Cytokinin, jasmonates and postharvest physiology of *Asparagus officinalis* L.

Nigel Esteven Gapper

A thesis submitted for the degree of

Master of Science,

in Plant Biology,

Massey University,

Palmerston North,

New Zealand

December, 1998

Abstract

The asparagus spear is a rapidly growing shoot, dependent on the crown and storage roots for substrate . Once harvested, spears have a very short shelf-life. Investigations to date point to a physiological cause of this deterioration rather than a pathogenic one. Since loss of membrane integrity is a notable feature of the postharvest deterioration, spears were treated immediately following harvest with cytokinin (which promotes membrane integrity), and jasmonic acid (produced by deteriorating membranes). Treated plant material was collected and monitored for physiological and compositional changes. Results show a reduction in postharvest elongation of spears treated with cytokinin, and a reduction of shelf-life of spears treated with jasmonic acid, when compared with control spears treated with water. Also an extension of shelf-life was observed for spears treated with cytokinin. We quantified jasmonates using ELISA in spears after harvest, and also in naturally senescing cladophylls. Jasmonate concentration increased in spears rapidly after harvest, which is most likely to be in response to wounding. Results also showed that jasmonates may be involved in desiccation stress and cessation of elongation in asparagus spears. Jasmonate production and metabolism appears to be more ordered during natural foliar senescence than during harvest induced senescence of the spear. Jasmonic acid and dihydrojasmonic acid are metabolised to cucurbic acid during the later stages of natural foliar senescence. The presence of jasmonates in asparagus spears was confirmed by electrospray ionisation mass spectrometry. This analysis also enabled to identify a novel jasmonate, tryptophan-dihydrojasmonic acid amino acid conjugate.

Acknowledgements

I would like to express my thanks to the many people who have helped me throughout the duration of my MSc program.

Firstly, I would like to express thanks to my two supervisors, Professor Paula Jameson (Institute of Molecular Biosciences, Massey University), and Dr Ross Lill (Crop & Food Research, Palmerston North), whose knowledge, patience, advice and encouragement was much appreciated.

Thank you Dr Gill Norris for the electrospray mass spectrometry work you did for me. Without your knowledge this section of the thesis would not have been achieved.

Thank you Dr John Koolaard, whose statistical knowledge helped me out no end.

To the people in the Plant Physiology Laboratory, Lyn Watson, Don Hunter, Trish Murray, Simone Downs, Celia Zhang, Deming Gong, San Dong Yoo, Rainer Hoffman thank you for the advice and for making the lab a happy environment to work.

A very special thanks to Huaibi Zhang, Marian McKenzie and Geraldine Wood of the cytokinin group. You all helped me very much in different ways and I probably would not have been able to get my project underway without your help and advice.

I would like to acknowledge the assistance I received from Dr Sean Clarke when setting up the ELISA for jasmonate quantification. I learnt a lot from you in the short time you were in Palmerston North.

Thank you to the staff of Crop & Food Research Levin, in particular I would like to thank Dr Jocelyn Eason, Dr Erin O'Donahgue, Sheryl Somerfield, Willy Borst and Jason Johnson. Thank you for the advice and encouragement while I was in Levin.

To my friends at Creative Sounds (The Stomach), and the wider live music scene here in Palmerston North ,thank you for giving me the opportunity to meet new people and forget about science for a while. In particular I would Like to thank Jarrod Love, Malcolm McKinnon, Rob Williams, Craig Black, Dave Bloxham, James Lissette, Mitchell and Ryan Watson and Kelvin Shearman.

Thank you to all the people of the Institute of Molecular Biosciences in particular the students and staff of the old Department of Plant Biology and Biotechnology.
I would like to express my gratitude to Crop & Food Research for financial assistance throughout the project. Without this funding I would not have been able to start this project.

Finally to my family, Mum, Dad, brother Leyton, and my two Nanas, thank you for being there when I needed you.

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Chapter One

INTRODUCTION

1.1 Overview

Fresh asparagus (*Asparagus officinalis* L.) is a sought after vegetable crop and only available during spring. As it has a high value to weight ratio it is a suitable crop for export to supply off season markets in the northern hemisphere but asparagus spears are metabolically very active and highly perishable during handling and storage (Lill 1980; King et al. 1988). The New Zealand industry favours a shelf-life of four to five days after three to four weeks of storage to enable sea freight of asparagus but this is not yet attainable due to tissue deterioration during shelf-life (Lill 1980; King et al. 1986, 1988).

The asparagus spear is an immature, fast growing shoot. After cutting at harvest the spear undergoes many physiological, biochemical and gene expression changes, many of which occur very rapidly (King et al. 1993). Altogether these changes lead to loss of visual appeal and freshness which are important attributes for the sale of fresh asparagus.

While the physiology, biochemistry and gene expression of senescing asparagus spears has been investigated (Lipton 1990; Hurst et al. 1994a; King et al. 1992, 1995), there has been little work on plant hormones in this process. The cytokinins and jasmonates were selected for this project because the harvested asparagus spear is a system where substantial membrane degradation occurs (Hurst et al. 1994b). Cytokinins have been shown to delay senescence in a number of crop plants, such as broccoli (Clarke et al. 1994), tobacco (Gan and Amasino 1995) and asparagus (Dedolph et al. 1961), by possibly protecting against oxidative reactions and maintaining membrane function (Grossman & Lesham 1978). Jasmonates have been found to occur naturally in a wide range of higher plants, and are believed to be derived from fatty acids, and produced

during lipid/membrane degradation. Jasmonates have not been reported in asparagus and although implicated in senescence there appear to be no studies of jasmonate levels over time in a senescing system. Furthermore, there are suggestions in the literature which indicate that cytokinins and jasmonates may have opposing effects on leaf senescence (Ueda and Kato 1982; Weidhase et al. 1987a; Parthier et al. 1992), that their modes of action are different and that they do not compete for the same target (Weidhase et al. 1987a). For example, the action of jasmonates and cytokinins were negatively correlated in studies comparing the effects of exogenously applied jasmonates and cytokinins in isolated barley segments (Weidhase et al. 1987a). It was thought that methyl jasmonate might act indirectly by accelerating chloroplast senescence. Furthermore, it has been suggested that jasmonates promote senescence processes and that the cytokinins partially counteract their effects (Hermann et al. 1992).

1.2 Postharvest deterioration of asparagus

The asparagus spear is a rapidly growing immature shoot capable of up to 70 mm growth per day at 20°C. While outwardly asparagus spears appear green, they are relatively non photosynthetic and thus dependant on the crown and storage roots for substrate. The spear also has a very high rate of respiratory metabolism, producing 9-40 μmol $CO_2 .g^{-1}$ $FW.h^{-1}$. Deterioration is rapid and spears can become unsaleable within five to six days after harvest if left under shelf-like conditions at 20°C (see Figure 1.1).

Deterioration coincides with a trait known as tip-rot or melting tip (King et al. 1993). Investigations to date point to a physiological cause of tiprot rather than a pathogenic one (Carpenter et al. 1988). Tiprot starts with a softening of the spear tip, the tip bracts then become grey and feathered, and the tip tissue becomes waterlogged. Soft rots, mainly of bacterial origin invade the affected tissue resulting in a slimy disintegration of the top 30-55 mm of the spear (Lill et al. 1996). Investigations of the disorder have shown that the rate of development is influenced by postharvest

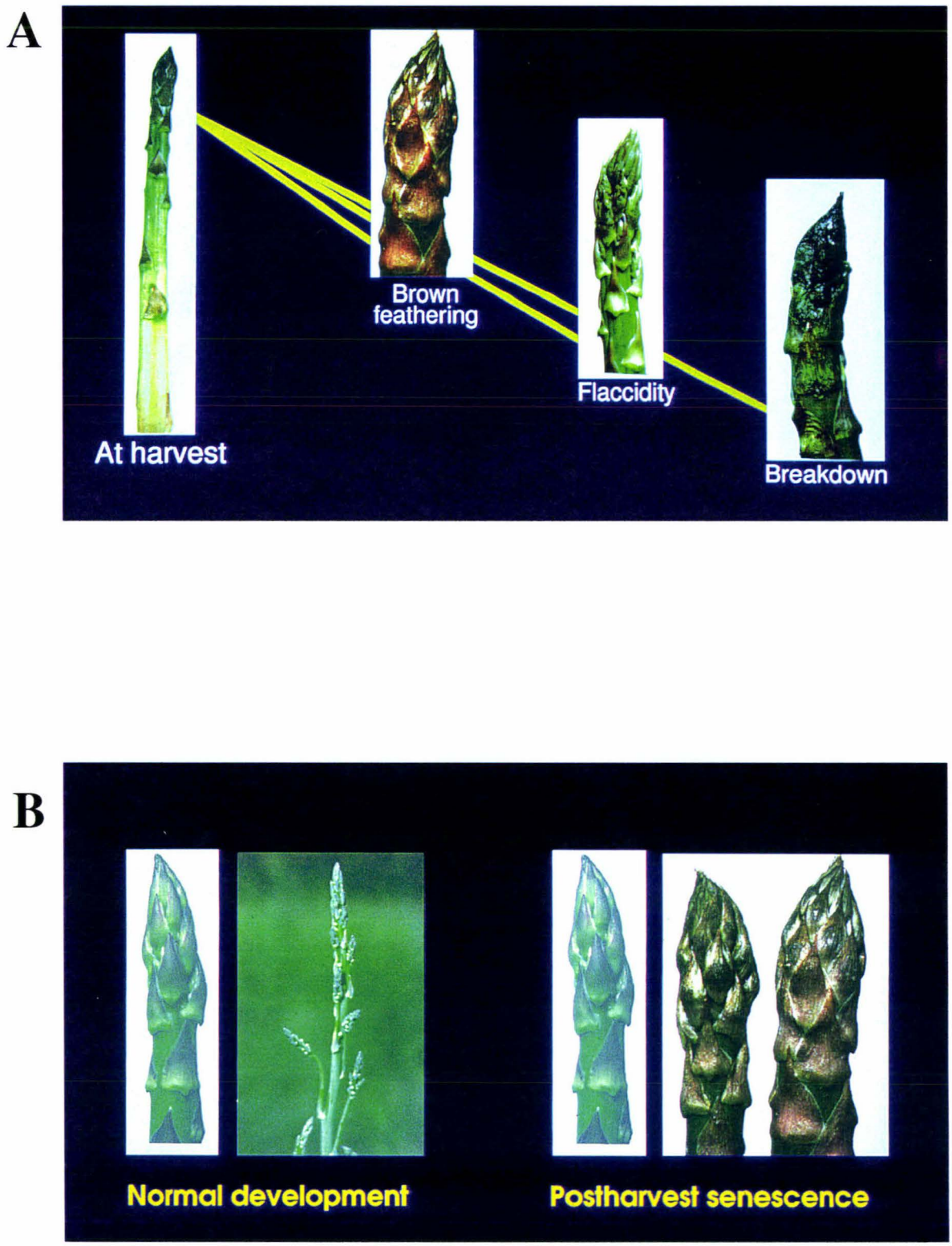


Figure 1.1 A. Photograph showing the deterioration in spear tips after harvest. B. Photograph showing interrupted development of asparagus following harvest.

temperature (King et al. 1993), genotype and concentration of soluble sugars (Lill et al. 1994) and physiological status of the spear at the time of harvest and preharvest temperature (Lill et al. 1996). Lill et al. (1998) noted that controlled atmosphere conditions (5 % O_2, 10 % CO_2) extended shelf-life significantly and prevented development of tiprot.

Biochemical changes in spears after harvest include degradation of chlorophyll (King et al. 1995), reduction in ascorbic acid (Scott and Kramer 1949; Lill 1980), accumulation of ammonia and free amino acids (in particular asparagine) (Lill et al. 1990), loss of proteins (King et al. 1990), loss of soluble sugars (Hurst et al. 1993a), and loss of lipids (Hurst et al. 1994b). Changes in gene expression in spears postharvest (consistent with the compositional changes seen) include up-regulation of the genes encoding asparagine synthetase (King and Davies 1992) and β-galactosidase (King and Davies 1994), and down-regulation of the gene encoding glutamine synthetase (Hurst et al. 1993c).

Consequently, because some of the same proteins expressed during natural foliar senescence are also present during the harvest-induced changes in spears, the deterioration in the spear has been considered to be due to a programmed senescence process which was induced by harvest. King et al. (1995) showed that the artificial postharvest-induced "senescence" of the spear had similarities to the natural foliar senescence in asparagus fronds. Transcripts for three harvest-induced cDNA clones were found to accumulate during natural foliar senescence, suggesting that the regulatory mechanisms may be similar in both situations. Two of the transcripts showed homology with ß-galactosidase and asparagine synthetase. Both of these enzymes are known to be involved with the remobilisation of carbon and nitrogen respectively. However, Hurst et al. (1993a) demonstrated that there were no differential effects on nitrogen metabolism in asparagus spear tips during early postharvest senescence, irrespective of whether spears were stored in the dark or the light. This differs markedly from other naturally senescing plant systems. They concluded that the observed shifts in nitrogen metabolism were storage-related rather than light-modulated.

1.3 Cytokinins and plant development

The cytokinins are a group of plant hormones which are involved with a number of plant growth and developmental processes (Mok 1994) including *de novo* bud formation during tissue culture (Stabel et al. 1990; Zhang 1998), germination and seed dormancy (Shultz and Small 1991), release of apical dominance (Wickson and Thimann 1958; Li and Bangerth 1992), leaf expansion (Brock and Cleland 1989), cell division (Miller et al. 1956; Lewis et al. 1996), reproductive development (van der Krieken et al. 1991) and delay of senescence (Clarke et al. 1994; Gan and Amasino 1995). Reviews by Horgan (1984) and Mok (1994) cover these topics in more detail.

1.3.1 Cytokinins and senescence

The cytokinins appear to be the major group of senescence-retarding hormones in plants. Many studies have reported that leaf senescence is usually correlated with a decrease in cytokinin activity in the leaves, and roots have been implicated as the major source of cytokinins in mature leaves (Nooden et al. 1990 and papers within). These root-produced cytokinins are carried through the xylem into the leaves with the transpiration stream. It is thought that cytokinins act by preventing oxidising reactions which produce free radicals (Grossman and Lesham 1978). Lipoxygenase mediated lipid oxidation produces free radicals, which are hyperactive and can cause damage to biological materials within plants, which is an essential facet of senescence. Grossman and Lesham (1978) further showed that exogenous cytokinin application to pea plants lowered lipoxgenase activity considerably. They suggested that lipoxygenase repression induced by cytokinin is a contributing factor to the overall anti-senescence action of the hormone.

Recently developed molecular biology techniques have enabled the possibility of genetically modifying cytokinin biosynthesis and metabolism to influence plant development. Genes controlling cytokinin biosynthesis have been isolated from *Agrobacterium tumefaciens*, the bacterium responsible for crown gall disease (McKenzie et al. 1994). The genes responsible for the regulation of cytokinin biosynthesis in plants is yet to be isolated. The bacterial cytokinin synthase gene (*ipt*)

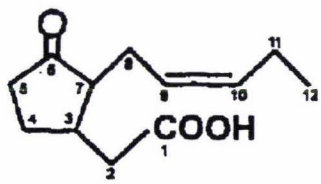
codes for the enzyme isopentenyltransferase and has been used to raise cytokinin levels in a number of transformed plants including tobacco, arabidopsis, petunia and kalanchoë (Medford et al. 1989; Smart et al. 1991; McKenzie et al. 1998). Increased cytokinin production in these plants caused a variety of growth responses including axillary bud release, reduced root growth and delayed senescence. Undesirable responses include inhibition of root growth and increased bushiness, a result of constitutive over-production of cytokinins. The development of artificially or developmentally regulated or tissue-specific gene promoters is being studied in the hope of better regulating the elements necessary to control the location and timing of gene expression (McKenzie et al. 1994; Gan and Amasino 1995).

1.4 Jasmonates

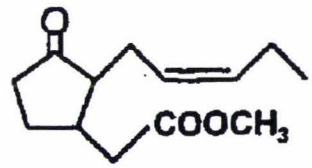
The jasmonates, namely, (-)-jasmonic acid and its methyl ester, methyl jasmonate, have been detected in a wide variety of plants (Vick and Zimmerman 1984) and in a wide variety of plant tissues (Meyer et al. 1984). Jasmonates are considered by some to be a new group of plant hormone for a number of reasons, including their ubiquitous occurrence in the plant kingdom (Meyer et al. 1984), specificity of structure in physiological responses, and their interaction with other phytohormones (Koda et al. 1992; Sembdner and Parthier 1993; Staswick et al. 1992). Other members of the group include cucurbic acid (6-hydroxy-jasmonic acid), 9,10-dihydrojasmonic acid and (+)-7-iso-jasmonic acid.

1.4.1 Jasmonate structure and biosynthesis

The structures of different jasmonates are shown in Fig. 1.2. Jasmonic acid comprises a cyclopentanone ring with two attached side chains. An acetic acid side chain is attached at C_3, and a pentenyl side chain is attached at C_7. The biosynthesis of jasmonic acid and its derivatives is shown in Fig. 1.3. Jasmonic acid is synthesised from linolenic acid. Lipoxygenase catalyses the oxygenation of polyunsaturated fatty

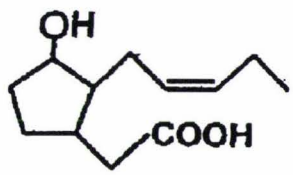


Jasmonic acid



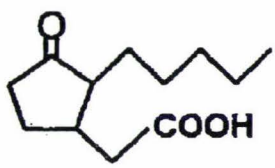
Methyl jasmonate

Reduced derivatives

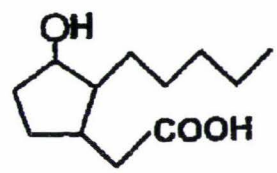


Cucurbic acid

Dihydro- derivatives

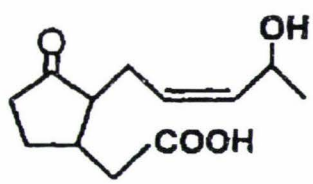


Dihydrojasmonic acid



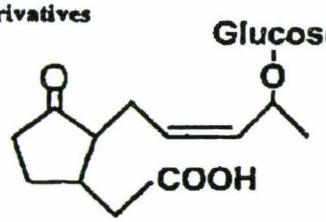
Dihydrocucurbic acid

Hydroxylated derivatives

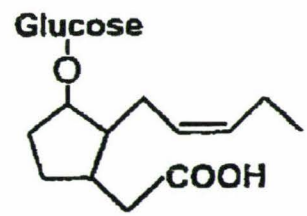


11-Hydroxyjasmonic acid

Glucosylated derivatives

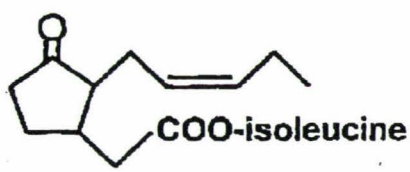


11-*O*-glucosyljasmonic acid



6-*O*-glucosylcucurbic acid

Amino acid derivatives



N-jasmonyl-isoleucine

Figure 1.2 Structure of jasmonic acid and some of its derivatives.

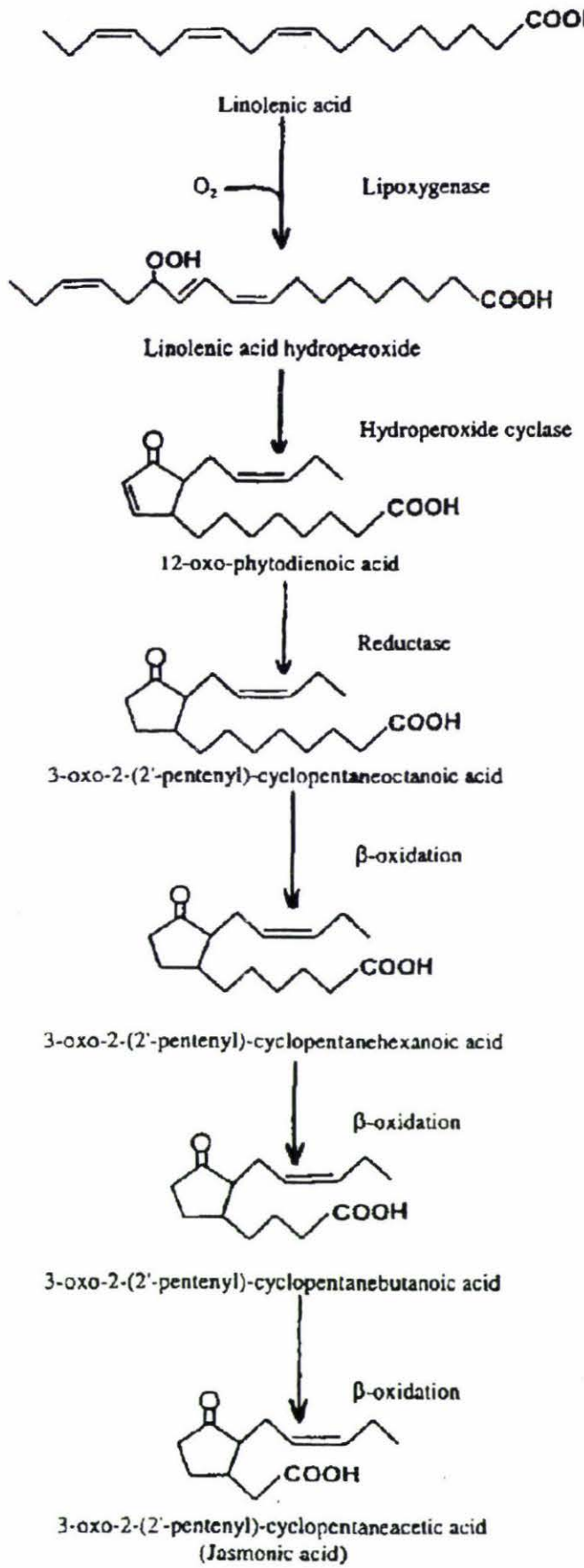


Figure 1.3 Biosynthetic pathway of jasmonic acid.

acids which contain a cis,cis-1,4-pentadiene system. The product of this enzymatic reaction is a highly reactive polyunsaturated fatty acid hydroperoxide. 13-hydroperoxylinolenic acid is then converted to an 18-carbon fatty acid containing a cyclopentanone ring by a hydroperoxide cyclase (Vick and Zimmerman 1984). The product 3-oxo-2-(2-pentenyl)-4-cyclopentene-octanoic acid was given the common name 12-oxo-phytodienoic acid (12-oxo-PDA).

