The plant world is not colored green...

it is colored morphine, caffeine, tannin, phenol, terpene, canavanine, latex, phytohaem-agglutinin, oxalic acid, saponin, L-dopa, etc...

Jansen, 1978

NOVEL INDUCIBLE PHYTOCHEMICAL DEFENCES AGAINST PLANT PARASITIC NEMATODES

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SUMMARY

The insect moulting hormone, 20-hydroxyecdysone (20E), is inducible in spinach and has been demonstrated to provide defence against insect herbivory. It is not known if such phytoecdysteroids are inducible by and defensive against plant parasitic nematodes. However, given that insects and nematodes belong to the same clade, the ecdysozoa, this is possible. Therefore, plants were tested for the presence of inducible phytoecdysteroids and effects on the nematodes tested.

Induction of possible defence compounds in common cultivars of spinach, two *Briza* spp., *B. maxima* and *B. minor*, oats and lucerne cultivars with varying degree of resistance to stem nematode, *Ditylenchus dipsaci*, was undertaken by treatment with methyl jasmonate and by challenging plants with nematodes. The influence of nematode inoculum density on the induction of phytoecdysteroid was also assessed in spinach. In addition, the relationship between the levels of inducible compounds and resistance response of lucerne cultivars to the stem nematode were evaluated.

Treatment with methyl jasmonate induced methanol extractable compounds in all plants tested. *Pratylenchus neglectus* induced the same compounds at levels equivalent to methyl jasmonate induction in all plants except lucerne, which was not tested. An inoculum rate of 500 to 10,000 *P. neglectus* induced similar levels of phytoecdysteroids in spinach. *Heterodera schachtii* induced phytoecdysteroids in both roots and shoots of spinach. *H. avenae* induced methanol extractable compounds in the roots of *B. minor* and shoot and roots of oats. *Meloidogyne javanica* was only found to increase levels of phytoecdysteroids in the shoots of spinach. Among the plants inoculated with the stem nematode, induced compounds were detected only in some lucerne cultivars resistant to the nematode.

The methanol extracts from the induced plants were further tested for biological activity using *Drosophila melanogaster* B_{II} cell microplate-based bioassay to screen and

detect biologically active ecdysteroids. The extracts were subjected to mass spectrometry to confirm the presence of ecdysteroids. The biological and chemical characterisation of the inducible compounds in the plants tested provided evidence that spinach, *Briza* spp. and lucerne contained the ecdysteroids 20E and polypodine B, which were biologically active based on the B_{II} cell bioassay, except for lucerne. Lucerne shoots appeared to contain compounds or conjugate groups that inhibit ecdysteroids. In addition to the ecdysteroids above, *B. maxima* also contained ecdysone. On the other hand, inducible flavonoids were observed in the shoots and roots of oats.

Two plant parasitic nematodes, *P. neglectus* and *Anguina tritici*, were examined for the presence of similar ecdysteroids induced in the plants tested. This information will corroborate the involvement of these compounds in plant defence against nematodes. Based on HPLC and mass spectrometry data, both nematodes did not contain the ecdysteroids induced in the plants. However, compounds with masses similar to 20,26-dihydroxyecdysone, 20,26-dihydroxyecdysone 22-acetate, makisterone A, and possibly an unreported ecdysteroid were observed in *P. neglectus*. No ecdysteroid was observed in *A. tritici,* which consisted only of second stage juveniles in the anhydrobiotic survival state as opposed to the presence of all stages of an actively developing population of *P. neglectus*.

In order to establish that ecdysteroids are potential defence compounds against parasitic nematodes, the effects of direct application of 20E on nematodes was assessed by treating cereal cyst nematode, *H. avenae*, juveniles with concentrations of 20E from 8.2×10^{-8} to 5.2×10^{-5} M before applying to wheat. *H. avenae*, *H. schachtii*, *M. javanica* and *P. neglectus* were treated with 5.2×10^{-5} 20E and incubated in moist sand. To test the protective effects of 20E in plants, *H. schachtii* and *H. avenae* were applied to spinach and quaking grass, respectively, and the latter two nematodes in both plants, in which elevated concentrations of 20E had been induced by methyl jasmonate. Abnormal moulting, immobility, reduced invasion, impaired development and death occurred in nematodes

exposed to 20E either directly at concentration above $4.2x10^{-7}$ M or in plants. Phytoecdysteroid induction apparently protected spinach and *B. maxima* from plant parasitic nematodes and may confer a mechanism for nematode resistance.

Green manure is an alternative option to deliver the defence compound, as high constitutive production in a crop plant might impose unacceptable metabolic cost. Induced spinach when applied as green manure suppressed invasion of *H. avenae* in wheat but the direct involvement of 20E was not established because of the highly toxic effects of the treatment on the nematode.

Three inducible compounds, isolated in methanolic root and shoot extracts of oats were identified as flavone-*C*-glycosides by mass spectrometry. The effect of the flavone-*C*-glycosides on the invasion by and development of cereal cyst nematode, *H. avenae,* was assessed using methanolic extracts of shoots and roots from methyl jasmonate treated plants. Both extracts impaired nematode invasion and development. When the extracts were fractionated by high voltage paper electrophoresis, only one flavone-*C*-glycoside, *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside, inhibited nematode invasion. The protective effect of the induction of flavone-*C*-glycosides in oats by methyl jasmonate was evaluated against *H. avenae* and *P. neglectus*. Treatment with methyl jasmonate reduced invasion of both nematodes and increased plant mass, compensating for damage caused by the nematodes, and is attributed to the active flavone-*C*-glycoside. The active compound, *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside, has not been implicated previously in plant defense against any pest or pathogen, and appears to provide protection against the major cereal nematodes *Heterodera* and *Pratylenchus*.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institute and, to the best of my knowledge and belief, contains no material previously published or written by any other person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Signed: Date: 28 October 2004

Imelda Rizalina Soriano

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CHAPTER 1. INTRODUCTION

Plant parasitic nematodes are economically damaging pests of global significance to agriculture (Whitehead, 1998). Their impact on yield losses has been estimated to be over US$78 billion annually for the major food and fibre crops (Barker *et al.*, 1994). Most of the damage is caused by a relatively small number of nematode genera that attack crops (Nickle, 1991), principally the sedentary root-knot (*Meloidogyne* spp.) and cyst (*Globodera* and *Heterodera* spp.) nematode, and also the migratory nematodes *Pratylenchus* and *Radopholus* spp. (Bird and Koltai, 2000). In 1982, 50 thousand tonnes of nematicide active ingredient were applied to crops in the United States, at a cost exceeding US$1 billion (Landels, 1989). In the Netherlands, nematicide application from 1986-1990 was more than three times the combined total of chemicals needed to control insects, fungi and weeds (Lewis *et al.*, 1997). However, chemical control is becoming increasingly proscribed, due to its detrimental effects on the environment and human health. Furthermore, in many countries, effective nematicides have been and continue to be deregistered (Thomason, 1987). Cultural practices such as crop rotation and application of organic amendments, and the use of resistant cultivars, when available, are currently popular nematode control strategies. However, crop rotation restricts the farmers because of economic returns and offers very few choices against some nematodes with broad host range. Natural resistance tends to fail if it has high parasite specificity, which provides no protection against new virulent nematode populations. These limitations justify the introduction of a novel control or resistance strategy against parasitic nematodes.

Since plants have efficient defence mechanisms for preventing parasitism and predation or at least limiting their effects despite continual exposure to a wide range of pests and pathogens, phytochemical-based strategies for control of nematodes has been an attractive option. Plant-nematode interaction is mediated by complex chemical interactions and a major offshoot of this interaction is the production of defence

compounds by plants. Plant defence may be in the form of structural or chemical barriers (Lucas, 1998), which could either be constitutive or induced (Hammerschmidt and Kuc, 1995; Deverall, 1976; Karban and Meyers, 1989). Considerable research has focused on exploiting the chemical defence strategies of plants, specifically the defensive nature of the extensive array of secondary compounds produced by plants.

Studies of plant secondary metabolites are often directed towards a specific biochemical target site within the pest for a strategic defence. For instance, in insects (Goosey, 1992), ecdysteroids are compounds present in all stages of development and regulate many developmental biochemical and physiological processes. Thus, the ecdysteroid receptor site has been targeted as a possible avenue for defence. Ecdysteroids are steroid hormones of all classes of arthropods and probably of other invertebrates (Koolman, 1989). Active analogues of the insect moulting hormone were detected in plants (Nakanishi, *et al.*, 1966; Galbraith and Horne, 1966) soon after its structure was described in insects (Hüber and Hoppe, 1965). These identical or analogous structures to the insect ecdysteroids, termed phytoecdysteroids, are widely distributed in plants (Lafont, 1997; Dinan, 2001).

Numerous findings support the hypothesis that phytoecdysteroids confer resistance against non-adapted phytophagous insects through disruption of the moulting cycle (Robbins *et al.*, 1970; Singh and Russell, 1980; Kubo, *et al.*, 1983; Lafont *et al.*, 1991; Dinan, 2001). Another indicator of the defensive nature of phytoecdysteroids was the rapid phytochemical induction of 20-hydroxyecdysone, an insect moulting hormone, in the roots of spinach following insect herbivory, mechanical damage or application of a signal molecule, jasmonic acid (Schmelz *et al.*, 1998, 1999). Many plant defence chemicals are induced by herbivory (Creelman *et al.*, 1992; Creelman and Mullet, 1995; Karban and Baldwin, 1997; Creelman and Mullet, 1997) which is a reaction induced by plant-derived wound signals arising from the site of damage (Pearce *et al.*, 1991; Davies *et al.*, 1991;

Baldwin *et al.*, 1997).

As insects and nematodes belong to the Ecdysozoa clade (Aguinaldo *et al.*, 1997), the likelihood that plant parasitic nematodes and insects possess the same ecdysteroid hormone system, but with substantially different organization and concentration, allows exploration as a basis for defence strategies against plant parasitic nematodes. This concept has not yet been explored for the nematode-plant system, despite the fact that nematodes undergo moulting in a similar way to insects.

This research is directed towards investigating the potential of phytoecdysteroids to defend plants against parasitic nematodes. No study has yet demonstrated the effective control of plant parasitic nematodes using the ecdysteroid system in plants as has been done in arthropods. The demonstration that ecdysteroids occur in nematodes, coupled with the possibility that these compounds may be essential regulators or hormones in their development, suggests that it may be possible to use this steroid system for control of these parasites. One advantage of pursuing this strategy for control is that it may be more difficult for nematodes to develop resistance to a phytochemical acting at the same site and in the same mode as one of their own natural hormones.

In the plant survey for inducible phytoecdysteroids, inducible compounds in oats were detected using the same system used for ecdysteroid elution. The properties of the compounds represent a strong indication of a flavone glycoside. Flavonoids, having both primary and secondary functions in plants (Croteau *et al.*, 2000), comprise a big group of compounds with a great influence in many plant-animal interactions. Various flavonoids have been studied extensively from the perspective of plant defence. Being inducible novel compounds implicated in defence in oats, it is worthwhile characterising these compounds, which may also occur in other major cereals.

Therefore, the project was undertaken to investigate the potential of phytoecdysteroids and flavone glycoside to provide defence against or resistance to plant

parasitic nematodes, with the following specific objectives:

- To determine the inducibility of possible plant defense compounds in response to invasion by parasitic nematodes with different feeding behavior and to application of an elicitor, methyl jasmonate.
- To detect the presence and identify phytoecdysteroid induced in plants, based on *Drosophila melanogaster* B_{II} cell bioassay and by chemical analysis.
- To determine the presence of ecdysteroids in plant parasitic nematodes.
- To examine the effects of the ecdysteroids 20E, polypodine B and ecdysone on migratory and sedentary endoparasitic nematodes in genera that are responsible for most economic damage, by direct application and through exposure to plants.
- To determine the effects of the inducible compounds in oats on the invasion by and development of cereal cyst nematode in wheat, by direct application of the methanolic extracts of oats and semi-purified compounds.
- To evaluate the protective effects of the inducible compounds in oats against nematodes *in planta*.

CHAPTER 2. REVIEW OF LITERATURE

2.1 INTRODUCTION

Plant parasitic nematodes have been recognised as agricultural pests over the last fifty years (Whitehead, 1998). Research on control of these parasites has increased greatly over time and the literature generated is extensive. This review discusses the potential of novel forms of nematode control using the plant ecdysteroids and inducible flavone glycosides. The first part of the review covers the importance of controlling nematode pests and a brief review of some recognized plant defences against nematodes. The second part discusses the existence of ecdysteroids in plants and their biological effects on arthropods. Studies on the occurrence and biological activity of ecdysteroids in nematodes are then presented. The last part presents a brief review of flavonoids and their involvement in plant defence against nematodes.

2.2 NEMATODES AS PESTS

Plant parasitic nematodes occur diversely and are associated with almost all plant species in cultivated and natural ecosystems. Plant parasitic nematodes represent one of the important groups of pests that affect the growth and yield of crop plants. Damage caused by plant nematodes is estimated to be one third of the overall losses attributed to pests and diseases, which is 10% of world crop production (Whitehead, 1998). Losses due to parasitic nematodes are estimated to be $78 billion per year for the major food and fibre crops worldwide and $8 billion per year in the USA (Barker *et al.*, 1994). Species of more than 20 genera of nematodes cause plant disease. Yield losses due to nematodes vary greatly from 10-50% in economically important crops worldwide (Sasser and Freckman, 1987; McSorley, 1987). This shows that plant parasitic nematodes are potential pests that could cause severe financial losses to farmers if not controlled.

2.3 NEMATODE CONTROL

To prevent unacceptable yield losses for a productive and sustainable agriculture, efforts are made to manage and control nematodes. A wide range of nematode management and control strategies to limit crop losses have been studied and adopted, such as cultural and land management practices, biological and chemical control measures, and exclusion (Whitehead, 1998). Among the control measures, the use of resistant cultivars has been widely adopted due to environmental concerns and its cost effectiveness. Host-plant resistance has been a major area of agricultural research for developing nematode management programs (Atkinson, *et al.*, 1994; Opperman *et al.*, 1994; Cai *et al.*, 1997). Studies of resistance responses have already identified a wide range of proteins, enzymes and genes suitable for detailed analysis of targeted signals. However, there is no study yet that convincingly demonstrates the mechanisms involved in resistance to nematodes. Basic information on host-nematode interaction may help in understanding the underlying mechanism(s) and will aid in the practical application of plant defences against nematodes.

2.4 PLANT DEFENCES AGAINST NEMATODES

Plants have evolved and developed defence mechanisms as a result of their constant exposure to pests and pathogens. Plant defence may be in the form of structural barriers that inhibit invasion or development of a pest in the plant, or biochemical reactions that occur in the cells or tissues of the plant producing substances, which either inhibit development or are toxic to the pest (Agrios, 1997). However, these categories are not mutually exclusive. For instance, some structural defences result from initial biochemical responses of the plants such as the deposition of calloses or formation of tyloses (Lucas, 1998).

Natural defences that protect plants against nematodes may result from compounds always present in plant tissues (constitutive) or compounds that are synthesized in response

to plant invasion (inducible). Both structural and biochemical forms of defences can be constitutive or inducible (Lucas, 1998). Pre-existing chemical defences may be in the form of inhibitors such as toxins, enzymes, anti-feedants, repellents and hormones or the lack of essential factors such as the recognition factors, host receptor sites and essential substances (Lucas, 1998; Gheysen, 1998). Some examples of induced chemical barriers are hypersensitive response, reinforcement of cell wall, detoxification of the pests' toxins and the presence of inhibitors (Lucas, 1998; Gheysen, 1998; Agrios, 1997).

In plants, a biochemical basis for plant resistance to nematodes is likely to be more common than structural defence (Zacheo *et al.*, 1997). In general, resistance seems to depend on many biochemical components. The elucidation of biochemical mechanisms in the plants' ability to resist nematode infection is crucial to the understanding and control of plant disease caused by nematode parasites.

2.4.1 HYPERSENSITIVE RESPONSE

Hypersensitive response (HR), one of the several possible plant defence mechanisms against nematodes, is the earliest mechanism for resistance described by Christie in 1949. It involves rapid metabolic changes and localized cell necrosis in tissue at the site of invasion by the nematode. Resistance, in this case, is associated with necrosis in which a limited number of plant cells in direct contact with the invading nematode dies rapidly. Hence, further growth and development of the nematode is prevented (Christie, 1949). However, Kiràly *et al.* (1972) claims that HR is a consequence and not the cause of plant resistance to infection. Induction of secondary metabolites such as phytoalexins, oxidized phenolics, lipid peroxides, hydrolytic enzymes and proteinase inhibitors that are toxic to the parasite is associated with the plant's necrotic reactions to nematodes (Zacheo *et al.*, 1997). An increase in the polyphenol oxidase and peroxidase, enzymes involved in the production of lignin and suberin and phenol metabolism, was observed in plant tissues

undergoing a HR (Lucas, 1998; Zacheo *et al.*, 1997). Hence, formation of structural barriers such as lignification and suberization, which prevents nematode invasion of plants, is also associated with HR. In addition, several enzyme systems such as NADPH-oxidases, peroxidases and lipoxygenases were reported to cause cell death similar to HR or induces lignification (Adam *et al.*, 1993; Baker and Orlandi, 1995).

2.4.2 PREFORMED TOXINS AND INHIBITORS

Plants with nematicidal or nematostatic properties are often detected because of their marked suppression of nematode population in the field (Zacheo *et al.*, 1997). A comprehensive review of the nematicidal compounds in plants has been published (Gommers, 1981, Chitwood, 1993; 2002). A list of over 100 plant species that includes the specific nematode antagonism and type of bioassay employed was consolidated by Akhtar and Mahmood (1994). It has yet to be determined if these constitutive nematicidal compounds confer resistance to nematodes (Gommers, 1981). However, Kaplan and Keen (1980) postulated that the resistance mechanism to a nematode occurs after infection. Hence, an active mechanism involves compounds produced after infection rather than being preformed plant products.

2.4.3 INDUCIBLE INHIBITORS

In plant-nematode interactions, several secondary metabolites have been shown to be induced in plants after nematode invasion (Zacheo and Bleve-Zacheo, 1995). The synthesis of low moleculer weight antibiotics, production of hydrolytic enzymes, rapid modification of existing cell walls are examples of inducible responses that may confer resistance (Cramer *et al.*, 1993).

Studies have also shown that the treatment of chemical agents or limited infection increases resistance to nematodes due to a systemic accumulation of phytoalexins and proteins (Ogallo and McClure, 1995; 1996).

Phytoalexins

Phytoalexins are low molecular weight plant antibiotics and their accumulation is determined by the release from conjugate precursors (Kuc, 1995). Many phytoalexins are flavonoids and these will be discussed in detail later in this chapter. Their action against nematodes and accumulation in host tissues has been reported by Rich *et al.* (1977). Evidence of the anti-nematode effect of the phytoalexin, glyceollin, has been presented and its possible mechanism for resistance suggested (Huang and Barker, 1991; Kaplan and Keen, 1980). Furthermore, the rapidity and magnitude with which phytoalexins are produced was considered important in nematode resistance. Despite the many studies on the role of phytoalexins in nematode resistance, knowledge of the action of phytoalexins and convincing link to resistance is still required.

Proteinase inhibitors

Proteinase inhibitors are widely distributed in plants and may have a protective function against herbivores, fungi and bacteria (Zacheo *et al.*, 1997). Information about the functions of proteolytic enzymes in plant parasitic nematodes is limited. Proteinases present throughout the development of plant parasitic nematodes were hypothesized to be involved in the pathogenic processes (Hepher and Atkinson, 1992; Koritsas and Atkinson, 1994; Perry *et al.*, 1992). It was reported that proteinase inhibitors serve as a protectant in potato against the nematodes, *Globodera pallida* and *Meloidogyne incognita* (Hepher and Atkinson, 1992). In addition, studies have assessed the potential of cystein proteinase inhibitors for the control of the nematode, *Meloidogyne hapla* (Vrain *et al.*, 1995).

However, the importance of these proteins for nematode resistance remains to be demonstrated.

Pathogenesis related proteins

Pathogenesis related proteins are plant proteins that are induced in response to pathological or related environmental challenge and are associated with necrotic reactions (Elad and Evensen, 1995; and Van Loon, 1999). Their application against pathogens has been well documented (Cutt and Klessig, 1992; Bowles, 1990; Malehorn *et al.*, 1993; Sela-Buurlage *et al.*, 1993). The direct involvement of pathogenesis related proteins in nematode control remains to be demonstrated. A study demonstrated the accummulation of pathogenesis related proteins similar to the polypeptides reported in other plant pathogen infection in the apoplast of potato leaves infected with *Globodera rostochinensis* in roots (Hammond-Kosack, 1989; Rahimi *et al.*, 1995). Chitinase and β-1,β glucanases, two pathogenesis related proteins, increased significantly in roots and leaves, respectively, of *G. pallida* infected potato cultivar (Rahimi *et al.*, 1995).

Phytohormones

It has been postulated that phytohormones, such as the growth regulators are involved in plant-nematode interaction (Giebel, 1974). An example of such is the increase in levels of ethylene and abscisic acid upon nematode invasion (Volkmar, 1991). In addition, there are several reports of the presence of indole compounds in nematode - induced galls, which were suggested to have come from the parasitising nematode and regulated by the host (Bird, 1962; Yu and Viglierchio, 1964; Viglierchio and Yu, 1968; Balasubramanian and Rangarwami, 1962). This claim was supported by the presence of auxins in the secretions of nematode enzymes that release free auxins from conjugates in plant roots (Giebel *et al.*, 1966). However, other observations suggested that the higher

auxin levels in galls might rather be the result of the retention of auxins due to the inhibition of transport of auxin to other parts of the plant (Vercauteren et al, 1995). There is a differential sensitivity in different plant-nematode interactions to specific auxins (Gheysen, 1998), for example, indoleacetic acid was detected in galls induced by *Meloidogyne javanica* but not by *M. incognita* in tomato (Bingefors, 1982). This phenomenon has been linked to plant resistance. Auxin-degrading enzymes have been found in solanaceae cultivars resistant to *G. rostochiensis* (Giebel, 1974) and cotton resistant to *M. incognita* (Bingefors, 1982).

Cytokinin activity was lower in resistant than susceptible tomato cultivars (Van Staden and Dimalla, 1977). When exogenous cytokinin was applied, there was a shift of response from resistant to susceptible. Resistance to *M. javanica* in peach rootstocks was, likewise, correlated with lower cytokinin levels (Kochba and Samish, 1972). However, when 6-benzylamino purine was applied to *M. incognita* resistant peach rootstock, the plant remained resistant (Huettel and Hammerschlag, 1986). Likewise, a mutant of *Arabidopsis thaliana* defective in either the biosynthesis of or response to auxin, abscissic acid, gibberellins and ethylene, was found to be susceptible to *H. schachtii* as well as *M. incognita*. Applying 2,4-dichlorophenoxyacetic acid decreased the resistance of oat but not of barley to *Ditylenchus dipsaci* (Webster, 1967).

Therefore, the role of the major phytohormones in plant defence is not well understood or convincingly established. According to Gheysen (1998), the involvement of phytohormones in the plant-nematode interaction will only be clarified once the molecular triggers that induce nematode-feeding cells are known.

2.5 ECDYSTEROIDS

The process of growth and development in arthropods are controlled primarily by a complex interacting association of three hormone systems namely, the brain, juvenile and

moulting hormones (Camps, 1991). Ecdysteroids are moulting hormones that are derivatives of insect phytosterol (Hikino and Hikino, 1970). Although regulating moulting, these steroids are present in all stages of insect development as well as in adults (Hoffmann *et al.*, 1984). The hormone acts upon the epidermal cells and initiates moulting. However, the kind of moult which results depends on the amount of juvenile hormone present at the same time (Wigglesworth, 1950). Juvenile hormone functions to modify the effect of ecdysteroid and does not affect the larva by itself.

The detection of active analogs in plants after the elucidation of the first insect moulting hormone resulted in advances in isolation and identification of more than 250 ecdysteroids in plants and animals (Koolman, 1989; Lafont and Horn, 1989; Lafont, 1997).

2.5.1 HISTORY

In 1917, Kopeć reported that the removal of the brain of an insect before a critical stage of growth prevented moulting, but that cutting of nerve cord did not yield the same effect. When caterpillars were tied with thread into sections at a time before the critical stage, only the anterior portion pupated. However, all sections pupated when the operation was carried out after the critical period. This led to a conclusion that the brain does not influence the general processes of metamorphosis through the nerves, but that it has the function of an organ of internal secretion. It affects the organism by means of a substance (or substances) which may be supposed to pass into the blood of the caterpillar from the brain at a certain stage of the larvae life (Kopeć, 1917).

The work of Wigglesworth (1934) confirmed the conclusion of Kopeć (1917), which showed that removal of the head of the bug, *Rhodnius*, before a critical period prevented moulting. In addition, he found that the blood of *Rhodnius* that had passed the critical period induced moulting in beheaded insects, which showed that the process was linked by a chemical means. Hanström (1938) suggested after locating neurosecretory cells

in the dorsum of the cerebrum that it was possible for these cells in the brain to be the source of hormone. The *Rhodnius* larvae moulted when this area of the brain was excised and implanted into decapitated larvae (Wigglesworth, 1940). However, in the experiment of Plagge (1938), implantation of brains failed to induce pupation in posterior segments of the ligated *Deilephila* caterpillars. Thus, showing that there are other factors besides the brain necessary for the initiation of pupation.

Fukuda (1940) showed convincingly that only the region of *Bombyx mori* larva that contained the prothoraic gland pupated and isolated abdomens were induced to pupate if the prothoracic glands were implanted into them The brain-prothoracic gland association in insect moulting was confirmed by Williams (1947) who showed that isolated abdomens could be induced to moult if both brain and prothoracic gland were implanted. It was further illustrated that the brain hormone controlled the secretory behaviour of the prothoraic gland.

The classical work of Possompès (1953) demonstrated that the large limb cells of the ring gland in Diptera have the same function as the prothoracic gland in Lepidoptera, which brought about pupation in Diptera. Karlson and Hanser (1953) showed that hormone extracts that were active in the *Calliphora* bioassay were also able to cause pupation in *Calliphora* larvae from which the ring gland had been removed. *Calliphora* pupae were first used as hormone source until later when it was found that silkworm pupae extracts were equally active and available in large quantities. From then on, the first isolation of 25 mg ecdysteroid, now called ecdysone, by Butenandt and Karlson (1954) from almost 500 kg of silkworm pupae has lead to studies on the significance of this hormone.

The discovery of Nakanishi *et al.* (1966) of ecdysone-like compounds in plants was another breakthrough in the ecdysteroid research. A mixture of terpenoids was extracted from *Podocarpus nakaii* during a search for anti-tumor compounds from plants. Although the leaf extract was negative for cytotoxicity assay, the structure of the compound was still

derived and was identified as ponasterone A, which was found to be active in *Calliphora* bioassay as the natural hormone. Nakanishi's discovery of ponasterone A led to several other studies on the extraction of compounds from plants with insect moulting activity.

2.5.2. CHEMISTRY AND STRUCTURE

The chemical structures of ecdysteroids are diverse (Fig. 2.1) and may vary depending on the number of carbon atoms which may range from 19 to 30, the number and the stereochemistry of hydroxyl groups, the presence of keto groups, the stereochemistry at C-5 (α/β), and the conjugation of different alcohol groups with either polar (phosphates, sulfates, sugars) or non-polar (acetates, glycolates, fatty acid esters, benzoates, cinnamates, coumarates etc.) moieties (Lafont, 1997). The side chain may have a 5-or 6-membered lactone ring involving a 26- (or 29-) COOH and a hydroxyl group in position 28 (or 22). The methyl groups at C-10 and C-13 of phytoecdysteroids have a β-configuration. The B/C- and C/D-ring junctions are always *trans*. The A/B-ring junction is normally *cis* (5β-H), and only rarely *trans* (5α-H) (Lafont, 1998; Dinan, 2001). The chemical structures of the major or common phytoecdysteroids; 20E, polypodine B and ecdysone, are presented in Fig. 2.2. These are also found in arthropods.

The diversity of ecdysteroids especially the ones isolated from plants has raised problems of defining precise limits to the ecdysteroid family (Lafont and Horn, 1989). Initially, ecdysteroids were defined as all compounds structurally related to ecdysone (Goodwin *et al.*, 1977). To distinguish between true ecdysteroids and ecdysteroid-related compounds, Lafont (1997) described ecdysteroids as cholesterol-derived molecules that are polyhydroxylated steroids bearing an A/B ring *cis* (5β) junction, a 7-ene-6-one chromophore and a 14α-OH moiety regardless of the biological activity in a moulting hormone assay. Some phytoecdysteroids are derived from phytosterols and thus possess a C28 or C29 skeleton with an alkyl group at C-24 (Lafont, 1997, 1998; Dinan, 2001).

Compounds which do not meet these criteria such as if they lack 14α-OH, or the 7-ene, or they have an additional 4- or 14-ene double bond etc. are arbitrarily considered ecdysteroid-related compounds (Lafont and Horn, 1989). However, ecdysteroid-related compounds may also be biologically active in insects, present in plants containing true ecdysteroids, and biogenetically related/connected to true ecdysteroids (Lafont, 1997). Although no study has yet contested the ecdysteroid classification and definition of Lafont (1997), he is aware that the present groupings and definition is not entirely satisfactory and has suggested a classification based on biological activity. In addition, it was recommended to include in the ecdysteroid group compounds that do not fit into the ecdysteroid definition but occur in plants together with phytoecdysteroids and are biochemically synthesized from ecdysteroid by further reactions (e.g. cheilanthones A and B, calonysterone, praemixisterone and stachysterone A). As there are so many borderline cases like 14α-hydroxypinnasterol, which do not occur in plants together with ecdysteroids, there are considerable difficulties in defining the ecdysteroid family no matter which criteria are used (Lafont and Horn, 1989). The availability of more sensitive and rapid techiniques in the detection and isolation of ecdysteroids has contributed to the growing list of ecdysteroids and ecdysteroid-related compounds, which makes classification and definition of ecdysteroids more complicated.

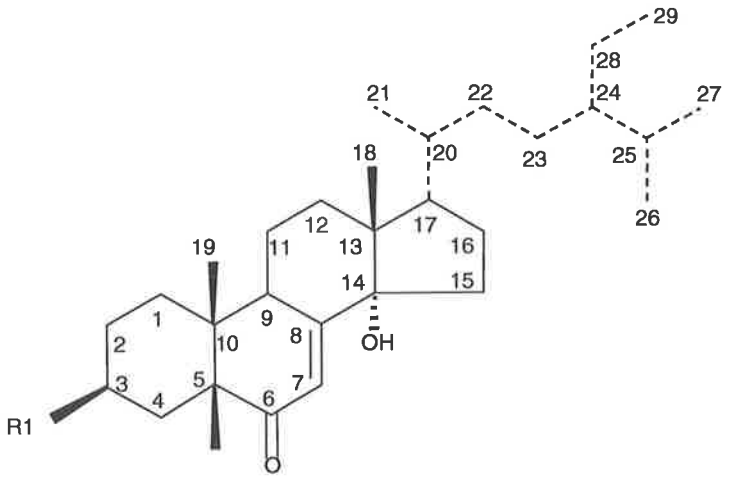

Fig. 2.1. Common structural feature of ecdysteroids (after Lafont *et al.*, 1991)

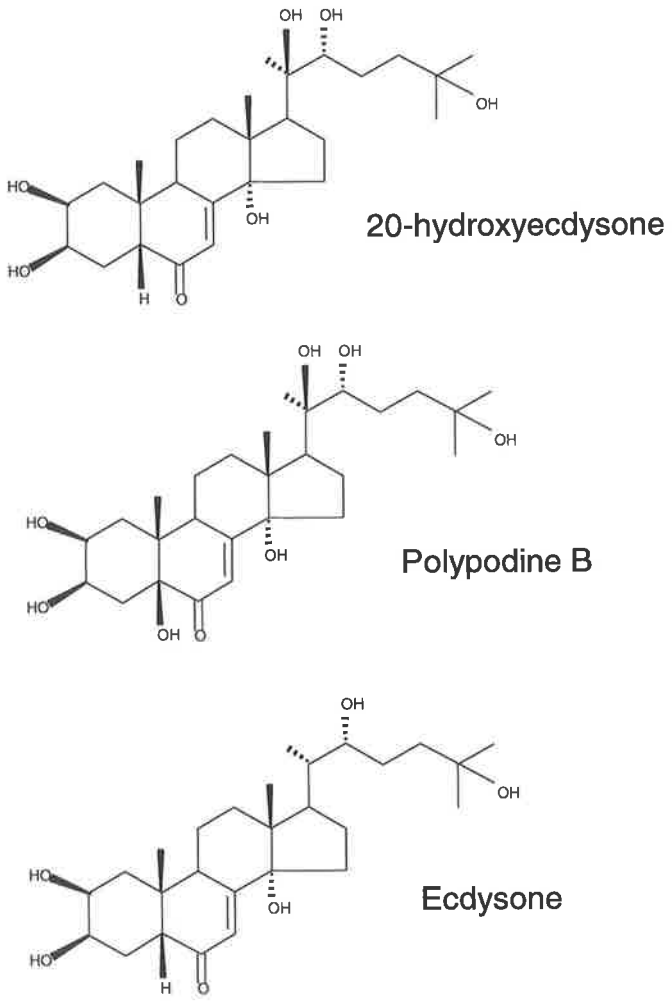

Figure 2.2 Common phytoecdysteroids

2.6 ECDYSTEROIDS IN PLANTS

2.6.1 DISTRIBUTION OF PHYTOECDYSTEROIDS

Phytoecdysteroids were earlier found in almost 100 plant families including fungi, pteridophytes, gymnosperms and angiosperms at varying quantities (Camps, 1991; Bergamasco and Horn, 1983; Lafont and Horn, 1989; Dinan, 2001). However, with increasing sensitivity of bioassay that could detect $5x10^{-9}$ mg of 20-hydroxyecdysone, the number of plant families observed to contain ecdysteroids has increased to 111 (Camps, 1991; Bergamasco and Horn, 1983, Horn and Bergamasco, 1985). Lafont (1997) even suggested that all plants possess the ability to produce ecdysteroids and the availability of an extremely sensitive detection technique can help prove this claim. Among the angiosperms in which phytoecdysteroids have been detected are *Ajuga* (Lamiaceae), *Achryanthes*, *Spinacea*, and *Chenopodium* (Amaranthaceae), *Vitex* (Verbenaceae), *Stachyurus* (Stachyuraceae), *Diploclisia* and *Abuta* (Menispermaceae), *Serratula* (Asteraceae), *Helleborus* (Ranunculaceae) and *Selene* (Caryophyllaceae) (Camps, 1991; Hikino and Takemoto, 1974; Miller *et al.*, 1985). Nevertheless, the probability of finding active plant species in Angiospermae is much lower than Pteridophyta or Gymnospermae. The random distribution of phytoecdysteroids elicited the argument of their function as general plant growth hormones (Sláma, 1979; Lafont *et al.*, 1991). However, since less than 2% of the world's flora (Dinan, 2001) has been investigated for the presence of ecdysteroids, it is not surprising that the distribution of ecdysteroid-containing species has appeared random. Recent studies focussing on significant numbers of species in specific families indicate that the occurrence of ecdysteroids can be related to phylogenetic position (Dinan *et al.*, 1998; Savchenko *et al.*, 1998; Zibareva, 2000).

Over 102 phytoecdysteroids are known in plants and occur typically at 0.1% or less of the dry weight of plants (Camp, 1991). However, ecdysteroids are greater in certain plant parts of some species, e.g. 3.2% in the stem of *Diploclisia glaucescens* (Bandara *et*

al., 1989). The concentrations of ecdysteroid vary with plant part, season, and habitat of the plant, vegetation period, and developmental stage (Yen *et al.*, 1974; Jones and Firn, 1978; Grebenok and Adler, 1991; Dinan, 1992). Evidence showed that ecdysteroids concentrations are highest in tissues which are most important for the survival of the plant or, in the case of annuals, of the species into the next generation (Dinan, 2001). Although not conclusive, the variations in ecdysteroids levels and profiles during development and between organs/plants can be most readily reconciled with a role in phytophagous predator deterrence (Dinan, 2001).

