ABSCISIC ACID METABOLISM IN A *CITRUS SINENSIS* FLAVEDO ENZYME SYSTEM

by

MALOJI KALALA (BOB)

Submitted in partial fulfilment of
the requirement for the degree of

MASTER OF SCIENCE IN AGRICULTURE

in the

Department of Horticultural Science
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ABSTRACT

This research project had its major objective the unequivocal demonstration that the plant growth regulator, abscisic acid could be produced *in vitro* from labelled precursors. In addition, this project was intended to demonstrate the metabolic relationship between β,β-xanthophyll turnover and ABA biosynthesis. Finally attempts were made to isolate the enzyme responsible for the cleavage of the immediate xanthophyll precursor to ABA, 9'-cis-neoxanthin. This was achieved using a cell-free system developed from *Citrus* flavedo which demonstrated formation of xanthoxal and abscisic acid from zeaxanthin, antheraxanthin, violaxanthin and neoxanthin. In addition product abscisic acid was shown to incorporate label from mevalonic acid lactone establishing the terpenoid origin of this plant growth regulator. 9'-*cis*-neoxanthin cleavage enzyme was present in the *Citrus* cell-free system, partially purified, and separated from lipoxygenase activity and shown to convert 9'-*cis*-neoxanthin into xanthoxal and related but unidentified neutral products

DECLARATION

I hereby declare that the results presented in this thesis are from my original investigation, except where acknowledged. I also declare that this work has not been submitted to any other university for a degree.

MALOJI KALALA (BOB)

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I would like to express my sincere gratitude to the following people;

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CHAPTER 1

GENERAL INTRODUCTION

Abscisic acid (ABA, Figure 1.1), is a naturally occurring plant growth substance present not only in higher plants but also in green algae, mosses, bacteria and some genera of fungi (Teitz et al., 1987; Giraudat et al., 1994; Marin et al.,1996). Originally believed to be a dormancy-inducing and abscission-accelerating substance, ABA has been since shown to have multiple roles during the life cycle of higher plants, and each of its functions is determined by the growth stage of the organ involved and the environment (Zeevaart and Creelman, 1988; Creelman, 1989; Davies and Jones, 1991; Hetherington and Quatrano, 1991).

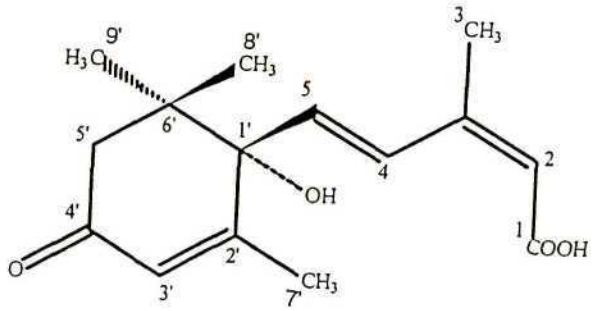

Figure 1.1 The structure of naturally-occurring (+)-S-abscisic acid

The influence of ABA on the typical life cycle of a higher plant is critical during seed development, when ABA levels increase markedly (Rock and Quatrano, 1995). This increase is part of a signal that initiates regulatory pathways which, in turn, promote seed maturation, acquisition of desiccation tolerance, and which also prevents precocious germination i.e vivipary (McCarthy, 1995). ABA also plays an important role in activating responses to environmental stress, such as extremes in osmotic and temperature stress (Ingram and Bartels, 1996). Because activation of the ABA signal transduction cascade occurs very early in response to biotic and abiotic changes, ABA acts as an internal chemical signal that enables the plant to adjust and adapt to new conditions (Davies et al., 1990; Griffiths and Bray, 1996; Dodd and Davies, 1996; Ingram and Bartels, 1996; Ivanona et al., 1997; Quatrano et al., 1997).

Although crucial in plant development, the use of ABA in agriculture and basic research application is very limited, despite considerable progress made in the identification of ABA-responsive genes, mutant characterization and signalling (Bray, 1993; Chandler and Robertson, 1994, Giraudat et al, 1994). This is because examination of mechanisms of ABA action, identification of receptor proteins, and cellular localisation of this plant growth regulator have been restricted by its rapid turnover (Abrams et al., 1997). Moreover, chemically synthesized ABA is very expensive making its use in practical farming difficult. The solution to this problem has been the development of synthetic ABA analogues which can be used to prolong ABA-like effects for agricultural and basic research application (Loveys, 1991; Lamb et al., 1996; Abrams et al., 1997).

Several ABA analogues are known and in most cases, these analogues are regarded as intermediates or catabolites on ABA biosynthetic pathways (Jones and Mansfield, 1972; Walton and Sondheimer, 1972; Sharkey and Raschke, 1980; Loveys and Milborrow, 1984, Li and Ho, 1986; Rademarcher et al., 1989, Loveys and Milborrow, 1991; Yamamoto and Oritani, 1995; Walker-Simmons et al., 1997; Balsevich et al., 1997). Although able to mimic ABA activities in some bioassays, several reports have indicated that these analogues are either unstable or show low activity in comparison to ABA (Rose et al., 1996, Suzanne et al.,1997). This is because a number of features of the ABA molecule (Figure 1.1) are essential for its activity (Milborrow, 1974b, 1978; Walker-Simmons, 1992, 1994; Perras et al., 1994; Rose et al., 1996). These include the carboxyl group, the presence of the 2-*cis* and 4-*trans* bonds in the dienoic acid side-chain and in the ring, the 4'-keto group and the 2'-double bond. These features are often rapidly removed by the presence of enzyme systems (Loveys and Milborrow,1984, 1991). Compounded to this problem is the fact that these analogues are chemically synthesized and would in principle be expensive. These facts taken together create the need to seek alternative ways which could be used in solving the problems of limited use of the phytohormone ABA for agricultural and basic research applications.

Cloning of enzymes using advanced biotechnology is one of many possibilities. The backbone of this industry is the use of recombinant proteins, obtained by inducing the host organism to express foreign proteins. The host organism can be a bacterium, a yeast, a fungus, an intact plant or animal (Meyers, 1995). One aspect of biotechnology, whole-cell bioreactors (Ogden and Bier, 1995) has been used as a cheap means for the

production of vitamins and antibiotics. This approach can also be used in solving the problem of limited use of ABA in practical agriculture. However identification of the compounds which are involved in ABA pathways and purification and characterisation of the enzymes which interconvert these compounds are a pre-requisite for using this approach. In the case of ABA, many of the constituents of the pathways have been unravelled but little is known about the enzymes involved (Walton and Li, 1995).

The enzymology of a compound can easily be studied in *vitro* using a cell-free system. Several cell-free systems have been developed and shown to contain the necessary enzymes for the biosynthesis of ABA. Recently, Richardson and Cowan (1996) developed a cell-free system from *Citrus* flavedo and its efficiency in the study of ABA biochemistry was enhanced by the use of an acetone powder. ABA concentrations reported in *Citrus* peel are among the highest (Goldschmidt et al., 1973; Brisker et al., 1976; Aung et al., 1991; El-Otmani et al., 1995) and this ABA occurs both as free and bound. It is therefore possible that the cell-free system from *Citrus* could contain all the required enzymes and the co-factors necessary for studying ABA biosynthesis.

1.1 METABOLISM OF ABA

ABA is a sesquiterpene which like other sesquiterpenoids is derived from mevalonic acid (MVA). There are two main routes whereby MVA could be converted to ABA (Figure 1.2). The direct route, also referred to as the C_{15} pathway suggests that ABA is formed by the cyclization of farnesyl pyrophosphate (FPP), the precursor of sesquiterpenoids. The alternative and more commonly accepted pathway is called the indirect or C_{40} pathway. This pathway involves the cleavage of a carotenoid such as violaxanthin (V) to yield a C_{15} compound such as xanthoxin (XAN), recently redefined as xanthoxal (Milborrow et al., 1997) which is then converted to ABA (Creelman, 1989; Parry, 1993; Giraudat et al., 1994; Walton and Li, 1995).

1.1.1 DIRECT PATHWAY

Early work on the biosynthetic pathway of ABA was based on the use of radioactive precursors such as [^{14}C]- and [^{3}H]-MVA and [^{14}C]-CO_2. Several authors reported

incorporation of label from MVA into ABA in different plant organs and tissues (Milborrow and Noddle, 1970; Milborrow and Robinson, 1973;Milborrow, 1974a; Loveys et al., 1975; Milborrow, 1983; Cowan and Railton, 1986).

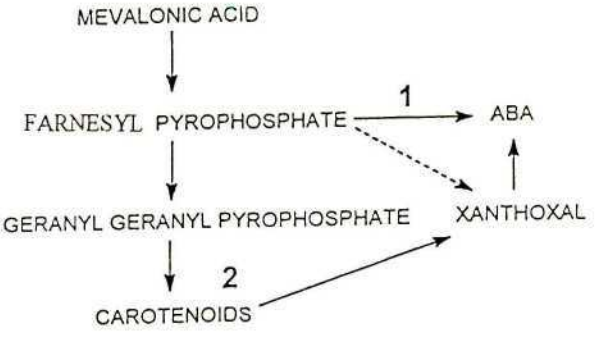

Figure 1.2. Schematic of the alternate pathways for ABA biosynthesis in plants. ① The direct pathway whereby ABA is metabolised from farnesyl pyrophosphate. ② The commonly accepted pathway involving cleavage of carotenoids (After Parry, 1993).

The major limitation in using this approach however was poor incorporation of labelled MVA into ABA (Li and Walton, 1989). This is because MVA is the precursor to many isoprenoid compounds (Figure.1.3), thus resulting in greater dilution of the radioactivity en route to ABA (Milborrow, 1969; Takahashi et al., 1986; Cowan and Railton, 1987b; Li and Walton, 1989; Milborrow, 1970; Abrams et al.,1997). Support in favour of this route has come from the fact the stereochemical retention in ABA, of hydrogens from MVA was similar to that observed for carotenoid biosynthesis (Milborrow, 1983). As a consequence, it has not been possible to distinguish between the C_{15} and C_{40} pathway using this approach.

ABA has been reported to be synthesised in several phytophathogenic fungi (Assante, et al., 1977; Bennet, et al., 1984, 1990; Yamamoto and Oritani, 1997) and its formation occurs *via* the direct pathway (Arigoni, 1975; Baker et al., 1975; Bradshaw et al., 1978; Cane et al., 1981; Cane 1983). Unlike results obtained with plants, both [^{14}C]-acetate and [^{14}C]-MVA were converted into ABA in some fungi (e.g *Cercospora cruenta, Cercospora rosicola*) with a reasonable yield (Bennet et al., 1981; Neill et al., 1984). When fed to fungi the radioactive compound, 1′-deoxy-ABA was isolated. This compound is

converted to ABA in good yield when re-fed to fungi. Other compounds related to 1' deoxy-ABA, such as -α, β or γ-ionylidene acetic acid and 4'-hydroxy-α-ionylidene acetic acid were also converted to 1'-deoxy-ABA and ABA in the fungus.

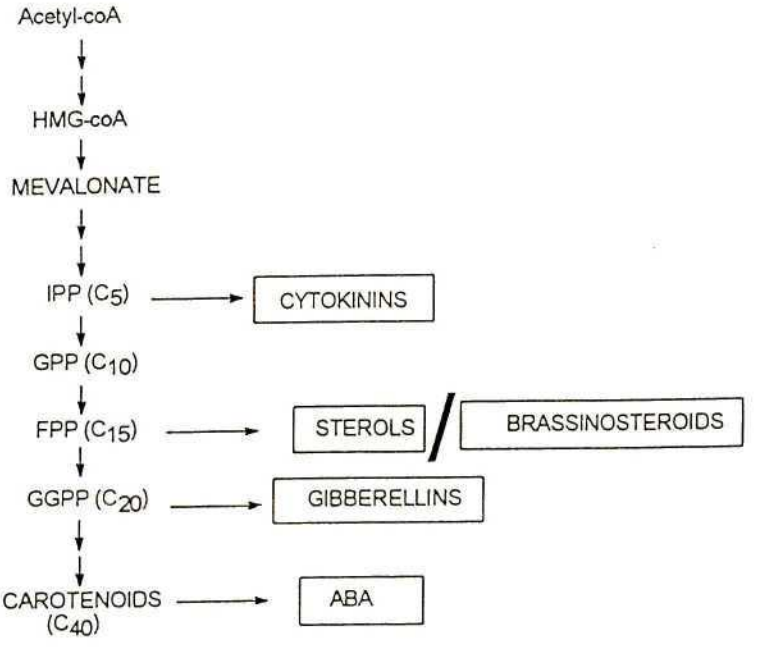

Fig. 1.3. Schematic representation of the isoprenoid biosynthetic pathways in the plants. (IPP: isopentenyl pyrophosphate; GPP: geranyl pyrophosphate; FPP: Farnesyl pyrophosphate; GGPP: geranyl geranyl pyrophosphate)

Inhibitors of carotenoid biosynthesis did not affect the accumulation of ABA in *C. cruenta* suggesting that the isoprenoid pathway from FPP was involved (Bennet et al., 1981, 1984; Oritani and Yashimita, 1985). In attempts to confirm the operation of this pathway in plants, α-ionylidene acetic acid and 1'-deoxy ABA were fed, in their radioactive form, to several plant tissues (Neill et al., 1984). Conflicting results were obtained and these seemed to depend on the tissue used. Taken together, these studies dashed all hope for similarity between the fungal and plant ABA pathways.

1.1.2 INDIRECT PATHWAY

Since the resurgence of research into ABA biosynthesis (Zeevaart and Creelman, 1988), there is overwhelming evidence which points to the direction that ABA biosynthesis in

plants occurs *via* the indirect pathway illustrated in Figure 1.4.

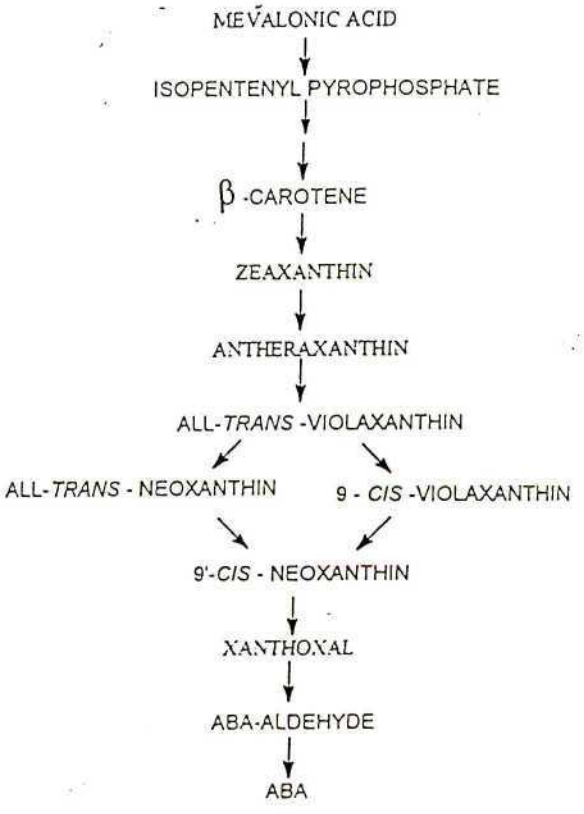

Figure 1.4. The indirect biosynthetic route of ABA from mevalonic acid as proposed by Zeevaart et al.(1989).

In many species of fungi, (*Cerscospora beticola, Cerscospora nicotianae, Cerscospora zeaemaydis*), β-carotene and other minor carotenes accumulate in mycelial cultures. In *Cerscospora rosicola,* ABA increases occurred concomitant with synthesis of carotenoids, in particular all-*trans*-β-carotene (Norman, 1991). It is therefore possible that in *Cerscospora* species, some of the ABA arises *via* the C_{40} pathway since the occurrence and rapid increase of β-carotene occurred simultaneously with increased ABA production.

Further evidence which supports carotenes as being ABA precursor comes from studies done using the microalgae *Dunalliela bardawill.* In this species, ABA has been characterised as an endogenous compound (Teitz et al., 1987) and its increase is accompanied by the increase of β-carotene (Cowan and Rose, 1991). In the same way,

the conversion of β-carotene to ABA and phaseic acid (PA) in cell-free extracts has been reported (Cowan and Richardson, 1993a, 1993b, 1997) confirming that β-carotene is an ABA precursor.

Supporting evidence in favour of the indirect pathway has come from (1) the use of inhibitors of carotenogenesis (Fong et al., 1983a, 1983b; Henson, 1984; Quarrie and Lister 1984; Moore and Smith 1985; Moore et al., 1985; Gamble and Mullet, 1986; Li and Walton 1987), (2) $^{18}O_2$ labelling studies employing a wide range of tissues (Creelman and Zeevaart, 1984; Zeevaart et al., 1989; Li and Walton, 1987; Parry et al., 1990; Zeevaart et al., 1991) and (3) ABA-deficient mutants (Giraudat et al., 1994; Marin and Marion-Poll, 1997; Leydecker et al.,1995; Schwartz et al., 1997). In addition, the discovery of possible by-products of the C_{40} pathway and observations that in water-stressed etiolated bean leaves, reduction in the level of putative xanthophyll precursors are stoichiometrically related to increases in the levels of ABA and its metabolites (Parry, 1993, Norman et al, 1990) support ABA biosynthesis from carotenoids.

Fluridone and norflurazon are known to inhibit carotenogenesis by blocking the dehydrogenation of phytoene to phytofluene (Ridley, 1982). Several authors have reported that numerous plant tissues treated with these carotenogenesis inhibitors were either ABA deficient or incapable of synthesizing ABA in response to stress (Moore and Smith, 1984; Henson, 1984; Quarrie and Lister, 1984; Moore et al., 1985; Li and Walton,1987). Normal seed development of wild-type maize treated with fluridone could be restored if sprayed with ABA (Fong et al., 1983a, 1983b). In an *in vitro* experiment, incubates of green *Phaseolus* leaves pre-treated with fluridone or water in a [^{14}C]-CO_2 environment for 24 hours and stressed in air for 14 hours, had similar reduction of specific activity (>90%) in both xanthophylls and ABA (Li and Walton, 1987). The incorporation of [^{14}C]-CO_2 into ABA and xanthophylls was equally inhibited suggesting that ABA has a carotenoid origin in plants. Since then a tremendous amount of evidence in support of the indirect pathway has come from the use of stable isotopes and mutants deficient in ABA.

1.1.2.1 Stable isotopes labelling studies

In order to understand how the concentration of ABA is regulated at the tissue and

cellular level, knowledge of its synthesis and degradation is essential. Thus it was envisaged that the use of $^{18}O_2$ would help to unravel the ABA biosynthetic pathway as the position of ^{18}O could be traced using advanced physico-chemical techniques (Neill and Horgan, 1987b, Parry and Horgan, 1992a). These studies showed that ABA extracted from stressed leaves of *Phaseolus vulgaris* and *Xanthium strumaruim* incubated in the presence of $^{18}O_2$, incorporated one atom of ^{18}O into the carboxyl (COOH) group (Creelman and Zeevaart, 1984; Netting et al., 1988; Creelman, 1989; Heath et al., 1990; Zeevaart et al, 1991). A similar labelling pattern was observed in several other tissues including non stressed leaves, roots, fruits and embryos (Gage et al., 1989; Zeevaart et al., 1989).

Zeaxanthin and its oxygenated products namely antheraxanthin (A) and all trans-violaxanthin (V) are photosynthetic pigments known to perform the important function of dissipating excess light energy within the photosynthetic pigment complexes (Demming et al.,1987). This dissipation mechanism known as the xanthophyll cycle, involves the light-induced interconversion of V to Z via A and occurs when plants are exposed to stress, particularly where the incidence of high light intensity occurs concurrently with a water deficit (Yamamoto and Bassi, 1996; Demming Adams and Adams, 1996; Demming Adams et al., 1997; Gilmore, 1997). Recent results of Marin et al. (1996), argue strongly for Z being an intermediate in the synthesis of ABA (Figure 1.5) and several authors have shown that there is a stoichiometric relationship between the amount of ABA made, measured as the increase in ABA plus its two main catabolites; phaseic acid (PA) and dihydrophaseic acid (DPA), and losses of certain xanthophylls (Gamble and Mullet, 1986; Li and Walton, 1990; Parry et al., 1990; Parry et al., 1991). It is therefore evident that manipulation of the xanthophyll cycle could be used in order to confirm which xanthophylls are ABA precursors. Li and Walton (1987) were able to do this for leaves of *Phaseolus* and replaced the epoxide oxygens of 40-45% of the V with $^{18}O_2$ in *vivo*. After the leaves had been stressed, analysis of the extracted, stress-induced ABA revealed that 10 to 15% was labelled in the 1'-hydroxyl group and had therefore been synthesized from labelled V. In the same way deuterium-oxide (^2H_2O) labelling experiments were carried out in an attempt to determine whether ABA was produced from a carotenoid precursor. Using etiolated *Phaseolus* seedlings, Parry et al. (1990) showed that deuterium from D_2O was incorporated into carotenoids. In addition, in extracts of etiolated leaves of *Phaseolus vulgaris* grown on 50% ^2H_2O, the extent of

deuteration of V, neoxanthin (N), lutein and ABA was very similar (12-18%). Although these results did not exclude the possibility of a C_{15} pathway, they were consistent with the operation of a C_{40} pathway and in agreement with the results obtained by Li and Walton (1987).

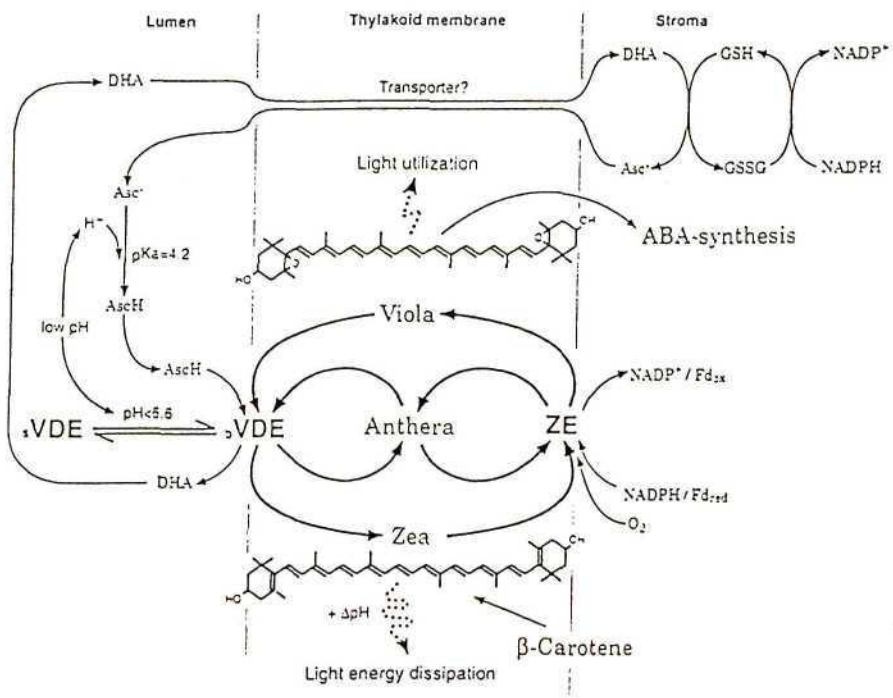

Figure 1.5. Model of the regulation of the xanthophyll cycle in relation to ABA synthesis. VDE, violaxanthin de-epoxidase, sVDE, soluble VDE; bVDE, bound VDE; DHE, dehydroascorbate; Asc$^-$, ascorbate; AscH, ascorbic acid; GSH, glutathione; Fd, ferredoxin (after Eskling et al., 1997).

1.1.2.2 Studies using mutants deficient in ABA

The characterisation of ABA-deficient mutants gave further evidence supporting the C_{40} pathway (Giraudat et al., 1994; Marin et al., 1996; Schwartz et al., 1997; Marin and Marion-Poll, 1997). These mutants display various abnormalities including precocious germination and a strong tendency to wilt, and can be restored to the wild-type phenotype by exogenous ABA. Among the best characterised mutants are the *viviparous* (*vp*) *Zea mays* (Neill et al., 1986), tomato *flacca, sitiens* and *notabilis* (Taylor et al., 1988), *Arabidopsis thaliana, aba* (Koorneef et al., 1982), potato *droopy* (Quarrie, 1982), pea

wilty (Wang et al., 1984), *Nicotiana plumbaginifolia*, *aba*1 (Taylor, 1991; Parry et al., 1991; Rousselin et al., 1992).

Each of these mutants is impaired in a specific step of the ABA biosynthetic route. The early stage of carotenoid biosynthesis is impaired by the *Zea mays* *vp2*,*vp5*, vp7, and *vp9* mutants (Moore and Smith, 1985; Neill et al., 1986). *Arabidopsis aba* mutants are impaired in the epoxidation of Z, which is considered as the first step in ABA formation (Duckham et al., 1991; Rock and Zeevaart, 1991). The tomato *flacca* and *sitiens* (Taylor et al, 1988) and potato *droopy*, barley *nar*2, *N. plumbaginifolia aba*1, *aba*2, *aba*3 (Parry et al, 1991a,b; Rousselin et al., 1992; Leydecker et al., 1995; Marin and Marion-Poll, 1997; Schwartz et al., 1997) mutants are all blocked in the final step(s) of ABA biosynthesis; i.e oxidation of XAN to ABA.

