2A).

The red culture was composed of mainly elongated cells. At log phase, both the white and red cells were actively dividing (Figure 4.2A and 4.2B). With the onset of the stationary phase, the white cultures started to turn pink in colour (Figure 4.2C) and proliferation of anthocyanin-synthesizing cells began. At this stage, lengthening of the cells occurred and they took on an elongated appearance. At the onset of the death phase, the red cultures started to brown and cellular aggregates showing signs of necrosis were dispersed amongst still viable red cells (Figure 4.2D). Rapid cell death was observed with the red cultures after 27 days of growth.

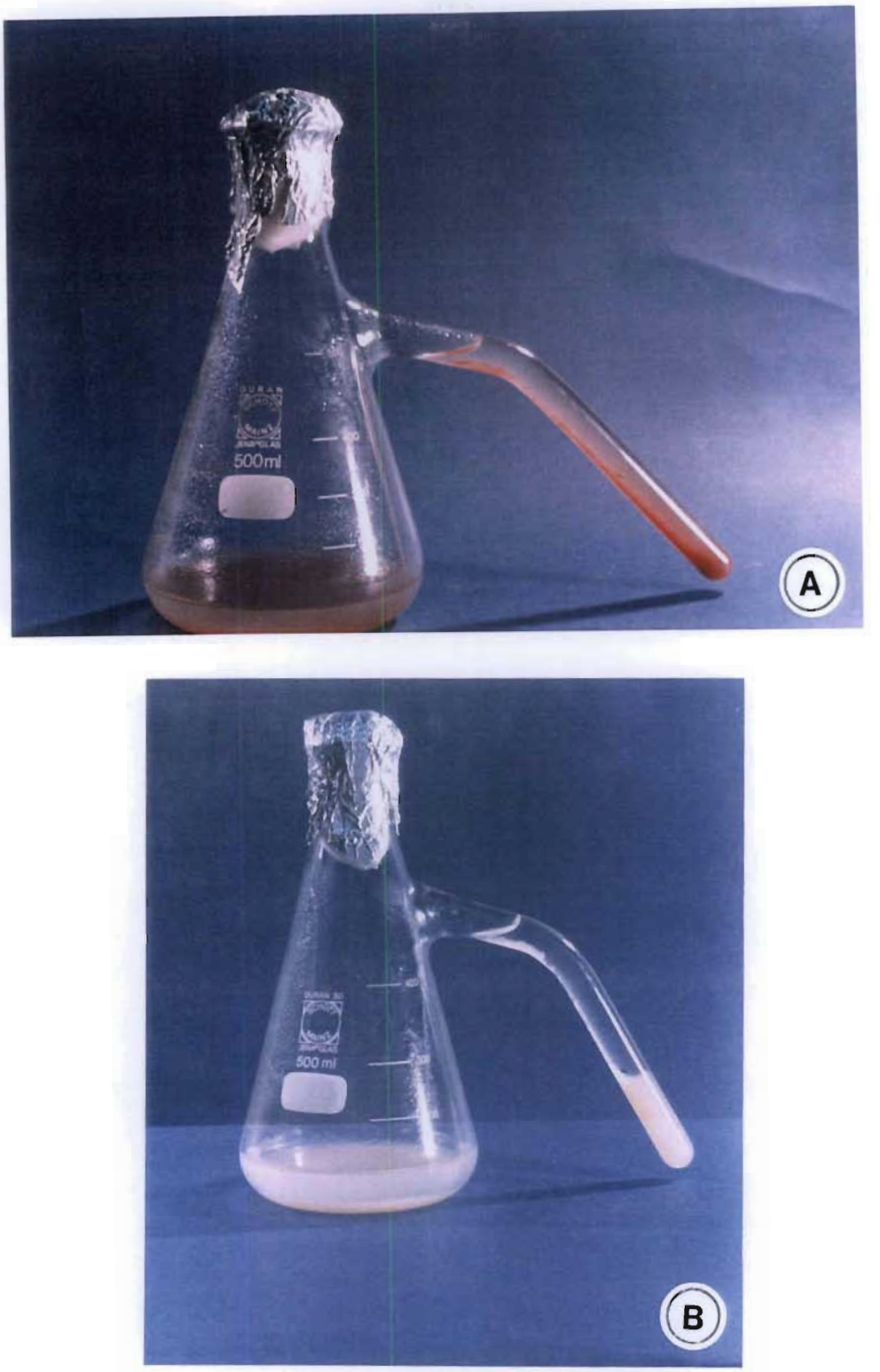


Figure 4.1: Liquid suspension cultures were established for the homogenous red and white callus lines of *O. reclinata* in sterile flasks. (A) Suspension culture of cells containing red anthocyanin pigment. (B) Suspension culture of non-pigmented cells

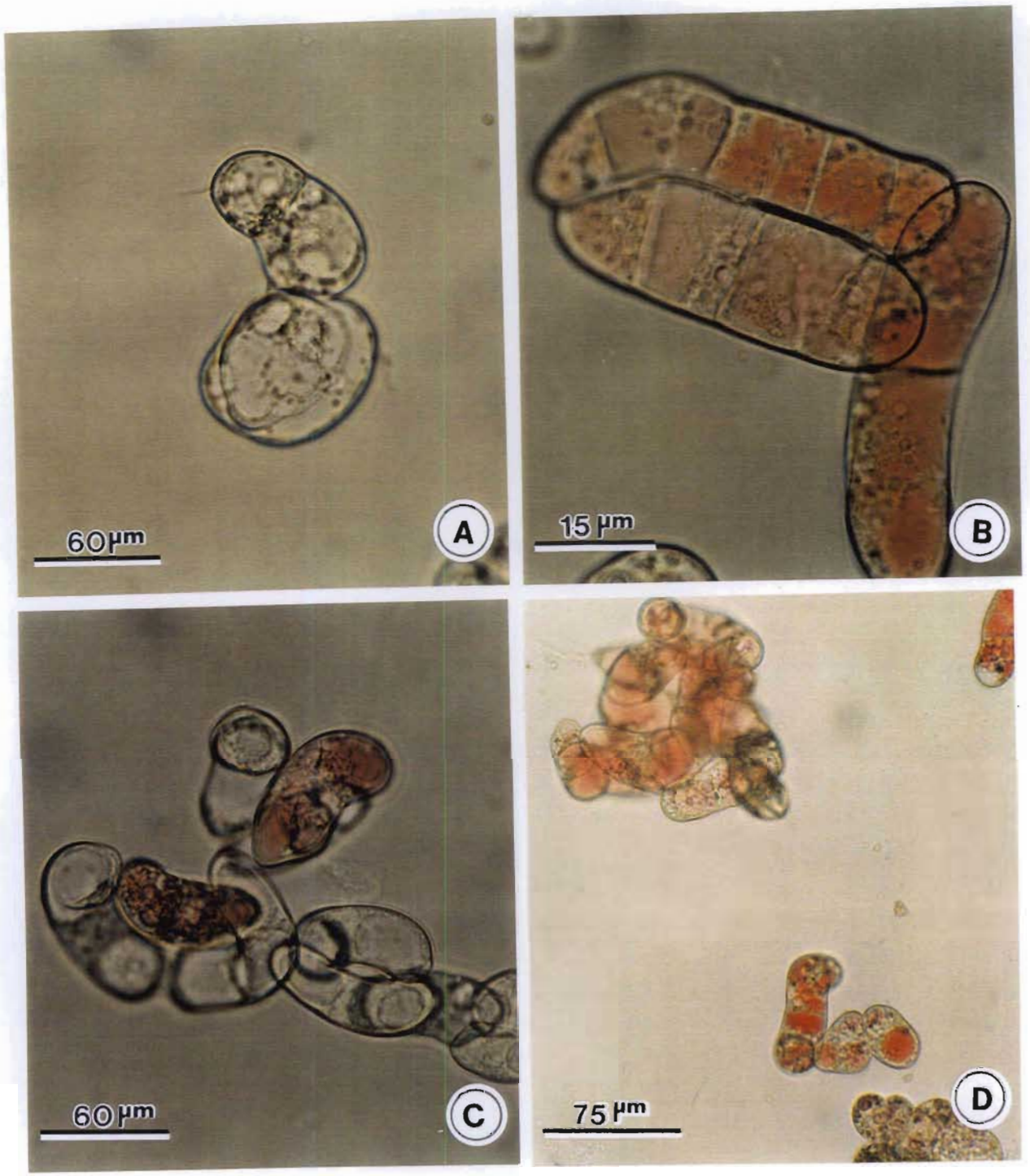


Figure 4.2: Cells isolated from suspension cultures of *O. reclinata* as viewed from a light microscope. (A) Cells from white callus were circular and had small vacuoles. (B) Elongated red cells had large vacuoles. (C) White cells accumulated red pigment towards the stationary phase of the growth cycle. A more heterogenous culture was formed at this time. (D) Browning of individual cells associated with the end of the growth cycle

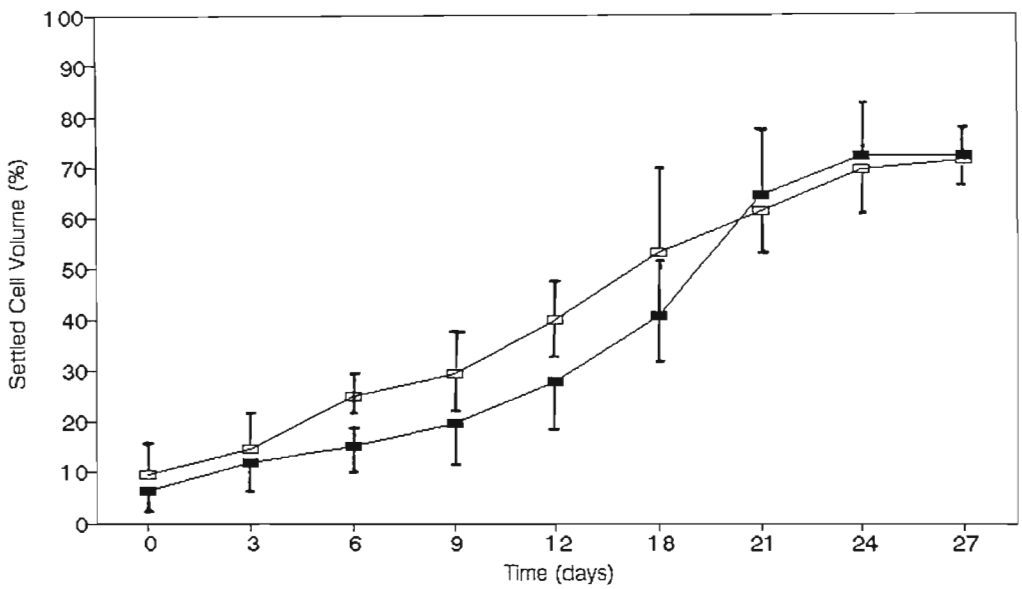


Figure 4.3: The growth curves for white (□) and red (■) cells of *O. reclinata* showing typical sigmoidal growth of liquid suspension cultures

4.4 DISCUSSION

Oxalis reclinata white and red callus responded favourably to the transfer from solid medium to liquid medium. Establishment of 'good' or 'ideal' suspension cultures was achieved for both red and white callus. According to DODDS & ROBERTS (1985) 'good' suspension cultures are those that contain a high number of single cells and small cell clusters. Both red and white cell suspension cultures had a high number of single cells. However, the red cultures had a higher percentage of clustered cells when compared with the white callus (Figure 4.3).

Initiation of suspension cultures requires a large amount of inoculum (DODDS & ROBERTS, 1985). The initial inoculum to establish cell suspension cultures for *Oxalis* was 30% (v/v). The growth cycle of these cultures was short and they required constant subculturing. Using pre-conditioned cultures, lower levels of inoculum were required to determine the growth response of red and white cells of *Oxalis* in suspension media. Sigmoidal growth curves constructed from the SCV for both red and white cell types (Figure 4.2) showed that, the white callus had a shorter lag phase compared to the red cells. The lag phase was a three day period for the white cells, whereas exponential growth for the red callus began after six days. The lag phase represents the stage where no apparent cell division occurs. Cell growth is characterized by a growth in size rather than in cell number through division. An exponential rise in cell number occurs after a lag period. Linear growth, representing an increase in the cell population for both red and white cell types was evident from day nine to 18 after subculturing. Levelling off of the growth curve (indicative of the stationary phase) was noted after 18 days for the white suspension cultures. The red cells grew for a longer period as they only reached the stationary phase after 21 days. The red and white cells had reached the same cell density (approximately 55% SCV) before the white cells went into the stationary phase. The red cells in suspension proceeded to accumulate in number and the cell density increased to 65% SCV. On solidified medium, the white callus

generated greater amounts of biomass compared the red callus. This may be due to red callus being inhibited by contaminants which are released by the agar into the culture environment as well as phenolic compounds which are secreted from the callus towards the end of the growth phase. Accumulation of such contaminants has been reported in agar-solidified media. In a liquid medium such problems are not encountered (DODDS & ROBERTS, 1985).

Light microscopic analysis of the red and white cells showed that anthocyanin accumulation occurs within the vacuole. The vacuole has previously been shown to be the main organelle for accumulation of many flavonoid compounds. Intensely pigmented vacuolar structures termed 'anthocyanoplasts' have been observed using light and electron microscopy. Their function and their mechanism of formation are not yet known (NOZUE, KUBO, NISHIMURA, KATOU, HATTORI, USUDA, NAGATA & YASUDA, 1993).

Expression of secondary metabolism is closely related with cell growth and differentiation. In cultured cells, maximal accumulation of secondary metabolites is observed during stationary phase. Therefore, accumulation of anthocyanin coincides with cessation of cell growth (KAKEGAWA, SUDA, SUGIYAMA & KOMAMINE (1995). White cells of *O. reclinata* were circular and accumulated little or no pigment (Figure 4.2B) during the lag or log phases. Coloured cells were observed at the stationary phase when cell division was reduced. KAKEGAWA, SUDA, SUGIYAMA & KOMAMINE (1995) suggested that high levels of phenylalanine accumulated as cell division declined in grape suspension cultures. Phenylalanine may act as a signal promoting transcription of anthocyanin biosynthetic genes.

Establishment of suspension cultures was achieved with ease for *O. reclinata*. Therefore, liquid cultures are a viable means of propagating red pigmented cells of *O. reclinata* for use in the food industry.