The ecdysteroids common to Pteridophyta, Gymnospermae and Angiospermae are 20-hydroxyecdysone, ponasterone, polypodine B, ecdysone and pterosterone, with 20-hydroxyecdysone often being present in the largest concentration (Camps, 1991; Lafont, 1997; Dinan *et al.*, 1999). These ecdysteroids were also found in arthropods but in much lower concentrations, 10^{-9} to 10^{-5} percent in insects (Camps, 1991). In fact, all of the 38 ecdysteroids isolated from arthropods have been found in plants (Lafont and Koolman, 1984; Camps, 1991). Although more ecdysteroids are still being discovered, a comprehensive list of these compounds isolated from different plants and plant parts is provided in Camps (1991) and Dinan (1995). An electronic database of the updated list of ecdysteroids from animals and plants is also available (Lafont *et al.*, 2002).

2.6.2 BIOSYNTHESIS OF ECDYSTEROIDS

The pathway for the biosynthesis of ecdysteroids is still not fully known despite the numerous studies performed in plants and insects. Current reviews done emphasized on synthesis in invertebrates for which more information is known (Warren and Hetru, 1990; Grieneisen, 1994; Rees, 1995; Lafont, 1997). Only the review by Adler and Grebenok (1995) deals specifically with the biosynthesis of phytoecdysteroids.

Early investigation with labelled cholesterol in an insect *Calliphora erytrocephala* demonstrated that cholesterol was the precursor of ecdysone (Karlson and Hoffmeister, 1963). In insects, 7-dehydrocholesterol is an intermediate in the ecdysteroids pathway (Grieneisen *et al.*, 1993) and the reactions for its formation from cholesterol are clear. However, reactions occurring in between this step and the last three hydroxylation still remain ambiguous (Koolman, 1990; Grieneisen, 1994; Lafont, 1997). Unlike insects, plants are capable of biosynthesising phytoecdysteroids from mevalonic acid and the precursor of the biosynthesis could either be cholesterol and/or lathosterol (Adler and Grebenok, 1995).

The presence of phytoecdysteroids in various parts of plants at different concentrations does not allow any conclusion to be drawn about where biosynthesis occurs. However, studies have suggested that active biosynthesis of phytoecdysteroids takes place in developing tissues (Grebenok and Adler, 1991, 1993) and transported to other parts of the plant (Ripa *et al.*, 1990; Grebenok and Adler, 1991; Tomás, *et al.*, 1993). In spinach, polyphosphorylated ecdysteroid conjugates are formed which may be involved in the down-regulation of ecdysteroid biosynthesis (Grebenok *et al.*, 1994). Enzymological study of ecdysteroid synthesis in spinach indicated that hydroxylation of ecdysone at C-20 position to form 20-hydroxyecdysone is a cytochrome P450-dependent reaction occurring predominantly in the microsomal fraction of spinach leaves (Grebenok *et al.*, 1996)

2.6.3 FUNCTION IN PLANTS

Some authors claimed that phytoecdysteroids may perform a direct physiological function in biochemical processes within plants such as storage, transport and metabolism of sterols, plant metamorphosis and other more specific physiological activities (Kaplanis *et al.*, 1967; Robbins *et al.*, 1968; Sláma, 1969; Heftman, 1970, 1975; Abubakirov, 1982).

Attempts to demonstrate phytoecdysteroids as plant hormones have been unconvincing (Carlisle *et al.*, 1963; Felippe, 1980; reviews in Dinan, 1998). A more significant and practical role of phytoecdysteroids as a defence mechanism in plants has gained much attention in insect-plant relationship studies. This is supported by the fact that the major phytoecdysteroid in most ecdysteroid-containing plants is 20-hydroxyecdysone, which is also the major ecdysteroid in insects (Dinan, 2001). Some studies suggest the possibility of allelochemical properties of phytoecdysteroids as hormonal disruptors and toxins for insects and other invertebrates (Arnault and Sláma, 1986; Camps and Coll, 1993; Savolainen *et al.*, 1995; Mondy *et al.*, 1997). The biological activity of some phytoecdysteroids has been tested in a wide variety of insect bioassays (reviewed in Bergamaso and Horn, 1980; Cymborowski, 1989; Dinan, 1985). Ecdysteroids incorporated in natural or artificial diet affected insect growth and reproduction (Robbins *et al.*, 1968; Williams, 1970; Singh and Russell, 1980; Whitehead, 1981; Camps *et al.*, 1982; Singh *et al.*, 1982; Kubo *et al.*, 1983; Arnault and Sláma, 1986; Melé *et al.*, 1992; Tanaka and Takeda, 1993; Blackford and Dinan, 1997; Mondy *et al.*, 1997). Ecological evidence demonstrated the defensive role of phytoecdysteroids against these herbivores (Gailbraith and Horn, 1966; Staal, 1967; Williams and Robbins, 1968; Herout, 1970; Swain, 1978; Camps, 1991) by acting as feeding deterrents against non-adapted insects or by interfering with the normal cycle of growth and development (Bergamasco and Horn, 1983; Lafont *et al.*, 1991; Blackford and Dinan, 1997). However, there are some highly polyphagous insect species, which develop normally on diets containing very high concentrations (400 ppm) of 20-hydroxyecdysone (Robbins *et al.*, 1968; Kubo *et al.*, 1987; Robinson *et al.*, 1987; Tanaka and Naya, 1995; Blackford *et al.*, 1996; Blackford and Dinan, 1997). Hence, the defensive nature of phytoecdysteroids does not apply to all herbivorous insects (Lafont and Horn, 1989).

The activity of phytoecdysteroids depends on the timing, affinity for, effective concentration, and target site which is the ecdysteroid receptor complex in insects (Dinan, 2001). Two types of hormones that play a key role in insect development are the juvenile hormones produced by the larvae and the hormone group ecdysteroids which act to initiate the cycles of ecdysis that occur within the developmental sequence. Hence, a timely exposure of the herbivores to the moulting hormone in the plant could initiate premature moulting within the developmental stage of the herbivore. Deterrent effects of 20-hydroxyecdysone on non-adapted insect species are observed at 2-25 ppm (Blackford and Dinan, 1997), which is well within the concentration range found in plants containing ecdysteroids. Therefore, the potency of phytoecdysteroids would depend on the amount ingested and the ability to deliver the detrimental amount to the critical site of metabolism.

2.6.4 INDUCTION OF PHYTOECDYSTEROIDS

The inducibility of phytoecdysteroids following insect herbivory and increase in production of phytoecdysteroids in response to mechanical damage reflected a plant defence function (Schmelz *et al.*, 1998, 1999; Karban and Baldwin, 1997). Schmelz *et al.* (1998) have demonstrated the rapid phytochemical induction of 20-hydroxyecdysone in the roots of spinach following insect or 70% mechanical damage. Plants, being active in responding to a wide range of stimuli (Silvertown and Gordon, 1989), produce enhanced levels of deterrent metabolites following herbivory and mechanical damage (Baldwin, 1994). These induced responses often deter subsequent insect herbivory (Karban and Myers, 1989; Tallamy and Raupp, 1991). The ability of the plant to protect itself through this manner suggests the occurrence of a built-in resistance mechanism inherent in the plant.

The regulation of phytoecdysteroid in plants has been studied using the synthetic hormones, jasmonic and salicyllic acid (Schmelz *et al.*, 1998). These are endogenous

signals implicated in eliciting plant resistance responses. Jasmonic acid is produced following cell damage via the octadecanoid pathway (Farmer and Ryan, 1992; Hamberg and Gardner, 1992) and its induced accumulation activates defence responses in plants. Addition of methyl jasmonate stimulated accumulation of phytoecdysteroids in roots of spinach in a dose-dependent manner similar in magnitude to the response induced by mechanical damage to root. In contrast, methyl salicylate inhibited the induction of 20-hydroxyecdysone in wounded roots in the same experiment. Salicyllic acid involves essentially disease resistance responses in its defence signaling (Klessig and Malamy, 1994). It was found partly to stimulate fungal and viral resistance by inducing pathogenesis-related proteins (Ward *et al.*, 1991). Hence, salicyllic acid is used to investigate both pathogen resistance responses and effects caused by inhibiting wound-induced jasmonic acid biosynthesis (Peña-Cortes *et al.*, 1993). It is worthwhile exploring the inducibility of phytoecdysteroids in plants to maximize its role in plant defence.

2.7 ECDYSTEROIDS IN NEMATODES

2.7.1 OCCURRENCE IN NEMATODES

Ecdysteroids have been detected and characterised in animal and free-living nematode species (Rees and Mendis, 1984). However, reports on the occurrence of ecdysteroids in nematodes are relatively few compared to arthropods. This explains the extensive use of the moulting hormone system in insects to illustrate the role of ecdysteroids in plants in the previous section of this review. The slow advancement may be due to the microscopic size of most nematodes, the difficulty in obtaining sufficient biological material for chemical analysis (particularly in plant parasitic nematodes) and the much lower concentrations of ecdysteroids in nematodes compared to insects (Rees and Mendis, 1984). These also limited the complete characterization of the compounds by physical techniques. Nevertheless, several reports indicated the presence of insect moulting hormone-like

materials in nematodes.

The detection of ecdysone and 20-hydroxyecdysone as major ecdysteroids in adult filarial nematode, *Dirofilaria immitis*, by high performance liquid chromatography in conjunction with radioimmunoassay (HPLC/RIA) established for the first time the occurrence of ecdysteroids in nematodes (Mendis *et al.*, 1983; Cleator *et al.*, 1987). Consequently, using the same technique, ecdysone and 20-hydroxyecdysone were also found in the free-living nematode, *Caenorhabditis elegans* (Mercer *et al.*, 1988), the swine parasite, *Ascaris suum* (Fleming, 1985; Cleator *et al.*, 1987) and the mycophagous species, *Aphelenchus avenae*. With the use of HPLC/Enzyme immunoassay, *Nippostrongylus brasiliensis* was found to contain both ecdysteroids (Nembo *et al.*, 1993). The two ecdysteroids were present in these nematodes as free compounds and as highly polar conjugates. Ecdysone-like substances were observed in *Phocanema depressum* (Rajulu *et al.*, 1972), *Haemonchus contortus* (Rogers, 1973) and *Panagrellus redivivus* (Dennis, 1977). *Ascaris lumbricoides* (Horn *et al.*, 1974) and *Trichinella spiralis* (Hitcho and Thorson, 1971) contained 20-hydroxyecdysone. O'Hanlon *et al.* (1991) found no evidence of 20-hydroxyecdysone production from ecdysone by either *A. suum* or *Parascaris equorum*. He concluded that if ecdysteroids are biologically important in nematodes, it is unlikely that 20-hydroxyecdysone is the major active form of hormone as in the case of insects. Ecdysone was the major free ecdysteroid detected endogenously in both nematodes.

2.7.2 ECDYSTEROID BIOSYNTHESIS

Although it has been established that ecdysteroids occur in nematodes, there is no strong evidence to support the hypothesis that nematodes can synthesize ecdysteroids from cholesterol or from any of the intermediates in the major biosynthetic pathway of insects (Mercer *et al.*, 1988; Chitwood and Feldlaufer, 1990; Kuervers *et al.*, 2003). An

alternative biosynthetic pathway may occur in nematodes or ecdysteroids may not be synthesized at all, but rather obtained passively from dietary sources. Moreover, the lack of the ecdysteroid receptor genes within the *C. elegans* genome (Sluder *et al.*, 1999) indicates that it is likely that processes such as moulting and reproduction are regulated by a different steroid hormone in *C. elegans* (Kuervers *et al.*, 2003). The requirement for cholesterol for growth, development and reproduction, and possibly moulting, suggests that, as in *D. melanogaster*, steroid hormones are required for these processes in *C. elegans* (Kuervers *et al.*, 2003). To establish the hormonal function for ecdysteroids in nematodes, it is necessary that the nematodes synthesize the hormone. A nematode tissue clearly responsible for synthesis of ecdysteroids has yet to be identified. The low concentration of ecdysteroids in nematodes may have rendered demonstration of this difficult.

In enzymology studies, a link between *D. melanogaster* ecdysone response and *C. elegans* has been demonstrated by the conversion of function of two orphan nuclear hormone receptors. βFTZ-F1 is an orphan nuclear receptor in *D. melanogaster* that is directly induced by another orphan nuclear receptor, DHR3, which in turn is directly induced in response to ecdysone. The *C. elegans* orthologues of these receptors, NHR-23 (DHR3) and NHR-25 (βFTZ-F1), are both required for reproduction, moulting and embryogenesis in the nematode. However, unlike βFTZ-F1, NHR-25 is not directly induced by NHR-23 indicating a divergence in the hormone regulated pathway (Kostrouchova *et al.*, 2001).

2.7.3 BIOLOGICAL ACTIVITIES

As in the case of insects, nematodes develop through successive moults and ecdysteroids have been proposed to also regulate moulting in nematodes (Willett, 1980). An indirect establishment of the involvement of ecdysteroids in nematode moulting was commonly used in previous studies where exogenous ecdysteroids were introduced to culture media of

nematodes. Ecdysone was found to stimulate moulting of fourth-stage juveniles of *Nematospiroides dubius* at 0.01 ng/ml (Dennis, 1976). The active moulting hormone of insects, 20-hydroxyecdysone, promoted moulting of *T. spiralis* juveniles at 15 μg/ml (Rogers, 1973) and *A. suum* juveniles at 5 ng/ml (Fleming, 1985). Dennis (1977) observed that more ecdysone-like material was present in moulting larvae than in adults of *P.redivivus* and *A. avenae*. An increase in the ecdysteroid titre immediately before ecdysis in the larval stages of *C. elegans* was noted by Mendis *et al.* (1983).

In other bioassay experiments, ecdysone has retarded the development of *Cephalobus* sp. at 0.1 μg/ml (Nelson and Riddle, 1984). Likewise, the development of the free-living stages of *H. contortus* was inhibited by ecdysone at 464 μg/ml and by 20-hydroxyecdysone at 480 μg/ml (Rogers, 1973). In the contrary, ecdysone and 20-hydroxyecdysone increased the length of the fourth-stage juveniles of *A. suum* at 0.05 ng/ml (Fleming, 1985).

Fleming (1993) indicated that ecdysteroids play generalised regulatory roles in embryogenesis and growth as well as specific functions in cuticle deposition and gametogenesis based on their concentration pattern throughout the development of *H. contortus*. The recovery of ecdysone and 20-hydroxyecdysone from the reproductive tract of *A. suum* adult female further strengthens their potential roles in gametogenesis, fertilization and embryogenesis (Fleming, 1985). Furthermore, ecdysteroids were observed to be localised in *A. suum* and *D. immitis* tissues as free ecdysteroids in the reproductive system and the body wall and as polar conjugates in gut and reproductive system for both sexes (Cleator *et al.*, 1987). Ecdysteroids were postulated to exert control over embryonic membrane or cuticle formation since these are located primarily in the ovaries and eggs developing *in-utero* of adult *D. immitis* (Mendis *et al.*, 1983).

In some studies, however, ecdysteroids did not affect the growth and development of *C. elegans*, *P. redivivus*, *Brugia pahangi* and *Heterodera schachtii* (Dennis, 1977;

Dropkin *et al.*, 1971; Hansen and Buecher, 1971; Johnson and Viglierchio, 1970; Ogura *et al.*, 1981). Obviously, the findings enumerated above regarding the effects of ecdysteroids in nematodes as moult-promoter or development enhancer/reducer are not sufficient to justify that ecdysteroids are endogenous nematode hormones. Moreover, most of the effects recorded are in response to relatively high concentrations of exogenous hormones used, although the concentration of ecdysteroids within the nematode tissue is still obscure. In addition, the number of compounds evaluated was generally few and the control treatments (if ever used) using non-hormonal substances resulted in similar activity to that of the putative hormone. Chitwood *et al.* (1985) commented that compounds with hormonal activity in other organisms must be regarded as being merely bioactive rather than hormonal within nematodes.

2.8 FLAVONOIDS IN PLANTS

Flavonoids constitute an enormous and diverse group of natural products synthesized from phenylpropanoid and acetate-derived precursors which play important roles in growth and development, and in defence against microorganisms and pests (Dixon *et al.*, 1999; Harborne and Williams, 2000; Martens *et al.*, 2003). Flavonoids consist of various groups of plant metabolites including chalcones, aurones, flavonones, isoflavonoids, flavones, flavonols, leucoanthocyanidins (flavan-3,4-diols), catechins, anthocyanins, procyanidins and condensed tannins (Harborne and Williams, 2000; Croteau *et al.*, 2000; Martens *et al.*, 2003). There is no obvious underlying structural feature of flavonoids that would lead to a specific biological function (Romeo *et al.*, 1996). The commonly cited functions of flavonoids are as ultraviolet filters, pollinator attractants, compounds necessary for pollen germination and pollen tube growth, messenger or signal molecules, oviposition stimulants, pigments insects and plants and defence agents (reviews in Bohm, 1998; Harborne and Williams, 2000; Harbone, 1999).

2.8.1 FLAVONOID BIOSYNTHESIS

The biosynthetic pathways for the production of the major classes of flavonoids are shown in Fig. 2.2 (Dixon *et al.*, 1999). All flavonoids are derived from a chalcone precursor, which is a product of the condensation of three molecules of acetate-derived malonyl-CoA and one molecule of *p*-coumaryl-CoA. The chalcones can then be converted into aurones, a subclass of flavonoids found in certain plants. The next step, which is shared by most of the flavonoid biosynthesis pathways, is catalysed by chalcone isomerase to form flavanones. The flavanones represent the most important branching point in flavonoid metabolism because isomerization of these compounds yields the phytoalexin isoflavonoids, compounds implicated mainly in plant defence. The introduction of a C-2-C-3 double bond results to flavones and flavonols. The second branching point involves the dehydration of naringenin at the C-2/C-3 position to generate flavones such as apigenin. The third branch point can result to flavanol such as quercetin through the hydroxylation involving an NADPH-dependent cytochrome P450 monooxygenase of naringenin to form dihydroquercetin. Subsequent species and tissue specific enzymatic conversions can create vast arrays of structurally diverse group of flavonoids (Croteau *et al.*, 2000; Dixon *et al.*, 1999).

2.8.2 FLAVONOIDS AS PLANT DEFENCE AGAINST NEMATODES

As flavonoids comprise a large group of versatile compounds in terms of functions and structures, only their involvement in plant defence against nematodes will be discussed. Flavonoids in plants have long been implicated as defence against nematodes. Some of the phytoalexins described earlier are flavonoids. The flavone, quercetin was found to inhibit reproduction of *M. javanica* as soil drench at 400 µg/ml (Osman and Viglierchio, 1988). The flavone glycosides, linaroside and lantanoside, from *Lantana camara* were lethal to

Meloidogye incognita juveniles at 1% (Begum *et al.*, 2000). The isoflavanone, rotenone was nematicidal to *C. elegans* (Stadler *et al.*, 1994).

The isoflavones, coumestrol and psoralidin are compounds produced by lima beans in response to infection by *Pratylenchus scribneri* (Rich *et al.*, 1977). The motility of the nematode was inhibited at 5-25 µg/ml coumestrol but was not effective against *M. javanica*. The incompatible response to root-knot nematodes during the accumulation of the phytoalexin glyceollin in soybean has been very well studied. At 15 µg/ml, the compound strongly inhibited the movement of *M. incognita in vitro* (Kaplan *et al.*, 1980b; Kanagy and Kaya, 1996). The isoflavanone, medicarpin, a constitutively expressed phytoalexin of lucerne, occurs in higher concentrations in resistant cultivars and inhibits mobility of *P. penetrans* (Baldridge *et al.*, 1998). Along with 4-hydroxymedicarpin, medicarpin was isolated as a nematicide in the roots of *Taverniera abyssinica* (Fabaceae) based on the biological activity against *C. elegans* in *in vitro* bioassay. Sakuranetin, and a related phenylpropanoid in the leaves of rice were induced by *Ditylenchus angustus* infestation and were considered to be responsible for the resistance of some cultivars (Plowright *et al.*, 1996).

Proanthocyanidins are formed by the polymerization of catechins and flavan 3,4-diols. The constitutive and induced levels of these compounds were higher in resistant than in susceptible *Musa* cultivars (Collingborn *et al.*, 2000). Complex condensed tannins from *Quercus petraea* bark and a fraction containing a mixture of a non-flavonoid, ellagitannins, inhibited the reproduction of *C. elegans* at 500 µg/ml (König *et al.*, 1994). Hatching of *Heterodera glycines* was stimulated at concentrations lower than 39 µg/ml while higher concentrations were inhibitory (Chen *et al.*, 1997).

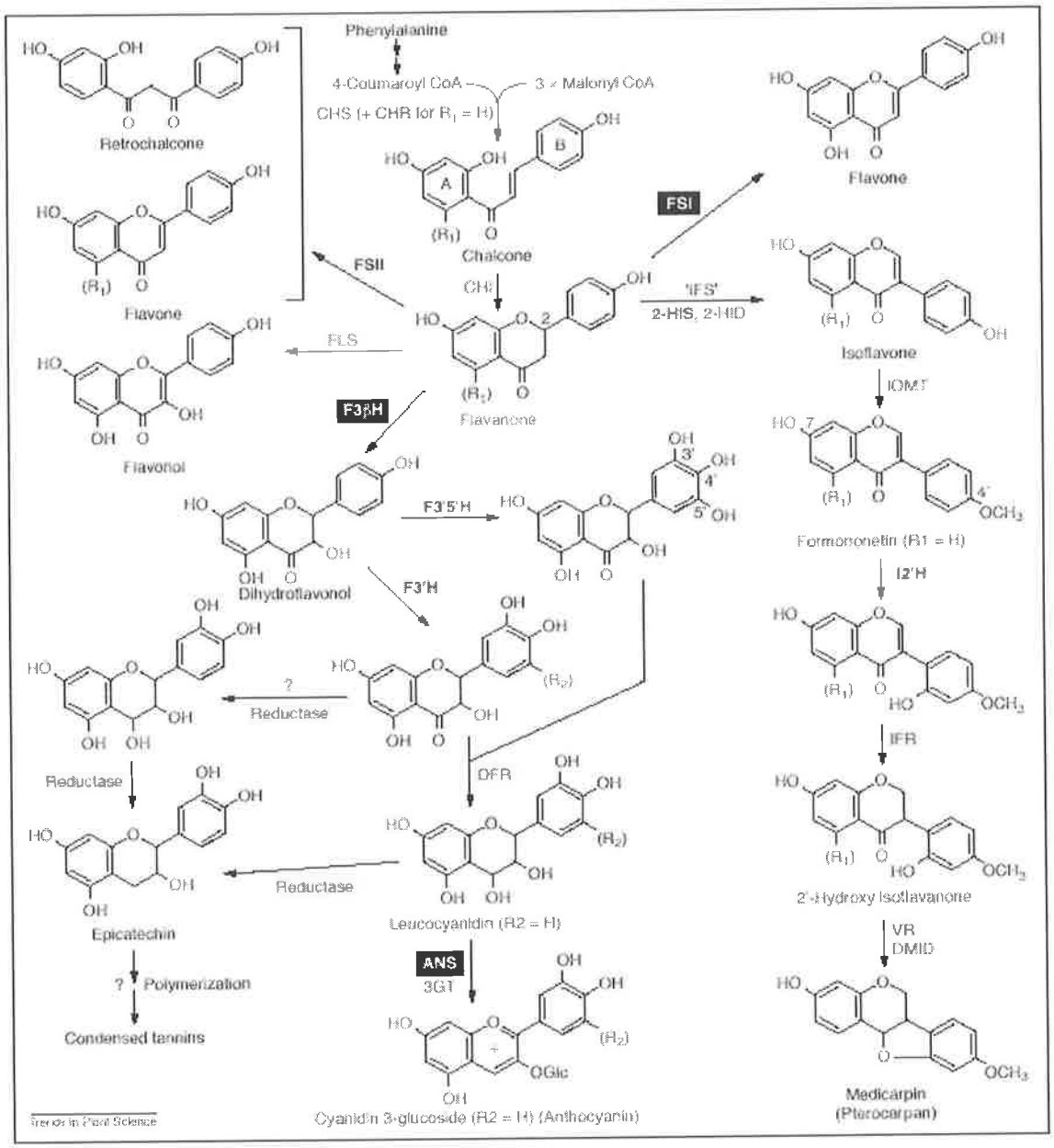

Fig. 2.2 Simplified biosynthesis pathways of the major classes of flavonoid derivatives. Enzymes in white are 2-oxoglutarate-dependent dioxygenases, in black bold are cytochrome P450s, and highlighted in grey are NADPH-dependent reductaces; CHS, chalcone synthase; CHR, chalcone reductase; CHI, chalcone isomerase; FSI, flavone synthase I; FSII, flavone synthase II; FLS, flavonol synthase; 'IFS', isoflavone synthase, consisting of 2-hydroxyisoflavanone synthase (2-HIS) and 2-hydroxyisoflavanone dehydratase (2-HID); F3βH, flavanone 3β hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydrolase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; 3GT, anthocyanidin 3-glucosyltransferase; IOMT, isoflavone O-methyltransferase; IFR, isoflavone reductase; VR, vestitone reductase; DMID, 7,2'-dihydroxy, 4'-methoxyisoflavanol dehydratase. Parallel pathways function in the formation of anthocyanins with mono- and tri-substituted B-rings. In the latter, F3'5'H can act at the level of the dihydroflavonol with a mono- or di-substituted B-ring. The pathway to epicatechin from a dihydroflavonol is shown to follow two routes, both via leucocyanidin. It is unclear whether there is a specific form of DFR that functions only in condensed tannin biosynthesis. (from Dixon and Steele, 1999)

2.9 SUMMARY

Ecdysteroids, also called the moulting hormones, are compounds involved in the regulation of many developmental and physiological processes in insects. The ecdysteroids are also known in other arthropods and moulting invertebrate organisms, such as nematodes, but their function is yet obscure. Active analogues of ecdysteroids are present in plants and are referred to as phytoecdysteroids. Phytoecdysteroids are chemically diverse, inducible and commonly occurring in plants. Although considered to be involved in plant growth and development, phytoecdysteroids have been reported to effect toxicity, feeding deterrent or ecdysis inhibition in insects. Hence, the presence of phytoecdysteroids confers resistance in plants against non-adopted phytophagous insects. The potential of phytoecdysteroids as plant defence against parasitic nematodes has not been explored despite the similarity of the moulting process of insects and nematodes. Ecdysteroids may have a hormonal role and biological functions in nematodes since these have been isolated from various species specifically from the animal parasites. Although not extensive, there is evidence that ecdysteroids may interfere with normal growth and development of the nematode and have antihelminthic effect in nematodes exposed exogenously. However, this has yet to be demonstrated for plant parasitic nematodes. To establish the importance of ecdysteroids in plant parasitic nematodes, their presence should be corroborated and the biological activity determined. Confirming that phytoecdysteroids participate in plant defence against parasitic nematodes will be an important finding as only few convincing biochemical bases of host resistance has been established. Furthermore, the inducibility of phytoecdysteroids will permit its manipulation in plants to maximise potential nematode control.

Flavonoids, on the other hand, comprise a large and diverse group of compounds common in plants as primary and secondary metabolites. The biosynthetic pathway of this group has been well defined and understood. Their involvement in plant defence against pathogen and pests has been given much attention among its many functions in plants. The

discovery of flavonoid compounds in plants with nematicidal properties has increased as methods for detection and bioassay have improved. Furthermore, induction of flavonoids in resistant plants compared to susceptible ones after inoculation is an indication of its link to plant defence against nematodes. The ubiquity of flavonoids suggests that they can be easily exploited for nematode control.

CHAPTER 3. GENERAL TECHNIQUES

3.1 PLANT MATERIALS AND MAINTENANCE

Steam pasteurised (65°C for 45 min) University of California (U.C.) mix (Appendix 1) and Tailem Bend sand (Appendix 2) were used as planting media. Unless specified, full strength Hoagland solution (Appendix 3) was applied every other day at the rate of 50 ml/plant. Plants were grown in a controlled environment growth room at 18°C, 85% relative humidity (RH) and with 12 h photoperiod unless stated otherwise. Fluorescent lighting was supplied approximately 145 μmol photons/sec/m^2 of photosynthetically active radiation at the leaf surface.

3.1.1 *SPINACIA OLERACEA*

The spinach (*S. oleracea*) cultivars Amsterdam, Sonata, Viking, and Winter Bloomsdale were purchased from New Gippsland Seeds & Bulbs and Winter Queen was from Yates (Australia). The variety Avon was acquired from W. Atlee Burpee Co., Warminster, Pennsylvania. Spinach seeds were germinated in U.C. mix topped with vermiculite and allowed to grow for 10 days. Seedlings at the cotyledon stage were transplanted into individual 300 ml pots filled with Tailem Bend sand and grown hydroponically for an additional 15 days before treatment. The plants were maintained in the growth room and were applied with Hoagland solution as described above.

3.1.2 *AVENA SATIVA*

The seeds of oat cultivars Swan and Quoll (*A. sativa*) were obtained from the South Australian Research and Development Institute (SARDI), Field Crops Pathology Group. The seeds were soaked overnight in continuously flowing water before being placed in a misting chamber for 24 h to germinate. The seedlings of approximately the same size and root length were transplanted individually onto 300 ml pots filled with Tailem Bend sand.

The plants were maintained hydroponically in a controlled environment growth room for 15 days before treatment.

3.1.3 *BRIZA* SPP.

The seeds of *Briza maxima* (quaking grass) were collected Southwest of Horsham, Victoria by Michael Moerkerk of the Department of Primary Industry, Victoria and *Briza minor* (lesser quaking grass) were collected from the Waite Arboretum, Urrbrae, SA. The seeds were sown in U.C. mix topped lightly with vermiculite. After 5 days, the grasses were transplanted onto 250 or 300 ml Tailem Bend sand contained in 4.2 cm diameter by 10.8 cm high polystyrene tubes or in 300 ml pots. The plants were grown in the growth room for another 10 days before treatment application in a hydroponic condition.

3.1.4 *MEDICAGO SATIVA*

Twelve lucerne cultivars (*M. sativa*), Hunterfield, Eureka, Super 7, Hunter River, Pioneer, Prime, Pioneer L6, Trifecta, Sceptre, Pioneer L9, Sequel HR and Siriver were obtained from SARDI Lucerne Breeding Unit. The seeds were scarified by scratching the seed coat with sand paper. Punnets were filled with 600 ml U. C. mix with OsmocotePlus® (Appendix 4), a granular, slow release fertilizer, incorporated at the rate of 4 g per kg soil. For each cultivar, 30 seeds per replicate were counted and sown onto the punnets. The plants were grown in a glasshouse at 22-25°C and watered as necessary.

3.1.5 *TRITICUM AESTIVUM*

Seeds of wheat cv. Egret (*T. aestivum*) were acquired from SARDI Field Crops Pathology Group. The procedure and conditions for germinating the seeds and for growing and maintaining the seedlings were similar to oats (Section 3.1.2). However, in some

experiments, pre-germinated seedlings were transplanted onto 250 ml Tailem Bend sand contained in polystyrene tubes.

3.2 NEMATODES

3.2.1 CULTURE

Pratylenchus neglectus

The inocula of the root lesion nematode were obtained from the SARDI Nematology Group. The nematode was multiplied on carrot callus cultures following the protocol described by Nicol and Vanstone (1993). The isolate was established by V. Vanstone (University of Adelaide) in 1991 from a population collected from Palmer, South Australia.

The carrot cultures were established by aseptically adding 10 surface sterilized female *P. neglectus* to a 2 µl drop of sterile reverse osmosis water on a carrot callus. Nematodes were surface sterilized in a solution of 1% streptomycin for 3 h then rinsed with sterile reverse osmosis water. The cultures were incubated in the dark at 20-22°C for up to 18 months to give about $1x10^6$ nematodes per culture.

Heterodera avenae

The cereal cyst nematode was also obtained from the SARDI Nematology Group. Soil with some root debris from a field infested with *H. avenae* in Yorke Peninsula, South Australia was air-dried. Matured cysts were separated from a 1 L of soil by mixing the soil thoroughly with approximately 3 L of water in a bucket. The soil sediments were allowed to settle at the bottom of the container for 2 min. Floating cysts, together with some organic materials, were collected by gently tipping off the water from the slurry through a 250 µm sieve carefully excluding the soil. The process was repeated and the collected materials were rinsed with tap water on the sieve. The collected cysts with associated

organic materials were placed onto bolting silk in a Whitehead tray (described in Section 3.2.2) and incubated at 10°C for about 8 weeks.

Heterodera schachtii

The initial inoculum of the sugar beet cyst nematode was from a field in Munster, Western Australia. The identity of the nematode was confirmed by Dr J. Nobbs, taxonomist of SARDI Nematology Group. Mature and healthy (no fungal infection) cysts were collected from the soil and were placed individually into a glass block with approximately 0.2 ml water enough for the cyst to be fully soaked. Each cyst was squashed gently with forceps to release the eggs. The eggs were allowed to hatch to second stage juveniles (J2) in an incubator set to 25°C before inoculating the J2s of a single cyst onto a 25-day old silverbeet grown in U. C. mix. The plants were grown in the glasshouse (22-25°C) for another 3-4 months before matured cysts could be collected in large amounts per plant. When J2s of the nematode were needed, the cysts and some associated organic matter were separated from the soil and roots of silverbeets from each pot following the procedure used for *H. avenae*. The eggs were hatched from the cysts in a Whitehead tray described in Section 3.2.2. Unlike *H. avenae*, the cysts and organic matter in the Whitehead tray set-up was changed regularly as fungi and bacteria multiplied rapidly in the temperature (20°C) at which the set-up was kept.

Meloidogyne javanica

The initial inoculum for the multiplication of the root-knot nematode was obtained from the cultures maintained for teaching purposes at the University of Adelaide. Females and J2s were collected to confirm the identity of the nematode. Single egg mass of the nematode was inoculated onto each one-month-old tomato cv. Grosse Lisse grown in U.C.

mix. The plants were maintained in the glasshouse at 22-25 °C. After three months, galls with egg masses were evident in the roots of the plant.

Ditylenchus dipsaci

Inocula of the stem nematodes (oats and lucerne strain) were obtained from the SARDI Nematology Group. These were cultured in carrot callus following the procedure described above for *P. neglectus*. The nematode cultures were incubated in the dark at 20-22°C for up to 6 months.

3.2.2 EXTRACTION

Misting

Carrots from the nematode cultures were cut into 10 mm sections. These were placed in 90 mm diameter by 70 mm high baskets made from PVC drain pipe covered at one end with plastic fly wire gauze. The baskets were rested on the inner of two funnels. The inner funnel had its neck removed to fit into the outer funnel to which clamped flexible tubing was fitted. The funnels were held in an upright position by PVC racks. The samples were misted for 15 s every 10 min for 24 h before 5-10 ml nematode suspensions per funnel were collected. Clamps were loosened to release nematodes that settled at the bottom of the tubing. The method was modified from Seinhort (1950).

Flotation and sieving method

In all experiments, nematodes were extracted from the sand using this method. The sand was collected in a 2 L polyvinyl beaker as it was washed off from the plants and pots. The water level was raised to about 1 l under strong pressure to thoroughly mix the sand. The sand was allowed to settle for a minute before pouring off the water into a 150 µm sieve over a 20 µm sieve. The nematodes collected in the fine sieve were concentrated in a

smaller area of the mesh using a wash bottle and were collected in screw cap tubs. The procedure was repeated with the same sample to maximise the recovery of nematodes (Byrd *et al.*, 1966).