In addition, the identification of several ABA-biosynthetic "by-products" in different plant tissues provided additional circumstantial evidence in favour of a C_{40} pathway (Taylor, 1987; Linforth et al., 1987a; 1987b; Milborrow et al., 1988). Oxidative cleavage of 9,9'-di-cis-V, across the 11,12 and 11' and 12' double bonds produced two XAN molecules and a central dialdehyde, C_{10} residue (2,7-dimethyl-2,4,6-octatrienedaildehyde). Taylor (1987) proposed that *in vivo*, the C_{10} dialdehyde would be oxidised to the corresponding dicarboxylic acid (2,7-dimethyl-2,4,6-octatrienedioic acid, OTA). In addition, an unknown compound, 2,7-dimethyl-2,9-octadienedioic acid (ODA) was identified in leaves of the wilty tomato mutant *flacca*. However, whether ODA or OTA are ABA byproducts is still a matter of debate (Koorneef, 1986; Neil and Horgan, 1987b). When wild-type tomato shoots were incubated in ^2H_2O for 6 days prior to severe wilting, the extracted ABA revealed the presence of up to 11 ^2H atoms while no label was detected in ODA suggesting that in wild type tomato ABA and ODA have different origins (Milborrow et al., 1988) .

1.1.2.3 Correlative changes in ABA and xanthophylls

Several authors have shown that there is a correlation between a decrease in xanthophyll content and amount of ABA synthesized. In many cases, the stochiometric decrease in xanthophylls which is accompanied by ABA synthesis, was due to a decrease in 9'-cis-N and 9'-cis-V, but more importantly to a decrease in all-trans-V (Parry, 1993). Following

dehydration of fluridone-treated etiolated seedlings of barley (*Hordeum vulgare*), Gamble and Mullet (1986) observed a decrease in the levels of V which occurred concomitantly with an increase in ABA. In addition, a comparison between *Phaseolus vulgaris* leaves grown under light and dark, showed that etiolated leaves synthesized large amounts of ABA in response to water stress although they had much reduced xanthophyll levels (Li and Walton ,1990; Parry et al., 1990). Similar findings were obtained in both soil-grown and hydroponic roots of *Lycopersicon esculentum* (Parry et al., 1991a). This author found that water stressed, soil grown plants of *Lycopersicon esculentum* had an increase in ABA levels which correlated with a decrease in xanthophyll levels in a ratio of 1:1. These findings are consistent with the operation of the pathways illustrated in Figure 1.4.

Despite reports by Willows et al. (1994), Netting and Milborrow (1994) that there are two precursor pools involved in ABA synthesis and that neither consists of carotenoids, and statements by Netting and Milborrow (1994) that the endogenous precursor of stress-induced ABA is an as yet unidentified compound, several lines of evidence clearly show that the ABA biosynthetic route in plants includes the oxidative cleavage of a xanthophyll to a C_{15} compound that is then metabolised to ABA (Rock and Zeevaart, 1990, 1991; Duckham et al., 1991; Li and Walton, 1990; Parry et al.,1990; Parry and Horgan, 1991a,b; Rock et al., 1992; Walton and Li, 1995; Cowan and Richardson, 1997). Recently, it was shown by Lee and Milborrow (1997b) that [^{2}H]-carotenoids added to a cell-free system prepared from avocado fruit gave rise to [^{2}H]-ABA. In agreement with these observations, it has been unequivocally established that 9'-cis-N synthesized from either all-trans-N or 9'-cis-V (Figure 1.5) is the major precursor of ABA in plants (Parry, 1993; Walton and Li , 1995; Cowan and Richardson, 1997).

1.1.2.4. Cleavage of 9'-cis-neoxanthin

By the early 1970's, XAN was regarded as the in *vivo* precursor of ABA which arose either by photolytic or enzymic xanthophyll breakdown (Taylor and Burden, 1970, 1972; Firn and Friend 1972). XAN occurs as a mixture of the 2-*cis* and 2-*trans* isomers in a ratio of 1:2 and 1:10 (Milborrow et al., 1997a). However, only the 2-*cis* isomer has been shown to be biologically active and converted to ABA, suggesting that the xanthophyll precursor to ABA has a *cis*-configuration to yield *cis*-XAN and subsequently ABA (Taylor and Burden, 1974, 1973).

Sindhu and Walton (1987, 1988) showed that cell-free systems from leaves of *Phaseolus vulgaris, Vigna radiata, Zea mays, Cucurbita maxima* and *Pisum sativum* and roots of *Phaseolus vulgaris* successfully converted XAN to ABA. Further evidence that XAN is an ABA precursor was obtained with cell-free systems from ABA-deficient mutants which convert XAN to ABA very poorly (Parry et al., 1988; Taylor et al., 1988). The outcome of these experiments was based on observations made when synthetic XAN was fed to these mutants. When [^{13}C]-XAN was fed to tomato mutants and the wild-type plant, wild type and *nor* mutants readily converted XAN into ABA but *flc* and *sit* plants converted only a small amount of applied XAN (Parry et al., 1991a). In all plants, t-XAN was not converted to ABA. Biosynthesis of ABA at the C_{15} level was investigated by feeding XAN to detached leaves of *Nicotiana plumbaginifolia*. Wild type leaves converted 9-19% of applied XAN to ABA while 1% only was converted in the mutants. Several other ABA-deficient mutant plants have been identified which are unable to oxidise the aldehydic group of XAN into the carboxylic group of ABA (Leydecker et al., 1995; Walker-Simmons et al., 1989; Sindhu and Walton, 1988).

Recently, Yamamoto and Oritani (1996) investigated the stereoselectivity of the conversion of XAN to ABA using XAN prepared from (*R*) and (*S*)-4-hydroxy-β-cycogeraniol via asymmetric epoxidation. They found that deuterium-labelled XAN fed to *Lycopersicon esculentum* Mill. was substantially incorporated into ABA both under non-stressed and stressed condition. Although, it is well established that XAN is an intermediate between xanthophylls and ABA, controversy still surrounds the identity of XAN metabolites. Currently, it is believed that ABA is biosynthesised from XAN via abscisic acid aldehyde (AB-ald)(Parry, 1993). However, the discovery of a shunt pathway between AB-ald and ABA (Sindhu et al., 1990; Taylor, 1987) coupled with the identification of ABA-diol as a naturally occurring compound (Parry et al., 1993; Vaughan and Milborrow, 1987; Richardson and Cowan. 1996), has left the post-XAN stages somewhat debatable. Based on these findings, several possible pathways for the conversion of XAN to ABA are presented (Figure 1.6).

In leaves of *flacc*a and *siti*ens mutants of tomato, a compound identified as 2-trans-ABA-alcohol (t-ABA-alc) was found to accumulate, especially after water stress, and this was proposed as an ABA precursor (Linforth et al., 1987b). Later, Taylor et al., (1988) and Duckman et al., (1989) reported that wild-type leaves converted AB-ald to ABA.

However, mutants impaired in the conversion of AB-ald to ABA i.e *flc* , *sit* in tomato and the *droopy* in potato mutants converted [^{2}H]-AB-ald to t-AB-alc. Several other mutants have been reported which are able to convert AB-ald to ABA (Parry et al., 1991; Linforth et al.,1987; Rock and Zeevaart 1990). Although these results were taken as evidence for AB-aldehyde being the immediate ABA precursor, Parry and Horgan (1991a,b) found that 1',4'-trans-diol-ABA was present in tomato wild-type and its mutants.

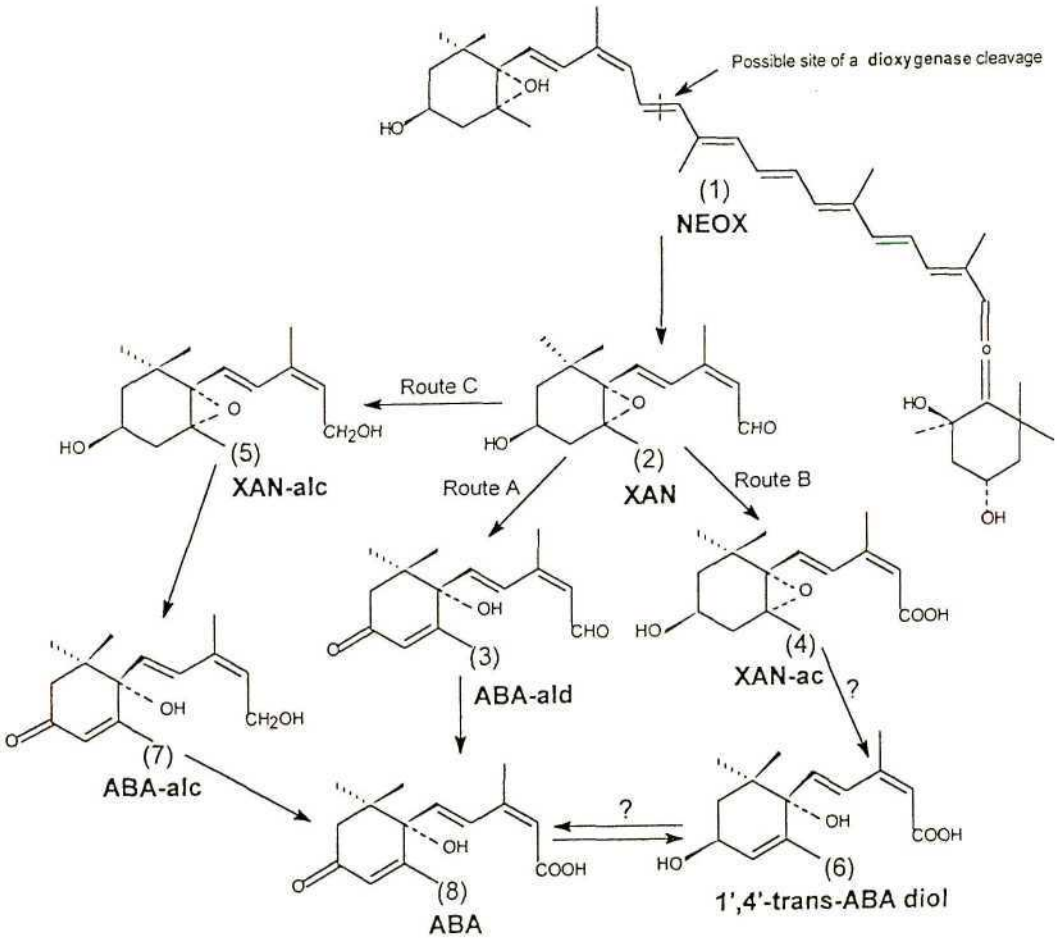

Figure 1.6. Post-N cleavage as proposed by Parry (1993) (A); and Richardson and Cowan (1997) (B). N (1) is cleaved by a dioxygenase enzyme to yield XAN(2). XAN is oxidised and isomerised to AB-aldehyde (3)which undergoes oxidation to yield ABA(8) (route A). Alternatively, XAN is converted to XAN-acid(4) and 1',4'-trans-ABA-diol (6) by two oxidation steps. 1',4'-trans-ABA diol is later oxygenated to (8) (route B). In addition, XAN has been suggested to be converted to (8) *via* XAN alcohol (5) and ABA-alcohol (route C)(Lee and Milborrow, 1997a).

The position of 1',4'-*trans* ABA diol in the biosynthetic route of ABA is controversial. When this compound was fed in the form of 1',4'-*trans*-[2-^{14}C, 4'-^{2}H]-diol to tomato shoots, the deuterium atom lost from the 4'-position of this substrate during conversion revealed that it is a catabolite (Vaughan and Milborrow, 1987). Similar observations were made by Rock and Zeevaart, (1990) using $^{18}O_2$ labelling studies in apple and Cowan and Richardson (1993a) reported that 1',4'-*trans*-ABA diol was obtained as a catabolite when R-[2-^{14}C]-mevalonic acid was fed to the *Citrus* cell-free system. In contrast, there are several reports suggesting that 1',4'-*trans*-ABA diol is a precursor of ABA (Vaughan and Milborrow, 1987; Okamoto et al, 1987; Parry et al., 1988, Richardson and Cowan, 1996, Cowan and Richardson, 1997). When FPP, GGPP and 9-*cis*-β-carotene were used as substrate in a *Citrus* cell-free system, Cowan and Richardson(1997), did not detect AB-ald rather XAN appeared to be converted to ABA via a compound tentatively identified as xanthoxic acid (XAN-ac) and 1',4'-*trans*-ABA diol (Figure 1.6, route B). The presence of XAN-ac in ABA biosynthetic pathway has been reported elsewhere (Milborrow and Garmston, 1973; Milborrow et al.,1997b).

1.3. THE ENZYMOLOGY OF ABA

The early part of ABA biosynthesis can be considered to be the conversion of all-*trans*-V to either all-*trans*- N or 9'-*cis*-V which is later converted to 9'-*cis* -N (Figure 1.4) (Parry, 1993). The nature of the enzymes responsible for this conversion (allene formation and isomerisation) are unknown. Parry (1993), suggested that these enzymes may exist in a dual-enzyme complex as all-*trans*-N does not accumulate during the conversion of all-*trans*-V in developing chloroplasts, or decrease following water stress. Nonetheless, taking into consideration that the immediate xanthophyll precursor in the ABA biosynthetic route is 9'-cis-N (Parry and Horgan, 1991a, 1991b; Parry, 1993; Cowan and Richardson, 1997) which is further metabolised to ABA via the aldehyde, XAN (Figure 1.6), a discussion on the putative enzymology, at least for this section of the ABA pathway is possible.

Since the pioneering work of Firn and Friend (1972), who showed that V is enzymatically cleaved by LOX to yield XAN and t-XAN, several workers have investigated the possible role of LOX in ABA and apo-carotenoid biosynthesis using LOX Inhibitors (Kleinig and Beyer, 1985; Parry and Horgan, 1991b; Creelman et al., 1992). Stress induced ABA

accumulation in soybean cell cultures and soybean seedlings was inhibited by specific LOX inhibitors (Creelman et al., 1992). Using a cell-free extract from *Citrus sinensis* flavedo, Richardson (1995), showed the presence of a protein which co-eluted with the standard soybean LOX under reducing conditions. In addition LOX-like activity has been detected in the *Citrus* cell-free system and preliminary studies indicated that the addition of naproxen, which inhibits LOX activity, retards conversion of *R*-[2-^{14}C]-MVA into ABA with a concomitant increase in label associated with the neutral, pigment containing fraction (Cowan and Richardson, 1997, Lee and Milborrow, 1997b).

With the advent of mutants deficient in ABA (Giraudat et al., 1994), several groups have reported on the cloning of genes for the oxidative cleavage of the enzyme involved in the cleavage of N (Schwartz et al., 1997; Tan et al., 1997; Burbridge et al., 1997). Using an ABA-mutant of maize, Schwartz et al.(1997), cloned the *vp*14 gene, and showed that its recombinant VP14 protein catalyses the cleavage of 9-cis-epoxy-carotenoids to form C_{25} apo-aldehydes and XAN. These results were confirmed by Tan et al. (1997), who found that there is a family of *vp* genes with protein sequences similar to bacterial lignostilbene dioxygenases (LSD's) which catalyse a double-bond cleavage reaction that is closely analogous to the cleavage reaction involving *cis*-xanthophylls (and carotenes). Similarly, Burbridge et al. (1997), isolated a tomato cDNA homologue of the maize VP14 sequence which has been referred to as neoxanthin cleavage enzyme (NCE). However, Burbridge et al. (1997) reported that the VP14 fusion protein was able to cleave 9'-cis isomers of Z, V, N. The former (i.e Z) is certainly not considered to be an immediate ABA precursor and has been shown to be converted to V *via* A by another enzyme, ZE (Marin et al., 1996). Therefore the substrate specificity of this cleavage enzyme in plants remains to be elucidated.

Several studies have implicated a molybdenum (Mo)-requiring oxidase enzyme as the terminal enzyme in ABA biosynthesis (Richardson and Cowan, 1996; Sindhu et al.,1990; Walker-Simmons et al., 1989). Sindhu et al. (1990) fractionated the "XAN oxidising" activity from *Phaseolus* leaves and showed that the activity of this enzyme requires a Mo-ion. Similarly, Richardson and Cowan (1997) have showed that Mo stimulated ABA-biosynthesizing activity in a cell-free preparation from *Citrus* flavedo. Furthermore, a requirement for NAD^+ or NADP (Sindhu and Walton, 1987) suggests that the enzyme concerned is a Mo-containing hydroxylase possibly of $NAD(P)^+$ Mo- requiring enzymes.

Because of the oxidative steps occurring during ABA biosynthesis, several authors have suggested the possible involvement of cytochrome P-450 mixed function oxygenases. It has been shown for example that the hydroxylation at the 3' position in β-carotene yields Z (Zairen et al., 1996; Misawa et al., 1995). The enzyme responsible for this reaction β-carotene hydroxylase has been shown to be dependent on molecular oxygen, stimulated by NADH, sensitive to CO, giving it all the characteristics of a monooxygenase (Sandman and Bramley, 1985). It is well established that the conversion of ABA to PA via 8'-hydroxy ABA is catalysed by ABA 8'-hydroxylase which is a cytochrome P-450 enzyme (Gillard and Walton, 1976; Zeevaart et al., 1989; Creelman and Zeevaart, 1984; Creelman et al., 1992; Richardson and Cowan, 1996; Windsor and Zeevaart, 1997; Cutler et al., 1997). Reports have shown that sterol cytochrome-P450-dependent enzyme inhibitors reduced ABA biosynthesis in the fungus *Cercospora rosicola* whereas inducers of cytochrome P-450 (eg, DTT) enhanced ABA synthesis (Norman et al., 1988). To obtain XAN oxidase activity from leaves of tomato, Sindhu and Walton (1988) had to include DTT in the extraction buffer. Using a cell-free system derived from imbibed barley embryos, Cowan and Railton, (1987a) showed that incorporation of label from MVA into ABA was inhibited by both anaerobiosis and CO. This suggests the participation of mixed function oxygenases in ABA biosynthesis. In addition the observation that ancymidol reduced incorporation of label into ABA in both the barley embryo and *Citrus* exocarp cell-free systems is further evidence supporting the involvement of a cytochrome P-450 in the ABA biosynthetic pathways (Cowan and Railton, 1987b; Richardson and Cowan, 1996).

1.4. CELL-FREE STUDIES IN ABA BIOSYNTHESIS

Until recently, most studies on the elucidation of the ABA biosynthetic pathway were carried out *in vivo* using intact plant tissues and in many cases, results were inconclusive. More recent experiments employed greater accuracy in the procedures used for the measurement of ABA levels and have made use of mutants deficient in ABA in order to determine both precursors and catabolites of this important plant growth regulator (Giraudat et al., 1994). In addition, the discovery of mutants impaired in one of the steps involved in ABA biosynthesis has shed more light on the understanding of the ABA biosynthetic pathway and its enzymology. Another avenue which has been used to study the biochemistry of ABA is the development of cell-free systems capable of

biosynthesizing ABA (Milborrow, 1974a; Hartung et al., 1981; Cowan and Railton, 1987a; Cowan and Richardson, 1993a, 1993b, 1997; Richardson and Cowan, 1997, Lee and Milborrow, 1997b).

Using cell-free extracts from *Cercospora rosicola*; Al-Nimri and Coolbaugh, (1991) showed that 1'-deoxy-^{2}H-ABA was easily converted to ^{2}H-ABA. This makes possible the characterization of the enzyme responsible for the conversion of 1'-deoxy-ABA to ABA in ABA-producing fungi. Until recently, only a few studies had attempted to demonstrate the cell-free biosynthesis of ABA and for the most part these were met with little success. Milborrow, (1974a), reported that a cell-free system of lysed chloroplasts biosynthesized ABA from MVA with low activity. However, subsequent work could not reproduce these findings (Cowan and Railton, 1986; Hartung et al., 1981). More recently a cell-free system derived from imbibed embryos of *Hordeum vulgare* was shown to synthesize a range of terpenyl pyrophosphates from labelled MVA which was subsequently transformed into an acid with similar chromatographic properties to those of ABA (Cowan and Railton, 1987a).

In addition the conversion of XAN to ABA in cell-free systems has been reported (Sindhu and Walton, 1987, 1988). These authors observed that cell-free extracts from *Phaseolus vulgaris* leaves and turgid or water-stressed leaves of wild type tomato (*Lycopersicon esculentum*) and its wilty mutants *sit, flc* and *not* contained XAN oxidising activity. This enzyme was shown to require NAD and NADP to be active.

Cowan and Richardson, (1993a, 1993b, 1997) successfully demonstrated that labelled MVA and all-*trans*-β-carotene were converted into a range of β,β-xanthophylls and ABA metabolites and catabolites in a cell-free system from *Citrus sinensis* exocarp. Similarly, Richardson and Cowan, (1996) reported that this cell-free system produced ABA from both a terpenyl pyrophosphate and carotenoids. The biosynthesizing activity of this system was shown to be stimulated by reduced nicotinamide nucleotides, molybdate, AMO 1618 and a cold-pool trap of (±)-ABA, but was inhibited by FAD. It is therefore evident that this cell-free system can be used to study the enzymes involved in the last step of ABA biosynthesis since it is enzymatically active and able to carry out the anabolism and catabolism of ABA yielding quantifiable amount of products.

1.5 OBJECTIVES

Now that the elucidation of the ABA biosynthetic pathway is almost complete and the control points identified, it should be possible to purify and characterise the enzymes responsible for either the cleavage of N or the oxidation of XAN from the *Citrus* flavedo cell-free system. Thus the present research project will utilize the cell-free system developed by Richardson (1995) to;

① confirm ABA biosynthesis via 9'-*cis*-N and XAN and evaluate the effects of chemical modifiers on this process,

② and attempt to purify and characterise xanthophyll cleavage enzyme.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CHEMICAL AND LABORATORY SUPPLIES

2.1.1.1 Radioactive and stable isotopes

DL-*cis*, *trans*-[2-^{14}C]-ABA (sp. act. 833 MBq mmol^{-1}) was obtained from Armersham International, Buckinghamshire, UK and 2-DL-[^{13}C]-mevalonolactone (MVL) was obtained from Euriso-Top, France.

2.1.1.2 Fine chemicals and cofactors

2-isopropyl-4'-trimethylammonium chloride-5'-methyl phenyl piperidine-1'-carboxylate (AMO 1618) and DL-dithiothreitol (DTT) were purchased from Calbiochem, Novabiochem, Corp, La Jolla, CA, USA. Adenosine tri-phosphate (ATP); the disodium salt ($C_{10}H_{14}N_5O_{13}P_3Na_2$), β-nicotidamide-adenine (NAD); the free salt ($C_{21}H_{23}N_7O_{14}P_3$), β-nicotidamide-adenine dinucleotide phosphate (NADP); the disodium salt ($C_{21}H_{26}N_7O_{17}P_3Na_2$) and β-nicotidamide-adenine dinucleotide phosphate reduced (NADPH); the tetra-sodium salt ($C_{21}H_{30}N_7O_{17}P_3Na_4$) were bought from Boehringer Mannhein,GmbH, Germany. Glutathione (GSH) and diethyl dithiocarbamic acid (DDC); the sodium salt ($C_5H_{10}NS_2Na$) were obtained from Sigma, St Louis. MO, USA.

Bovine serum albumin (BSA), *cis*-9-*cis*-12 octadecadienoic acid (linoleic acid), lipoxidase, 2,6-di-*t*-butyl-*p*-cresol (BHT), 4-hydroxypyrazolo[3,4-d]pyrimidine; HPP (allopurinol) were obtained from Sigma, St Louis. MO, USA. Serva blue, was obtained from Feinbiochemica, GmbH&Co. Triton X-100, ethylene diamine tetraacetic acid, (EDTA) were obtained form BDH Chemicals Ltd, Poole, England.

Polyoxyethylene sorbitan monolaurate (Tween 20) and polyoxyethylene sorbitan monooleate (Tween 80) were from E. Merck, Darmstadt, Germany. Pico Fluor 40 scintillation cocktail was purchased from Packard Instrument Company, USA . Colbatous sulphate ($CoSO_4.7H_2O$) and sodium molybdate (Na_2MoO_4)AR grades were supplied by Associated Chemicals Enterprises, RSA. Nickel sulphate ($NiSO_4.6H_2O$) and sodium chloride (NaCl) were obtained from uniLAB, SAARCHEM, RSA, While potassium tungstate (K_2WO_4) was purchased from Aldrich Chemical Co., USA. Phenyl-Sepharose CL-4B was obtained from Sigma Chemical Company, St Louis, Mo, USA.