12-oxo-PDA is saturated to 3-oxo-2-(2'-pentenyl)-cyclopentaneoctanoic acid (OPC-8:0). This is followed by three cycles of oxidation, each being a chain shortening reaction. Vick and Zimmerman (1984) observed that oxidation produced fatty acids with only even numbers of carbons meaning β-oxidation was occurring and not α-oxidation. OPC-8:0 undergoes β-oxidation to produce 3-oxo-2-(2'-pentenyl)-cyclopentanehexanoic acid (OPC-6:0), which in turn undergoes β-oxidation to form 3-oxo-2-(2'-pentenyl)-cyclopentanebutanoic acid (OPC-4:0), which is finally converted to 3-oxo-2-(2'-pentenyl)-cyclopentaneacetic acid (OPC-2:0 or jasmonic acid).

The site of jasmonate biosynthesis is still unknown, although lipoxygenase has been found to have a plasma membrane binding motif and its activity increases following disruption of the plasma membrane (Parthier 1990). Further, it has been suggested that jasmonic acid is spontaneously produced in the cytosol and the conversion of linolenic acid to its fatty acid hydroperoxidase is the rate limiting step of the biosynthesis. However, Vick and Zimmerman (1987) reported that some steps were catalysed by membrane bound enzymes in chloroplasts. Isotope labelling experiments *in vivo* have shown jasmonate biosynthesis to occur in fruits, cotyledons and leaves (Vick and Zimmerman 1984).

1.4.2 Metabolism

The major metabolites which form during jasmonic acid metabolism are described in detail in reviews by Sembdener et al. (1988), Parthier et al. (1992) and Sembdener

and Parthier (1993). Metabolic routes of jasmonates detected by Sembdener and Parthier (1993) are illustrated in Fig. 1.4. Biotransformation of dihydrojasmonic acid and jasmonic acid is characterised by four major reactions:

- Hydroxylation, normally at C-11, sometimes at C-12, giving either 11-OH or 12-OH derivatives
- *O*-glucosylation of the hydroxylated metabolites gives either *O*(11)- or *O*(12)-glucosides, or *O*(6)-glucosides of the cucurbic acid related compounds.
- Reduction of the C-6 keto group resulting in formation of cucurbic acid related compounds.
- Conjugation at C-1 with amino acids leucine, isoleucine and valine of (the non-metabolised) dihydrojasmonic acid and jasmonic acid, their 11-OH and 12-OH metabolites and the cucurbic acid related compounds (Brückner et al. 1986; Brückner et al. 1988; Sembdner et al. 1988).

Conjugation of jasmonates with amino acids was found to be widespread in plants but was not found in cell suspension cultures by Sembdner and Parthier (1993). Instead, conjugation at C1 with sugars took place in cell suspension cultures of tomato and potato, giving jasmonic acid glucosyl and jasmonic acid gentiobiosyl as the major metabolites. Neither of these metabolites has been found in plant tissues, but all other jasmonates detected after exogenous application of jasmonic acid are known to occur as endogenous metabolites in plants (Sembdner and Parthier 1993).

None of the enzymes involved with jasmonate metabolism has been isolated (Helder et al. 1993; Parthier et al. 1992). Furthermore, the biological functions of all these jasmonates is yet to be determined (Staswick 1995). Of all of these compounds only the *O*-12-glucoside of jasmonic acid (tuberonic acid) has been given a role (in tuberisation) (Helder et al. 1993). Furthermore, Helder et al. (1993) concluded that the enzyme responsible for the hydroxylation of jasmonic acid to tuberonic acid is probably important for tuberisation in potatoes. Further work is needed to elucidate the biological functions of the many jasmonates present in plant tissues, and the enzymes controlling their production.

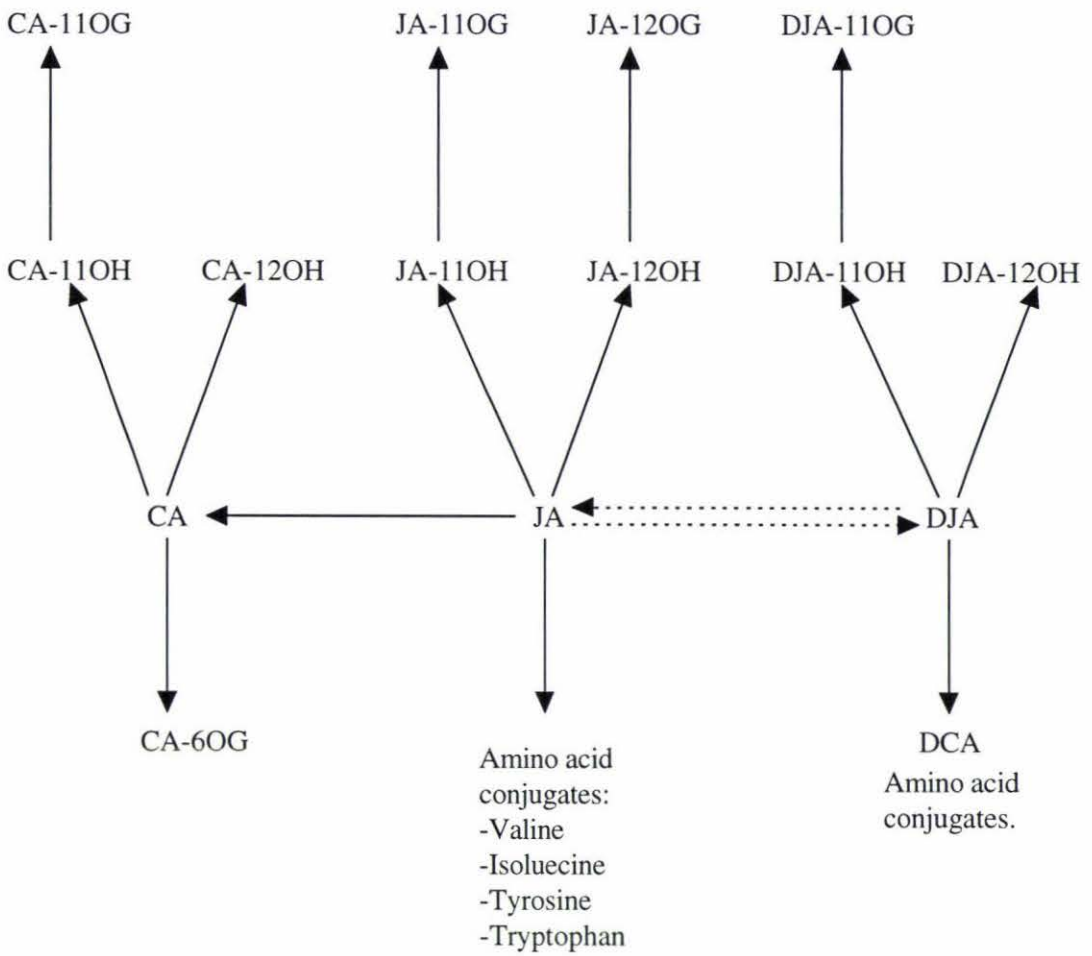


Figure 1.4 Metabolism of jasmonic acid and dihydrojasmonic acid.

1.4.3 Jasmonates and plant development

Since their discovery over 30 years ago jasmonates have been found in a number of plant tissues including tubers (Helder et al. 1993), hypocotyls (Creelman et al. 1992), flowers (Knöfel et al. 1990), and seeds (Ranjan et al. 1994). Jasmonates have been implicated in a number of developmental processes in plants including tendril coiling (Weiler et al. 1993), tuberisation (Helder et al. 1993), seed dormancy (Ranjan et al. 1994), plant defence (Farmer and Ryan 1992; Nojiri et al. 1996), osmotic stress (Parthier et al. 1992), desiccation stress (Xin et al. 1997), abscission and cell elongation (Ueda et al. 1996; Miyamoto et al. 1997), wounding (Creelman et al 1992; Pena-Cortes et al. 1993; Hildmann et al. 1992) and senescence (Ueda and Kato 1980; Parthier et al. 1992).

Jasmonates and senescence

The ability of jasmonates to promote senescence has been reported previously (Ueda and Kato 1980, 1981). Application of jasmonate caused a marked loss of chlorophyll (Ueda and Kato 1980, 1981; Weidhase et al. 1987b). In ripening tomato fruits methyl jasmonate prevented lycopene accumulation and stimulated β-carotene synthesis (Saniewski and Czapski 1983). Other typical senescence symptoms including cellular respiration, and proteolytic as well as peroxidase activities, increased in leaf segments treated with methyl jasmonate (Weidhase et al. 1987b). Structural damage to chloroplasts (U. zur Nieden, unpublished results [from Parthier 1990]) and the reduction of photosynthetic activity (markers of normal leaf senescence) were also observed following treatment of jasmonate (Popova et al. 1988). Also a rapid decline in the activity of and an increase in the protein degradation of ribulose-1,5-bisphosphate carboxylase has been observed (Weidhase et al. 1987b) as well as the cessation of ribulose-1,5-bisphosphate carboxylase synthesis (Popova and Vaklinova 1988; Weidhase et al. 1987b).

These phenomena resemble symptoms of natural senescence (Thimann 1985; Thomas and Stoddart 1980; Parthier 1988; Woolhouse 1984), although functional damage and dismantling of chloroplasts is regarded as representing late stages in senescence.

Further, Parthier (1990) suggested that there may be differences between jasmonate-induced senescence and naturally occurring senescence even in the early stages of the process.

Lesham (1987) suggested that sufficient endogenous jasmonates could be produced to induce senescence, as the production of jasmonic acid may be a consequence of a cascade process triggered by membrane peroxidation, the production of linolenic acid and subsequently jasmonic acid.

Another idea for the role of jasmonates refers to the jasmonate-induced changes in gene expression of specific genes resulting in the synthesis of novel polypeptides (Parthier 1990). Parthier (1990) found that jasmonate-induced proteins (JIPs) accumulated in the leaf tissues of a large number of monocotyledonous and dicotyledonous plants in response to jasmonate treatment. This indicates a dramatic alteration in gene expression in these tissues. These proteins were not induced by salt stress, ethylene-treatment, or anaerobic conditions. JIPs are synthesised *de novo*, as demonstrated by both labelling and inhibitor experiments (Weidhase et al. 1987a). The number and relative molecular masses of JIPs can differ in various plant species. Furthermore, Hermann et al. (1989) reported that even barley cultivars differed in their JIP patterns.

In recent reviews Creelman and Mullet (1997 a and b) suggested that there is little evidence supporting a causal role for jasmonates in senescence. Kinetics of ^{35}S-methionine-labelling experiments, as well as *in vitro* translation of JIP mRNAs, indicate a molecular response within 3-5 h after methyl jasmonate treatment of barley leaf segments (Mueller-Uri et al. 1988). Although JIPs are detectable much earlier than many of the senescence symptoms, a causative role for JIPs in the induction of senescence has yet to be established (Parthier 1990).

Jasmonates and wounding

Early jasmonate studies focused on their potential role in plant growth and development. However, there has been a resurgence in interest in recent years due to the discovery that jasmonates regulate gene expression during plant defence responses

(Farmer and Ryan 1992; Nojiri et al. 1996). Increasing amounts of evidence support the hypothesis that jasmonates are involved with stress responses in plants. One stress worthy of mention is that of wounding. During harvest asparagus is wounded and this must trigger a stress response within the spear.

Jasmonates have been reported to be involved in the wound stress signal by many authors (Creelman et al. 1992; Conconi et al. 1996; Ohnmeiss et al. 1997). Almost immediately following wounding, jasmonate concentration increases in the wounded tissue (Creelman et al. 1992; Conconi et al. 1996; Clarke 1996). Changes in gene expression takes place including induction of proteinase inhibitor II (*pin2*) in tomato and potato leaves (Peña-Cortés et al. 1993) and the accumulation of vegetable storage proteins (VSPs) in soybean hypocotyls (Creelman et al. 1992). Jasmonates have been observed to induce the expression of both *pin2* (Farmer and Ryan 1990; Farmer et al. 1992; Peña-Cortés et al. 1992) and *vsp* genes *in vivo* (Staswick 1994 and papers therein), so it is postulated that elevated levels of jasmonates due to wounding induced this gene expression. In some plants jasmonates also stimulated the production of lipoxygenase enzyme, a key enzyme in the jasmonic acid biosynthesis pathway. This may suggest a jasmonate signal amplification mechanism. Small increases in jasmonate levels could stimulate the jasmonic acid biosynthesis pathway, causing further induction of jasmonate-inducible genes (Staswick 1994 and papers therein). Although wounding induces VSP gene expression, there is no indication that VSPs are involved in plant defence as are the wound-inducible proteinase inhibitors (Staswick 1994).

As already mentioned jasmonic acid is synthesised in plants by an oxidative pathway that starts with linolenic acid produced by deteriorating membranes. This pathway has similarities to the pathways which produce prostaglandins in animals, both having lipoxygenase-dependant pathways (Anderson 1989). Furthermore, jasmonates are similar in structure to mammalian eicosanoids (prostaglandins), which are potent modulators of smooth muscle contraction and inflammatory responses (Creelman et al. 1992). These similarities in structure, biosynthesis and function or response have led to the idea that both situations share a common ancestral mechanism for defence responses (Peña-Cortés et al. 1993). Furthermore, they reported that aspirin, a drug

which prevents inflammatory responses in mammalian systems, also prevented wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis.

The asparagus spear is an ideal system in which to study endogenous jasmonates. As senescence progresses a significant amount of lipid breakdown occurs in asparagus spears (Hurst et al. 1994). Further, the process of harvesting should cause a substantial wound response. Monitoring endogenous jasmonate levels in asparagus spears after harvest may help elucidate whether jasmonates are involved in senescence and/or wounding.

1.4.4 Interactions between jasmonates and other plant hormones

Although the biological role of jasmonates is not yet completely elucidated, their interactions with other plant hormones should not be neglected. Only a few reports evaluate the interactions between jasmonates and cytokinins. Ueda and Kato (1982) observed the effects of jasmonate treatment on cytokinin-induced callus growth of soybean. Both jasmonic acid and methyl jasmonate treatment caused inhibition of callus growth induced by cytokinin whilst abscisic acid was only inhibitory at high concentrations. They suggested that jasmonates possibly strongly inhibit cellular metabolism in relation to the actions of cytokinin. Weidhase et al. (1987a) demonstrated a reversal of methyl jasmonate-induced degradation of ribulose-1,5-bisphosphate carboxylase in senescing barley leaf segments, by counteraction with cytokinin. Cytokinin treatment before MeJA treatment could not protect tissues against senescence promoting actions of methyl jasmonate. Also cytokinin treatment can stop or even restore the chlorophyll loss caused by methyl jasmonate only when cytokinin is added after methyl jasmonate has disappeared as an active compound. These observations suggest different modes of action for the two substances rather than competition for the same target (Weidhase et al. 1987a).

Dermatsia et al. (1994) suggested that jasmonates may have a role in the regulation of cytokinin metabolism. Although the total amount of cytokinins in jasmonate treated potato plantlets *in vitro* did not change compared to control plantlets *in vitro*, a

change in cytokinin metabolism was seen. Jasmonate treatment caused the accumulation of the cytokinin ribosides, a reduction in the cytokinin free bases, and a reduction in the cytokinin 9-glucosides. In contrast to the metabolically active ribosides and free bases, 9-glucosides are regarded as the non-active forms of cytokinins. The ratio between active and non-active forms of the cytokinins increased from 1.2 in control plantlets, to 2.1 in jasmonic acid- treated plantlets. Although the molecular basis of the jasmonate/cytokinin interaction is not known there is evidence that jasmonic acid-induced growth responses are associated with increased levels of cytokinin ribosides. Jasmonates have been reported to interact with ethylene as well as cytokinin during senescence. Tsai et al. (1996) demonstrated that an increase in ethylene sensitivity was associated with jasmonate-promoted senescence of detached rice leaves.

Jasmonates also interact with other plant hormones during wounding. Sano et al. (1996) demonstrated that levels of jasmonic acid increased in transgenic tobacco plants expressing a gene for a small GTP-binding protein, eighteen hours earlier upon wounding than in wild-type tobacco plants. These transgenic plants constitutively produced four- to six-fold higher amounts of endogenous cytokinins than wild-type plants. Cytokinin treatment of wounded tobacco plants also caused early accumulation of jasmonic acid, and addition of a cytokinin antagonist (2-chloro-4-cyclo-hexylamino-6-ethylamino-s-triazine) erased these effects (Sano et al. 1996). They concluded that cytokinins were indispensable for the control of endogenous jasmonates. Jasmonates have also been postulated to interact with abscisic acid upon mechanical wounding. Hildmann et al. (1992) demonstrated that treatment of both wild-type and ABA-deficient plants with jasmonates caused expression of transcripts of ABA-responsive genes. Wounding, however, did not cause transcription of ABA-responsive genes in ABA-deficient plants. These results support the hypothesis that jasmonic acid is an intermediate in the signalling pathway that leads from ABA accumulation in response to wounding, to the transcriptional activation of the genes.

1.4.5 Jasmonate identification and measurement

Jasmonates are present at trace levels in plant tissues so extensive separation and purification is essential to ensure against interference of substances during immunoassay. Since losses of jasmonates during purification can be relatively high, it is preferable to use an internal standard so that losses can be accounted for (Knöfel 1984). In this study, radiolabelled cucurbic acid (^{3}H-cucurbic acid) was used as an internal standard to monitor recovery.

Extraction:

Jasmonates have been extracted into a number of different solvents including ethanol (Gundlach et al. 1992), hexane (Creelman et al. 1992) and methanol (Clarke 1996). In this thesis, jasmonates along with an internal standard were extracted into 80% methanol (Meyer et al. 1984).

Purification:

Jasmonates are most commonly purified by partitioning steps, using carbonate and chloroform phases (Miersch et al. 1991; Clarke 1996). During partitioning jasmonic acid and methyl jasmonate move into different phases. Frequently, the extract is methylated and combined as a total. In this thesis, we wished to monitor methyl jasmonate and jasmonic acid separately. Jasmonates were separated from phenolic compounds and pigments by combined polyvinylpolypyrrolidone (PVPP) and DEAE cellulose (DE52) anion exchange column chromatography (Glenn et al. 1972; Clarke 1996). Low pressure C_{18} column chromatography was then used to separate jasmonates from more polar compounds (Gundlach et al. 1992; Nojiri et al. 1996).

Separation:

Separation of jasmonic acid from other analogues has routinely been achieved by thin layer chromatography (TLC) (Miersch et al. 1991; Clarke 1996). In this thesis jasmonates were separated by high performance liquid chromatography (HPLC). Separation of individual jasmonate derivatives is crucial as each has differing biological activity and differing cross reactivity in the immunoassay, due to differences in structure. HPLC has been previously used for the separation of individual

jasmonates (Bruckner et al. 1986; Weiler et al. 1993; Nojiri et al. 1996).

Detection:

Jasmonates have been commonly detected by enzyme linked immunosorbent assay (ELISA) (Clarke 1996) and radioimmunoassay (RIA) (Knöfel 1984). In this thesis, jasmonates were quantified by ELISA. Antibodies raised against jasmonic acid (Clarke 1996) were used in a competitive binding reaction between jasmonic acid bound to the well walls of the ELISA plate and an unknown amount of jasmonate in the sample. ELISAs are fast, simple and very specific, do not involve the use of radioactivity, and for jasmonic acid have detection limits as low as 10 pmol.

Identification/confirmation:

Occasionally jasmonates have been identified by use of combined gas chromatography/mass spectrometry (GC/MS) (Miersch et al. 1991). GC/MS uses a gas phase so jasmonates must be methylated, to become volatile, but only jasmonic acid and methyl jasmonate are usually identified by this method (Miersch et al. 1991). However, during this project electrospray mass spectrometry has been used to identify jasmonates. Electrospray MS/MS uses a liquid phase so no methylation is required. Jasmonic acid and jasmonic acid amino acid conjugates have been identified using electrospray mass spectrometry (Schmidt et al. 1995). Mass spectrometry provides structural identification of the derivatives cross reactive in the ELISA.