Whitehead tray

The method, modified from Whitehead and Hemming (1965), used 290x360x55 mm seedling trays lined with bolting silk to hold the samples (organic matter with cysts of *Heterodera* spp.) which were placed in 310x450x65 mm trays. Tap water was added to the trays to wet but not to cover the sample. The trays were incubated in the dark at 10°C for *H. avenae* and 20°C for *H. schachtii*. When *H. avenae* J2s were required, the temperature was increased to 15°C as an increase in the rate of hatching of eggs occurs after the low temperature exposure (Banyer and Fisher, 1971). *H. schachtii* J2s were collected everyday and stored at 5°C, as J2s of the nematode become inactive in higher temperatures. The nematode suspension was concentrated by passing the water in the photographic tray through a 20 μm sieve and washing the nematodes off the sieve in smaller volume of water.

Extraction of Meloidogyne egg masses

The method was adopted from Hussey and Barker (1973). Infested roots of tomato from the nematode culture were recovered from soil and debris using a gentle stream of water and then vigorously shaken by hand in 250 ml of 1% sodium hypochlorite (NaOCl) solution in a 500 ml container for 4 min. The NaOCl solution was then quickly passed through a 75 μm over a 20 μm sieve. The eggs collected in the 20 μm sieve were rinsed with water to remove the NaOCl because long exposure to NaOCl could be detrimental to the nematode (Vrain, 1977). The roots were washed 3 more times with tap water to remove additional eggs which were collected by sieving. The eggs were allowed to hatch

in a smaller scale Whitehead tray described above. Petri dishes (150 mm) fitted with stainless wire mesh with a 8 mm stand (made from 20 mm diameter PVC pipe) and lined with 2 sheets of Kimwipe® were used to hatch the eggs. The eggs with some debris were evenly distributed over the Kimwipe® and tap water was added into the petri plate to wet the eggs during incubation at 25°C. Only the J2s that hatched within 48 h were used.

The resulting nematode suspensions were adjusted to the required numbers of nematodes by diluting the nematode suspension after direct counting to estimate the nematode numbers. The nematode suspensions obtained from the sand of the experiments were assessed as described below.

3.2.3 ASSESSMENT

Direct counting

All parts of the nematode suspension from the soil of the experiments was placed in an open counting dish and examined under a stereoscopic microscope at 50X magnification, illuminated by transmitted light. Occasionally, nematodes were allowed to settle and excess water was carefully aspirated away. The number of live, dead and abnormal (any deviation from a healthy nematode) nematodes was recorded.

Root staining

Washed roots were soaked in 1.5% NaOCl for 4 min with occasional agitation and rinsed in running water. The bleached roots were allowed to soak in tap water for 15 min to remove NaOCl. The roots were then boiled in a solution of equal volumes of glycerol, lactic acid and RO water with 0.05% acid fuchsin for 3-4 min. The roots were washed with running water to remove excess stain before clearing in a solution of equal volumes of glycerol and RO water acidified with a few drops of lactic acid. The destained roots were then pressed between two rectangular (90x180 cm) glass slabs secured with bulldog clips

at both ends. The stained nematodes were counted and observed for abnormalities which were recorded. The method was modified from Bridge *et al.* (1982).

Vitality test

Nematodes recovered from the sand were tested for vitality by staining with 0.01% new blue R (GT Gurr Ltd., United Kingdom). The nematodes were soaked in a 0.05% aqueous solution of new blue R for 24-48 h. The dead nematodes became darkly stained but the live ones remained unstained (Shepherd, 1962).

3.3 EXTRACTION OF METHANOL-INDUCIBLE COMPOUNDS

The frozen root and shoot samples were freeze-dried and ground to a fine powder (c. 850 μm mesh). For each sample, 60 mg was extracted in 10 ml methanol for 48 h. Methanol is commonly used as a general solvent for the extraction of secondary metabolites such as phenolics from plants (Waterman and Mole, 1994). To remove the lipids in the extract collected, an aliquot of 7 ml of the methanol extract was diluted with water to make a 70% methanol extract solution and partitioned with 10 ml hexane for 48 h. The aqueous methanol layer (8 ml) was removed and evaporated to dryness by subjecting each tube to a stream of air using a manifold fitted with tubings to distribute air supplied by a centralised air system. In some cases, the speed vacuum was used for drying. The tubes were placed in a water bath at 45°C while drying. The residue was dissolved in 5 ml water and partitioned against 5 ml butanol. A 4 ml aliquot of the butanol phase was evaporated using the system described earlier and reconstituted in 0.4 ml 45% methanol. The extracts were dispensed in Waters HPLC vials prior to analysis.

3.4 DETECTION OF COMPOUNDS

The methanol-extractable compounds in shoot and root extracts were assayed using a reverse-phase high performance liquid chromatography (RP-HPLC) with a C-18 column (Waters Sperisorb ODS-2, 4.6mm x 150 mm, 5 μm particle column). Unless specified, isocratic elution was performed with water-methanol (55:45 v/v) as mobile phase at 1 ml/min at room temperature with detection at UV 254 nm.

CHAPTER 4. INDUCTION OF COMPOUNDS

4.1 INTRODUCTION

Inducible defences provide excellent models for investigation of the role of specific defence mechanisms in resistance because products of such defences are absent from healthy plants or present in detectable amounts during expression of resistance. Increases in plant defence metabolites are induced by abiotic and biotic stresses (Dixon and Paiva, 1995). Many of these inducible defence compounds are synthesized in plants in response to damage inflicted by pest and pathogen attack. The accumulation of plant defence chemicals is often signalled by plant derived wound signals that initiate from the site of damage (Baldwin *et al.*, 1997; Bergey *et al.*, 1996; Pearce and Ryan, 2003). Signal transduction pathways associated with plant defence response against herbivore and pathogen attack or severe wounding in plants are activated by various signal molecules, including jasmonic acid (Farmer and Ryan, 1990), oligogalacturonides (Reymond *et al.*, 1994), abscisic acid (Peña Cortes *et al.*, 1995), polypeptides (Pearce and Ryan, 2003) and electrochemical signals (Wildon *et al.*, 1992). The net effect of this process is the reduction of damage to or an increase in immunity of the plant, resulting in protection or resistance against pests and pathogens.

Jasmonic acid, a naturally occurring growth regulator found in higher plants, is one of the endogenous signals implicated in eliciting plant resistance responses which is produced following cell damage through the octadecanoid pathway (Farmer and Ryan, 1992). Induced accumulation of jasmonic acid activates a variety of insect and microbial defence responses in plants. Some products induced by jasmonic acid include proteinase inhibitors, benzophenanthridine alkaloids, nicotine, phenolics and flavonoids (Farmer and Ryan, 1990; Mueller *et al.*, 1993; Baldwin *et al.*, 1997; Mizukami *et al.*, 1993; Gundlach *et al.*, 1992).

In addition to the jasmonic acid-inducible metabolites above, rapid phytochemical induction of phytoecdysteroid in the roots of spinach following damage and application of methyl jasmonate has been reported (Schmelz *et al.*, 1998). Aside from the roots, mature spinach leaves are also known to be active sites for the biosynthesis of the phytoecdysteroid 20-hydroxyecdysone (20E), although young apical leaves are not biosynthetically active (Schmelz *et al.*, 1999). High levels of 20E are accumulated through transport from other tissues (Grebenok and Adler, 1991; 1993). Increases in root 20E induced by damage could result from basipetal transport of shoot 20E to the roots or by increases in *de novo* biosynthesis in the roots (Schmelz *et al.*, 1999). The cultivar Avon (W. Atlee Burpee Co., Pennsylvania,USA) was used for the studies on the inducibility of 20E in spinach. It is not known if variation exists in the constitutive and inducible levels of 20E in spinach varieties. Hence, 20E in seven varieties of chemically induced and uninduced spinach was compared.

Schmelz *et al.* (2000) produced convincing results on the possible defence function of the inducible phytoecdysteroid in spinach. However, it is not known if 20E is induced by nematodes, which belong to the same clade as insects (Aguinaldo *et al.*, 1997). Insects, along with mechanical damage, are known to induce 20E in spinach (Schmelz *et al.*, 1999). Hence, another aim of the study is to determine if 20E in spinach is inducible by nematodes with different feeding behaviour and if inoculum density has an influence on the induction of 20E.

The presence of possible defensive compounds inducible by nematodes and the wound hormone, methyl jasmonate, in plants other than spinach was tested. Two of which, *Briza maxima* and *B. minor*, were reported to contain 20E (Savchenko *et al.*, 1998). Oats were also tested for compounds, possibly 20E, inducible by nematodes and methyl jasmonate since oats are known to have resistance to a wide range of pest and pathogens (Schrickel, 1986). Different cultivars of lucerne with varying degree of resistance were

included in the test for compounds inducible chemically or by nematodes. The relationship between the levels of inducible compounds and the resistance response of the cultivars to the stem nematode, *Ditylenchus dipsaci* (lucerne strain), was also evaluated to determine if the compound(s) are linked to resistance in the plant.

The major questions investigated in this chapter were as follow: Do nematodes induce secondary metabolites in plants? If so, does inoculum density affect induction of the compounds and does the induction of metabolites occur at the right time and location in the plant to be effective as a resistance mechanism? Is the induction of metabolites by nematode invasion/infection associated with resistance in plants? Also, do the constitutive and chemically induced 20E levels differ in common cultivars of spinach?

4.2 MATERIALS AND METHODS

4.2.1 SPINACH

20E induction in different varieties of spinach

Six cultivars of spinach: Amsterdam, Sonata, Viking, and Winter Bloomsdale (New Gippsland); Winter Queen (Yates); and Avon (Burpee) were grown and maintained as described in section 3.1. Plants with uniform growth were treated with 10 ml 10^{-4} M methyl jasmonate (Sigma) as a soil drench 15 days after transplanting to induce 20E levels. Untreated plants were included as controls. Sampling for 20E quantification in the roots and shoots was done 3 and 6 days after treatment. Sand was washed from the roots of each plant and these were snap frozen in liquid nitrogen. The samples were stored at –80 C until phytoecdysteroid extraction was done. The extraction procedure and quantification of 20E followed the protocol described in the general methods (Sections 3.3 and 3.4).

20E Induction by nematode infestation of spinach

Attempts were made to induce 20E in spinach cv. Avon by inoculation of *D. dipsaci* (Lucerne race), *H. schachtii*, *M. javanica* and *P. neglectus* at the rate of 600 nematodes/plant 15 days after transplanting. The cultivar Avon was used because the 20E stability in this cultivar had been tested in previous studies (Schmelz *et al.*, 2000; Schmelz *et al.*, 1998). Ten plants were inoculated with each nematode genus to give five plants each for 20E quantification and nematode assessment. For comparison of response, another group of individual spinach seedlings was treated with 10 ml 10^{-4} M methyl jasmonate as soil drench 15 days after transplanting. Uninoculated or untreated plants were included as controls. Plants were grown and maintained as described previously (Section 3.1). To determine 20E levels in the roots and shoots, the sand was washed from each plant without damaging the roots, and the roots were snap frozen in liquid nitrogen. Phytoecdysteroid extraction and quantification from roots and shoots were done as outlined in Section 3.3 and 3.4.

Effects of nematode inoculum levels on 20E induction in spinach

Spinach cv. Avon were grown in individual pots and maintained as in Section 3.1. Four inoculum levels of *P. neglectus*, 100, 500, 1,000 and 10,000 nematodes/plant, were tested in the study. *P. neglectus* was selected because of its ability to induce 20E in spinach and because it can be readily cultured. Application of different inoculum levels of the nematode was done 15 days after transplanting of spinach. For comparison of response, the sand of a group of plants was drenched individually with 10 ml 10^{-4} M methyl jasmonate 15 days after transplanting. Uninoculated and untreated control plants were included. Roots of plants were washed carefully for 20E quantification at 3, 6 and 9 days after treatment or inoculation. Shoot and roots of plants were snap frozen in liquid

nitrogen immediately after washing, and 20E was extracted from these as in Section 3.3 and was quantified (Section 3.4)

4.2.2 OATS

To determine the inducibility of compounds in oats, an experiment as in Section 4.2.1.2 was conducted, using oats instead of spinach. Oats were grown and maintained as outlined in Section 3.1. The nematodes tested to induce compounds were *D. dipsaci* (Oats race), *H. avenae* and *P. neglectus*. They were applied at the rate of 500 nematodes/plant. For comparison of the response, the sand of some oats seedlings was drenched with 10 ml 10^{-4} M methyl jasmonate. The treatments were applied to oat seedlings with uniform growth 15 days from sowing. Uninoculated or untreated plants were included as controls. Sampling of plants for quantifying induced compounds and for assessing nematodes was done three days after treatment application following the procedure above (Section 4.2.1). The induced compounds were then extracted from the root and shoot samples (Section 3.3) and were quantified (Section 3.4).

4.2.3 *BRIZA* SPP.

The test plants were grown and maintained as described previously (Section 3.1). The treatments used for inducing compounds in *Briza maxima* and *Briza. minor*, were nematodes and methyl jasmonate treatment. *H. avenae*, *P. neglectus* and *M. javanica* were inoculated into the sand adjacent to the roots of *B. maxima* and *B. minor*. For comparison of response, 10 ml 10^{-4} M methyl jasmonate was applied as soil drench around some plants. Treatments were administered to the *Briza* spp. 15 days after sowing. Plants were sampled as above (Section 4.2.2) to quantify the inducible compounds and to assess nematode counts three days after treatment. Extraction and quantification of inducible compounds from the root and shoot samples were performed as in Section 3.3 and 3.4.

4.2.4 LUCERNE

Twelve lucerne cultivars were grown and maintained as described previously (Section 3.1). A group of plants was inoculated with *Ditylenchus dipsaci* (lucerne strain) at the rate of 1,000 nematodes/pot. Another group of plants was treated with 10 ml 10^{-4} M methyl jasmonate as a soil drench around the plants. Treatments were applied 5 days after sowing. Inoculated plants were evaluated for resistance 30 days after treatment, using the following disease rating scale for lucerne (Elgin *et al*, 1984):

Description	Proportion of healthy plants
High resistance (HR)	>50%
Resistant (R)	31-50%
Moderate resistance (MR)	15-30%
Low resistance (LR)	6-14%
Susceptible (S)	<6%

Sampling of plants for quantifying the induced compounds was done by washing the sand carefully from the roots. The roots and shoots were snap frozen in liquid nitrogen immediately after washing. Extraction and quantification of inducible compounds from the root and shoot samples were performed as in Section 3.3 and 3.4.

4.2.5 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

All plants of uniform height and vigour were arranged in a randomised complete block design with six replicates before treatments were applied. Nematode counts were transformed (logarithmic transformation) to normalize the data for analysis. Analysis of variance was used to analyse the data for significant treatment effects and means were compared using the Least Significant Difference test (LSD). The simple linear regression

analysis was used to estimate and test the significance of the relationships between levels of inoculum and induced compounds, and between resistance in lucerne cultivars and levels of induced compounds in the plant.

4.3 RESULTS

4.3.1 SPINACH

20E induction in different varieties of spinach

Treatment with methyl jasmonate increased the concentrations of 20E in the roots of all spinach cultivars after 3 and 6 days except for cultivar Viking after 3 days (Fig. 4.1). However, after 6 days, the 20E concentration in MJ-treated Viking increased 12.5 fold compared to the untreated plants (Fig. 4.1). This indicates a slow induction in this cultivar. After 3 days, Avon had the lowest constitutive 20E concentration in the roots but induction by MJ increased the 20E concentration by 12.72 and 11.76 fold compared to the untreated plants after 3 and 6 days, respectively (Fig. 4.1). The increase in 20E concentration after 3 days in the rest of the plants ranged from 1.5 to 3.7 fold only after 3 days. The cultivars Winter Bloomsdale, Viking and Winter Queen had the highest constitutive level of 20E 3 days after MJ application. After 6 days, only Winter Bloomsdale maintained its high constitutive level of 20E and Sonata had a similar level of 20E increase (Fig. 4.1). However, the increase in 20E concentration when induced by methyl jasmonate was only 2.16 and 1.79 fold compared to the constitutive levels 3 and 6 days after treatment. Methyl jasmonate treatment did not affect dry matter accumulation.

20E Induction by nematode herbivory in spinach

Except for *D. dipsaci*, the nematodes tested increased 20E levels in the roots and shoots of spinach cv. Avon, which were not significantly different from those induced by the wound hormone (Fig. 4.2). In comparison to the constitutive level 5 and 10 days after

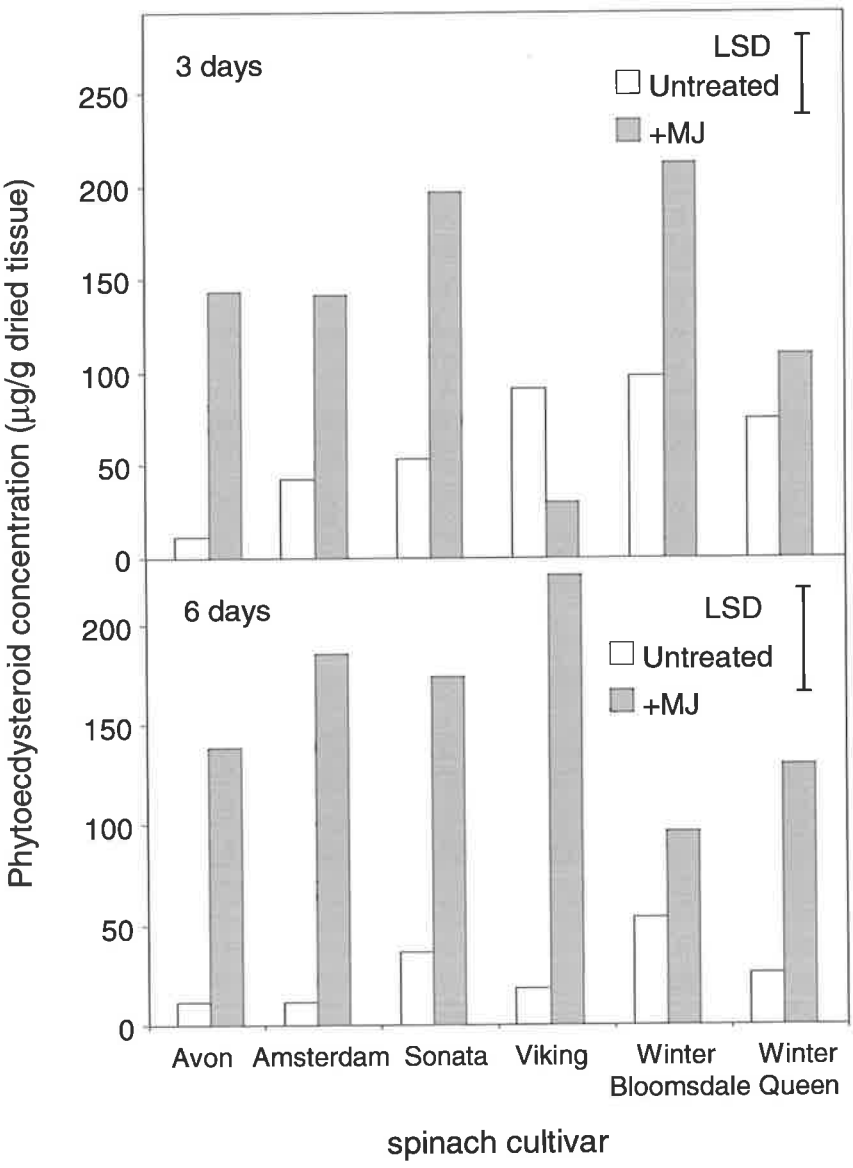

Figure 4.1. Concentration of phytoecdysteroids in the roots of different varieties of spinach 3 and 6 days after treatment of methyl jasmonate (+MJ); treated = shaded, untreated = unshaded; bar indicates LSD at $P < 0.01$

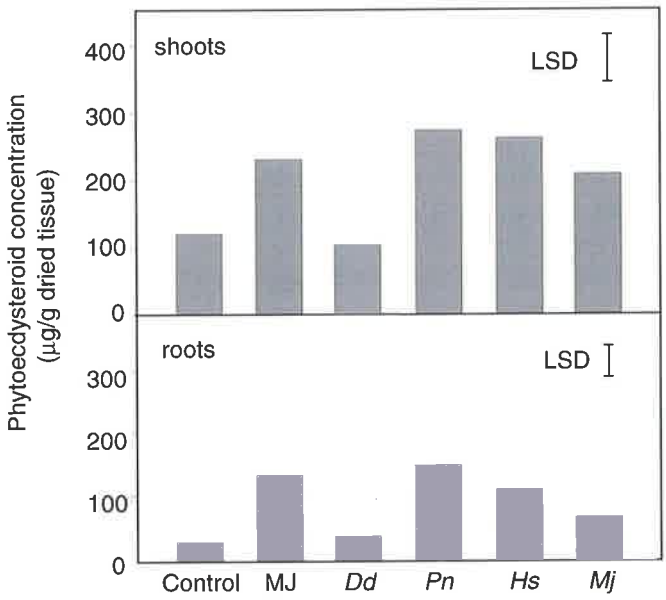

Figure 4.2. Concentration of phytoecdysteroids in the shoots and roots of spinach 5 days after inoculation of *Ditylenchus dipsaci* (Dd), *Pratylenchus neglectus* (Pn), *Heterodera schachtii* (Hs) and *Meloidogyne javanica* (Mj), and treatment of methyl jasmonate (MJ); control is untreated plants; bar indicates LSD at $P < 0.01$

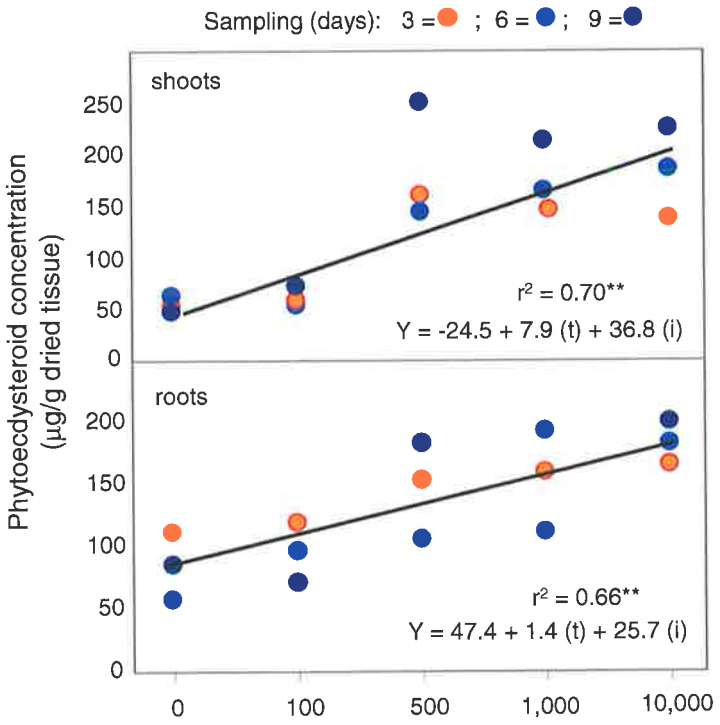

Figure 4.3. Relationship between the phytoecdysteroid concentrations in the roots and shoots of spinach, and inoculum levels (i) of *Pratylenchus neglectus* at 3, 6 and 9 days (t) after inoculation. Each point represents the mean of phytoecdysteroid concentration for each inoculum and sampling time interaction (t x i); double asterisk after the *r* value indicates significance at $P < 0.01$, n = 15

inoculation, *P. neglectus* induced the highest level of 20E in spinach roots (109 and 81% increase, 5 and 10 days after inoculation, respectively), followed by *H. schachtii* (86 and 74% increase) and *M. javanica* (65 and 60% increase). This was not significantly different from roots treated with MJ which increased levels of 20E by 92 and 79% 5 and 10 days after treatment, respectively.

Levels of 20E in the shoots increased by 79 and 60% when inoculated with *H. schachtii*, 79 and 62% with *P. neglectus* and 58 and 52% with *M. javanica* 5 and 10 days after treatment, respectively (Fig. 4.2). This was not significantly different from the 75% (5 days) and 61% (10 days) increase in 20E levels in the shoots of MJ treated plants (Fig. 4.2).

Effects of nematode inoculum levels on 20E induction in spinach

A significant positive relationship between the nematode inoculum levels and the 20E concentrations in the shoots ($r^2 = 0.70$, $P < 0.01$; n = 15) and roots ($r^2 = 0.66$, $P < 0.01$; n = 15) of spinach was observed (Fig. 4.3). The sampling time did not affect the 20E levels in both shoots and roots. The inoculum level 100 *P. neglectus*/plant did not increase 20E levels in the shoots and roots at all sampling times (Fig. 4.4). The 20E levels in the shoots and roots of spinach were increased by inoculation with 500, 1,000 and 10,000 *P. neglectus* per plant (Fig. 4.4). There was no difference in the increase of 20E levels induced by these three inoculum levels in spinach roots at any sampling time (Fig. 4.4). However, differences in the effects of these inoculum levels were observed in the shoots 6 days after inoculation where 10,000 *P. neglectus*/plant increased 20E levels by 73 and 56% over plants with 500 and 1,000 *P. neglectus*/plant (Fig. 4.4), respectively.

Similar to the observations in the earlier section, 20E levels in the roots of spinach were increased after treatment with methyl jasmonate. This increase in 20E levels in the roots after treatment with methyl jasmonate was greater by 109, 106 and 144% (3

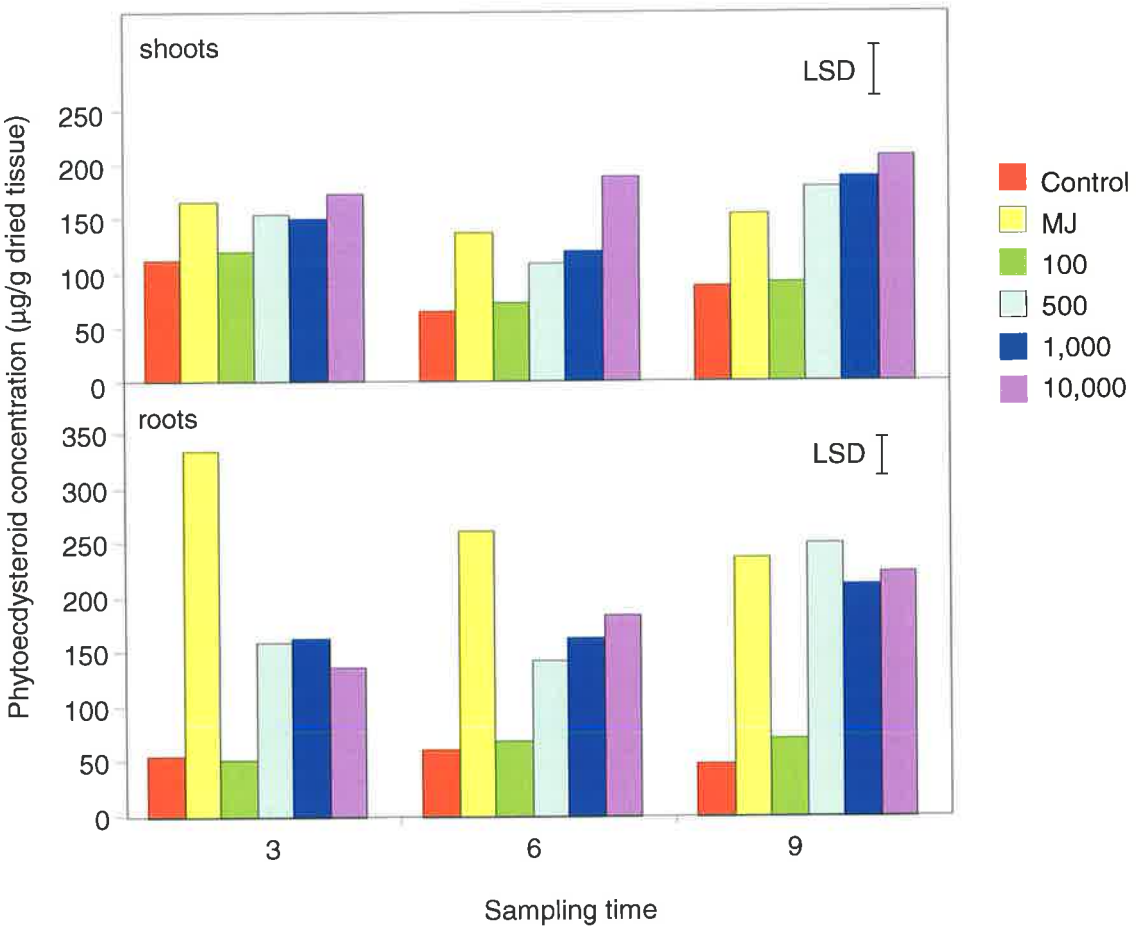

Figure 4.4 Concentration of phytoecdysteroids in shoots and roots of spinach at 3, 6 and 9 days after inoculation of *Pratylenchus neglectus* at the rate of 100, 500, 1,000 and 10,000 nematodes/plant. Plants treated with methyl jasmonate (MJ) and untreated/uninoculated (Control) were included for comparison of response; bar indicates LSD at $P < 0.05$ for shoots and $P < 0.01$ for roots (n = 6)

days after treatment), and 84, 60 and 42% (6 days after treatment) compared to the increase of 20E levels by inoculation of 500, 1,000 and 10,000 nematodes/plant, respectively (Fig. 4.4). There was no difference in the 20E levels in the roots treated with methyl jasmonate or nematodes 9 days after treatment or inoculation.

The 20E levels in the shoots were higher by 38 and 34% when inoculated with 10,000 nematodes/plant than when treated with methyl jasmonate at 6 and 9 days after inoculation (Fig. 4.4), respectively. The increase in the 20E levels in the shoots 3 days after treatment or inoculation was not different.

4.3.2 OATS

Treatment of two oat cultivars with methyl jasmonate (Fig. 4.5) increased concentrations of the methanol extractable compounds detected by HPLC in aqueous methanol extracts in the roots of cv. Swan (149.12%) and cv. Quoll (140%) as well as in the shoots of these cultivars (274 and 84%, respectively). Inoculation with the nematodes, *H. avenae* and *P. neglectus* increased concentrations of these compounds in the roots of cv. Swan by 95 and 112% and in the shoots by 249 and 293%, respectively (Fig. 4.5). Similarly, the concentrations of these compounds were increased in cv. Quoll after inoculation with *H. avenae* and *P. neglectus* (52 and 83% in roots and 59 and 75% in shoots, respectively). *D. dipsaci* did not increase the concentration of the compounds in the shoots or roots (Fig. 4.5). No other methanol extractable compound was detected to be inducible by methyl jasmonate or nematodes. Methyl jasmonate treatment did not affect plant growth. Details and identification of these compounds is discussed in Chapter 9.

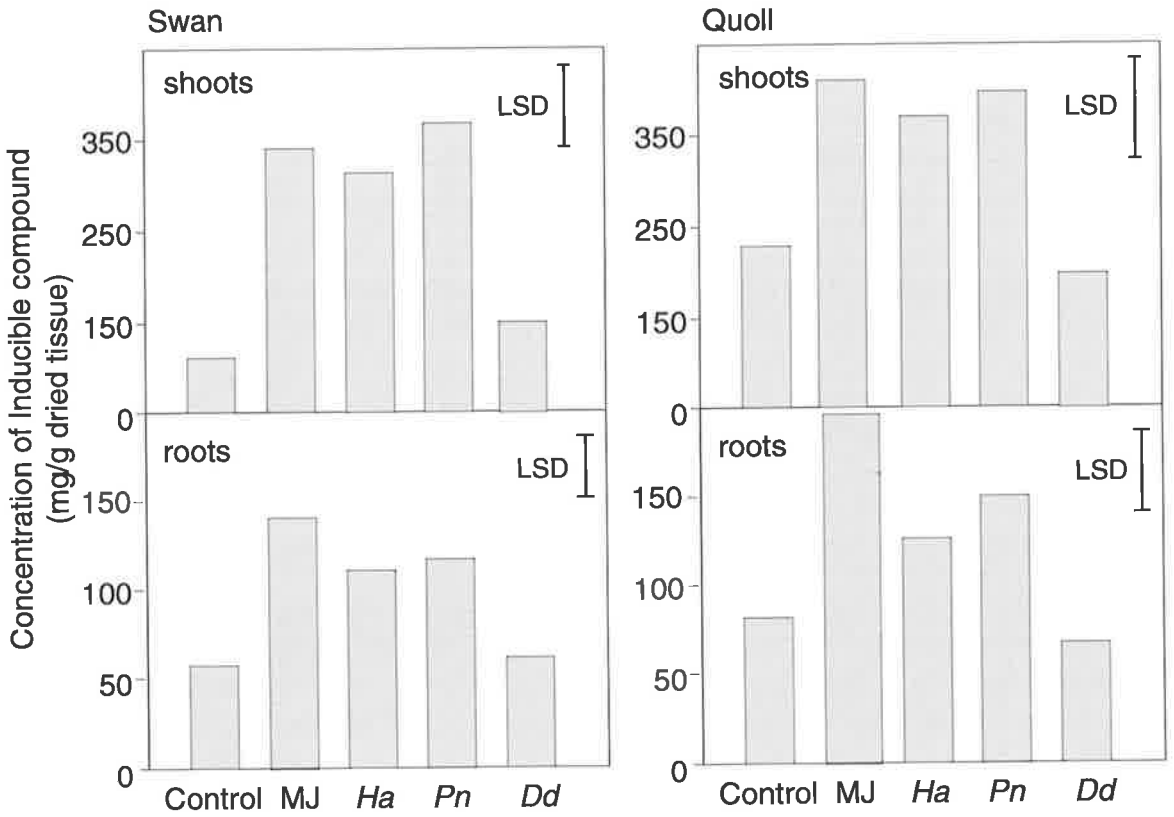

Figure 4.5 Concentration of inducible compounds in shoots and roots of two oat cultivars (Swan and Quoll) 5 days after inoculation of *Heterodera avenae* (Ha), *Pratylenchus neglectus* (Pn), and *Ditylenchus dipsaci* (Dd), and treatment of methyl jasmonate (MJ); control was untreated plants; bar indicates LSD at $P < 0.01$ for shoots and $P < 0.05$ for roots

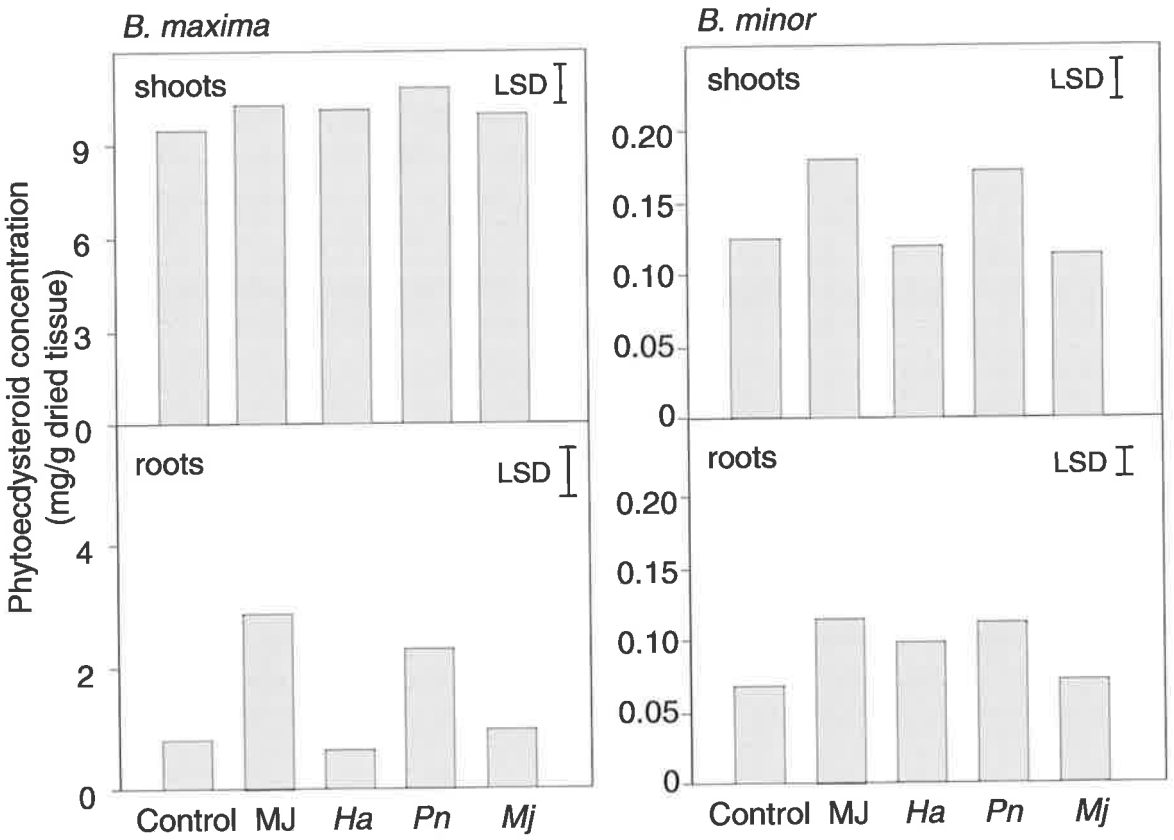

Figure 4.6 Concentration of phytoecdysteroid-like inducible compounds in shoots and roots of *Briza* spp. (*B. maxima* and *B. minor*) 5 days after inoculation of *Heterodera avenae* (*Ha*), *Pratylenchus neglectus* (*Pn*), and *Meloidogyne javanica* (*Mj*), and treatment of methyl jasmonate (MJ); control was untreated plants; bar indicates LSD at $P < 0.01$ for shoots and $P < 0.05$ for roots

4.3.3 *BRIZA* SPP.