2.1.1.3 Solvents

High Performance Liquid Chromatography (HPLC) grade solvents (acetonitrile; UV cutoff 189 nm, ethyl acetate; UV cutoff 253 nm and methanol; UV cutoff 193 nm) were obtained from Burdick and Jackson, USA. The AR grade solvents were supplied either by NT Laboratory (Pty) Ltd., RSA or Associated Chemical Enterprises, RSA. Glacial acetic acid (100%) was purchased from Merck, Germany.

2.1.1.4 Chromatographic media

For thin layer chromatography (TLC) plates of required thickness (0.20 mm or 0.25 mm) were obtained ready for use from Merck, Darmstadt, Germany. For high performance liquid chromatography (HPLC) analysis, columns were as follow: for analysis of pigments, a C_{18} ODS Zorbax column (4.6 mm x 25 cm) supplied by Phenomenex, Torrance, CA was used, and for analysis of XAN and ABA either a 5µm C_{18} ODS2 Spherisorb column (250 x 4.60 mm) purchased from Deeside Ind. Est., Queensferry, Clwyd, UK, or a 5µm C_{18} ODS Sphereclone (250 x 10 mm i.d.) purchased from Phenomenex Torrance, CA was used. For gas chromatography-mass spectrometry (GC-MS), a capillary column (15 mm x 0.25 mm i.d.) with 0.3 µm coating of SPB-1 was purchased from SUPELCO, Bellefonte, CA, USA was used.

2.1.2 PREPARATION OF REAGENTS AND STANDARDS

2.1.2.1 Buffered linoleate-carotene solution

Buffered carotene-linoleate solution was prepared as follows: linoleic acid (1 mL) in ethanol (7.5%, w/v) was mixed with 0.3 mL of Tween 80 in ethanol (10%, v/v). Aqueous EDTA (5 mL, 0.5 %) was added and the pH adjusted to 9 by dropwise addition of 0.1 M NaOH, the volume was then made up to 10 mL with distilled water and stored under nitrogen for up to a week (solution A). β-carotene (25 mg) and 0.9 mL of Tween 80 were dissolved in 25 mL of chloroform. One mL of this solution was evaporated to dryness using a rotary evaporator and the residue immediately dissolved in 10 mL of EDTA solution (0.25%) (Solution B). One mL of solution A was mixed with one mL of solution B and the volume made up to 10 mL with 0.2 M citrate-phosphate buffer (pH 7.0). The buffered carotene-linoleate solution was prepared immediately before use and contained 750 µg of linoleic acid, 10 µg of β-carotene, 0.66 µL of Tween 80 and 0.5 mg of EDTA per mL.

2.1.2.2 Bradford reagent

Bradford dye-binding-reagent was prepared by dissolving 500 mg of Serva blue in 250 mL of 95% ethanol. Concentrated o-phosphoric acid (500 mL) was added followed by distilled water to a final volume of 1 L (Bradford, 1976). This mixture was stirred overnight and filtered through Whatman N° 1 filter paper. This solution was stored in a brown bottle at 4° C and diluted five times before determination of protein. If precipitation was visible before use, the reagent was filtered.

2.1.2.3 Preparation of ethereal diazomethane

Ethereal diazomethane was generated at room temperature without co-distillation, by hydrolysis of N-nitroso-N- methyl urea (obtained from Sigma Chemicals) with NaOH (5 N) in a Wheaton Diazomethane Generator (Pierce Chemical Co., Rockford, ILL, USA) using the small scale technique described by Fales et al. (1973). Basically 133 mg N-nitroso-N- methyl urea and 0.5 mL distilled water used to dissipate heat generated, were

placed inside the tube while 3.0 mL dry diethyl ether was placed in the outer tube. The two parts were assembled and held together with a pinch-type clamp and then placed in an ice-bath. Thereafter 0.6 mL 5 N NaOH injected through the Teflon rubber septum. The reaction was allowed to proceed for more or less 45 min or until the ether developed a deep yellow colour.

2.2 METHODS

2.2.1 PREPARATION OF CELL-FREE SYSTEM

2.2.1.1 Preparation of cell-free extracts

Aliquots of the dried *Citrus* flavedo acetone powder prepared and stored at 4^{0}C (Cowan and Richardson, 1993; Richardson and Cowan, 1996), were homogenised in 0.1 M of Tris-HCl buffer (pH 7.5) containing 10 mM DTT, 5 mM $MgCl_2$ and 0.1 % detergent (Tween 20, Tween 80 or Triton X100). The slurry was filtered through a double layer of Miracloth (Calbiochem, La Jolla, CA, USA) and centrifuged at 23 500 g for 30 min at 2 ^{0}C using a Hitachi CR 20B refrigerated centrifuge. The supernatant was used to determine protein concentration and assay ABA biosynthesis.. The supernatant was also used to measure LOX-like enzyme activity and neoxanthin cleavage enzyme (NCE) activity following fractionation by ammonium sulphate [$(NH_4)_2SO_4$] precipitation at 30 %, 45 %, 65 % and 100 %. The pellet was resuspended in Tris-HCl buffer and stored at -70 ^{0}C.

2.2.1.2 Determination of protein concentration

To determine protein concentration, Bradford dye-binding reagent (1 mL) was mixed with different concentrations of the proteins and the volume made up to 5 mL with distilled water. The mixtures were vortexed and the colour allowed to develop at room temperature for 5 min. The absorbance was then measured at 595 nm using a Metertek SP-850 spectrophotometer. A standard curve was constructed using several concentrations of the BSA. Unknown protein concentrations were established from linear regression analysis of calibration curves. All assays were done in triplicate.

2.2.1.3 Protein purification by low pressure hydrophobic column chromatography

Low pressure hydrophobic chromatography was used to separate protein required to study the activity LOX enzyme and NCE. For LOX enzyme activity, $(NH_4)_2SO_4$ precipitated protein pellets were directly applied to a phenyl Sepharose CL-4B column (0.5 x 7 cm), with a bed volume of 5.5 mL, equilibrated with 0.5 mM of potassium phosphate (K_2PO_4) buffer (pH 7.5) containing 0.5 M of $(NH_4)_2SO_4$. After removal of unbound inactive proteins with high salt buffer, proteins of interest were eluted using a decreasing linear gradient of 0.5- 0 M of the equilibration buffer [K_2PO_4 + 0.5 M of $(NH_4)_2SO_4$ buffer (pH 7.5)] at a flow rate of 1 mL min^{-1} maintained by a Miniplus 3 Peristaltic pump. Absorbance at 280 nm was determined using a Beckman DU spectrophotometer and the same instrument was used to measure LOX-like activity at 460 nm. For studies related to NCE, $(NH_4)_2SO_4$ precipitated and phenyl Sepharose CL-4B purified proteins were first dialysed in Tris-HCl buffer.

2.2.2 REACTION INCUBATIONS

Aliquots of the crude enzyme extract and $(NH_4)_2SO_4$ precipitated fractions prepared as described in section 2.2.1.1 were incubated as described by Cowan and Richardson (1993a). Routinely these incubates contained 2.0 mL of 23500 g supernatant (equivalent to ± 2.5 mg protein) in 50 mM of Tris-HCl buffer (pH 7.5) containing glutathione (5 mM), sodium fluoride (5 mM), ATP (5 mM), AMO1618 (1 mM), $MgCl_2$ (1.25 µM) , NAD^+, NADH, NADPH (0.125 mM) and either sodium or ammonium molybdate (1 µM). In addition, $NiCl_2$, K_2WO_4, $CoSO_4.7H_2O$ and allopurinol (0.1 mM) were added singly or in combination and the volume adjusted to 4 mL with the homogenizing buffer (0.1 M of Tris-HCl buffer (pH 7.5) containing 10 mM of DTT, 5 mM of $MgCl_2$). Where $(NH_4)_2SO_4$ precipitated fractions were used as source of enzyme, these fractions were first dialysed against 0.1 M Tris-HCl buffer (pH 7.5) in the ratio 1:200 (v/v) for 2 h. Following addition of substrate, reactions were initiated by addition of enzyme and allowed to proceed for 2 h at 37 °C under laboratory light in a water bath unless otherwise specified. On completion, reactions were terminated by heating to 100 °C for 3 min and allowed to cool to room temperature. Protein was then precipitated by the addition of an equal volume of ice-cold methanol containing DCC (200 mg L^{-1}) and/or BHT (100 mg L^{-1}) as

antioxidant(s). Where non-labelled substrates were used, radioactive [^{14}C]-ABA was added to correct for losses during extraction and purification.

For XAN assays, the 45% $(NH_4)_2SO_4$ precipitated fraction was used. After purification of protein by low pressure hydrophobic chromatography, high salt [0.5 M of $(NH_4)_2SO_4$], gradient [0.5 to 0 M $(NH_4)_2SO_4$] and low salt [0 M $(NH_4)_2SO_4$] fractions were respectively pooled together, dialysed and incubated in 50 mM of Tris-HCl buffer (pH 7.5) containing glutathione (5 mM), sodium fluoride (5 mM), ATP (5 mM), AMO1618 (1 mM), $MgCl_2$ (1.25 µM) , NAD^+, NADH, NADPH (0.125 mM) and either sodium or ammonium molybdate (1 µM). Following addition of 9'-*cis*-N, reactions were initiated by the addition of enzyme (dialysed pooled fractions) and allowed to proceed for 15 min at 37 ^{0}C. On completion, reactions were terminated by addition of an equal volume of ice-cold methanol containing DCC (200 mg L^{-1}) and/or BHT (100 mg L^{-1}) as antioxidants and stored at - 20 ^{0}C.

2.2.3 ASSAY FOR LIPOXYGENASE BLEACHING

Carotenoid bleaching was determined as per Ben-Aziz et al.(1971). Crude extracts and ammonium sulfate precipitates were used as enzymatic sources to determine LOX-like activity present in the *Citrus* cell-free system. The reaction mixture contained 1.5 mL of buffered carotene-linoleate, enzyme, BHT in alcohol and distilled water. Reactions were carried out at ± 25 °C in a water bath, initiated by addition of enzyme and allowed to proceed for 1 min before being terminated by the addition of an equal volume of BHT in ethanol (0.106 mg mL^{-1}) to give a total volume of 3 mL. The degree of bleaching was determined spectrophotometrically at 460 nm using a Backmann DU 65 spectrophotometer. One unit of activity was defined as the amount of enzyme (mg) that bleached 1 µmol of β-carotene min^{-1} at ± 25 °C.

2.2.4 EXTRACTION AND PURIFICATION OF PRODUCTS

2.2.4.1 Xanthophylls and xanthoxin

Precipitated protein was removed by centrifugation and the pellet extracted three times with equal volume (4 mL) of methanol containing BHT as antioxidant. Combined organic

extracts were reduced to a small aqueous volume at 35 °C in *vacuo* and the residue resuspended in 4 mL of 0.5 M phosphate buffer (pH 7.5). These fractions were partitioned three times against equal volumes of diethyl ether to collect neutral and basic compounds. The diethyl ether fractions were reduced in *vacuo*, and stored dry under nitrogen (N_2) until analysis on HPLC.

After removal of excess diethyl ether these fractions were partitioned at pH 7.5 three times against equal volume of ethyl acetate. The ethyl acetate fractions were reduced in *vacuo*, resuspended in small volume and chromatographed on thin layers of silica gel GF_{254} (20 cm x 20 cm, 0.5 mm thick) in hexane:ethyl acetate (1:1, v/v). The UV absorbing band corresponding to published R_f for XAN ($R_f = 0.21$) in this solvent system (Taylor and Burden, 1972), was recovered into water-saturated-ethyl acetate and further purified on thin layers of silica gel (GF_{254}) by development in light petroleum:acetone (3:1, v/v). Xanthoxin was eluted from the silica gel with water saturated-ethyl acetate. Ethyl acetate fraction containing XAN were collected, reduced in vacuo and stored at -20 ^{0}C until analysis on HPLC. Alternatively, the aqueous fractions (pH 7.5) were partitioned twice against equal volume of hexane to remove lipids and subsequently against equal volume of ethyl acetate for XAN. The XAN containing fractions were reduced in *vacuo* and directly analysed on HPLC.

2.2.4.2 ABA and related acids

The pH of the aqueous fraction was adjusted to 2.5 using HCl and partitioned three times against equal volumes of ethyl acetate. The acidic ethyl acetate fraction was reduced in vacuo at 35 ^{0}C and the soluble acids either analysed immediately by HPLC or stored at -20 ^{0}C. Where required the acidic ethyl acetate acid fraction was further purified by TLC. The sample was resuspended in a small volume of methanol:ethyl acetate (1:1, v/v), applied to TLC plates GF_{254} (20 cm x 20 cm, 0.5 mm thick) which were developed twice in the solvent system toluene:ethyl acetate:acetic acid (25:15:2, v/v/v). UV absorbing bands, co-chromatographing with authentic ABA and its acidic products PA and DPA (kindly supplied and prepared by Professor A.K.Cowan, Department of Horticultural Science, Faculty of Agriculture, University of Natal) were eluted with ethyl acetate, reduced in *vacuo* at 35 °C and stored at -20 ^{0}C until analysis on HPLC and/or GC-MS.

2.2.5 ANALYSIS AND QUANTIFICATION OF COMPOUNDS

2.2.5.1 Xanthophylls

Carotenoid-containing, diethyl ether extracts were reduced to dryness under N_2 and analysed by reserved-phase HPLC using a C_{18} Zorbax 5 µm column (2.5 mm x 4.6 cm i.d.) eluted isocratically at 26 °C with methanol:acetonitrile (9:1, v/v) containing 0.1% of triethylamine at a flow rate of 1 mL.min^{-1}. Carotenoids were identified by on-line scanning in the range of 350-550 nm and compared with absorption maxima with those of standards (Figure 2.1). In addition, compounds of interest were detected using a SpectraSystem UV 3000 scanning detector and quantified by peak integration at 460 nm after calibration with authentic standards (kindly supplied by Professor P. Molnar , Department of Medical Chemistry, University of Hungary, Pecs, Hungary) (Figure 2.2).

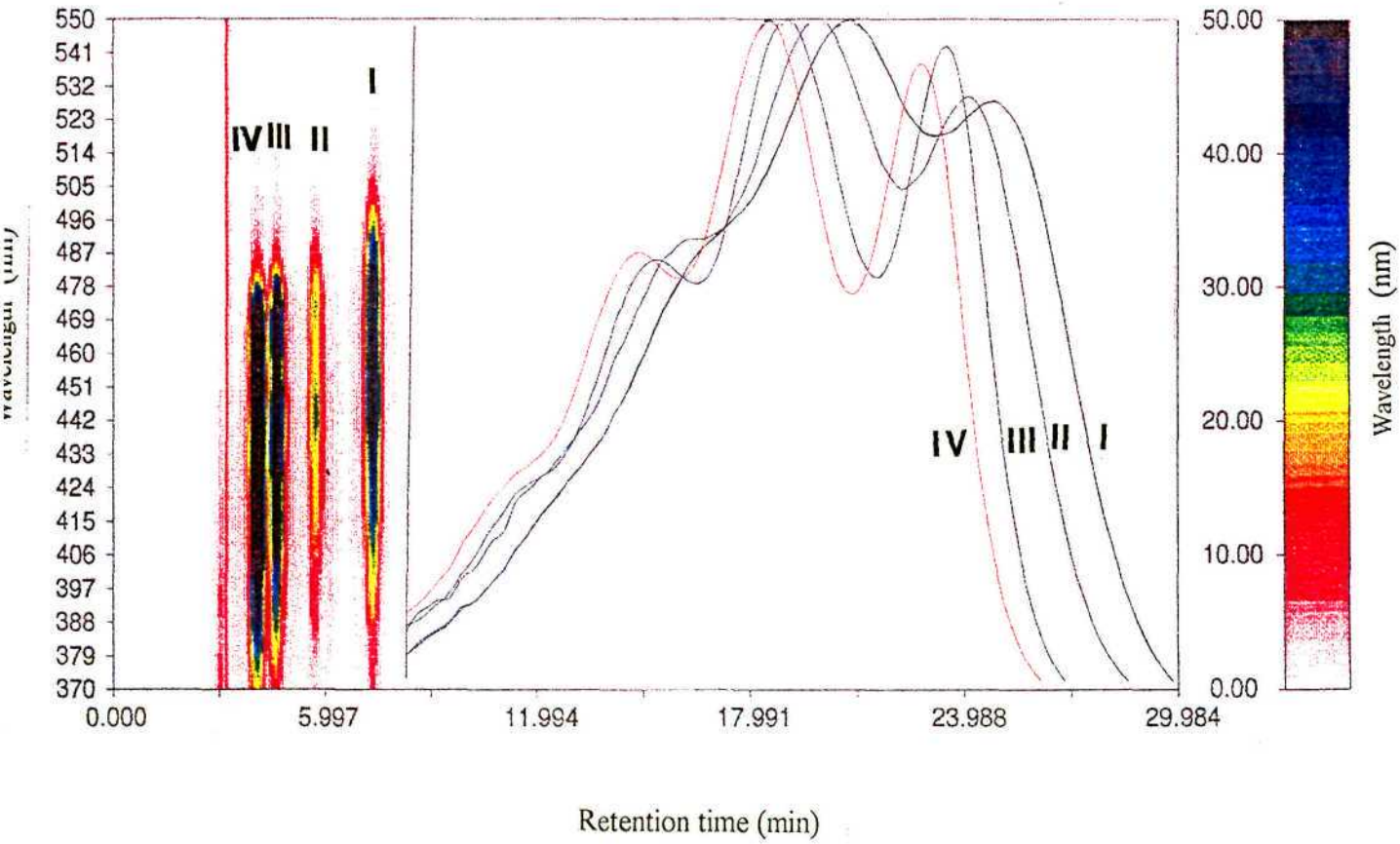

Figure 2.1. Spectral characteristics of authentic Z (I), A (II), V (III) and 9'-*cis*-N (IV) which were dissolved in CH_3CN/MeOH (9:1)/CH_2Cl_2[7:3, v/v] and analysed isocratically as described in Section 2.2.5.1.

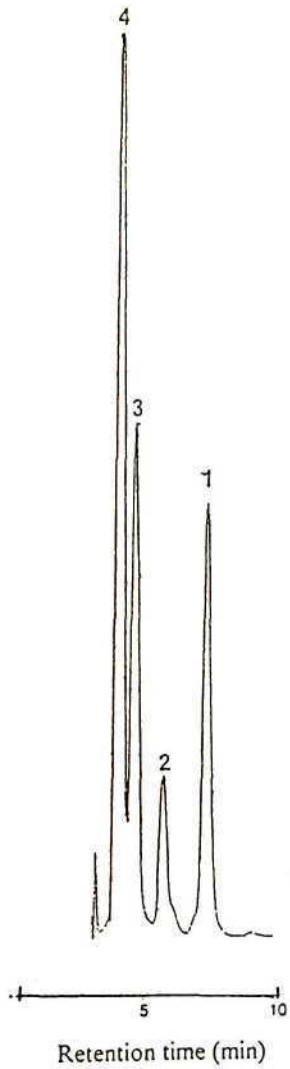

Figure 2.2 Chromatographic separation of a mixture containing authentic Z (1), A (2), V (3) and 9'-*cis*-N (4). Xanthophylls were reconstituted in CH_3CN/MeOH (9:1):CH_2Cl_2 (7:3, v:v) and analysed isocratically at 460 nm as described in Section 2.2.5.1.

2.2.5.2 Xanthoxin, ABA and related compounds

Neutral, ethyl acetate fractions containing XAN and acidic ethyl acetate-soluble acid fractions containing ABA and related acids, were separated by reversed-phase HPLC using a linear gradient of either 0 -100 % methanol in 0.5 % aqueous acetic acid over 60 min on a 5 μm C_{18} ODS Spherisorb column (250 x 4.60 mm i.d.) at a flow rate of 1.0 mL min^{-1} or 20 - 80 % methanol in 0.5 % aqueous acetic acid over 60 min on a 5 μm C_{18} ODS1

Phenomenex column (250 x 10 mm i.d.) at a flow rate of 2 mL min^{-1}. Metabolites were quantified at 260 nm using SpectraSystem UV/VIS 1000 detector. Where necessary, XAN was resolved using a linear gradient of 20 - 100 % methanol in water over 45 min on a 5 µm C_{18} Phenomenex column (250 x 10 mm i.d.) at a flow rate of 2 mL min^{-1}.

Alternatively, products were resolved isocratically on a 5µm C_{18} ODS Spherisorb column (250 x 4.60 mm i.d.) eluted water: methanol: acetic acid (60:40:1, v/v/v) at 1 mL min^{-1}. Metabolites were quantified using a SpectraSystem UV 3000 detector by scanning in the range 190 - 360 nm and quantified by peak integration after calibration with authentic standards (kindly supplied and prepared by Professor A.K.Cowan, Department of Horticultural Science, Faculty of Agriculture, University of Natal). Typical HPLC profiles illustrating the separation achieved are shown in Figures 2.3 and 2.4.

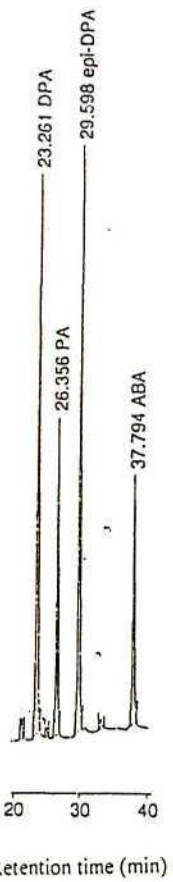

Figure 2.3 High-performance liquid chromatography profile of standard ABA and related acids showing retention time (min). ABA and related acids were separated on linear gradient of 20 - 80 % methanol in 0.5 % aqueous acetic acid over 60 min at 260 nm on a 5 µm C_{18} ODS1 Phenomenex column (250 x 10 mm i.d.) at a flow rate of 2 mL min^{-1}.

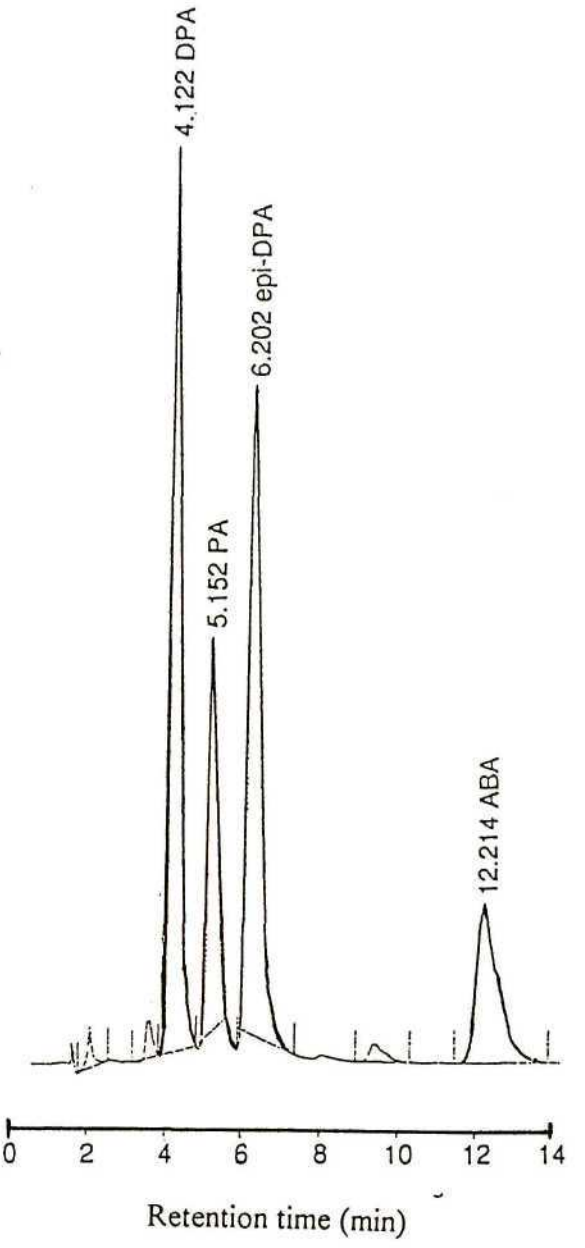

Figure 2.4 Reversed-phase HPLC chromatogram of ABA and related acids showing the retention time (min). ABA and related acids were isocratically separated at 260 nm on a 5µm C_{18} ODS Spherisorb column (250 x 4.60 mm i.d.) eluted with water: methanol: acetic acid (60:40:1, v/v/v) at 1 mL min^{-1}.

2.2.6 LIQUID SCINTILLATION SPECTROMETRY

The amount of radioactivity associated with either thin layer or HPLC-purified compounds was determined by liquid scintillation spectrometry after addition of 2 mL Pico-Fluor 40 scintillation cocktail (supplied by Analytical and Diagnostic Products, RSA)

using a Packard Tri-Carb liquid scintillation counter, with automatic quench correction. By using Pico-Fluor 40 scintillation liquid the amount of radioactive present in the solvent is determined as light or photons. The light is created by solvent molecules which are excited by radioactive emissions and transfer acquired energy to the floor which re-emits it as "light" which are detected by the photomultiplier tubes in the scintillation spectrometer and converted to analogue data.