1.5 Objectives

This project was a component a FRST-funded programme, Biochemistry and Genetics of Vegetable Quality (Crop & Food Research). The overall objective of the programme was to determine the biochemical and genetic factors affecting quality attributes of vegetable crops so that improved cultivars can be developed. This project is part of a study of how shelf-life of very perishable vegetables is controlled. Consequently the aims of this project were:

- To determine the effects of exogenous treatment of cytokinin and jasmonic acid after

harvest on shelf-life, tiprot, spear extension postharvest, chlorophyll loss, soluble sugar loss and asparagine accumulation.

- To determine the effects of exogenous treatment of cytokinin and jasmonic acid on endogenous jasmonate concentration in spear tips.
- To determine the effects of harvest and dry or wet storage of spears on the endogenous jasmonate concentration in tips, mid sections and bases of spears.
- To determine the jasmonate concentration during natural foliar senescence of the asparagus frond and compare the pattern of harvest-induced changes in jasmonates with those occurring during foliar senescence.

Chapter Two

MATERIALS AND METHODS

2.1 Plant material

Spears of *Asparagus officinalis* L. var. Jersey Giant from a commercial crop located at Crop & Food Research in Levin were harvested at 180 to 240 mm in length. The spears were washed with water and cut to 180 mm in length. They were then subjected to a number of different postharvest treatments for physiological analyses, and 30 mm spear tips were quartered longitudinally and stored at -80°C for later analysis. Spears analysed in Year 1 were harvested from four year old plants between November 25 and December 3, 1996. Spears analysed in Year 2 were harvested from a mixture of four year old and five year old plants between November 10 and December 13, 1997. Spears harvested in Year 2 for endogenous jasmonate analyses were harvested at 180 to 240 mm in length and stored either untreated or using the water-vase technique in water (see section 2.2, Fig. 2.1B). Tips, mid sections and bases of spears were then excised as shown in Fig. 2.1A. Naturally senescing foliar tissue was harvested from the plants in the autumn of 1996, at six different stages of senescence (King et al. 1995).

2.2 Plant growth regulator treatments

Spears were treated with exogenous cytokinin and jasmonate, using the "water vase" technique (Fig 2.1B). This technique involved the treatment of spears standing in plastic 1 litre buckets, with 250 ml of solution (giving a basal depth of 20-25 mm), which was replenished on a daily basis. Two control treatments were also used, a water control, and a non treated dry control. In year 1 all spears were treated in a temperature controlled room at 20°C in the light with plastic film draped over the top. In year 2 all spears were treated in a temperature controlled room at 20°C in the dark. Jasmonic acid (JA; 50 nM and 50 μM) and dihydrozeatin riboside (DZR; 2 nM and 2 μM) were used for treatment of spears.

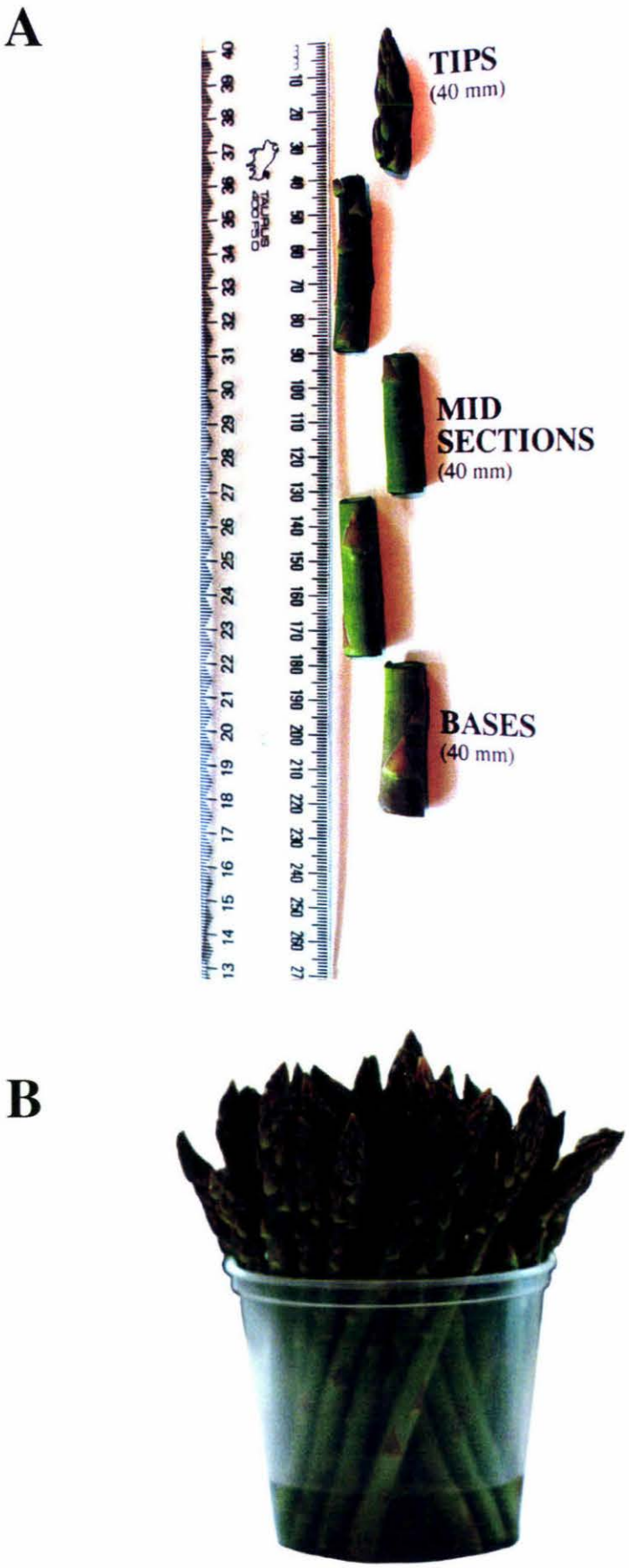


Figure 2.1 A. Photograph showing tips, mid sections and bases of spears harvested in Year 2 for jasmonate analysis. B. Photograph showing water-vase technique.

2.3 Spear quality

Spear quality is a visual assessment for determining shelf-life. Spear quality was visually assessed and ranked to a scheme developed by R. Lill. (Table 2.1) Spears were ranked, from 1 through to 9, where 1 indicates freshly harvested, and anything scoring over 6 is unsaleable.

Table 2.1 Visual assessment scale of spear quality. Scale adapted from Lill [1980].

Score	Description
1	fresh
3	slight wilt, very slight wrinkle on stem
5	browning of stem bracts, more pronounced wilting
7	soft rots starting to develop, some browning of spears, wilting and feathering of bud bracts, occasional spears with stem collapse
9	more extensive rotting and stem collapse, bud wilting, browning of spears

2.4 Tiprot

The proportion of spears which had tiprot was determined after 9 d of treatment. Spears with tiprot collapse under a slight downward pressure exerted by the finger on the spear tip. Spears were scored either with or without tiprot.

2.5 Spear extension

Spear extension was measured during treatment to provide an estimate of growth rate.

2.6 Extraction and quantification of soluble sugars

Soluble sugars were extracted as described by Irving and Hurst (1993). Samples (10 mg) of freeze dried tissue were extracted with 1 ml 62.5% (v/v) methanol at 55°C for 15 min. This extraction was followed by precipitation of interfering substances from the supernatant with 10 ml saturated lead acetate (Haslemore & Roughan 1976). Sugars (mg/g dry weight) were determined enzymatically with sucrose/D-fructose/D-glucose kits (Boehringer Mannheim Biochemicals). The methodology was altered to use micro amounts using a plate reader instead of a spectrophotometer (Dr Jocelyn Eason, unpublished).

Determination of D-glucose and D-fructose

Sample (10 µl) was mixed with 154 µl milli-Q water and 100 µl of solution 2 (triethanolamine buffer, pH 7.6, NADP 2.4 mg/ml, ATP 5.8 mg/ml), and left to incubate for 3 min at room temperature. Absorbance was read at 340 nm (A_1). Suspension 3 (hexokinase and glucose-6-phosphate dehydrogenase), diluted 10-fold, (20 µl) was then added and mixed. When the reaction was complete (10-15 min), the absorbance was again read at 340 nm (A_2). Suspension 4 (phosphoglucose isomerase), diluted 10-fold (20 µl) was added and mixed. After completion of the reaction absorbances were again read at 340 nm (A_3). A standard curve was made and sample amounts were calculated from the standard.

$A_2 - A_1$ = D-glucose Δ A

$A_3 - A_2$ = D-fructose Δ A

Determination of sucrose

Sample (10 µl) was mixed with 20 µl solution 1 (β-fructosidase). This reaction hydrolysed sucrose to form D-glucose and D-fructose. The above protocol for D-glucose determination was then followed to determine sucrose concentration and a sucrose standard was used to calculate sucrose content.

$A_2 - A_1$ = D-glucose Δ A = sucrose Δ A

2.7 Chlorophyll extraction and quantification

Chlorophyll was extracted from the asparagus spear tips by the method described by Moran and Porath (1980), who used dimethyl formamide (DMF) rather than other methods which are more time consuming and require more in the way of chemicals and materials.

Year 1

Spear tips were stored at -80°C for approximately two months. Spear tips (upper 30 mm; 75 mg FW total) was extracted in 1.5 ml DMF for 24 h at 4°C. Samples were then centrifuged briefly to separate out debris.

Year 2

Spear tips were stored for a maximum of 5 d at -80°C. Spear tips (upper 20 mm; 4 g FW total) was extracted in 5 ml DMF for 48 h at 4°C. Samples were centrifuged briefly to separate out debris.

Absorbances were taken at wavelengths 625, 647, and 664.5 nm, corresponding to protochlorophyll (Pchl), chlorophyll b (Chl *b*), and chlorophyll a (Chl *a*), respectively. The formulae used by Inskeep et al. (1985) to estimate total chlorophyll, can also be used to estimate the components of Pchl, Chl *b* and Chl *a*. Chlorophyll content was measured in mg/g FW.

$$\text{Chl } a. = 12.65\,A_{664} - 2.99\,A_{647} - 0.04\,A_{625}$$
$$\text{Chl } b = -5.48\,A_{664} + 23.44\,A_{647} - 0.97\,A_{625}$$
$$\text{Pchl} = -3.49\,A_{664} - 5.25\,A_{647} + 28.3\,A_{625}$$

$$\text{Total chlorophyll} = 27.29\,A_{625} + 15.2\,A_{647} + 3.68\,A_{664}$$

2.8 Asparagine

2.8.1 *Extraction of amino acids*

Amino acids were extracted by a procedure developed by Mr Jason Johnson (personal communication). Amino acids were extracted from 10 mg freeze dried material in 4.5 ml methanol/chloroform/water (MCW), (12:5:3, v/v/v), at 4°C. The extract was centrifuged at 4000 g for 10 minutes, and the supernatant collected. Chloroform (1.5 ml) was then added to the supernatant. Milli-Q H_2O (3 ml) was added to the pellet, which was vortexed then heated at 55°C for 10 min, cooled and centrifuged at 4000 g for 10 min. The supernatents were combined and two phases were created. These were vortexed and again centrifuged at 4000g for 5 min. The upper aqueous phase (amino acids and sugars) was aspirated away and stored at -20°C. The lower chloroform phase (lipids and phospholipids)was also retained and stored at -20°C. The aqueous phase was subsequently dried by Speed Vac (Savant SC200 Speed Vac & Savant RT4104 Refrigerated Condensation Trap) and resuspended in 0.5 ml milli-Q H_2O. The resuspension was then centrifuged in a bench top microfuge for 3 min, and the supernatant filtered through a 22 µm syringe tip filter. The filtrate was then stored at - 20°C until required for HPLC analysis.

2.8.2 *Pre-column derivatisation of amino acids*

Amino acids were determined in subpicomole amounts by the pre-column fluorescence derivatisation with o-phthaldahyde (OPA) as described by Lindroth and Mopper (1979). The OPA (270 mg) was dissolved in 5 ml ethanol. 2-Mercaptoethanol (200 µl) was added. The volume was then made up to 50 ml with 0.4 M borate buffer pH 9.5, (pH to 9.5 with 1M NaOH). The OPA derivatising mixture was then aged for 48 h before use. Reagent strength was maintained by addition of 20 µl 2-mercaptoethanol every 3-4 d, and was stable for up to two months at 4°C. The OPA derivatisation agent (100 µl) was mixed with 20 µl of amino acid solution, (standard or sample), and incubated at room temperature for 2 min. Aliquots (25 µl) were then injected into the HPLC via the injection loop.

2.8.3 *High performance liquid chromatography (HPLC)*

The HPLC used was a Waters 600 multisolvent controller with a Waters U6K injection loop and a Waters 490E programmable multiwave UV detector. UV absorbance was measured at 335 nm and all solvents used were of HPLC grade. All solvents were filtered through a 0.45 µm filter and degassed prior to use. Separation of amino acids was achieved on an octadecyl silica C_{18} column, (Phenomenex Prodigy 5µm ODS (3) 100A 150 x 4.6 mm), using a phosphate buffer/methanol gradient. (Refer to Appendix II for gradient table). The separation of asparagine from other amino acids was verified using four amino acid standards, asparagine, serine, glutamine and 1-aminocyclopropane-1-carboxylic acid (ACC). The HPLC gradient table is shown in Appendix II.

2.9 Jasmonic acid purification and detection

2.9.1 *Plant material*

Plant material came from the same crop as described in section 2.1. Year 1 material came from spear tips (upper 30 mm). Year 2 spear material was harvested slightly differently. Spears were harvested between 180 and 240 mm in length, but were not trimmed to 180 mm nor were they washed in water before treatment and sampling. Spear material came from spear tips (upper 40 mm), mid section (mid section 90-130 mm from top of tip), and base section (lower 40 mm, thus from 140 - 180 mm thru to200 - 240 mm). Spear material was harvested differently in Year 2, because we were interested in the initial wound response at harvest in the field, and also needed to reduce the amount of plant material harvested.

2.9.2 *Extraction of jasmonates*

Plant material (10 g FW) was snap frozen at harvest in liquid nitrogen, and ground into a fine powder. Jasmonates were extracted using 100 ml 80% methanol for 48 hr at 4°C. A known amount of radioactive internal standard (^{3}H-cucurbic acid) was

added. The extract was then centrifuged at 3000 g for 10 min. The supernatant was decanted off and stored at 4°C. The pellet was resuspended in a further 100 ml of 80% methanol for 48 h at 4°C. The extract was again centrifuged at 3000 g for 10 min, and the supernatants were combined. The volume of the extract was then reduced in the Speed Vac or by rotary evaporation, to ca. 10 ml aqueous.

2.9.3 *Chromatography systems*

Combined PVPP+DE52 column chromatography: The PVPP and DE52 columns were prepared as described in Appendix III. The column system was equilibrated with 0.1 M phosphate buffer pH 6.5. The jasmonate extract was loaded onto the PVPP+DE52 combined column system and washed through with 15 ml phosphate buffer. The PVPP column was then removed and a further 20 ml phosphate buffer was washed through the DE52 column. Jasmonates eluted off in the wash, and were collected. The volume was then reduced to 5 ml using a Speed Vac. The concentrate was then acidified to pH 2.5 using dropwise addition of conc HCl.

Low pressure C_{18} chromatography: The low pressure C_{18} column was pre-equilibrated in 100% methanol, then 1% acetic acid, (10 column volumes of each). The sample was then loaded and washed through with 15 ml 1% acetic acid, 15 ml 1% acetic acid in 30% methanol, and 30 ml 1% acetic acid in 60% methanol. Jasmonates eluted in the 60% methanol wash and were collected. The eluent was then dried completely, and then resuspended in 70 µl 0.05% Trifluoroacetic acid in 40% acetonitrile (methyl cyanide, MeCN), prior to HPLC injection.

2.9.4 *High performance liquid chromatography of jasmonic acid and derivatives*

The HPLC used was a Waters 600 multisolvent controller with a Waters U6K injection loop and a Waters 490E programmable multiwave UV detector. UV absorbance was measured at 218 nm and all solvents used were of HPLC grade. All solvents were filtered through a 0.45 µm filter and degassed prior to use. Separation of jasmonates was achieved on an octadecyl silica C_{18} column, (Beckman ultrasphere 7 µm, 250 mm x 4.6 mm). (Refer to Appendix II for gradient table). Solvent bottles

were placed in a water bath at 20°C to stabilise retention times. The separation of jasmonates was verified using four jasmonate standards; jasmonic acid, methyl jasmonate, dihydrojasmonic acid and cucurbic acid.

2.9.5 Purification of antiserum

The antiserum was a gift from Dr Sean Clarke. It had been produced at the University of Otago by the inoculation of a New Zealand white rabbit with jasmonic acid conjugated to the immunogen keyhole limpet haemocyanin (KLH-JA). Ovalbumin - jasmonic acid conjugate was used in the competitive immunoassays. The blood serum was stored in 50% glycerol at 4°C, and purified as described by Clarke (1996).

Blood serum (1 ml) was diluted with 9 ml H_2O. The antiserum was then precipitated by dropwise addition of 10 ml saturated ammonium sulphate. The precipitate was then centrifuged at 3000 g for 15 min, and the pellet resuspended in half strength PBS buffer. (PBS buffer: 1.4 mM KH_2PO_4, 8.0 mM Na_2HPO4, 0.14 M NaCl, 2.6 mM KCl, 0.2% NaN_3). The resuspension was then dialysed against half strength PBS buffer for 48 h at 4°C. DE23 (1 g) was equilibrated overnight in half-strength PBS buffer. The dialysed antibody solution was loaded then washed through the column with half strength PBS buffer. Five to seven fractions (2 ml) were collected and the concentration of antibody determined and altered to 1 mg/ml using a spectrophotometer at 280 nm with crystal cuvettes. Sodium azide NaN_3 (0.2%) and 5 mg bovine serum albumin (BSA) were then added.

2.9.6 *Preparation of JA:OVA conjugate*

OVA (10 mg) was dissolved in 1 ml water. Jasmonic acid (10 mg) was then dissolved in 2.5 ml of conjugation buffer (0.1 M MES, 0.9 M NaCl, 0.02% NaN_3, pH 4.7). The two solutions were combined and 10 mg of EDC was added and left at room temperature for 4 h. The solution was then dialysed against MES buffer (0.1 M MES, 0.9 M NaCl, 0.02% NaN_3, pH 4.7) for 12 h followed by three 12 h changes of milli-Q H_2O. After dialysis was complete, 0.02% NaN_3 was added.

2.9.7 ELISA for jasmonic acid

The ELISA for jasmonic acid was developed by Clarke (1996). OVA-JA (100µl), diluted 1:1000 using coating buffer (Appendix I) was added to the ELISA well for 48 h at 4°C. Plates were washed five times with PBST buffer (Appendix I), and 50 µl jasmonic acid (diluted in ELISA buffer (Appendix I) to give a standard curve ranging from 0.001 to 100 nmol JA) was added to each well. Sample (50 µl) was added to remaining wells. Anti-JA IgG (50 µl) diluted 1:500 was then added to each well. The plate was then incubated at room temperature for 18 h. Plates were washed five times with PBST buffer. Anti-rabbit IgG conjugated to alkaline phosphatase (100 µl) (Sigma Immunochemical), diluted 1:2000 with ELISA buffer, was added to each well and incubated at 37°C for 1 h. The plate was then washed five times with PBST buffer and 100 µl of substrate was added to each well (0.5 mg/ml p-nitrophenol phosphate (Sigma Chemicals) in substrate buffer (Appendix I)). The plates were then incubated at room temperature for 1 h and absorbance measured using the ELISA microplate reader at 405 nm.

2.9.8 *Cross-reactivity of jasmonates*

Jasmonates were tested for cross-reactivity with the anti-JA IgG previously (Clarke 1996). Cross-reactivity of jasmonates is shown in Appendix IV.

2.9.9 *Immuno-affinity of jasmonates for mass spectrometry*

Fractions were collected every 30 sec during HPLC separation of jasmonates extracted from sample. The fractions containing individual jasmonates were reduced to dryness in a speed vac, and resuspended in 1 ml anti-JA IgG (diluted 1:50) in ELISA buffer. The resuspended jasmonates (0.5 ml) were transferred to micro concentrators with a protein cut off of 10 K (Nanosep™, PALL FILTRON), and spun at 12000 rpm for 15 min. The remaining 0.5 ml was then placed in the concentrators and again spun at 12000 rpm for 15 min. The micro concentrators were washed and spun with 0.5 ml ELISA buffer (twice), and then washed again with 0.5 ml water (twice). Methanol (0.5 ml twice) was added to the washed micro

concentrators and spun at 12000 rpm for 20 min, and the elutates were collected. The elutates were reduced to near dryness and resuspended in 0.2 ml methanol (HPLC grade), ready for electrospray mass spectrometry analysis.