CHAPTER 5

PROTEIN STUDIES ON ANTHOCYANIN PRODUCTION

5.1 INTRODUCTION

In the past, analysis of molecular events resulting from changes made to increase secondary product formation, was often limited to measurements of end product accumulation. End product based studies do not reveal the total attainable capacity for secondary metabolite production. This approach is undoubtably valuable for preliminary optimisation of plant tissue culture conditions, but it overlooks endogenous regulatory controlling mechanisms of secondary metabolism. Therefore, it is necessary to ascertain the factors that control enzymic activity both *in vitro* and *in vivo*. It is important to elucidate the effect of positive and negative effectors on transcription, translation and post-translational modification (DIXON & BOLWELL, 1986).

The two molecular techniques employed to analyze results obtained during the tissue culture of *O. reclinata* were:

1) Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of proteins, and

2) *In vitro* translation.

The use of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is unmatched in its ability to resolve proteins and polypeptides in complex protein mixtures (O'FARRELL, 1975). The method based on that of O'FARRELL (1975) using denaturing conditions increases protein resolution capacity. Its

adaptability to a wide variety of samples with differing solubility properties is commendable when compared to previously attempted separations under non-denaturing conditions. Two-dimensional electrophoresis under non-denaturing conditions is limited to the analysis of soluble proteins (DUNN & PATEL, 1987). Two-dimensional electrophoresis has become one of the most powerful tools for the separation and quantification of proteins from complex mixtures. It employs separation of proteins according to two different parameters: isoelectric point and molecular weight (POLLARD, 1984). The method first involves the separation of proteins according to their isoelectric points, by isoelectric focusing in a first dimensional tube gel. This is followed by a separation according to protein molecular weight in a second dimensional gel using a sodium dodecyl sulphate (SDS) slab gel. The power of the method arises from the combination of high resolution (first gel) with a high separation capacity (second gel). The method is relatively fast and easy to carry out once the technique is optimised (BAUW, VAN MONTAGU & INZE, 1992). It has been used extensively to study proteins whose expression is changed by external stimuli or which are developmentally regulated. The technique allows for several gene products to be shown simultaneously. For this reason, the technique is effective in studying changes in genome expression (BAUW, VAN MONTAGU & INZE, 1992).

This method has been widely used in the analysis of proteins from bacteria, several animal systems, insects, nematodes and fungi. The use of 2D-PAGE, in the past, has often been restricted to non-pigmented plant tissues such as seeds and hypocotyls. Pigmented tissue extracts pose several problems because they contain phenolic compounds which can interact with proteins and nucleic acids and change their characteristics. Electrophoresis of proteins from extracts that are not treated to remove the pigments and other phenolic compounds give unreliable results (HARI, 1981).

Phenolic compounds interact with proteins to form insoluble complexes which interfere with conventional assays for proteins. Proanthocyanidin-protein

interactions result in insoluble complexes being formed. These interactions result in a irreversible precipitation step of the insoluble complex during protein extraction procedures (HAGERMAN & BUTLER, 1981). According to LOOMIS (1974) the reaction of phenolics with proteins falls into four main classes:

1) Hydrogen bonding: isolated phenolic hydroxyl groups form very strong hydrogen bonds with the oxygen atoms of peptide bonds. This is one of the strongest hydrogen bonds known, and cannot be broken by conventional techniques, namely, dialysis or gel filtration;

2) Browning (oxidation) reactions in plant tissues and extracts is principally caused by quinone oxidation which is then followed by covalent coupling reactions or protein functional groups being oxidised by the quinone. Quinones are known to be powerful oxidising agents. They have a tendency to polymerise and condense readily with reactive groups of proteins through -SH and $-NH_2$ groups;

3) Ionic interactions: phenolic hydroxyl groups in general have pKa values of 8.45 or higher. At higher pH's they may form salt linkages with the basic amino acid residues of proteins. Plant phenolic compounds of the phenylpropanoid group contain carboxyl groups as well, and may be negatively charged even at a neutral pH or below; and

4) Hydrophobic interactions: phenolic compounds possess hydrophobic aromatic ring structures which have an affinity for other hydrophobic compounds. Phenolic compounds may interact with hydrophobic regions of proteins.

When isolating proteins from plant tissues that are rich in phenolic compounds and other secondary products, removal of these compounds before formation of covalent complexes is necessary. This is best accomplished by the addition

of adsorbents or protective agents that compete with reactive phenolics. Prevention of phenolic oxidation must be maintained simultaneously.

Polyphenols may be removed from crude plant extracts by complexing with polyvinylpyrrolidone (PVP) or at times by gel filtration. The insoluble form of PVP, termed polyvinlypoly pyrrolidone (PVPP) may be added to the extraction buffer and hydrated. The phenolic adsorbent, PVPP, (1.5% [w/v]) is usually used to inactivate polyphenols (GEGENHEIMER, 1990). Polyvinylpyrrolidone bears some structural resemblance to proline. Proline has a pyrrolidine ring which resembles the heterocyclic vinyl pyrrolidine subunits of PVP. Proteins with high proline contents have been shown to have a higher affinity for flavonoid compounds (HAGERMAN & BUTLER, 1981). Polyvinlypoly pyrrolidone has strong binding ability to polyphenolics through its CO-N= group. The phenolic adsorbent (PVPP) has been shown to bind compounds with free aromatic hydroxyl groups and as the number of aromatic groups increases, PVPP binding capacity increases (WANG & VODKIN, 1994). For plant tissue extracts with high levels of procyanidins and other phenolic substances, addition of other polyphenol-binding agents (0.2 M sodium tetraborate) or antioxidants to inhibit phenol oxidase activity, such as 0.25 M sodium carbamate, 0.02 M sodium metabisulfite, may prevent formation of insoluble protein-phenolic complexes (GEGENHEIMER, 1990).

Secondary phenolic compounds can interact with RNA and DNA. Therefore, it is necessary to inactivate polyphenolics or to prevent interactions of phenolics with nucleic acids. TODD & VODKIN (1993) showed the ability of a dihydroxylated proanthocyanidin to bind to both proteins and RNA. Association of phenolics with nucleic acids and proteins results in changed electrophoretic mobility and a changed absorption spectra in these compounds (WANG & VODKIN, 1994).

Polypeptide differences between red and white tissue callus cultures of *O. reclinata* were investigated using two-dimensional electrophoresis. This study

was undertaken with the hope of acquiring greater insight into the molecular mechanisms involved in the control of anthocyanin biosynthesis.

5.2 MATERIALS AND METHODS

5.2.1 Reagents

All chemicals used were purchased from BDH Chemicals Ltd, Poole England; Oxoid Unipath Ltd, England; Unilab, Saarchem, Ltd, South Africa; Boehringer Mannheim, Johannesburg, South Africa; Merck Darmdstadt, Germany; Sigma Chemical Co., U.S.A. and Kleber Chemicals Ltd, South Africa. Ampholytes were purchased from Pharmacia for iso-electric focusing.

5.2.2 Plant material

Oxalis reclinata anthocyanin-containing callus and non-pigmented callus were generated using the methods described by CROUCH, VAN STADEN, VAN STADEN, DREWES & MEYER (1993). The two main callus lines (light-grown red and dark-grown white callus, simply referred to as red and white) were maintained on MURASHIGE & SKOOG (1962) medium (MS) (0.8% agar [w/v]; pH 5.8) supplemented with 5 mg ℓ^{-1} NAA, 0.5 mg ℓ^{-1} BA, 30 g ℓ^{-1} sucrose and 0.1 g ℓ^{-1} myo-inositol. Stocks were subcultured at four-weekly intervals.

5.2.3 Protein isolation

Four methods were used in an attempt to isolate intact proteins from pigmented *Oxalis* tissues. Protein was isolated from the four callus types generated (Section 3.2.1). One gram of callus was ground to a fine powder with liquid N_2 in a pre-cooled mortar and pestle. Proteins were extracted with 4 mℓ of 200 mM potassium phosphate buffer (KPi, pH 6.8) containing 2 mM 2-merçaptoethanol and 40 mM ascorbic acid. The protease inhibitors included in the extraction buffer were 1 mM ethylene diamine tetra-acetic acid (EDTA) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The samples were centrifuged

at 10 000 rpm (SS-34 rotor head, Sorvall RC-5 Superspeed Centrifuge) for 20 minutes. The supernatant was collected and proteins were precipitated overnight at -20°C with an excess amount of pre-chilled acetone. The proteins were recovered by centrifugation at 12 000 rpm for 10 minutes in a microfuge (Sigma-113). The Biorad Protein Assay modified from the original BRADFORD (1976) assay was used to quantify total protein.

No measurable amounts of protein were recovered with this method from the red callus. A second method was attempted and proteins were extracted from *O. reclinata* callus with a buffer containing 200 mM potassium phosphate (pH 6.8), 50 mM NaCl, 1 mM PMSF, 1 mM EDTA, and 14 mM 2-merçaptoethanol. The buffer to sample ratio was 1 : 1 (v/w). The extracts were incubated with 2 mg $m\ell^{-1}$ protamine sulfate for 10 minutes at room temperature on an orbital gyratory shaker and were shaken gently at 80 rpm at room temperature. Samples were then centrifuged for 20 minutes at 10 000 rpm (SS-34 rotor head, RC-5 Sorvall Centrifuge) to remove cell debris. The pellets were discarded and the supernatants were chromatographed through a Sephadex G-25 column to monitor colour and protein separation (GLEITZ & SEITZ, 1989). Fractions of 500 $\mu\ell$ were collected and spectrophotometrical readings then taken at 260 and 280 nm. The fractions were collectively assayed using the Biorad Protein Assay modified from BRADFORD (1976). Extracted proteins were electrophoresed on polyacrylamide gels (Section 5.2.5).

A third method was also used for protein extraction. Callus (1 g) was ground in 0.1 g PVPP in the presence of liquid N_2 to a fine powder and homogenised in 4 $m\ell$ extraction buffer (0.1 M potassium phosphate, pH 6.8), 1.4 mM 2-merçaptoethanol, 40 mM ascorbic acid, 3 mM EDTA, 0.2 M PMSF and 10% glycerol (v/v). Protamine sulfate (2 mg $m\ell^{-1}$) was added and the samples were shaken gently for 10 minutes at room temperature followed by 20 minutes centrifugation at 14 000 rpm (SS-34 rotor head, RC-5 Sorvall Centrifuge). Proteins were measured using the Biorad Protein Assay with modifications to the BRADFORD (1976) method.

Proteins were successfully isolated from red and white calls of *O. reclinata* when the effect of proanthocyanidins on proteins was minimized The phenolic adsorbent, PVPP (1.5% [w/v]) was incubated overnight in extraction buffer at 4°C. The extraction buffer (0.1 M potassium phosphate [pH 6.8]), 1.4 mM 2-merçaptoethanol, 40 mM ascorbic acid, 3 mM EDTA, 0.2 M PMSF and 10% glycerol [v/v]) was modified to include sodium salts, namely, 20 mM sodium diethyl-dithiocarbamate, 20 mM sodium metabisulphite, and 200 mM sodium tetraborate. These sodium salts were included to adsorb phenolic compounds and to inhibit phenol oxidase activity. Callus (1 g) from light-grown red callus and dark-grown white callus was extracted by grinding to a fine powder in liquid N_2 in a pre-cooled mortar and pestle. Five millimolar dithiothreitol (DTT) and 1.4 mM merçaptoethanol were compared as reductants. Due to DTT proving to be a better reducing agent, all subsequent protein isolations were performed using 5 mM DTT.

5.2.4 The effect of anthocyanins on proteins

An experiment was conducted to determine the effect of anthocyanins on the isolation of total protein. At first, proteins were extracted from dark-grown white callus, dark-grown red callus, light-grown white callus and light-grown red callus using 4 mℓ extraction buffer (0.1 M potassium phosphate [pH 6.8], 1.4 mM 2-merçaptoethanol, 40 mM ascorbic acid, 3 mM EDTA, 0.2 M PMSF and 10% glycerol [v/v]). Secondly, proteins were extracted from a mixture of light-grown red callus and dark-grown white callus (1:1; w/w). Bovine serum albumin (10 mg) and 1 g of light-grown red callus were placed in a mortar and pestle, and proteins isolated from this mixture. Protein extractions in the presence of 1.5% PVPP were also performed from dark-grown white and light-grown red callus types. Extracted proteins were quantified and electrophoresed using a one-dimensional polyacrylamide gel.

5.2.5 Polyacrylamide gel electrophoresis of proteins

Protein isolated using the four methods outlined above was electrophoresed on polyacrylamide gels. One-dimensional gel electrophoresis and two-dimensional gel electrophoresis were utilized to determine the efficiency of the different isolation methods employed.

Following quantification of proteins using a standard curve, 10 μg of protein was electrophoresed on a one-dimensional polyacrylamide gel consisting of a stacking gel (pH 6.8) and a separating gel (pH 8.8) according to the basic method described by LAEMMLI (1970). A stock mixture composed of acrylamide and N'N'methylenebisacrylamide (bisacrylamide) (30 : 0.8 w/v respectively) was made and stored at 4°C for a period of no longer than 30 days. A 12% polyacrylamide running gel was prepared by making a gel solution composed of 18 mℓ acrylamide stock, 11.75 mℓ 1.5 M Tris (pH 8.8) with 40% SDS and 15.25 mℓ deionized H_2O. The gel was degassed using a vacuum pump, and polymerization of the gel solution was initiated by the addition of 75 $\mu\ell$ 20% ammonia persulfate (APS) and 30 $\mu\ell$ NNN'N'tetra methylethylenediame (Temed). The gel was poured between two clean glass plates and left to polymerize for an hour after the addition of an overlay of water-saturated butanol. Once polymerization had taken place, the overlay solution was removed and the top of the gel was rinsed with 0.5 M Tris buffer (pH 6.8) and the stacking gel was poured. A comb was placed so that loading wells could form. The 6% stacking gel was made from a degassed gel solution composed of 1.95 mℓ acrylamide stock, 3.75 mℓ 0.5m Tris (pH 6.8) containing 40% SDS and 9.15 mℓ dH_2O polymerized by the addition of 30 $\mu\ell$ 20% ammonium persulfate (APS) and 15 $\mu\ell$ NNN'N'tetramethylethylenediamine (Temed). Protein samples stored at -20°C were thawed and 10 μg protein was denatured by boiling for 10 minutes in sample buffer containing 2.5% SDS (w/v), 2% 2-merçaptoethanol (v/v), 10% glycerol (v/v) and 0.1% bromophenol blue (w/v) as the tracker dye. Samples were loaded in the wells and electrophoresed at 15 mA through the stacker gel,

and at 30 mA through the separating gel until the bromophenol blue marker dye had reached the end of the gel. A running buffer (5x normal strength) consisting of 7.2 g glycine, 1.5 g Tris and 0.5 g SDS dissolved in 100 mℓ was diluted to a 1x solution prior to electrophoresis.

5.2.6 Two-dimensional electrophoresis

Two-dimensional electrophoresis was carried out according to O'FARRELL (1975) using the modifications made to the basic O'FARRELL method by MAYER, HAHNE, PALME & SCHELL (1987).