2.2.7 COMBINED CAPILLARY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Samples were analysed using a Hewlett-Packard 5898A gas chromatography/mass spectrometry system (GC/MS) fitted with a fused-silica column of OV1 (15 cm x 0.25 mm i.d., 0.3 μm coating). Gas chromatography was carried out under temperature programming (40 °C x 1 min, ramped at 20 °C min^{-1} to 300 °C) with He as carrier gas (1.5 to 20 mL min^{-1}) and an injector temperature of 220 °C. Total run time was 14 min. Electron impact mass spectra (EI-MS) were recorded at 70 eV and a typical full spectrum of ABAMe is shown in Figure 2.5. Where specified, positive ion-chemical ionisation-mass spectra (PI-CI-MS) were generated at an ionizing temperature of 220 °C using methane (1 Torr) as ionizing gas. Detailed spectra of the molecular ion regions of c,t-ABAMe and t,t-ABAMe are shown in Figure 2.6 and 2.7.

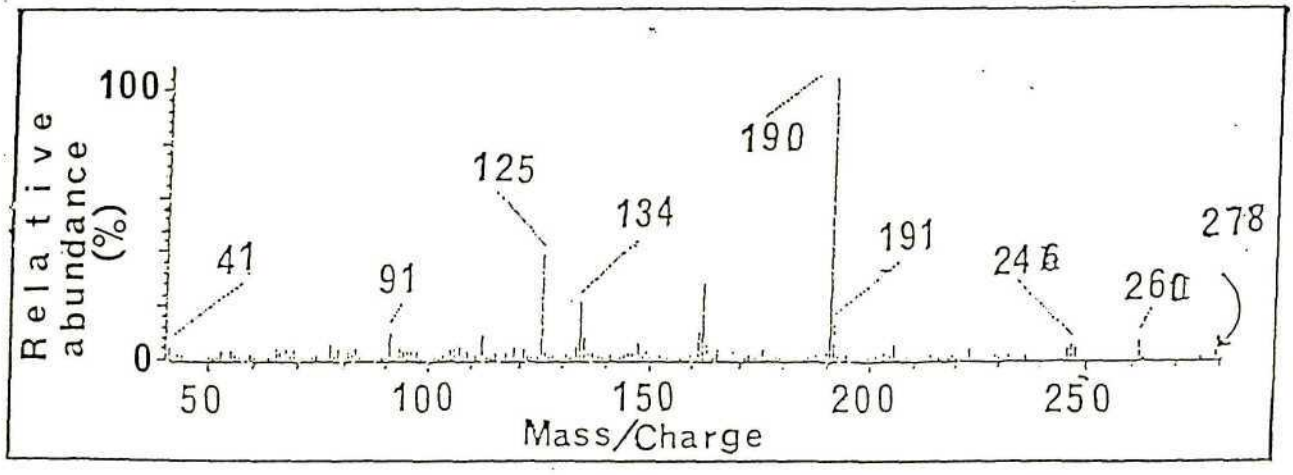

Figure 2.5 Electron impact-mass spectrum of ABAMe as proposed by Netting et al. (1988). Sample was analysed using a Hewlett-Packard 5898A. Mass spectrum was recorded as described in section 2.2.7.

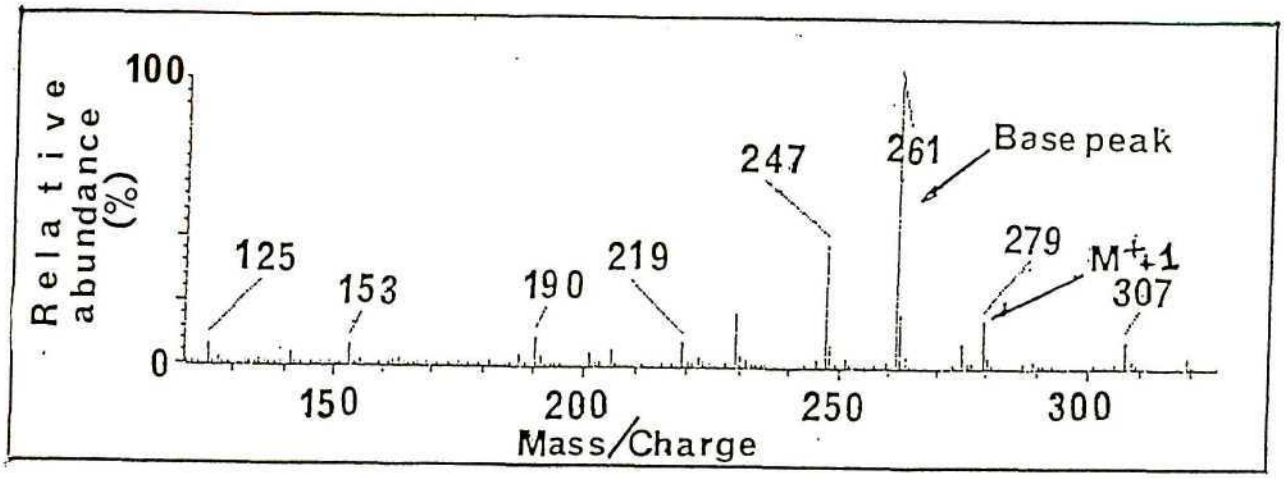

Figure 2.6 Methane positive ion-chemical ionisation mass spectrum of *c,t*-ABAMe Netting et al.(1988). Conditions were as described in Section 2.2.7.

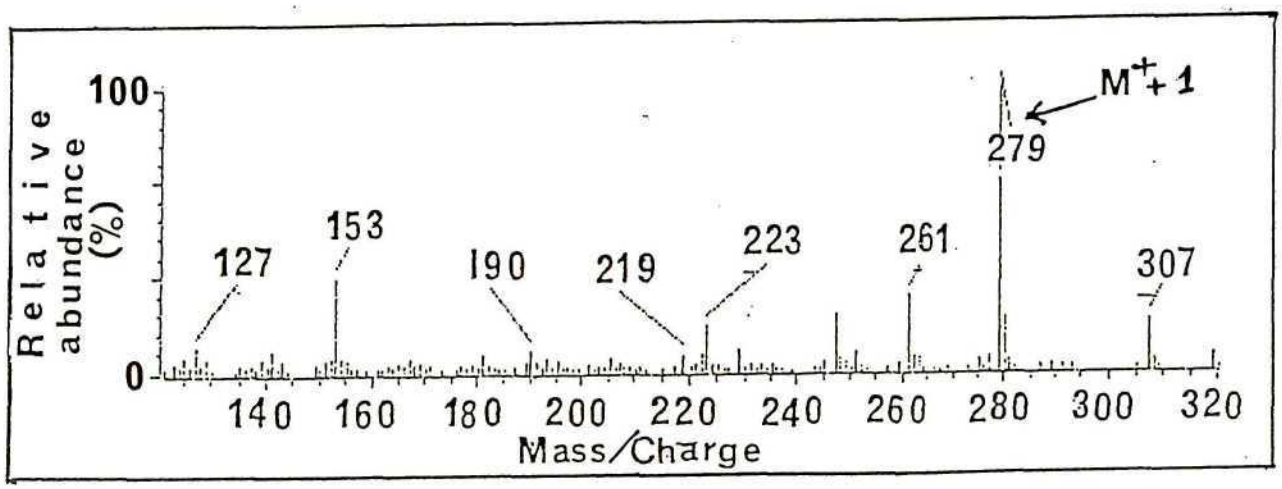

Figure 2.7 Methane positive ion-chemical ionisation mass spectrum of *t,t*-ABAMe Netting et al. (1988). Conditions were as described in Section 2.2.7.

CHAPTER 3

CONFIRMATION OF ABA BIOSYNTHESIS IN THE *CITRUS* FLAVEDO CELL-FREE SYSTEM

3.1 INTRODUCTION

A cell-free system capable of synthesizing ABA from MVA, IPP, FPP, GGPP and β-carotene has been developed (Cowan and Richardson, 1993a; 1997; Richardson and Cowan, 1996). In addition, these workers demonstrated that crude homogenates of *Citrus* flavedo converted 1',4'-*trans*-ABA diol into ABA (Richardson and Cowan 1996). These observations, coupled with the isolation of a product which when analysed by combined capillary GC-MS produced a spectrum consistent with XAN-ac methyl ester, lead Cowan and Richardson (1997) to propose that ABA was synthesized in the *Citrus* cell-free system along the pathway involving 9'-*cis*-N → XAN → XAN-ac → 1',4'-*trans*-ABA diol → ABA. Although elegant, this work contains several conspicuous omissions. Firstly, it was never unequivocally demonstrated that the ABA formed in the *Citrus* cell-free system in fact arose from the supplied substrates (eg. 3R-[2-^{14}C]-MVA, [^{14}C]-β-carotene or [^{14}C]-9'-*cis*-N). Secondly, although ABA seemed to be formed from either terpenyl pyrophosphates or carotenes *via* β,β-xanthophylls, there were no indications that the *Citrus* cell-free system metabolised these substrates *via* Z, A, V and N.

The key reaction in ABA biosynthesis has been established to be the conversion of 9'-*cis*-N to XAN, which is later converted to ABA (Rock et al., 1992; Parry, 1993; Richardson and Cowan, 1996; Cowan and Richardson, 1997; Schwartz et al., 1997; Lee and Milborrow, 1997). The conversion of XAN to ABA is catalysed by an aldehyde oxidase enzyme requiring a molybdenum co-factor (Mo-co). Mo-co enzymes are inhibited by tungsten (Rajagopalan and Johnson, 1992) and belong to the family of xanthine oxidase enzymes. Xanthine enzymes have been shown to be activated and/or inhibited by allopurinol (Montalbini and Della-Torre, 1995). In addition most studies have shown that

the biosynthesis of ABA in plants either *in vivo* or *in vitro* occurs at the same time as ethylene (Lee and Milborrow, 1997b). The biosynthesis of ethylene has been shown to be affected by several metal ions. For instance, cobalt inhibits production of ethylene by blocking the conversion of 1-aminocyclopropane-1-carboxylic acid oxidase enzyme (ACC oxidase), the enzyme responsible for the conversion of ACC to ethylene(Yu and Yang, 1979). Thus an investigation into the effect of different chemical modifiers on ABA biosynthesis would be appropriate in order to elucidate their possible effects on the enzymes involved in the latter stages of ABA biosynthesis. Using the *Citrus* flavedo cell-free system, this chapter intends to show;

① that ABA is formed *de novo* from labelled substrates,

② that interconversion between carotenoids of the xanthophyll cycle are required for the formation of 9'-*cis*-N and ABA, and

③ that the post-cleavage enzyme-catalysed-reactions can be modified by both metal ions and organic inhibitors.

3.2 RESULTS

3.2.1 BIOSYNTHESIS OF ABA FROM LABELLED MVA

Previous studies were apparently unable to confirm the incorporation of label from radioactive substrates into ABA, in reactions catalysed by the *Citrus* flavedo cell-free system (Cowan and Richardson, 1993a, 1996 and 1997). To unequivocally demonstrate the synthesis of ABA by this cell-free enzyme system, [2-^{13}C]-MVA was prepared from stock of D,L-[^{13}C]-MVAL and used as substrate. The acidic ethyl acetate soluble fraction obtained from 2 h incubations, was purified by TLC on thin layers of silica gel GF_{254} and the zone co-chromatographing with authentic ABA recovered into water-saturated ethyl acetate. After removal of water, the sample was dried, resuspended in methanol and methylated with excess ethereal diazomethane. The methyl ester derivative was further purified by TLC and then analysed by combined capillary GC-MS in both PI-CI and EI modes. Methane positive ion-chemical ionization mass spectrometric (PI-CI-MS) analysis of the molecular ion region of the methyl ester of putative ABA derived from [2-^{13}C]-MVA in the *Citrus* cell-free system, revealed the spectrum shown in Figure 3.1.

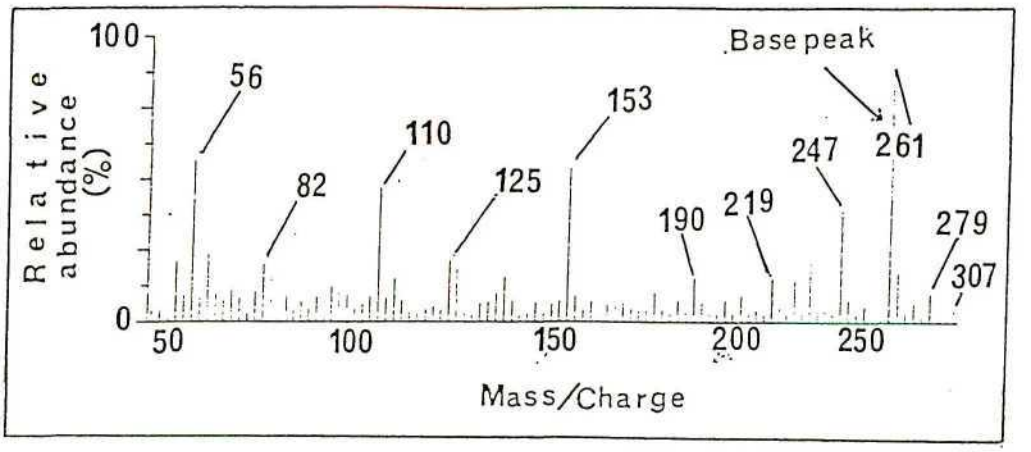

Figure 3.1 Methane PI-CI-MS of c,t-ABAMe derived from [2-^{13}C]-MVA incubates. ABA was extracted as per Section 2.4.2.1, purified on TLC, methylated and analysed by GC-MS as described in section 2.2.7. The spectrum obtained was compared to the standard spectrum published in the literature (Netting et al., 1988).

This spectrum is consistent with the methane PI-CI-MS published for ABAMe (Netting et al., 1988). Closer scrutiny of the molecular ion region of the spectrum shown in Figure 3.1, and when compared to the spectrum of non-labelled ABAMe, revealed enrichment by three atomic mass units attributable, to three atoms of [^{13}C] (Figure 3.2). These data strongly suggested that the labelling pattern in ABA produced *de novo* in the *Citrus* cell-free system was consistent with its formation from [2-^{13}C]-MVA via the terpenoid biosynthetic pathway.

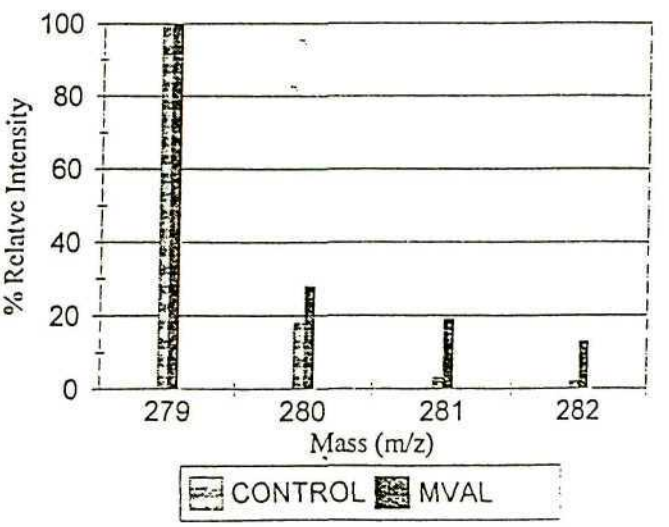

Figure 3.2 Enrichment of the molecular ion (m/z 279) of ABAMe isolated from *Citrus* cell-free system supplied with [2-^{13}C]-MVA. Analysis was by PI-CI-MS as described in Section 2.2.7.

In an attempt to confirm this observation, the identical samples were analysed by electron impact-mass spectrometry. Unfortunately the spectrum obtained (results not shown) bore no resemblance to that of authentic ABAMe. The reason for this anomalous result remains unresolved. Even so, based on PI-CI-MS results it is concluded that label from MVA is incorporated into ABA in a manner consistent with the incorporation of [^{14}C]-MVA into ABA (Milborrow, 1975).

3.2.2 INTERCONVERSION OF β-β XANTHOPHYLLS

The immediate oxy-carotenoid precursor of ABA, 9'-*cis*-N, is purportedly derived from the metabolism of xanthophyll cycle carotenoids and in particular all-*trans*-V. In addition, since several reports indicate a biosynthetic or metabolic relationship between turnover of the xanthophyll cycle and the formation of ABA (Parry et al., 1990; Parry et al., 1991), the relationship between β-β xanthophyll interconversion and ABA biosynthesis was investigated using the *Citrus* cell-free system.

3.2.2.1 Interconversion between all-*trans*-isomers of β-β xanthophylls

All-*trans*-isomers of Z, A , and V were supplied to incubates and the reactions allowed to proceed for 2 h. Thereafter, pigment fractions were prepared and analysed by reversed-phase HPLC with on line spectral scanning and the results are presented in Figure 3.3.

Analysis of the neutral diethyl ether fraction from incubates supplied with all-*trans*-Z, by reversed-phase HPLC, revealed the presence of two major components (Figure 3.3.A). By on-line spectral analysis and with reference to standards, the component at retention time R_t= 7.4 min was identified as Z, and the component at R_t = 4.2 min was identified as A. Similar metabolic studies using A as substrate revealed that this xanthophyll was converted primarily into Z (Figure 3.3.B). A minor component of longer retention time than the substrate (R_t= 5.9 min) was present, but attempts made to identify this compound were not successful.

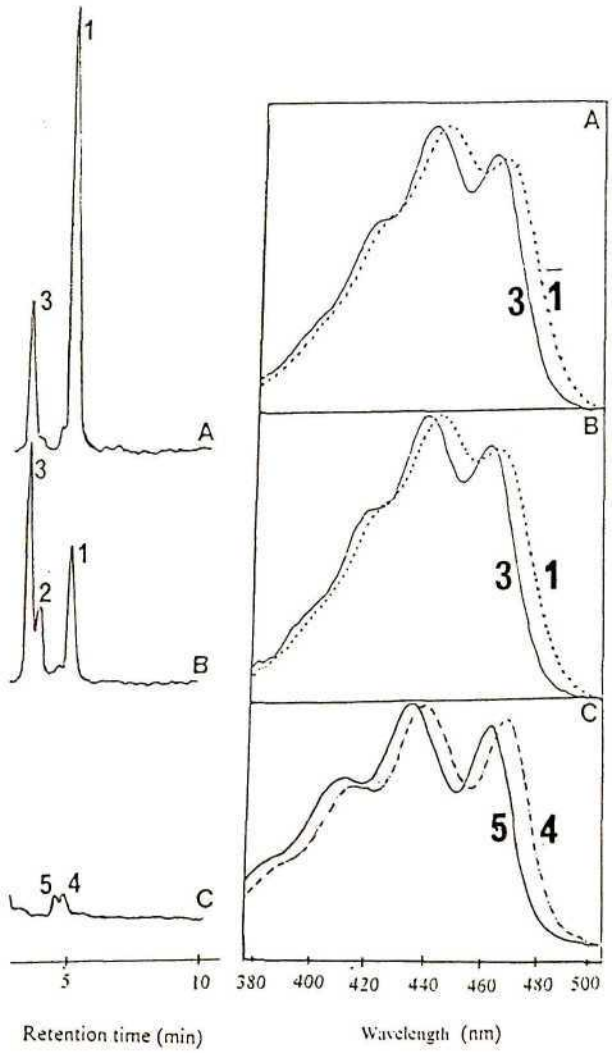

Figure 3.3 Reversed-phase HPLC traces of the products of Z (A), A (B), and V (C) metabolism in the *Citrus* cell-free system. Enzyme protein equivalent to 2.5 mg was incubated with Z (200 µg), A (200 µg) and V (200 µg) respectively in their all-*trans*-forms as described in section 2.2.2. Xanthophylls were extracted as per section 2.2.4.1, separated and analysed at 460 nm as described in Section 2.2.5.1. 1. All-*trans*-Z, 2. Unknown, 3. All-*trans*-A, 4. All-*trans*-V, 5. 9'-*cis*-V. (See insets).

In contrast to the interconversions referred to above, all-*trans*-V was not converted back to Z as proposed in the xanthophyll cycle. Rather, this compound was preferentially metabolised into one component of shorter retention time (Figure 3.3.C). This compound when analysed by on line spectral analysis revealed that it was the *cis*-isomer of the substrate.

Since the conversion of Z to other xanthophylls is an enzyme-catalysed process, the metabolic interrelationship between Z and other β,β-xanthophylls was determined by determining the kinetics of all-*trans*-Z conversion. Results obtained from these studies are presented in Figure 3.4.

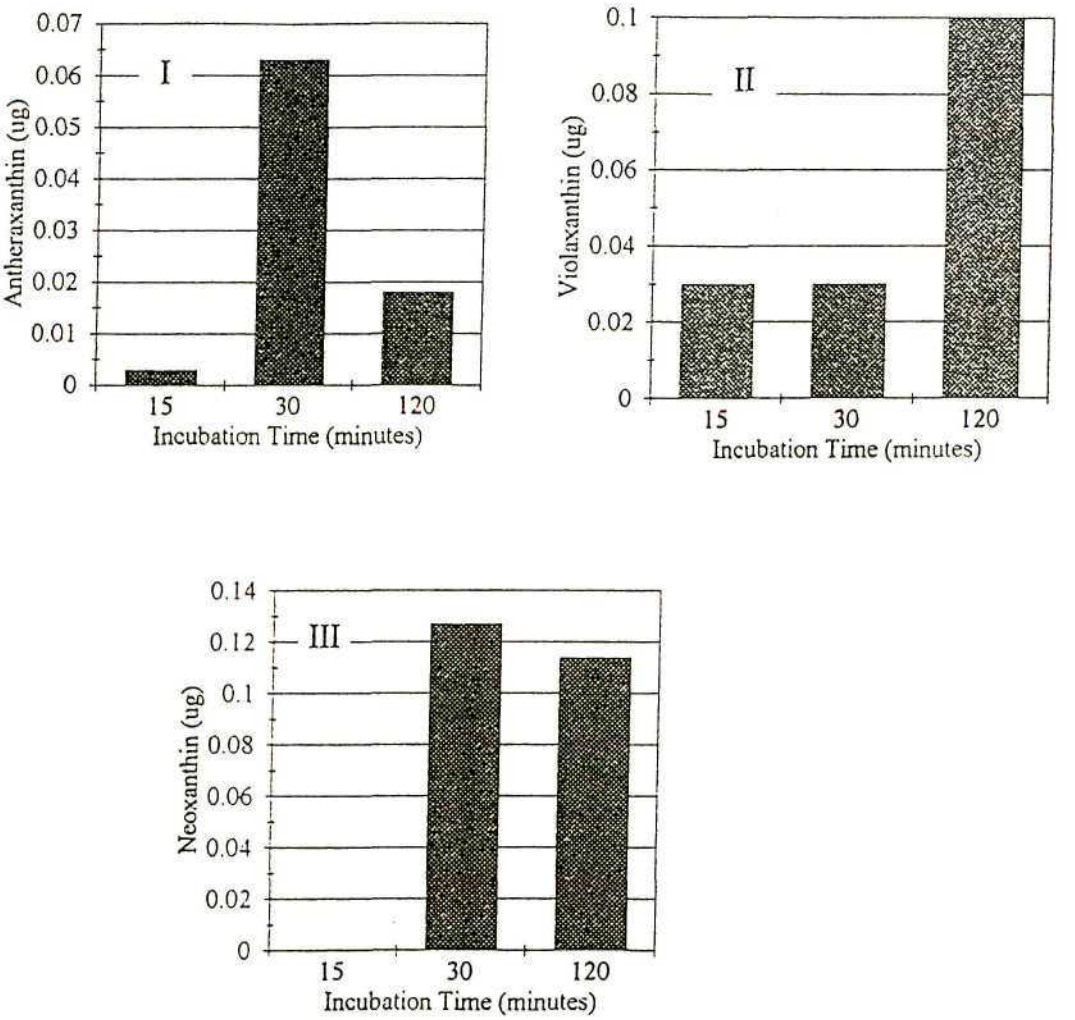

Figure 3.4 Time course of conversion of Z into A (I), V (II) and N (III). Enzyme protein equivalent to 2.5 mg and Z (200 μg) were incubated as described in section 2.2.2. Xanthophylls were extracted as per section 2.2.4.1, separated and analysed as described in Section 2.2.5.1.

Results (Figure 3.4) indicate that A formed was at maximum levels by 30 min and thereafter declined (Figure 3.4. I). Violaxanthin synthesized from Z reached maximum at 2 h of incubation (Figure 3.4. II). The diethyl ether fractions from all-*trans*-Z incubates analysed for N revealed that there was no significant difference between the amount of N

formed at either 30 min or 2 h (Figure 3.6 . III). These observations clearly indicate that the direction of the reaction of catabolism of Z in the *Citrus* cell-free system occurs from Z → A → V → N and that Z was preferentially converted to N after 2 h of incubation.