2.9.10 *Electro-spray mass spectrometry*

Electrospray (ES) mass spectrometry measurements were carried out on a PE Sciex API 300 triple quadruple mass spectrometer fitted with articulated ionspray plenums and atmospheric pressure ionisation sources. The abundance of low-mass jasmonate marker ions in electrospray mass spectra was enhanced by collision-induced decomposition of the parent ions. This was accomplished by stepping the potential, which controls the extent of the collision-induced decomposition of source-produced ions from a high voltage, to maximise fragment-ion production during acquisition of the low *m/z* ions, to a lower voltage to yield intact ionised molecules during the remainder of the scan (Carr et al. 1993; Huddleston et al. 1993). All samples were infused into the mass spectrometer at 5 $\mu l\ min^{-1}$ by a syringe pump. Methyl jasmonate, cucurbic acid and the unknown jasmonate (dwell time 1 ms each) were monitored in positive ion mode, with an orifice potential of 15 V, ring potential of 90 V and ionisation potential of 4800 V, and scans were acquired at *m/z* 100 - 600 (0.1 amu steps). Jasmonic acid and dihydrojasmonic acid (dwell time 1 ms each) were monitored in negative ion mode, with an orifice potential of -60 V, ring potential of -200 V and ionisation potential of-5000 V, and scans were acquired at *m/z* 100 - 600 (0.1 amu steps).

2.9.11 *Statistical analysis*

The data were analysed using standard analysis of variance (ANOVA) procedures on the Genstat 5 statistical package.

Chapter Three

RESULTS

3.1 Physiological changes postharvest

3.1.1 *Spear quality*

Year 1

The influence on spear quality of postharvest treatment with cytokinin and jasmonic acid for spears harvested in Year 1 is shown in Fig. 3.1A. Spears with a spear quality of 6 and higher become unsaleable (Lill 1980). Taking a horizontal line across the graph at a spear quality of 5-6 it can be seen that spears subjected to dry storage deteriorated faster than all other wet treatments having a shelf-life of around 4.5-5 d. Spears treated with jasmonic acid deteriorated faster than the water control and both of the cytokinin treatments, having a shelf-life of 5.5-6.5 d. Spears subjected to water treatment and both cytokinin treatments showed an extended shelf-life to around 7-7.5 days.

Year 2

The influence of cytokinin and jasmonic acid treatment on postharvest spear quality for spears harvested in Year 2 is shown in Fig. 3.1B. Again focussing on a spear quality of 5-6 it can be seen that spears subjected to dry storage deteriorated at a similar rate to those in Year 1, but the spears subjected to wet treatments appeared to have an extended shelf-life compared to wet treated spears in Year 1. Shelf-life of dry control spears was only around 4.5-5 days. Spears treated with the high concentration of jasmonic acid or with water share a shelf-life of around 7-7.5 d. Spears treated with both cytokinin treatments share had an extended shelf-life of around 8-8.5 d, compared to spears treated with the low concentration of jasmonic acid which had a shelf-life of 7.5-8 d.

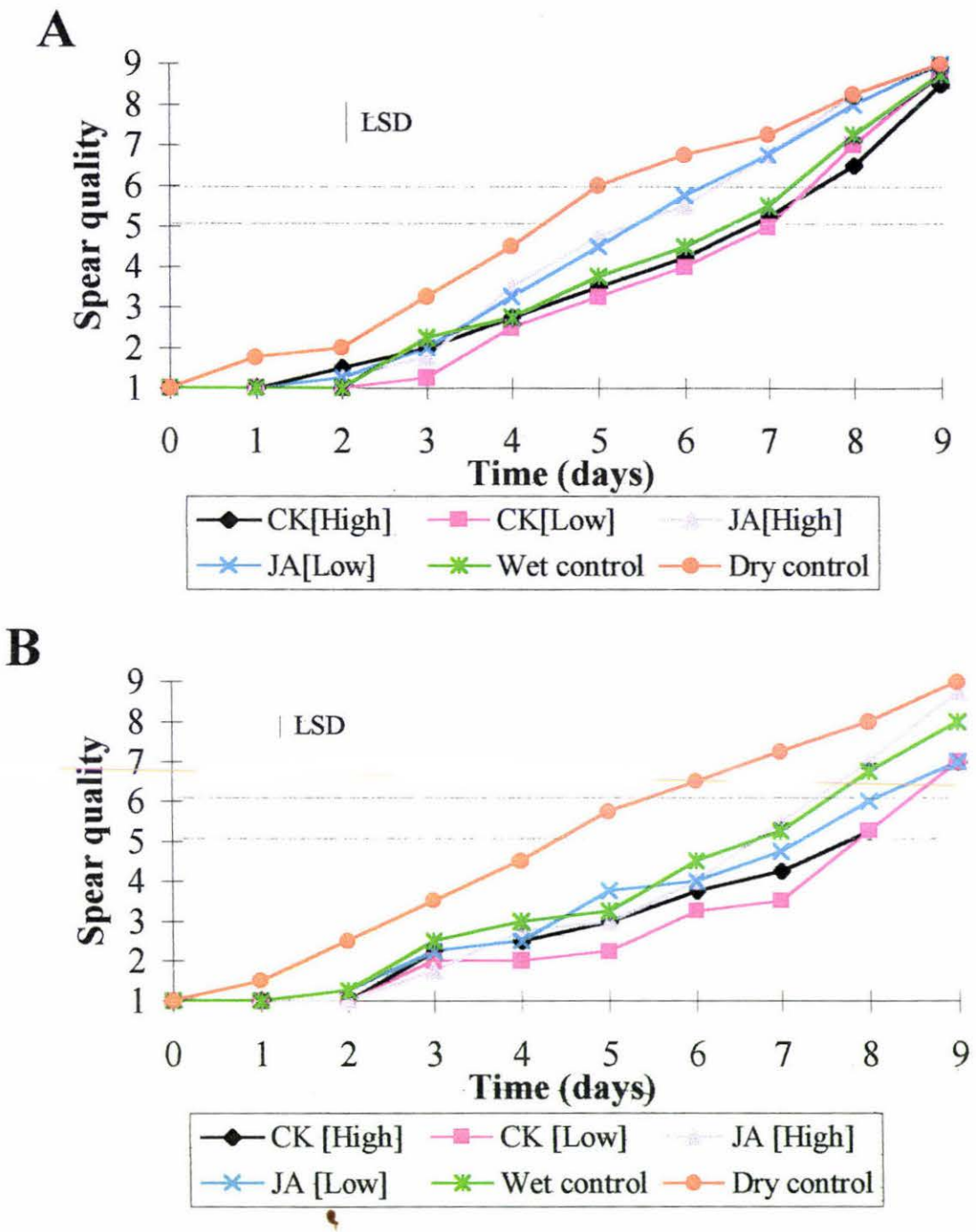


Figure 3.1 Influence of exogenous cytokinin and jasmonic acid treatment on shelf-life of asparagus spears postharvest. A = spears harvested in Year 1. B = spears harvested in Year 2. (CK [High] = 2 µM DZR, CK [Low] = 2 nM DZR, JA [High] = 50 µM JA, JA [Low] = 50 nM JA, wet control = H_2O, dry control = untreated). Least significant differences (LSD {p = 0.05}) for the whole analysis are given as vertical bars. Each point in the above graphs represents the mean value of four individual observations.

3.1.2 *Tiprot*

Year 1

The effect of postharvest treatment of spears with cytokinin and jasmonic acid in relation to tiprot for Year 1 is shown in Fig 3.2A. Approximately 60 % of spears treated with cytokinin contracted tiprot. 72 % of spears treated with the high concentration of jasmonic acid contracted tiprot. Tiprot was contracted in approximately 70 % of spears in both control groups, and 65 % of spears contracted tiprot that were subjected to the low concentration jasmonic acid postharvest.

Year 2

The effect of postharvest treatment of spears with cytokinin and jasmonic acid in relation to tiprot for Year 2 is shown in Fig 3.2B. The proportion of spears having tiprot in year two was lower than that of year one. 18 % of spears treated with high concentration cytokinin contracted tiprot, compared to spears treated with low concentration jasmonic acid with 40 % contracting tiprot. The low cytokinin, high jasmonic acid, and dry control treatments all led to between 32-34 % of spears acquiring the disorder, whilst 24 % of the wet control treated spears had the disorder.

3.1.3 *Spear extension*

The effect of postharvest cytokinin and jasmonic acid treatment on spear extension is shown in Fig 3.3. All spears subjected to wet treatments tended to grow or extend after harvest at least 25 mm more than the dry untreated spears. The spears treated with a low concentration of cytokinin were slightly, but not significantly, shorter after treatment than the rest of the wet-treated spears.

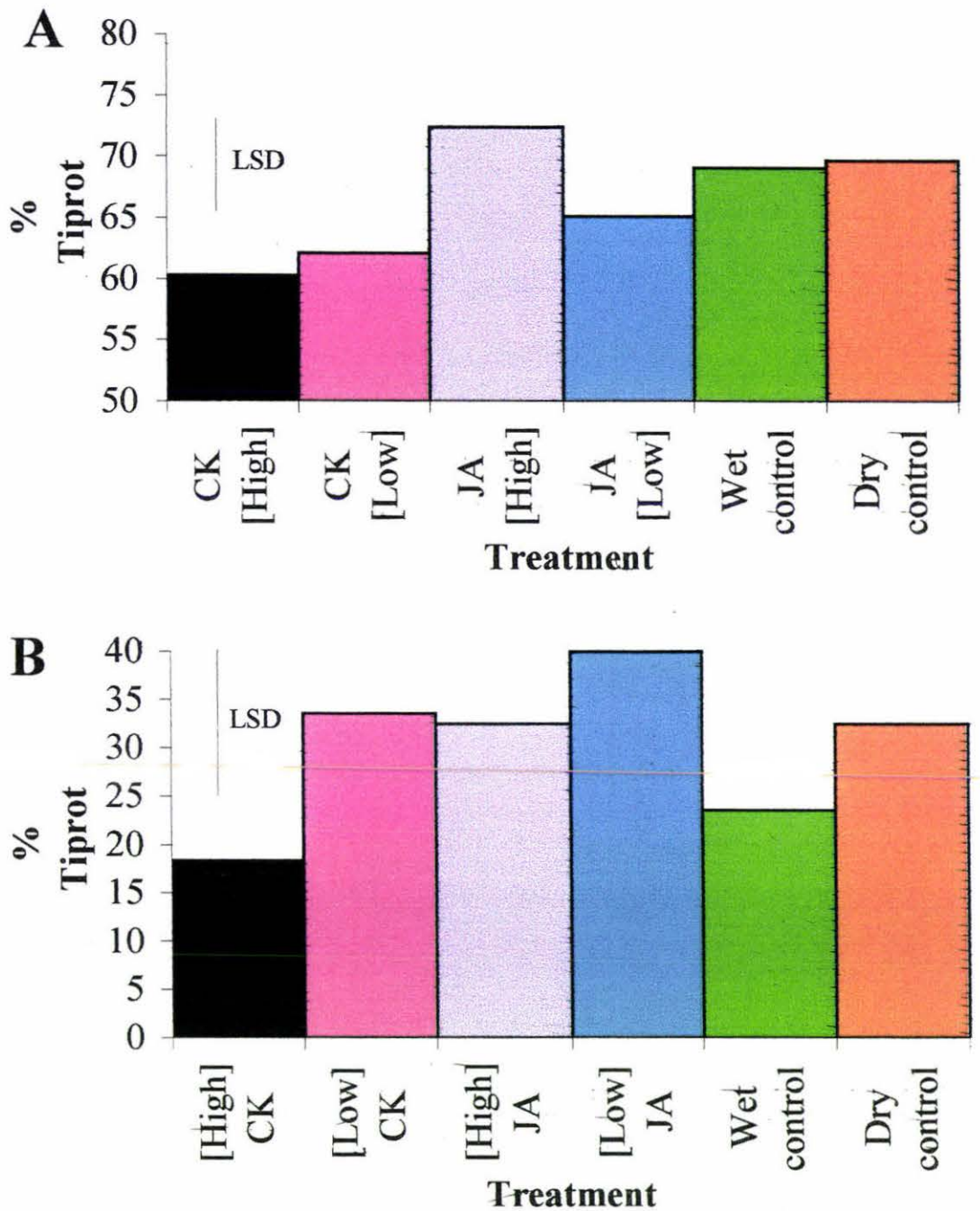


Figure 3.2 Influence of exogenous cytokinin and jasmonic acid treatment on tiprot of asparagus spears postharvest. A = spears harvested in year one. B = spears harvested in year two. Plant hormone concentrations are shown in Fig. 3.1. LSDs (p = 0.05) for full analysis are given as vertical bars. Each bar in the above graphs represents the mean value of four individual observations.

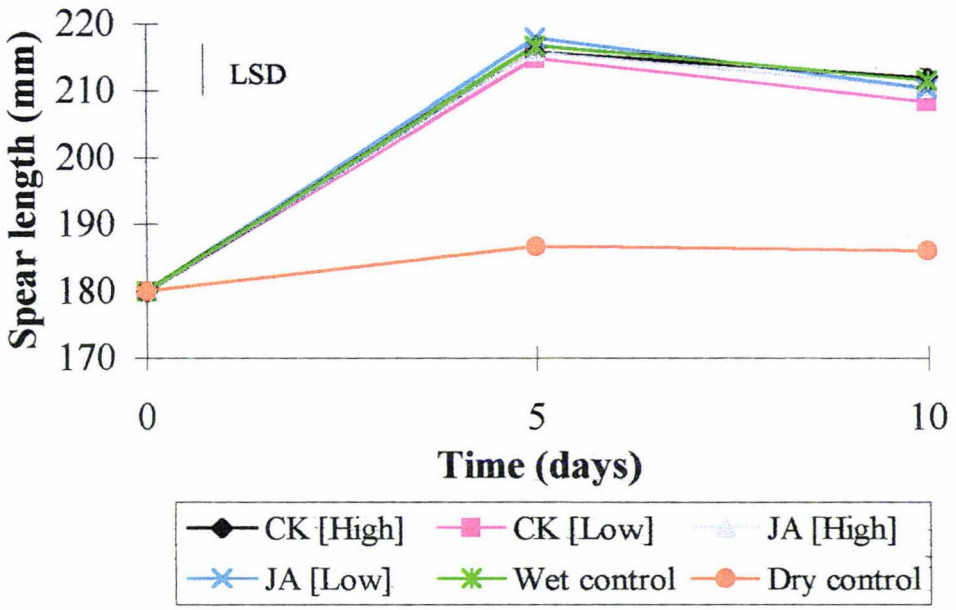


Figure 3.3 Influence of exogenous cytokinin and jasmonic acid treatment on extension of asparagus spears postharvest. Spears harvested in year one. Plant hormone concentrations are shown in Fig. 3.1. LSD ($p = 0.05$) for full analysis is given as vertical bar. Each point in the above graph represents the mean value of four individual observations.

3.2 Biochemical changes postharvest

3.2.1 *Chlorophyll*

The influence of exogenous cytokinin and jasmonic acid treatment after harvest on chlorophyll content is shown in Fig. 3.4.

Year 1

The graph and statistics clearly show that there is a high degree of variability within samples. However, the overal trend is a decline in chlorophyll content over time, in all treatments except the dry (control) spears. The downward trend in chlorophyll content seen was due to the loss of chlorophyll a (data not shown).

Year 2

The chlorophyll content in spears harvested in year two was also variable. Chlorophyll content rose in some treatments and then declined two or three days after harvest. All samples were held at -80°C for no longer than five days before extraction of chlorophyll to stop degradation occurring. Contents of chlorophyll a and protochlorophyll declined whilst chlorophyll b content increased slightly (data not shown).

3.2.2 *Soluble sugars*

Sucrose

The effect of exogenous cytokinin and jasmonic acid treatment on sucrose content in spear tips is shown in Fig. 3.5A. Sucrose content declined rapidly in all spear tips within the first 24 h after harvest. Sucrose levels declined faster in all spear tips subjected to wet treatment compared to dry storage. By two days after harvest sucrose levels in spear tips had declined to around the same level in all treatments. The standard curve for sucrose is shown in Appendix VI.

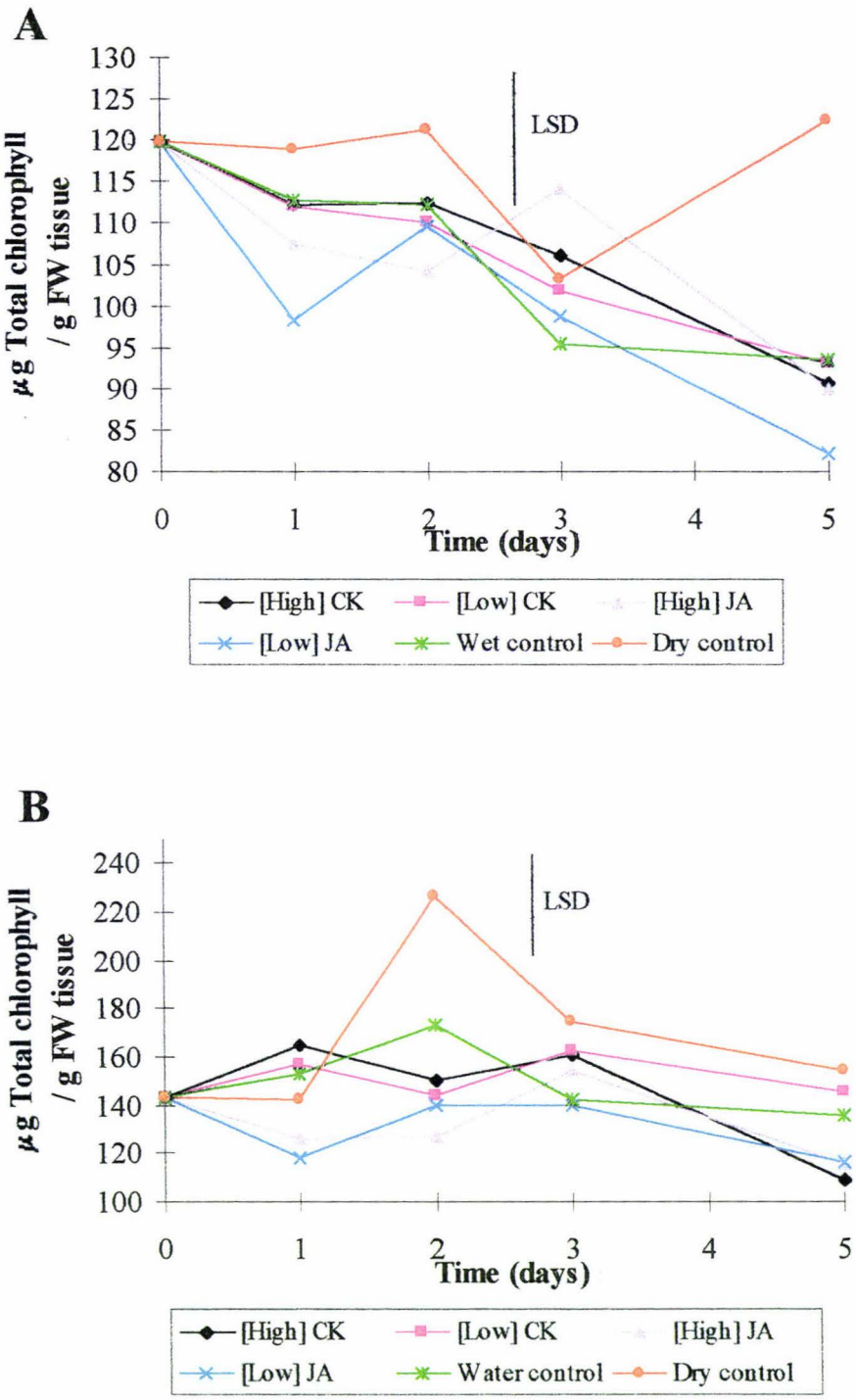


Figure 3.4 Influence of exogenous cytokinin and jasmonic acid treatment on chlorophyll content in asparagus spears postharvest. A = spears harvested in year one. B = spears harvested in year two. Plant hormone concentrations are shown in Fig 3.1. LSDs (p = 0.05) for full analysis are given as vertical bars. Each point in the above graphs represents the mean value of four individual observations performed in duplicate.

Glucose

The effect of exogenous cytokinin and jasmonic acid treatment on glucose content in spear tips is shown in Fig 3.5B. Glucose content declined rapidly in all spear tips within the first 24 h after harvest. The standard curve for glucose is shown in Appendix VI.

Fructose

The effect of exogenous cytokinin and jasmonic acid treatment on fructose content in spear tips is shown in Fig 3.5C. Fructose content in spear tips declined immediately after harvest but at a much slower rate compared to sucrose and glucose. Again, as with the sucrose decline, the untreated dry control spears showed a slower decline in fructose over the first 24 h than all other wet treated spear tips. The low concentration jasmonic acid treatment appears to have caused a slowing in the decline of fructose from spear tips five days after harvest.

3.2.3 *Asparagine*

The effect of exogenous cytokinin and jasmonic acid treatment on asparagine content in spear tips is shown in Fig 3.6. Asparagine accumulated in spear tips immediately after harvest in all treatments. The untreated dry control spears accumulated asparagine faster than all other wet treatments after the first 24 h of treatment, but reached a peak and leveled off three days after harvest. A chromatogram from HPLC separation of amino acids and standard curve for asparagine are shown in Appendix V.