Glass tubes were cleaned by soaking in chromic acid for three hours, rinsed with 2 g KOH made up in 95% ethanol, followed by several rinses in distilled water (dH_2O). The glass tubes were then dried thoroughly in a drying oven prior pouring of polyacrylamide gels.

Iso-electric focusing (IEF) gels (4.2%) were prepared with acrylamide / N'N'methylenebisacrylamide stock (29.16 : 1.33 [w/v]), 5% ampholytes (Pharmacia, 3-10), 4% nonidet P-40 (NP-40) and 9.0 M urea. The gel solution was filtered through a 0.22 μm millipore filter and polymerization was initiated with the addition of 2 $\mu\ell$ 20% APS $m\ell^{-1}$. The gel solution was poured into the IEF glass tubes and overlaid with water. Temed was not essential for the polymerization reaction but 1 $\mu\ell$ Temed was utilized on occasion to increase the polymerization rate of the gel. Degassing of the gel prior addition of APS was not necessary after filtering. All solutions for IEF reagents were made with redistilled deionized H_2O.

To prepare extracted proteins for IEF, urea was added to a concentration of 9.0 M, and 4% NP-40 (v/v) was added to the extracts. Alternatively, precipitated proteins were solubilized in IEF sample buffer of 9.0 M urea and 4% NP-40. Protein (10 to 25 μg) was routinely applied to the basic end of the IEF gels. Once the gels were loaded, IEF was initiated. Focusing of proteins was

performed overnight. The voltage was increased in steps from 200 V (20 minutes), 300 V (20 minutes) and 400 V until a total of 20 000 V h was achieved. The cathode and anode electrolytes were 0.1 M NaOH and 0.11 M H_3PO_4 respectively. The focused gels were equilibrated for three minutes in equilibration buffer (0.5 M Tris-Cl [pH6.8] 2.5% SDS [w/v], 0.1% DTT [w/v] and 10% glycerol [v/v]) prior storage at -70°C or subjection to the second dimension. The basic end of the gel was marked by injection of equilibration buffer containing 0.025% bromophenol blue in the glass rods containing the gel before extrusion of the IEF gel by gentle pressure.

Two-dimensional polyacrylamide gel electrophoresis was conducted using basically the same method as described for one dimensional gel electrophoresis with modifications. If IEF gels were frozen, they were subsequently thawed at room temperature in equilibration buffer and then subjected to electrophoresis. The acrylamide gel solutions and conditions for gel polymerization were maintained as previously described for one-dimensional electrophoresis. The electrophoretic conditions were 25 mA until the marker dye (bromophenol blue) reached the end of the stacking gel, whereafter it was increased to 45 mA in the running gel. The gels were run at room temperature until the tracker dye had reached the end of the running gel. Following electrophoresis, the gels were subjected to silver staining (MORRISSEY, 1981). Once the extraction method for anthocyanin-rich callus was established, protein molecular weight markers (Amersham) were run next to the IEF gels.

5.2.7 Detection of electrophoresed proteins

To detect electrophoresed proteins, one-dimensional and two-dimensional polyacrylamide gels were subjected to silver staining (MORRISSEY, 1981). Firstly, the gels were prefixed for 30 minutes in an acidic-alcoholic solution of 50% methanol - 10% acetic acid, followed by a second pre-fixation step in 5% methanol - 7% acetic acid for 30 minutes. The proteins were then fixed to the

gels by washing in 10% glutaraladehyde for 30 minutes. The gels were then washed in several changes of deionised dH_2O for three hours or left to wash overnight with gentle shaking in deionised dH_2O. Following the water wash, gels were subjected to 25 mgℓ^{-1} DTT for 30 minutes and silver stained for 30 minutes with 0.1% $AgNO_3$. After silver staining, gels were rinsed for 30 seconds in deionised water. The silver staining was developed by soaking gels in 3% sodium carbonate containing 5 $\mu\ell$ 37% formaldehyde solution. Citric acid (2.3 M) was used to stop the developing reaction after 10 minutes. Polypeptide patterns on gels were photographed using 100 ASA Pan F black and white film.

5.3 RESULTS

The amount of total protein isolated from the different callus types using the four attempted methods (Section 5.2.3) were compared (Table 5.1). The best method to isolate proteins from anthocyanin containing tissue was the fourth method attempted.

Comparing the amount of protein isolated from light-grown red callus and dark-grown white callus, lower protein yields were observed with the pigmented red callus (Table 5.1). It was thought that one of the enzymatic differences between the red and white callus might be the presence of a larger pool of proteases in the pigmented callus. An experiment to test the effect of protease inhibitors was conducted. For all the callus types, less protein was isolated in the absence of inhibitors. In the presence of one inhibitor (EDTA), isolated protein for the red callus increased approximately four-fold. An increase in the amount of protease inhibitors in the extraction buffer, increased the amount of protein isolated. Three protease inhibitors were included in the extraction buffer for subsequent experiments, namely, EDTA and PMSF. Total protein isolated from the red and white callus using method number one (Section 5.2.3), were visualized by silver staining after electrophoresis. No proteins were seen on silver stained gels for the red callus (Figure 5.1B).

Table 5.1: The effect of extraction buffer components on protein yields ($\mu g\ g^{-1}$ fresh weight) isolated from *O. reclinata* callus

Extraction Method	Light Grown Red Callus ($\mu g\ g^{-1}$ FW)	Dark Grown White Callus ($\mu g\ g^{-1}$ FW)
Extraction buffer (EB)*	18.69	49.80
EB + EDTA	61.00	67.78
EB + protamine sulfate	43.93	54.98
EB + EDTA + protamine sulfate	87.60	93.38
EB + EDTA + protamine sulfate + MEC (sephadex column)	15.8	88.00
EB + EDTA + protamine sulfate + PVPP	66.00	119.60
EB + EDTA + protamine + PVPP + sodium salts #	107.80	110.80
EB + EDTA + protamine + PVPP + sodium salts + DTT	110.30	172.10
EB + EDTA + protamine + PVPP + sodium salts + 2-merçaptoethanol	115.95	137.80

* Extraction buffer composed of 200 mM KPi (pH 6.8), 1.4 mM 2-merçaptoethanol, 50 mM NaCl and 1 mM PMSF.

\# sodium salts included were 20 mM sodium diethyl-dithiocarbamate, 20 mM sodium metabisulphite and 200 mM sodium tetraborate.

Proteins were successfully isolated from the white callus (Figure 5.1A).

GLEITZ & SEITZ, 1989, monitored the separation of colour and protein using Sephadex G25 columns before crude protein extracts, obtained from anthocyanin accumulating *D. carota* callus lines, were separated on IEF gels. This procedure was attempted with red and white cultures of *O. reclinata*. As previously noted, proteins were isolated successfully from the dark-grown white callus only (Figure 5.1A). For this callus line, better protein quantities were obtained when extracts were not passed through a column (Table 5.1).

Equal amounts of protein were loaded and electrophoresed on a one-dimensional SDS-PAGE gel (Figure 5.2). Clearly, anthocyanins interfered with the isolation of proteins from red callus lines (Figure 5.2, lanes LR and DR respectively). No distinct banding pattern was visualised after silver staining. By contrast, proteins were successfully isolated from white callus kept in the dark as intense bands were observed (Figure 5.2, lane labelled DW). This callus remains anthocyanin-free at all stages of the growth cycle. Light-grown white callus (Figure 5.2, lane labelled LW), on the other hand accumulated anthocyanins in small quantities and the presence of phenolics resulted in proteins being lost during the extraction procedure as only five bands were detected.

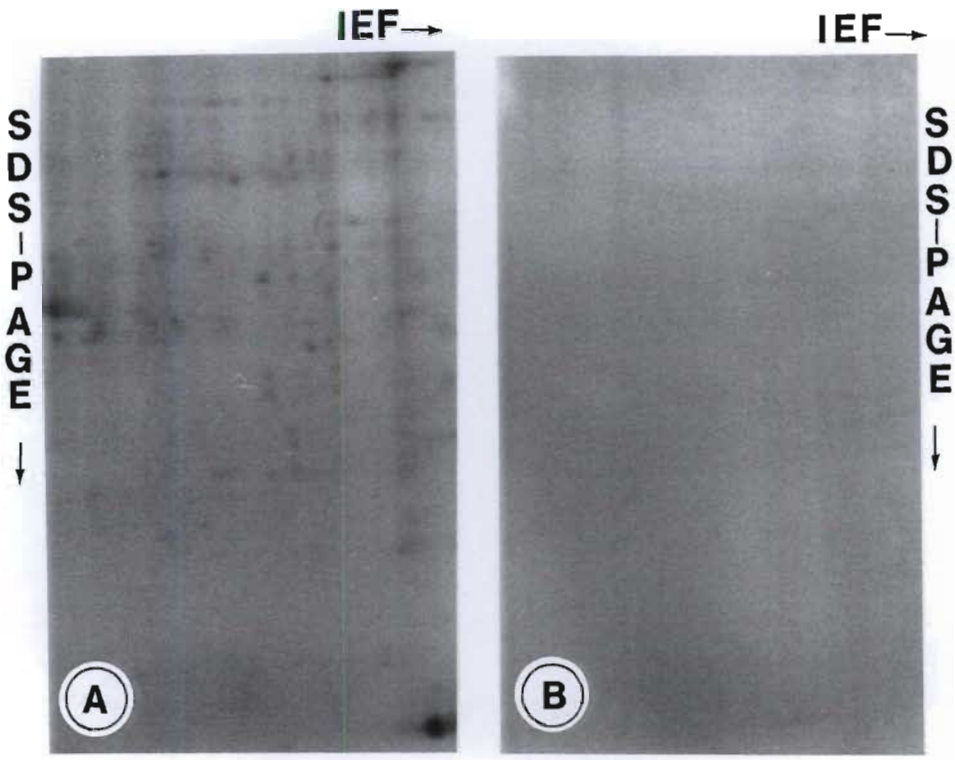


Figure 5.1: Two-dimensional gels of *O. reclinata* callus proteins stained by silver staining. (A) Polypeptide pattern of electrophoresed proteins isolated from white callus. (B) Proteins were not successfully isolated from the red callus. Black arrows indicate the direction of IEF and SDS-PAGE

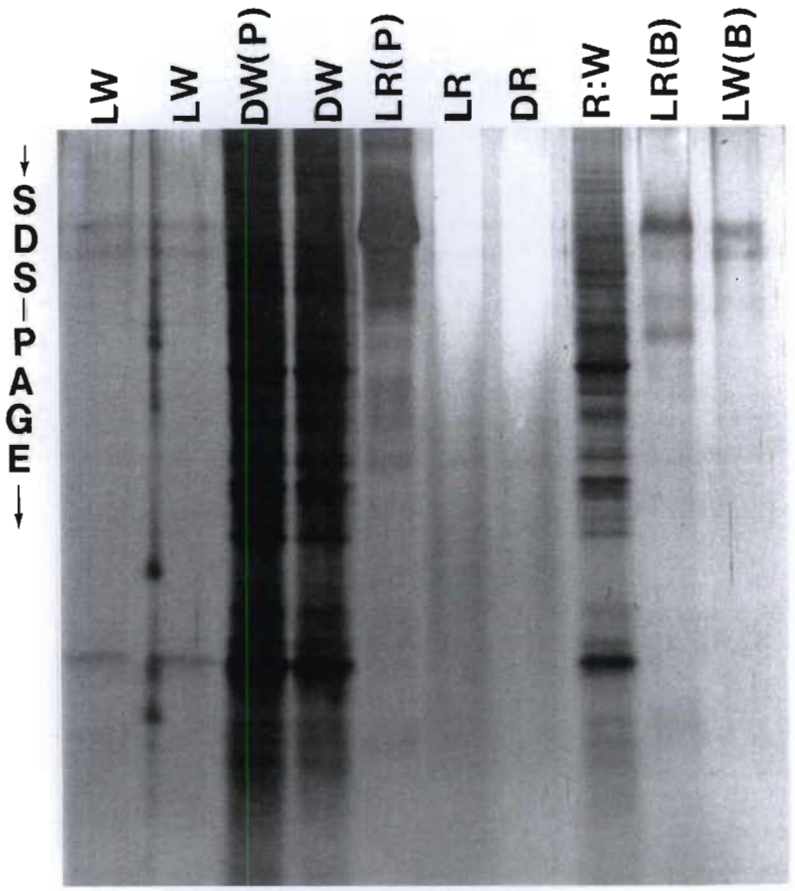


Figure 5.2: One-dimensional gel of *O. reclinata* proteins stained by silver staining. Proteins were recovered as described in Section 5.2.4. Key to Figure, LR, proteins extracted from light-grown red callus; LW, proteins extracted from light-grown white callus; DR, proteins extracted from dark-grown red callus; DW, proteins extracted from dark-grown white callus; LR(P), proteins extracted from light-grown white callus; DW(P), proteins extracted from dark-grown red callus; R:W, proteins extracted from a mixture of light-grown red callus and dark-grown white callus (1:1, w/w); LR(B), proteins extracted from light-grown red callus in the presence of 1% BSA and LW(B), proteins extracted from light-grown white callus in the presence of 1% BSA. Black arrows indicate the direction of SDS-PAGE

The inclusion of PVPP in the extraction improved the amount and the quality of proteins extracted from the red callus (Figure 5.2, lane designated LR (P)). However, PVPP did not stop all the interactions between proanthocyanidins and proteins occurring. To further elucidate the effect pigment had on protein extraction efficiency, proteins were isolated from a mixture of light-grown red and dark-grown white callus (1:1, w/w). The presence of pigment caused proanthocyanidin-protein complexes to form and these insoluble complexes were removed by centrifugation during extraction. More protein was isolated form the dark-grown white callus compared to the light-grown red and dark-grown white callus mixture (lane designated R:W). This indicated the binding capacity of proanthocyanidin to proteins.

Total protein isolated from red and white callus (lanes designated LR(P) and DW(P), respectively) were run on 2D gels. The buffer used to extract protein contained PVPP. As usual, dark-grown white proteins focused and ran on a second dimension polyacrylamide gel successfully (Figure 5.3A). Only one acidic protein polypeptide was detected on the 2D-PAGE gel in the light-grown red callus (Figure 5.3B).

Successful 2D gels were obtained when proteins were isolated in the presence of sodium salts which acted as phenol oxidase inhibitors and phenolic adsorbents (Figure 5.4). The effect of commonly used reducing agents was tested. Dithiothreitol (DTT) was found to be a better reductant and all subsequent extractions were performed in its presence. Samples extracted with 2-merçaptoethanol yielded fewer spots on the 2D gels (Figure 5.4B).