Five inducible compounds were detected in the shoot and root methanolic extract of *B. maxima* using both isocratic and gradient elution methods. One of the inducible compounds, which was considered a major component of the methanolic extract because of its high concentration compared to the other components, has a similar retention time to 20E. As 20E, along with polypodine B, was designated as major compound in *B. maxima* and *B. minor* (Savchenko *et al.*, 1998) and well studied in spinach, this component was quantified and referred to as the inducible compound in this plant.

The level of the inducible compound was increased 1.5 fold in the roots of *B. maxima* by *P. neglectus*. *H. avenae* and *M. javanica* did not induce the compound in this plant (Fig. 4.6). Application of methyl jasmonate increased the level of the compound by 2 fold in the roots. This compound was not induced in the shoots by either the nematodes or methyl jasmonate application.

B. minor was found to have three inducible compounds, one of which had a similar retention time of 20E (Fig. 4.6). The level of this compound, referred to as the inducible compound in *B. minor*, increased in the roots by 64 and 36% after inoculation of *P. neglectus* and *H. avenae*, respectively (Fig. 4.6). Likewise, application of methyl jasmonate increased the level of the compound in the roots by 70%. Inoculation of *P. neglectus* and application of methyl jasmonate increased levels of the inducible compound in the shoots by 39 and 45%, respectively. The level of this compound in the shoots of *B. minor* inoculated with *H. avenae* and *M. javanica* did not increase (Fig. 4.6).

4.3.3 LUCERNE

Two compounds with retention time of 6.36 and 8.76 min were found to be inducible in lucerne. The first compound (LIC 1) has similar retention time (6.36 min) and properties as 20E and was greater in the shoots than in the roots of most cultivars. Conversely, the

second compound (LIC 2) was greater in concentration in the roots than in the shoots in most of the cultivars.

Application of methyl jasmonate increased the concentration of LIC 1 by 1 – 9 fold and LIC2 by 2 - 6 fold in the roots of all cultivars. Shoots of 4 lucerne cultivars had 0.6 – 1.2 and 1 – 5 fold increase of LIC 1 and 2, respectively. Inoculation of *D. dipsaci* increased the levels of the LIC 1 in the roots of 3 lucerne cultivars by 1, 2 and 3 fold, respectively, and two of the shoots (0.8 and 0.9 fold) of the lucerne cultivars. On the other hand, the level of LIC 2 was increased in the roots of all lucerne cultivars by 2 – 5 fold and in the shoots of 4 cultivars by 1 – 3 fold.

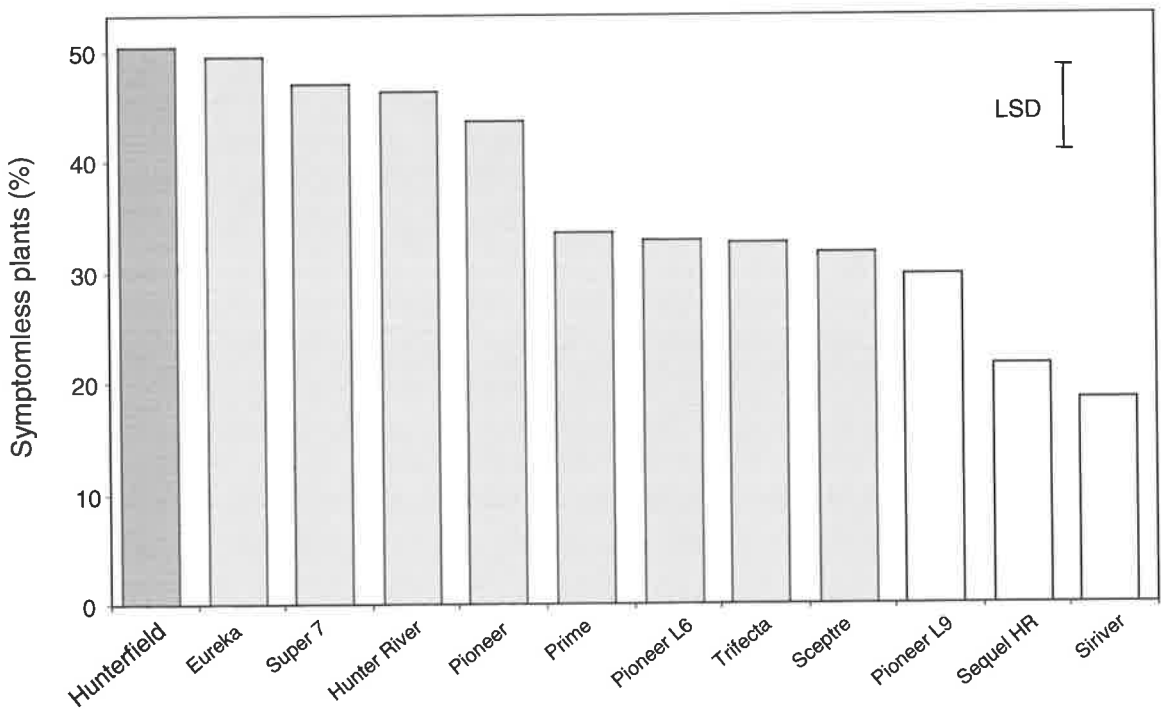

Figure 4.7 Proportion of resistant lucerne cultivars to *Ditylencus dipsaci* (lucerne strain); Similarly shaded and unshaded bars are considered resistant and moderately resistant using the standard rating scale for lucerne against the nematode; bar indicates LSD at $P < 0.05$

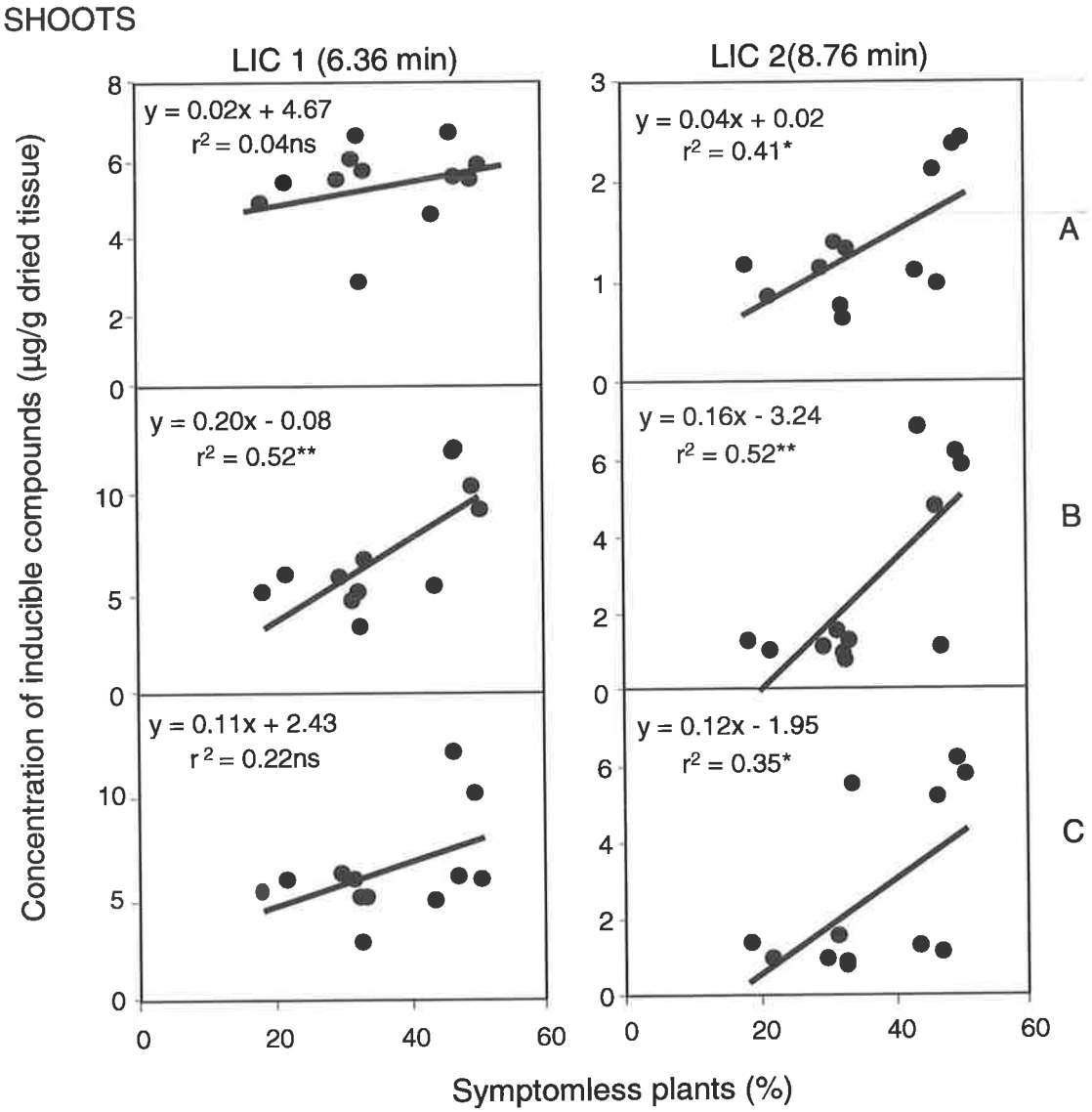

Figure 4.8 Relationship between the concentration of the two inducible compounds (LIC 1 and LIC 2) in the shoots of 12 cultivars of lucerne as induced by *Ditylenchus dipsaci* (lucerne strain) and application of methyl jasmonate, and the proportion of resistant plants; Panel A. untreated plants; B. methyl jasmonate treatment; C. inoculation of *D. dipsaci*. Each point represents the average for each variety (n = 6)

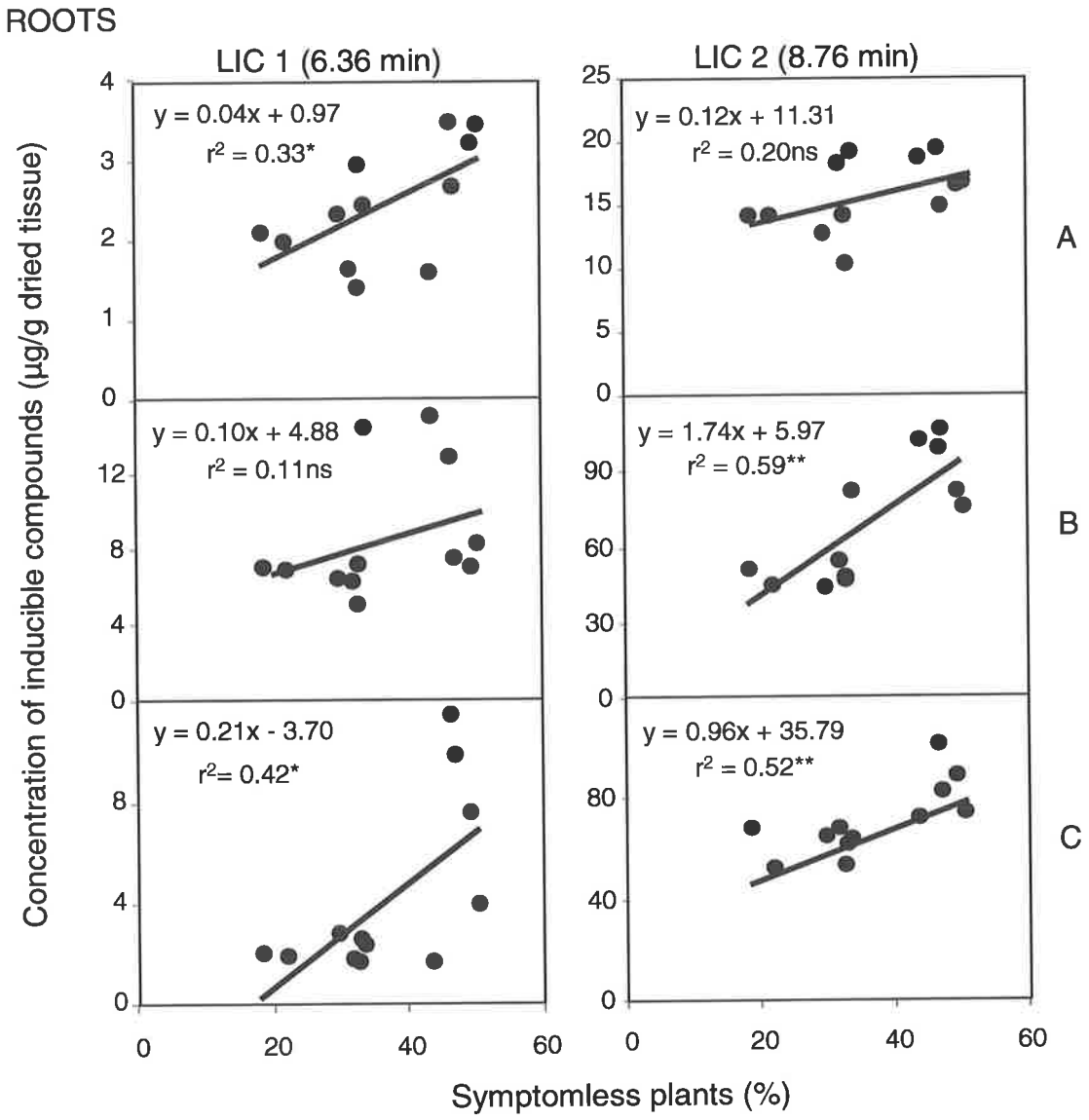

Figure 4.9 Relationship between the concentration of the two inducible compounds (LIC 1 and LIC 2) in the roots of 12 cultivars of lucerne as induced by *Ditylenchus dipsaci* (lucerne strain) and application of methyl jasmonate, and the proportion of resistant plants; Panel A. untreated plants; B. methyl jasmonate treatment; C. inoculation of *D. dipsaci*. Each point represents the average for each variety (n = 6)

The resistance response to *D. dipsaci* varied among the 12 lucerne cultivars (1 highly resistant, 8 resistant, and 3 moderately resistant) based on the standard disease rating scale for lucerne against *D. dipsaci* (Elgin *et al.*, 1984). However, statistical analysis assigned the highly resistant cultivar, Hunterfield, as not significantly different from four of the resistant cultivars (Fig. 4.7). One of the moderately resistant cultivars (Pioneer LR) was likewise not significantly different from four of the resistant cultivars (Fig. 4.7). A significant positive relationship between the proportion of resistant lucerne cultivars and the constitutive levels of the LIC 2 in the shoots ($r^2 = 0.41$, $P = < 0.05$, n = 12) and LIC 1 in the roots ($r^2 = 0.33$, $P = < 0.05$, n = 12) was observed (Figs. 4.8 and 4.9). The levels of both compounds in the shoots ($r^2 = 0.52$, $P = < 0.01$, n = 12) after treatment of methyl jasmonate were significantly related to the proportion of resistant plants (Fig. 4.8). This was also the case in the roots ($r^2 = 0.59$, $P < 0.01$; n = 12) but with LIC 2 only (Fig. 4.9). A significant relationship was observed between the resistant ratings and the levels of LIC 1 ($r^2 = 0.42$, $P = < 0.05$, n = 12) and LIC 2 ($r^2 = 0.52$, $P = < 0.01$, n = 12) in the roots of lucerne cultivars inoculated with *D. dipsaci* (Fig. 4. 9). The same case is true with the LIC 2 in the shoots ($r^2 = 0.42$, $P = < 0.05$, n = 12) of the cultivars (Fig. 4.8).

4.4 DISCUSSION

The experiments on induction of compounds in plants led to the conclusion that nematode parasitism can induce secondary metabolites such as the phytoecdysteroid, 20E, in the roots and shoots of plants. In plant-nematode interactions, several secondary metabolites have been shown to be induced in plants after nematode invasion (Zacheo and Bleve-Zacheo, 1995) and were linked to plant resistance responses (Giebel, 1974; Veech, 1982).

The migratory endoparasitic nematode *P. neglectus* induced methanol-extractable compounds in the plants tested and the induction of these compounds was usually higher than the level induced by other nematodes. The random and destructive nature of the

nematode's feeding behaviour in the roots may have predisposed the plant to stimuli that activated signal molecules from the site of damage. All stages of *P. neglectus* freely invades and leaves roots, feeding preferentially on root hairs and also attacks cortical tissue in lateral roots (Zunke, 1990). As the nematode migrates intracellularly, it kill cells in contact extending the damage to adjacent cells (Zunke, 1990; Townshend *et al.*, 1989) which prompt plants to induce defence responses such as polyphenols and lignin-like substances (Townshend and Stobbs, 1981; Townshend *et al.*, 1989).

Heterodera spp. are sedentary endoparasitic nematodes that were also able to induce methanol-extractable compounds in some inoculated plants in this study. *H. schachtii* increased the levels of 20E in the roots and shoots of spinach. Inoculation of *H. avenae* caused increase of compounds in the roots of *B. minor* and in the roots and shoots of oats. *Heterodera* migrates intracellularly like *P. neglectus* but moves directly towards the differentiating vascular tissue at the root tips where it establishes a permanent feeding site (Wyss and Zunke, 1986).

In contrast, *M. javanica*, a sedentary endoparasitic nematode, increased levels of 20E in the shoots of spinach only. The feeding behaviour of the root-knot nematode may be one of the factors that caused the failure to induce compounds in spinach roots. The invasive stage (J2) of the root-knot nematode migrates intercellularly through the cortex of the roots until a developing vascular tissue is reached causing limited damage (Paulson and Webster, 1970). Upon injection of secretions through the stylet of the J2 into the developing xylem parenchyma cells, the affected cells of the plant undergo a dramatic transformation, becoming giant, multinucleated cells on which the nematode non-destructively feeds (Huang, 1985). In the case of the *Briza* spp., there was no root invasion by *M. javanica* nor induction of methanol-extractable metabolites. Hence, the non-induction of compounds in the incompatible interaction between *Briza* spp. and the root-knot nematode as opposed to induction of compounds with methyl jasmonate and

compatible interaction with *P. neglectus* indicates that wound signals may be responsible for the induction of these compounds. The study demonstrated that accumulation of defence compounds occur not only in incompatible interactions (Smith, 1996) but significant accumulation of these compounds could also occur in the compatible interaction.

The stem nematode, *D. dipsaci* did not increase levels of methanol-extractable compounds in inoculated plants except in some of the lucerne cultivars which were also rated as resistant to the nematode. *D. dipsaci*, is a migratory endoparasite that feeds on the contents of host plant cells (Hooper, 1972). Accumulation of phytoalexins in roots of lucerne following infection by *D. dipsaci* (Edwards *et al.*, 1995) and in rice stem by *Ditylenchus angustus* (Plowright *et al.*, 1996) was previously reported.

Nematodes as biotic elicitors of compounds in plants have been reported previously (Veech, 1982). Most of these compounds were linked to resistance in plants against the invading pest although in some cases, the induced compounds were not shown to be related to resistance. The first plant-nematode interaction study that specifically reported the induction of compounds was that of Abawi *et al.* (1971) where the phytoalexin, phaseolin, was extracted from kidney beans (*Phaseolus vulgaris*) 5 days after inoculation of *Pratylenchus penetrans*. However, while phaseolin was synthesized by the host plant in response to nematode infection, exogenous application of this compound failed to exhibit antihelminthic activity. In contrast, biological activity was reported in other cases such as in resistant lima bean (*Phaseolus lunatus*) infected by *Pratylenchus scribneri*, which was found to produce coumestrol (Rich *et al.*, 1977). Likewise, soybean (*Glycine max*) infected by *Meloidogyne incognita* (Kaplan *et al.*, 1980a,b) or *Heterodera glycines* (Huang and Barker, 1991) produced glyceollin. Cotton (*Gossypium hirsutum*) produced terpenoid aldehydes upon infection with *M. incognita* (Veech, 1977). However, in some cases, nematodes failed to induce detectable secondary metabolites in plants. Baldridge *et al.*

(1998) reported that *P. penetrans* infection in alfalfa (*Medicago sativa*) had no effect on isoflavonoid levels although constitutive levels were highest in the roots of two resistant plants.

In this study, an inoculum of 500 nematodes or more per plant was able to induce the 20E levels in the shoots and roots of spinach while an inoculum of 100 *P. neglectus* and probably below this inoculum level per plant did not induce 20E in spinach. However, Plowright *et al.* (1996) demonstrated the sensitivity of the resistant rice cultivar Rayada 16-06 to *D. angustus* where low numbers, 10 and 200 nematodes/plant, induced metabolites in rice at the rate of 8 and 13 μg/g leaves, respectively. Rich *et al.* (1977) inoculated very high numbers (3,000-7,000) of *Pratylenchus scribneri* onto lima bean to induce coumestrol and psoralidinin at 89 and 39 μg/g fresh weight, respectively. In red kidney beans, 59 μg phaseolin/g root was induced after inoculation of 10,000 *P. penetrans* (Abawi *et al.*, 1971). In this study, inoculum rate is positively correlated to the 20E levels induced regardless of sampling period. This is the first study to demonstrate the effective inoculum density for induction of 20E. It is also taken into account that these data may vary depending on the nematode and plant.

In addition to its role in plant growth and development, jasmonate has been proposed as a key regulator of responses to pathogens and pests (Ryan, 1992). The wound signal analogues, jasmonic acid or methyl jasmonate, are signal molecules in plant defence responses (Gundlach *et al.*, 1992; Reinbothe *et al.*, 1995; Creelman and Mullet, 1995). In the study, methyl jasmonate significantly increased levels of methanol-extractable compounds like 20E and flavonoids (identity of compounds presented in Chapter 5) in the roots and shoots of the plants tested. Whether these compounds are involved in plant defence or are products of plant defence response can not be established at this point. However, as these compounds are also induced by nematode invasion, it is likely that they are associated with the plants' defence response to the pest. Low concentrations of

jasmonate were found to induce enzymes involved in flavonoid biosynthesis (chalcone synthase and phenylalanine ammonia lyase, Creelman *et al.*, 1992; Gundlach *et al.*, 1992), sesquiterpenoid biosynthesis (hydroxymethylglutaryl CoA reductase, Choi *et al.*, 1994), genes encoding proteinase inhibitors (Farmer and Ryan, 1992) and defence associated proteins (Andersen *et al.*, 1992; Gundlach *et al.*, 1992; Xu *et al.*, 1994; Mueller *et al.*, 1993).

This study also demonstrated that the 20E in different cultivars of spinach is inducible by methyl jasmonate. Apparently, the time of induction appeared to vary among varieties. For example, it was 6 days before an induction of 20E was evident in Viking which was slower than the other cultivars tested. The varieties Winter Bloomsdale, Viking, Winter Queen and Sonata had higher constitutive levels of 20E than Avon and Amsterdam. However, application of methyl jasmonate greatly increased the 20E level in Avon at 3 and 6 days and Amsterdam at 6 days after induction. Although 20E accumulation and stability in spinach had been well established making it a good model plant (Grebenok and Adler, 1993; Schmelz *et al.*, 2000), the lack of spinach resistant germplasm to nematodes is a hindrance in attempts to link the phytoecdysteroid to resistance. However, the varietal tests in this study showed that 20E is present in common cultivars of spinach.

Another key finding from this study is the correlation between inducible compounds, and response of lucerne to *D. dipsaci*. Subsequent to infection by *D. dipsaci*, the secondary chemistry of resistant cultivars of lucerne was altered except for the first compound (LIC 1) in the shoots. The second compound (LIC 2) in lucerne could have a functional role in resistance.

The lucerne cultivars tested were chosen due to their varying degree of resistance to the nematode *D. dipsaci*. Interestingly, the compounds with the lower constitutive concentration in the shoots (LIC 2) and roots (LIC 1) were observed to have a significant

relationship with resistant ratings of the cultivars while compounds that were constitutively higher in concentration were not functionally related. Application of wound elicitor like methyl jasmonate increased the concentration of the inducible compounds in the roots and shoots (except LIC 1 in the roots) as the proportion of resistant plants increased. This points to a possible involvement of these inducible compounds in resistance. Likewise, the concentrations of inducible compounds were higher in cultivars with higher proportions of resistant plants when inoculated with *D. dipsaci*, which further strengthen the role of these compounds in nematode resistance in lucerne. The induction of compounds after treatment of methyl jasmonate was similar to that of *D. dipsaci* inoculation. The increase of these compounds in the roots of cultivars treated with methyl jasmonate or inoculated with *D. dipsaci* suggests a general wounding response. However, reaction in the shoots would be more strongly linked to the resistance response of the plants as *D. dipsaci* invades the shoots and not the roots. The induction of compounds in the roots of all cultivars and in the shoots of only four lucerne cultivars was not surprising since methyl jasmonate was applied as soil drench although the signal molecule is highly volatile.

The accumulation of the compounds in the roots of lucerne when inoculated with *D. dipsaci* was similar to the systemic induction of isoflavonoid observed when alfalfa seedlings were infected with alfalfa race of *D. dipsaci* (Edwards *et al.*, 1995). Isoflavonoid conjugates accumulated in the roots but not in the foliage where the nematodes were localised. Likewise, a resistant alfalfa cultivar accumulated higher concentrations of the conjugates of formononetin, medicarpin and coumestrol in the roots than a susceptible cultivar and this was associated with an increase in the specific activity of the enzyme phenylalanine ammonia lyase in the roots (Dixon, 1986). This differs from the results obtained by Cook *et al.* (1995) where infection with *D. dipsaci* in resistant and susceptible white clover cultivars elicited a localised accumulation of isoflavonoids and their conjugates.

In summary, this chapter describes an increase in the level of secondary metabolites in plants challenged by nematodes or treated with a wound signal analogue, methyl jasmonate. Secondary metabolites are usually synthesized in plants following stress such as nematode infection. This study may provide evidence of the involvement of these compounds in plant defence which will be confirmed in later chapters. The association of a compound in lucerne with resistance to the foliar nematode possibly indicates a resistance mechanism to nematodes.

CHAPTER 5. BIOLOGICAL AND CHEMICAL CHARACTERISATION OF INDUCIBLE COMPOUNDS IN CRUDE EXTRACTS

5.1 INTRODUCTION

In earlier studies, phytoecdysteroids were mainly associated with ferns and gymnosperms (Dinan *et al.*, 1998). However, with the advent of bioassays (Cymborowski, 1989), immunological methods (Reum and Koolman, 1989) and reverse and normal phase-separation, more phytoecdysteroids were detected from a wider range of plants (Dinan *et al.*, 2001).

The *Drosophila melanogaster* B_{II} cell microplate-based bioassay is generally used to screen and detect biologically active ecdysteroids, non-steroidal agonist and anti-ecdysteroids in plant extracts (Clement *et al.*, 1993; Dinan, 1998). It has the advantage of being rapid, simple and reproducible. The major criterion for activity is the affinity of the interaction with the ecdysteroid receptor. The B_{II} cell line is known to possess ecdysteroid receptors and active ecdysteroid compounds will interact with this protein without the cell line metabolising the ecdysteroid (Dinan, 1985). Hence, by using the B_{II} cells, problems of penetration, sequestration and excretion are minimized. The B_{II} cells respond to ecdysteroids with reduction in cell density and cell clumping which may be assessed turbidometrically through the absorbance at an appropriate wavelength (Clement *et al.*, 1993; Clement and Dinan, 1991; Dinan, 1985).

The RP-HPLC coupled with the photodiode array detection is important for the examination and isolation of ecdysteroids in plant extracts (Lafont *et al.*, 1982). Ecdysteroids have strong ultraviolet absorption near 242 nm, which makes them appropriate for the UV detection (Morgan and Wilson, 1989). The elution order of ecdysteroids in RP-HPLC depends upon the molecular structure, in which the position of the hydroxyl groups is the most important molecule factor, and is also strongly affected by the stationary and mobile phases. Acetonitrile, methanol and tetrahydrofuran are among the commonly used solvents (Wilson *et al.*, 1982).

In Chapter 4, *Spinacia oleracea* (spinach), *Briza maxima* (quaking grass), *Medicago sativa* (lucerne) and *Avenae sativa* (oats) were found to contain inducible compounds. It was not known if the inducible compounds were biologically active ecdysteroids. Hence, the presence of inducible biologically active ecdysteroids in spinach, quaking grass, lucerne and oat root and shoot extracts were examined and characterized using the BII cell line bioassay, RP-HPLC, UV absorbance and mass spectrometry.

5.2 METHODS

5.2.1 PREPARATION OF PLANT EXTRACTS

Spinach cv. Avon, quaking grass, lucerne cv Eureka and Oats cv Quoll were grown and maintained as described in section 3.1. Methyl jasmonate (10 ml 10^{-4} M) was applied as soil drench around the plants 15 days after transplanting for spinach and 15 days after sowing for the other plants. Five days after treatment, sand was washed carefully from roots of plants. Shoots and roots were snap frozen in liquid nitrogen right after washing and lyophilised in a freeze drier. Inducible compounds were extracted from the roots and shoot using the extraction procedure outlined in Section 3.3 and were detected and quantified using an isocratic elution RP-HPLC described in Section 3.4.

5.2.2 MICROPLATE-BASED BIOASSAY FOR ECDYSTEROID-LIKE OR ANTI-ECDYSTEROID ACTIVITIES

Culture of B_{II} tumorous blood cell line

A stock culture of B_{II} tumorous blood cell line was stored in Schneider's medium (Sigma) with 15% (v/v) DMSO at –80 C until use. It was rapidly thawed and 4 ml Schneider's medium was added dropwise. The Schneider's medium (500 ml) was supplemented with 50 µg/ml penicillin/streptomycin, 50 µg/ml gentamycin, 5 ml glutamax and 25 ml foetal calf serum. The B_{II} cells were then centrifuged at $1.5x10^4$ rpm for 5 min

and the supernatant was replaced with 10 ml Schneider's medium. Centrifugation was repeated and 5 ml Schneider's medium was added after discarding the supernatant. The B_{II} cell suspension was dispensed gently in 50 ml polystyrene cell culture flasks. The cell cultures were incubated in the dark at 25°C until the dividing cells were semiconfluant. At this stage, the medium in which the cells were growing was discarded and subsequently replenished with new Schneider's medium at 3-day intervals throughout the incubation period.

Microtitre plate assay (Clement and Dinan, 1991)

Logarithmically increasing concentrations of 20E were initially tested in the bioassay. Concentrations of 20E were prepared from a stock solution of $1.0x10^{-5}$ M 20E (10 mg 20E/ml methanol). Serial dilutions were made to obtain the following concentrations: $2.1x10^{-6}$, $4.2x10^{-7}$, $1.7x10^{-8}$, $8.3x10^{-8}$, $3.3x10^{-9}$ and $6.7x10^{-10}$ M 20E. For the subsequent experiment, the treatments used were as follow: (*i*) B_{II} cells only; (*ii*) B_{II} cells with test plant extract; (*iii*) B_{II} cells with 20E ($1x10^{-7}$ M); (*iv*) B_{II} cells with 20E ($1x10^{-7}$ M) and test plant extract. A 20 µl aliquot of the different concentrations of 20E, plant extracts diluted with methanol (1:1 v/v) and $1x10^{-7}$ M 20E were dispensed into the wells of flat-bottomed sterile microtitre plates (Cell-Cult) according to the treatments above. These were allowed to dry in a laminar flow hood. Meanwhile, B_{II} cells were harvested from the tissue culture flasks and the cell density was adjusted to $2x10^5$ cells/ml Schneider's medium. After the solvent had evaporated from the microtitre plates, 200 µl of the B_{II} cells were added into each well. The microplates were then covered and incubated in the dark at 25°C with high relative humidity (85%) to reduce evaporation from the wells. All the steps were performed aseptically in a laminar flow hood. After 6 days of incubation, the absorbance at 405 nm of each well relative to the control (200 µl Schneider's medium alone) was determined using a microtitre plate reader (Bio-Rad

Model 2550 EIA reader) to assess the cell density. Cells were also observed *in situ* in the microtitre plate wells using an inverted microscope (Leica DM IL) to check for ecdysteroid and cytotoxic or cytostatic effects.

5.2.3 GRADIENT ELUTION RP-HPLC

For UV-photodiode array detection (range: 190 – 750 nm), RP-HPLC analysis of the plant extracts was performed on a Hewlett Packard 1100 Series HPLC system with diode array detector equipped with gradient controller. Separation was carried out using a LiChroCART® 250-4 reverse phase C_{18} column (5 μm, 150x3.9 mm I.D.; Merck). A guard column (LiChroCART® 4-4 RP-18; 5 μm; Merck) was used to protect the analytical column. Elution was carried out at a flow rate of 0.65 ml/min with the following solvent system: (A) water + formic acid (pH 3.0), (B) 80% acetonitrile + 15%water + 5% formic acid; 0 to 80% B in 60 min., then 0 B until 70 min. Separation was monitored at three signals, 280, 254 and 340 nm. The absorbance spectra of the compounds were recorded using an HP-1050 photodiode array detector (Hewlett-Packard) with 20E, polypodine B and ecdysone as standards.

5.2.4 MASS SPECTROMETRY

For structural analysis, HPLC-mass spectrometry (LC-MS) was performed. Separation of inducible compounds was achieved on a reversed-phase column (SYNERGI Hydro-RP Phenomenex, 4 micron, 80A, 150 x 2 mm) with a flow rate of 180 μl/min and injection volume of 20 μl. Gradient elution was run with solvent A (1:19 formic acid:water) and solvent B (1:3:16, formic acid:water:acetonitrile) following a schedule of 10 to 35% solvent B for 35 min. then 35 to 60% B for 25 min. The HPLC column was connected to a UV-*Vis* detector (HP1100, Hewlett Packard) monitoring at 280 and 340 nm, followed by a mass spectrometer with an ion spray ion source (API-300, PE Sciex, Thornhill, Ontario,

Canada). The mass spectrometer was operated in positive ion mode and was scanned from m/z 250 to m/z 1000 in 1.88 s. Ion spray and orifice potentials were set at 5.5 kV and 30 V, respectively. Curtain and nebuliser gases were nitrogen and air, respectively. All mass spectral data were processed using Bio-Multiview software (version 1.2ß3, PE Sciex).

5.2.5 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

The dilutions for the B_{II} cell dose response to 20E were dispensed in microtitre plates in six replicates. Likewise, the treatments (*i-iv*) for ecdysteroid detection in plant extracts were applied in six replicates. Analysis of variance was used to analyse the data for significant treatment effects and means were compared using the Least Significant Difference test (LSD).