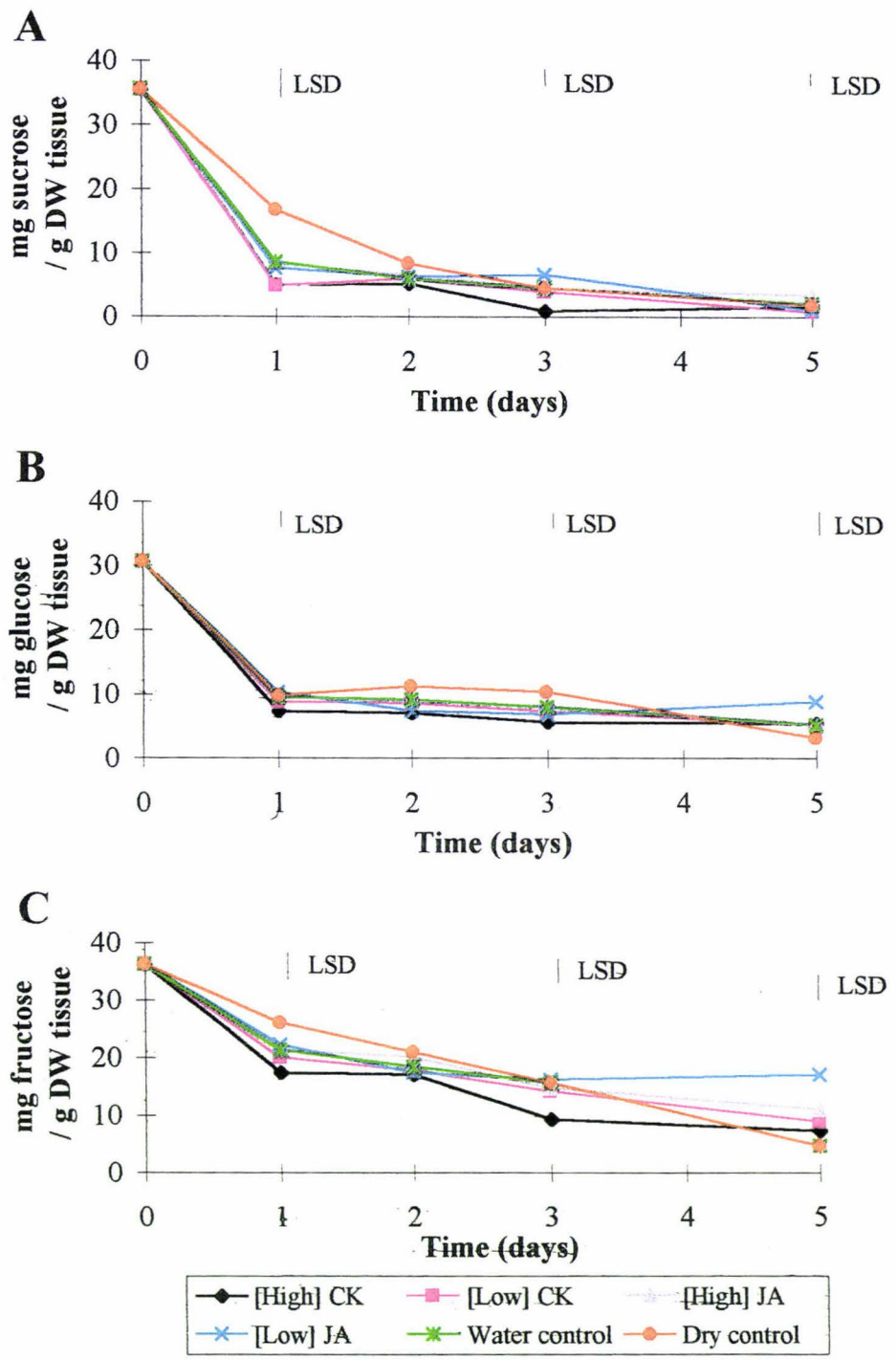


Figure 3.5 Influence of exogenous cytokinin and jasmonic acid treatment on soluble sugar content in asparagus spears postharvest. Spears harvested in year one. A = sucrose, B = glucose and C = fructose contents. Plant hormone concentrations are shown in Fig. 3.1. LSDs (p = 0.05) for individual time points (1, 3 and 5 d after harvest) are presented as vertical bars. Each point in the above graphs represents the mean value of four individual observations performed in duplicate.

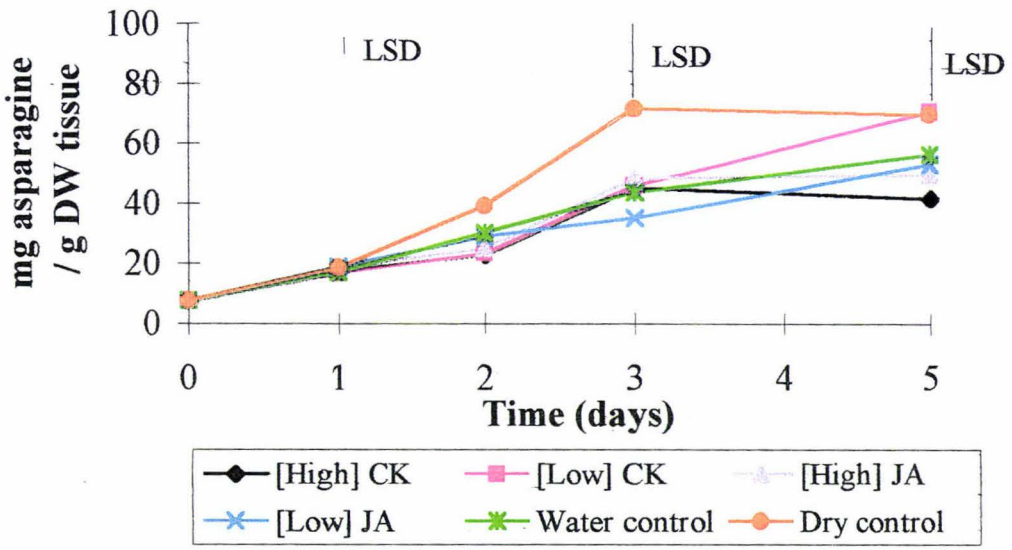


Figure 3.6 Influence of exogenous cytokinin and jasmonic acid treatment on asparagine content in asparagus spear tips postharvest. Spears harvested in year one. Plant hormone concentrations are shown in Fig. 3.1. LSDs ($p = 0.05$) for individual time points (1, 3 and 5 d after harvest) are given as vertical bars. Each point in the above graphs represents the mean value of four individual observations.

3.3 Changes in endogenous jasmonates postharvest

3.3.1 *High performance liquid chromatography*

The separation of jasmonates on the HPLC C_{18} column is shown in the chromatogram in Fig 3.7. Jasmonic acid derivatives were separated in the following order: cucurbic acid (CA) was first to elute in two peaks, followed by jasmonic acid (JA), dihydrojasmonic acid (DJA) and finally methyl jasmonate (MeJA). [^{3}H]CA eluted with the same retention times as the CA standard also showing as two adjacent peaks.

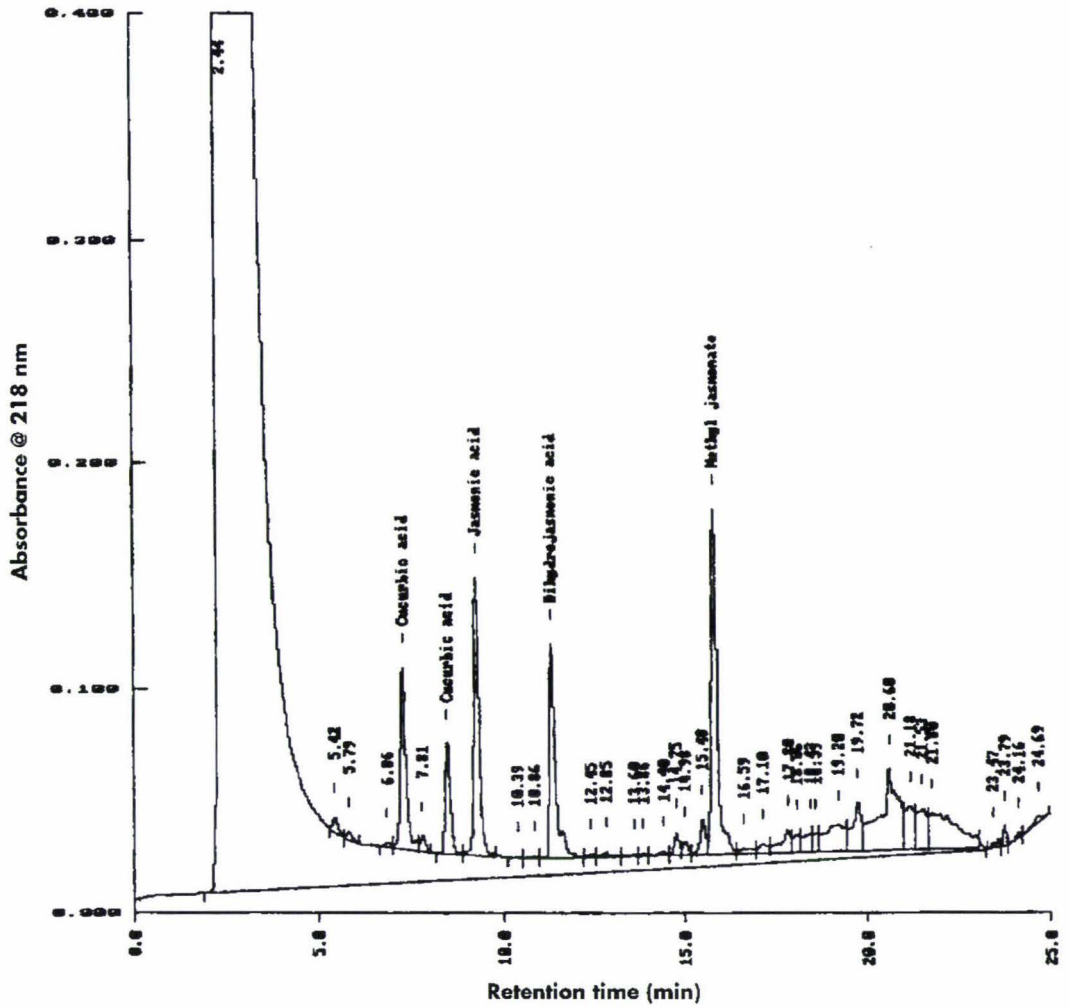


Figure 3.7 HPLC separation profiles of jasmonate standards. Separation of jasmonates using a reverse phase octadecyl silica C_{18} column. Solvents used were 0.05% trifluoroacetic acid (TFA), methanol, 0.05% TFA in acetonitrile and the gradient used is shown in Appendix II. Retention times for jasmonates are marked.

3.3.2 *Optimization of ELISA*

Anti-jasmonic acid antibody blood serum was gifted to Professor Paula Jameson by Dr Sean Clarke from the University of Otago. Dilutions of purified antibody (Section 2.9.5) were tested against dilutions of JA:OVA conjugate (see section 2.9.6) bound to the ELISA plate well and shown in Fig 3.8. The anti-JA IgG was detected by anti-rabbit IgG conjugated to alkaline phosphatase.

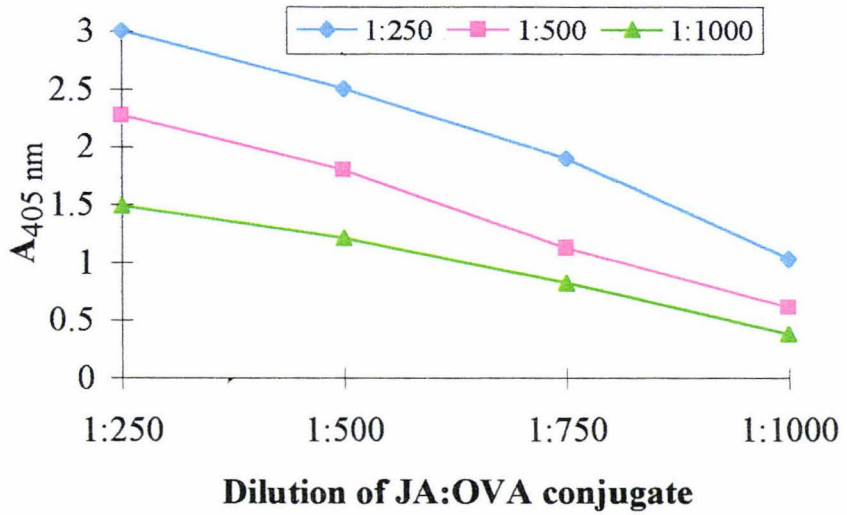


Figure 3.8 Anti-jasmonic acid IgG titer. Antibodies (diluted 1:250 ◆, 1:500 ■, 1:1000 ▲) and tested against jasmonic acid conjugated to ovalbumin (OVA:JA) diluted 1:250, 1:500, 1:750, 1:1000. Antibodies were detected using an anti-rabbit antibody conjugated to alkaline phosphatase.

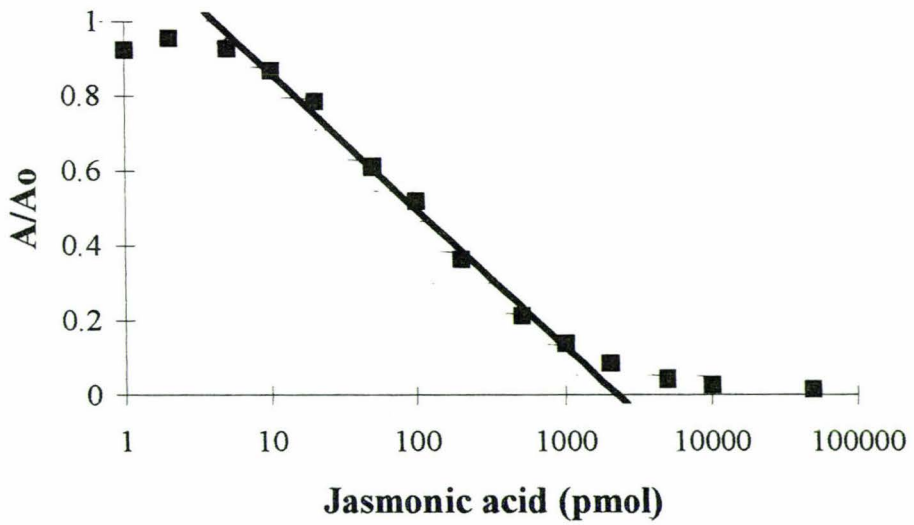


Figure 3.9 ELISA standard curve for jasmonic acid (JA) cross reacted with anti-JA antibodies. Anti-JA IgG was detected using an anti-rabbit antibody conjugated to alkaline phosphatase. A = absorbence in presence of JA, A_0 = absorbence in absence of JA. X axis is a log scale. A representative standard curve

for jasmonic acid is shown in Fig. 3.9. The A/A_0 values were plotted against concentration of jasmonic acid (pmol) and sample values were read from the straight line region of the curve.

3.3.3 *Effect of harvest on endogenous jasmonates*

Jasmonates were detected in spear tips following harvest. Examples of ELISA profiles from HPLC fractions are shown in Fig. 3.10. The data are from spear tips at the time of harvest and 30 h after harvest. The values shown have been corrected for plant weight and % recovery, but have not been corrected for cross reactivity. Cross-reactive compounds can be seen with similar retention times to cucurbic acid and jasmonic acid.

Year 1

Results from year one jasmonate extractions are shown in Table 3.1. These values have been corrected for plant weight and % recovery but have not been corrected for cross reactivity. The spears harvested and held in water for 5 d showed a peak of jasmonic acid in the tips 1 d after harvest. Spears treated with jasmonic acid postharvest did not accumulate any of the forms of jasmonate detectable in the ELISA until 5 d after harvest (although a poor recovery may be why no peak was detected 1 d after harvest). Spears treated with cytokinin postharvest accumulated jasmonic acid in their tips at 2 d after harvest compared to the water treated tips which accumulated jasmonic acid just 1 d after harvest. Spears subjected to dry storage postharvest had little jasmonic acid present until 5 d after harvest.

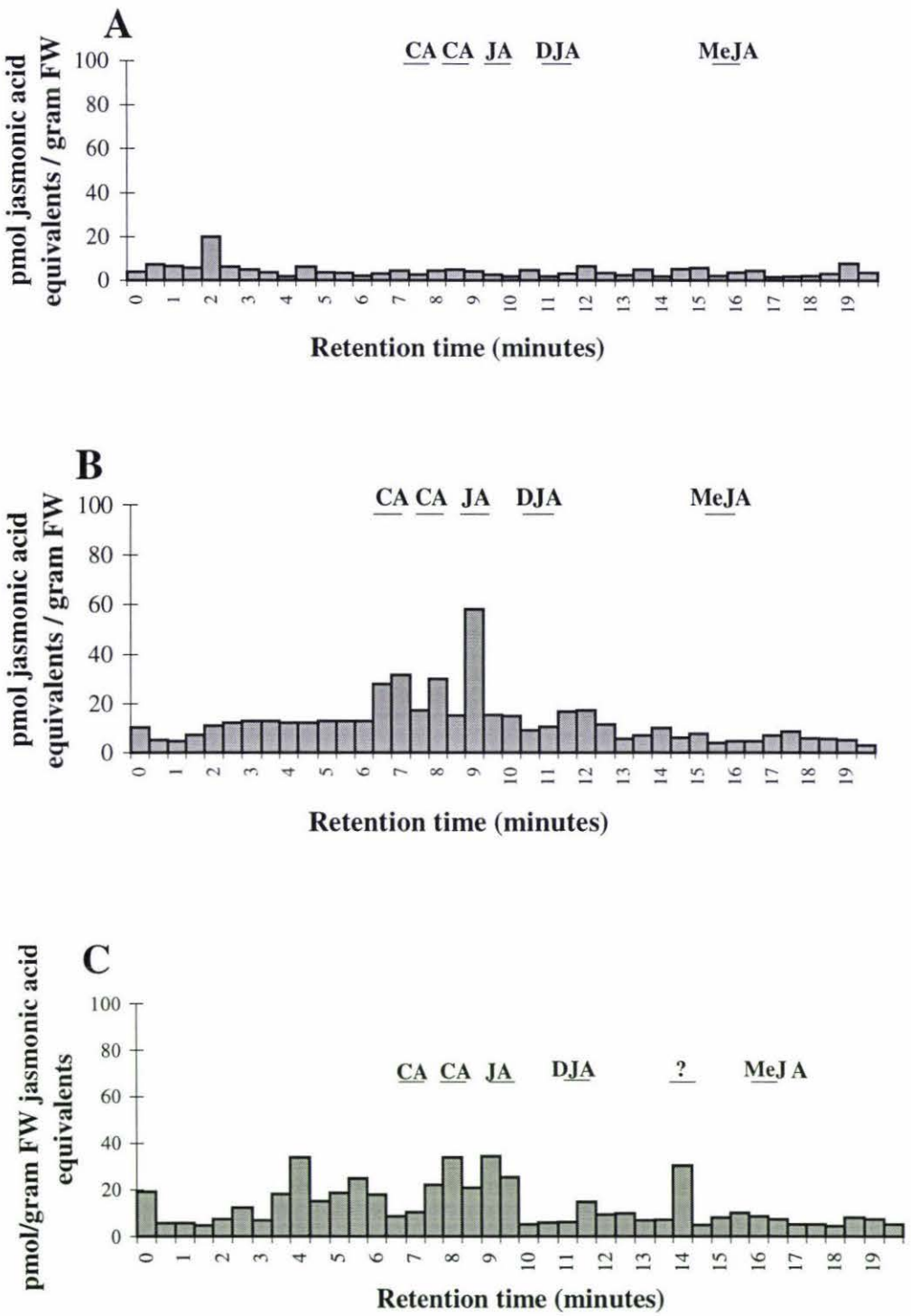


Figure 3.10 Jasmonate profiles of asparagus spear tips. A. At time of harvest. B. 30 h after harvest. C. 36 h after harvest. Data shown are the cross-reactivity of the individual samples with the antibody used in the ELISA after separation by HPLC. CA = cucurbic acid, JA = jasmonic acid, DJA = dihydrojasmonic acid, ? = unknown and MeJA = methyl jasmonate.

Table 3.1 Effect of harvest and exogenous cytokinin and jasmonic acid treatment postharvest on endogenous jasmonate levels within asparagus spear tips. (nd = not detected; - = not analysed; * = no recovery of internal standard).

pmol jasmonic acid equivalents / gram FW

Influence of water treatment postharvest on endogenous jasmonate levels in spear tips						
	0hrs + 2	**0 h rs**	**1 day**	**2 day**	**3 day**	**5 day**
CA	23	100	16	trace	nd	16
JA	23	71	156	nd	trace	trace
DJA	nd	22	39	nd	nd	28
MeJA	nd	22	nd	nd	nd	nd
Influence of jasmonic acid (50 μM) treatment postharvest on endogenous jasmonate levels in spear tips						
	0hrs + 2	**0 h rs**	**1 day**	**2 day**	**3 day**	**5 day**
CA	44	171	nd*	trace	-	26
JA	trace	20	nd*	nd	-	43
DJA	nd	10	nd*	nd	-	30
MeJA	83	trace	nd*	trace	-	29
Influence of cytokinin (2 μM) treatment postharvest on endogenous jasmonate levels in spear tips						
	0hrs + 2	**0 h rs**	**1 day**	**2 day**	**3 day**	**5 day**
CA	44	171	49	64	-	42
JA	trace	20	nd	227	-	35
DJA	nd	10	123	70	-	166
MeJA	83	trace	trace	26	-	trace
Influence of dry storage postharvest on endogenous jasmonate levels in spear tips						
	0hrs + 2	**0 h rs**	**1 day**	**2 day**	**3 day**	**5 day**
CA	23	100	13	-	-	35
JA	23	71	trace	-	-	18
DJA	nd	22	16	-	-	42
MeJA	nd	22	trace	-	-	48

Year 2

The influence of harvest on endogenous jasmonate levels in asparagus spears is shown in Figs. 3.11-3.14 (the data presented are corrected for plant weight, % recovery and differential cross reactivity against the anti-JA antibody). In spear tips JA levels were 40 pmol / g FW at the time of harvest. Immediately after harvest JA levels dropped to trace levels followed by a rapid upsurge in both dry and wet treated spears by 12 h (wet) and 15 h (dry) after harvest. This peak was followed by a decline in JA levels by 15 h (wet) and 24 h (dry) after harvest and another rapid rise at 36 h after harvest to levels of around 100 pmol (wet) and 80 pmol (dry). This second peak was followed by another fall in JA levels by 48 h after harvest. JA levels increased significantly again only in the dry treated spear tips between 7 and 10 d after harvest to a level of around 120 pmol / g FW.