3.2.2.2 Conversion of 9'-*cis*- violaxanthin to 9'-*cis*- neoxanthin

Current thinking suggests that the xanthophyll precursor to ABA, 9'-*cis*-N is derived from either all *trans*-N or 9'-*cis*-V. To confirm if 9'-*cis*-V is a precursor of 9'-*cis*-N, an experiment was carried out whereby the neutral diethyl ether fractions containing products of 9'-*cis*-V and 9'-*cis*-N incubates were extracted and analysed. Results obtained are presented in Figure 3.5 and 3.6 respectively.

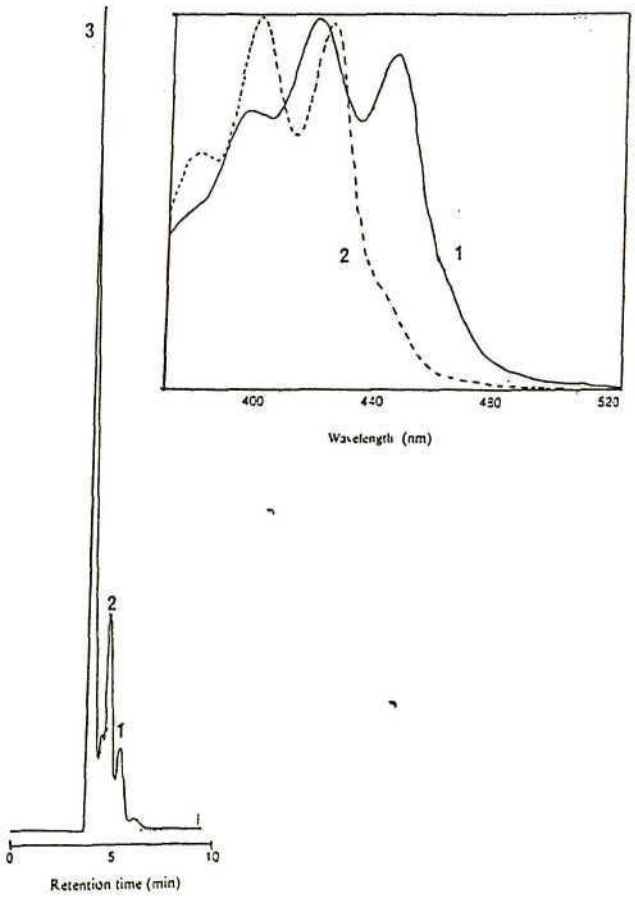

Figure 3.5 Reversed-phase HPLC trace of the products of 9'-*cis*-V metabolism in the *Citrus* cell-free system. Enzyme protein equivalent to 2.5 mg was incubated with of 9'-*cis*-V (200 µg) as described in section 2.2.2. Xanthophylls were extracted as per section 2.2.4.1, separated and analysed at 460 nm as described in Section 2.2.5.1. 1. Auroxanthin, 2. Shamoutichrome = Trollichrome, 3. Unknown. (See inset).

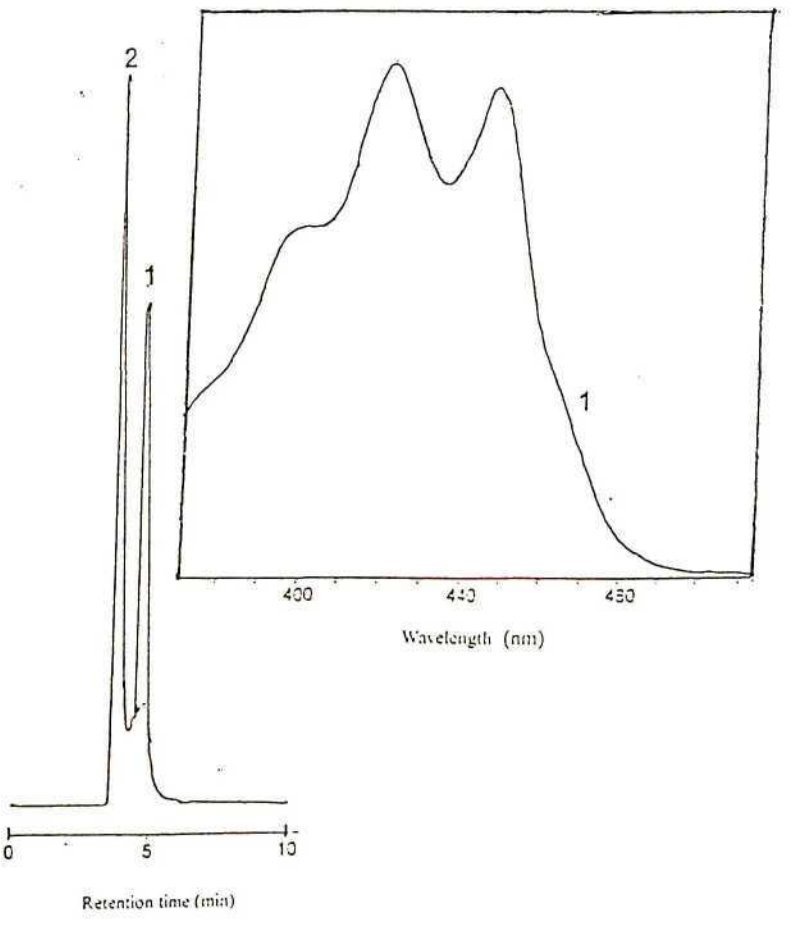

Figure 3.6 Reversed-phase HPLC separation of the pigment fraction products of 9'-*cis*-N metabolism in the *Citrus* cell-free system. Enzyme protein equivalent to 2.5 mg was incubated with of 9'-*cis*- N (200 µg) as described in section 2.2.2. Xanthophylls were extracted as per section 2.2.4.1, separated and analysed as described in Section 2.2.5.1. 1. Shamoutichrome, 2. Unknown. (See inset)

Three major components were present in the neutral diethyl ether fraction from incubates supplied with 9'-*cis*-V (Figure 3.5). The component at R_t= 5.6 min was identified as trollichrome (5,6-dihydro-β,β-carotene-3,3'-5,6-tetrol) while the one at R_t= 4.95 min was identified as auroxanthin. Trollichrome has been found as a minor carotenoid present in extracts prepared from several cultivars of *Citrus*. The trivial name of this compound has subsequently been changed to shamoutichrome (Gross, 1987). Shamoutichrome is a somewhat unknown tetrol (tetra-hydroxy derivate of β-carotene) containing a trihydroxylated ring.

High performance liquid chromatography analysis of incubates supplied with 9'-*cis*- N (Figure 3.6) revealed the presence of two components of which one was identified as trollichrome. Like the compound at R_t = 3.8 min in Figure 3.5, the second component of a shorter retention time (R_t = 3.75 min) remains unidentified because the absorption spectrum was not characteristic of any known carotenoid. However this does not preclude either compound from being an apocarotenoid. The two components identified and derived from 9'-*cis*- V and 9'-*cis*- N respectively, might represent novel apocarotenoids of shamoutichrome.

3.2.3 BIOSYNTHESIS OF ABA FROM ZEAXANTHIN, ANTHERAXANTHIN, VIOLAXANTHIN AND NEOXANTHIN

3.2.3.1 Biosynthesis of ABA from zeaxanthin, antheraxanthin and violaxanthin

The biosynthetic pathway of ABA in plants appears to involve the xanthophyll cycle components Z, A and V (see Section 3.2.2.1). In order to confirm the involvement of these β-β xanthophylls in the biosynthetic route of ABA and determine the metabolic interrelationship between these carotenoids with respect to ABA formation, acidic fractions from incubates supplied with all-*trans* Z, A, and V were extracted and analysed for ABA. Results obtained are presented in Table 3.1.

Table 3.1 ABA formation from the metabolism of Z (200 μg), A (200 μg), and V (200 μg) in the *Citrus* cell free system. Incubates were carried out as described in section 2.2.2. ABA was extracted and analysed by HPLC as described in Section 2.2.5.2. Data are the mean of three replicates ± S.E.

Substrate	ABA (μg.mg. protein^{-1})(% conversion)
V	16.88 ± 1.98 (8.4)
A	9.08 ± 1.06 (4.5)
Z	4.70 ±1.01 (2.4)

Analysis of the pH 2.5 fractions of incubates supplied with all-*trans* of Z, A and V by HPLC revealed that ABA was synthesized from each of these xanthophylls. More ABA was, however; biosynthesized from incubates supplied with V (8.4 % conversion) compared to those supplied with A and Z (4.5 % and 2.4 % conversion respectively). These results therefore indicate that there is a metabolic relationship between β-β xanthophylls and ABA. The results also suggest that V is the last of xanthophyll cycle components to be involved in the biosynthetic route of ABA.

3.2.3.2 Biosynthesis of ABA from neoxanthin

In order to determine which of all-*trans*-N and 9'-*cis*-N is the immediate carotenoid precursor to ABA, these substrates were incubated in the *Citrus* cell-free system and any ABA and related acids formed were isolated and quantified by peak integration after calibration with authentic standards, together with co-chromatography. Results obtained are presented in Table 3.2

Table 3.2 ABA formation from metabolism of all-*trans*-N and 9'-*cis*-N (50 µg) into ABA in a *Citrus* cell-free system. Incubations were carried out as described in Section 2.2.2. ABA and related acids were purified as per Section 2.2.4.1 and analysed by HPLC under conditions described in Section 2.2.5.2

Substrate	Experiments	ABA (µg.mg.protein^{-1}) (% converted)
All-*trans*-N	Experiment 1	1.46 (2.92)
	Experiment 2	1.20 (2.40)
	Mean	1.33 (2.66)
	S.E	0.18 (0.37)
9'- *cis* -N	Experiment 1	2.25 (4.50)
	Experiment 2	2.40 (4.80)
	Mean	2.33 (4.65)
	S.E	0.10 (0.21)

Results presented in Table 3.2 confirm several earlier reports which suggested that 9'-*cis*-N is the immediate precursor to ABA. More ABA was produced from 9'-*cis*-N (4.65 ± 0.21 % conversion) compared to all-*trans*-N (2.66 ± 0.37 % change).

The kinetics of ABA production from 9'-*cis*-N (Figure 3.7) revealed that the biosynthesizing activity was sustained for 2 h. Thereafter ABA levels declined due to its further conversion, presumably to PA and DPA. This result confirmed that the production of ABA in the *Citrus* cell-free system arose due to enzyme catalysed biosynthesizing activity and negated any possibility of facile conversion or cleavage of 9'-*cis*-N to compound that could be converted to ABA.

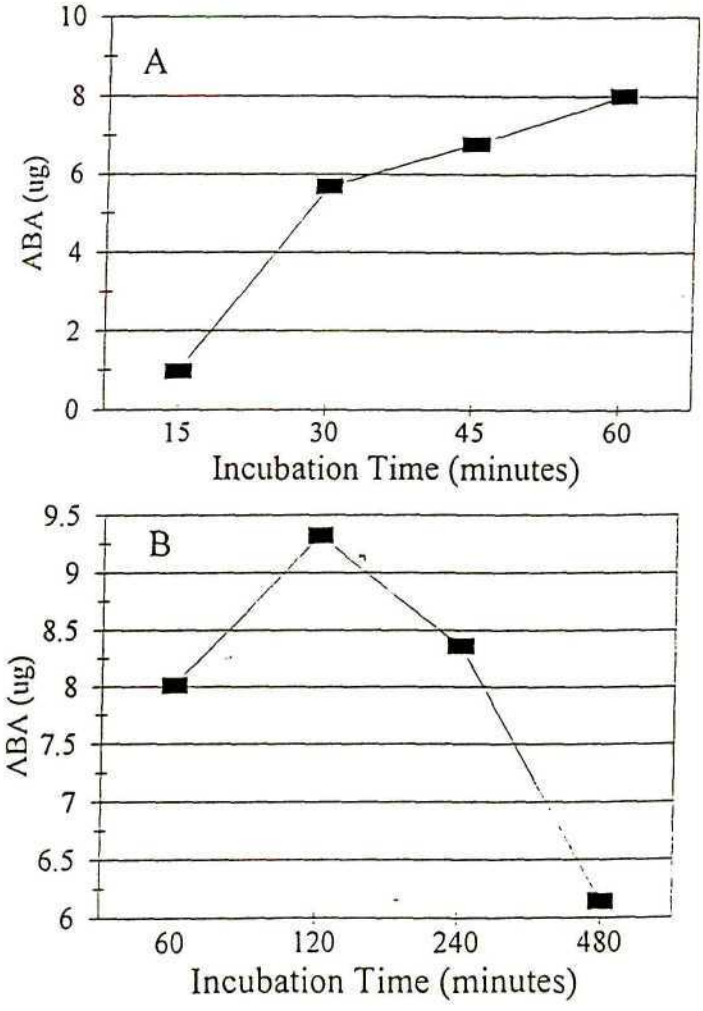

Figure 3.7 Short (A) and long (B) reaction kinetics of 9'-*cis*-N metabolism in a cell-free system. Incubations were carried out as described in Section 2.2.2. ABA and related acids was purified as per Section 2.2.4.1 and analysed by HPLC under conditions described in Section 2.2.5.2.

3.2.3.3 Effects of cobalt, nickel, tungstate ions and allopurinol on the conversion of 9'-*cis*-N to ABA

Activity of many plant enzymes is influenced by different organic and inorganic chemicals which act as inhibitors and /or activators by behaving as co-factors or co-substrates. Thus an investigation was carried out to determine the effect of the metal ions cobalt, nickel and tungstate and the organic compound, allopurinol on the enzymatic network involved in the biosynthetic route of ABA in the *Citrus* cell-free system, using 9'-*cis*-N as substrate. Results obtained from these studies are presented in Table 3.3.

Table 3.3 Effect of nickel, cobalt, tungstate ions, and allopurinol (0.1 M) on the formation of ABA from 9'-*cis*- N (50 µg). Incubations were carried out as described in Section 2.2.2. ABA and related acids were purified as per Section 2.2.4.1 and analysed by HPLC under conditions described in Section 2.2.5.2. Data are the mean of three replicates ± S.E.

Treatment	ABA ($\mu g.mg^{-1}$.protein) (% conversion)	% of control
Control	1.33 ± 0.18 (2.66)	100
Nickel	1.87 ± 0.22 (3.74)	141
Cobalt	3.73 ± 0.01 (7.46)	281
Tungstate	0.37 ± 0.01 (0.74)	28
allopurinol	0.65 ± 0.17 (1.31)	49

The results in Table 3.3 indicate that more ABA was produced from incubates treated with cobalt and nickel (% change of 281 and 141 respectively) suggesting that these metals have a stimulating effect on the enzymatic system involved in the conversion of 9'-*cis*-N to ABA in the *Citrus* cell-free system. Similar metabolic studies undertaken using tungstate revealed that ABA biosynthesis from 9'-*cis*-N was inhibited (28 % conversion of the substrate). These results confirm the earlier report that the biosynthesis of ABA in

plant tissue is blocked by tungstate. Similar observations were made with the organic compound allopurinol (49 % change).

3.3 SUMMARY OBSERVATIONS

To summarise, the results presented in this chapter indicate that the *Citrus* cell-free system has the following characteristics;

- ★ ABA is formed *de novo* from MVA confirming its isoprenoid origin.
- ★ β-β xanthophyll interconversion does occur and is similar to that reported for higher photosynthetic tissue.
- ★ Zeaxanthin, A and V are indeed precursors to ABA and the order of efficacy is Z > A > V.
- ★ 9'-*cis*-V is not the immediate precursor of 9'-*cis*-N.
- ★ 9'-*cis*-N is the immediate precursor to ABA and its conversion is enzymatically mediated.
- ★ Nickel and cobalt stimulate ABA production from 9'-*cis*-N whereas tungsten inhibits this process.
- ★ Allopurinol inhibits ABA formation from 9'-*cis*-N.

CHAPTER 4

ATTEMPTS TO PURIFY 9-*CIS*-NEOXANTHIN CLEAVAGE ENZYME ACTIVITY FROM THE *CITRUS* FLAVEDO CELL-FREE SYSTEM

4.1 INTRODUCTION

Biochemical studies and analysis of mutants deficient in ABA have shown that the biosynthesis of this plant growth regulator occurs *via* oxidative cleavage of epoxy-carotenoids (Tan et al., 1997). This oxidative cleavage, currently accepted as the key regulatory step in ABA biosynthesis, has been mediated by an inducible enzyme which acts specifically to cleave a 9'-*cis*-xanthophyll or xanthophylls (Sindhu and Walton, 1987; Li and Walton, 1989, 1990; Zeevaart and Creelman, 1988; Parry, 1993). The products of this cleavage reaction (Figure 4.1) are formed by the incorporation of molecular oxygen into 9'-*cis*-xanthophylls after breakage of the 11-12 (11'-12') double bond of 9'-*cis*-N or 9'-*cis*-V (Creelman et al., 1992). This type of reaction has been shown to be catalysed by dioxygenase enzymes (Siedow, 1991; Gardner, 1995; Prescott and John, 1997; Hedden and Kamiya, 1997).

Nonheme iron-containing dioxygenases are involved in the biosynthesis of many important compounds, including ethylene, gibberellins and jasmonates. They comprise two major groups: LOX and 2-oxoglutarate-dependent dioxygenases (2-ODD's). Several reports have indicated the presence of a dioxygenase that has LOX-like properties in the biosynthetic pathway of ABA (Creelman et al., 1992; Parry, 1993; Richardson, 1995). Confirmation that a dioxygenase enzyme is responsible for the cleavage of a C_{40}-xanthophyll into C_{15} compounds has come from studies using tomato and *Zea mays* mutants deficient in ABA. Recently, the VP14 protein cloned from the *Zea mays* mutant *vp*14 was shown to be related to lignostilbene dioxygenase, a bacterial enzyme catalysing a double-bond cleavage reaction that is chemically similar to the *cis*-carotenoid cleavage step in ABA biosynthesis. The same protein has also been shown to catalyse the cleavage of 9'-*cis*-N (Burbridge et al., 1997; Schwartz et al., 1997; Tan et al., 1997). A cDNA encoding a similar protein has been isolated from a wilt-related tomato library and was

library and was shown to encode the message for a putative neoxanthin cleavage enzyme (NCE) (Thompson et al.,1998).

Despite progress made in the isolation and characterization of ABA biosynthesis genes using ABA deficient mutants, little is known about the specificity of the NCE. In fact, the NCE has been reported to cleave Z (Burbridge et al., 1997). This therefore creates the need to find alternative techniques that can be used to study the biochemistry of 9'-*cis*-N cleavage enzyme. Several plant LOXs have been isolated and characterised by biochemical means (Siedow, 1991, Gardner, 1995). The *Citrus* cell-free system, which metabolised 9'-*cis*-N to ABA, has been shown to contain a 66 kDa protein which co-migrated with soybean lipoxidase using polyacrylamide gel electrophoresis (SDS-PAGE) (Richardson, 1995). Using the same system, this chapter is intended to develop the methodology for the isolation and purification of the putative 9'-*cis*-N cleavage enzyme.

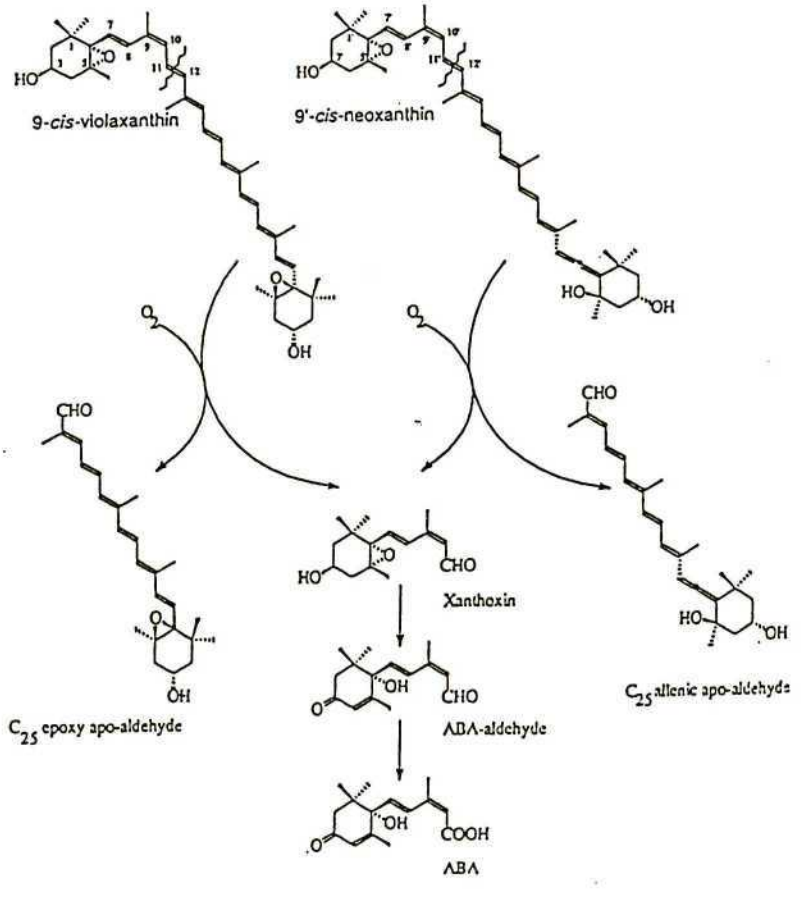

Figure 4.1 Scheme illustrating the possible formation of XAN and C_{25} epoxy and allenic apo-aldehydes from oxidative cleavage of 9'*cis*-N and 9'-*cis*-V, reactions believed to be mediated by a LOX-like dioxygenase as proposed by Schwartz et al. (1997).

4.2 RESULTS

4.2.1 ASSAYS FOR LIPOXYGENASE AND NEOXANTHIN CLEAVAGE ENZYME ACTIVITY IN PRECIPITATES OF THE *CITRUS* CELL-FREE SYSTEM

Prior to attempts to purify and to isolate and purify the NCE, it was desirable to develop systems in which to assay for both LOX and NCE. As demonstrated in chapter 3; Section 3.2.3.2, NCE activity can be determined by monitoring the production of ABA from substrate 9'-*cis*-N in the presence of the *Citrus* cell-free enzyme extract. However, since NCE is responsible for catalysing the conversion of 9'-*cis*-N to XAN, it seemed more appropriate to determine NCE activity by measuring the amount of XAN produced per mass of protein. In contrast, LOX activity was typically been measured by monitoring the rate of carotenoid bleaching spectrophotometrically (Ben-Aziz, 1971). Since N is metabolised to ABA in the *Citrus* cell-free system simple measurement of N was clearly inadequate. Thus LOX activity was determined by monitoring the bleaching of all-*trans*-β-carotene as described by Ben-Aziz (1971).

To attempt to isolate and purify the NCE, it was necessary to first fractionate the crude 23500 g *Citrus* cell-free supernatant using $(NH_4)_2SO_4$ and determine the ability of each fraction to bleach N or β-carotene. After removal of an aliquot of the crude enzyme preparation (control), the supernatant was further fractionated into 30%, 45 %, 65% and 100% fractions and incubated in the presence of β-carotene for the determination of LOX or 9'-*cis*-N for NCE activity. For NCE activity, the neutral ethyl acetate fractions containing XAN were analysed by HPLC, XAN tentatively identified by co-chromatography, and quantified by peak integration after calibration with authentic ABA-Me standard.

Table 4.1 Lipoxygenase and 9'-*cis*-NCE activity of different ammonium sulphate fractions expressed as μmol β-carotene bleached min^{-1} mg^{-1} protein at 25 ^0C and μg XAN produced mg^{-1} protein respectively. Incubations were carried out as per Section 2.2.2.

$(NH_4)_2SO_4$ (%)	LOX activity (units mg^{-1}) [A]	NCE activity (μg.XAN.mg^{-1}protein)
Control (0)	2.00	5.32
30	6.02	1.57
45	17.15	7.65
65	3.47	0.26
100	1.47	0.01

[A] Units: μmol of β-carotene bleached min^{-1}at 25^0C.

The results in Table 4.1 show that more β-carotene was bleached by the 45 % fraction (17.15 units mg^{-1}). This represented an increase in LOX activity of 9 fold. The same fraction yielded the highest amount of XAN mg^{-1} protein illustrating an approximation of 1.5 fold increase in NCE activity. Together these results indicated that 45 % ammonium fraction contained both LOX and NCE activity. Thus, methods were sought with which these activities could be adequately separated.

4.2.2 PURIFICATION OF LIPOXYGENASE AND NEOXANTHIN CLEAVAGE ENZYME ACTIVITY PRECIPITATES OF THE *CITRUS* CELL-FREE SYSTEM

Because 30 % and 45 % $(NH_4)_2SO_4$ precipitates fractions had high LOX enzyme and NCE activity, it was decided to attempt to purify them further using hydrophobic chromatography. A single 45 % fraction was prepared using $(NH_4)_2SO_4$ and directly loaded on phenyl Sepharose CL-4B column previously equilibrated with potassium phosphate buffer containing 0.5 M of $(NH_4)_2SO_4$. After removal of unbound proteins with the equilibration buffer (referred to as high salt = HS), proteins of interest were eluted from the column with a linear decreasing gradient of 0.5 to 0 M of $(NH_4)_2SO_4$ in potassium phosphate buffer (referred to as gradient = GR). The remaining proteins were removed

from the column with the equilibrium buffer (referred to as non salt = NS). Results of hydrophobic column chromatography separation of the proteins present in the 45 % $(NH_4)_2SO_4$ fraction from the *Citrus* cell-free system are shown in Figure 4.2.