5.3 RESULTS

5.3.1 MICROPLATE-BASED BIASSAY

20E response curve

In the absence of 20E, the B_{II} cell density increased steadily over 6 days. However, in the presence of increasing concentration of 20E, the absorbance value decreased significantly ($P < 0.05$ for the lowest concentration and $P<0.01$ for the rest) compared to the control (B_{II} cells only) by 5.56, 6.36, 44.64, 170.26, 184.35 and 263.48% for $6.7x10^{-10}$, $3.3x10^{-9}$, $8.3x10^{-8}$, $1.7x10^{-8}$, $4.2x10^{-7}$ and $2.1x10^{-6}$ M 20E, respectively after 6 days (Fig. 5.1). Cellular elongation and clumping of the cells was evident when cells were treated with 20E, and the effects were more pronounced as the concentration increased.

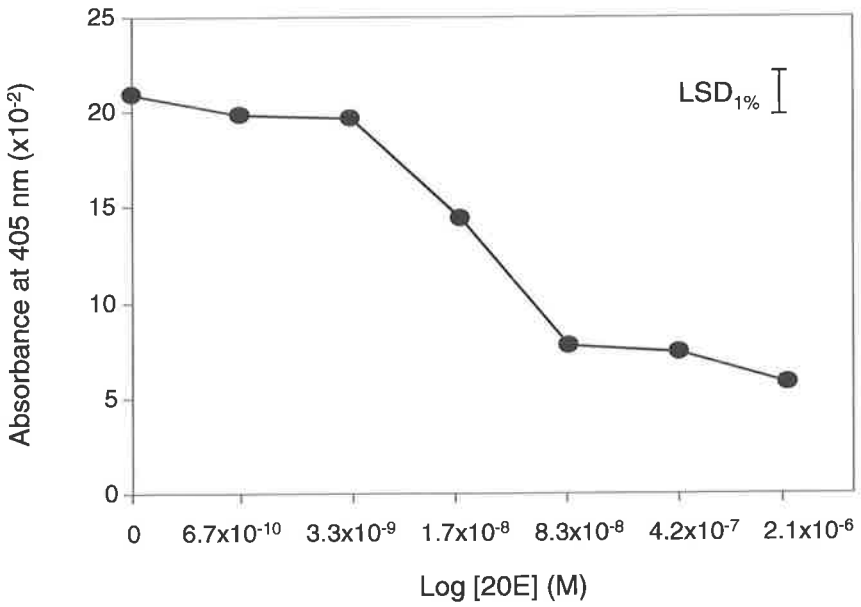

Figure 5.1 Growth of *Drosophila melanogaster* B_{II} cell line monitored by the absorbance reading of the microtitre wells as affected by different concentrations of 20-hydroxyecdysone (20E)

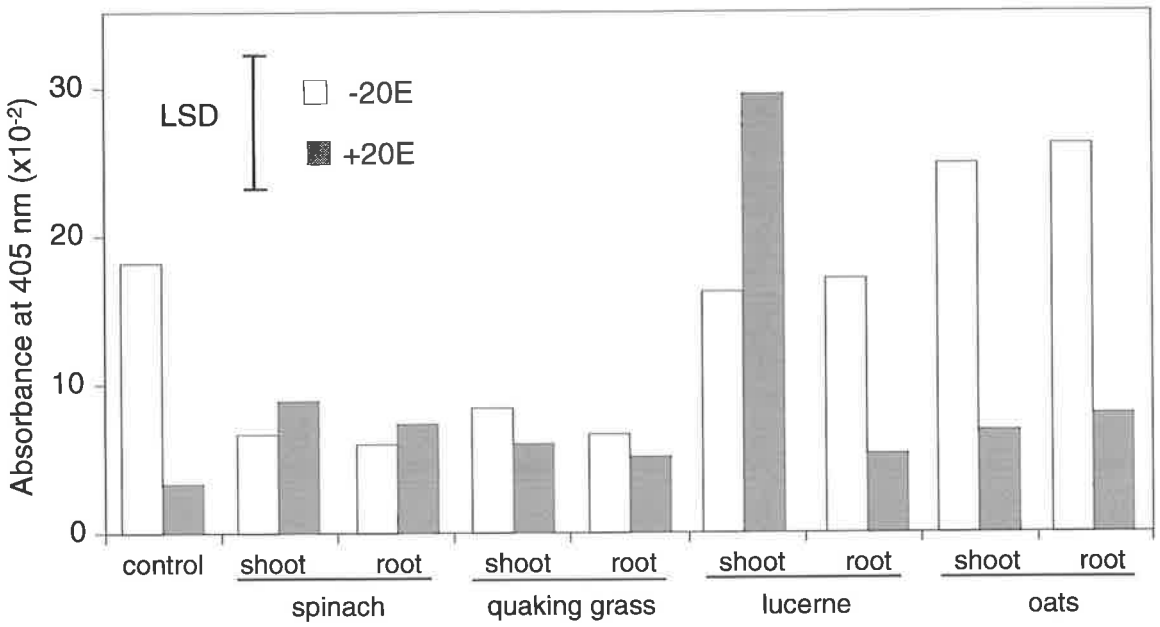

Figure 5.2 Growth of *Drosophila melanogaster* B_{II} cell line monitored by the absorbance reading of the microtitre wells as affected by shoot and root extracts of selected plants with and without the presence of $1x10^{-7}$ M 20-hydroxyecdysone (20E); control is B_{II} cells with or without 20E application

Plant extracts

Spinach and quaking grass shoot and root extracts caused inhibition of growth to B_{II} cells which was evident from the low absorbance values that were significantly different ($P < 0.05$) from the control (B_{II} cells only) and also from oats and lucerne extracts (Fig. 5.2, Table 5.1). When the cells were treated with root and shoot extracts of lucerne, the absorbance values were not significantly different from the control (B_{II} cells only). On the other hand, oat root extract increased cell growth since the absorbance values of cells treated with oat root extract were 45% higher than the control. The effect of oat shoot extract on the B_{II} cells was not significantly different from the root extract and control (B_{II} cells only). Elongation and clumping of cells was observed when treated with spinach and quaking grass extracts (Fig.5.3).

When 20E was applied (Fig. 5.2), only lucerne shoot extract had an antagonistic effect on the activity of 20E, which is reflected by the high absorbance value that was significantly different ($P < 0.05$) from the rest of the treatments including the control (B_{II} cells with 20E). Cell elongation and clumping was evident in all 20E with plant extract treated cells (Fig. 5.3).

Addition of 20E to cells treated with shoot and root extracts of lucerne and oats decreased the absorbance value significantly ($P < 0.01$, Fig. 5.2). On the other hand, the addition of 20E to B_{II} cells treated with shoot and root extract of spinach and quaking grass did not affect the absorbance value.

5.3.2 RP-HPLC

Using the isocratic RP-HPLC, the inducible compounds in all plant extracts except for oats had retention times similar to those of the three standards, 20E, polypodine B and ecdysone, used (Table 5.1). The gradient RP-HPLC separated some of the inducible

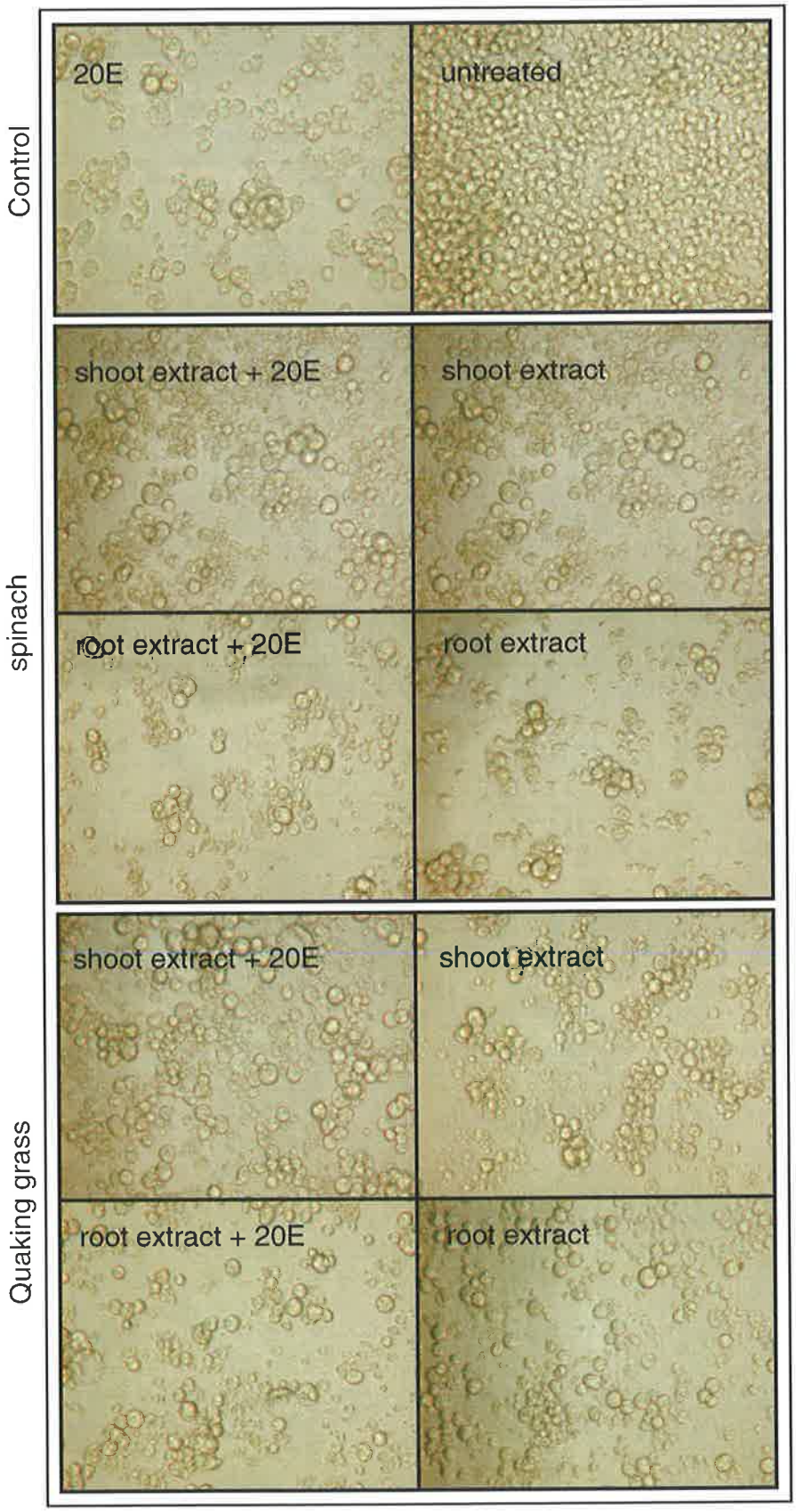

Figure 5.3 Growth of *Drosophila melanogaster* B_{II} cell line as affected by the treatment of shoot and root extracts of spinach and quaking grass (*Briza maxima*) alone or together with $1x10^{-7}$ M 20-hydroxyecdysone (20E); controls were 20E treated or untreated B_{II} cells; cell elongation and clumping are evident in the presence of ecdysteroids whether inherent in plants or applied

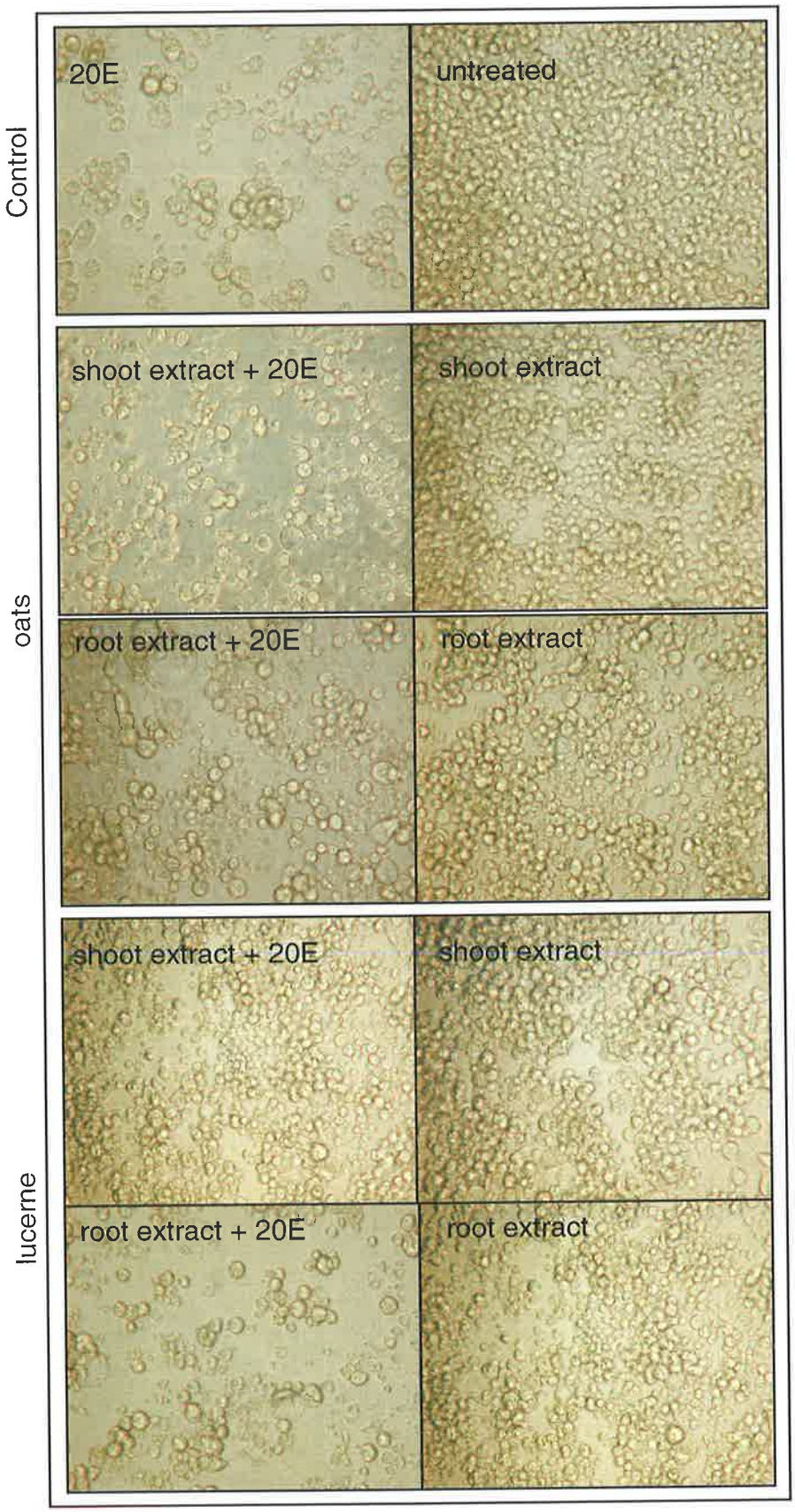

Figure 5.4 Growth of *Drosophila melanogaster* B_{II} cell line as affected by the treatment of shoot and root extracts of oats and lucerne alone or together with 1x10-7 M 20-hydroxyecdysone (20E); controls were 20E treated or untreated B_{II} cells

Table 5.1 Analysis of the inducible compounds in the shoots and roots of selected plants for the presence of phytoecdysteroids by B_{II} cell bioassay, RP-HPLC and UV absorption spectrum

Plant extract	B_{II} cell bioassay[a]	RP-HPLC			UV spectrum (nm)[c]
		Peak	Retention time (min)	Amount (mg eq./g dwt)[b]	
Spinach					
shoots	+	1	34.28	1.44	**254**, 280[d]
		2	38.86	0.18	**254**, 280[e]
roots	+	1	34.28	0.87	**254**, 280[d]
		2	38.86	0.07	**254**, 280[e]
Quaking grass					
shoots	+	1	34.42	3.11	**254**, 280[d]
		2	38.33	0.56	**254**, 280[e]
		3	32.84	0.12	**254**, 280[f]
		4	31.41	0.16	**254**, 280[g]
		5	36.40	1.14	**254**, 280[g]
roots	+	1	34.64	1.46	**254**, 280[d]
		2	38.95	0.30	**254**, 280[e]
		3	32.86	0.09	**254**, 280[f]
		4	31.38	0.05	**254**, 280[g]
		5	36.35	0.59	**254**, 280[g]
Lucerne					
shoots	-*	1	33.53	1.21	254, 280, **340**[h]
		2	34.61	0.69	**254**, 280[d]
		3	37.56	0.56	**254**, 280[g]
		4	38.77	0.47	**254**, 280[e]
roots	-	1	36.62	6.59	**254**, 280[d]
		2	40.31	1.62	**254**, 280[e]
Oats					
shoots	-	1	20.89	29.23	254, 280, **340**[h]
		2	23.21	269.00	254, 280, **340**[h]
		3	24.43	49.76	254, 280, **340**[h]
roots	-	1	20.95	66.97	254, 280, **340**[h]
		2	23.43	2.44	254, 280, **340**[h]
		3	24.65	40.73	254, 280, **340**[h]
Standards					
20E	+	1	34.45	1 mg/ml	**254**, 280
Polypodine B	nd	1	38.75	1 mg/ml	**254**, 280
Ecdysone	nd	1	32.89	1 mg/ml	**254**, 280

[a] +/- = positive or negative for the presence of biologically active ecdysteroid in the B_{II} cell bioassay; * = with anti-ecdysteroid effect; nd = not done; response is for the whole root and shoot extract irregardless of the peak or compound

[b] amount of inducible compound based on the respective standard equivalent per gram dry weight of the sample

[c] absorbance spectra at which the peak was detected; peaks were most evident (highest level) in the absorbance spectra in bold

[d] 20E

[e] polypodine B

[f] ecdysone

[g] unidentified putative ecdysteroids

[h] inducible compounds that were not ecdysteroids

compounds that co-eluted in the isocratic run. For instance, a single peak observed in the isocratic run of spinach shoot and root extract was resolved as two compounds in the gradient RP-HPLC run (Table 5.1). Similarly, the single peak of oat shoot and root extracts was separated as three compounds in the gradient RP-HPLC. The isocratic and gradient RP-HPLC elution of the lucerne root extract resulted in two major peaks and one of them was similar to 20E, based on retention time (Table 5.1). A minor peak similar to polypodine B was detected in the gradient elution (Table 5.1). The isocratic elution of lucerne shoot revealed two major peaks but the gradient elution resulted in four major peaks and one of them had similar retention time as 20E (Table 5.1). Polypodine B was also detected in the lucerne shoot extract but in minute amount. The isocratic and gradient elution for shoot and root extracts of quaking grass indicated the presence of 20E, ecdysone and polypodine B (gradient run only) from the five major peaks obtained (Table 5.1).

5.3.3 UV-*VIS* SPECTRA OF COMPOUNDS

Table 5.1 summarizes the characteristics of the inducible compounds in the plant extracts, based on the peaks observed from the gradient RP-HPLC elution. The absorbance maxima observed from the gradient RP-HPLC of the standards, 20E, polypodine B and ecdysone were compared with the inducible compounds in the plant extracts. All the plant extracts except oats contained compounds with UV-*Vis* spectra similar to the ecdysteroid standards. The absorbance spectra of the inducible compounds in the shoots and roots of oats and an inducible compound in the shoot of lucerne and quaking grass had spectra with absorbance maxima at 254, 280 and 340 nm, which were similar to the spectrum of the flavone glycoside, rutin.

Table 5.2 Fragmentation spectrum (EI-MS) and relative intensity (%) of the daughter ions of the inducible compounds in the extracts of selected plants and the known major ecdysteroids

Plant Extract	Retention time (min)	EI-MS *m/z* (relative intensity, %)
Spinach		
shoots	34.28	480(100), 462(72), 444(56), 426(17), 408(6), 390(3), 370(65), 302(15), 283(2), 264(2), 164(10)
	38.86	496(100), 478(47), 442(18), 424(15), 362(2)
roots	34.28	480(100), 462(36), 444(86), 426(36), 405(14), 370(86), 346(14), 325(14), 279(18), 225(14), 192(18); 164(29)
	38.86	496(100), 478(19), 442(35), 424(3), 362(18)
Quaking grass		
shoots	34.42	480(100), 462(85), 444(74), 426(45), 408(20), 390(35), 370(52), 346(2), 328(2), 302(10)
	38.33	496(100), 478(25), 442(38), 424(20), 386(18), 362(10)
	32.84	446(100), 428(80), 410(22), 393(14), 371(14), 354(2)
	31.41	480(100), 462(24), 444(68), 426(45), 408(20), 362(15), 328(2)
	36.40	496(100), 472(18), 460(12), 424(10), 363(12), 351(10), 345(40), 301(15), 141(36)
roots	34.64	480(100), 462(77), 444(68), 426(28), 408(6), 390(3), 370(70), 346(12)
	38.95	496(100), 478(10), 442(15), 424(5), 386(1),
	32.86	446(100), 428(60), 410(2), 393(10), 330(20), 312(3)
	31.38	480(100), 462(5), 444(15), 362(8), 328(2)
	36.35	496(100), 472(25), 460(5), 424(5), 363(12), 351(4), 345(10)

continued

Lucerne		
shoots	33.53	516(100), 494(20), 471(18), 453(30), 436(10), 426(10), 408(15), 381(5), 364(40)
	34.61	480(32), 462(14), 444(46), 396(20), 370(50), 328(20), 283(24), 218(72), 174(100)
	37.56	446(100), 408(14), 373(34), 338(36), 318(10)
	38.77	496(100), 478(50), 442(42)
roots	36.62	480(75), 462(50), 444(100), 408(30), 370(100), 315(80), 258(90)
	40.31	496(100), 478(22), 442(48), 424(39), 386(28)
Oats		
shoots	20.89	580(100), 519(10), 448(96), 430(35), 412(24), 394(15), 382(26), 352(30), 328(38), 298(10), 218(5), 168(10)
	23.21	564(100), 503(5), 432(70), 414(30), 396(20), 378(16), 366(22), 336(20), 312(26), 282(15), 169(2)
	24.43	592(100), 530(2), 446(45), 410(5), 363(5), 326(2), 284(22), 178(5)
roots	20.95	580(100), 519(2), 448(50), 430(15), 412(12), 394(4), 382(5), 352(16), 328(28), 298(5)
	23.43	564(100), 503(2), 432(85), 414(46), 396(5), 378(6), 366(18), 336(34)
	24.65	592(100), 530(5), 446(72), 410(20), 363(15), 326(4)
Standards		
20E	34.45	480(100), 462(77), 444(68), 426(28), 408(6), 390(3), 370(70), 346(12), 328(3), 302(7), 283(2), 264(1), 164(13)
Polypodine B	38.75	496(100), 478(13), 442(26), 424(7), 386(1), 362(7)
Ecdysone	32.89	446(100), 428(78), 410(8), 393(3), 371(2), 354(3), 330(27), 312(3)

5.3.4 MASS SPECTROMETRY

The mass spectrometry results confirmed the presence of 20E and polypodine B in the plant extracts except in oats (Table 5.2). Ecdysone was detected in quaking grass only. The ionization patterns of the peaks obtained were similar to the standards (Table 5.2). The amount of the ecdysteroids in spinach and quaking grass shoots were greater than in the roots (Table 5.1 and 5.2). In contrast, the concentrations of these ecdysteroids were greater in the roots than in the shoots of lucerne. In fact, polypodine B was hardly detected in the shoots. The quaking grass contained two other ecdysteroid-like compounds, which have a mass charge of 481 and 497 *m/z*.

5.4 DISCUSSION

The dose response curve to 20E was established to determine the optimum concentration of 20E that will elicit a full response effect on the *D. melanogaster* B_{II} cells and to test the efficiency of the system (Fig. 5.1). The growth of B_{II} cells in the wells of the microtitre plates was influenced by the concentration of 20E. The cell density in the presence of 20E ($\geq 6.7 \times 10^{-10}$M) decreased after six days and 45% inhibition was observed at concentration 1.7×10^{-8} M 20E. An optimum response, i.e. full inhibition, was at a concentration of 8.3×10^{-8} M 20E where the cell density was lower than the seeding density (2×10^5 cells/ml). Clement and Dinan (1991) observed 50% and maximum inhibition at 10^{-8} and 10^{-7} M 20E, respectively. In another paper of Clement *et al.* (1991), a full response was achieved with 5×10^{-8} M 20E and a significantly detectable response was obtained with 10-fold lower concentrations. They also found that ecdysone was less active than 20E by 100-fold, which was not tested in this study. Together with the fact that 20E was shown to be inducible (Schmelz *et al.*, 1999), stable (Schmelz *et al.*, 2002) and common (Lafont *et al.*, 1991; Dinan *et al.*, 2001), this was another reason for the strong emphasis given to 20E among other ecdysteroids in this study. The morphological responses of the B_{II} cells to

20E includes cellular elongation of a large portion of the cells followed by retraction of the processes and clumping of the cells (Clement and Dinan, 1991) which was evident after 6 days in 20E treated cells in this study.

The inducible compounds in spinach, quaking grass, lucerne and oat shoot and root extracts were tested using the B_{II} cell bioassay to assess ecdysteroid-like activity, since the bioassay proved to be efficient and robust. The extracts with ecdysteroid-like activity will reduce cell density in wells treated with the test extract (treatment *ii*) similar to cells treated with 20E (treatment *iii*) and relative to cells alone (treatment *i*). Moreover, the extracts with ecdysteroid-like activity will further depress the cell density in cells treated with 20E and plant extract (treatment *iv*) relative to treatment *iii*. Based on these criteria, the root and shoot extracts of spinach and quaking grass were positive for ecdysteroid-like activity. There was no difference between cells treated with 20E and cells treated with these extracts. The characteristic morphological responses (cell elongation and clumping) induced by 20E were also observed in cells treated with spinach and quaking grass extracts. Analysis of seed samples of quaking grass tested positive for the presence of phytoecdysteroids even in 100-fold dilution of the extract using the B_{II} cell bioassay (Savchenko *et al.*, 1998). The RP-HPLC chromatograms of these extracts, as compared to the standards, corroborated the findings that the ecdysteroids 20E and polypodine B are present in both spinach and quaking grass and ecdysone in quaking grass only. Spinach is known to contain 20E and polypodine B (Bathory *et al.*, 1982; Grebenok *et al.*, 1991; Dinan and Sehnal, 1995) while six ecdysteroids, ecdysone, 20E, polypodine B, abutasterone, pterosterone and sidisterone, have been identified in quaking grass (Savchenko *et al.*, 1998; Dinan, 1998).

In another premise, the plant extract was considered anti-ecdysteroid when cells treated with the extract (treatment *ii*) yield similar cell density as the untreated cells (treatment *i*). Anti-ecdysteroids enhanced the density of B_{II} cells treated with 20E and

plant extract (treatment *iv*) relative to cells treated with 20E (treatment *iii*). Oat shoot and root extracts did not lower the density of the B_{II} cells relative to the control (treatment *i*). Cells treated with oat roots even had higher cell density than the B_{II} cells alone. This indicated, in agreement with the HPLC data, that oats do not contain ecdysteroid-like compounds. Furthermore, the oat extracts cannot be considered anti-ecdysteroid because the oat extracts did not inhibit the effect of 20E on the cells when both were applied together (treatment *iv*). While HPLC spectroscopic data suggested that the major inducible compounds in oats were flavonoids, a detailed description of the identity of these inducible compounds is presented in Chapter 9. Although lucerne root extract reacted similarly to the oat extracts based on the criteria used, the chromatogram and UV absorption spectra of the inducible compounds in lucerne root extracts were characteristic of ecdysteroids but not 20E, polypodine B or ecdysone. The ecdysteroids present in lucerne root extracts may be less active in the B_{II} cell bioassay than 20E. Hence, the extract did not inhibit the cell density. Clement and Dinan (1991) claimed that the potency of each ecdysteroid represents its affinity for the ecdysteroid binding site on the receptor in the B_{II} cells. Although interaction with the ecdysteroid receptor appears to be the major determinant of biological potency, Dinan (1998) proposed that possible artefacts such as metabolism of test compounds (resulting in enhanced or decreased in activity) or lack of entry into the B_{II} cells, as may be the case with the charged carboxylated ecdysteroid derivatives, may confound the interaction.

Based on the UV absorption spectra and the chromatogram, lucerne shoot extract was found to contain 20E, polypodine B, an unidentified possible ecdysteroid, and a flavonoid. However, in spite of the presence of ecdysteroids in lucerne shoot extract, the density of B_{II} cells treated with this was comparable to the untreated cells and there was no evident morphological response characteristic of ecdysteroid activity on the cells. Furthermore, an anti-ecdysteroid effect was observed when cells were treated with both

20E and lucerne shoot extract. The presence of the extract overrode the effects of 20E and the cell density was even higher when cells were treated with both 20E and the lucerne shoot extract than when cells were treated with 20E or the extract alone. The lucerne shoot extract appeared to contain compounds or conjugate groups that inhibit the action of ecdysteroids, even those inherent in the plant.

There are conjugate groups linked to ecdysteroids, which affect binding to ecdysteroid receptor in the B_{II} cell bioassay (Harmatha and Dinan, 1997), thus reducing the biological activity of the ecdysteroid. The presence of glucosides in the 20E molecule reduces its activity by 1,000 fold (Harmatha and Dinan, 1997). The low activity of glucosides in ecdysteroids was attributed to their roles as inactivation and storage products in insects (Harmatha and Dinan, 1997). The presence of 11α-hydroxyl, 16β-hydroxyl, 24β-hydroxyl and 22,23-epoxide groups reduced the biological activity of ecdysteroids by 258, 1,200, 17 and 260 fold, respectively (Harmatha and Dinan, 1997). Other compounds or compound conjugates could have contributed to the antagonist effect of the lucerne shoot extract to 20E in the bioassay, considering that the foliar part of plants accumulate a wide array of compounds compared to the roots (Dixon and Paiva, 1995). Further characterization of compounds in lucerne would seem to be warranted.

The biological and chemical characterization of the inducible compounds in the test plants provided evidence that roots and shoots of spinach and quaking grass contained ecdysteroids that are biologically active. Lucerne contained ecdysteroids in the shoots and roots but those in the shoot were not active due to the presence of an ecdysteroid antagonist. Contrastingly, the inducible compounds in oats were flavonoids, which do not interfere with the activity of ecdysteroids.

CHAPTER 6. ECDYSTEROIDS IN PLANT PARASITIC NEMATODES

6.1 INTRODUCTION

Detection of compounds in nematodes that are hormones in related animals can facilitate development of management strategies with novel sites of action. Since moulting in insects is regulated by ecdysteroids and juvenile hormones (Hoffmann *et al.*, 1984), efforts have been made to isolate or detect these compounds in animal and free-living nematodes (Hitcho and Thorson, 1971; Horn *et al.*, 1974; Rogers, 1973; Bottjer *et al.*, 1984; Dennis, 1977; Fleming, 1985; Moeller and Koolman, 1986; Mendis *et al.*, 1983).

Although small amounts of ecdysone and 20-hydroxyecdysone have been identified in the free-living nematode *Caenorhabditis elegans* (Mercer *et al.*, 1988), there is as yet no evidence for ecdysteroid synthesis (Chitwood and Feldlaufer, 1990) or ecdysone receptor genes (Sluder *et al.*, 1999) in the nematode. However, the nematode is unable to survive in the absence of cholesterol (Brenner, 1974) or exhibits moulting defects upon reduction in cholesterol level (Kuervers *et al.*, 2003; Yochem *et al.*, 1999). Ecdysteroids are polyhydroxylated derivatives of cholesterol containing a C-7 double bond and a keto group at C-6 (Koolman, 1989). The requirement for cholesterol for growth, development, reproduction, and moulting denotes that steroid hormones are required for these processes in *C. elegans* (Merris *et al.*, 2003; Yochem *et al.*, 1999) and possibly in other nematodes as in insects (Kozlova and Thummel, 2000).

Whether nematodes biosynthesize ecdysteroids from cholesterol or obtain them from their diet or host is unclear but that ecdysteroids occur in nematodes is unequivocal. No attempt has been made in isolating or detecting ecdysteroids in plant parasitic nematodes to this date. An indication of the presence and significance of ecdysteroids in plant parasitic nematodes would give valuable information in the formulation of a sound control strategy. Hence, this part of the study aims to detect ecdysteroids in two plant parasitic nematodes, *P. neglectus* and *Anguina tritici*.

6.2 METHODS

6.2.1 PREPARATION OF NEMATODES

P. neglectus was multiplied as described in Section 3.2.1 until an estimated 2 g dry weight of the nematode was obtained (Fig 6.1). *A. tritici* was extracted from infested inflorescence of wheat by breaking up the galls and soaking them in a Whitehead tray (Section 3.2.2). The process was repeated until a dry weight of 0.7 g of the nematode was collected. After every collection, the nematodes were surface-sterilised to exclude and prevent development of bacteria during the accumulation of required amounts of nematodes. Sterilisation was performed on 0.65 μm filters with Hibitane® (I.C.I. Australia) at 0.5% a.i. for 15 min then rinsed three times with sterile reversed osmosis (RO) water. These were then soaked in a solution of 0.1% (w/v) penicillin G and streptomycin sulphate (Sigma) overnight at 20°C. The nematodes were rinsed with sterile RO water three times before soaking again in Hibitane® for 15 min. After rinsing the nematodes three times with sterile RO water, they were transferred into lyophilisation tubes and centrifuged at 10,000 g for 5 min. Water was aspirated from each tube and the nematodes were snap frozen and lyophilized. The lyophilized nematodes were stored in -20°C until extraction for ecdysteroids.

6.2.2 EXTRACTION OF ECDYSTEROIDS

Methanolic extracts of the nematodes were prepared using modified method of Mendis *et al.* (1983). *P. neglectus*, divided into three 0.7 g (dry weight) and *A. tritici* were suspended in methanol (3:1, w/v) and homogenised together with 0.05 g 500 mesh carborundum for 5 min at room temperature. Methanol was added into the tubes of homogenised nematodes

Figure 6.1. Lyophilised *Pratylenchus neglectus* (2 g) used in the extraction of ecdysteroids

to make 1:20 (w/v) suspension and was mixed continuously in a rotary mixer for 48 h. The suspension was centrifuged at 15,000 g for 10 min and 70% of the total volume of the methanol supernatant was obtained. Filtered (0.45 µm) RO water was added to the methanol supernatant to make 70% methanol extract and partitioned with an equal volume of hexane to remove fats and lipids for 48 h in a rotary mixer. An 80% volume of the 70% methanol extract was obtained and dried under vacuum at 5°C. The extract was redissolved in 5 ml filtered (0.45 µm) RO water and partitioned with 5 ml butanol in a rotary mixer overnight. After allowing the water and butanol to settle separately, 4 ml of the butanol phase was obtained and dried under vacuum at 5°C. The dried extracts were resolublised in 0.40 ml of 45% methanol and transferred in HPLC vials.

As controls, carrots and nematode-free galls of wheat inflorescence from where the nematodes were collected were extracted for ecdysteroid using procedure described in Section 3.3.

6.2.3 DETECTION AND QUANTIFICTION OF ECDYSTEROIDS

The methanolic extracts of the nematodes and controls were subjected to RP-HPLC gradient elution described in Section 5.2. Mass spectrometry (LC-MS) as in Section 5.3 was performed to confirm the presence of ecdysteroids. The standards used were 20E, ecdysone and polypodine B.

6.3 RESULTS

The evident peaks (referred to as major peaks) that were detected in the chromatogram of *P. neglectus* extracts including the controls have elution times that do not correspond to the standards, 20E, ecdysone and polypodine B (Fig. 6.2). However, major peaks with an absorbance maximum of approximately 254 nm, which is the wavelength commonly observed for ecdysteroids (Section 5.3), were detected in *P. neglectus* extract but not in the diet (carrots) extract. Major peaks in carrots were observed in the absorbance spectrum 280 and 340 nm (Fig. 6.3). No major peaks were detected in *A. tritici* extract. The LC-MS of the nematode methanolic extracts confirmed the absence of 20E, ecdysone and polypodine B in the nematodes. The major peaks in *P. neglectus* have molecular weights (*m/z*) of 453, 494, 496 and 538 (Table 6.1). The ecdysteroid database (Ecdybase, Lafont *et al.*, 2002) was scanned for known ecdysteroids and the compounds with 494, 496 and 538 *m/z* have similar mass and fragmentation pattern to makisterone A, 20,26-dihydroxyecdysone and 20,26-dihydroxyecdysone 22 acetate, respectively.