In spear mid sections and bases JA levels were around 200 pmol / g FW at the time of harvest. This was followed by a rapid decline in JA to trace levels in both mid sections and bases, of both wet and dry treated spears by 1 h after harvest. JA levels immediately rose again in both mid sections and bases, with the mid sections from dry spears accumulating higher concentrations of JA. The peak JA concentration in mid sections (30 h) occured later than in the bases (6 h).

The influence of harvest on endogenous dihydrojasmonic acid (DJA) levels in asparagus spears is shown in Fig. 3.12. The pattern of DJA accumulation in spears postharvest is similar to the accumulation of JA, but differs in the amount of DJA accumulated (3-4 times as much DJA than JA). In tips of spears DJA levels were low at harvest and increased to peak twice in dry treated material (15 h and 36 h to 500 and 300 pmol DJA / g FW respectively). Although only one peak in DJA occurred in wet treated spears (30 h to 120 pmol DJA / g FW), two peaks were seen for this material in JA accumulation. Also similar to the JA accumulation, DJA levels accumulated in only dry treated spear tips 7-10 d after harvest. In mid sections and bases of spears DJA levels declined initially, and then fluctuated in the same manner as for JA.

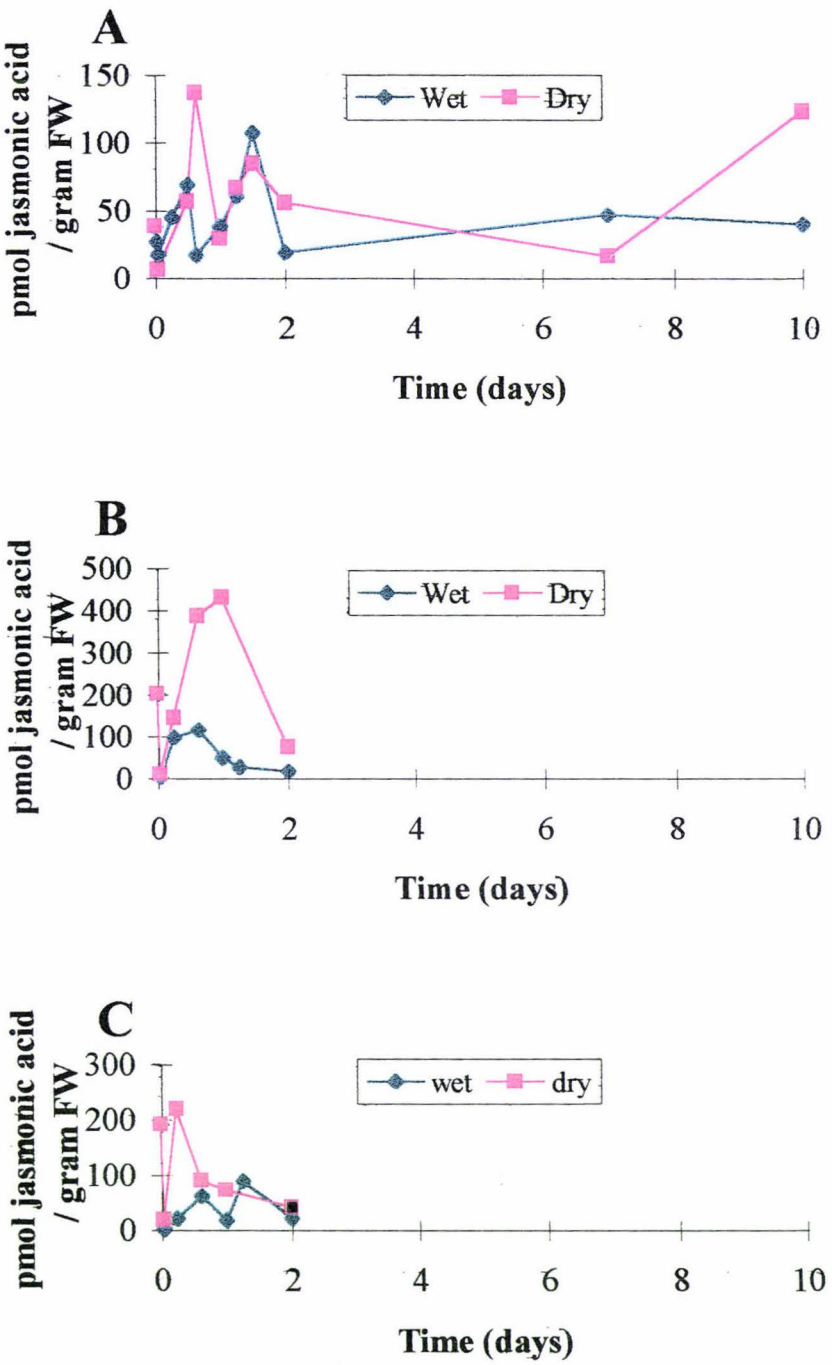


Figure 3.11 Changes in endogenous jasmonic acid in spears of asparagus. A. Changes in jasmonic acid in tips of spears. B. Changes in jasmonic acid in mid sections of spears. C. Changes in jasmonic acid in bases of spears.

The influence of harvest on endogenous cucurbic acid (CA) levels in asparagus spears is shown in Fig. 3.13. CA accumulated in tips of spears in a similar way to JA and DJA after harvest although to much higher levels than all other jasmonates quantified. In tips of spears CA levels were low at harvest and increased to peak twice in dry treated material (15 h and 36 h to 5.5 and 1.6 nmol CA / g FW respectively). Again like DJA, only one peak in CA occured in wet treated spears (30 h to 2.5 nmol CA / g FW). Also similar to the JA and DJA accumulation was the accumulation of CA in dry treated spear tips 7-10 d after harvest. In mid sections and bases of spears CA levels declined initially, and then fluctuated in the same manner as for JA and DJA.

The influence of harvest on endogenous methyl jasmonate (MeJA) levels in asparagus spears is shown in fig. 3.14. Very small amounts of methyl jasmonates were detected in the 60 samples extracted compared to the other jasmonates extracted. Levels never rose over 40 pmol MeJA / g FW, although some peaks were detected at times when peaks were detected for other jasmonates. Contrary to the other jasmonates, MeJA concentrations were usually higher in the samples from the wet as oppossed to the dry treatment.

3.3.4 *Effect of natural foliar senescence on endogenous jasmonates*

Changes in jasmonates during natural foliar senescence of asparagus fern are shown in Fig. 3.15. These values have been corrected for plant weight and % recovery, but not for cross reactivity. Jasmonic acid and dihydrojasmonic acid levels rose between stages 3 and 4, and then fell through to stage 6 of senescence. Cucurbic acid levels rose sharply between stages 4 and 5, and then slowly leveled off through to stage 6 of senescence. The concentration of methyl jasmonate stayed at trace levels throughout the six stages of senescence.

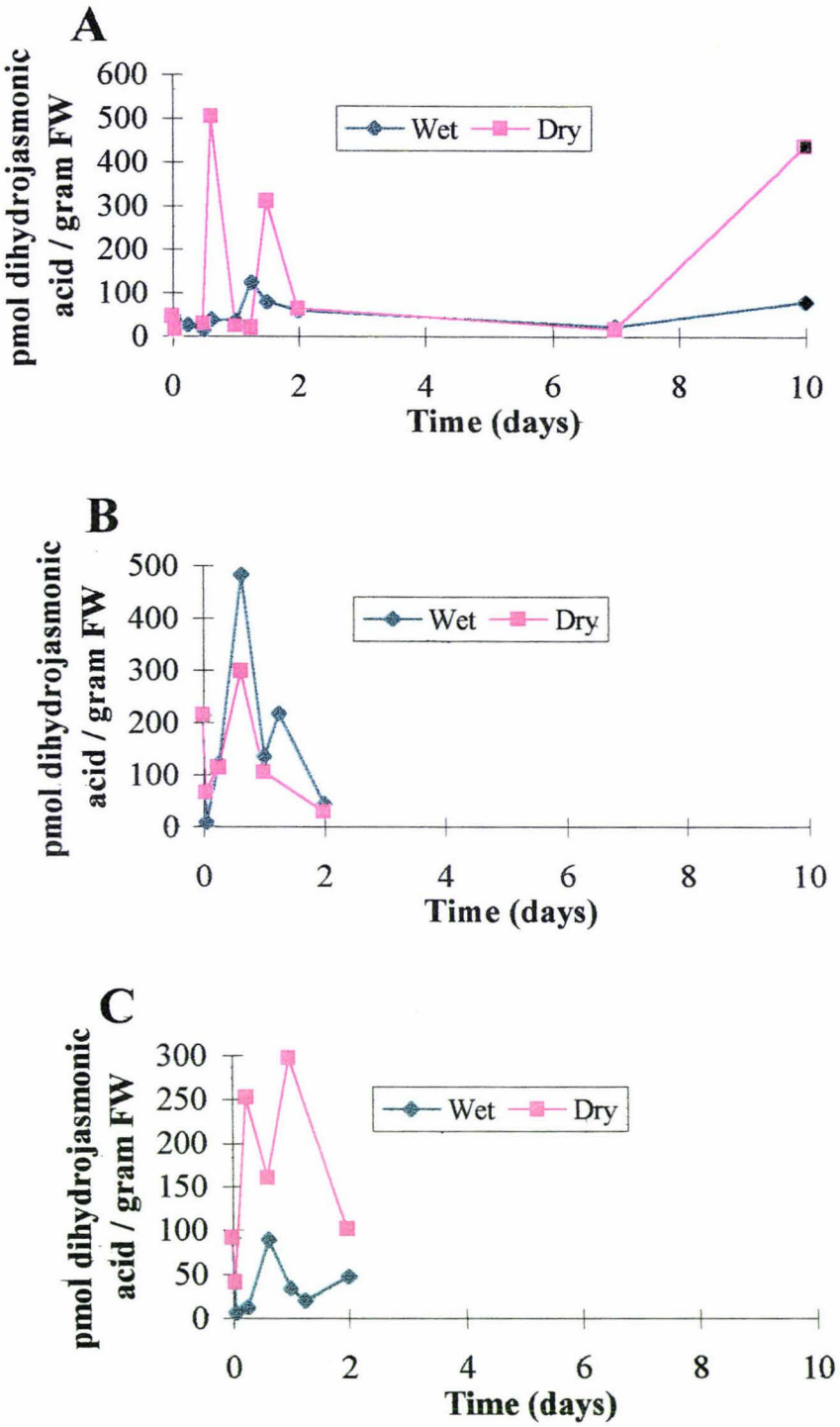


Figure 3.12 Changes in endogenous dihydrojasmonic acid in spears of asparagus.

A. Changes in dihydrojasmonic acid in tips of spears. B. Changes in dihydrojasmonicacid in mid sections of spears. C. Changes in dihydrojasmonic acid in bases of spears.

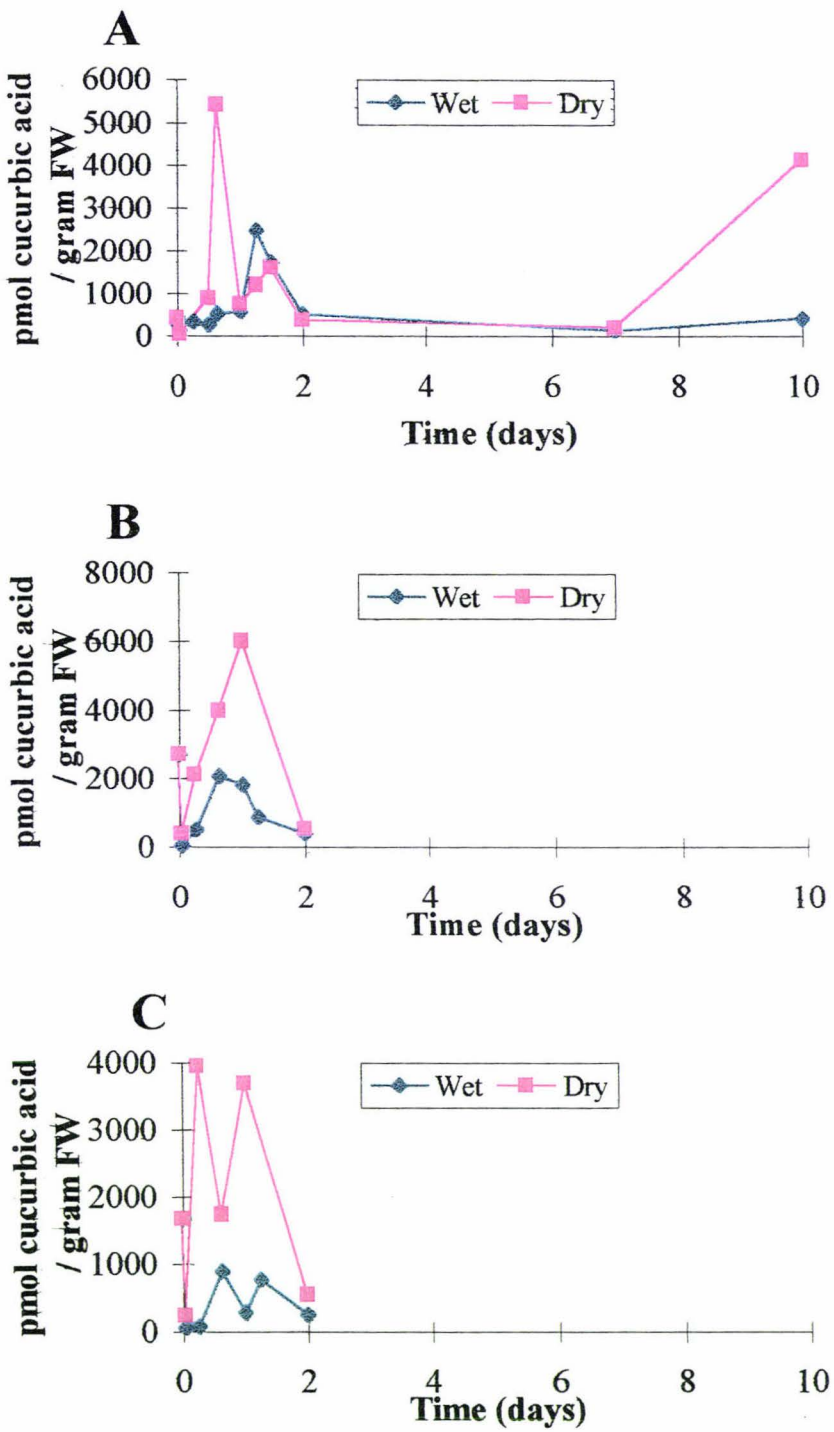


Figure 3.13 Changes in endogenous cucurbic acid in spears of asparagus. A. Changes in cucurbic acid in tips of spears. B. Changes in cucurbic acid in mid sections of spears. C. Changes in cucurbic acid in bases of spears.

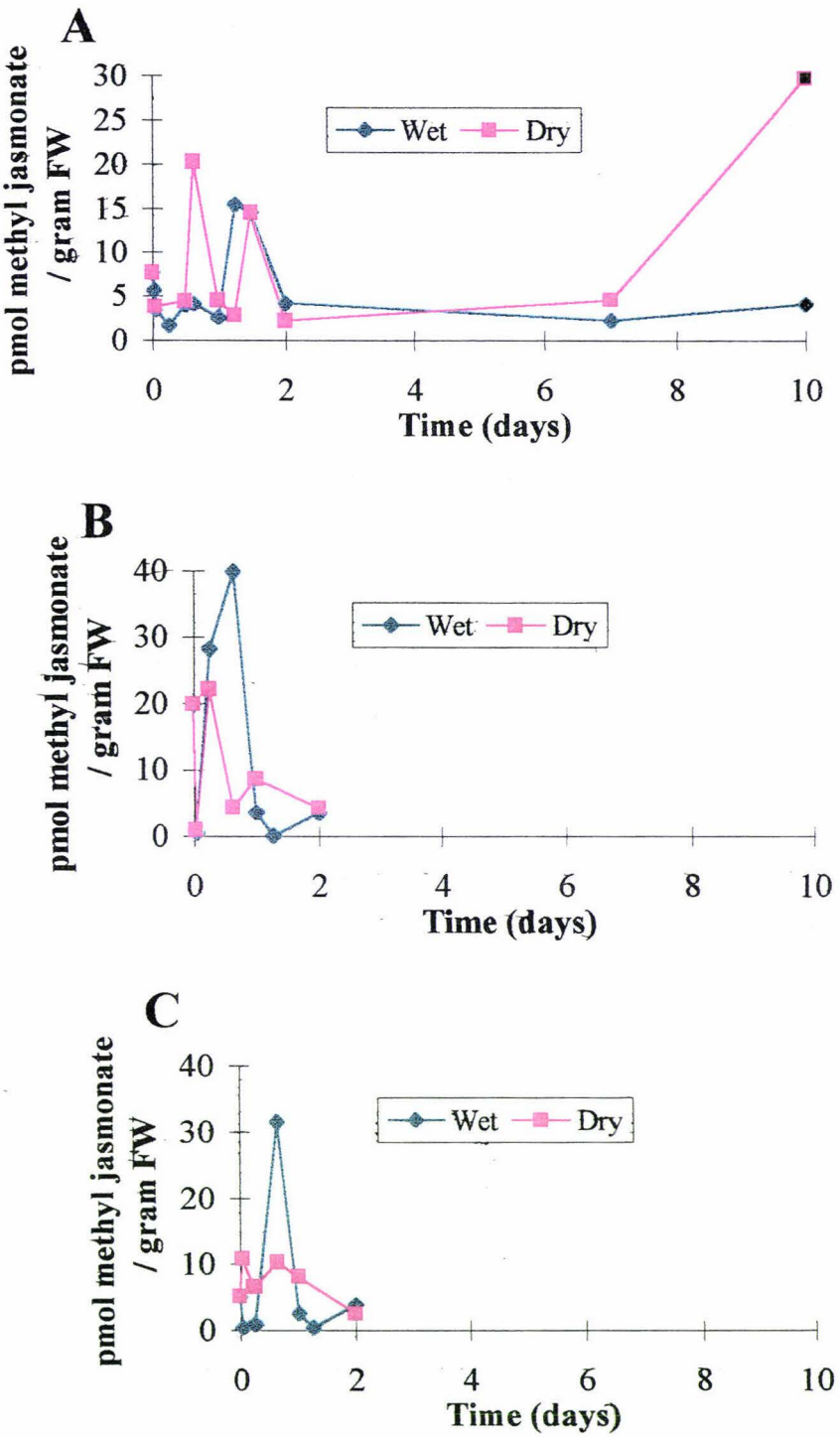


Figure 3.14 Changes in endogenous methyl jasmonate in spears of asparagus. A. Changes in methyl jasmonate in tips of spears. B. Changes in methyl jasmonate in mid sections of spears. C. Changes in methyl jasmonate in bases of spears.

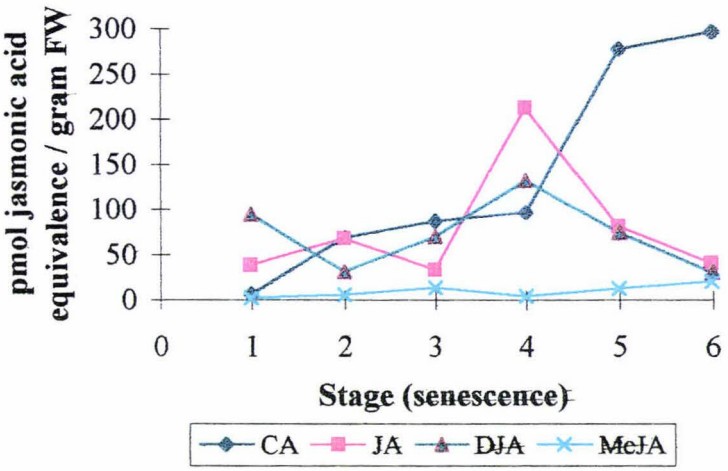


Figure 3.15 Changes in endogenous jasmonates in naturally senescing foliar tissue of asparagus. Stage 1 = dark green (mature green), stage 2 = half tone, stage 3 = light green, stage 4 = yellow (fully senescent), stage 5 = yellow/orange tinge, stage 6 = yellow/orange/brown.