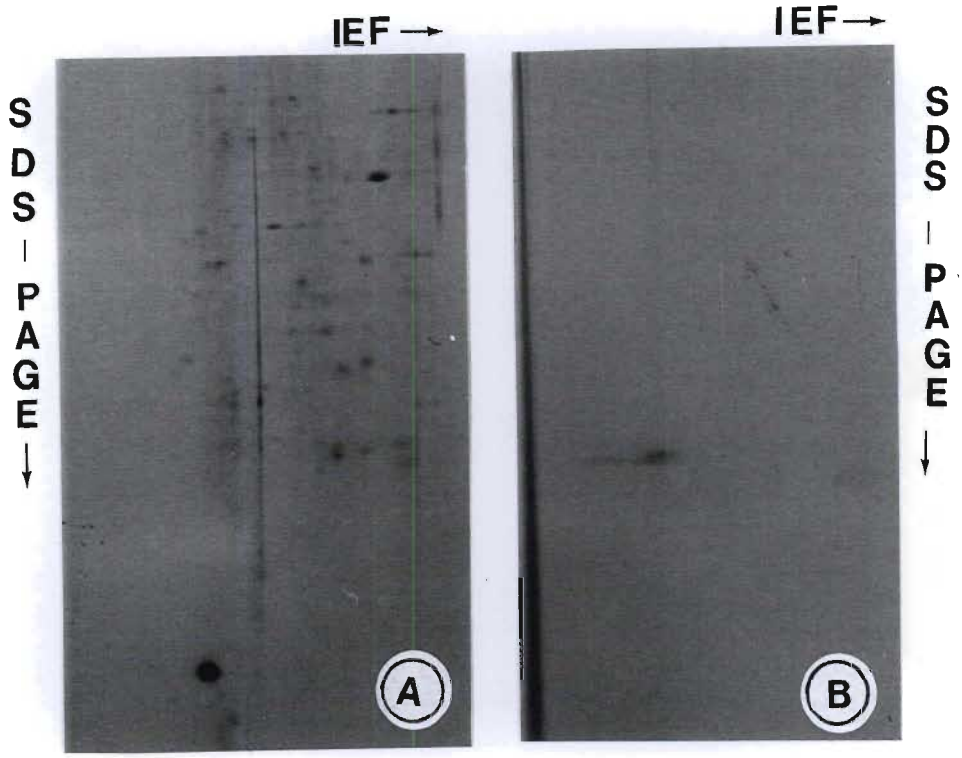


Figure 5.3: Two-dimensional gels of *O. reclinata* proteins stained by silver staining. (A) White callus proteins were isolated with extraction buffer containing protease inhibitors and the phenolic adsorbent, PVPP. (B) A basic polypeptide isolated from red callus. Black arrows indicate the direction of IEF and SDS-PAGE

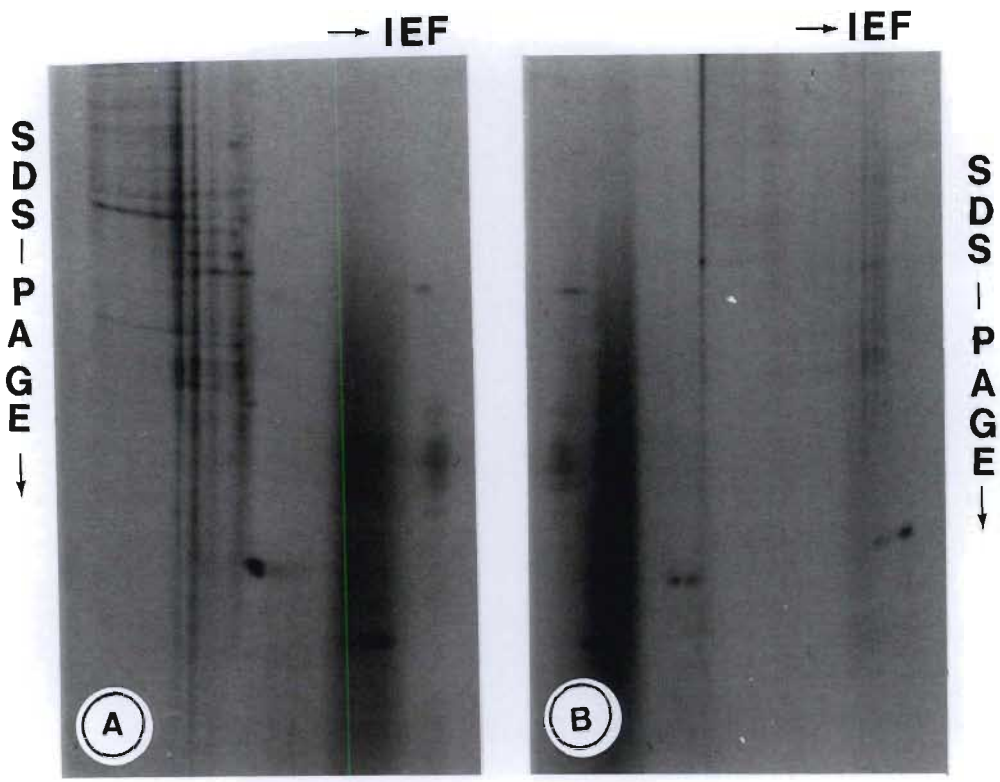


Figure 5.4: Two-dimensional gels of *O. reclinata* red callus proteins stained by silver staining. (A) The effect of DTT on isolation of proteins. (B) The effect of 2-merçaptoethanol as a reducing agent. Black arrows indicate the direction of IEF and SDS-PAGE

The differences in polypeptide composition between the white callus (DW) and the red callus (LR) are shown in Figure 5.5. There was an overall increase in polypeptide number of the red callus. More neutral to acidic proteins had accumulated in the red callus grown in the light (Figure 5.5B) as compared to the dark-grown white callus (Figure 5.5A). Synthesis of these more acidic proteins appeared to be associated with anthocyanin synthesis. The light-grown white callus showed an overall increase in neutral and acidic proteins (Figure 5.6A). This callus line was made of red and non-pigmented cells. It accumulated anthocyanin pigments (Section 3.3, Figure 3.6C)

A reduction in basic proteins was noted for the light-grown white callus. In Figure 5.5A, polypeptides 1-3 were recognized as being unique to the dark-grown white callus. Some polypeptides of the dark-grown white callus were markedly reduced in intensity when compared to light-grown red callus polypeptides (Figure 5.5, shown by black and white arrows). The dark-grown red callus was morphologically different from the other callus types. It had a tendency to be softer, mucilaginous and grey in colour. The protein composition of this callus appears to be dramatically different as compared to the other types (Figure 5.6B). A general loss in polypeptide number was noted. The complete disappearance of the intense (< 20 kilodalton [KDa]) polypeptide present in the other callus types was observed (Figure 5.5).

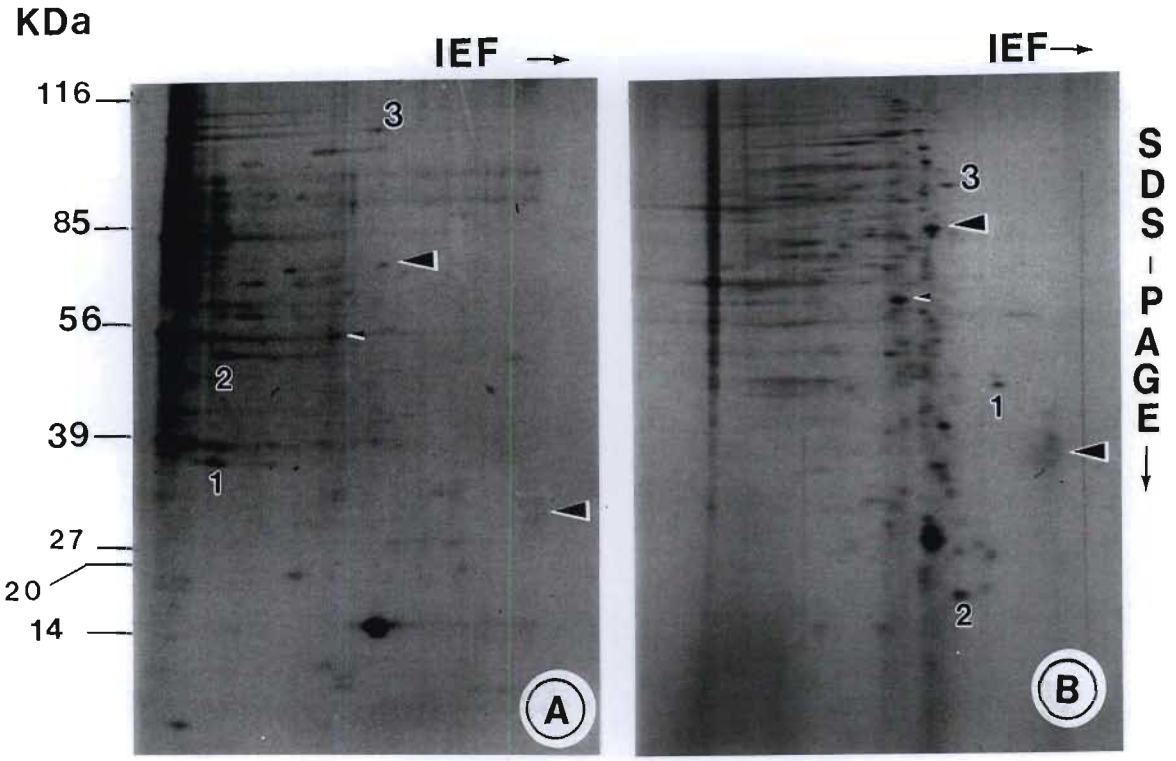


Figure 5.5: Two-dimensional gels of *O. reclinata* callus proteins visualised by silver staining. (A) Polypeptide patterns of proteins isolated from dark-grown white callus. (B) Polypeptide patterns of proteins isolated from light-grown red callus. Black arrows indicate the direction of IEF and SDS-PAGE

5.4 DISCUSSION

The use of two-dimensional electrophoresis to analyze polypeptide mixtures has often been restricted to unpigmented tissues in plant species (HARI, 1981). It was necessary to optimise the isolation methods for pigmented *Oxalis* tissues. Isolation of proteins from the light-grown red and dark-grown white callus lines of *O. reclinata* proved to be most successful when phenol oxidase inhibitors and phenolic adsorbents were included in the extraction buffer (Figures 5.5 and 5.6). When preventative measures were not taken to reduce or inhibit proanthocyanidins from coupling with proteins, little or no protein was isolated from anthocyanin-rich *O. reclinata* callus.

Difficulties experienced with the extraction of proteins from the red callus may be ascribed to the interference of proanthocyanidins present in the callus. These compounds are polyphenolic compounds that bind proteins and form insoluble complexes. During homogenization, they are released from cell vacuoles where they are compartmentalized. Once released, they react with proteins and nucleic acids (WANG & VODKIN, 1994). During extraction release of phenolics from the red callus occurred and resulted in the formation of insoluble phenolic-protein complexes were being formed.

These protein-phenolic complexes would be discarded with cellular debris during the centrifugation steps of the extraction process. Phenolic-protein interactions result in changed electrophoretic mobility and absorption spectra of proteins and nucleic acids. In plant extracts, phenolics interfere seriously with several protein determination methods which were originally described for animal tissues. These popular methods often give plant protein values that are in error by orders of magnitudes as plant phenolics absorb strongly at 260 nm as was observed with spectrophotometrical analysis of isolated proteins from red callus of *Oxalis* (Table 5.1).

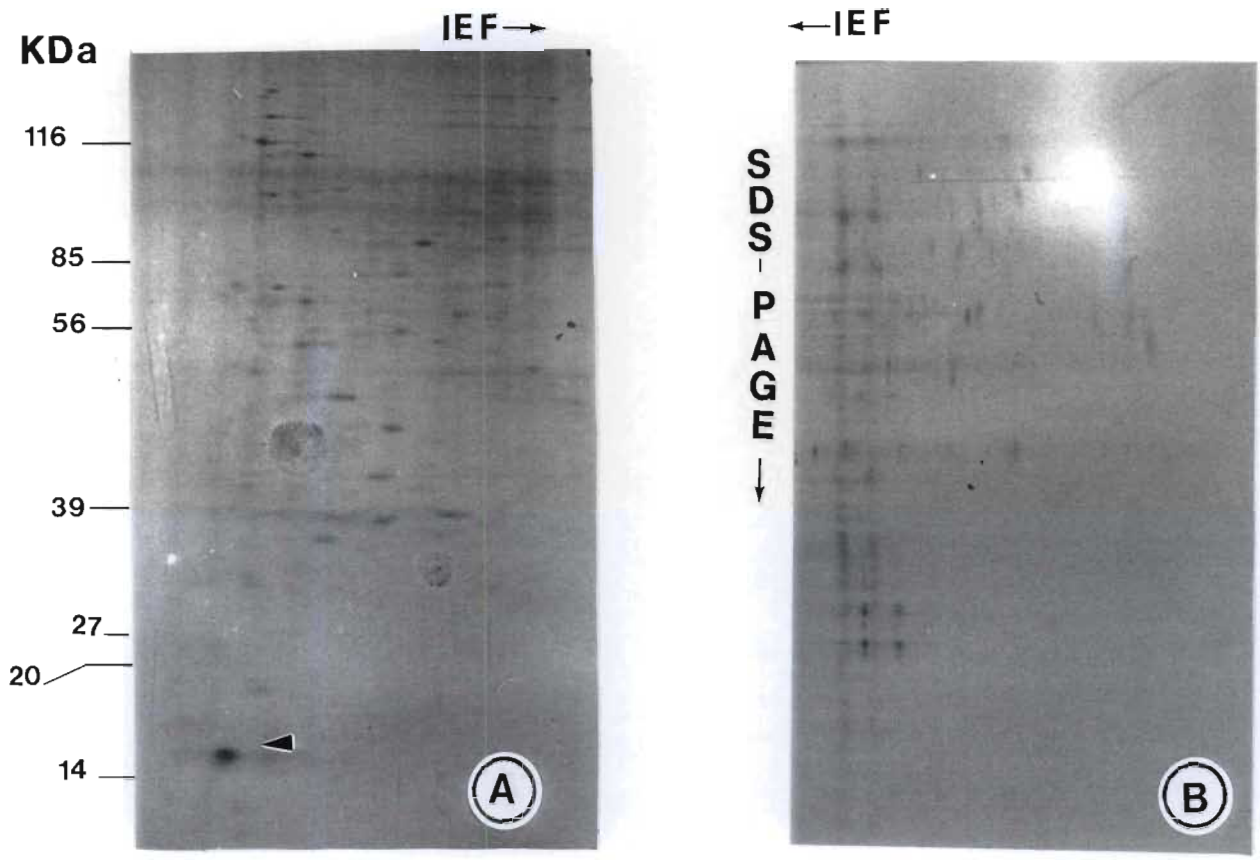


Figure 5.6: Two-dimensional gels of *O. reclinata* callus proteins stained by silver staining. (A) Polypeptide patterns of proteins isolated from light-grown white callus. (B) Polypeptide patterns of proteins isolated from dark-grown red callus. Black arrows indicate the direction of IEF and SDS-PAGE

Therefore, absorption at 260 nm is mainly due to phenolic compounds rather than nucleic acids or proteins (LOOMIS, 1974).