6.4 DISCUSSION

This study confirms that the free forms of the major ecdysteroids 20E, ecdysone and polypodine B were not present in *P. neglectus* or *A. tritici* at the nematode stages used in the study. As there is no evidence of ecdysteroid synthesis in nematodes, the possibility exists that the ecdysteriod-like substances observed in the animal and free-living

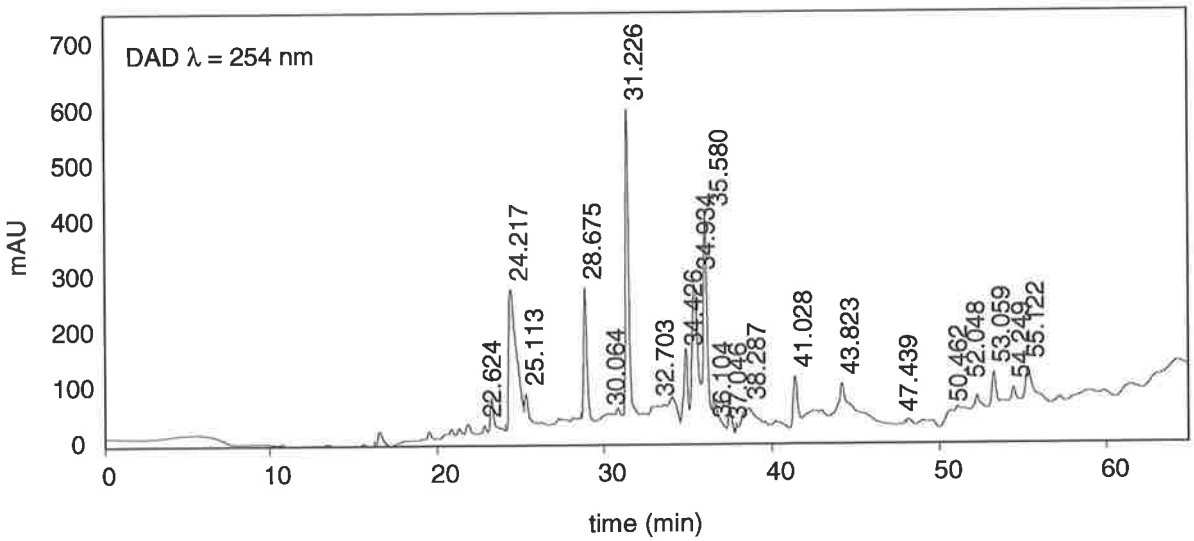

Figure 6.2. Chromatogram resolved by acetonitrile/water gradient elution using methanolic extract of *Pratylenchus neglectus* detected at 254 nm; DAD = photodiode array detection

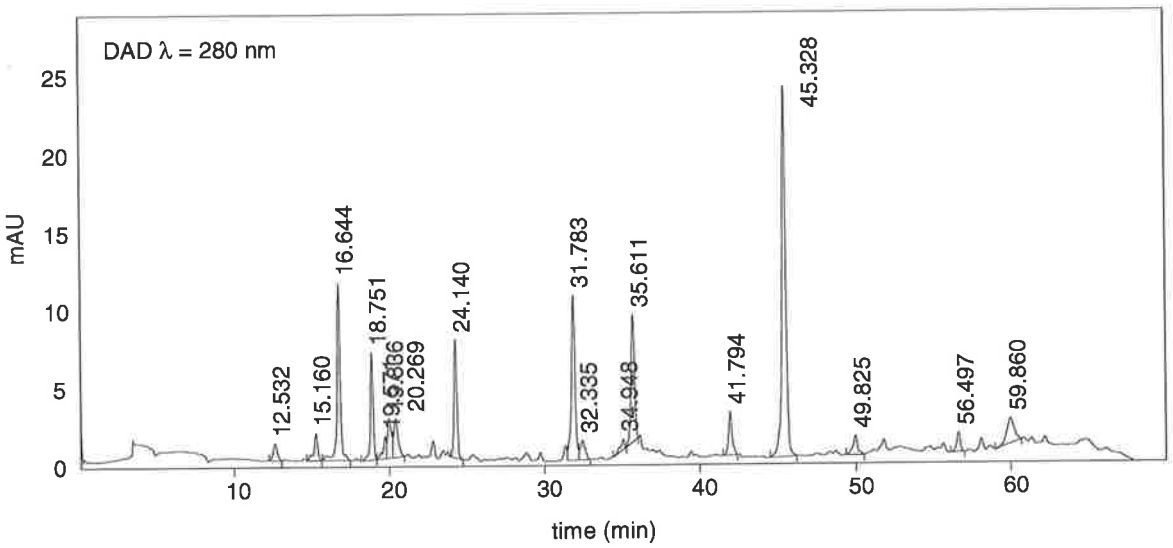

Figure 6.3. Chromatogram resolved by acetonitrile/water gradient elution of the methanolic extract of carrots used to multiply cultures of *Pratylenchus neglectus* detected at 280 nm; DAD = photodiode array detection

Table 6.1. Fragmentation spectrum (EI-MS) of the putative ecdysteroid compounds in the methanolic extract of *Pratylenchus neglectus* and the relative intensity (%) of the daughter ions compared to known ecdysteroids with similar fragmentation

Peaks detected & similar ecdysteroid[a]	Retention time (min)	EI-MS *m/z* (relative intensity, %)[b]
Peak 1	24.22	**494**(50), 471(60), 453(90), 426(10), 408(68), **363**(100), **345**(85), 336(45), 318(60), 274(5)
Makisterone A		**494**(100), **363**(100), **345**(78), 131(24), 113(59)
Peak 2	31.23	**539**, **521**, **503**, **485**, **461**, **425**, 380, 363…
20,26Di20E-22A		**539**[c], **521**, **503**, **485**, **461**, 443, **425**, **407**…
Peak 3	35.58	**496**, 478, **460**, **442**, **363**, **345**
20,26Di20E		**496**[d], **460**, **442**, 427, 409, 391, **363**, **345**, 133, 115
Peak 4	28.64	452(60), 425(30), 405(38), 390(42), 363(50), 330(100), 314(42), 300(38), 258(55), 240(45)

[a]Putative ecdysteroid compounds from *P. neglectus* assigned as peaks (unshaded); known ecdysteroids (shaded) are makisterone A, 20,26Di20E-22A = 20,26 dihydroxyecdysone 22-acetate, and 20,26di20E = 20,26 dihydroxyecdysone

[b]Matching base peaks and daughter ions of the known ecdysteroids and the peaks obtained from the nematode extract are indicated in bold values

[c]$(M+H)^+$

[d]$(M-2x18)^+$

nematodes mentioned earlier were obtained from the host. However, the putative ecdysteroid-like compounds found in *P. neglectus*, which were not present in the host, were apparently produced endogenously.

The presence of ecdysteroid-like compounds in *P. neglectus* and not in *A. tritici* may have been because the *P. neglectus* sample was actively developing and contained all stages including nematodes that would have been or nearing moulting. In contrast, *A. triciti* sample was dormant for a long time. The population profiles of *Aphelenchus avenae* and *Panagrellus redivivus* in the study of Dennis (1977) suggest that high levels of ecdysteroid-like materials were present in actively developing populations of *A. avenae* compared to other static nematodes. It was also observed that a high juvenile to adult and

fourth stage juvenile to second stage juvenile ratios result in higher titres of ecdysteroid-like materials.

Of the three putative ecdysteroids in *P. neglectus*, only 20,26-dihydroxyecdysone was recorded in nematodes such as *C. elegans* (Rees and Mendis, 1984), *Dirofilaria immitis* (Mendis *et al.*, 1983), and *Ascaris suum* (Cleator *et al.*, 1987). The 20,26-dihydroxyecdysone and makisterone A occur as minor ecdysteroids in both plants and insects (Lafont, 1997). The occurrence of 20,26-dihydroxyecdysone 22-acetate has not been reported yet in plants (Lafont, 1997). The unknown compound in *P. neglectus* with molecular weight of 453 *m/z* may be classified as new ecdysteroid as this does not appear yet in the Ecdybase (Lafont *et al.*, 2002) or it may just belong to another group of compounds, which like ecdysteroids, has an absorbance maxima of 254 nm.

Absence of 20E in *P. neglectus*, may indicate that plant parasitic nematodes have evolved moulting hormones that are not abundant and not the main ecdysteroids found in most plants as a means to overcome plant defence strategies. However, 20,26-dihydroxyecdysone and 20,26-dihydroxyecdysone 22-acetate are derivatives of 20E and, therefore, 20E may be essential or detrimental at certain concentrations to plant parasitic nematodes.

CHAPTER 7. PROTECTIVE ROLE OF PHYTOECDYSTEROIDS AGAINST PLANT PARASITIC NEMATODES

7.1 INTRODUCTION

The impact of plant parasitic nematodes on global food production is well recognised. Efforts to control this major pest group include selection, breeding and, more recently, molecular engineering for host resistance along with cultural, chemical and biological methods (Whitehead, 1998). Host resistance has proven to be an economic and environmentally attractive approach applicable to any scale of production from subsistence farming to highly mechanised broad-acre farming. Despite the identification, incorporation, genetic characterization and application of nematode resistance genes, the biochemical basis of nematode resistance remains largely unknown (Roberts *et al.*, 1998).

Several secondary metabolites are induced in plants following nematode invasion (Zacheo and Bleve-Zacheo, 1995). The responses directly induced by nematodes include synthesis of phytoalexins, production of hydrolytic enzymes, rapid modification of existing cell wall materials and systemic responses such as accumulation of proteinase inhibitors (Cramer *et al.*, 1993). However, beside the phytoallexin, glyceollin, a convincing link to mechanisms of nematode resistance remains to be demonstrated for these nematode induced responses in plants. Glucosinolates in *Brassica* provide resistance to plant parasitic nematodes (Potter, *et al.*, 1999). However, these compounds are enzymatically converted to broad-spectrum biocides, not specific to nematodes, and are largely restricted to Brassicaceae. The limited scope of these findings clearly shows that there is much to be discovered about how plants defend themselves against nematodes.

Ecdysteroids are highly polar steroidal hormones responsible for growth and development in many arthropods. Surveys conducted after their discovery in plants (termed as phytoecdysteroid) along with numerous biologically active analogues (Galbraith and Horn, 1966; Nakanishi *et al.*, 1966) indicated that phytoecdysteroids, a diverse group of steroidal compounds, may be present in a wide range of plants (Dinan and

Sehnal, 1995) including many used for food (Imai *et al.*, 1969). Phytoecdysteroids induce abnormal moulting in many arthropods with lethal effect (Robbins *et al.*, 1970; Bergamasco and Horn, 1983; Kubo *et al.*, 1983; Lafont *et al.*, 1991; Dinan, 2001), indicating a possible defensive role in plants. The observation that phytoecdysteroid concentrations increase in response to mechanical damage, insect herbivory and application of wound hormone, methyl jasmonate, (Schmelz *et al.*, 1999) is consistent with the notion that phytoecdysteroid induction can protect plants from insect attack (Schmelz *et al.*, 2002).

As nematodes are placed in a clade of moulting metazoans, the Ecdysozoa, it is likely they have similar hormonal regulation of ecdysis (Aguinaldo *et al.*, 1997). Since ecdysteroids possess biological activity in free-living and animal nematodes (Rogers, 1973; Dennis, 1976, 1977; Mendis *et al.*, 1983; Nelson and Riddle, 1984; Fleming, 1985; Davies and Fisher, 1994), phytoecdysteroids may also provide an important plant defence against nematodes and, thus, may serve as basis for the development of resistant cultivars.

The phytoecdysteroid, 20-hydroxyecdysone (20E), is a major (Lafont *et al.*, 1991; Schmelz *et al.*, 1999; Dinan, 2001) and stable (Schmelz *et al.*, 2000) ecdysteroid inducible (Schmelz *et al.*, 1999) in *Spinacia oleracea* (spinach) and *Briza maxima* (quaking grass) as shown in Chapter 4. Therefore, the effects of 20E on migratory (*Pratylenchus*) and sedentary (*Heterodera* and *Meloidogyne*) endoparasitic nematodes in genera that are responsible for most economic damage was examined by direct application and through exposure within spinach and quaking grass. Spinach and quaking grass was used as a model plants because elevated levels of 20E can be induced predominantly in these plants (Schmelz *et al.*, 1999; Chapter 4) and it is parasitised by species of important phytophagous nematode genera. Moreover, wound-induced root 20E accumulations were found to be the result of increased *de novo* 20E synthesis in spinach roots (Schmelz *et al.*, 1999), which may confer enhanced resistance to nematodes. Since spinach and quaking

grass also contains a minor phytoecdysteroid, polypodine B, and ecdysone, in quaking grass, the effect of these compounds applied exogenously on *Heterodera avenae* was determined.

7.2 METHODS

7.2.1. EXOGENOUS ECDYSTEROID

Second stage juveniles (J2) of *Heterodera avenae* (cereal cyst nematode), were treated with different concentrations of 20E and a single concentration of polypodine B and ecdysone to examine the effect on invasion and development in wheat (*Triticum aestivum*). *H. avenae* was used because a uniform invasive stage can be easily obtained and only one life cycle is completed in wheat. The nematodes were incubated for 24 h at 15°C in 2 ml of the following concentrations of 20E (Sigma, 98.8% purity) in 10% methanol: $8.2x10^{-8}$, $4.2x10^{-7}$, $2.1x10^{-6}$, $5.2x10^{-5}$ and $1.0x10^{-5}$ M. Some nematodes were also treated with 2 ml $5.2x10^{-5}$ M polypodine B (Sigma, 95% purity) and ecdysone (Sigma, 90% purity) before incubating under the same condition. Both water and 10% methanol controls were included. After treatment, nematodes were rinsed with three changes of water. The treated and controls were visually examined for vitality before 600 J2/plant were applied to 10-day old wheat cv. Egret seedlings grown hydroponically in 250 ml pots (Section 3.1). Wheat was used because it did not contain any inducible ecdysteroid-like compounds. Plants were maintained in a growth room at optimum conditions (15°C; 85% RH; 12 h day and night) for the nematode (Mor *et al.*, 1992). Nematodes extracted from the sand by flotation and sieving method (Section 3.2.2) and stained in roots (Section 3.2.3) were counted 30 days after inoculation. Moulting nematodes were also recorded and tested for vitality (Section 3.2.3).

Mixed stages of *Pratylenchus neglectus* (root lesion nematode) and J2 of three sedentary endoparasitic nematodes, *Meloidogyne javanica* (root-knot nematode), *H. avenae* and *Heterodera schachtii* (beet cyst nematode), were treated with 2 ml $5.2x10^{-5}$ M

20E. The nematodes were incubated for 24 h at 15°C, except for *M. javanica,* which was kept at 25°C. Known numbers of the treated nematodes were dispensed in 100 g sand in 70 ml polystyrene screw cap containers after rinsing them as described above. Each container was watered to and maintained at field capacity after adding 300 nematodes/container. Nematodes from each container were extracted from the sand by flotation and sieving (Section 3.2.2) and evaluated for vitality (Section 3.2.3) and abnormal moulting after incubation for two weeks.

7.2.2 ENDOGENOUS PHYTOECDYSTEROID

The effect of endogenous 20E in spinach cv. Avon and quaking grass on plant parasitic nematodes was assessed. Spinach and quaking grass was grown as described in Section 3.1. To induce 20E production, plants were drenched with 10 ml 10^{-4} M methyl jasmonate 26 and 15 days after sowing (referred to as "treated plants") of spinach and quaking grass, respectively. Three days following induction, treated and untreated spinach and quaking grass were inoculated with *H. schachtii* (spinach) or *H. avenae* (quaking grass), *P. neglectus* and *M. javanica* at the rate of 600 nematodes/plant. Uninoculated controls of both induced and untreated plants were included. Plants from each treatment were sampled, and roots were washed thoroughly 5 and 10 days after inoculation. Extraction of phytoecdysteroids from shoots and roots were performed following the procedure in Section 3.3. Nematodes were extracted and counted from both the sand and the roots as described in Section 3.2.2 and 3.2.3. Moulting nematodes were recorded and tested for vitality (Section 3.2.3).

7.2.3 PHYTOECDYSTEROID QUANTIFICATION

Quantification of 20E in shoot and roots, as described in Schmelz *et al.* (1999), used reverse-phase high performance liquid chromatography (RP-HPLC) with a C-18 column

(Waters Sperisorb ODS-2, 4.6mm x 150 mm, 5 μm particle column). Isocratic elution was performed as in Section 3.4.

7.2.4 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

Plants of uniform height and vigour were arranged in a randomised complete block design with 10 replicates before treatments in both experiments were applied. Analysis of variance was used to analyse the data and means were compared by using Tukey's test.

7.3 RESULTS

7.3.1 EFFECT OF EXOGENOUS ECDYSTEROID

Immediately following nematode treatment and just before inoculation, no effect of 20E on the condition of *H. avenae* J2 was evident. Also, water and 10% methanol controls did not differ significantly in any parameter measured, so only 10% methanol controls are presented. After 30 days, the total number of treated nematodes that failed to invade the plants increased linearly ($r^2 = 0.98$) with 20E concentration above 4.2×10^{-7} M (Fig. 7.1). The number of *H. avenae* with adverse morphological defects in the sand increased linearly ($r^2 = 0.95$) with 20E concentration above 4.2×10^{-7} M (Fig. 7.1). Exposure to 20E caused incomplete apolysis, shortening of juveniles and loosening of cuticle sections of nematodes recovered from the sand as well as in those that invaded the roots (Fig. 7.2). The number of nematodes in the roots decreased ($r^2 = 0.91$) with increasing concentration of 20E and the proportion that developed beyond the invasive stage was reduced to less than 20% when treated with 2.1×10^{-6} M 20E or more ($P < 0.01$, Fig. 7.1). In contrast, equivalent numbers of polypodine B (64%) and ecdysone (70%) treated and untreated (62%) nematodes invaded the wheat roots and development to adults was largely unimpaired.

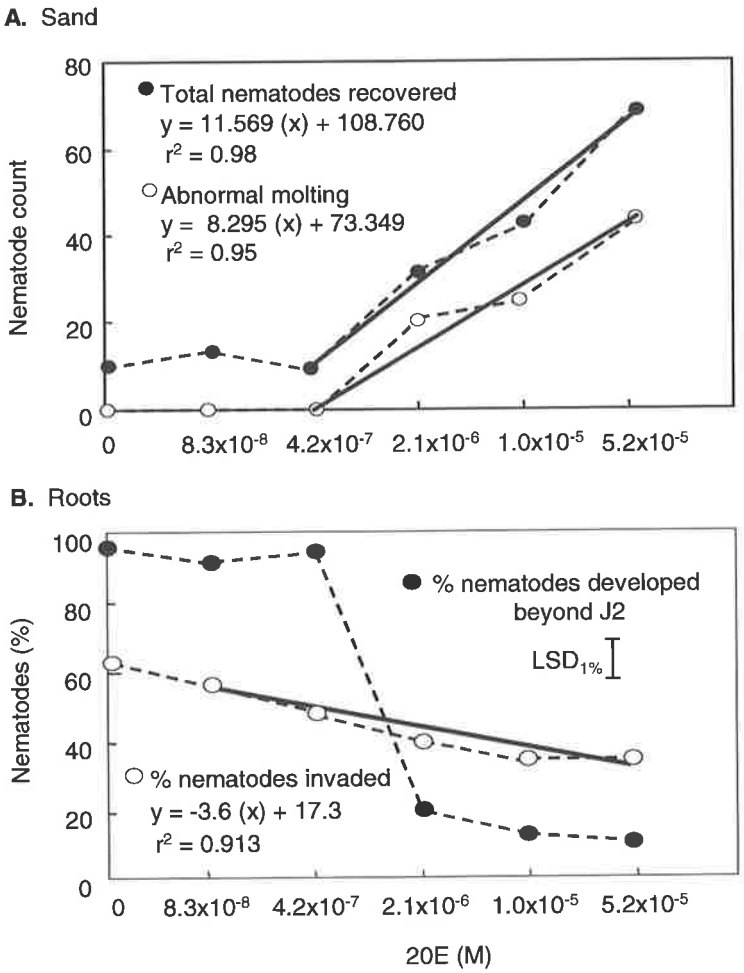

Figure. 7.1 Effect of exogenous 20-hydroxyecdysone (20E) on invasion by and development of *Heterodera avenae* in wheat cv. Egret 30 days after inoculation following treatment of the nematodes with varying concentrations of 20E in 10% methanol. **A.** Invasive stage (J2) of *H. avenae* recovered from sand and the number exhibiting abnormal moulting. **B.** Percentage of inoculated nematodes that invaded roots and percentage of invading nematodes that developed beyond the invasive stage

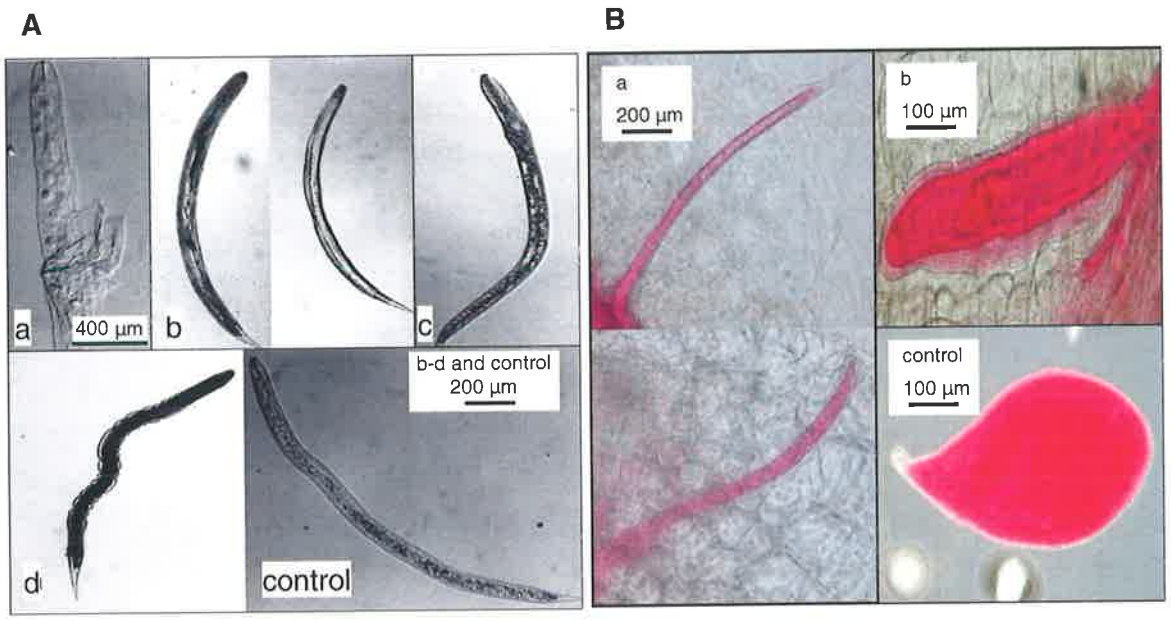

Figure 7.2 Detrimental effects of exogenous 20E on *Heterodera avenae* including **A.** incomplete apolysis (a), reduction in size (b), partial bloating (c) and random detachment of cuticle (d) of invasive stage from the sand, and **B.** abnormal moulting of invasive stage (a) and third stage juveniles (b) in wheat cv. Egret roots 30 days after inoculation

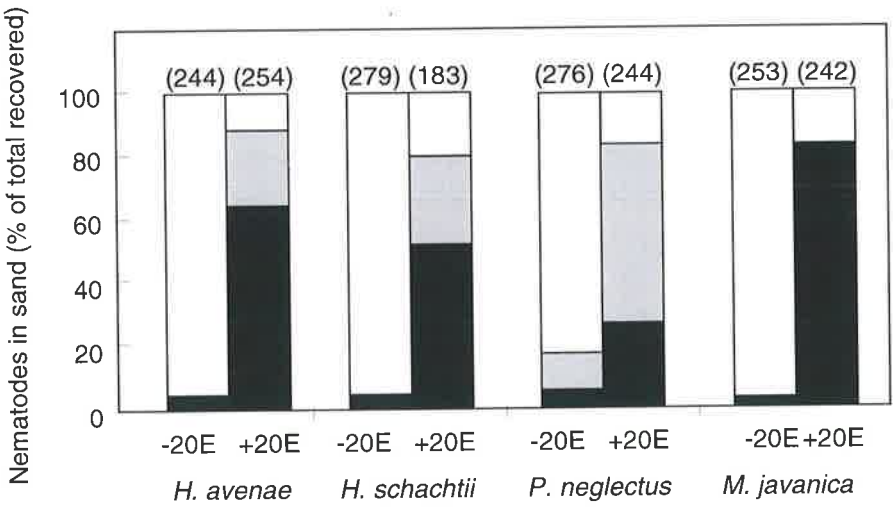

Figure 7.3 Proportion (%) of active (white; LSD 1% = 48.1), abnormally moulting (shaded; LSD 1% = 31.5) and immobile/dead (black; LSD 1% = 52.9) *H. avenae, H. schachtii, P. neglectus* and *M. javanica* after two weeks of incubation in moist sand following treatment with (+20E) and without (-20E) 20E; total number of nematodes recovered shown in brackets. LSD = least significant difference

In the subsequent experiment, in which four nematode species were treated with 5.2 x 10^{-5} M 20E, abnormal moulting and mortality was observed in *H. avenae*, *H. schachtii* and *P. neglectus* (Fig. 7.3). In contrast, for *M. javanica*, only increased mortality was observed with most juveniles being immobilised by the treatment even before inoculation. Since the effects of water and 10% methanol controls did not differ significantly, only the 10% methanol control was used for comparison.

In the case of the two *Heterodera* species (Fig. 7.3), about 26% of the treated nematodes moulted abnormally and 80% or more were dead (as shown by vitality staining, all abnormally moulting nematodes had died). In the control, no abnormal moulting was observed and only 5% of the nematodes were dead. Treatment of *P. neglectus* resulted in 58% of nematodes moulting mostly abnormally and compared to 12% in the control showing normal moulting (Fig. 7.3). Again, about 80% of treated nematodes died compared to 15% in the control. Likewise, 80% of the treated *M. javanica* recovered were dead compared to only 15% in the control. In all cases, the treatment effects differed from the respective controls by probabilities less than 0.01.

7.3.2 EFFECT OF ENDOGENOUS ECDYSTEROID

Compared to the control or uninduced/uninoculated plants, treatment of spinach with MJ resulted in increased concentrations of 20E in the roots (353%, $P < 0.01$) and shoots (163%, $P < 0.05$). Likewise, compared to the untreated plants, treatment of quaking grass with MJ increased concentration of 20E in the roots (212%, $P < 0.01$) but not in the shoots which had already a high constitutive level of 20E relative to spinach. The increase in 20E affected the invasion and development of the nematodes applied. MJ treatment did not affect plant growth.

Spinach

In the case of *H. schachtii*, induction with MJ had no effect on the number of nematodes found in the sand or the roots (Fig. 7.4). However, 5 days after inoculation, 54% of the nematodes that invaded roots of treated plants were moulting prematurely but none were in untreated plants (Fig. 7.4). Ten days after inoculation, normal moulting was detected in untreated plants, but 32% more *H. schachtii* were moulting in the treated plants (Fig. 7.4).

Five days after inoculation of untreated plants, *H. schachtii* increased 20E concentration in the shoots and roots by 250% compared to the untreated/uninoculated control (Fig. 7.5A). This concentration was equivalent to that induced by MJ treatment. Induction by combination of MJ treatment and inoculation, however, did not increase 20E concentration over that of inoculation alone.

Elevating 20E before inoculation protected spinach from nematode damage, resulting in growth that was equivalent to uninoculated controls. Inoculation of untreated plants reduced dry root and shoot mass by 53% and 56% (Fig. 7.5B and 7.6), respectively, compared to untreated /uninoculated and treated/inoculated spinach.

In the case of *P. neglectus*, greater numbers of nematodes were recovered from the sand of treated plants (37% and 28% of inoculum applied 5 and 10 days after inoculation, respectively, Fig. 7.4), but there was no effect on the number found in the roots (Fig. 7.4). However, the proportion of moulting nematodes had increased in treated plants (Fig. 7.4). Only normal moulting was found among nematodes in untreated plants. By contrast, in treated plants, a large proportion of nematodes of all stages moulted abnormally.

Five days after inoculation of untreated plants, *P. neglectus* had induced a 250% and 500% increase in 20E in shoots and roots, respectively, compared to the untreated/uninoculated control (Fig. 7.5A). The induction of 20E by *P. neglectus*

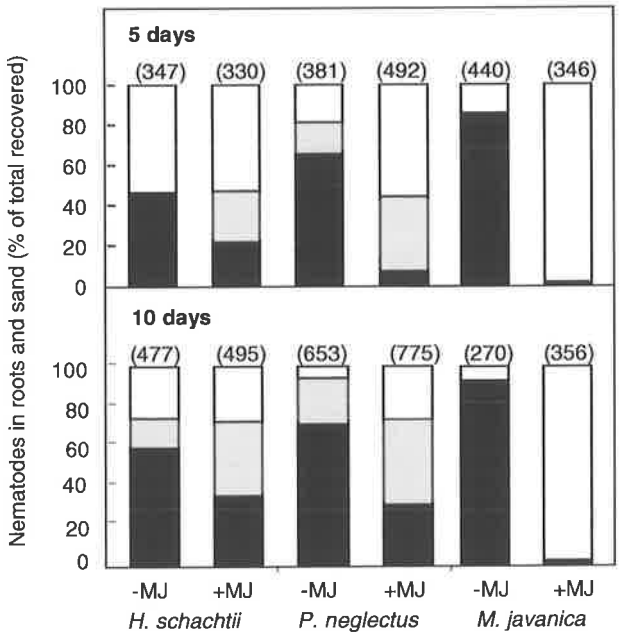

Figure 7.4 Effect of induction of 20E in spinach cv. Avon by methyl jasmonate (MJ) on the proportion (%) of total nematodes recovered from the sand (white; -MJ: LSD = 16.18, 5 days; 11.77, 10 days; +MJ: LSD = 18.13, 5 days; 23.59, 10 days) and roots (normal, black; LSD = 29.92, 5 days; 32.95, 10 days; moulting, shaded; LSD = 15.61, 5 days; 27.91, 10 days) 5 and 10 days after inoculation of *H. schachtii*, *P. neglectus* and *M. javanica*, in treated (+MJ) and untreated (-MJ) spinach; numbers in brackets are total number of nematodes recovered

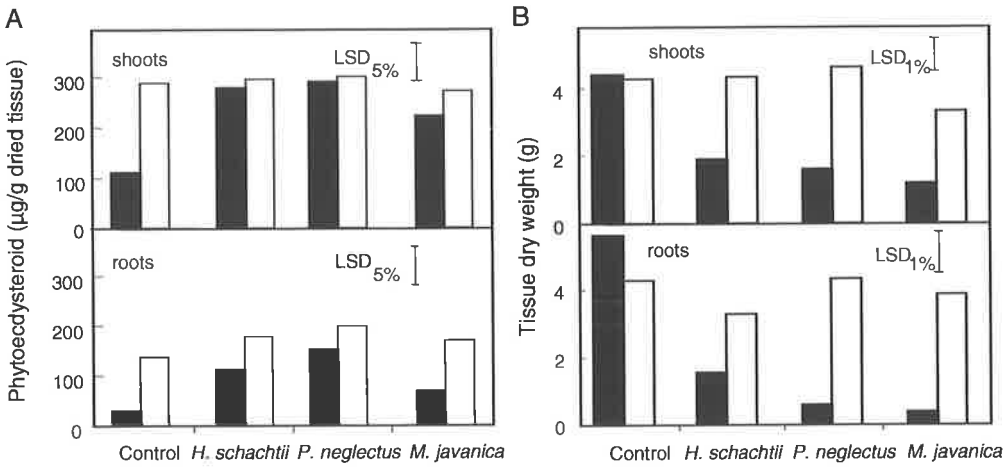

Figure 7.5 **A**. Concentration of 20E in shoots and roots of methyl jasmonate-induced (white) and untreated (black) spinach 10 days after inoculation of *Meloidogyne javanica*, *Pratylenchus neglectus* and *Heterodera schachtii*. **B**. Shoot and root dry mass of methyl jasmonate-induced (white) and untreated (black) spinach 10 days after inoculation of *Meloidogyne javanica*, *Pratylenchus neglectus* and *Heterodera schachtii*

Figure 7.6 Effects of induction using methyl jasmonate of endogenous 20-hydroxyecdysone on the growth of spinach inoculated with *Heterodera schachtii*, *Pratylenchus neglectus* and *Meloidogyne javanica* (-MJ, uninduced plants; +MJ, 20E induced)

inoculation was equivalent to that induced by MJ treatment, but again combined treatment and inoculation did not increase 20E concentration over that of inoculation alone.

Inoculation of untreated spinach with *P. neglectus* reduced root and shoot dry weight 86% and 65%, respectively, compared to untreated and uninoculated controls (Fig. 7.5B and 7.6). In contrast, pre-inoculation induction of 20E protected the plant from damage.

Greater numbers of *M. javanica* applied were recovered from the sand of induced plants (47% and 55%, 5 and 10 days after inoculation, respectively, Fig. 7.4), but there was no effect on the number found in roots (Fig. 7.4). However, only 1% and 2% of the *M. javanica* inoculated were found in the treated spinach roots, compared to 63% and 42% in untreated plants 5 and 10 days after inoculation, respectively (Fig. 7.4). No morphological abnormalities were observed in nematodes from the sand or roots. Moulting nematodes were only observed in untreated plants and they appeared normal. Moulting was not observed in nematodes from the sand.

Five days after inoculation of untreated plants, *M. javanica* had induced a 58% and 50% increase in 20E in roots and shoots, respectively, compared to the untreated/uninoculated control (Fig. 7.5A and Fig. 7.6). The concentration of 20E induced by *M. javanica* inoculation alone was less than that induced by MJ but still greater than the untreated controls.

Inoculation of untreated spinach with *M. javanica* damaged plants reducing root and shoot dry weight by 90% and 65%, respectively compared to untreated/uninoculated controls (Fig. 7.5B and 7.6). The damage was sufficient to cause death of some plants. *M. javanica* inoculation did not cause damage to MJ treated plants.

*Quaking grass (*Briza maxima*)*

Induction with MJ had no effect on the number of *H. avenae* in the roots of quaking grass as well as in the sand wherein only 46 and 42% were able to invade the roots of untreated and treated plants, respectively (Fig. 7.7). Five days after inoculation, abnormal moulting of the nematodes that invaded the roots was observed in both treated (50%) and untreated (18%) plants (Fig. 7.7). After 10 days, abnormal moulting had increased by 20% in untreated and treated plants.

In the case of *P. neglectus*, a greater proportion of nematodes remained in the sand of treated plants (85 and 61%, 5 and 10 days after inoculation, respectively, Fig. 7.7) as compared to untreated plants. Of the nematodes that invaded the roots, abnormal moulting was observed in both treated (50 and 48% of the nematodes in roots, 5 and 10 days, respectively) and untreated plants (52 and 48%, 5 and 10 days, respectively).

M. javanica, on the other hand, failed to invade the roots of quaking grass (Fig.7.7). The nematodes recovered from the soil were mostly immobile and did not exhibit abnormal moulting.

Similar to the previous findings in the study (Chapter 4), only *P. neglectus* induced an increase in 20E level (125%) in the roots of quaking grass which was equivalent to that induced by MJ treatment (Fig. 7.8A). However, none of the nematodes or the MJ treatment induced the 20E level in the shoot.