3.3.5 *Electrospray mass spectrometry*

Jasmonate standards jasmonic acid, dihydrojasmonic acid, cucurbic acid and methyl jasmonate, and HPLC samples containing putative jasmonates were subjected to E/S mass spectrometry. The positive ion E/S mass spectra of methyl jasmonate and cucurbic acid were characterised by a $[M + H]^+$ ion, although other fragment ions were also present some of which were probably impurities(Table 3.2). Likewise, the negative ion E/S mass spectra for jasmonic acid and dihydrojasmonic acid showed a $[M - H]^-$ ion which was prominent although other fragment ions were also present, again some of which were probably impurities(Table 3.3). Compounds of the correct molecular weight were detected in the appropriate HPLC fractions except for methyl jasmonate. Samples had the same fragment ions present as the relevant standard. In addition, jasmonate samples bound a free sodium ion only in positive ionisation mode ($[M + H + Na^+]^+$). The E/S mass spectra of a jasmonic acid sample is shown in Figure 3.16A. The E/S mass spectra of an unknown cross-reactive compound (HPLC retention time 14 - 14.5 min, Fig. 3.10) was characterised by a $[M + H]^+$ ion and also other fragment ions (Table 3.4, Fig. 3.16B). The collision-induced decomposition (CID) mass spectra of this $[M + H]^+$ ion produced the prominent fragment ion *m/z* 159 (Fig. 3.18).

Table 3.2. Key ions in the E/S mass spectra of the $[M + H]^+$-ion of methyl jasmonate (MeJA) and cucurbic acid (CA) obtained by E/S mass spectrometry using positive ionisation mode (*m/z*).

Ion	**MeJA standard**	**MeJA sample**	
$[M + H]^+$	225	nd	
$[M + H - CH_3COOHCH_3]^+$	151	nd	
$[M+H-CH_3CH_2CHCHCH_2COCH_3]^+$	99	nd	
Ion	**CA standard**	**CA_1 sample**	**CA_2 sample**
$[M + H]^+$	213	213	213
$[M + Na^+]^+$	nd	235	235
$[M + H - HCOOH]^+$	167	167	167

nd = not detected.

Table 3.3. Key ions in the E/S mass spectra of the $[M - H]^-$-ion of jasmonic acid (JA) and dihydrojasmonic (DJA) acid obtained by E/S-MS using negative ionisation mode (*m/z*).

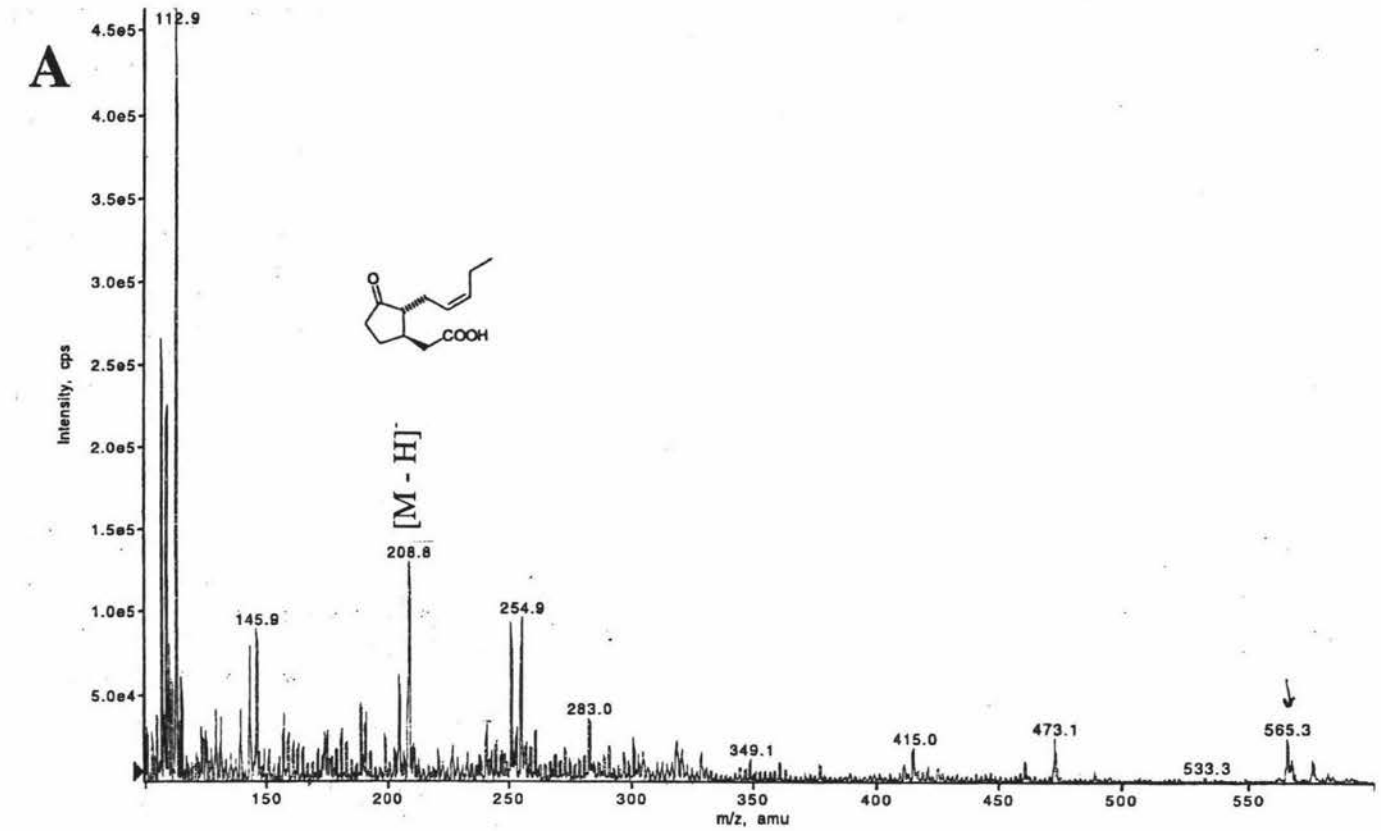
Ion	**JA standard**	**JA sample**
$[M - H]^-$	209	209
Ion	**DJA standard**	**DJA sample**
$[M - H]^-$	211	211
$[M - H - CH_3COO]^-$	152	152

nd = not detected.

Table 3.4. Key ions in the E/S mass spectra of the $[M + H]^+$-ion of an unknown jasmonate obtained by E/S mass spectrometry using positive ionisation mode (*m/z*).

Ion	**Unknown sample**
$[M + H]^+$	399
$[M + H - HCOOH - H_2O]^+$	335
$[a + 2H]^+$	205
f	203
e	159

nd = not detected.



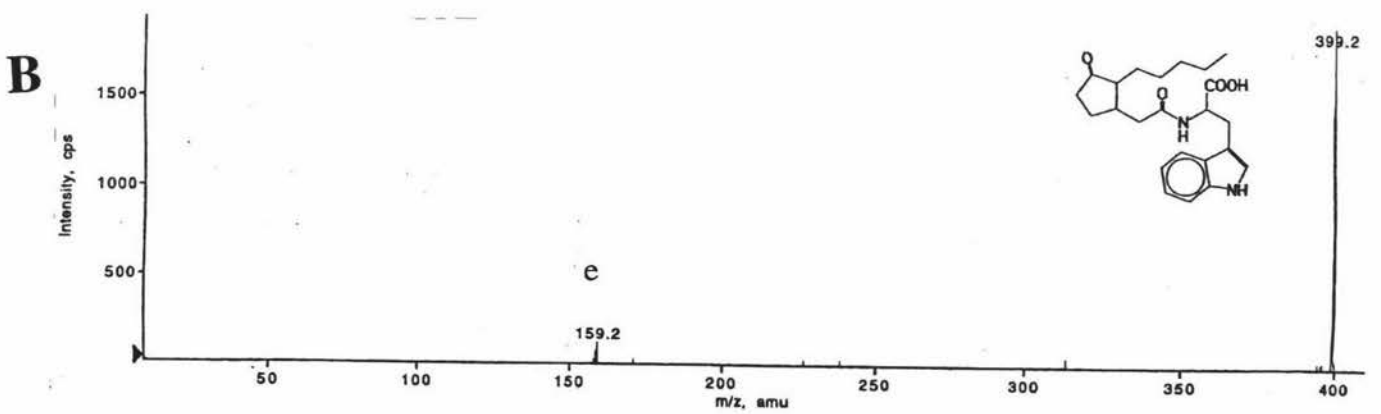


Figure 3.16 Electrospray mass spectra of jasmonate samples. A. E/S mass spectra of a jasmonic acid sample. B. E/S M/SM/S of an unknown cross-reactive jasmonate with an HPLC retention time of 14 min.

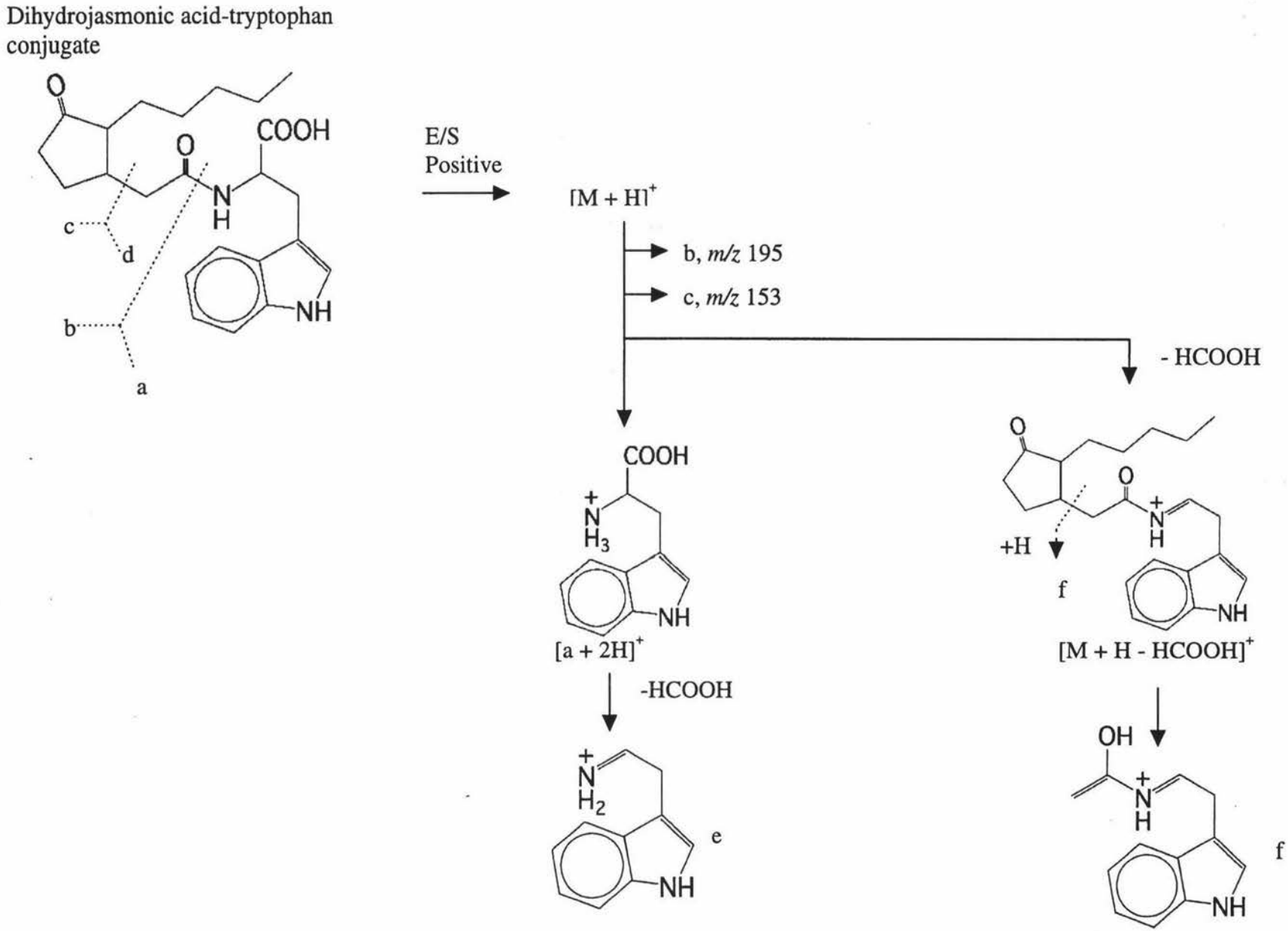


Figure 3.17 Proposed fragmentation pattern of dihydrojasmonic acid tryptophan conjugate obtained from the [M + H]+ ion using E/S mass spectrometry.

Chapter Four

DISCUSSION

4.1 Cytokinin and jasmonate-induced physiological and biochemical changes

Physiological, biochemical and gene expression changes in asparagus spears occur rapidly after harvest (King et al. 1993). However, little is known about the effects of plant hormones on these processes. In the first part of the project, the effects of exogenous cytokinin and jasmonic acid on various parameters were investigated.

Perhaps the most important marker of shelf-life and harvest-induced "senescence" of asparagus is estimation by visual assessment of spear quality. The effect of cytokinin and jasmonic acid treatment on spear quality is shown in Fig. 3.1. Spears were visually ranked to a scheme developed by Lill (1980). The dry stored spears deteriorated faster than all other wet treatments and thus had a shorter shelf-life. Jasmonic acid treatment had some negative effects on spear quality, reducing spear shelf-life compared to the water treated spears. The trend for the cytokinin treated spears to show an increase in shelf-life in Year 1, was confirmed in Year 2, with a statistically significant increase in shelf-life compared to water-treated spears. However, in Year 2, spears treated with the low concentration of jasmonic acid also showed a statistically significant increase in shelf-life compared to water-treated spears. This was a little unexpected, although there have been reports that low concentrations of jasmonate can stimulate several physiological processes in potato plants *in vitro* (Dermatsia et al. 1994).

The effects of harvest and of exogenous cytokinin and jasmonic acid treatment on the chlorophyll content in tips of spears are shown in Fig 3.4. A general trend in chlorophyll degradation after harvest was observed, but there were no significant effects of treatment. Any treatment effects may have been masked by the variability within the samples. There are only a few reports of chlorophyll content in asparagus spears after harvest. Lee (1981) reported chlorophyll contents of 397 $\mu g.g^{-1}$ FW in

spear tips at the time of harvest, compared to 150 $\mu g.g^{-1}$ FW reported here in this thesis. Scott and Kramer (1949) reported chlorophyll contents of 248 $\mu g.g^{-1}$ FW, but this value came from an average of spears stored postharvest for 0, 1, 3, or 7 d at 0, 4.5, 10, or 21°C. The initial value for chlorophyll content would probably have been higher. Neither author reported from which cultivar of asparagus chlorophyll was extracted. In this work, the cultivar 'Jersey Giant' was used. The appearance of Jersey Giant is deep green, but anthocyanin pigment may add to the depth of colour perceived (Ross Lill personal communication). Scott and Kramer (1949) further reported that smaller spears contained about 20 % less chlorophyll than larger spears, but no diameters were reported in their study. Time of harvest might also influence chlorophyll content in asparagus spears. I suggest spears harvested later in the season from crowns which are nearly depleted of soluble substrate may contain more chlorophyll due to their need to become autotrophic. Furthermore higher levels of chlorophyll could also be influenced by higher temperatures and the higher light levels available later in the picking season. Wang (1971) observed that time of day influenced the chlorophyll content of asparagus, with higher levels present in the afternoon than in the morning.

The many variables affecting the chlorophyll content in asparagus spear tips may be why there are few published results: chlorophyll content may not be a good biochemical marker of shelf-life and harvest-induced "senescence" in asparagus. In fact, although spears appear green, they are relatively non-photosynthetic as only the outer layers of the spear contain chlorophyll.

Soluble sugar loss and accumulation of asparagine in spear tips of asparagus have repeatedly been used as biochemical markers of shelf-life and harvest-induced "senescence". Soluble sugar loss (shown in Figure 3.5) occurred in spear tips immediately following harvest. None of the postharvest treatments caused any significant differences in either sucrose, glucose or fructose contents in spear tips after harvest, although all spears subjected to wet treatments lost sucrose and fructose at a faster rate from their tips compared to the dry treated spears.

The initial amounts of sucrose, glucose and fructose reported here are similar to those reported previously. At harvest the sucrose, glucose and fructose contents were 36, 31 and 36 $mg.g^{-1}$ DW respectively. Sucrose, glucose and fructose contents declined to approximately 25 %, 33 % and 60 % respectively, of their initial value just 24 h after harvest. These figures are comparable with those published by Davies et al. (1996). They reported sucrose, glucose and fructose contents in asparagus spear tips to be 50, 30 and 40 $mg.g^{-1}$ DW respectively at the time of harvest. They also showed that sucrose, glucose and fructose contents fell to approximately 20 %, 30 % and 50 % respectively, of their initial values just 24 h after harvest, and that most of the soluble sugar loss occurred within 6 h after harvest. The data reported here do not show this rate of loss because analyses were not conducted until 24 h after harvest. Further, Hurst et al. (1996) showed that continuous feeding of water to spears using the water vase technique reduced the loss of fructose after 24 h of treatment. Glucose content in spear tips declined in a similar way to those subjected to dry storage. These results are similar to those presented in this study.

Lipton (1990) reported that the sugar content of freshly harvested whole spears declined by up to 50 % during a picking season. Consequently, the date of harvest has an effect on soluble sugar content in spear tips. This could explain any small differences in soluble sugar content reported in the literature compared to the data presented here. This decline over the picking season coincides with a rapid decline in storage root reserves (Shelton and Lacy 1980; Pressman et al. 1993).

Amino acids, in particular asparagine, accumulate rapidly in asparagus spear tips following harvest. This accumulation coincides with a decrease in protein content in spear tips (Hurst et al. 1996). Asparagine accumulation in spear tips postharvest is used frequently as a biochemical marker for estimation of shelf-life and harvest-induced "senescence". The influence of exogenous after harvest treatment of cytokinin and jasmonic acid on asparagine accumulation in spear tips is shown in Fig. 3.6. Asparagine accumulated in spear tips following harvest in all treatments. Spears subjected to dry storage accumulated asparagine faster than all other wet treatments, reaching a peak asparagine content of 75 $mg.g^{-1}$ DW just three days after harvest.

King et al. (1990) published results similar to those shown in Fig. 3.6. Spears were subjected to dry storage at 20°C. At the time of harvest the asparagine content in the spear tips was 6.5 $mg.g^{-1}$ DW and increased to 20.8 $mg.g^{-1}$ DW 24 h after harvest. After 48 h of dry storage at 20°C asparagine levels in spear tips had reached 28.6 $mg.g^{-1}$ DW. Hurst et al. (1996) published results which are a little different. Although accumulation of asparagine took place in both cases, Hurst et al. (1996) in contrast, reported asparagine levels of a different magnitude to those presented in this thesis and those reported by King et al. (1990). Using the water vase technique, spears were continuously fed water during storage at 20°C. The asparagine content in spear tips at the time of harvest was 7 $mg.g^{-1}$ DW and increased very steadily over time to reach 152 $mg.g^{-1}$ DW after 5 d of treatment in water. These values are approximately three times greater than those presented in this thesis, although Hurst et al. (1996) did not compare water fed spears with dry stored spears, and the cultivar they used was 'Limbras 10' not 'Jersey Giant' as used in this study.

Spear extension was measured five and ten days after harvest (Fig. 3.3). From visual observation, almost all of the spear extension took place within the first 24 h of harvest. Spears in all wet treatments grew more following harvest than the dry untreated spears. Spears treated with the low concentration of cytokinin appeared to be slightly shorter than all other wet treated spears although this observation is not statistically significant. However, this observation is consistent with the findings of Dedolph et al. (1961). They treated asparagus spears postharvest with N^6-benzylamino purine (6-BAP) and observed a 15 % reduction in spear length compared to non-treated spears. They concluded that this reduction in spear length was due to a reduction in respiration in the cytokinin-treated spears.

Tiprot is a disorder of asparagus which limits the shelf-life of the harvested crop (Fig. 3.2). Spears harvested in Year 1 were treated slightly differently than Year 2 spears. Year 1 spears were draped in plastic film to reduce water loss due to transpiration, and also to mimic asparagus packaging. This could have hastened deterioration, as damage could have taken place when removing and replacing plastic film. Also condensation which built up inside the plastic film was in contact with spear tips and

this could have increased chances of soft rots developing within the tips of spears. This observation appears to be important for tiprot analyses, as Year 1 spears overall had a higher proportion of tiprot than Year 2 spears (Fig. 3.2). However, many factors have been reported to influence the occurrence of tiprot including the temperature of both the crown and the spear (Lill et al. 1996), soluble sugar and protein content at harvest, the microenvironment of the plant, genetic and seasonal variability (Lill et al. 1994), and harvest date (Hurst et al. 1993b).

In both years, spears treated with cytokinin had a reduction in tiprot disorder compared to control spears. Also in both years, spears treated with jasmonic acid had an increase in the tiprot disorder compared to control. This may be because cytokinins have been noted to maintain membrane integrity (Grossman and Lesham 1978), whilst jasmonates are produced by deteriorating membranes and can lead to further membrane disruption (Parthier 1990).