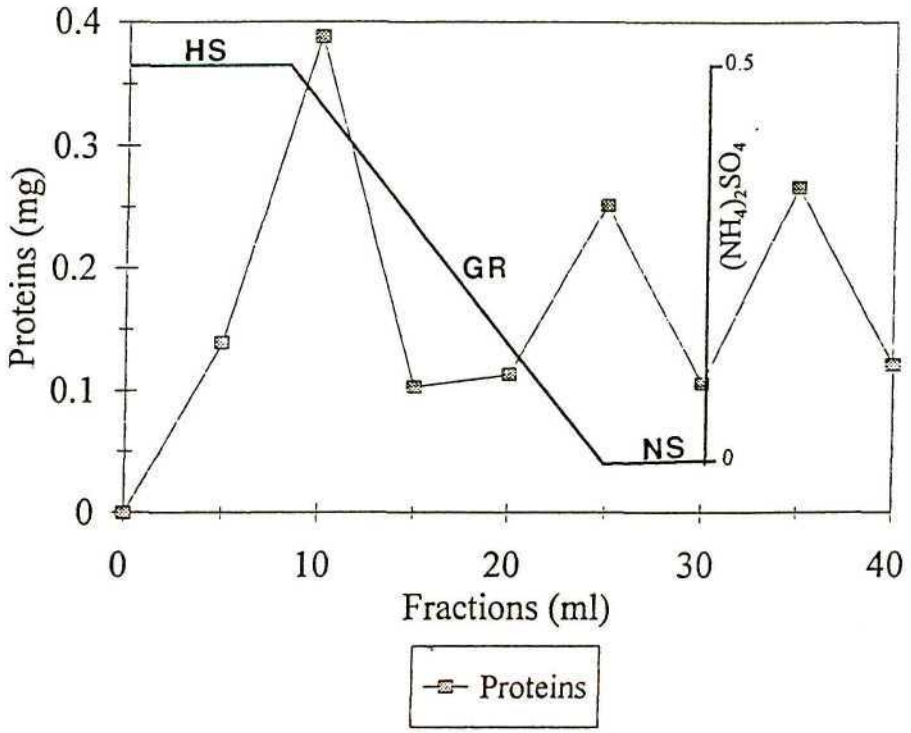

Figure 4.2 Elution profiles of proteins eluted from the single 45% $(NH_4)_2SO_4$ precipitate. Proteins were separated by hydrophobic chromatography on the phenyl Sepharose CL-4B column as described in Section 2.2.1.3.

The presence of 3 broad peaks of proteins which eluted at different levels of salt concentration are clearly evident.

In order to test which of these protein fractions contained either LOX or NCE activity or both, the fractions were collected and assayed for β-carotene bleaching activity and the NCE activity. A summary of these results (Table 4.2) indicated that proteins present in the fractions eluted with high salt containing buffer catalysed 15.96 µg XAN mg^{-1}.protein from 9'-*cis*-N which represented a 300 % increase in the purification of NCE. About 3.89 µmol of β- carotene was bleached min^{-1} mg^{-1} protein at 25 ° C, indicating an increase of 194 % in the purification of LOX activity. These results clearly indicated that the activity of LOX enzyme and NCE which were present in the *Citrus* cell-free system, could be successfully separated using hydrophobic chromatography.

Table 4.2 9'-*cis*-NCE and LOX enzyme activities in the 45 % $(NH_4)_2SO_4$ precipitated fraction purified on phenyl Sepharose. Incubations were carried out as per Section 2.2.2.

Phenyl Sepharose fractions	Total protein (mg)	LOX activity (units mg^{-1})[A]	NCE activity (μgXANmg $^{-1}$ protein)
HS[a]	0.53	0.38 (19 %)	15.96 (300 %)
GR[b]	0.84	3.89 (194 %)	2.09 (39 %)
NS[c]	0.12	0.19 (10 %)	0.02 (0 %)

[a] Fractions collected at high salt concentration (5 M) of $(NH_4)_2SO_4$.
[b] Fractions collected at decreasing salt (0.5 - 0 M) of $NH_4)_2SO_4$.
[C] Fractions collected at non salt concentration (0 M) of $NH_4)_2SO_4$.
[A] Units: μmol of β-carotene bleached min^{-1}at 25°C.

4.3 SUMMARY OBSERVATIONS

In summary, data presented in this chapter illustrate the following:

- ★ The *Citrus* cell-free system contains both LOX enzyme and NCE activity,
- ★ Both LOX and NCE activity could be precipitated at 45 % $(NH_4)_2SO_4$,
- ★ LOX and NCE activity were separated chromatographically using a column placed with phenyl Sepharose , and
- ★ Fractions purified on Phenyl Sepharose columns showed a 300 % increase of NCE activity.

CHAPTER 5

DISCUSSION AND CONCLUSIONS

A cell-free system with which to study detailed aspects of the biochemistry and enzymology of ABA biosynthetic pathway was developed earlier in our laboratory form an acetone powder of *Citrus sinensis* flavedo (Richardson, 1995; Richardson and Cowan, 1996). Detailed metabolic studies revealed that in this system, ABA was in all probability derived from MVA along a pathway involving β-carotene, 9'-*cis*-N, XAN, XAN-ac and 1',4'-*trans*-ABA diol as intermediates (Cowan and Richardson, 1993(a), 1993(b), 1997). Although ABA was characterized as a product when β-carotene was used as substrate, incorporation of isoprenoid precursors was **not** unequivocally established by these authors. In Addition, recent studies using mutants deficient in ABA have suggested that Z is an intermediate (Rock and Zeevaart, 1991;Taylor , 1991, Marin et al., 1996) which implies that the *Citrus* cell-free system capable of synthesizing ABA, should be able to carry out β,β-xanthophyll interconversion.

5.1 STUDIES ON THE ABA BIOSYNTHETIC PATHWAY

It has postulated that ABA is formed directly from MVA *via* terpenoids (Milborrow and Robinson, 1973; Milborrow, 1983; Cowan and Railton, 1987; Cowan and Richardson, 1993a; Lee and Milborrow, 1997). In most cases, studies undertaken to investigate the operation of this pathway have used labelled MVA which has been shown to be incorporated into ABA and reports suggest that this incorporation is enhanced by NADPH (Cowan and Railton, 1987). In agreement with previous findings, metabolic studies described in chapter 3 in which [2-^{13}C]-MVA was used as substrate have shown that ABA is produced *de novo* in the *Citrus* cell-free system. This observation strongly supports a terpenoid origin for ABA at least in *Citrus*. Although no result was obtained using EI-MS, PI-CI-MS of ABAMe obtained from [2-^{13}C]-MVA incubates indicated the presence of a strong base peak (m/z 261, $[MH-H_2O]^+$) due to expulsion of water from the tertiary

hydroxyl (Netting et al.,1988). A second characteristic ion in the PI-CI-MS of ABAMe m/z 247, $[MH\text{-}MeOH]^+$ has been proposed to arise by the loss of methanol from the ester methyl. $[MH\text{-}H_2O]^+$ and $[MH\text{-}MeOH]^+$ lose methanol and water respectively to give rise m/z 229, $[MH\text{-}MeOH\text{-}H_2O]^+$. Since these ions were clearly present in the PI-CI-MS of ABAMe, coupled with a molecular ion at m/z 279 $[M^+H]$, it is concluded that the compound under investigation was indeed ABAMe (Gray et al.,1974). Unfortunately, the exact position of [^{13}C]-labelling could not be determined in the present study due to insufficient mass of product ABA. Nevertheless, theoretical evidence indicates that ABA produced from radioactive MVA is labelled at the carbon 4 of the dienoic acid side-chain and carbon 3' and 7' of the ring (Milborrow, 1975)(Figure 5.1) that is three atomic mass units extra. The present study has confirmed the enrichment ABAMe, isolated from *Citrus* cell-free system supplied with [2-^{13}C]-MVA with three atomic mass units. Results obtained in the present study are therefore in accord with these observations and demonstrate that [2-^{13}C]-MVA was incorporated in ABA. Since conversion of MVA to ABA occurs *via* isoprenoids, the present study unequivocally establishes the incorporation of this compound into ABA.

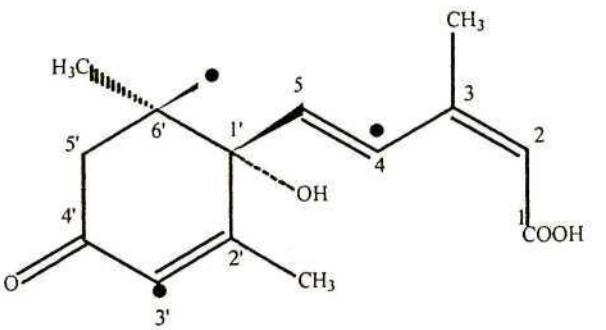

Figure 5.1 The labelling pattern of ABAMe derived from [R ^{14}C]-MVA as proposed by Milborrow (1975).

It is largely accepted that the biosynthesis of ABA occurs indirectly *via* the carotenoid pathway. Currently the accepted pathway appears to involve the cleavage of a 9'-*cis*-xanthophyll to yield XAN which is then converted to ABA *via* AB-ald (Sindhu et al., 1990; Rock and Zeevaart, 1990; Parry, 1993). Several mutants deficient in ABA have been reported (Giraudat, 1994). In most of these mutants there is a blockage at an early step in the carotenoid pathway which results in little or no ABA being biosynthesized. The C_{40}-carotenoid pathway has been shown to involve the xanthophyll cycle, a process involving

interconversions between Z, A, and V occur. These interconversions (epoxidation of Z to V *via* A, and the de-epoxidation of V back to Z) are important since they can become limiting factors in the formation of ABA. Metabolic studies described in the present work indicated that Z was converted to A ,V and N with large amounts of the substrate preferentially metabolised to 9'-*cis*-N. In agreement with several earlier reports, these results indicate that 9'-*cis*-N is the immediate xanthophyll precursor to ABA (Parry et al., 1990; 1991; Li and Walton, 1990; Parry, 1993; Cowan and Richardson, 1997, Schwartz te al, 1997; Thompson et al.,1998; Tan et al., 1997, Burbridge et al, 1997).

It is hypothesized that, under photoinhibitory conditions associated with strong oxidative stress, V is used in large part not for the xanthophyll cycle reaction but for the synthesis of growth inhibitory substances such as ABA (Eskling et al., 1997; Ederli, 1997). An investigation into the efficiency of the production of ABA from Z, A and V indicated that more ABA was made from V compared to Z and A. These results clearly indicate that V is closer to ABA along the pathway. In addition, the cDNA protein encoding for VDE, the enzyme responsible for the de-epoxidation of V to Z has been shown to be inhibited by DTT which was used in the present research. The sulphydryl group of DTT prevents the cysteine rich N-terminal of enzymes to form disulfide bonds and thus deactivates VDE activity favouring the conversion V to ABA (Yamamoto and Kalmite, 1972). It has been demonstrated that in plants V occurs as the *trans* and *cis*-isomer (Li and Walton, 1990; Parry ,1993). Currently it is accepted that 9'-*cis*-N, the immediate xanthophyll precursor to ABA, is derived either from all-*trans*-N or 9'-*cis*-V both originating from all-*trans*-V. Our study has shown that 9'-*cis*-V is not the precursor of 9'-*cis*-N and suggests that 9'-*cis*-N is preferentially synthesized from all-*trans*-V *via* all-*trans*-N. In addition, the suggestion that 9'-*cis*-N is synthesised from all-*trans*-N is consistent with previous hypotheses regarding N formation (Goodwin, 1980; Parry et al., 1990; Parry , 1993).

It has been postulated that 9'-*cis*-V could be the immediate xanthophyll precursor of ABA along with 9'-*cis*- N, and that it is directly converted to XAN by an oxidative cleavage (Li and Walton, 1990). Metabolic studies carried out in the present research indicated that other xanthophylls are produced from 9'-*cis*-V. The present studies have indicate that V is converted to auraxanthin (Figure 5.2.A) by isomerisation of the 5,6 epoxide of 9'-*cis*-V into 5,8 epoxide, a process which apparently occurs rapidly, particularly in the presence of trace amounts of dilute acids (Gross,1987). Auraxanthin has been shown to be

converted to shamoutichrome (Figure 5.2.B). These two apocarotenoids have been found to be present in *Citrus* fruit peel. Another apo-carotenoid closely related to shamoutichrome; shamoutixantin (previously called trollixanthin) (Figure 5.2.C) has been shown to have one terminal ring that has the structure of the epoxy ring of V while the other ring is equivalent to ABA diol (i.e the 1',2'-epoxy group has been isomerised to a 1'-hydroxy,2'-ene and the 4'-hydroxyl is retained). Even so the role for these xanthophylls in ABA synthesis seems doubtful but awaits thorough investigation.

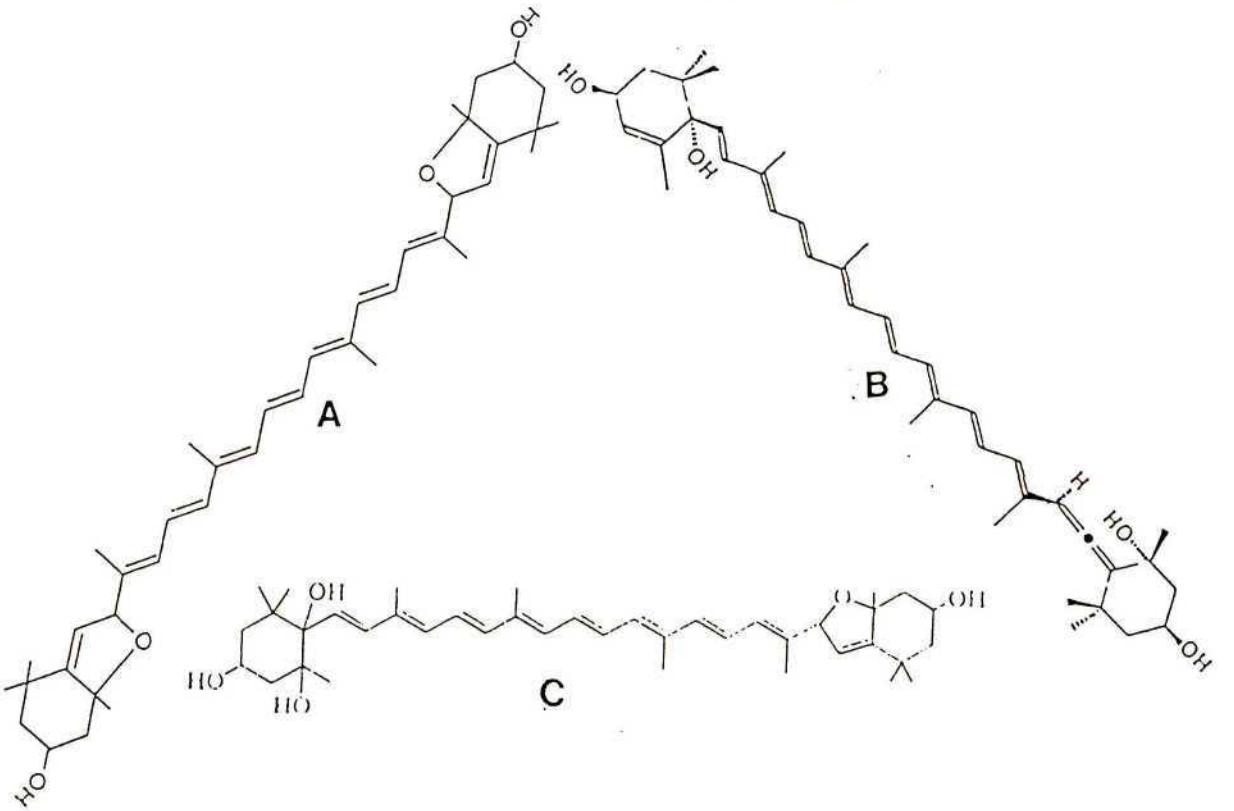

Figure 5.2. structure of *Citrus* apocarotenoids that could be involved in ABA biosynthesis. (A) Auraxanthin. (B) shamoutichrome. (C) shamoutixanthin.

Despite tremendous evidence that is available in support of the carotenoid origin of ABA, there is still the possibility that ABA does not solely arise from β,β-xanthophylls in plants. One of the mutants used to study the biosynthetic pathway of ABA, the *vp*12 mutants of *Zea mays* is ABA deficient at most of its developmental stages (Maluf et al., 1997) not because ABA is not biosynthesized in this plants but rather due to the presence of a leaky mutation in terms of ABA. In another mutant of the same plant, the *vp*15, low levels of ABA accumulate under stress (Saab et al., 1990). These observations support the hypothesis that ABA biosynthesis can be sustained in these mutants despite low levels of

carotenoid precursors. Yet another ABA deficient mutant, *aba*-2 of *Nicotiana plumbaginifolia* (Marin et al., 1996) had 23 - 48 % of the wild type content of ABA which was surprising as data indicate that this is a single copy gene mutant. One possible reason to explain these observations is the operation of a completely different ABA pathway in these mutants. Further evidence in support of an alternative route for ABA biosynthesis might be the presence of C_{30} apo-carotenoids in extracts of plants and *Citrus* peel. With regard to the biosynthesis of apo-carotenoids, several pathways have been proposed (Gross, 1987). In fruits, it has been observed that these compounds are formed by the enzymic reduction at carbons 9-10,9'-10' and 11-12, 11'-12 of C_{40} carotenoids resulting in the formation of C_{27} and C_{25} apoaldehydes and C_{15} compounds. This possibility was also suggested by Milborrow and co-workers who found that ABA biosynthesis in plants does not involve carotenoids (Netting and Milborrow, 1994; Willows et al., 1994).

It is well established that 9'- *cis*-N is the immediate xanthophyll precursor of ABA and that its conversion occurs *via* XAN. Despite tremendous evidence that is available in support of this pathway, there is a possibility that XAN is not solely derived from 9'-*cis*-N. For example , Wagner and Elstner (1989) showed that oxidative cleavage of xanthophylls was not a major biosynthetic route for the formation of XAN. In addition , when contrasting the incorporation of [^{2}H] from [^2H_2O] into ABA, XAN and carotenoids in tomato shoots, it was found that only a single [^{2}H] atom was incorporated into ABA, ruling out XAN as an ABA precursor (Nonhebel and Milborrow, 1987). Demonstrating a role of XAN in the inhibition of pea seedling growth under red light, Anstis et al. (1975) suggested that since more XAN was biosynthesized compared to the isolated V, there was an alternative pathway which triggers XAN formation from lower molecular weight precursors. Furthermore, despite earlier reports confirming that XAN occurs naturally in a variety of plants and plant parts (Taylor and Burden, 1970, 1972; Firn and Friend, 1972), most of the experiments carried out to demonstrate the conversion of XAN into ABA have been done *in vitro* using exogenous XAN. No attempt has been made to demonstrate the conversion of XAN to ABA in *vivo*. These observations cast some doubt on the validity of the implication of XAN as the cleavage product of 9'- *cis*-N in ABA biosynthesis in plants. Despite this controversy, the outcome of the present study is in line with the currently accepted origin of XAN. Xanthoxal was found to be the immediate catabolite of 9'-*cis*-N in the *Citrus* cell- system, confirming earlier report by Cowan and Richardson (1997).

It had previously been suggested that AB-ald was the immediate precursor of ABA (Sindhu and Walton, 1988; Taylor et al., 1988). In the current research project, AB-ald was not found. In addition, Richardson and Cowan (1996) reported that the biosynthesis of ABA from *9'*-cis-N in the *Citrus* cell-free system does not seem to occur *via* ABA-ald but rather that XAN was converted to ABA *via* a compound that was tentatively identified as XAN-ac. In addition, these authors reported that the naturally occurring 1',4' ABA-*trans*-diol was the immediate catabolite of XAN-ac. This led the authors to propose of the following pathway **9'-cis-N→XAN→XAN-ac →1',4'-*trans*-diol ABA→ABA**. The present study has shown that the conversion of 9'-*cis*-N to ABA occurs *via* XAN, unfortunately no intermediates between XAN and ABA were found. Nonetheless this does not preclude the presence of such compounds.

Evidence that AB-ald is the intermediate between XAN and ABA is supported by the discovery of *flacca* and *sitiens* tomato mutants and a molybdopterin-deficient mutant of barley which are unable to convert AB-ald to ABA (Rock and Zeevaart, 1990). However, evidence is also available to show that aldehyde oxidase enzymes are unspecific and can oxidize a range of substrates (Leydecker et al, 1995; Walker-Simmons et al., 1989; Willows and Milborrow, 1990; Netting and Milborrow, 1994). Aldehyde oxidase enzymes are inhibited by allopurinol and are sensitive to tungstate. Both allopurinol and tungstate inhibited ABA synthesis from 9'-*cis*-N in the present work. Since XAN was the major product of N cleavage, it is likely that these inhibitors negatively affect XAN oxidation. This aspect has been elegantly confirmed in recent studies using avocado (Lee and Milborrow, 1997b). Thus, XAN appears to be the substrate for aldehyde oxidase involved in ABA biosynthesis. As stated by Lee and Milborrow (1997b) "there is no *a priori* reason why XAN cannot be converted to AB-alc", which might imply that XAN-alc is also involved in ABA synthesis. If XAN-alc is a catabolite en route to ABA, this makes XAN-ac the immediate precursor of ABA. The presence of XAN-alc and XAN-ac in the biosynthesis of ABA has been reported by several authors (Parry et al, 1990; Cowan and Richardson , 1997, Milborrow et al., 1997b). In addition the conversion of XAN-ac to ABA has been demonstrated (Milborrow and Noddle, 1970; Milborrow and Garmston, 1973; Lee and Milborrow, 1997; Milborrow et al., 1997b). Using the *Citrus* cell-free system, Richardson and Cowan (1996) reported the presence of an acidic compound which they tentatively identified as XAN-ac. The following pathway is therefore proposed as an alternative to current opinion: **9'-cis-N → XAN → XAN-alc → XAN-ac →ABA**

5.2 ENZYMATIC CONVERSION OF 9'-*CIS*-NEOXANTHIN TO ABA AND XANTHOXIN

It is currently accepted that 9'-*cis*-N is converted to XAN by the addition of molecular oxygen. Earlier observations by Firn and Friend (1972) suggested that V was enzymatically cleaved to produce XAN and t-XAN. Thus the possibility that LOX is involved in ABA biosynthesis has been investigated using inhibitors of this enzyme (Parry and Horgan, 1991b; Creelman et al., 1992; Lee and Milborrow, 1997). The presence of LOX in the *Citrus* cell-free system has been reported (Richardson, 1995). In addition Schwartz et al.(1997) cloned a protein with a sequence similar to that of a dioxygenase from mutants deficient in ABA and showed that expression of this protein is developmentally regulated and induced in leaves exposed to drought stress (Tan et al., 1997, Thompson et al., 1998). Studies represented in chapter 4 confirm that the cleavage of 9'-*cis*-N to XAN occurs enzymatically in the *Citrus* cell-free system. Attempts made to purify the NCE from *Citrus* flavedo extracts revealed that this enzyme is present and that its activity can be successfully separated from the LOX. The present studies therefore establish NCE activity in the *Citrus* cell-free system.

Several studies have indicated that the activity of several enzymes can be enhanced by other substances through co-oxygenation or co-oxidation (Núñez-Delicado et al., 1997). Perhaps, LOX involved in ABA biosynthesis, requires the activity of a hydroperoxidase. For instance, complex nonstoichiometric co-oxidation and co-oxygenation of several lipid related compounds which are not hydropreoxide co-substrates is a well-studied phenomenon in animals (Pace-Asciak and Smith, 1993; Núñez-Delicado et al., 1997). The insertion of molecular oxygen observed with diphenylisobenzofuran and benzo(a)pyrene derivatives in human is achieved by PGD synthase which co-oxidises with hydrogen peroxidase (Pace-Asciak and Smith, 1993). Furthermore the oxidation of diethylstilbesterol (DES) to DES quinone requires a lipoxygenase with hydroperoxidase activity (Núñez-Delicado et al., 1997). In both cases, the by-product of the reaction is a free radical believed to be carcinogenic. The presence of C_{25}-allenic apocarotenoids in the biosynthetic route ABA can justify the possibility of having a LOX which requires hydroperoxidase activity in ABA biosynthesis. In fact, Richardson and Cowan showed the presence the 53 kDa protein with peroxidase activity characteristic of a cytochrome

P-450. The presence of this enzyme implies that the biosynthesis of ABA involves at least one cytochrome P-450 catalysed reaction.