Inoculation of untreated quaking grass with *P. neglectus* reduced the shoot and root weight by 88 and 75%, respectively, compared to untreated/uninoculated controls (Fig. 7.8B and 7.9). Similarly, inoculation of *H. avenae* reduced the shoot and root dry weight by 41 and 52% (Fig. 7.8B and 7.9), respectively. Both nematodes did not cause reduction in shoot and root weight of MJ treated plants. *M. javanica*, on the other hand, did not affect plant weight or cause damage to either treated or untreated quaking grass.

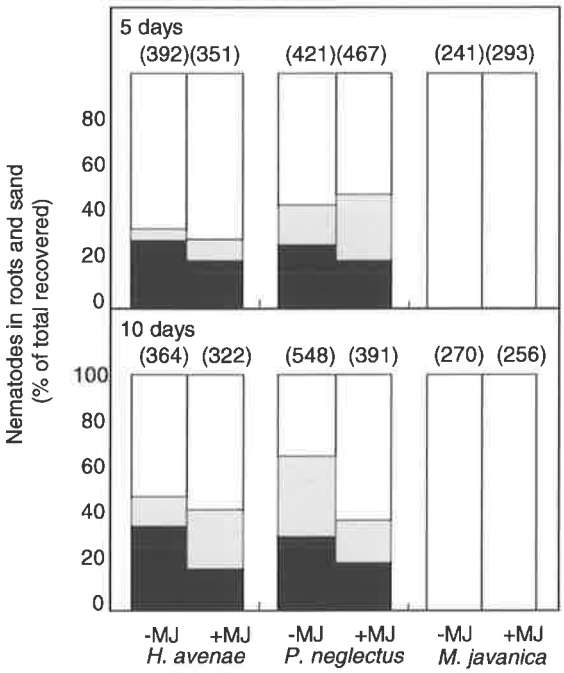

Figure 7.7 Effect of induction of 20E in *Briza maxima* by methyl jasmonate (MJ) on the proportion (%) of total nematodes recovered from the sand (white; -MJ: LSD = 8.14, 5 days; 5.40, 10 days; +MJ: LSD = 8.96, 5 days; 13.12, 10 days) and roots (normal, black; LSD = 14.27, 5 days; 12.68, 10 days; moulting, shaded; LSD = 6.44, 5 days; 14.28, 10 days) 5 and 10 days after inoculation of *H.avenae*, *P. neglectus* and *M. javanica*, in treated (+MJ) and untreated (-MJ) *B. maxima*; numbers in brackets are total number of nematodes recovered

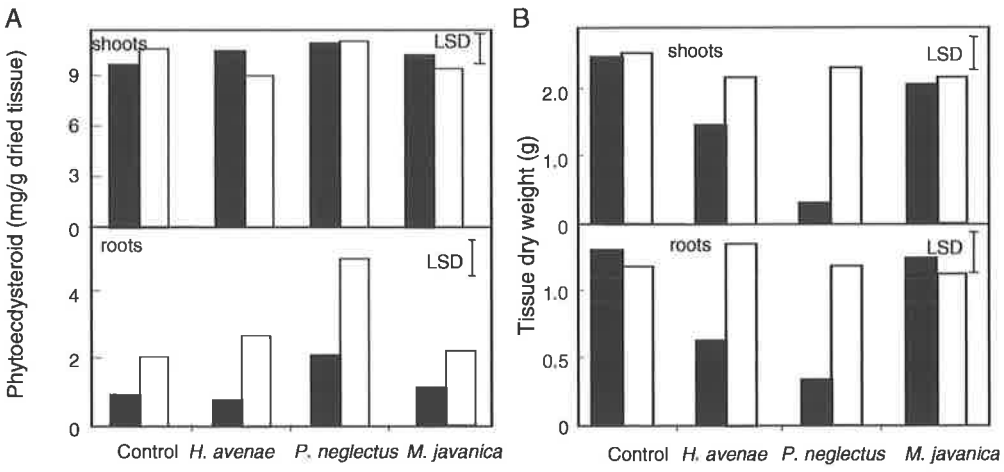

Figure 7.8 **A**. Concentration of 20E in shoots and roots of methyl jasmonate-induced (white) and untreated (black) *Briza maxima* 10 days after inoculation of *Meloidogyne javanica*, *Pratylenchus neglectus* and *Heterodera avenae*. **B**. Shoot and root dry mass of methyl jasmonate-induced (white) and untreated (black) *B. maxima* 10 days after inoculation of *Meloidogyne javanica*, *Pratylenchus neglectus* and *Heterodera avenae*

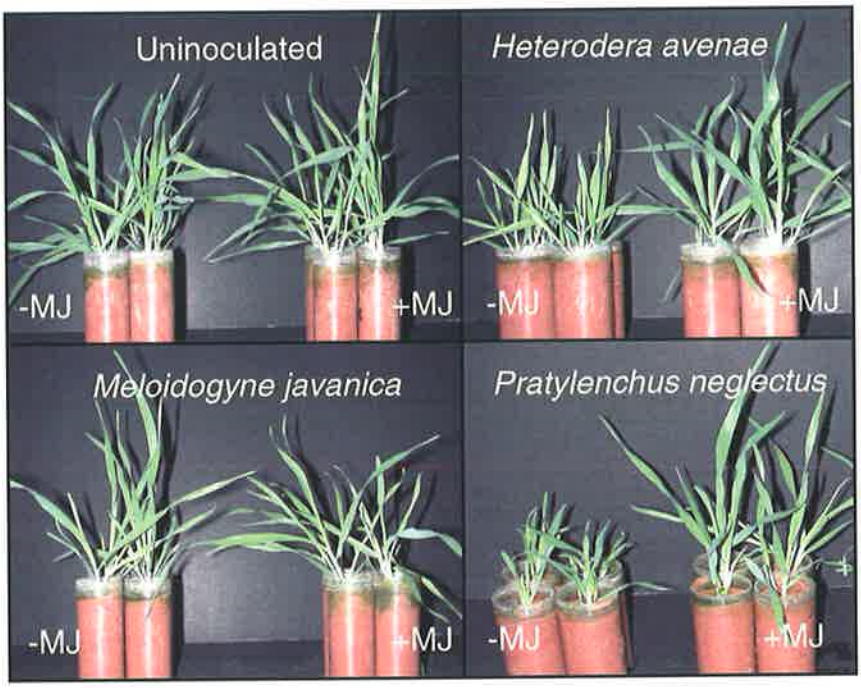

Figure 7.9 Effects of induction using methyl jasmonate of endogenous 20-hydroxyecdysone on the growth of *Briza maxima* inoculated with *Heterodera avenae*, *Pratylenchus neglectus* and *Meloidogyne javanica* (-MJ, uninduced plants; +MJ, 20E induced)

7.3 DISCUSSION

The first part of the study demonstrated biological activity of exogenous 20E in plant parasitic nematodes. The most evident physiological response was abnormal moulting, resulting in immobility and death or in the case of *M. javanica*, just immobility and death. In *H. avenae*, the consequence was impaired root invasion and development. *Heterodera* second stage juveniles moult only after invasion of the roots (Wyss and Zunke, 1986), so premature moulting of the invasive stage is clearly disruptive. All stages of *Pratylenchus* survive in the sand and, apart from eggs, can invade roots. However, increased and abnormal moulting in 20E-treated *P. neglectus* would have likewise affected host invasion.

This is the first evidence of an adverse effect of exogenous 20E on plant parasitic nematodes, which adds to the few known reports of activity in animal parasites, *viz. Ascaris suum* (Fleming, 1985) at 1 x 10^{-8} M and *Trichinella spiralis* (Rogers, 1973) at 3.12 x 10^{-5} M, and the fungal-feeding nematode, *Aphelenchus avenae* (Davies and Fisher, 1994) at 2 x 10^{-3} M.

The ecdysteroids, recognised as natural hormones regulating moulting and metamorphosis in insects (Butenendt and Karlson, 1954), are biologically active in similar ways in nematodes. Whether the ecdysteroids of insects are the same or similar to the moulting hormones regulating similar physiological processes in nematodes is unknown. However, the close parallel of their physiological actions indicates that they might share similar chemistry. The externally applied ecdysteroid concentration required to induce moulting and disrupt development in *H. avenae* exceeded expected physiological concentrations. The high polarity of ecdysteroids, however, may severely limit their absorption through the nematode cuticle and only a minimal increase in internal 20E concentrations might happen. In adult *Dirofilaria immitis*, for instance, ecdysone was sparingly absorbed through the cuticle when the nematode was incubated with the hormone, but once absorbed, it was metabolised readily to several less polar products

(Mercer *et al.*, 1989). Nonetheless, moulting of the third-stage larvae of *D. immitis* was stimulated by 20E, at both $1x10^{-5}$ and $1x10^{-7}$ M (Barker *et al.*, 1990).

The adverse responses to application of ecdysteroid are of greater significance in this chapter than the hormonal function in nematodes. The effects of exogenous 20E indicate a plant defence function, given that phytoecdysteroids occur widely and that the biologically active concentrations we found were well within the range occurring in plants (Dinan, 2001).

The effects of endogenous 20E in spinach and quaking grass on the three genera of phytophagous nematodes further indicate a plant defence role. Given the similarities with the effects of exogenous application of 20E on the nematodes tested, i. e. abnormal moulting of *H. schachtii*, *H. avenae* and *P. neglectus* and immobilization of *M. javanica* leading to impaired invasion, the ecdysteroid, 20E, is strongly implicated as the principal compound induced by MJ that affects nematodes. Polypodine B, which is also present in spinach and co-eluted with 20E, did not affect *H. avenae* when applied as exogenous treatment. Likewise, ecdysone was also not effective against the cyst nematode.

Jasmonic acid induces genes involved in the biosynthesis of compounds that are involved in plant defence (Creelman and Mullet, 1995, 1997; Baldwin, 1998). Therefore, the possibility of other methyl jasmonate inducible compounds in spinach that were not detected by our system is not being excluded. Although methyl jasmonate may have other unmeasured effects, its induction of 20E in high concentrations and the physiological and direct inhibitory effect of 20E on the nematodes are sufficient to account for the protection observed.

The most abundant phytoecdysteroid in plants is 20E although it may be accompanied by one or more related major phytoecdysteroids (Dinan, 2001; Lafont *et al.* 1991). Thus, its occurrence as the predominant phytoecdysteroid in plants as well as in insects, and its induction by both insects and nematodes, supports its defensive role.

Schmelz *et al.*, (2000) demonstrated through radioactive labeling of 20E that ecdysteroids are metabolically stable in spinach, which further suggests a role in plant defence.

The results of this study support the findings of Schmelz *et al.* (1998) that induced accumulations of 20E are caused by active root processes in spinach. Such a finding was also observed in quaking grass inoculated with *P. neglectus* or treated with MJ. The root synthesis of 20E has also been established in other systems (Grebenok and Adler, 1993; Tómas, *et al.*, 1993). However, the mechanism responsible for generating induced accumulations of root phytoecdysteroids is unknown, although the modulation of gene expression and *de novo* synthesis has been predicted (Creelman *et al.*, 1992; Gundlach *et al.*, 1992). The exact location of 20E in roots has not been established, but since abnormal moulting of *P. neglectus* and *H. schachtii* occurred in the cortical cells, 20E must be present in this tissue. The pre-invasion effect of *M. javanica* indicates also that 20E diffuses into the rhizosphere.

The failure of *H. avenae* and *M. javanica* to induce 20E in quaking grass may be due to the inherently high levels of the ecdysteroid in the plant. Abnormal moulting of *H. avenae* in both treated and untreated plants may also be attributed to this. *M. javanica*, which was sensitive to induced levels of 20E in spinach, was also inhibited in untreated quaking grass. Hence, it is possible that resistance to *M. javanica* and the reduced invasion of *H. avenae* in quaking grass relative to spinach were due to the high constitutive levels of 20E in the plant.

In summary, pre-inoculation induction of 20E in spinach and quaking grass partially inhibited invasion of *P. neglectus* and markedly inhibited that of *M. javanica*, which was also inhibited in untreated quaking grass. Abnormal moulting occurred in *Heterodera* spp. and *P. neglectus*, and development of the sedentary nematodes (*Heterodera* spp. and *M. javanica*) was halted. Development of the migratory *P. neglectus*, applied as mixed stages, was not directly assessed, but the abnormal moulting in

this species indicates that impaired development was likely. In all cases, plants were protected from nematode damage. The ability of the nematode species to induce 20E was again demonstrated. However, nematode-induced 20E appeared not to have been sufficiently rapid to prevent damage at the inoculation rate used or to affect the nematodes within the period assessed.

The effects of endogenous 20E in spinach and quaking grass on *P. neglectus* and the *Heterodera* spp. paralleled the effects of exogenous 20E. These nematodes failed to develop in spinach and quaking grass roots because of the onset of physiological abnormalities (abnormal moulting) after invasion. The pre-inoculation induction of 20E did not reduce the invasion by these nematodes. The intracellular movement of *P. neglectus* (Zunke, 1990) and the *Heterodera* spp. (Wyss and Zunke, 1986) within the root cortex may have exposed them to high 20E concentration in this tissue. *P. neglectus*, an endoparasitic nematode invades roots randomly at any point and migrates intracellularly (Zunke, 1990). The cyst nemtodes also migrates intracellularly but moves directly towards the differentiating vascular tissue at the root tips where it establishes a permanent feeding site (Wyss and Zunke, 1986). In contrast to *M. javanica*, a sedentary endoparasitic nematode that migrates intercellularly through the cortex causing limited damage (Wyss *et al.*, 1992), no abnormal moulting of the few that invaded the roots was observed. Rather, the invasion of the plants by *M. javanica* was impaired by the induction of 20E, which indicates sensitivity to extracellular 20E and the movement of the ecdysteroid into the rhizosphere. An increased concentration of 20E in the roots appears to protect the plant from *M. javanica*, providing pre-invasion resistance, which is uncommon; the majority of recorded resistance occurs post invasion (Kaplan and Keen, 1980a; Trudgil, 1991; Niebel *et al.*, 1993; Sijmons, 1993). The immobilization of *M. javanica* pre-invasion is consistent with the effect of exogenous 20E on this nematode. It appears that *M. javanica* is more

sensitive to 20E than the *Heterodera* spp. and *P. neglectus*, as these are only affected post invasion and develop morphological changes (abnormal moulting) before they die.

The uniqueness of the observed defence provided by 20E against plant parasitic nematodes is that it protects against nematode species with differing parasitic behaviour and operates both pre- and post-invasion. Known nematode resistance in crop plants is stage and species specific (Kaplan and Keen, 1980; Trudgill, 1991; Roberts *et al.*, 1998; Opperman, 2001). The 20E defence in spinach and quaking grass imparts tolerance to the three nematodes tested by reducing their damaging impact on yield, and resistance by reducing the nematode capacity to reproduce, an obvious consequence of the effects observed.

Previous studies have indicated an allelochemical role for phytoecdysteroids as hormonal disruptors and toxins for insects and some other invertebrates (Robbins *et al.*, 1970; Kubo *et al.*, 1983; Arnault & Sláma, 1986; Camps & Coll, 1993; Savolainen *et al.*, 1995; Blackford & Dinan, 1997; Mondy *et al.*, 1997). This study is the first to implicate phytoecdysteroids as a resistance mechanism against plant parasitic nematodes, and, thus, adds to the few known induced plant defences against them (Kaplan and Keen, 1980; Huang and Barker, 1991; Oka *et al.*, 2000; Kempster *et al.*, 2001).

Our studies reveal a defensive role for phytoecdysteroids against certain plant parasitic nematodes and may reveal a more general resistance mechanism in plants against multiple arthropod pest species. Deliberate induction or breeding for constitutive levels of phytoecdysteroid in economically important plants may be useful for nematode population management.

CHAPTER 8. GREEN MANURE CONTAINING 20E FOR NEMATODE CONTROL

8.1 INTRODUCTION

The earlier part of the study has shown the potential of 20E as a defence against nematodes. Since affordable synthetic production is unlikely, incorporation of 20E in plants by deliberate breeding may be an option. However, activation of this defence compound may entail a high metabolic costs to the plant potentially compromising yield. Hence, another possible mode of deployment of the defence compound is through green manure.

In addition to being grown as a vegetable, spinach is one of the plant species traditionally grown as green manure for nitrogen source in temperate farming systems to improve the growth of the main crop (Lehmann *et al.*, 1991; Elers and Hartmann, 1988). It has not been recognised as a control option against pathogens or pests such as nematodes. Suppression of pests and pathogens related to the addition of organic matter has been attributed to an increase in microbial activity or release of toxic compounds (Widmer and Abawi, 2000; Widmer *et al.*, 1998; Hoitink *et al.*, 1993; Hoitink and Fahy, 1986). Since spinach contains 20E which has the potential to reduce nematode populations, the delivery of this defence compound through green manure could be an attractive option.

Therefore, the use of spinach as green manure to control nematodes particularly *H. avenae* was evaluated. The possible involvement of 20E in the mechanism of nematode suppression in green manure application was also determined.

8.2 MATERIALS AND METHODS

Spinach cv. Avon were grown (Section 3.1) for 25 days before treatment with 10 ml 10^{-4} M methyl jasmonate. The plants were harvested after three days and chopped roughly into 10 mm pieces separating the roots and the shoots. Two batches each of 1 kg pasteurised

Tailem Bend sand was prepared. Chopped spinach shoots (530 g) and roots (810 g) were incorporated into each batch of the previously prepared sand to have an estimated 25 mg phytoecdysteroid per kg soil or an equivalent of $5.2x10^{-5}$ M 20E. The amended sand was mixed thoroughly and dispensed in 42 mm diameter by 108 mm high polystyrene tubes. Unamended sand was also dispensed in similar tubes. Uniform size and vigour 10-day old wheat cv. Egret seedlings, which were previously grown in U.C. potting mix contained in seedling trays, were transplanted into each tube. The plants were watered at field capacity before being inoculated with 500 *H. avenae* J2. Uninoculated plants in amended and unamended sand were included as controls. The plants were grown in a controlled environment growth room at 15°C, 85% RH and 12 h photoperiod. The roots were harvested 5 and 10 days after treatment, stained (Section 3.2.3) and nematodes counted. Nematodes were also extracted from the sand using Whitehead tray (Section 3.2.2) and counted.

Statistical analysis

Six replicates of each treatments and controls were arranged in a randomised complete block design. Nematode counts were transformed (log10[x + 1]) to normalise the data for analysis. The effects of the treatments were analysed using analysis of variance and means were compared using the least significant difference.

8.3 RESULTS

Majority of the nematodes in the amended treatment remained in the sand and only few *H. avenae* J2 penetrated wheat roots, 2% in shoot and 6% in root-amended sand (Fig. 8.1). None of the nematodes in the roots had moulted abnormally. The nematodes recovered from the sand were immobile and abnormal moulting was uncommon (< 1%).

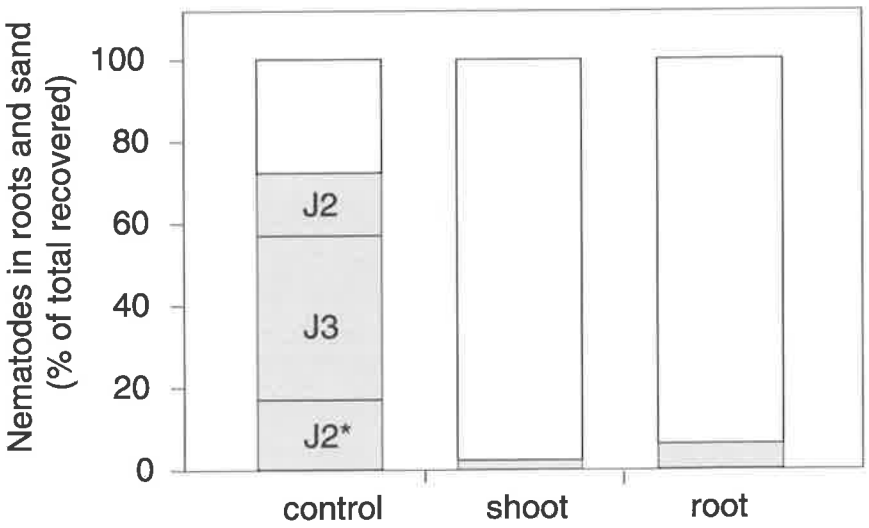

Figure 8.1 Effect of spinach green manure on the proportion (%) of total nematodes recovered from the sand (white; LSD = 39.30) and roots (shaded, J2, LSD = 39.30; J2* = normally moulting J2; J3, LSD = 23.09; $P < 0.001$)

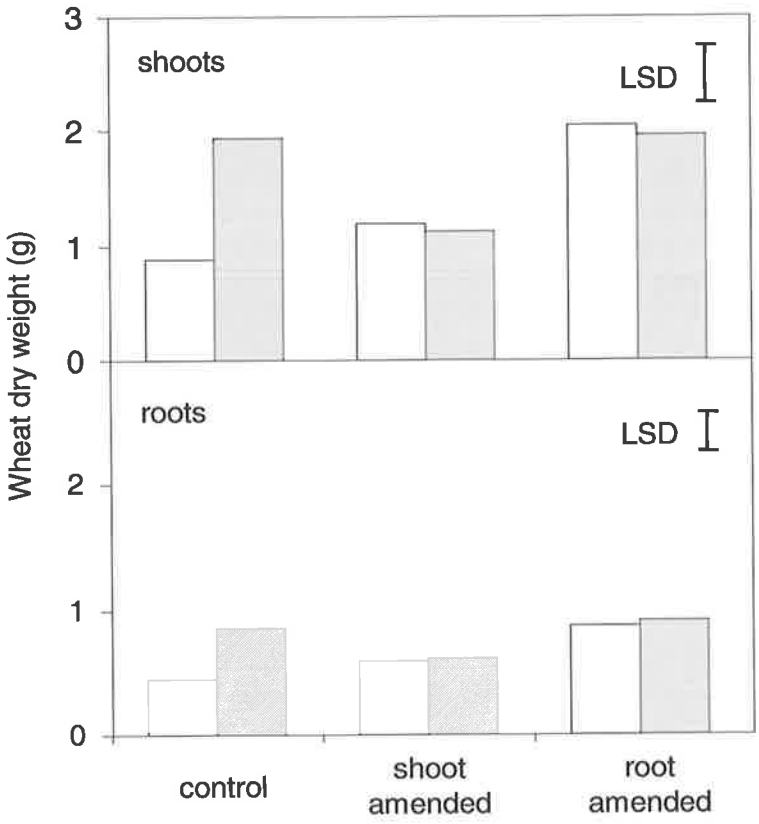

Fig. 8.2 Shoot and root dry mass of *Heterodera avenae* inoculated (unshaded) and uninoculated (shaded) wheat grown in spinach shoot and root amended sand for 10 days; control is unamended Tailem Bend sand; $P < 0.001$

Fig. 8.3. Wheat grown in spinach shoot and root amended sand 10 days after inoculation with *Heterodera avenae*; Control is Tailem Bend sand only

In contrast, 72% of the *H. avenae* J2s penetrated the roots of wheat in untreated sand and 23% of those that invaded were moulting normally while 56% were beginning to swell or at the early J3 stage (Fig. 8.1). No abnormal moulting was observed.

The shoot and root dry weight of inoculated or uninoculated wheat in spinach root amended sand were not significantly different from the uninoculated unamended control (Fig. 8.2 and 8.3). However, the shoot and root dry weights of inoculated plants in shoot-amended sand were reduced by 38 and 32%, respectively, compared to the uninoculated unamended control (Fig. 8.2). This was not significantly different from the uninoculated plants in shoot amended sand. These plants (shoot-amended) were stunted but were greener and more robust than the inoculated plants grown in unnamended sand (Fig. 8.3). The inoculated plants in unamended sand had the poorest growth with 54 and 48% reduction in shoot and root dry weight, respectively as compared to those grown in uninoculated unnamended sand.

8.4 DISCUSSION

The approximate concentration of 20E in the amended sand was $5.2x10^{-5}$ M which was the concentration active against nematodes when applied exogenously (Chapter 7). Therefore, it was assumed that if the plant tissues were able to release the same amount of 20E, then the effects similar to those due to 20E application would have been observed. However, no abnormally moulting nematodes were observed. The amount of spinach incorporated into the sand caused rapid death to majority of the inoculated nematodes. The nematicidal effects of spinach when used as green manure may have prevented abnormal moulting. It is not known if 20E caused the toxic effect on the nematodes or some other compounds released into the sand during the breakdown of the green manure. An increase in microbial activity as mechanism for control is unlikely because the sand used was sterile and the growth room temperature was relatively low (15°C) for a rapid build up of the microbial population to effect competition or antagonism against the nematodes. The inhibitory effect on the nematodes was more likely a function of the breakdown of products released from the green manure.

The incorporation of spinach shoots in the sand stunted the growth of wheat but the effect of an increase in nitrogen was evident in the colour and vigour of the plant despite the stunting effect on the plant itself. Nitrogen uptaken from the soil by spinach is stored primarily in the shoots and only in small amounts in the roots (Lehmann *et al.*, 1991; Elers and Hartmann, 1988). This may explain the nutritional effect in shoot amended sand.

In contrast, the amendment with spinach roots protected wheat from nematode invasion without apparent effect on the plant. The effect of an additional source of nitrogen was not evident because the plants were similar to the uninoculated unnamended sand. The amendment of spinach roots proved to be more effective for nematode control than the shoot amendment.

In addition to the known fertilizer effect of spinach when incorporated as green manure, this part of the study reveals that the use of spinach as green manure suppresses invasion of the nematode *H. avenae* through the release of deleterious compounds. However, the direct involvement of 20E could not be established. As the concentration of 20E in the sand with the amendments was at a biologically active concentration, it may have contributed to the effect. Further work is needed for confirmation. Such further studies could examine the effects of lower rates of incorporated spinach because abnormal moulting may occur if the direct toxic effects are less. Varying amounts of the shoots and roots of spinach incorporated into the sand will also determine the threshold level of control. The use of spinach cultivars with low levels of 20E in comparison to ones with inherently higher levels of the phytoecdysteroid is another strategy that could be used to confirm the involvement of 20E. There is great potential for this green manure application system to be used as an effective and sustainable management option to control nematodes in intensive agriculture.

CHAPTER 9. PROTECTIVE EFFECTS OF THE FLAVONE-*C*-GLYCOSIDES IN OATS

9.1 INTRODUCTION

Plant defence responses to parasitic nematodes have the potential to become part of management strategies that increase agricultural productivity (Cramer *et al.*, 1993; Niebel *et al.*, 1994; Sijmons, 1993; Trudgill, 1991). Both constitutive and induced defence mechanisms contributing to disease resistance are observed in plants (Kaplan and Keen, 1980; Oka *et al.*, 2000; Trudgill, 1991). In plant-nematode interactions, a range of secondary metabolites is induced in plants after nematode invasion (Zacheo and Bleve-Zacheo, 1982). Induction of phytoalexin synthesis in the roots in response to nematode parasitism has been documented (Veech, 1982). Proteins and enzymes, such as peroxidases of the phenylpropanoid pathway leading to the synthesis of lignin, suberin, flavonoids, phytoalexins and coumarins, accumulate in plants indicating an involvement in nematode resistance (Brueske, 1980, Dean and Kuc, 1987, Edens *et al.*, 1995; Oka *et al.*, 1997; Zacheo *et al.*, 1982). Biosynthesis and accumulation of phenolic compounds in plant tissues following nematode infection is also considered a defence response (Giebel, 1974). Although various compounds are associated with plant resistance to nematodes, the phytoalexin glyceollin in soybean provides the most convincing link for resistance mechanism against nematodes in plants (Huang and Barker, 1991; Kaplan *et al.*, 1980a). The limited information on direct involvement of phytochemicals in nematode resistance compared to insects and other pathogens is a major barrier for the development of novel nematode management strategies and highlights the need for further studies.

The induction of secondary metabolites by pests and pathogens, which limits further herbivory or infection (Baldwin, 1998; Chitwood, 2002; Friend, 1979; Harbone and Williams, 2000), is indicative of their defence function. Therefore, induction of metabolites by mechanical damage or chemical treatment (e.g. jasmonic acid) is a widely

used approach for the discovery of unknown defence compounds. Well-known potential defence compounds induced by jasmonic acid, are proteinase inhibitors, benzophenanthridine alkaloids, nicotine, and phenolics such as flavonoids (Baldwin *et al.*, 1997; Farmer and Ryan, 1990; Gundlach *et al.*, 1992; Mizukami *et al.*, 1993; Mueller *et al.*, 1993).

Oats (*Avena sativa*) are recognised among other cereals for their resistance to pests and diseases and are often grown in crop rotation to break the cycles of soil borne pests and diseases (Schrickel, 1986). Hence, it is possible that this species possesses secondary metabolites that impart resistance to nematodes. The presence of saponins, such as the avenacins, which are restricted to the genus *Avena*, has been implicated in disease resistance in oats (Osbourn, 2003). However, oats also produce and accumulate other secondary metabolites such as flavonoids, sterols and other phenols (Knogge and Weissenböck, 1986; Peterson, 2001; Popovici *et al.*, 1977), which are not characterised in the context of a possible involvement in resistance to nematodes. Accumulation of some compounds following nematode invasion has been reported and a possible involvement in plant defence has been suggested (Baldridge *et al.*, 1998; Chitwood, 2002; Cook *et al.*, 1995; Edwards *et al.*, 1995; Giebel, 1974; Oka *et al.*, 1997; Plowright *et al.*, 1996; Veech, 1982).

In this study, the inducibility of possible defence compounds in oats in response to invasion by parasitic nematodes with different feeding behavior and to application of methyl jasmonate was examined. Oat seedlings were challenged with *Pratylenchus neglectus* (root lesion nematode), a migratory endoparasite of roots, *Heterodera avenae* (cereal cyst nematode), a sedentary endoparasite of roots, and *Ditylenchus dipsaci* (stem nematode), a migratory endoparasite of shoots. The compounds induced in oats by nematodes and methyl jasmonate were extracted, characterised and identified. To determine the biological activity of the inducible compounds, a bioassay was conducted by

assessing the effects of shoot and root extracts from oats induced with methyl jasmonate on the invasion by and development of cereal cyst nematode, *Heterodera avenae*, in wheat. The protective effects of the inducible compounds in oats against *H. avenae* and the root lesion nematode, *Pratylenchus neglectus*, were also evaluated *in vivo*.

9.2 MATERIALS AND METHODS

9.2.1 PLANT AND NEMATODE CULTURE

Individual oat (*Avena sativa* cv. Quoll) or wheat (*Triticum aestivum* cv. Egret) seedlings were grown in 300 ml Tailem Bend sand (Section 3.1). The nematodes *D. dipsaci* (oat race), *H. avenae*, and *P. neglectus* were used in the study. Mixed stages of *D. dipsaci* and *P. neglectus* and J2s of *H. avenae* were obtained as described in Section 3.2.2.

9.2.2 INDUCTION OF COMPOUNDS IN OATS

Oat seedlings with uniform growth and vigor were treated with a soil drenching of 10 ml 10^{-4} M methyl jasmonate or inoculated with the nematodes, *D. dipsaci*, *H. avenae* and *P. neglectus*, 15 days after sowing. Uninoculated and untreated plants were included as controls. Roots and shoots were sampled 3 days after treatment or inoculation by carefully washing off the soil from the roots of each plant and snap freezing the samples in liquid nitrogen. Methanol-extractable compounds from shoots and roots were detected and quantified as described below.

9.2.3 EXTRACTION OF INDUCIBLE COMPOUNDS

The frozen root and shoot samples were freeze-dried. These were then ground to fine powder (c. 850 μm mesh) and 60 mg of each sample was extracted in 10 ml methanol for 48 h. Methanol is commonly used as a general solvent for the extraction of secondary metabolites such as phenolics from plants (Waterman and Mole, 1994). To remove the lipids in the extract collected, an aliquot of 7 ml of the methanol extract was diluted with

water to make a 70% methanol extract solution and partitioned with 10 ml hexane for 48 h. The aqueous methanol phase (8 ml) was removed and evaporated to dryness. The residue was dissolved in 5 ml water and partitioned against 5 ml butanol. A 4 ml aliquot of the butanol phase was evaporated and reconstituted in 400 µl 45% methanol prior to analysis.

9.2.4 CHEMICAL ANALYSES

The methanol-extractable compounds in shoot and root extracts were assayed using reversed-phase high performance liquid chromatography (RP-HPLC) with a C-18 column (Waters Sperisorb ODS-2, 4.6 mm x 150 mm, 5 µm particle column). Isocratic elution was performed with water-methanol (55:45 v/v) as mobile phase at 1 ml/min at room temperature with detection at UV 254 nm. Rutin was used as a reference standard. Inducible compounds detected were collected and quantified using an UV-Vis spectrometer (Perkin-Elmer Lambda 5) with rutin as standard.

For structural analysis, HPLC-mass spectrometry (LC-MS) was performed. Separation of flavonoids was achieved on a reversed-phase column (SYNERGI Hydro-RP Phenomenex, 4 micron, 80A, 150 x 2 mm) with a flow rate of 180 µl/min and injection volume of 20 µl. Gradient elution was run with solvent A (1:19 formic acid:water) and solvent B (1:3:16 formic acid:water:acetonitrile) following a schedule of 10-35% solvent B (in water) for 35 min. then 35-60% B for 25 min. The HPLC column was connected to a UV-Vis detector (HP1100, Hewlett Packard) monitoring at 280 and 340 nm, followed by a mass spectrometer with an ion spray ion source (API-300, PE Sciex, Thornhill, Ontario, Canada). The mass spectrometer was operated in positive ion mode and was scanned from m/z 250 to m/z 1000 in 1.88 s. Ion spray and orifice potentials were set at 5.5 kV and 30 V, respectively. Curtain and nebuliser gases were nitrogen and air, respectively. All mass spectral data were processed using Bio-Multiview software (version 1.2ß3, PE Sciex).

9.2.5 FRACTIONATION OF SHOOT AND ROOT EXTRACTS OF OATS

Preparative high voltage paper electrophoresis (HVPE) was performed to separate the inducible compounds in both oat shoot and root methanolic extracts into fractions as well as to accumulate a sufficient amount of the inducible compounds to test the biological activity against nematodes. One ml of oat shoot and root extracts containing 268 and 124 mg of inducible compounds, respectively, in 45% methanol were blotted evenly along the central axis on a sheet of Whatman 3mm Chr chromatography paper (200 x 500 mm). Rutin was used as standard. Electrophoresis was carried out using borate buffer at pH 9.4 for 1.5 h at 400V. Fraction sections were made based on electrophoretic mobility of the compounds in the extracts. Fluorescent illumination helped in the identification of bands. Each band was eluted in 45% methanol and passed through Sep-Pak® C18 cartridges (Waters) to remove the borate. Several HVPE runs were performed and all the fractions collected were analysed by RP-HPLC using the gradient run method in order to pool similar fractions from different runs. Prior to testing for biological activity, pooled fractions were evaporated and reconstituted in 1 ml 10% methanol.

9.2.6 TEST FOR BIOLOGICAL ACTIVITY OF INDUCIBLE COMPOUNDS

Approximately 3,000 active invasive stage (J2) of *H. avenae* were treated for 24 h with 1 ml shoot and root extracts of oats. The oat shoot and root extracts were prepared by drying 50 µl of the extracts used in the preparation of fractions described previously. These were resuspended in 1 ml 10% methanol before treating the nematodes. The shoot and root extracts contained 3.39 and 0.16 mg inducible compounds after diluting to 1 ml. The diluted amount was used for treating the nematodes because the concentrations of the inducible compounds were close to the adjusted concentrations of the pooled compounds A and B (1.04 and 0.82 mg, respectively) obtained from the fractionation by HVPE. The 16 fractions in 1 ml 10% methanol obtained by HVPE were also used to treat nematodes. Preparation of the fractions was as described previously. Both water and 10% methanol

controls were included. After treatment, the nematodes were rinsed with three changes of water. Known number (500 nematodes/plant) of treated and control nematodes were applied to 15-day old wheat seedlings grown as described above. Nematodes extracted from the sand by a flotation and sieving method (Byrd *et al.*, 1966) and stained in roots were counted 30 days after inoculation. Nematodes recovered from the soil were tested for vitality (Shepherd, 1962) by staining with 0.01% new blue R (GT Gurr Ltd. UK).