Inhibitory compounds can often be removed, by passage over a column of BioGeL P-6DG or Sephadex G-50, from plant extracts high in phenolics (LOOMIS, 1974) Molecular exclusion chromatography was not able to reduce binding of phenolics to proteins in this study. Gel filtration was not sufficient in preventing proanthocyanidin-protein complexing from occurring as only one polypeptide was noted after two-dimensional electrophoresis for the red callus line (Figure 5.2A). However, two-dimensional analysis of dark-grown white callus proteins gave a typical pattern of polypeptides (Figure 5.2B). The inclusion of the phenolic adsorbent, PVPP, gave similar results for both callus lines.

Rapid procedures when dealing with plant material high in polyphenolics is recommended. Rapid measures reduce the time period for which secondary products are in contact with proteins and nucleic acids. Procedures used to prepare samples for gel filtration were rapid. Precautions were taken during this study, such as, grinding the callus in liquid N_2 and mixing the powder with the extracting medium rapidly. However, the time period taken for the samples to pass through Sephadex columns could have allowed for phenolic-protein bonds to form resulting in only one polypeptide being visible after silver-staining.

One-dimensional gels were run to confirm the effect of pigment production on the methods used to extract proteins (Figure 5.3). Some insight into secondary metabolism was gained. With the presence of pigment, which indicates biosynthesis of anthocyanins, little or no proteins were obtained using conventional methods to isolate proteins. This was due to formation of phenolic-protein complexes, as previously mentioned. The white callus grown in the dark accumulates a negligible amount of anthocyanin (less than 0.002 mg g^{-1} FW, Section 3.3). This callus grows significantly faster than the red callus in the light. Primary metabolic activity is greater than secondary

metabolic activity in the white callus. Therefore, it is not necessary to take precautions against phenolic interference when isolating total protein from this type of callus. Darkly stained bands were present when total protein (10 μg) isolated from this callus were run on a one-dimensional gel (Figure 5.3). Inclusion of PVPP, a phenolic adsorbent, in the extraction buffer increased the intensity of the bands slightly. Therefore, production of secondary products was minimal as basically no difference was observed with the addition of PVPP. On the other hand, when secondary metabolic activity is taking place, no proteins were isolated in the absence of PVPP (Figure 5.3., lane LR). The inclusion of this phenolic adsorbent improved protein recovery to a great extent (Figure 5.3., lane labelled LR(P)).

Light was demonstrated to play a major role in the induction of anthocyanin biosynthesis as the light grown white callus was induced to accumulate pigment. With the initiation of anthocyanin biosynthesis, callus growth slowed and energy was shunted towards pigment production. The initiation of anthocyanin production is reflected by the inability to isolate proteins from this heterogenous callus line. Placing the red callus in the dark is paralleled by a decline in anthocyanin biosynthesis. Dark-grown red callus accumulated significantly lower levels of anthocyanin (0.003 mg g^{-1} FW). Thus, showing a reduction in the activity of flavonoid biosynthetic enzymes. Although anthocyanin production was reduced, binding of the already stored phenolic compounds to proteins was observed and isolating proteins from this callus using the conventional methods was impossible. No proteins were successfully isolated from this callus in the absence of phenolic adsorbents and inhibitors of phenolic oxidases.

Total protein isolated from a 1:1 mixture (v/v) of light grown red and dark grown white callus mixture gave a less intense banding pattern as compared to the dark grown white callus (Figure 5.3., lane R:W). This confirmed the interference of phenolics with protein isolation procedures. From spectrophotometric quantification of protein, equal amounts of protein were

supposed to have been loaded. However, the resultant banding patterns were not of the same intensity.

Prevention of phenolic-protein interactions was achieved by the inclusion of phenolic adsorbents and phenol oxidase inhibitors. Phenolic adsorbent, PVPP, (1-2%) has been utilized by many researchers to control polyphenolic-protein binding (LOOMIS, 1974). The inclusion of this phenolic adsorbent in the extraction buffer (Section 5.2.3.) slightly improved the amount of protein isolated. Bands were observed after silver staining of one-dimensional gels (Figure 5.3., lanes labelled R(P)). The phenolic adsorbent, PVPP, acts by forming strong H-bonded complexes with phenolic complexes. One of the ways in which phenolic compounds interact with proteins is through H-bonding (LOOMIS, 1974; GEGENHEIMER, 1990). Therefore, PVPP acts by competitively binding to phenolics. However, on two-dimensional gels one polypeptide (< 20 KDa) was noted, indicating that irreversible interactions between proteins and procyanidins were still taking place (Figure 5.2B).

Bovine serum albumin is a protein that can be included in extraction buffers to act as a phenolic adsorbent. It acts by binding to phenols through H-bonds to the peptide-bond oxygens. Bovine serum albumin was observed to have a very small effect on protein isolation from red calli, only a few bands were observed when comparing its effect to that of PVPP (Figure 5.3., lane designated LR(B)).

Phenol oxidase activity may be inhibited by anti-oxidants such as mercaptobenzothiazol and metabisulfites. These antioxidants provide a strong reducing environment to counteract the effect of phenol oxidases. Phenol oxidases allow for interactions to occur between proteins and phenolic compounds. Proteins isolated in the presence of these sodium salts (e.g. sodium metabisulfite and sodium tetraborate) from the red callus were successfully run on two dimensional gels and patterns of polypeptides were observed (Figure 5.4).

Metabisulfites are thought to protect plant enzymes through complex reactions. They are known to react with a wide variety of plant secondary products such as phenolics, coumarins and quinones. Borate acts by complexing with compounds such as *o*-diphenols and it inhibits diphenol oxidase activity. In the presence of PVP, borate has been shown to give higher yield of soluble protein. This effect may be assigned to phenolic compounds that have highly interacting hydroxyl and carbonyl groups forming stable compounds with borate and which are less active in forming hydrogen bonds with proteins (LOOMIS, 1974).

Several proteins were unique to the red callus grown in the light (Figure 5.6B). These proteins were thought to be involved with the synthesis of anthocyanins. The white callus which is grown in the light has a patchy appearance showing initiation of anthocyanin synthesis, as a result there are polypeptides which are shared between the red callus and this white callus line (Figure 5.7A). The polypeptide which is less than 20 KDa is common to three callus lines of *O. reclinata*, namely, light-grown red callus, dark-grown white callus and light-grown white callus. It may be assumed to be associated with primary metabolic events.

Two-dimensional studies were not conclusive and further molecular analysis was required to elucidate mechanisms involved in anthocyanin production.

CHAPTER 6

IN VITRO TRANSLATION

6.1 INTRODUCTION

The technique of *in vitro* translation provides information about protein synthesis mechanisms. It allows for the identification of mRNA molecules and the study of the properties for which they code (BROWN, 1990). Cell-free protein-synthesising systems are now available commercially and have been extensively used for the translation of eukaryotic mRNA's. The two most commonly utilized *in vitro* translation systems are the rabbit reticulocyte lysate and the wheat germ extract (CLEMENS, 1984). Attention will be paid to the wheat germ extract as it was utilized in this study. The manufacturer, BOEHRINGER MANNHEIM (1994) claims that the wheat germ translation kit is an ideal source of ribosomes because of the low levels of endogenous mRNA activity. The cell-free translation system allows for the performance of the three basic steps involved in translations, namely, initiation, elongation and termination. The kit is intended for protein synthesis in the presence of [^{35}S]-methionine or [^{3}H]-leucine. The labelled protein product may be examined by trichloro-acetate (TCA) precipitation and gel electrophoresis, followed by autoradiography (Figure 6.1).

The wheat germ extract has several advantages. In comparison to the reticulocyte system, its preparation, and therefore cost, are easier and lower respectively. Wheat germ extracts are sensitive to stimulation of overall protein synthesis by exogenous mRNAs without micrococcal nuclease treatment. Under optimal conditions, mRNA may stimulate amino acid incorporation during *in vitro* translation as much as 400-fold. The wheat germ system is capable of synthesizing high molecular weight polypeptides (ANDERSON, STRAUS, &

DUDOCK, 1983). Optimisation of cell-free translation using the wheat germ system may be time consuming as the ionic optima for translation are sensitive to the nature and the concentration of mRNA. It may be necessary to determine the ionic optima for various templates (CLEMENS, 1984).

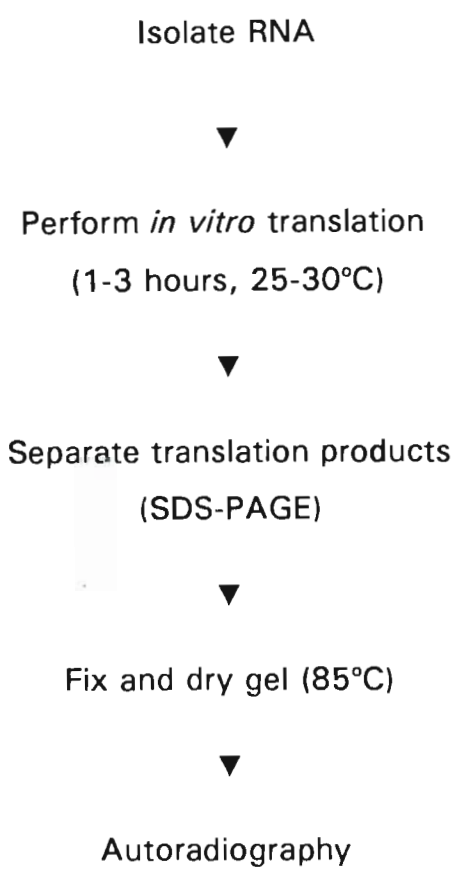


Figure 6.1: Flow chart of basic steps involved in *in vitro* translation assays

In vitro translation using the wheat germ system was performed with the objective of analysing the differences in gene expression at the translational level between the red and white callus lines of *O. reclinata.*

6.2 MATERIALS AND METHODS

6.2.1 RNA isolation

Two methods were attempted to isolate RNA from light-grown red callus and dark-grown white callus types of *O. reclinata*. Prior to preparation of reagents, a 1% solution of diethylpyrocarbonate (DEPC) was prepared and stirred overnight at room temperature in a glass Schott bottle. The DEPC solution was then used to remove RNases from all glassware and plasticware. The DEPC was inactivated by autoclaving for an hour. Initially, RNA was isolated from a small amount of tissue (0.1 g) by grinding in liquid N_2 in a pre-cooled mortar and pestle to a fine powder. The powder was transferred to a centrifuge tube with 1.5 mℓ Tris-saturated phenol (pH 5.0) and 1.5 mℓ extraction RNA buffer (0.2 M Tris, pH 9.0; 0.4 M LiCl, 25 mM EDTA and 1% SDS) and vortexed well. The extract was centrifuged at 3 000 rpm (Sigma-113) for five minutes and the aqueous phase transferred to another tube and precipitated with 1/10 volume 3 M sodium acetate (pH 5.2) and 2 volumes absolute ethanol by placing at -20°C for two hours, followed by centrifugation at 8 000 rpm (Sigma-113 microfuge) for 15 minutes. The recovered pellet was resuspended in 0.3 mℓ sterile RNase-free dH_2O, vortexed well, and 30 $\mu\ell$ 3 M sodium acetate (pH 5.2) and 600 $\mu\ell$ absolute ethanol and RNA was precipitated at -70°C for 15 minutes. The pellet was washed with 80% ethanol twice and RNA was dissolved in 50 $\mu\ell$ of water. Quantification of RNA was performed using 5 $\mu\ell$ of the extract. RNA was quantified in $\mu g\ m^{-1}\ell$ using a nucleic acid calculator (Genequant, Pharmacia). To bind phenolics PVPP (2%) was added to the extraction buffer.

The second method used was described by WANG & VODKIN (1994) for anthocyanin-containing tissues. For this study, the method was modified as large amounts of heparin are required and this makes the original method expensive. Bovine serum albumin (5%), which acts as a phenolic adsorbent, was incubated in Extraction Buffer A (100 mM Tris-Cl, pH 9.0, 200 mM NaCl,

20 mM EDTA, 4% sarkosyl [v/v]) containing 16 mM 2-merçaptobenzothiazol (a RNase inhibitor) and 1.5% PVPP, for a period of two hours to overnight. Callus material (0.2 - 0.5 g) was ground to a fine powder in a pestle and mortar using liquid N_2. The fine powder was transferred to a centrifuge tube, containing 2 mℓ of extraction buffer (EB) A, before it began to thaw. Extraction buffer B (6.5 mℓ) which had the same chemical composition as extraction buffer A but excluding BSA and PVPP was added to the centrifuge tube and 200 $\mu\ell$ Proteinase K (10 mg mℓ^{-1}, Boehringer Mannheim) added, and the homogenate left on ice in the combined extraction buffers for one minute. The extract was then incubated at 37°C in a waterbath, and gently shaken in rotary motion at 80 rpm for 20 minutes. The protease enzyme was used to digest BSA which competitively binds anthocyanins. The BSA and cell debris were removed by centrifugation at 10 000 rpm (SS-34 rotor head, RC-5 Sorvall Centrifuge) for 10 minutes at 4°C. The supernatant was transferred to a new centrifuge tube and extracted in an equal volume of Tris-saturated phenol (pH 7.5) at 8 000 rpm for 10 minutes (SS-34 rotor head, RC-5 Sorvall Centrifuge). The supernatant was extracted twice with phenol at 20°C. Chloroform-isoamyl alcohol extractions were performed following phenol extractions using Sevag solution (Chloroform : isoamyl-alcohol, 24 : 1 [v/v]) by centrifugation of supernatant at 8 000 rpm (SS-34 rotor head, RC-5 Sorvall Centrifuge) for 10 minutes at 20°C. The supernatant was measured and 1/3 volume of LiCl (8 M) was used to precipitate RNA overnight at 4°C. The RNA was recovered by centrifugation at 8 000 rpm in a microfuge (Sigma-113) for 20 minutes. The pellet was washed twice in 2 M LiCl and RNA was dissolved in deionized dH_2O and 1/10 volume of 2.5 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol were used for the final RNA precipitation at 20°C overnight, or at -70°C for 15 minutes. The RNA samples were centrifuged at 8 000 rpm for 20 minutes, in a microfuge (Sigma-113) and vacuum dried for three minutes. Pellets were resuspended in distilled water, or TE (0.1 M Tris, 0.01 M EDTA [pH 8.0]) and stored as 10 $\mu\ell$ aliquots at -70°C until needed. All glassware and plasticware

was treated with 0.1% DEPC overnight (as previously described), to remove RNases.