Although extension of shelf-life and reduced tiprot were observed in spears treated after harvest with cytokinin, these benefits were not observed in the biochemical analyses. Likewise, decreased shelf-life and increased tiprot was observed in spears treated postharvest with jasmonic acid, but these negative effects were not observed in the biochemical analyses. As one would imagine, the process of harvesting induces considerable stress on the immature, rapidly growing spear, which until harvest had been totally reliant on the crown and storage roots for substrate. Once this substrate source is taken away changes occur very rapidly. Based on the feeding experiments with phosphinothricin (Eason et al. 1996) and other protein synthesis inhibitors (Hurst et al. 1996), I suggest that exogenous cytokinin and jasmonic acid might not have arrived in the spear tip until 6 h after application to the base of spear. There was, therefore, sufficient time for changes in gene expression to take place within the spear tip after harvest before the exogenous phytohormones could have had an effect. Up to 50 % of soluble sugars are lost within the first 6 h of harvest (Davies et al. 1996) and this rapid loss of soluble sugar causes carbohydrate stress and induces the expression of asparagine synthetase (Davies and King 1993). Transcripts for asparagine synthetase which are barely detectable in growing spear tips begin to accumulate to relatively high levels just 2 h after harvest (Davies and King 1993). These very early

changes in sugar content and gene expression possibly over-ride any effects the phytohormones could induce on primary metabolism.

4.2 Postharvest changes in endogenous jasmonates

The quantification of jasmonates following harvest of spears was the second aim of this thesis. An ELISA, developed by Clarke (1996), for jasmonic acid and derivatives of jasmonic acid was used for this purpose. The standard curve produced from the ELISA for jasmonic acid (Figure 3.9) was very similar to that published by Clarke (1996) and the RIA standard curve of Knöfel et al. (1990). The ELISA standard curve is sensitive over the range 10 to 1000 pmol JA whereas the RIA standard curve was sensitive over the range 5 to 1000 pmol JA.

An extensive purification process was used which included combined PVPP + DE52 column chromatography and low pressure C_{18} column chromatography instead of the solvent partitioning steps employed by most other laboratories around the world. This enabled us to also quantify methyl jasmonate levels separately.

HPLC separation for jasmonic acid and derivatives of jasmonic acid is widely used (Yamane et al. 1981; Nojiri et al. 1996; Sano et al. 1996) and is a lot less time consuming than thin layer chromatography that also has commonly been used to separate individual jasmonates (Meyer et al. 1984; Miersch et al. 1989; Knöfel et al. 1990). The HPLC separation used in this thesis was developed following advice from Dr Robin Mitchell. This HPLC protocol enabled good separation of the four jasmonic acid derivatives cross reactive in the JA ELISA (Figure 3.7).

The influence of harvest on endogenous jasmonates in asparagus spears (or any other postharvest system) has not been reported in the literature previously. Furthermore, although jasmonates have been implicated in senescence (Parthier 1990), there appears to be no studies on jasmonate levels in a senescing system over time. Moreover the roles of dihydrojasmonic acid and cucurbic acid in wounding and/or senescence is not well understood.

Creelman et al. (1992) showed that jasmonic acid and methyl jasmonate levels in soybean hypocotyls increased to 1900 pmol JA.g^{-1} FW and 860 MeJA pmol.g^{-1} FW in the 12 h following wounding. Clarke (1996) showed that jasmonic acid levels rose to 150 pmol.g^{-1} FW 12 h after wounding in both water- and white clover mosaic virus-treated bean leaves. By 24 h after wounding jasmonic acid had again fallen to basal levels. Jasmonic acid levels again rose only in virus inoculated leaves and by day five had peaked at 180 pmol JA.g^{-1} FW. Conconi et al. (1996) showed that jasmonic acid levels in tomato leaves rose in response to wounding and this increase was correlated with a rise in linolenic acid. They also showed that levels of jasmonic acid peaked at 400 pmol.g^{-1} DW (ca. 40 pmol.g^{-1} FW) just one hour following wounding and dropped rapidly to basal levels by 24 h post-wounding.

These reports are consistent with the amounts of jasmonate detected in asparagus spears. In all spears treated with water or subjected to dry storage jasmonic acid levels rose within the first 24 h after wounding at harvest in the tips, mid sections and bases of spears. The rise in levels of jasmonic acid in the bases of spears preceded the initial increase in jasmonic acid concentration in the mid sections and tips of spears. The levels of the other jasmonates quantified, CA, DJA and MeJA also fluctuated in the same manner. Furthermore, the initial rise in jasmonic acid levels occurred in the tips of spears treated with water 3 h sooner than the peak is detected in the tips of dry stored spears. This would suggest jasmonic acid produced by wounding at harvest may be translocated up the spear towards the tip. However, there appear to be no reports of jasmonate movement within plants. Feeding experiments using radiolabelled jasmonate standards could be used to follow the movement of jasmonates in plants. However, this could be a difficult study to undertake as we found exogenous jasmonic acid was probably metabolised rapidly after application, since the concentration of jasmonates detected in spear tips (Table 3.1) during this study did not increase upon feeding with jasmonic acid. Non-polar substances are frequently glycosylated in plants to make them more polar to facilitate transport and phloem loading. Helder et al. (1993) suggested that jasmonates might become glycosylated just before phloem loading. Such glycosylated jasmonates may not have been detected in this thesis because the antibody used may not have cross reacted with these forms.

While it is accepted that jasmonate levels rise in response to wounding (Creelman et al 1992; Conconi et al 1996; Clarke 1996), jasmonate levels may also rise in response to water stress. Xin et al. (1996) reported that endogenous jasmonic acid levels rose in both roots and shoots of maize plantlets in response to water stress. Jasmonic acid levels rose from 1.4 to 5.3 nmol JA $.g^{-1}$ DW in roots and from 1.1 to 4.2 nmol JA $.g^{-1}$ DW in shoots of maize plantlets 13 h after water-stress induction. These results are supportive of those presented in this thesis. In most cases (with exception of methyl jasmonate) jasmonate levels rose to higher levels in the dry stored spears than the water-treated spears within the first 24 h after harvest. Furthermore, jasmonate levels only rose in dry-treated spears between 7 and 10 d after harvest. These data suggest that the longer-term increase of jasmonate levels in asparagus spears may also be in response to water stress.

Jasmonates exert inhibitory effects on auxin-induced cell elongation (Miyamoto et al. 1997). These authors suggested that jasmonates exert their inhibitory effects on cell elongation by affecting the metabolism of cell wall polysaccharides but only in monocotyledons. In this study, in both dry- and wet-treated spears, jasmonate levels were high at the time of harvest in mid sections and bases. All mid sections and bases were below the zone of cell elongation, which stops 70-75 mm below the apex of the spear (Ross Lill, personal communication). This suggests that jasmonates may play a role in cessation of cell elongation in non-apical regions of the spear. Furthermore, jasmonate levels rose again after the initial wound-induced increase 30-36 h after harvest in both wet-and dry-treated spear tips. Spears continue to grow for the first 24-48 h after harvest, and cell elongation then stops. The increase in jasmonate levels 30-36 h after harvest thus may inhibit cell elongation in spear tips after harvest.

In this study a third rise in JA, DJA, CA and MeJA occurred from seven days after harvest in dry-treated spear tips only. Conconi et al. (1996) demonstrated that linolenic acid produced by deteriorating membranes was quickly converted to JA and DJA. Jasmonates have been reported to induce senescence (Meyer et al. 1984; Dathe et al. 1991). The data presented here suggest that jasmonates could trigger the early signals involved with harvest-induced "senescence" in asparagus spears. Although

jasmonates do increase in concentration in spears late after harvest, this increase is probably a consequence of senescence rather than being a direct cause of senescence, as many of the deteriorative processes precede this accumulation of jasmonate seven days after harvest.

Natural foliar senescence, though, maybe somewhat different. Levels of JA and CA started to increase early during natural foliar senescence (Figure 3.15). This increase in JA and CA coincided with a reduction in DJA concentration. Mid way through senescence DJA and JA levels increased and in the later stages DJA and JA levels decreased as CA levels increased dramatically. This suggests that JA and DJA are metabolised into CA during the later stages of senescence. The fluctuation of jasmonate concentrations during natural foliar senescence appears to be much more ordered than during the harvest-induced "senescence" of the spear. The spear deteriorates rapidly after harvest and may not be developmentally mature enough to control jasmonate production as seen in the naturally senescing fern.

Spears treated with exogenous jasmonic acid did not accumulate jasmonates until five days after harvest (this increase is only minimal), although jasmonates may have been present one day after harvest but were not detected. Jasmonates may have been lost from this sample during purification as the recovery of the internal standard was low. Jasmonic acid levels rose in tips one day later in cytokinin-treated spears than in water-treated spears. This suggests that treatment with cytokinin reduces (for a short time) the stresses imposed on the spear at harvest. A possible mode of action in this case may be the capability of cytokinins to maintain membrane integrity (Grossman and Lesham 1978). If no membrane deterioration occurs within the first 24 h after harvest, no linolenic acid is produced, thus the biosynthetic pathway for jasmonic acid is blocked, and therefore no jasmonic acid is present in spear tips immediately following harvest.

The identities of the compounds tentatively ascribed to jasmonic acid, dihydrojasmonic acid and cucurbic acid were confirmed by E/S mass spectrometry (Tables 3.2, 3.3). The presence of methyl jasmonate was not confirmed by E/S mass spectrometry, possibly because it was only detected at low concentrations in the

ELISA. A novel jasmonate was also present in samples, whose chromatographic properties differed from any of the standards available (Fig. 3.10C).

It is unlikely that this novel compound is a jasmonate glucoside as glycosylation makes these jasmonates more polar (Helder et al. 1993). It would thus have eluted with an earlier retention time during HPLC separation. It is probable that this novel compound is a hydrophobic amino acid conjugate. Schmidt et al. (1995) synthesised amino acid conjugates of jasmonic acid and investigated their ion fragmentation pattern by electrospray MS/MS. One of these conjugates, tryptophan-jasmonic acid, fragmented in a similar way to the novel jasmonate in this work, although has a molecular weight two hydrogen ions lower. Since amino acid conjugates of dihydrojasmonic acid have been reported previously (Parthier et al. 1992), but not amino acid conjugates of cucurbic acid, we propose that this novel jasmonate compound is a tryptophan-dihydrojasmonic acid conjugate. Also since cucurbic acid eluted during HPLC showing two peaks, it is probable that any cross-reactive conjugate of cucurbic acid would also elute in two peaks.

4.3 Summary and conclusion

In summary exogenous cytokinin treatment of spears after harvest led to a modest extension of shelf-life and slightly reduced tiprot, although it had no effect on chlorophyll, soluble sugar and asparagine contents in tips of spears. Exogenous jasmonic acid treatment of spears after harvest caused spears to deteriorate faster after harvest and increased tiprot, although it had no effect on chlorophyll, soluble sugar and asparagine contents in tips of spears.

Spears of asparagus responded to wounding which occurred at harvest by production of jasmonate with a typical wound signal as reported before in the literature, although how these jasmonates are metabolised is unclear. Jasmonates (produced by wounding at harvest) may be translocated in an upward direction to the tip of the spear. The data also suggest that asparagus may respond to water stress by production of jasmonates, and that jasmonates may be involved with cessation of cell elongation by affecting the metabolism of cell wall polysaccharides. Jasmonate production and

metabolism appears to be more ordered during natural foliar senescence than during the harvest or wound-induced "senescence" of the spear. It appears that jasmonic acid and dihydrojasmonic acid are metabolised to cucurbic acid during the later stages of natural foliar senescence. Jasmonates were confirmed by E/S mass spectrometry, one of these was a novel form not reported in the lierature previously.

There are only modest effects of cytokinin and jasmonic acid treatment on spears, so there may be little value in further pursuing this line of research for methods to reduce postharvest deterioration. The future may lie with improved postharvest handling and packaging technologies, such as controlled atmosperes reported to extend shelf-life (Lill et al. 1998), or with genetic manipulation of genes controlling the metabolic processes associated with senescence (King et al. 1993).

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Appendix I

COMMONLY USED BUFFERS

A. Phosphate buffered saline (PBS)

8 mM Na_2HPO_4

0.14 M NaCl

1.4 mM KH_2PO_4

2.6 mM KCl

0.2 % NaN_3

B. ELISA buffer

PBS buffer containing: 2% Polyvinylpyrrolidone

0.2% Bovine serum albumin

0.01% Tween20

C. PBSTween20 (PBST)

PBS buffer containing: 0.01% Tween20

D. Coating buffer

15 mM Na_2CO_3

35 mM $NaHCO_3$

0.2% NaN_3

pH 9.6

E. Substrate buffer

0.92 M Diethanolamine

pH 9.8 (using HCl)

Appendix II

HPLC GRADIENTS

1. C_{18} HPLC (for jasmonates)

Time	Flow rate	%A	%B	%C	%D
initial	1.00	60	32	8	0
7.00	1.00	45	47	8	0
11.00	1.00	40	52	8	0
16.00	1.00	20	72	8	0
18.00	1.00	0	0	100	0
28.00	1.00	0	0	100	0
32.00	1.00	60	32	8	0

Column: Altex 5 µm, 250 x 4.6 mm, Beckman

Solvents:		
	A	0.05% trifluoroacetic acid in MilliQ water
	B	0.05% trifluoroacetic acid in acetonitrile (HPLC grade)
	C	Methanol (HPLC grade)
	D	Spare

2. C_{18} HPLC (for amino acids)

Minute	Flow rate	%A	%B	%C	%D
initial	1.00	0	90	10	0
1.00	1.00	0	65	35	0
13.00	1.00	0	60	40	0
14.00	1.00	5	0	95	0
19.00	1.00	5	0	95	0
20.00	1.00	0	90	10	0

Column: Phenomenex Prodigy 5 µm ODS (3) 100A (150 x 4.6 mm)

Solvents:		
	A	MilliQ
	B	50 mM Phosphate buffer
	C	Methanol (HPLC grade)
	D	Spare

Appendix III

CHROMATOGRAPHY PREPARATION

A. Preparation of PVPP for jasmonate purification

A bulk quantity of polyvinylpolypyrrolidone (PVPP) was prepared. PVPP was suspended in 0.1 M sodium phosphate buffer (pH 6.5) and stored at 4°C overnight. The PVPP was then washed with 0.1 M sodium phosphate buffer until the pH was stable at 6.5.

B. Preparation of DEAE cellulose (DE52) for jasmonate purification

A bulk quantity of diethylaminoethyl cellulose (Whatman DE52) was prepared. DE52 was suspended in 0.1 M sodium phosphate buffer (pH 6.5) and stored at 4°C overnight. The DE52 was then washed with 0.1 M sodium phosphate buffer until the pH was stable at 6.5.

C. Preparation of octadecyl silica C_{18} for jasmonate purification

Dry C_{18} (Varian Analytichem Bondesil 40 µm preparative grade) was packed into a 5 ml polypropylene syringe to a final column volume (cv) of 3 ml. On the day of use the dry C_{18} was wet with methanol and then washed with 20 cv of HPLC grade methanol, and then 20 cv of 0.2% acetic acid (HPLC grade).

Appendix IV

CROSS-REACTIVITY OF JASMONATES

The cross-reactivity of the anti-JA antibody used in this work, with various jasmonates are outlined in the table below.

The work to determine these values was carried out by Dr. S. Clarke.

Table 1 Cross-reactivity of anti-JA antibody with various jasmonates and other plant hormones.

Compound	**% Cross-reactivity**
Methyl jasmonate	123
Jasmonic acid	100
Dihydrojasmonic acid	42.8
Cucurbic acid	7.5
Dihydrocucurbic acid	0
Abscisic acid	0
Gibberellic acid	0
Dihydrozeatin	0
Dihydrozeatin riboside	0
Indole-3-acetic acid	0

Appendix V

QUANTIFICATION OF ASPARAGINE

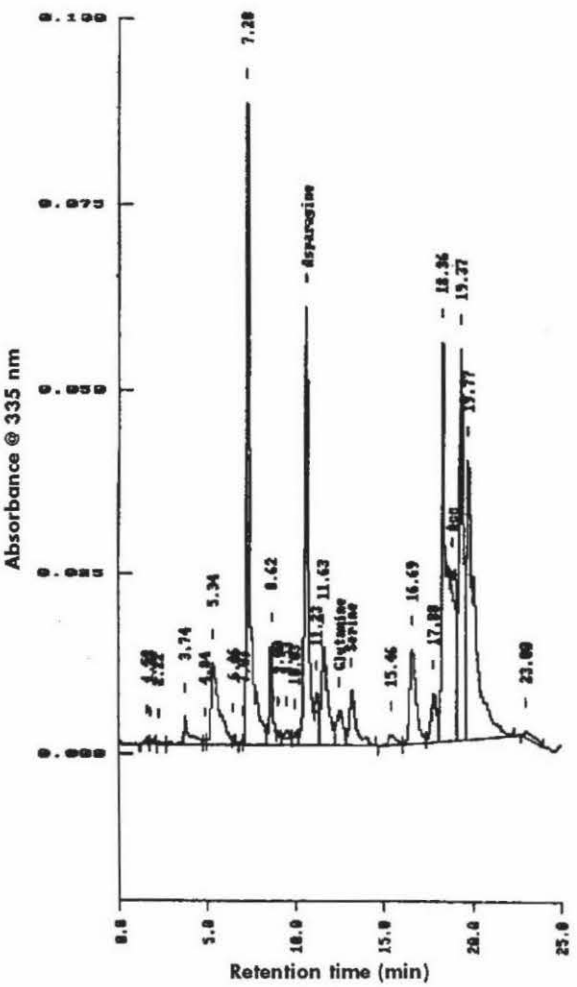


Figure 1 HPLC separation profile of amino acids. HPLC separation profiles of amino acids quantified using pre-column derivatisation from dry untreated control spear tips 2 days after harvest. Amino acids eluted in the following order: asparagine (10.6 min), glutamine (12.6 min), serine (13.3 min) and ACC (18.9 min). Standard curves were made for asparagine (see below) and all had an R^2 of no less than 0.998.

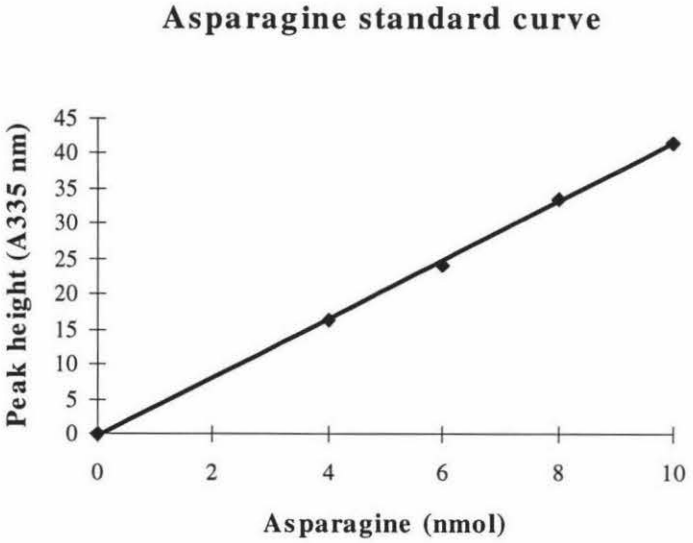
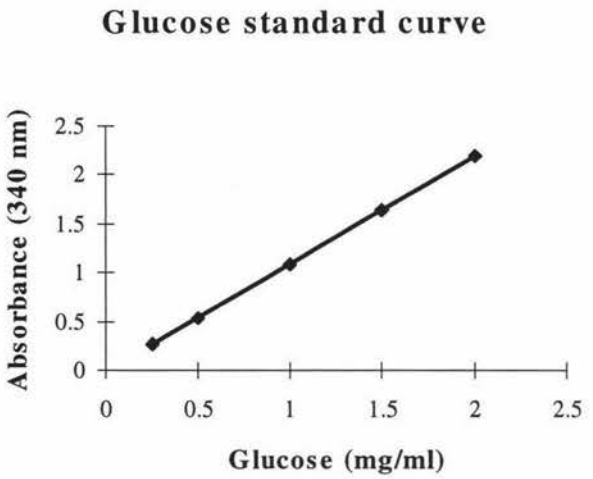


Figure 2 Standard curve for asparagine.

Appendix VI

STANDARD CURVES FOR SOLUBLE SUGARS



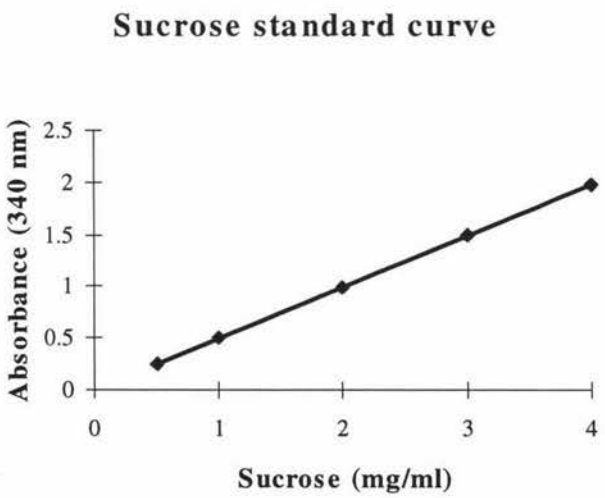


Figure 3 Standard curves for soluble sugars.

All glucose and sucrose standard curves used in this thesis had values for R^2 no less than 0.9988.