As mentioned earlier, the conversion of XAN to ABA occurs in the cytosol and this reaction is mediated by an aldehyde oxidase. Initially purified as XAN oxidising activity (Sindhu et al., 1990), this enzyme has been shown to require NAD/NADP, characteristics confirmed in the present research. Xanthoxal oxidizing activity has also been shown to require a molybdenum co-factor (Mo-co) (Walker-Simmons et al., 1989; Leydecker et al., 1995) and ABA biosynthesis in the *Citrus* cell-free system is enhanced by the inclusion of Mo in the homogenizing buffer (Richardson and Cowan ,1995, Cowan and Richardson, 1996, 1997). Aldehyde oxidase has been shown to be inhibited by tungsten (Lee and Milborrow, 1997). Similar observations were made in this study. The mechanism of inhibition of Mo-co requiring enzymes by tungstate is not understood. A possible explanation could be that since these metals belong to the same group in the periodic table (IVb), tungsten could substitute for molybdenum as the metal ion coordinating the ligand molybdopterin (Coughlan, 1980) and so be a specific inhibitor of aldehyde oxidase.

Inclusion of allopurinol as a chemical modifier in the metabolism of 9'-*cis*-N to ABA did not stimulate ABA biosynthesis (49 % change). Allopurinol is a substrate and potent inhibitor of the metalloflavoprotein, xanthine oxidoreductase and aldehyde oxidase. This peroxidase enzyme contains a molybdenum component in its structure and has been classified as a Mo-co enzyme (Kisker et al., 1997; Mendel,1997). Recent studies have indicated that *in vivo* treatment of tobacco tissue with allopurinol resulted in the inhibition of tobacco xanthine dehydrogenase (Montalbini and Della Torre, 1995). Allopurinol does so by tight binding to the active site of xanthine oxidase by re-oxidation of the molybdenum component of the enzyme. The inactivation of the oxidase enzyme by allopurinol is mediated through its conversion to oxypurinol which is subsequently phosphorylated to oxypurinol ribonucleoside. Oxipurinol ribonucleoside is the compound that re-oxidizes the molybdenum component of the enzymes. Because xanthoxal oxidase requires molybdenum in its structure, a similar mechanism of action might explain the observed reduction in ABA biosynthesis in the *Citrus* cell-free system incubates containing allopurinol when 9'-*cis*-N is used as substrate.

It has been suggested that nickel (Ni^{+}) and cobalt (Co^{2+}) have multiple effects on physiological processes during the life cycle of plant(Singh et al., 1994; Vieira and Barros, 1994; Dube et al., 1993; Pretruzelli et al., 1995). Most of these influences are manifested by their effect on the regulation of enzymic network. A well studied case is the influence of Co^{2+} and Ni^{+} on ethylene biosynthesis. Both metal ions have been shown to inhibit ethylene biosynthesis at certain concentrations and appear to do so by blocking the activity of the ethylene forming enzyme, ACC oxidase (Hoffman and Yang, 1985; Yu and Yang, 1979). Ethylene forming enzyme has an oxidative activity associated to it which is impaired by ABA. As proposed by Dube et al. (1993) and McKeon et al. (1979), the mechanism of inhibition of ACC oxidase implies either the binding of metal ions to the SH group involved in the structural integrity of the enzyme, or the complexion of the metal ions with the carboxylic acid group of the enzyme. The results obtained in this study show that Co^{2+} and Ni^{+} stimulated ABA biosynthesis in the *Citrus* cell-free system. This means that the presence of Co^{2+} and Ni^{+} ions enhanced substantially the activity of the enzyme(s) responsible for the conversion of 9'-*cis*-N to XAN and ABA. Whether these metals act as co-substrate or as co-enzyme in the biosynthesis of ABA in the *Citrus* cell-free system is unknown at this stage. Perhaps they inhibit some regulator molecule (enzyme) that represses ABA synthesis as is the case with the E8 protein involved in ethylene biosynthesis. Interestingly, this gene, shown to control fruit ripening by controlling ethylene evolution after the onset of ripening, has been suggested to be a member of the dioxygenase family of enzymes (Picton et al., 1995). Although it is well documented that a dioxygenase with LOX-like activity is responsible for 9'-*cis*-N cleavage and the protein encoding the neoxanthin cleavage enzyme cloned (Schwartz et al., 1997, 1998; Tan et al., 1997; Burbridge et al., 1997), this enzyme still needs to be isolated, characterized and purified to homogeneity.

5.3 CONCLUSION

In the light of the present study, further work should aim at investigating the required co-factors and co-enzymes which might enhance or repress the activity of the NCE. In addition co-oxidation and co-oxygenation of LOX involved in the cleavage of 9'-*cis*-N with other oxido-reductase enzymes such as peroxidase and hydroperoxidase enzymes

should be studied. Further purification of LOX should be attempted using other chromatographic techniques such as ion exchange. The purification, isolation and characterization of enzymes involved in the ABA biosynthesis will in all probability lead to the cloning of genes which could prove beneficial in the use of ABA as an agrochemical.

REFERENCES

Abraham, G.D., Abrams, S.R, Nelson, L.A.R., and Gusta L.V. (1990). Total synthesis of (+) phaseic acid, Tetrahedron, 46: 5543-5554.

Abrams, S.R., Rose, P.A., Cutler, A.J., Balsevich, J.J., Lei, B. and Walker-Simmons, M.K. (1997), 8'-Methylene abscisic acid, an effective and persistent analog of abscisic acid. Plant Physiology 1: 89-97.

Al-Nimri, L. and Coolbaugh, R.C (1991). Conversion of 1'-desoxy-[^{2}H]-ABA to [^{2}H]-ABA in cell-free extracts from *Cercospora rosicola,* Journal of Plant Growth Regulation, 10: 63-66.

Anstis, P.J.P., Friend, J. and Gardner, C.J. (1975). The role of xanthoxin in the inhibition of pea seedling growth by red light, Phytochemistry, 14: 31 - 35.

Arigoni, D. (1975). Stereochemical aspects of sesquiterpene biosynthesis, Pure and Applied Chemistry, 41: 219-245.

Assante, G., Merlini, L., and Nasini, G. (1977). (+)-Abscisic acid, a metabolite of the fungus *Cercospora rosicola,* Experimentia, 33: 1556-1557.

Aung, L.H., Houck, L.G., and Norman, S.M. (1991) The abscisic acid content of *Citrus* with special reference to lemon, Journal of Experimental Botany, 42: 1083-1088.

Baker, F.C., Brooks, C.J.W., and Hutchinson, S.A. (1975). Biosynthesis of capsidiol in sweet peppers (*Capsicum frutescens*) infected with fungi: Evidence for methyl group migration from ^{13}C by nuclear magnetic resonance spectroscopy, Journal of the Chemical Society: Chemical Communication, 293-294.

Balsevich, JJ., Bishop, G.G. and Banowetz, G.M. (1997). Activity and utility of abscisic acid having a 3' thioesther linked arm, Phytochemistry, 44: 215-220.

Ben-Aziz, A., Grossman, S., Ascarelli, I. and Budowski, P. (1971). Carotene-bleaching activities of lipoxygenase and *haem* proteins as studied by a direct spectrophotometric method, Phytochemistry, 10: 1445-1452.

Bennet, R.D., Norman, S.M. and Maier, V.P. (1990) Intermediate steps in the biosynthesis of abscisic acid from farnesyl pyrophosphate in *Cercospora rosicola*, Phytochemistry, 29: 3473-3478.

Bennet, R.D., Norman, S.M. and Maier, V.P. (1984). Biosynthesis of abscisic acid from farnesyl derivatives in *Cercospora rosicola*, Phytochemistry, 23: 1913-1915.

Bennet, R.D., Norman, S.M. and Maier, V.P. (1981). Biosynthesis of abscisic acid from [1,2-$^{13}C_2$] acetate in *Cercospora rosicola*, Phytochemistry, 20: 2343-2344.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical Biochemistry, 72: 248-254.

Bradshaw, A.P.W., Hanson, J.R., and Siverns, M. (1978). Biosynthesis of illudin sesquiterpenoids from [1,2-$^{13}C_2$]-acetate, Journal Chemical Society Chemical Communication. 303-304

Bray, E.A. (1993). Molecular responses to water deficient, Plant Physiology, 103: 1035-1040.

Brisker, H.E., Goldschmidt, E.E., and Gore, R. (1976). Ethylene-induced formation of ABA in *Citrus* peel as related to chloroplast formations, Plant Physiology, 58: 377-379.

Burbridge, A., Grieve, T.M., Terry, C., Thompson, A. and Taylor, I.B. (1997). Structure and expression of a CDNA encoding Zeaxanthin epoxidase, isolated from a wilt-tomato (*Lycopersicon esculentum* Mill.) Library, Journal of Experimental Botany, 48: 1749-1750.

Burden, R.D. and Taylor, HF (1972). Xanthoxin, a recently discovered plant growth inhibitor, Procure of the Royal Society of London, 180: 317-346.

inhibitor, Procure of the Royal Society of London, 180: 317-346.

Cane, DE (1983). Cell-free studies of monoterpene and sesquiterpene biosynthesis, Biochemical Society Transaction, 11: 473-483.

Cane, DE, Haler, H., and Materia, J. (1981). [^{2}H]-NM determination of the stereo chemistry of an allylic displacement in the biosynthesis of virescenol B., Journal of the Chemical Society: Chemical Communication. 280-282.

Chandler, P.M. and Robertson, M. (1994). Gene expression regulated by abscisic acid and its relation to stress tolerance, Annual Review of Plant Physiology, 45: 113-141.

Couglan, M.P. (Ed) (1980). Molybdenum and molybdo-containing enzymes. Pergamon Press. Oxford.

Cowan, A.K. and Railton, I.D. (1987a). Cytokinins and Ancymidol inhibit abscisic acid biosynthesis in *Persea gratissima*. Journal of Plant Physiology 130: 273-277.

Cowan, A.K., and Railton, I.D. (1987b). The biosynthesis of abscisic acid in a cell-free system from embryos of *Hordeum vulgare*, Journal of Plant Physiology, 131: 423-431.

Cowan, A.K., and Railton, I.D. (1986). Chloroplasts and the biosynthesis and catabolism of abscisic acid, Journal of Plant Growth Regulation, 4: 211-224.

Cowan, A.K. and Richardson, G.R. (1993a). 1', 4'-*trans*-[^{14}C]-Abscisic Acid Diol: A major product of R-[2-^{14}C]-mevalonic acid metabolism in extracts of *Citrus sinensis* exocarp, Journal of Plant Physiology, 142: 730-734.

Cowan, A.K. and Richardson, G.R. (1993b). The biosynthesis of abscisic acid from *all-trans*-β-carotene in a cell free system from *Citrus sinensis* exocarp, Plant Cell Physiology, 34(6): 969-972.

Cowan, A.R., and Richardson, G.R. (1997). Carotenogenic and abscisic acid biosynthesizing activity in a cell-free system, Physiologia Plantarum, 99: 1-8.

Cowan, A.K., and Rose, P.D. (1991). Abscisic acid metabolism in salt stressed cells of *Dunaliella salina*, Possible interrelationship with β-carotene accumulation, Plant Physiology, 97: 798-803.

Creelman, R.A. and Zeevaart, J.A.D. (1984). Incorporation of oxygen into abscisic acid and phaseic acid from molecular oxygen, Plant Physiology, 75: 166-169.

Creelman, R.A. (1989). Abscisic acid physiology and biosynthesis in higher plants, Physiologia Plantarum, 75: 131-136.

Creelman, R.A., Bell, E. and Mullet, J.E. (1992). Involvement of a lipoxygenase-like enzyme in abscisic acid biosynthesis, Plant Physiology, 99: 1258-1260.

Cutler, A.J., Squires, T.M., Loewen, M.K. and Balsevich, J.J (1997). Induction of (+)-abscisic acid in cultured maize cells, Journal of Experimental Botany, 48: 1782 -1795.

Davies, WJ., Mainfield, T.A, and Hetherington, A.M. (1990). Sensing of soil water status and the regulation of plant growth and development, Plant Cell and Environment, 13: 709-719.

Davies, W. J. and Jones, H.G. (1991). Abscisic acid: physiology and biochemistry. Bios Scientific Publishers, Oxford, UK.

Demming, B., Winter, K., Krügger, A., and Czygan, F.C. (1987). Photoinhibition of zeaxantin formation in intact leaves, Plant Physiology, 84: 218-224.

Demming-Adams, B., Adams, W.W. I., and Grace, S.C. (1997). Physiology of light tolerance in plants, Horticultural Reviews, 18: 15-246.

Demming-Adams, B. and Adams, W.W.I (1996). The role of xanthophyll cycle carotenoids in the protection of photosynthesis, Trends in Biochemical Science, 1: 21-26.

Dodd, I.C. and Davies, W.J. (1996). The relationship between leaf growth and ABA accumulation in the grass leaf elongation zone, Plant Cell and Environment, (in press).

Dorffling, K. and Tietz, D. (1983). Methods for the detection and estimation of abscisic acid and related compounds. In: Addicott, F.T. (ed), Abscisic Acid,, Praeger, New York' Pp 25-77.

Dube, A., Barthi, S. and Laloraya (1993). Inhibition of anthocyanin synthesis in the first internodes of *Sorghum bicolor* by cobaltous ions, The site of action of cobalt, Physiologia Plantarum, 87: 444-446.

Duckham, S.C., Taylor, I.B., Linforth, R.S.T., Al-Naieb, R.J., Marples ,B.A. and Bowman, W.R. (1989). The metabolism of *cis*- ABA aldehyde by the wilty mutants of potato, pea and *Arabidopsis thaliana*, Journal of Experimental Botany, 40: 901-905.

Duckham, S.C., Linforth, R.S.T., and Taylor, I.B. (1991). Abscisic acid deficient mutants at the *aba* gene locus of *Arabidopsis thaliana* are impaired in the epoxidation of Zeaxanthin, Plant Cell and Environment, 14: 601-606.

Ederli, L., Pasqualini, S., Batini, M. and Antoneilli, M. (1997). Photoinhibition and oxidative stress: effects on xanthophyll cycle, scavenger enzymes and abscisic acid content in tobacco plants, Journal of Plant Physiology, 151: 422 - 428.

El-Otmani, M., Lovatt, C.J., Coggins, C.W.-Jr., and Agusti, M. (1995). Plant growth regulators in citriculture: factors regulating endogenous levels in *Citrus* tissues, Critical Reviews in Plants Sciences, 14 (5): 367-412.

Eskling, M., Arvidsson, P.D., and Åkerlund, H.E. (1997). The xanthophyll cycle, its regulation and components, Physiologia Plantarum, 100: 806-816.

Fales, H.M., Jaouni, T.M., and Babashak, J.F. (1973). A simple device for preparing ethereal diazomethane without resorting to co-distillation, Analytical Chemistry, 45(13): 2302-2303.

Firn, R.D. and Friend, J. (1972). Enzymatic production of the plant growth inhibitor, xanthoxin, Planta, 103: 263-266.

Fong , F., Koehler, DE, and Smith, J.D. (1983a). Fluridone induction of vivipary during maize seed development. In: Kruger, J.E. and LaBerge, D.E (eds), The third international symposium on pre-harvest sprouting in cereals, Westview Press Boulder, Colorado, Pp188-196.

Fong, F., Smith, J.D., and Koehler, J.D. (1983b). Early events in maize seed development, Plant Physiology, 73: 899-901.

Gage, D.A., Fong, F., and Zeevaart, J.A.D. (1989). Abscisic acid biosynthesis in isolated embryos of *Zea mays* (L), Plant Physiology, 89: 1039-1041.

Gamble, P.E., and Mullet, J.E. (1986). Inhibition of carotenoid accumulation and abscisic acid biosynthesis in fluridone-treated dark-grown barley, European Journal of Biochemistry, 160: 117-121.

Gardner, H.W. (1995). Biological roles and biochemistry of lipoxygenase pathway, Hortscience, 30: 197 - 205.

Gillard, D.F. and Walton, D.C. (1976). Abscisic acid metabolism by a cell-free preparation from *Echinocystis lobata* liquid endosperm, Plant Physiology, 58:790-795

Gilmore, A.M. (1997). Mechanistic aspects of xanthophyll cycle-dependent photoprotection in higher plant chloroplasts and leaves, Physiologia Plantarum, 99: 197-209.

Giraudat, J., Parcy, F., Bertauche, N., Gosti, F., Leung, J., Morris, P C., Bouvier-Durand. M., and Vartanian, N. (1994). Current advances in abscisic acid action and signalling, Plant Molecular Biology, 26:1557-1577.

Goldschmidt, E.E., Goren, R., Even-Chen, Z. and Bittner, S. (1973). Increase in free and bound abscisic acid during natural and ethylene-induced senescence in *Citrus* fruit peel, Plant Physiology, 51: 879-882.

Goodwin, T.W. (1980). The biochemistry of the Carotenoids. Volume I: plants, Biosynthesis of Carotenoids , Chapman and Hall, London, Pp 33 - 76

Gray, R.T., Mallby, R., Ryback, G. and Williams, V.P. (1974). Mass spectra of methyl abscisate and isotopically-labelled analogues, Journal of the Chemistry: Chemical Society, 2: 919-924.

Griffiths, A. and Bray, E.A. (1996). Shoot induction of ABA- requiring genes in response to soil drying, Journal of Experimental Botany, 47: 1525-1531.

Gross, J. (1987). Pigments in fruit. In: Shweigert, B.S. (Ed.), Food Science and Technology, A series of Monograms, Harcourt Brace Jovanovich, London, Pp 127-137.

Hartung, W., Heilmann, B. and Gimmler, H. (1981). Do chloroplasts play a role in abscisic acid synthesis?, Plant Science Letter, 22: 235-242.

Heath, T.G., Gage, D.A., Zeevaart, J.A.D., and Watson, J.T. (1990). Role of molecular oxygen in fragmentation processes of abscisic acid methyl ester in electron capture negative ionization, Organic Mass Spectrometry, 25: 655-663.

Hedden, P. and Kamiya, Y.(1997). Gibberellin biosynthesis: enzymes, genes, and their regulation, Annual Review of Plant Physiology and Plant molecular Biology, 48: 431-460.

Henson, I.E. (1984). Inhibition of abscisic acid accumulation in seedling shoots of pearl millet, *Pennisetum americanum* (L) Leeke following induction of chlorosis by norflurazon, Zeïtschrift für Pflanzenphysiologie, Bd. 1145: 35-43.

Hetherington, A. M. and Quatrano, R. S. (1991). Mechanisms of action of abscisic acid at the cellular level, New Phytology, 119: 9-32.

Hoffman, L.Y. and Yang, S.F. (1985). Promotion by ethylene of the capability to convert 1-aminocyclopropane-1-carboxylic acid to ethylene in pre-climacteric tomato and cantaloupe fruits, Plant Physiology, 77: 407-411.

Ingram, J. and Bartels, D. (1996). The molecular basis of dehydration tolerance in plants. Plant Molecular Biology, 47: 377- 403.

Ivanova, A., Willianov, D., Onckelen, H.V. and Atanassov, A. (1997). Abscisic acid changes in osmotic stressed leaves of *Alfafa* genotypes varying in drought tolerance, Journal of Plant Physiology, 150: 224-227.

Jones, R.J. and Mansfield, T.A. (1972). Effects of abscisic acid and its esters on stomatal aperture and the transpiration ratio, Physiologia Plantarum, 26: 321-327.

Kisker, C., Schindelin, H. and Rees, C.D. (1997). Molybdenum cofactor containing enzyme: structure and mechanism, Annual Review of Biochemistry, 66: 233 - 267.

Kitagwa,Y.,Yamamoto, H. and Oritani, T. (1995). Biosynthesis of abscisic acid in the fungus *Cercospora cruenta*: stimulation of biosynthesis by water stress and isolation of a transgenic mutant with reduced biosynthetic pathway, Plant and Cell Physiology, 36: 557 - 567.

Kleinig, H. and Beyer, P. (1985). Carotene synthesis in spinach, *Spinacia oleracea* (L) chloroplasts and daffodil, *Narcissus pseudonarcissus* (L) chromoplasts, Methods in Enzymology, 110: 267-273.

Koorneef, M. (1986). Genetic aspects if abscisic acid. In Blonstein, A.D. and King, P.J.(eds), A Genetic Approach to Plant Biochemistry, Springer Press, Pp 35-54.

Koorneef, M., Jorna, M.L., Brinkhorst-Van der Swan, D.L.C. and Karssen, C.M. (1982). The isolation of abscisic acid deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of *Arabidopsis thaliana* (L) Heynh, Theoretical and Applied Genetics, 61: 385-393.

Lamb, N., Wahab, N., Rose, P.A., Shaw, A.C., Abrams, S.R., Cutler, A.J., Smith P.J., Gusta, L.V. and Ewan, B.(1996). Synthesis, metabolism and biological activity of a deuterated analogue of the plant hormone 5-(+)-abscisic acid, Phytochemistry, 41: 23-28.

Lee, H.S. and Milborrow, B.V. (1997a). Endogenous biosynthesis precursors of (+)-abscisic acid: V. Inhibition by tungstate and its removal by cinchronine shows that xanthoxal is oxidised by a molybdo-aldehyde oxidase, Australian Journal of Plant Physiology, 24: 727-732.

Lee, H.S. and Milborrow, B.V. (1997b). Endogenous biosynthesis precursors of (+)-abscisic acid: IV. Biosynthesis of ABA from [^2H_n] carotenoids by a cell-free system from Avocado, Australian Journal of Plant Physiology, 24: 715-726.

Leydecker, M.T., Maureaux, T., Kreapiel, Y., Schnorr, K., and Caboche, M. (1995). Molybdenum cofactor mutants, specifically impaired in xanthine dehydrogenase activity and abscisic acid biosynthesis, simultaneously over express nitrate reductase, Plant Physiology, 107: 1427-1431.

Li, Y. and Walton, D.C. (1990). Violaxanthin is an abscisic acid precursor in water-stressed dark-grown bean leaves, Plant Physiology, 92: 551-559.

Li, Y. and Walton, D.C. (1989) ABA is produced from xanthophylls in water-stressed bean leaves, Plant Physiology, 89: Suppl 182.

Li, Y. and Walton, D.C. (1987). Xanthophylls and abscisic acid biosynthesis in water-stressed bean leaves, Plant Physiology, 85: 910-915.

Lin, L-S. And Ho, T-H. D. (1986) Mode of action of abscisic acid in barley aleurone layers, Plant Physiology, 82: 289-297.

Linforth, R.S.T., Bowman, W.R., Griffin, D.A., Hedden, P. and Taylor, I.B. (1987a). 2,7-Dimethytocta-2,4-dienedioic acid, a possible by product of abscisic acid in the tomato. Phytochemistry 26: 1631-1634.

Linforth, R.S.T., Bowman, W.R., Griffin, D.A., Marples, B.A., and Taylor, I.B. (1987b). 2-*trans*-ABA-alcohol accumulation in the wilty tomato mutants *flacca* and *sitiens*, Plant Cell and Environment, 10: 599-606.

Linforth, R.S.T., Taylor, I.B. and Hedden, P. (1987). Abscisic acid and C_{10} dicarboxylic acids in wilty tomato mutants, Journal of Experimental Botany, 38: 1734-1740.

Loveys, B.R., Brien, C.J., and Kriederman, P.E. (1975). Biosynthesis of abscisic under osmotoic stress, studies based on a duel labelling technique, Physiologia Plantarum, 33: 166-170.

Loveys, B.R. and Milborrow, B.V. (1991). Hydroxylation of abscisic acid methyl ether and the formation of three β-glucosides, Phytochemistry, (In press).

Loveys, B.R. and Milborrow, B.V. (1984). Metabolism of abscisic acid. In: Crozier, A. and Hillmann, J.R.(eds), The Biosynthesis and Metabolism of Plant Hormones, Cambridge University Press, Cambridge, Pp 288.

Loveys, B.R. (1991). How useful is a knowledge of ABA physiology for crop improvements. In: Davies, W.J. and Jones, H.G. (eds.), Abscisic acid: Physiology and Biochemistry, Bios Scientific Publishers, Oxford, Pp 245-260.

Maluf, M.P., Saab, I.M., Wurtzel, T.E. and Sachs, M.M. (1997). The viviparous in maize mutant is deficient in abscisic acid, carotenoids, and chlorophyll synthesis, Journal of Experimental Botany, 311: 1259-1268

Marin, E. and Marion-Poll, A. (1997). Tomato *flacca* mutant is impaired in ABA aldehyde oxidase and xanthine dehydrogenase activities, Plant Physiology and Biochemistry.35: 369-372.

Marin, E., Nussaume, L., Quesada, A., Gonneau, M., Sotta, B., Hugueney, P., Frey,A., and Marion-Poll, A. (1996). Molecular identification of zeaxanthin epoxidase of *Nicotiana plumbaginifolia*, a gene involved in abscisic acid biosynthesis and corresponding to the ABA1 locus of *Arabidopsis thaliana*, Embo Journal 15: 2331-2342.