The method described by WANG & VODKIN (1994), proved to be the most successful. Several other modifications were also attempted to the WANG & VODKIN (1994) protocol. They were as follows:

i) Extraction was performed in the absence of 16 mM merçaptobenzothiazol (RNase inhibitor);

ii) BSA was excluded from Extraction Buffer A; and

iii) Addition of phenol oxidase inhibitors and a phenolic adsorbent to the two extraction buffers were attempted. These were 0.2 M sodium tetraborate, 0.02 M sodium metabisulfite, and 0.002 M sodium diethyldithiocarbamate.

6.2.2 RNA analysis

The isolated RNA was quantified prior to storage using an automated nucleic acids calculator (Genequant, Pharmacia). RNA (2 μg) was electrophoresed in 1.5% agarose gel under non-denaturing conditions at 56 V (5 V cm^{-1}) for two hours to establish its integrity. Electrophoresis was performed using 1X TAE (0.04 M Tris-Acetate, 0.002 M EDTA, pH 8.5). This running buffer was made from a 50X TAE buffer stock solution (121 g Tris, 28.55 ml glacial acetic acid, 50 ml 0.5 M EDTA and 421 ml H_2O). To monitor the electrophoretic process, RNA samples were dissolved in a loading buffer containing bromophenol blue. The loading buffer (10X stock solution) was made of 50 mM NaOH, 1 mM EDTA, 2.5% glycerol and 0.025% bromophenol blue) and was stored at 4°C. Once electrophoresis was complete, gels were stained by submersion in a 0.5 μg ml^{-1} solution of ethidium bromide (EtBr) for 10 minutes. When necessary gels were destained using the previously described agarose electrophoresis

running buffer (TAE [pH 8.5]). The RNA was visualised using UV-illumination (Spectroline UV-Transilluminator, TC-312A) after staining with EtBr.

6.2.3 Non-radioactive *in vitro* translation

Non-radioactive translation is not conventionally utilized. However, as part of this study it was necessary to employ this method for the reasons listed below:

1) The wheat germ kit initially used for translation was 'old'. It had been stored at - 70°C for a year. Non-radioactive translation was used to test the activity of the kit;

2) It was regarded as a cheaper means of optimizing cell-free translation as radioactively labelled methionine is expensive and the wheat germ system is sensitive to potassium and magnesium concentrations;

3) This system would be able to provide sufficient information with respect to gene expression and translation mechanisms of pigment synthesizing callus, provided that several controls were set up to eliminate background proteins which form part of the wheat germ kit and;

4) The hazards encountered with radioactive experimental procedures would be eliminated.

A stock solution of 125 mM methionine was made and stored at -70°C. Prior to use, the stock solution was diluted 100-fold in RNase-free water. Translation reactions were set up as described in Table 6.1.

The reaction volume adjusted to 25 $\mu\ell$ with RNase-free dH_2O. Cell-free translation was initiated by incubating reaction tubes in a waterbath at 30°C. After three hours, the tubes were removed from the waterbath and placed on ice prior to electrophoresis of translation products using one-dimensional PAGE

(Section 5.2.5). The gels were silver stained to detect *in vitro* synthesized polypeptides (Section 5.2.7).

Table 6.1: Components added to reaction vessels for non-radioactive *in vitro* translation according to the Boehringer Mannheim protocol

Reagent	Stock concentration	Volume ($\mu\ell$)	Final concentration
Translation mixture (contains 19 amino acids)	25 μM	5 μl	5 μM
Potassium acetate	2,5 M	1 $\mu\ell$	100 mM
Magnesium acetate	25 mM	1 $\mu\ell$	1 mM
Redistilled dH_2O	-	variable	
▲ [1 & 2] RNA	variable	variable	1 μg
methionine	12.5	0.14 $\mu\ell$	2.5 μM
■Wheat germ extract	* See below	7.5 $\mu\ell$	

* Contains ribosomes and proteins (enzymes) required for translation, concentration not given by manufacturer.

▲ [1] Control reactions translated with tobacco mosaic virus (TMV) RNA or ∝-globin RNA according to manufacturer's instructions.

▲ [2] Two negative control reactions were set up as follows:
a) components necessary for translation were included in the reaction except for the translation mix; and
b) the wheat germ extract was excluded from the reaction.

■ Wheat germ extract which contains the enzymes responsible for protein synthesis was added last to initiate translation.

6.2.4 Radio-active *in vitro* translation

The Boehringer-Mannheim protocol based on the wheat germ system was utilized. One microgram of RNA isolated from red and white callus types of *O. reclinata* was translated according the manufacturer's instructions (Boehringer Mannheim) in microfuge tubes. The basic reaction is tabulated in Table 6.2.

Table 6.2: Components added to reaction vessels for radioactive *in vitro* translation according to the Boehringer Mannhein protocol

Reagent	Stock concentration	Volume	Final concentration
Translation mixture (contain 19 amino acids)	25 μM	5 $\mu\ell$	5 μM
Potassium acetate	2,5 M	1 $\mu\ell$	100 mM
Magnesium acetate	25 mM	1 $\mu\ell$	1 mM
Redistilled deionised water	-	variable	
▲ mRNA	variable	variable	1 μg
●[^{35}S]-Methionine, aqueous solution		2 $\mu\ell$	
■Wheat germ extract	* See below	7.5 $\mu\ell$	

* Contains ribosomes and proteins required for translation

▲ Control reactions translated with tobacco mosaic virus (TMV) RNA or α-globin according to manufacturer's instructions.

■ Wheat germ extract added last to initiate translation.

● [^{35}S]-methionine (> 37 TBq mmol^{-1}; 15 mCimℓ^{-1}) purchased from Amersham.

The reaction volume was adjusted to 25 $\mu\ell$ with redistilled water. The reaction tubes were incubated for three hours at 30°C. Attempts to optimise cell-free translation were made. These involved manipulating mRNA concentrations of the basic standard assay and varying the concentration of magnesium and potassium ions.

6.2.4.1 Trichloroacetic acid (TCA) precipitation

Assay mixture (3 $\mu\ell$) was mixed with 147 $\mu\ell$ redistilled water and placed on ice. From the diluted assay mixture 5 $\mu\ell$ were pipetted onto a dry GFC-filter (Whatman). The filters were left to dry for 10 minutes at 65°C and counted with TCA precipitated radiolabelled proteins using a liquid scintillation counter (Beckman LS 600LL). Precipitation with TCA of radioactive translation products was performed by mixing the *in vitro* translation (50 $\mu\ell$) with 500 $\mu\ell$ 0.1 M NaOH and incubating for 10 minutes at 37°C. After incubating, 400 $\mu\ell$ TCA (50% [w/v]) containing casein hydrolysate (2% [w/v]), was added and the reaction was placed on ice for 30 minutes. GFC-filters (Whatman) previously soaked for 30 minutes in TCA solution (5% [w/v]), containing 0.01 M $KH_4P_2O_7$, were used to filter the mixture. The filters were washed three times in a solution of 2.0 M TCA and 2.0 M $Na_4P_2O_7$. The filters were dried for 10 minutes at 65°C after being washed.

6.2.4.2 Quantification of translation products

The dried filters were placed in scintillation counting vials with 5 mℓ of scintillation cocktail (Beckman Ready Value™) and counted on a liquid scintillation counter (Beckman LS 6000LL). The efficiency of translation was represented as the percentage incorporation. This value was derived by counting both precipitated and unprecipitated assay mixtures simultaneously. The percentage incorporation of [^{35}S]-methionine was calculated according to the following formula:

$$\% \text{ incorporation} = \frac{\text{TCA precipitated products (cpm } \mu\ell^{-1})}{\text{Unprecipitated products (cpm } \mu\ell^{-1})}$$

6.2.5 Electrophoresis of translation products

The assay mixture (10 $\mu\ell$) was run on a 6% stacking gel and 12% running gel, as described in Section 5.2.5. Once gel electrophoresis was complete, the gel was rinsed in a solution of 10% acetic acid (to fix proteins) containing 1% glycerol for 30 minutes. The gel was placed onto filter paper (Whatman 1), transferred to a gel drying system (Slab Gel Dryer SE1160, Hoefer Scientific) and vacuum dried for two-and-a-half hours at 80°C. The dried gel was then exposed to autoradiographic film (Hyperfilm α-max, Amersham) for a period of 12 - 24 hours. The film was then developed.

6.3 RESULTS

The quality of RNA's isolated using the methods described in Section 6.2.1. are compared in Figure 6.2. The type of degraded RNA recovered when interference derived from phenolic compounds has not been minimized during isolation is shown in Figure 6.2A. Therefore, standard phenol-LiCl methods were not effective for extracting RNA from the pigmented callus. Mostly, no RNA was recovered by these methods or RNA was degraded and appeared as smears on 1.5% non-denaturing agarose gels. Several other problems were encountered with conventional methods for isolating RNA. Gelatinous and pigmented pellets were formed (Table 6.3). These pellets were difficult to resuspend in water or TE buffer (pH 6.8). The texture of the pellets was probably a reflection of large amounts of polysaccharides which were not removed during the extraction. Bovine serum albumin was included in the extraction buffer to competitively bind procyanidins. Heparin, in large amounts, is required to act effectively as a RNase inhibitor because the BSA which is required to competitively bind proanthocyanidins is not free of RNase activity (WANG & VODKIN, 1994). This makes the WANG & VODKIN (1994) method expensive. The method was modified by excluding heparin from the buffer. The phenolic adsorbent, BSA, was then incubated in EB containing the RNase inhibitor, 2-merçaptobenzothiazol, for a longer time period (two hours to

overnight) at 4°C. This proved to be the most successful method to isolate RNA from anthocyanin containing callus tissues.

Proteinase K (Boehringer Mannheim) was the preferred proteinaceous enzyme as Pronase (Boehringer Mannheim) yielded intact white callus RNA but the red callus RNA was degraded. When extracting RNA, it was necessary to incubate the extracts with one of these enzymes to remove residual BSA unbound by proanthocyanidins. Digestion with Proteinase K resulted in discolouration of red extracts from red to cream and intact RNA was always recovered. By contrast, Pronase did not cause a colour change during the digestion. Pink to purple pellets were always recovered. This reflected the association of phenolic compounds with RNA. Absorbance values obtained with treatments without 2-merçaptobenzothiazol (Treatments 1 - 3), were always low. Comparing readings obtained with Pronase (treatment 4) and Proteinase K (Treatment 5), the former resulted in RNA with lower A_{260} values (data not shown). In general, RNA yields obtained with the red callus were far lower than those obtained with the white callus. Intact RNA obtained with Proteinase K containing EB (Treatment 4) was represented by the appearance of two bands, the major 28S and 18S rRNAs after electrophoresis in a non-denaturing 1.5% agarose gel (Figure 6.2B). The degradation of the two major ribosomal RNA bands was always seen as smears on 1.5% agarose gels (Figure 6.2A).

Non-radioactive translation was successful as revealed by one-dimensional and two-dimensional PAGE (Figures 6.3 and 6.4). Comparing the banding pattern observed for the wheat germ extract proteins (Lane labelled A) with the banding pattern observed after non-radioactive translation of white and red callus RNA (Lanes F-G and D-E, respectively), the *in vitro* translated samples showed an increase in the number of bands.

Table 6.3: Modifications made to the phenol-LiCl method described by WANG & VODKIN (1994) for RNA extraction from pigmented plant tissues

Extraction buffer (EB) composition	Observations and RNA quality
1) Basic EB	Basic buffer without RNase inhibitors, pigmented pellets, RNA degraded
2) EB + PVPP + sodium salts (phenol oxidase inhibitors added)	No RNase inhibitors, pigmented pellets, RNA degraded
3) EB + PVPP + BSA (phenolic adsorbent) + Pronase	BSA was not RNase free, RNA degraded
4) EB + PVPP + BSA + Pronase + merçaptobenzothiazol	Slightly pigmented pellets (grey), RNA degraded
5) EB + PVPP + BSA + Proteinase K + merçaptobenzothiazol	BSA incubated for < two hours, Proteinase K was RNase free, intact RNA

This was taken as an indication of translation having taken place and synthesis of new polypeptides having occurred. A larger number of small polypeptides was synthesized for both callus types (Figure 6.3, indicated by the arrows). Doubling the concentration of the template, as specified by the kit, increases translation efficiency. The intensity of the bands was greater for reactions set up with 2 μg of RNA isolated from both red and white callus. The resolution of the one-dimensional gels was poor due to the camouflaging effect of the proteins common to the kit. This made interpretation of one-dimensional gels difficult. It was difficult to differentiate between neo-synthesized polypeptides and those that form part of the wheat germ kit.

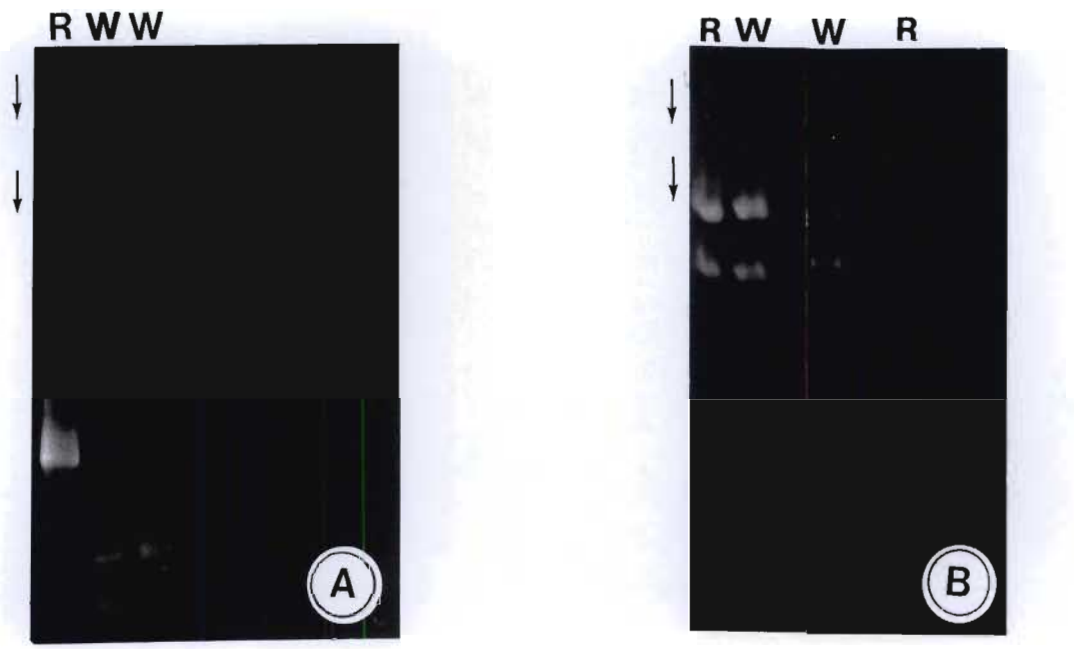


Figure 6.2: Comparison of RNA quality extracted from white and red callus lines of *O. reclinata* fractionated on a 1.5% non-denaturing agarose gels. (A) Conventional methods yielded poor quality RNA which was degraded. (B) Good quality RNA was extracted according to the modified WANG & VODKIN (1994) method.