McCarthy, D.R. (1995). Genetic control and integration of maturation and germination pathways in seed development, Annual Review of Plant Physiology Plant Molecular Biology, 46: 71-93.

McKeon, T.A., Fernăndez-Maculet, J.K and Yang, S.F (1995). Biosynthesis and Metabolism of Ethylene. In Davies, P. J.(ed), Plant Hormones, Physiology, Biochemistry and Molecular Biology, Kluwer Academic Publishers, Pp 671-697.

Mendel, R.R. (1997). Molybdenum cofactor of higher plants: Biosynthesis and Molecular biology, Planta, 203: 399 - 405.

Meyers, R.A. (1995). Molecular Biology and Biotechnology: a comprehensive desk reference, 1st ed.,VCH Publishers, Cambridge, Pp 106 - 110.

Milborrow, B.V., Burden, R.S. and Taylor, H.F. (1997). Xanthoxal: a revision of the nomenclature of ABA precursor Xanthoxin, Phytochemistry, 44: 977-978.

Milborrow, B.V. and Garmston, M. (1973). Formation of (-)-1',2'-epi-2-*cis* xanthoxin from a precursor of abscisic acid, Phytochemistry, 12: 1597-1608.

Milborrow, B.V. (1983). The reduction of (+)-[2-^{14}C] abscisic acid to the 1', 4'-*trans*-diol by pea seedlings and the formation of 4'-deoxy ABA as an artefact, Journal of Experimental Botany, 34:303-308.

Milborrow, B.V. (1975). The stereochemistry of cyclization in abscisic acid, Phytochemistry, 14: 123-128.

Milborrow, B.V. (1974a). Biosynthesis of abscisic acid by a cell-free system, Phytochemistry, 13: 131-136.

Milborrow, B.V. (1974b). The chemistry and physiology of abscisic acid, Annual Review of Plant Physiology, 25: 259-307.

Milborrow, B.V. (1974c). The chemistry and biochemistry of abscisic acid. In: Runeckles, V.C., Sondheimer, E. and Walton, D.C. (eds), The Chemistry and Biochemistry of Plant Hormones, Recent Advances in Phytochemistry, Pergamon Press, Oxford, Pp 57-91.

Milborrow, B.V. (1970). The metabolism of abscisic acid, Journal of Experimental Botany, 21(66):17-29.

Milborrow, B.V. (1969). Identification of "Metabolite C" from abscisic acid and a new structure for phaseic acid, Chemistry Communication, 966-967

Milborrow, B.V., Burdern, R.S. and Taylor, H.F. (1997a). Xanthoxal: a revision of the nomenclature of the ABA precursor xanthoxin, Phytochemistry, 44: 977-978.

Milborrow, B.V., Burdern, R.S. and Taylor, H.F. (1997b). The conversion of 2-cis-[^{14}C]-xanthoxic acid into [^{14}C]-ABA, Phytochemistry, 45: 257-260.

Milborrow, B.V., and Robinson, D.R. (1973). Factors affecting the biosynthesis of abscisic acid, Journal of Experimental Botany, 24: 537-548.

Milborrow, B.V., Nonhebel, H.M., and Willows, R.D. (1988). 2,7-Dimethylocta-2,4-dienedoic acid is not a by-product of abscisic acid biosynthesis, Plant Science, 56: 49-53.

Milborrow, B.V., and Noddle, R.C. (1970). Conversion of 5-(1',2'-epoxy-2,6,6-tri-methylcyclohexyl)-3-methylpenta-*cis*-2-*trans*-4-dienoic acid into abscisic acid in plants, Biochemical Journal, 119: 727-734.

Misawa, N., Yosihiko, S., Kondo, K.,Yokoyama, A.,Kajiwara, S., Saito, T., Ohtani, T. and Miki, W. (1995). Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at gene level, Journal of Bacteriology, 177: 6575 -6584.

Montalbini, T. and Della Torre, G. (1995). Allopurinol metabolites and xanthine accumulation in allopurinol treated tobacco, Journal of Plant Physiology, 147: 321-327.

Moore, R., and Smith, J.D. (1985). Graviresponsiveness and abscisic-acid content of roots and carotenoid-deficient mutants of *Zea mays* L, Planta, 164: 126-128.

Moore, R. and Smith, J.D. (1984). Growth graviresponsiveness and abscisic-acid content of *Zea mays* seedlings treated with fluridone, Planta 164: 126-128

Moore, R. , Smith, J.D., and Fong, F. (1985), Gravitropism in abscisic-acid deficient seedlings of *Zea mays*, American Journal of Botany, 72: 1311-1313.

Neill, S.J., Horgan, R., and Parry, A.D. (1986). The carotenoid and abscisic acid content of viviparous kernels and seedlings of *Zea mays* L., Planta 169: 87-96.

Neill, S.J., Horgan, R., and Walton, D.C. (1984). Biosynthesis of abscisic acid. In Crozier, A. and Hillman, J.R. (eds), The Biosynthesis and Metabolism of Plant Hormones, Cambridge University Press, Cambridge, 23: 43-70.

Neill, S.J. and Horgan, R. (1987a). Abscisic acid production and water relations in wilty tomato mutants subjected to water deficiency, Journal of Experimental Botany, 36: 1222 -1231.

Neill, S.J. and Horgan, R. (1987b). Abscisic acid and related compounds, In: Rivier, L. and Crozier, A. (eds), The Principles and Practices of Plant Hormone Analysis, Academic Press, London, Pp 111-167.

Netting, A.G. and Milborrow, B.V. (1994). Endogenous biosynthetic precursors of (+)-abscisic acid, II. Incorporation of isotopes from (+)-[^{2}H] abscisic aldehyde, [$^{18}O_2$] and [$H_2{}^{18}O$], Australian Journal of Plant Physiology, 21: 345-357.

Netting, A.G., Milborrow, B.V., Vaughan, G.T., and Lidgard, R.O. (1988). The fragmentation of methyl abscisate and its 2E isomer in methane positive and negative chemical ionization mass spectrometry, Biomedical and Environmental Mass Spectrometry, 15: 375-389.

Nonhebel, H.M and Milborrow, B.V. (1987). Contrasting incorporation of [^{2}H] from [$^{2}H_2O$] inti ABA, xanthoxin and carotenoids in tomato shoots, Journal of Experimental Botany, 191: 980 - 991.

Norman, S.J., Maier, V.P. and Po, D.L. (1990). Abscisic acid accumulation and carotenoid and chlorophyll content in relation to water stress and leaf age of different types of *Citrus*, Journal Agriculture and Food Chemistry 38, 1326-1334

Norman, S.M. (1991). β-Carotene from the abscisic acid producing strain of *Cercospora rosicola*, Journal Plant Growth Regulation, 10: 103-108.

Norman, S.J., Maier, V.P. and Po, D.L.(1988). Abscisic acid biosynthesis in *Cercospora rosicola*: sensitivity to inhibitors of sterol biosynthesis, Agricultural Biology and Biochemistry, 52: 1039-1310.

Núñez-Delicado, E., Súnchez-Ferrer, A. and Garcia-Carmona, F. (1997). Hydroperoxidative oxidation of diethylstilbestrol by lipoxygenase, Archives of Biochemistry and Biophysics, 348: 411-414.

Ogden, K.L. and Bier, M. (1995). Bioprocess Engineering. In Meyers, R.A.(ed.), Molecular Biology and Biotechnology: a comprehensive desk reference, 1st ed.,VCH Publishers, Cambridge, Pp 106 - 110.

Okamoto, M., Hirai, N. and Koshimizu, K. (1987). Occurrence and metabolism of 1', 4'-*trans*-diol of abscisic acid, Phytochemistry, 26: 1269-1271.

Oritani, T., Niitsu, M., Kato, T. and Yamashita, K. (1985a). Isolation of (2Z, 4E-*y*-ionylidene ethanol from *Cercospora cruenta* , a fungus producing (+)-abscisic acid, Agricultural Biology and Chemistry, 49: 2819-2822.

Oritani, T., and Yamashita, K. (1985). Conversion of (2Z,4E)-5(1',2'-epoxy-2',6',6'-trimethylcyclohexyl)-3-methyl-2, 4-pentadienoic acid to xanthoxin acid by *Cercospora cruenta*, a fungus producing (+)-abscisic acid, Phytochemistry, 24: 1957-1961.

Pace-Asciak, C.R. and Smith, W.L. (1993). Enzymes in the Biosynthesis and Catabolism of the Eicosanoids: Prostagladins, Thromboxanes, Leukotriens and Hydroxy Fatty Acids, In Boyer,P.D. (ed), The enzymes, 3rd edition, Lipid Enzymology; Fatty acids, Glycerides, Phospholipids, Glycolipids, Cholestrol, Academic Press, New York, Pp 543 - 603.

Parry, A.D. and Horgan, R. (1992a). Physico-chemical methods in ABA research. In: Davies, W.J. and Jones, H.G. (eds), Abscisic Acid Physiology and Biochemistry, Bios Scientific Publishers, Oxford, Pp.5-21.

Parry, A.D. and Horgan, R. (1992b). Abscisic acid biosynthesis in higher plants, In:Karssen, C.M., Van Loon, L.C. and Vreugdenhik, D. (eds), Progress in Plant Growth Regulation, Pp 160-172.

Parry, A.D. and Horgan, R. (1991a). Carotenoid metabolism and the biosynthesis of abscisic acid, Phytochemistry, 30 (3): 815-821.

Parry, A.D. and Horgan, R. (1991b). Carotenoids and ABA biosynthesis, Journal Experimental Botany, 42 (238S): 8.

Parry, A.D., Neilll, S.J. and Horgan, R. (1988). Xanthoxin levels and metabolism in the wild-type and wilty mutants of tomato, Planta, 173: 397-404

Parry, A.D. (1993). Abscisic acid metabolism. Methods in Plant Biochemistry, 9: 381-402.

Parry, A.D., Blonstein, A.D., Babiano, M.J., King, P.J. and Horgan, R. (1991c). Abscisic-acid metabolism in a wilty mutant of *Nicotiana plumbaginifolia*, Planta, 183: 237-243.

Parry, A.D., Griffiths, A. and Horgan, R. (1992). Abscisic acid biosynthesis in roots, II. The effects of water-stress in wild type and abscisic acid-deficient mutant (*notabilis*) plants of *Lycopersicon esculentum* Mill, Planta, 187: 192-197.

Parry, A.D., Babiano, M.J., and Horgan, R. (1990). The role of cis-carotenoids in abscisic acid biosynthesis, Planta, 182:118-128.

Perras, M.R., Abrams, S.R. and Balsevich, J.J. (1994). Characteristics of abscisic acid carriers in suspension of cultured barley cells, Journal of Experimental Botany, 280: 1565 - 1573.

Picton, S., Gray, J.E. and Gregson, D. (1995). Ethylene Genes and Fruit Ripening. In Davies, P. J.(ed), Plant Hormones, Physiology, Biochemistry and Molecular Biology, Kluwer Academic Publishers, Pp 372 - 394.

Pretruzelli, L., Harden, F.; Perrons, L. and Reiss, J. (1995). On the role of seed germination and early root growth of *Pisum sativum*, Journal of Plant Physiology, 145:83 - 86.

Prescott, A.G. and John, P. (1997). Dioxygenases: molecular structure and in role plant metabolism, Annual Review of Plant Physiology Plant Molecular Biology, 47: 245-271.

Quarrie, S.A., and Lister, P.E. (1984). Evidence of plastid control of abscisic acid accumulation in barley, *Hordeum vulgare* (L), Zeïtschrift für. Pflanzenphysiologie, Bd. 1145: 295-308.

Quarrie, S. (1982). *Droopy*: a wilty mutant of potato deficient in abscisic acid, Plant and Cell Environment, 5: 23-26.

Quatrano, R.S., Bartels,D., Ho, T-H.D. and Pagès, M. (1997). New insights into ABA mediated processes, The Plant Cell, meeting report, 470 - 475.

Rademarcher, W., Maisch, R., Liessegang, J. and Jung, J. (1989). New synthetic analogues of abscisic acid: their influence on water consumption and yield formation in crop plants. In: Kreeb, K.H., Richeter, H. And Winckley, T.M. (eds), Structural and Functional Responses to Environmental Stresses: Water shortage, SPB Academic Publishing, The Hague, Pp 147-154.

Rajagopalan, K.V. and Johnson, J.L (1992). The pterin molybdenum cofactors, Journal Biological Chemistry, 267: 10199-10202.

Richardson, G.R. and Cowan, A.R. (1996). Development of an abscisic acid biosynthesizing cell free system from flavedo of *Citrus sinensis* fruit, Journal of Experimental Botany, 47 (296): 459-464.

Richardson, G.R. (1995). Cell-Free biosynthesis of abscisic acid in extracts of flavedo from *Citrus sinensis* (L) Osbeck, Phd thesis, University of Rhodes.

Ridley, S.M. (1982). Carotenoids and herbicide action. In: Britton, G. And Goodwin, T.W. (eds), Carotenoid Chemistry and Biochemistry, Pergamon Press, Oxford, Pp 353-369.

Rock, C.D. and Zeevaart, J.A.D. (1991). The aba mutant of *Arabidopsis thaliana* is impaired in epoxy carotenoid biosynthesis, Proceedings of the National Academy of Science, USA, 88: 7496-7499

Rock, C.D., Bowlby, N.R., Hoffmann-Benning, S. and Zeevaart, J.A.D. (1992). The aba mutant of *Arabidopsis thaliana* (L) Heynh has reduced chlorophyll florescence yields and reduced thylakoid stacking, Plant Physiology, 100: 1796-1801.

Rock, C.D. and Zeevaart, J.A.D. (1990). Abscisic (ABA)-aldehyde is a precursor to, and 1', 4'-*trans*-diol a catabolite of ABA in apple, Plant Physiology, 93: 915-923.

Rock, C.D., Heath, T.G., Gage, D.A. and Zeevaart, J.A.D. (1991) Abscisic (ABA)-alcohol is an intermediate in ABA biosynthesis in a shunt pathway from abscisic aldehyde, Plant Physiology, 97: 670-676.

Rock, C. D. and Quatrano, R. S. (1995). The role of hormones during seed development. In Davies, P. J.(ed), Plant Hormones, Physiology, Biochemistry and Molecular Biology, Kluwer Academic Publishers, Pp 671-697.

Rose, P.A., Lei, B., Shaw, A.C., Barton, D.L., Walker-Simmons, M.K. and Abrams, S.R. (1996). Probing the role of the hydroxyl group of ABA: analogs with a methyl ether at C-1', Phytochemistry, 41: 1251-1258.

Rose, P.A., Cutler, A.J., Loewen, M.K., Hogge, L.R., Abrams, S.R. (1996). Metabolism and biological activity of (+) and (-)-C-1'-O' methyl ABA in maize suspension cell cultures, Phytochemistry, 42: 3, 575-579.

Rousselin, P., Kraepiel, Y., Maldiney, R., Miginia, E., and Caboche, M. (1992). Characterization of three hormone mutants of *Nicotiana plumbaginifolia*: evidence for a common ABA deficiency, Theoritical and Applied Genetics, 85: 213-221.

Saab, I.N., Sharp, R.E., Portchurd, J. and Voetberg, G.S. (1990). Increased endogenous ABA maintains primary roots growth and inhibits shoot growth of maize seedlings at low water potentials, Plant Physiology, 93: 1329-1336.

Sandmann, G. and Bramley, P.M. (1985). The *in vivo* biosynthesis of β-cryptoxanthin and related xanthophylls with alphanocapsa membranes, Biochemica et Biophysica Acta, 843:73-77.

Schwartz, S.H., Tan, B.C., Gage, D.A., Zeevaart, J.A.D., and Melarty, D.R. (1997). Specific oxidative cleavage of carotenoids by VP14 of maize, Science 276: 1872-1874.

Sharkey, T.D. and Raschke, K. (1980). Effects of phaseic acid and dihydrophaseic acid on stomata and photosynthetic apparatus, Plant Physiology, 65: 291-297.

Siedow, J.N. (1991). Plant lipoxygenase: Structure and function, Annual Review of Plant Physiology Plant Molecular Biology, 42: 145-188.

Sindhu, R.K. and Walton, D.C. (1987). Conversion of xanthoxin into abscisic acid by cell-free preparations from bean leaves, Plant Physiology, 85: 916-921.

Sindhu, R.K. and Walton, D.C. (1988). Xanthoxin metabolism in cell-free preparations from wild type and wilty mutants of tomato, Plant Physiology, 88: 178-182.

Sindhu, R.K., Griffin, D.H. and Walton, D.C. (1990). Abscisic acid aldehyde is an intermediate in the enzymatic conversion of xanthoxin to abscisic acid in *Phaseolus vulgaris* L leaves, Plant Physiology, 93: 689-694.

Singh, Z., Sing, L., Arrora, C.L and Dhillon, B.S. (1994). Effects of cobalt, cadmium, and nickel as inhibitors of ethylene biosynthesis on floral malformation, yield and fruit quality of mango, Journal of Plant Nutrition, 17 (10), 1659-1670.

Suzanne, R.A., Rose, P.A., Cutler, J.A., Balsevich, J.J., Lei, B. and Walker-Simmons, M.K. (1997). 8'-Methylene abscisic acid, an effective and persistent analog of abscisic acid. Plant Physiology 114: 89-97.

Takahashi, S., Oritani, T. and Yamashita, K. (1986). Total synthesis of (+) methyl phaseates. Agricultural Biology and Chemistry, 50: 1589-1595.

Tan, B.C., Schwartz, S.H., Zeevaart, J.A.D. ,and McCarthy, R.D. (1997). Genetic control of abscisic acid biosynthesis in maize, Proceedings of National Academy of Science, USA, 94: 12235-12240

Taylor, H.F. and Burden, R.S. (1974). The biochemistry of xanthoxin and its relationship to abscisic acid. In: Schreiber, K. (ed), Biochemistry and Chemistry of Plant Growth Regulators, Academic Science, GDR Halle (Saale), Pp 187-196.

Taylor, H.F. and Burden, R.S. (1973). Preparation and metabolism of 2-[^{14}C]-*cis, trans*-xanthoxin, Journal Experimental Botany , 24: 873-880.

Taylor, H.F. and Burden, R.S. (1972). Xanthoxin, a recently discovered plant growth inhibitor, Proceedings of Royal Society London ,180: 317-346.

Taylor, H.F. and Burden, R.S. (1970). Identification of plant growth inhibitors produced by photolysis of violaxanthin, Phytochemistry, 9: 2217-2223.

Taylor, I.B., Linforth R.S.T., Al-Naieb, R.J., Bowman, W.R., and Marples, B.A. (1988). The wilty tomato mutants *flacca* and *sitiens* are impaired in the oxidation of ABA-aldehyde to ABA, Plant and Cell Environment, 11: 739-745.

Taylor, I.B. (1987). ABA deficient mutants. In: Thomas, H. and Grierson, D. (eds). Developmental mutants in higher plants, CUP Cambridge, Pp 197-217.

Taylor, I.B.(1991). Genetics of ABA synthesis. In:Davies, W.J. and Jones, H.G.(eds). Abscisic Acid Physiology and Biochemistry,Bios Scientific Publishers, Oxford, Pp 23-37.

Teitz, A., Kholer, R., Rüttkowski, U. and Kaspric, W. (1987). Further investigations on the occurrence and effects of abscisic acid in algae. In Abstracts, General Lectures, Symposis Papers and Posters, 14th International Botany Congress, Berlin, Pp 135.

Thompson, A.J., Jackson, A.C., Wellesbourne, A., Grieve, T.M., Parker, R.A. and Taylor, I.B. (1998). Regulation and expression of abscisic acid biosynthesis genes in tomato, Journal of Experimental Botany, 49, Supplement, Pp 16 (Abstracts).

Vaughan, G.T. and Milborrow, B.V. (1987). The occurrence and metabolism of the 1',4'-diols of abscisic acid, Australian Journal of Plant Physiology, 14: 593-604.

Vieira, H.D. and Barros, S.R. (1994). Responses of seed of *Stylosanthes humilis* to germination regulators, Physiologia Plantarum, 92: 17-20.

Wagner, G.R. and Elstner, E. (1989). Oxidative xanthophyll degradation is not a major biosynthetic pathway for xanthoxin formation, Journal of Plant physiology, 134: 47 - 53.

Walker-Simmons, M.K., Anderberg, R.J., Rose, P.A, and Abrams, S.R. (1992). Optical pure abscisic acid analogs- tools for relating germination inhibition and gene expression in wheat embryos, Plant Physiology, 2: 501- 507.

Walker-Simmons, M., Kudrna, D.A. and Warner, R.L. (1989). Reduced accumulation of ABA during water stress in a molybdenum cofactor mutant of barley, Plant Physiology, 90: 728-733.

Walker-Simmons, M.K., Rose, P.A, Shaw, A.C. and Abrams, S.R. (1994). The 7'-methyl group of abscisic acid is critical for biological activity in wheat embryo germination, Plant physiology, 106: 1279 - 1284.

Walker-Simmons, M.K., Holappa, L.D., Abrams, G.D. and Abrams, S.R. (1997). ABA metabolites induce group 3LEA MRNA and inhibit germination in wheat, Physiologia Plantarum, 100(3): 474-480.

Walton, D.C. and Sondheimer, E. (1972). Metabolism of 2-^{14}C (+)-abscisic acid in excised bean axes, Plant Physiology 49: 285-289.

Walton, D.C. and Li, Y. (1995). Abscisic Acid Biosynthesis and Metabolism. In: Davies, P.J. (ed), Plant Hormones Physiology, Biochemistry and Molecular Biology, Kluwer Academic Publishers, London, Pp 140-157.

Wang, T., Donkin, M., and Martin, E. (1984). The physiology of wilty pea: abscisic acid production under water stress, Journal of Experimental Botany, 35: 1222-1232.

Willows, R.D. and Milborrow, B.V.(1990). Abscisic acid: a new synthesis, isotopic labelling exchange reactions and oxidation, Phytochemistry, 31:2649-2653.

Willows, R.D., Netting ,A.G. and Milborrow, B.V. (1994). Endogenous biosynthetic precursors of (+)-Abscisic acid, 1, Incorporation of isotopes from [$^{2}H_2$] ,[$^{18}O_2$] and [5-^{18}O] mevalonic acid, Australian Journal of Plant Physiology, 21: 327-343.

Windsor, M.L. and Zeevaart,J.A.D. (1997). Induction of ABA 8'-hydroxylase by (+)-S-, (-)-L- and 8',8',8'-trifluoro-S-abscisic acid in suspension cultures of potato and *arabidopis*, Phytochemistry, 1997, 45: 931- 934.

Yamamoto, H.Y. and Kalmite, L. (1972). The effects of dithiothreitol on violaxanthin deepoxidation and absorbance changes in the 500 nm region, Biochemica et Biophysica Acta, 267: 538-543.

Yamamoto, H. and Oritani, T. (1995). Derivatization and deuterium labelling of xanthoxin, Tetrahedron letters, 36: 5797-5800.

Yamamoto, H. and Oritani, T. (1996). Stereoselectivity in the biosynthesis conversion of xanthoxin into Abscisic acid, Planta, 200: 319-325.

Yamamoto, H. and Oritani, T. (1997). Incorporation farnesyl derivatives into abscisic acid and its intermediates in *Cercospora cruenta*, Bioscience biotechnology and Biochemistry, 61: 5821 - 5824.

Yamamoto, H.V. and Bassi, R. (1996). Carotenoids: Localization and function. In: Ort, R.R. and Yocum, C.F. (eds), Oxygenic Photosynthesis: The Light Reactions, Kluwer Academic Publishers, Dordrecht, Pp. 539-563.

Yu, Y.-B. and Yang, S.F. (1979). Auxin-induced ethylene production and its inhibition by amino-ethoxyvinylglycine and cobalt ions, Plant Physiology, 64: 1074-1077.

Zairen, S., Gantt, E., Cunning H. Fx. Jr. and Sun, Zr. (1996): cloning and functional analysis of the beta-carotene hydroxylase of *Arabidopsis thaliana*, Journal of Biological Chemistry, 24349-24352.

Zeevaart, J.A.D. and Milborrow, B.V. (1976). Metabolism of abscisic acid and the occurrence of epi-dihydrophaseic acid in *Phaseolus vulgaris*, Phytochemistry, 15: 493-500.

Zeevaart, J.A.D., Rock, C.D., Fantauzzo, F., Heath, T.G., and Gage, D.A. (1991). Metabolism of ABA and its physiological implications. In: Davies, W.J. and Jones, H.G. (eds), Abscisic Acid Physiology and Biochemistry, Bios Scientific Publishers, Oxford, Pp 39-52.

Zeevaart, J.A.D., Heath, T.G., and Gage, D.A.. (1989). Evidence for a universal pathway of abscisic acid biosynthesis in higher plants from ^{18}O incorporation patterns, Plant Physiology, 91: 1594-1601.

Zeevaart, J.A.D. and Creelman, R.A. (1988). Metabolism and physiology of abscisic acid, Annual Review of Plant Physiology Plant Molecular Biology, 39: 439-473.