Key to lane labels: R, RNA extracted from red callus; W, RNA extracted from white callus. Black arrows indicate the direction of electrophoresis

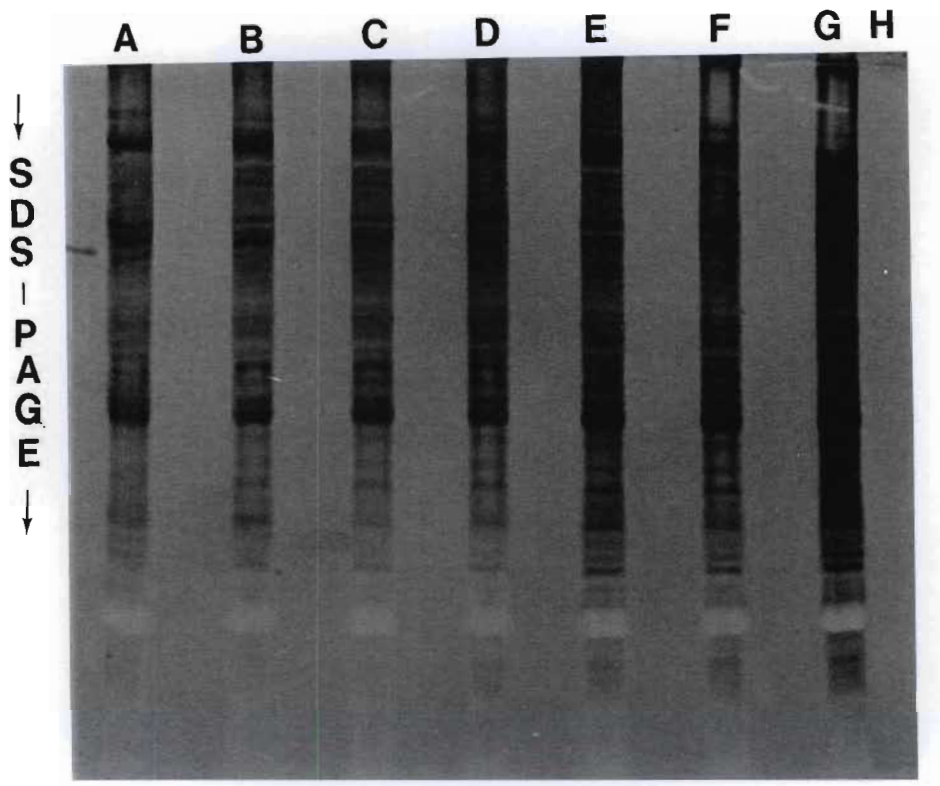


Figure 6.3: One-dimensional gel of non-radioactively synthesized *in vitro* translation products visualized by silver staining. Lane A shows proteins associated with the wheat germ extract when the amino acid translation mixture was excluded from the translation assay. Lane B represents a control reaction, where no RNA was included in the translation reaction. Lane C represents bands visualized after non-radioactive translation of β-globin. Lanes D-E show proteins obtained from translation of red callus RNA and wheat germ extract proteins. Lanes F and G represent translation products of white callus RNA and wheat germ extract proteins. No bands were visualized when the wheat germ extract was omitted from the translation assay (Lane H)

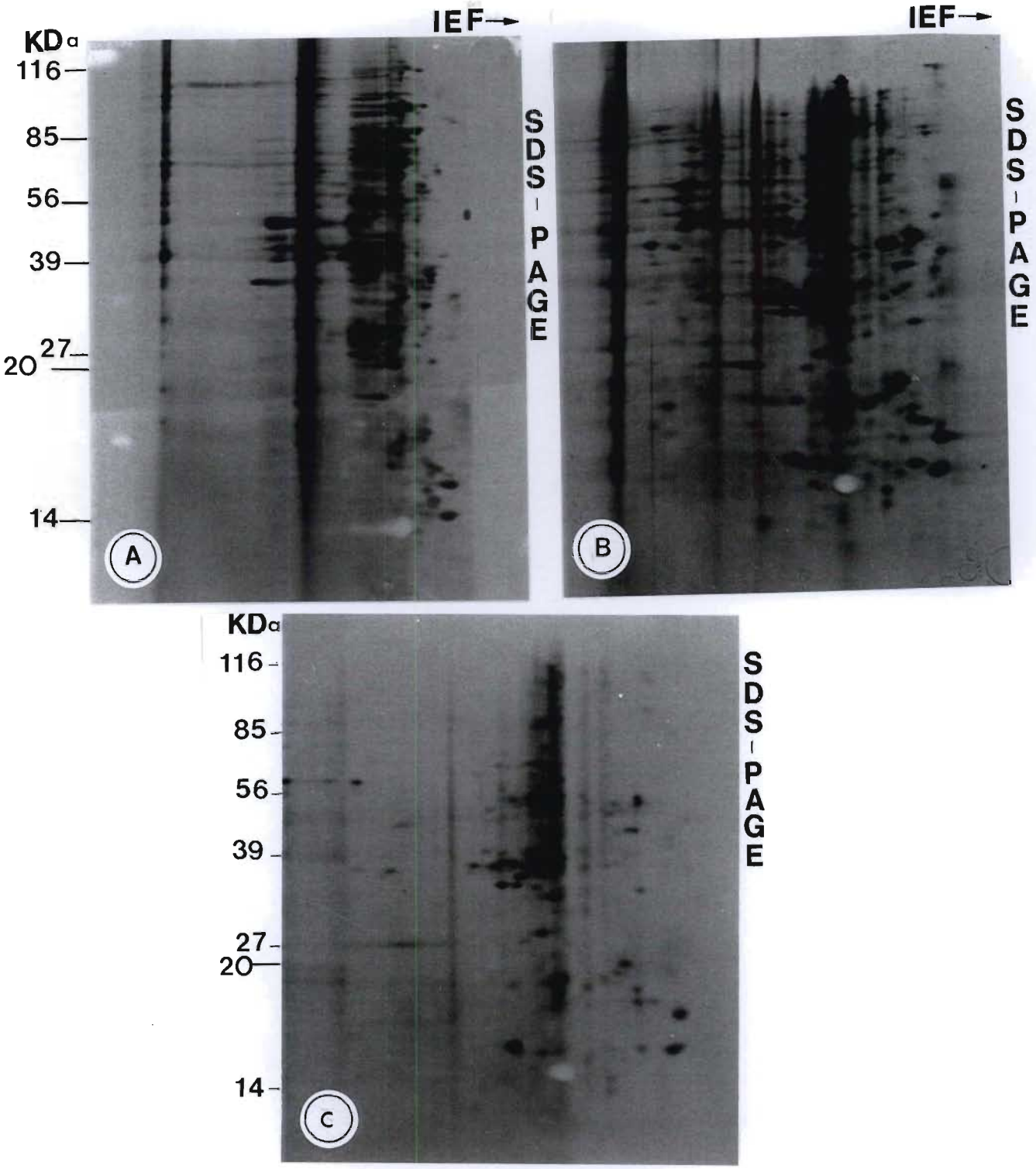


Figure 6.4: Comparison of silver-stained polypeptide patterns obtained from non-radioactive *in vitro* translation total RNA isolated from callus types of *O. reclinata* (A) A mixture of polypeptides synthesized from non-radioactive cell-free translation of white callus total RNA and wheat germ extract polypeptides. (B) A mixture of polypeptides synthesized from non-radioactive cell-free translation of red callus total RNA and wheat germ extract polypeptides. (C) Polypeptide pattern obtained from two-dimensional electrophoresis of wheat germ extract proteins

Two-dimensional gels confirmed that translation had taken place. The number of polypeptides for red and white translated samples (Figure 6.4B and A, respectively) exceeded that of proteins associated with the wheat germ system (Figure 6.4C). The red callus was associated with a larger subset of unique basic polypeptides (indicated by arrows). Overall, a greater amount of proteins was neo-synthesized for the red callus compared the white callus.

The main difference between the red and the white callus was the presence of more basic proteins translated from RNA isolated from red callus. Overall, more proteins were synthesized from the total RNA of the red callus of *O. reclinata*. Small-sized proteins, belonging to the red and white samples, which electrophoresed closer to the dye-front were visualized following silver staining of the gel. Newly-synthesized large proteins were closely packed at the top of the gel (Lane E) for the red callus.

Translation with [^{35}S]-methionine using the wheat germ kit was successfully achieved with the control RNA, TMV and β-globin RNA. Even though the kit was over a year old, translation products of TMV RNA (seen as distinct bands) were detected on 12% polyacrylamide gels after a 12 hour exposure on autoradiographical film (Figure 6.5, Lane A). The β-globin RNA translation product was seen as one band which was located 3/4 away from the front. The translation products were not sized as the experiment was conducted to test the efficiency and activity of the wheat germ kit. The arrow shows smears which obscures the banding pattern. These smears are speculated to be due to unincorporated amino acids and an insoluble complex formed between 40S ribosomes, peptidyl t-RNA and radioactive methionine (40S-[^{35}S]Met-tRNA complex). Attempts to translate white and red callus total RNA, yielded a stronger banding pattern for the white callus RNA and faint smears for the red callus on one-dimensional gels. The insoluble 40S-[^{35}S]Met-tRNA complex camouflaged some of the translation products. The complex had a strong signal and it was impossible to expose the gels to autoradiographical film for a longer time.

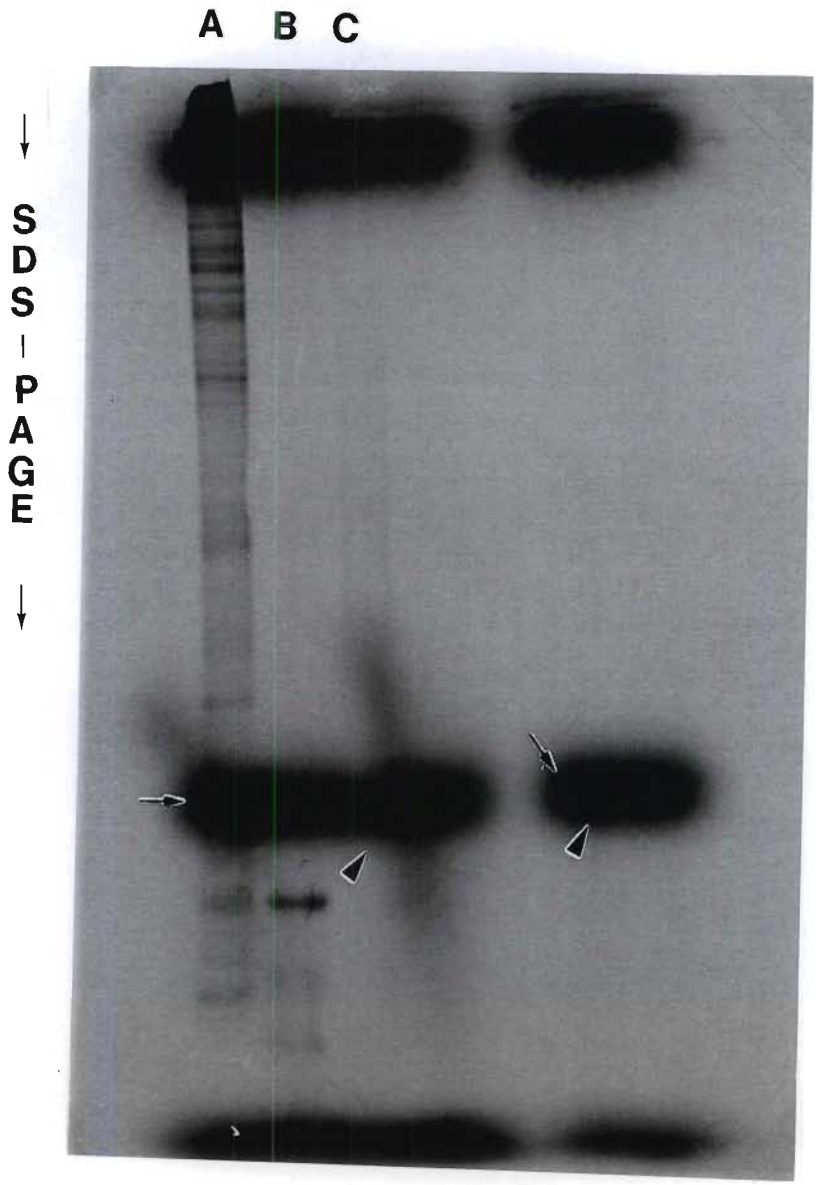


Figure 6.5: Autoradiogram of SDS-PAGE of translation products of control RNA provided with the wheat germ kit. Lane A represents TMV RNA translation products. Lane B represents the translation products of β-globin RNA. Lane C represents a faint smear of translation products of white callus RNA. Black and white arrows point to unincorporated amino acids and an insoluble 40S-[^{35}S]Met-tRNA complex. Black arrows indicate the direction of electrophoresis

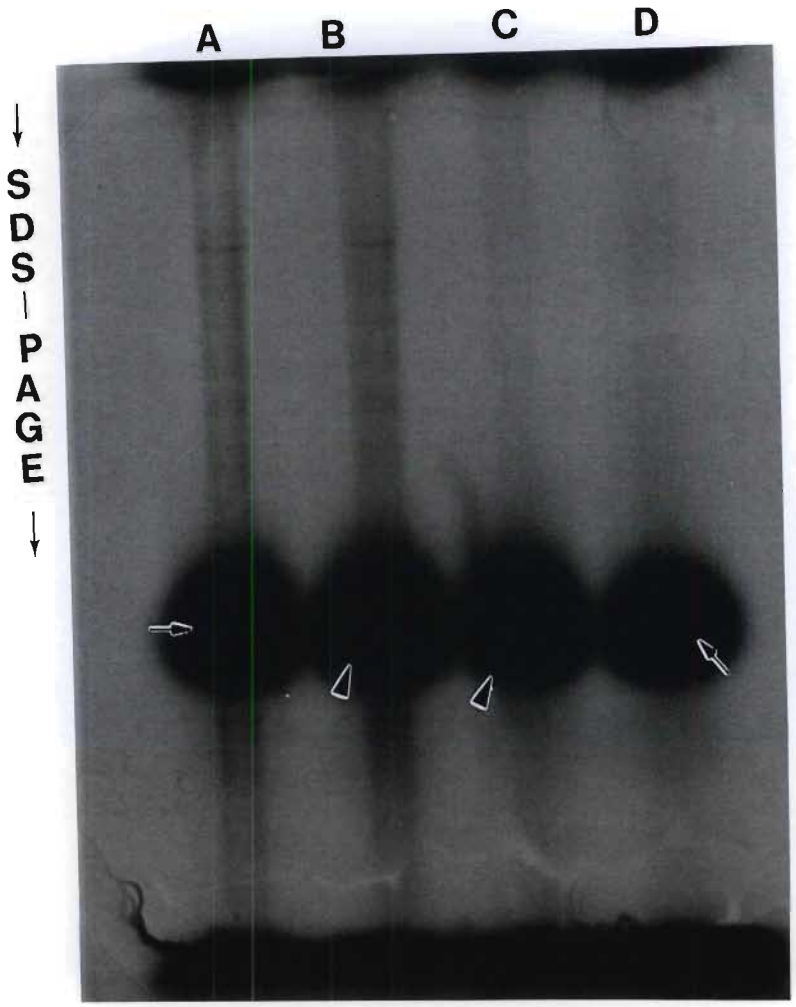


Figure 6.6: Autoradiogram of SDS-PAGE of translation products of total RNA isolated from callus types of *O. reclinata*. Lanes A and B represent faint bands of white callus translation products. Lanes C and D represent smears of translation products of red callus. Black and white arrows point to unincorporated amino acids and an insoluble 40S-[^{35}S]Met-tRNA complex. Black arrows indicate the direction of SDS-PAGE

6.5 DISCUSSION

Phenolic compounds have been shown to form insoluble complexes with nucleic acids and proteins. Common methods are not effective to isolate RNA from tissues with high levels of proanthocyanidins (TODD & VODKIN, 1993; WANG & VODKIN, 1994). Conventionally, RNA is usually extracted using slightly alkaline buffers at pH 9. The use of high pH's which is necessary to reduce RNase activity most probably contributes also to phenolics binding to the RNA. At higher pH's activity of phenol oxidase activity is increased. Although, alkalinity of the extraction buffer may reduce interactions via hydrogen bonding as phenols are ionized considerably and do not form strong interactions, the effectiveness of phenol adsorbents (such as PVPP) is also reduced (LOOMIS, 1974).

The inclusion of BSA in the extraction protocol to bind phenolics required the removal of excess protein using proteolytic enzymes. Pronase always resulted in slightly pigmented pellets which were difficult to resuspend and RNA that was degraded. By contrast, the use of Proteinase K yielded intact RNA that was pigment free for the both types of callus. Proteinase K is classified as a subtilin-related protease. It is therefore not inactivated by metal ions, chelating agents (EDTA), sulfhydryl reagents or trypsin and chymotrypsin inhibitors. It is also stable over a wide pH range (4-12,5). Due to the enzyme being effective against native proteins, it can inactivate endogenous nucleases such as RNases and DNases. On the other hand, what is termed Pronase by the manufacturer (Boehringer Mannheim) is a mixture of several unspecific endo- and exoproteases which digest proteins to single amino acids. These proteases are not as effective as Proteinase. Some of these proteases are most probably inactivated by chelators such as EDTA and mercaptobenzothiazol. They are not as effective on RNases as Proteinase K which is active in the presence of these compounds. Inclusion of phenolic adsorbents, BSA and PVPP, minimized interference from proanthocyanidin. These compounds provide a large amount of alternative substrate for which proanthocyanidins bind, thereby reducing the

their levels to bind RNA. This increases RNA yield and purity. Quality of RNA is important, if the RNA is to be manipulated further. Intact RNA (Figure 6.2B) was seen as two bands, 28S and 18S rRNA molecules for both red and white callus types (Lanes labelled R and W, respectively). This RNA was shown to translate effectively in a non-radioactive wheat germ system. More proteins were synthesized from the red callus compared to the white callus. This indicated that the red callus was more active at the gene level. Gene expression was presumed to increase to accommodate production enzymes involved in the secondary process of anthocyanin production. The vacuole has been shown to be the organelle where anthocyanins accumulate in *O. reclinata* callus cells (Section 4.2.2). Accumulation of anthocyanin in the vacuole is most probably associated with production of proteins involved in transport mechanisms. Proteins associated with anthocyanin biosynthesis and accumulation appear to be basic in nature. The non-pigmented white callus RNA did not translate into basic protein (Figure 6.4).

Radioactive translation studies would have been easier to interpret as the background proteins of the wheat germ extract would be eliminated as they do not become radioactively labelled during translation. Several attempts were made with this technique. The results were masked by unincorporated amino acids and formation of 40S[^{35}S]Met-tRNA complexes. Formation of these radioactive complexes occurs during translation and the compounds necessary for their formation are provided by the extract. Preparation of the wheat germ extract involves the release of polysomes, ribosomes, ribosomal units, aminoacyl-tRNA synthases, tRNA and translational factors. The system involves the incorporation of radioactive amino acid (such as leucine or methionine) into protein when incubated with ATP, GTP and amino acids. Ribonucleoprotein particles exist as 40S, 60S and 80S subunits after the extract has been treated with microccocal nuclease (ANDERSON, STRAUS & DUDOCK, 1983; CLEMENS, 1984). The wheat germ extract has several other disadvantages. The wheat germ system is said to have low endogenous translational activity as compared to the reticulocyte lysate system and that

translation in the wheat germ extract is far more dependent on the added mRNA (CLEMENS, 1984). The wheat germ extract has been criticized for its tendency to produce incomplete products due to premature termination and the release of peptidyl-tRNA. This factor may be problematic with large mRNA coding for polypeptides which are larger than 60 000 daltons. The polyamines, spermine and spermidine may overcome this problem by lowering the Mg^{2+} optimum as there is endonucleolytic cleavage of large mRNAs and this increase the probability of ribosomes completing the synthesis of full-length products before the degradation of the template (CLEMENS, 1984). It remains necessary to optimise translation conditions for RNA isolated from red and white callus cultures of *Oxalis* and this would involve the manipulation of Mg^{2+} and K^{2+} ion as the ionic optima for translation are sensitive to the nature and concentration of the mRNA. Inclusion of RNase inhibitors, such as RNasin (Promega) might prevent degradation of the template by RNases and thus, allow for completion and full-length synthesis of translation polypeptides. Digestion of translated samples with 200 μg $m\ell^{-1}$, in the presence of 200 mM EDTA or other protease inhibitors (e.g. PMSF), prior to electrophoresis may remove unincorporated amino acids. The translation products may be precipitated out with 90% acetone. If the proteins under study are soluble in acetone it may be necessary to precipitate the proteins using 10% TCA (ANDERSON, STRAUS & DUDOCK, 1983).

In vitro translation was initiated to study changes in genome expression which are induced or influenced by environmental stimuli as several gene products are shown at the same time. Using this technique, it was discovered that basic difference between red and white cultures was the synthesis of non-radioactive translation products which were basic in nature. Optimisation of radioactive translation will shed further insight to the mechanisms controlling anthocyanin biosynthesis at the enzymic level.

CHAPTER 7

CONCLUSIONS AND FUTURE PROSPECTS

7.1 CONCLUSIONS

The research conducted in this investigation has illustrated the benefits of using plant tissue culture systems in studying the effects of environmental factors on the production of economically important secondary metabolites, such as, anthocyanins. To reiterate, the main objectives of the study were to determine the inducer of anthocyanin biosynthesis in callus of *O. reclinata*, and to optimize pigment production, once anthocyanin synthesis had been stimulated. Accumulation of red pigment was hypothesized to be in response to external stimuli. It is well documented that production of anthocyanins in non-expressing plant tissues is usually under environmental control (DOONER, ROBBINS & JORGENSEN, 1991). Using *in vitro* culture techniques, light was recognized as a major inducing factor of anthocyanin biosynthesis in callus cultures of *O. reclinata*. Up-regulation of the genes encoding key enzymes involved in the flavonoid pathway, namely, PAL and CHS, has been reported to occur once plant tissues are illuminated. *De novo* synthesis of mRNAs encoding PAL and CHS takes place in response to UV and high-light (BATSCHAUER, ROCHOLL, KAISER, NAGATANI, FURUYA & SCHAFER, 1996). The spectral sensitivity of anthocyanin production differs in different plant species. However, UV and blue light have often been found to play the most significant role in stimulating pigment production. It has been suggested that, anthocyanin genes are expressed in response to light in culture as anthocyanins act as screening pigments in nature (TAKEDA, 1988; BATSCHAUER, ROCHOLL, KAISER, NAGATANI, FURUYA & SCHAFER, 1996).

The hormone, 2,4-D, had the most negative effect on production of anthocyanins. It resulted in significantly lower anthocyanin yield but increased

callus growth. This auxin most probably exerts its effect by increasing primary metabolic activity. It has been speculated that, this hormone acts on the phenylpropanoid pathway. Substrates for phenylpropanoid metabolism are the end products of primary metabolic activity. Precursors for anthocyanin biosynthesis are indirectly provided by primary metabolism. Therefore, 2,4-D has its influence on the pathway that links primary metabolism to secondary metabolism. Primary metabolic activity is most probably increased by 2,4-D and less energy is spent on secondary metabolic production. This hormone may increase the incorporation rate of amino acids into proteins and as a result decrease phenylalanine accumulation. The reduction of the phenylalanine pool would cause a decrease in phenylpropanoid metabolism. This in turn would reduce production of flavonoid pigments (SATO, NAKAYAMA & SHIGETA, 1996). Accumulation of phenylalanine due to cessation of cell division results in elevated PAL and CHS mRNA, leading to the induction of anthocyanin biosynthesis KAKEGAWA, SUDA, SUGIYAMA & KOMAMINE (1995).

Suspension cultures are a valuable means of propagating plant cells and are potentially useful for generation of secondary products for industrial purposes (SATO, NAKAYAMA & SHIGETA, 1996). This was the main motivation for investigating whether establishment of suspension cultures was possible with the red and white callus cultures of *O. reclinata*. Establishment of liquid cultures was performed with ease. A change from solid medium to liquid medium may result in the loss of secondary metabolite production by *in vitro* propagated plant cells, in certain species (BECKER, 1987). This was not the case with pigment producing cells of *O. reclinata*. Light microscopy showed that, this species maintained anthocyanin biosynthesis and accumulation of pigments took place in vacuoles. It may be concluded that suspension cultures are a viable means of mass producing anthocyanin accumulating cells of *O. reclinata*. Therefore, this study has shown the potential use of this culture method to propagate red cells for use in the food industry.

Plant tissue culture studies are invaluable for the optimisation of culture conditions for anthocyanin biosynthesis. However, their greatest limitation is that, they fail to recognize endogenous factors that control secondary metabolism (DIXON & BOLWELL, 1986). Molecular studies were conducted to gain insight into anthocyanin biosynthesis at the level of gene expression. The results obtained from two-dimensional electrophoresis showed that polypeptide differences exist between red and white callus of *O. reclinata*. Overall, the red callus was more active genetically as a larger subset of proteins was produced. Therefore, two-dimensional protein studies showed an increase in gene expression by the red callus of *O. reclinata*. These findings were further endorsed by results obtained with non-radioactive *in vitro* translation. These two techniques showed results which were similar in nature. Although, non-radioactive methods are not conventionally used for cell-free translation purposes, information concerning the differences in gene expression between red and white callus was extractable using this method. Translation was confirmed to have taken place for the red and white callus RNA. Polypeptide patterns showed a far greater number of total polypeptides and their intensity was far stronger compared to the polypeptide pattern of wheat germ extract proteins.

Attempts to optimise radio-active translation were in vain. Due to the expensive nature of the technique of *in vitro* translation, establishment of non-radioactive cell-free protein synthesis was regarded as a more cost effective method. Non-radioactive protein synthesis allowed for the study of proteins whose expression is dependent on external stimulus. Differences in genome expression between the white and red callus were observed. Some of the genes whose expression was induced or increased may be associated directly with the anthocyanin biosynthesis pathway or they may be involved in accumulation of anthocyanin pigments in vacuoles. However, the observed changes in polypeptide patterns do not reveal the effects of post-translational processing of neosynthesized proteins through glycosylation and/or phosphorylation.

7.2 FUTURE PROSPECTS

This study has shown that, in *O. reclinata* callus cultures, production of pigment is induced by high-light illumination. It would be of interest to investigate which part of the light spectrum is involved in controlling expression of anthocyanin genes. Many of the genes involved in the flavonoid pathway have been cloned and characterized from a range of plant species. Due to the availability of heterologous probes encoding anthocyanin genes, northern hybridization studies could be conducted to gain further insight into the control of anthocyanin gene expression.

Polypeptide differences between the red and white callus of *O. reclinata* were noted using the techniques of two-dimensional SDS-PAGE and non-radioactive *in vitro* translation. Optimisation of radioactive translation would yield more conclusive results. The major problems experienced with this work was the production of insoluble 40S[^{35}S]Met-tRNA complexes and the termination of translation before completion of synthesis of polypeptides. Removal of insoluble complexes by dialysis or by digestion with protease-free RNase A could be attempted. Inclusion of commercially available RNase inhibitors in translation reaction vessels might prevent degradation of the RNA template before translation is complete. With the optimisation of *in vitro* translation, identification of specific genes which are under regulatory control with hybrid arrested translation could be performed.

Long term objectives foreseen for this project would be to isolate and characterize some of the genes which encode key enzymes of anthocyanin biosynthesis. Structural comparisons could be made with genes isolated from other plant species. It has been shown that the key enzymes are encoded by multigene families in most plant species. It would be interesting to investigate whether this holds true for *O. reclinata*.

Regulatory genes acting upon structural genes of the anthocyanin synthesis pathway have been identified in all plants where anthocyanin genetics is established. These regulatory genes encode nuclear proteins which interact with DNA and act as transcriptional factors (BODEAU & WALBOT, 1993). Future research should concentrate on the controlling mechanisms of anthocyanin biosynthesis in *O. reclinata*. Manipulation of this pathway requires a greater knowledge about the positive and negative effectors which regulate anthocyanin production.

In conclusion, the molecular basis for co-ordinate induction of anthocyanin biosynthesis in plants remains poorly understood. This area should be a subject for further study.

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