9.2.7 PROTECTIVE EFFECTS OF THE METHANOL-EXTRACTABLE COMPOUNDS

Oat seedlings were treated with a soil drench of 10 ml 10^{-4} M methyl jasmonate 15 days after sowing (referred to as "treated plants") to induce the compounds. Three days following induction, treated and untreated plants were inoculated with 500 each of *H. avenae* J2 and mixed stages of *P. neglectus* per plant. Uninoculated controls of both treated and untreated plants were included. Plants from each treatment were sampled and roots were washed thoroughly 5 and 10 days after inoculation. Nematodes were extracted and counted from both the soil and the roots as previously described. The vitality of nematodes recovered from the soil was tested. Additional plants were harvested to determine the fresh and dry weights of shoots and roots and for chemical analysis.

9.2.8 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

Plants of uniform height and vigor were arranged in a randomised complete block design with six replicates before treatments in the experiments were applied. Data were transformed (logarithmic or arc sine transformation) when necessary for analysis. Analysis of variance was used to analyse the data and means were compared using Tukey's test.

9.3 RESULTS

9.3.1 INDUCIBILITY OF COMPOUNDS

Treatment of oats with methyl jasmonate (Fig. 9.1) increased concentrations of the methanol-extractable compounds from the roots (140%, $P < 0.01$) and shoots (131%, $P < 0.05$). The inducible compounds in the shoots and roots eluted as a single peak, $R_t = 4.34$ min, during isocratic RP-HPLC using 45% methanol (Fig. 9.2a). However, RP-HPLC gradient elution revealed the presence of three compounds (Fig. 9.2b and c). The three compounds, A, B and C were separated using acetonitrile with retention times (R_t) of 20.89, 23.21 and 24.43 min for the shoot extract (Fig. 9.2b) and 20.95, 23.43 and 24.65 min for the root extract (Fig. 9.2c), respectively. Compound A, B and C comprised 77.3, 14.3 and 8.4% of the total amount of inducible compounds in oat shoot extract and 1.6, 37.2 and 61.2% of the total amount of inducible compounds in oat root methanolic extract, respectively.

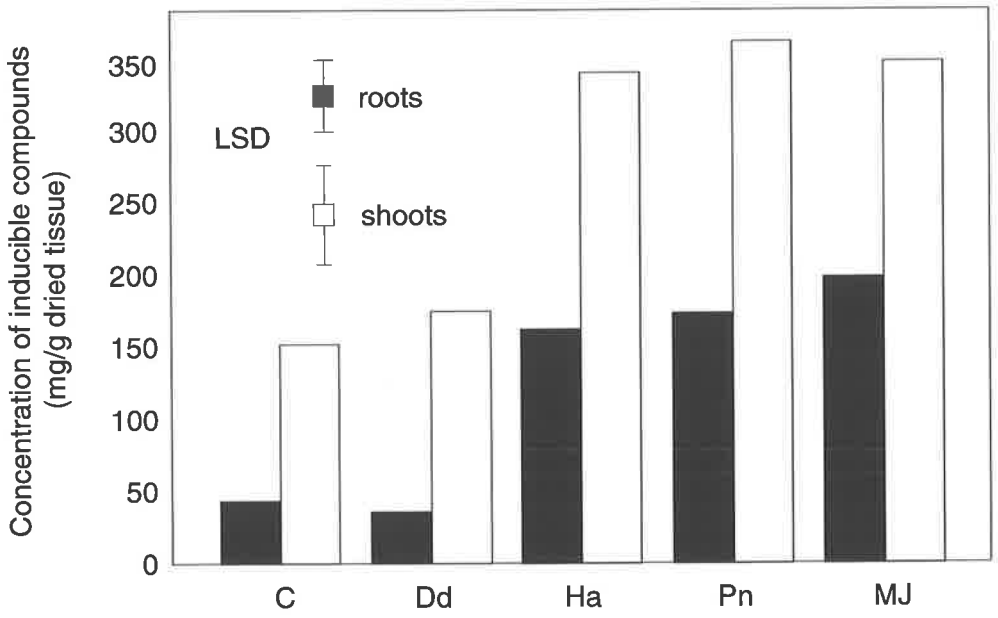

Figure 9.1 Concentration of the inducible compounds in oat shoot (open) and roots (solid) after induction using nematodes, *Ditylenchus dipsaci* (Dd), *Heterodera avenae* (Ha), and *Pratylenchus neglectus* (Pn), and methyl jasmonate (Mj) as detected by reverse phase HPLC; control (C)

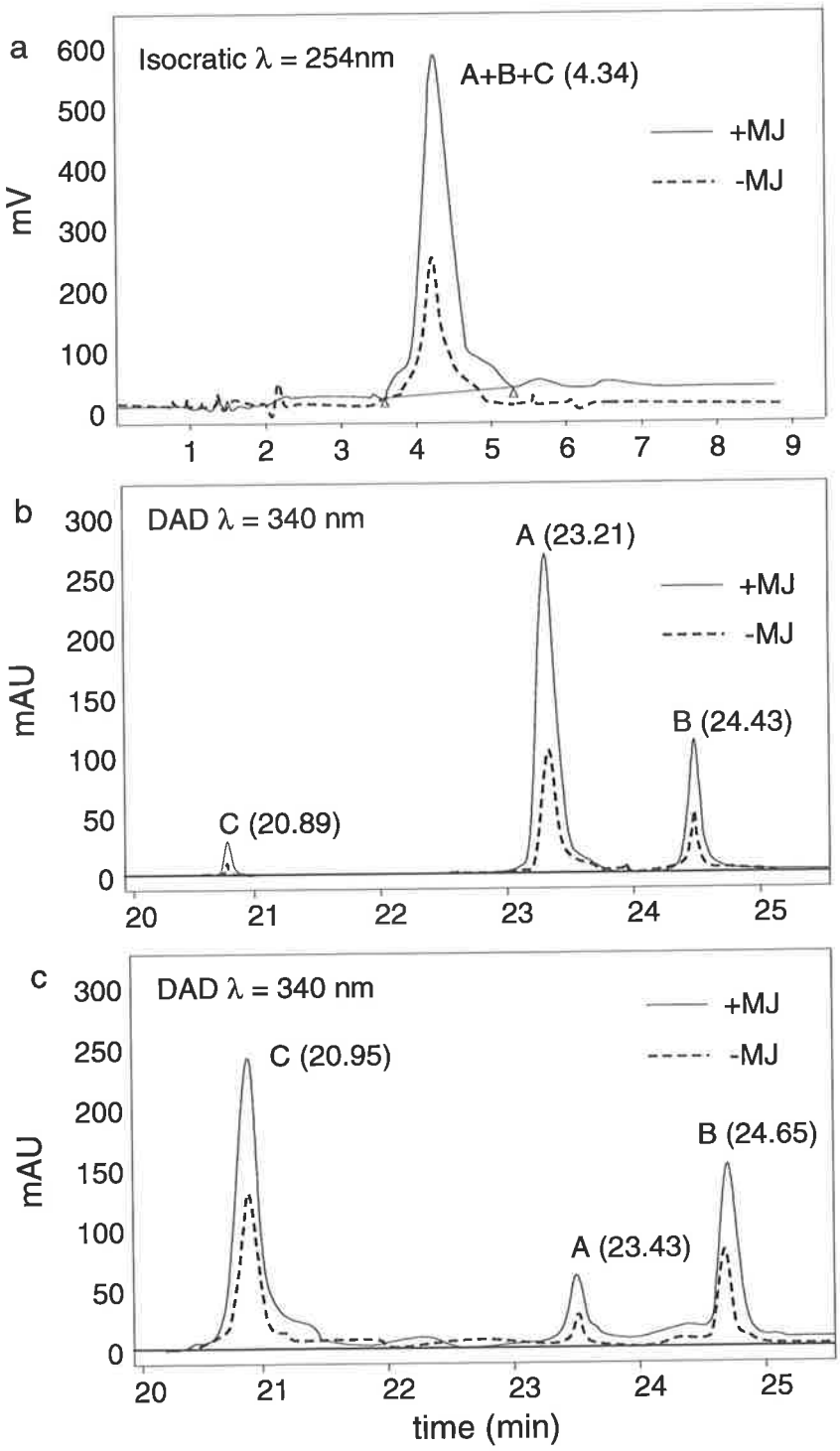

Figure 9.2 Chromatograms of the inducible compounds (A, B and C) in oats with (+MJ) and without (-MJ) methyl jasmonate treatment resolved by isocratic run using 45% methanol of shoots (a), and by acetonitrile/water gradient run of shoots (b) and roots (c)

Inoculation with the nematodes *H. avenae* and *P. neglectus* increased concentrations of these compounds in the roots by 288 and 305% ($P < 0.01$) and in the shoots by 127 and 141% ($P < 0.05$, Fig. 9.1), respectively. In contrast, *D. dipsaci* had no significant effect on the concentration of the compounds in the shoots or roots (Fig. 9.1). No other methanol-extractable compound was found to be inducible by methyl jasmonate or nematodes.

9.3.2 BIOASSAY OF CRUDE EXTRACT

The root and the shoot extracts were tested for biological activity against nematodes. The recovery from the roots of *H. avenae* treated with the methanolic shoot and root extracts was only 2 and 26%, respectively, compared to the 65 and 59% for nematodes treated with water and MeOH, respectively (Fig. 9.3). Of the shoot and root extract-treated nematodes that invaded the roots (2 and 26%, respectively), only 2.8 and 5.4%, respectively, developed beyond the invasive stage (J2), in comparison to about 60% developing beyond J2 or early adult stage (J3-J4) in the controls (data not shown).

9.3.3 BIOLOGICAL ACTIVITY OF THE INDUCIBLE COMPOUNDS

Preparative HVPE of shoot extracts after induction by methyl jasmonate yielded 16 fractions. The compounds A and B were detected through RP-HPLC from the fractions obtained (Fig. 9.4). The average concentration of compounds A and B obtained from each electrophoresis run was 0.21 and 0.04 mg, respectively. Compound C, on the other hand, was not recovered. Two fractions, containing the inducible compound B, detrimentally affected the invasion of *H. avenae* in roots (Fig. 9.4). Only 28 and 34% of the nematodes treated with these fractions invaded the roots. Although the fractions containing compound A had a slight inhibitory effect, they were not significantly different from the controls, as with the remaining fractions (Fig. 9.4). The inducible compounds were not

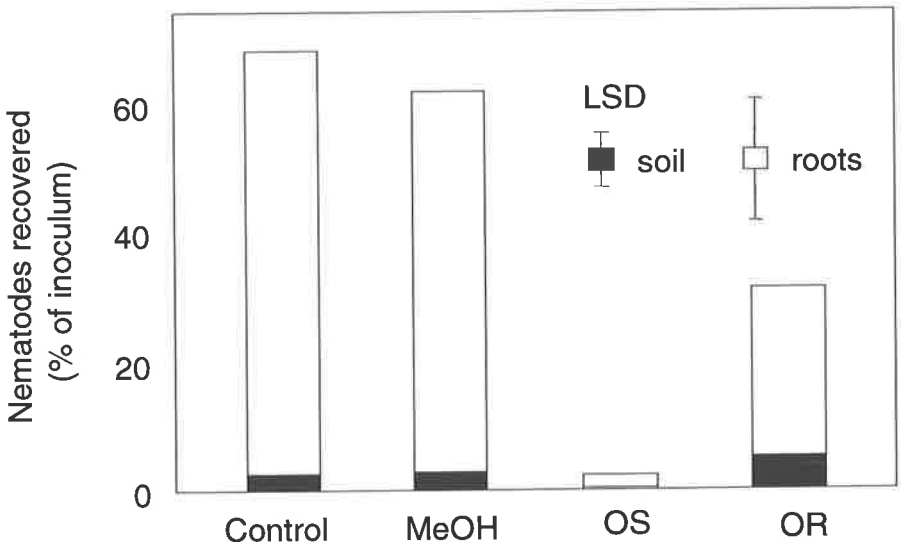

Figure 9.3 Proportion (%) of *Heterodera avenae* (inoculum = 500 nematodes/plant) recovered from soil (solid) and roots (open) of wheat cv. Egret 30 days after inoculation following treatment with water (control), 10% methanol (MeOH) and induced oat shoot (OS) and root (OR) extracts

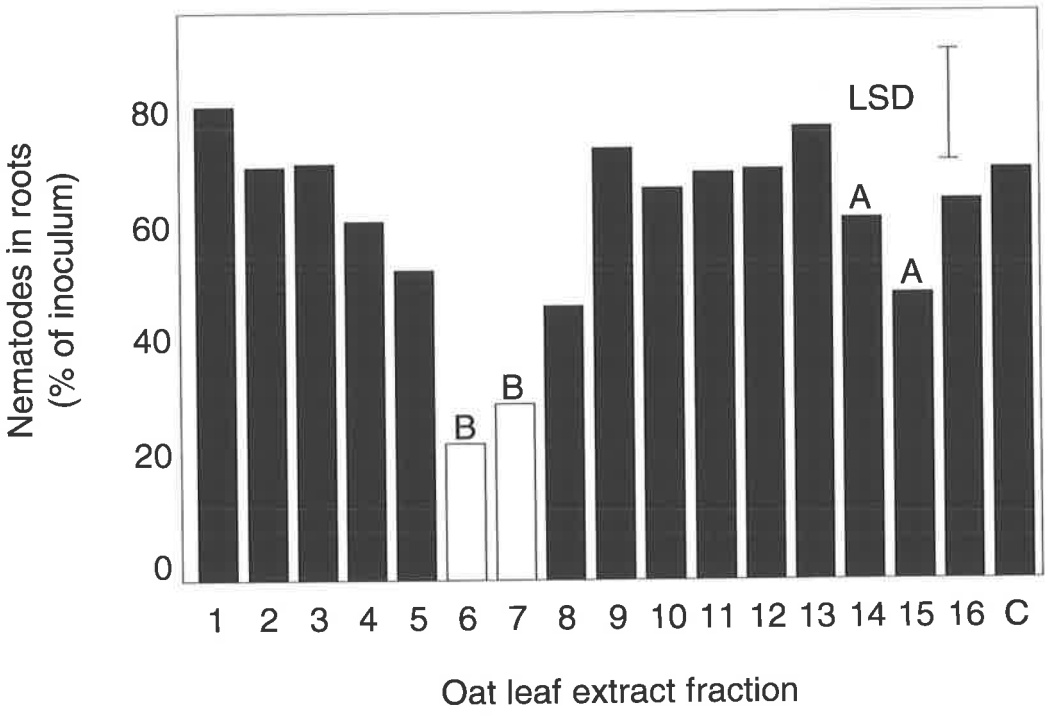

Figure 9.4 Proportion (%) of *Heterodera avenae* (inoculum = 500 nematodes/plant) recovered from wheat cv. Egret roots 30 days after inoculation following treatment with fractions (1-16) of induced oat shoots and control (C); open bars are biologically active fractions; fractions containing compounds A and B are labeled accordingly

detected from fractions of the oat root extracts obtained by preparative HVPE and these fractions did not significantly inhibit nematode invasion.

9.3.4 PROTECTIVE EFFECT OF INDUCIBLE COMPOUNDS

The protective effects of induction of oats against *H. avenae* and *P. neglectus* were assessed. Methyl jasmonate treatment of oats affected the invasion of the nematodes into the roots (Fig. 9.5). A comparative proportion of nematodes, *H. avenae* (56%, $P < 0.01$) and *P. neglectus* (84%, $P < 0.01$), failed to invade and were recovered from the soil of treated vs. untreated plants 10 days after inoculation. The proportion of nematodes that invaded the roots of treated plants decreased by 50% ($P < 0.01$) and 48% ($P < 0.01$) for *H. avenae* and *P. neglectus*, respectively, compared to untreated control plants. By contrast, the majority of the nematodes, *H. avenae* (68%, $P < 0.01$) and *P. neglectus* (79%, $P < 0.01$), were observed in the roots of untreated oats.

Ten days after inoculation of untreated plants, *P. neglectus* had induced 176% and 383% increase in methanol-extractable compounds in shoots and roots, respectively, compared with the untreated/uninoculated control (Fig. 9.6A). Likewise, *H. avenae* increased the concentration of the inducible compounds in the shoots and roots by 148% and 283%, respectively, compared with the untreated/uninoculated control 10 days after inoculation of untreated plants. The induction of the inducible compounds by inoculation with *P. neglectus* and *H. avenae* was equivalent to that induced by methyl jasmonate treatment, but combined treatment and inoculation did not increase concentrations over that of inoculation alone.

Inoculation of untreated oats with *P. neglectus* reduced root and shoot dry weight by 74% and 52% ($P < 0.01$), respectively, compared to untreated and uninoculated controls (Fig. 9.6B). Similarly, *H. avenae* in untreated oats reduced root and shoot dry weight by 42% and 54% ($P < 0.01$), respectively. In contrast, pre-inoculation induction of the

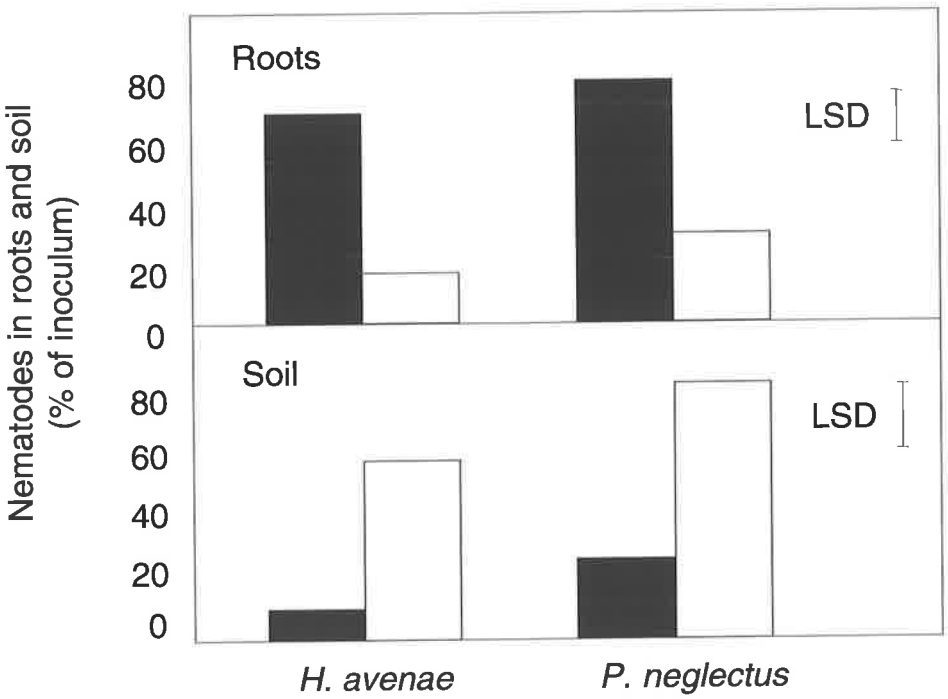

Figure 9.5 Proportion (%) of *Heterodera avenae* and *Pratylenchus neglectus* (inoculum = 500 nematodes/plant) recovered from the roots and soil 10 days after inoculation of methyl jasmonate-treated (open) and untreated (solid) oat seedlings cv. Quoll

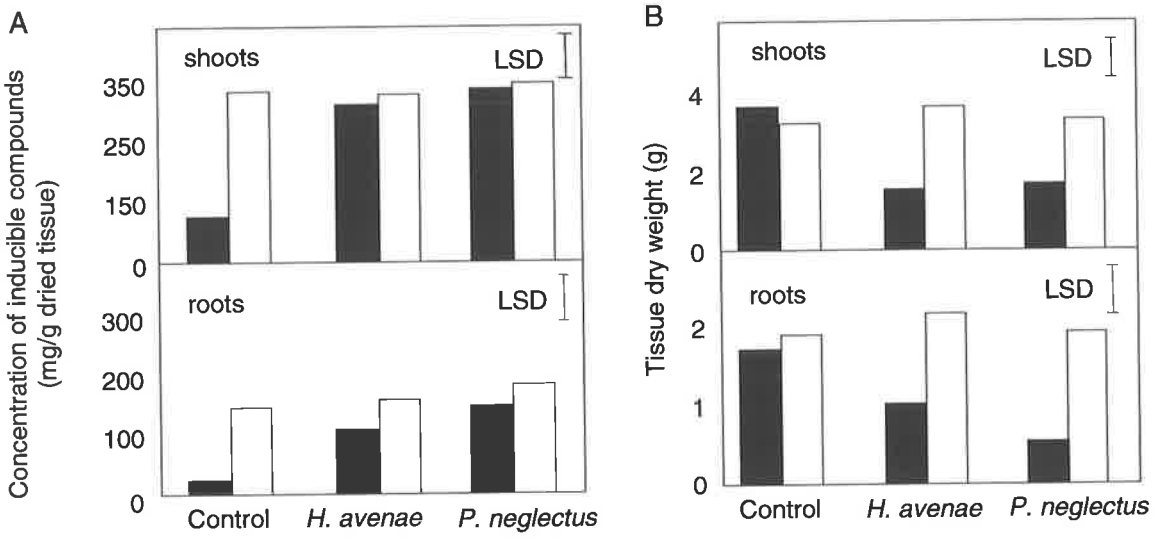

Figure 9.6 A. Concentration of inducible compounds in shoots (a) and roots (b) of methyl jasmonate-treated (open) and untreated (solid) oat seedlings cv. Quoll 10 days after inoculation of *Heterodera avenae* and *Pratylenchus neglectus*. B. Shoot (a) and root (b) dry mass of methyl jasmonate-treated (open) and untreated (solid) oat seedlings cv. Quoll 10 days after inoculation of *Heterodera avenae* and *Pratylenchus neglectus*

compounds protected the plant from damage resulting in growth equivalent to the uninoculated controls.

9.3.5 IDENTIFICATION OF THE INDUCIBLE COMPOUNDS

The absorbance spectra of the three compounds were typical of flavone glycosides (Mabry *et al.*, 1970). The λ_{max} (267, 336 nm) of A and B correspond to apigenin, while λ_{max} (267, 291, 349 nm) of C is similar to luteolin (Fig. 9.7).

Compounds A, B and C have a mass ($M+H^+$) charge (*m/z*) of 565, 593 and 579, respectively (Fig. 9.8). Compounds A and C have similar ionisation patterns (Fig. 9.8A and C), typical of a flavone-*C*-bisglycoside where the neutral loss of 132 *m/z* from the parent ions indicated the presence of an *O*-pentoside (Arpino, 1989; Arpino, 1990; Chopin and Bouillant, 1975; Stobiecki, 2000). The resulting ions of *m/z* 433 (A) and 449 (C) represent flavone-*C*-monoglycosides, were 162 *m/z* mass units greater than apigenin (*m/z*, 271 $M+H^+$) and luteolin (*m/z* 286, $M+H^+$), which is consistent with the presence of a hexose. The successive loss of three molecules of water from *m/z* 433 (A) and 449 (C) as reflected by the major ions *m/z* 415 (433 - H_2O), 397 (433 - $2H_2O$) and 379 (433 - $3H_2O$) for compound A and *m/z* 431 (449 - H_2O), 413 (449 - $2H_2O$), and 395 (449 - $3H_2O$) for compound C, and the ions *m/z* 283 (433 less 150) and 299 (449 less 150) for compounds A and C, respectively, representing the methylene flavone ion were indicative of flavone-*C*-monoglycosides (Chopin and Bouillant, 1975).

Compound B, on the other hand, has a different ionisation pattern from compounds A and C. A neutral loss of *m/z* 146 from the parent ion (*m/z* 593) corresponds to the presence of a deoxyhexose. The remaining mass (*m/z* 447) is 14 *m/z* units greater than compound A (*m/z* 433), which suggests that compound B has also the *O*-methyl group like compound A. The typical flavone-*C*-glycoside ionisation pattern exhibited by compounds A and C was not observed. However, other major ions at 327 *m/z* (447-120), 299 *m/z*

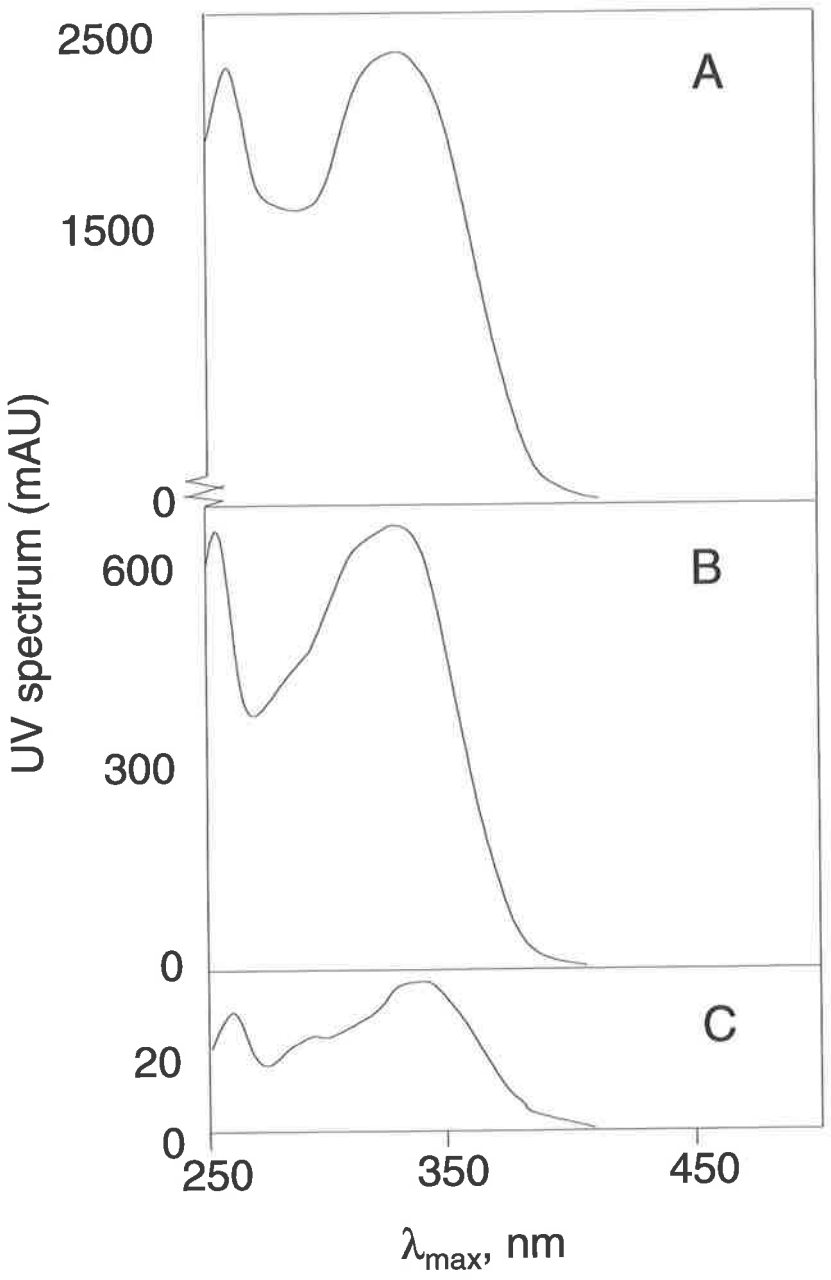

Figure 9.7 Absorbance spectra of purified compounds A, B, and C from oat seedlings cv. Quoll

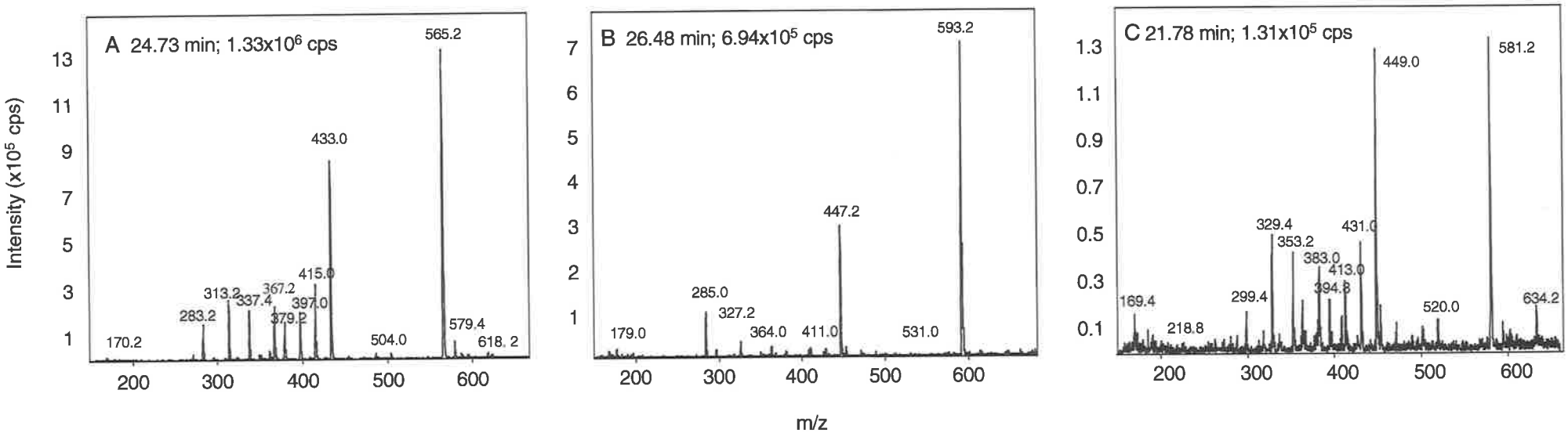

Figure 9.8 Fragmentation spectrum (MS-MS) of the $[M+H]^+$ ion at m/z 565 (A), 593 (B) and 579 (C) of compounds A, B and C, respectively.

(447-148) and 285 *m/z* (299-14) were consistent with apigenin-8-*C*-glycoside with an O-methyl group on the C7 position (Chopin and Bouillant, 1975).

These data suggest that compounds A, B and C were apigenin-*C*-hexoside-*O*-pentoside, *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside and luteolin-*C*-hexose-*O*-pentoside, respectively. As the compounds were all flavone glycosides with related chromophores, the concentration of these compounds may be expressed as rutin equivalents since rutin is a commercially available flavone glycoside.

9.4 DISCUSSION

Our study revealed that three flavone-*C*-glycosides, *O*-methyl-apigenin-*C*-hexoside-*O*-deoxyhexoside, apigenin-*C*-hexoside-*O*-pentoside and luteolin-*C*-hexoside-*O*-pentoside, were induced in oats by methyl jasmonate and nematode parasitism. Flavonoid glycosides are ubiquitous in the plant kingdom and are associated with a wide range of functions in plants (Harborne, 1999). Aside from constitutive levels in plants, flavone-*C*-glycoside accumulation in response to attack of herbivores makes them candidates for defence components of plants (Grayer and Harborne, 1994; Harborne, 1999). For example, levels of the flavonoid apigenin, as free aglycone, increased in herbivore-damaged plants (Agrell *et al.*, 2003) rendering them unpalatable to the herbivorous insect, *Spodoptera littoralis*. A constitutive flavone-*C*-glycoside in rice phloem sap has been implicated in resistance to the brown planthopper, *Nilaparvata lugens* (Grayer *et al.*, 1994). Hence, the accumulation of flavone-*C*-glycosides in oats can be interpreted as possible defence response of oats and may indicate resistance in plant. The damage in tissues of oats caused by the nematodes in this study may be similar in some ways to herbivory of insects, which results in synthesis of flavonoid derivatives in plants including apigenin and luteolin glycosides (Harborne and Williams, 2000; Harborne and Grayer, 1994).

Although flavonoids are generally accepted as defence metabolites of plants against both arthropod and mammalian herbivory (Harborne, 1999), most of the studies only report an increase in the level of the flavonoids in challenged plants and do not provide direct evidence of the involvement of flavonoids in plant defence. Our study has demonstrated that an inducible flavone-*C*-glycoside is toxic to nematodes and supports the view that it is involved in plant defence and in resistance against nematodes. This was demonstrated by the inactivation of *H. avenae* upon treatment with the induced flavone-*C*-glycosides in oat shoot and root extracts. In this bioassay, the crude shoot extract immobilised most nematodes resulting in limited invasion of roots. The crude root extract, on the other hand, had less dramatic effects but significantly impaired development of nematodes in wheat roots. The difference in the activity of the shoot and root crude extract was due to the higher concentrations of the active compound in the shoots than in the roots.

Flavonoid phytoalexins, other than flavone-*C*-glycosides have been reported to be involved in plant defence against nematodes. Coumestrol and psoralidin were compounds produced by lima beans in response to invasion by *Pratylenchus scribneri* and coumestrol was found to impair motility of the nematode at 5-25 µg/ml (Rich *et al.*, 1977). The phytoalexin, medicarpin, occurs in higher concentrations in resistant alfalfa cultivars and immobilised *P. penetrans in vitro* (Baldridge *et al.*, 1998). The same compound and 4-hydroxymedicarpin from *Taverniera abyssinica* were found to have nematicidal properties in bioassay with *C. elegans* (Stadler *et al.*, 1994). Another isoflavonoid, glyceollin has been implicated in soybean resistance to *Meloidogyne incognita* and *H. glycines* (Huang and Barker, 1991; Kaplan *et al.*, 1980a). Glyceollin inhibited *in vitro* oxidative respiration and motility of *M. incognita* (Kaplan *et al.*, 1980b) and adversely affected egg hatching and juvenile motility of *H. glycines* at 100 or 200 µg/ml (Trivedi *et al.*, 1984).

While the flavonoid content of oat leaves has been studied extensively (Chopin *et al.*, 1977; Popovici *et al.*, 1977), the flavonoids present in oat roots have not received much

attention. There were four major flavone glycosides reported in oat leaves: 2"-*O*-rhamnosyl-8-*C*-glucosylapigenin, 7-*O*-methyl-2"-*O*-rhamnosyl-8-*C*-glucosylapigenin, 2"-*O*-arabinosyl-6-*C*-glucosylapigenin, and 2"-*O*-arabinosyl-6-*C*-glucosylluteolin (Effertz and Weissenbock, 1980). The mass spectra of the latter three flavone glycosides were consistent with the three observed in this study. Thus, it is suggested that *O*-methyl-apigenin-*C*-hexoside-*O*-deoxyhexoside is equivalent to 7-*O*-methyl-2"-*O*-rhamnosyl-8-*C*-glucosylapigenin, apigenin-*C*-hexoside-*O*-pentoside to 2"-*O*-arabinosyl-6-*C*-glucosylapigenin and luteolin-*C*-hexoside-*O*-pentoside to 2"-*O*-arabinosyl-6-*C*-glucosylluteolin.

The analysis of the flavonoid profile obtained from RP-HPLC and mass spectrometry of the methanol extract of the shoot and roots of oats cv. Quoll showed that the concentration of apigenin-*C*-hexoside-*O*-pentoside was greatest in shoots and least in roots compared to the two other flavonoids. Luteolin-*C*-hexoside-*O*-pentoside was the reverse with lowest concentration in the shoots and highest in the roots and *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside reached moderate concentrations in both shoots and roots.

Of the three inducible compounds, *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside was found to be biologically active using the fractions from semipreparative HVPE. Apigenin-*C*-hexoside-*O*-pentoside was found to be inactive despite its high concentration in the shoots. The activity of luteolin-*C*-hexoside-*O*-pentoside could not be established because it was not recovered from shoot extracts in sufficient quantity. Preparations from roots, where luteolin-*C*-hexoside-*O*-pentoside was predominant, did not yield sufficient material for the components to be tested. When the biological activity of the crude extracts from roots and shoots and the fractions from shoots containing *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside is considered, it is likely that this is the sole active component in our study. Further studies are required to confirm the biological

activity of luteolin-*C*-hexoside-*O*-pentoside. The biological activity of *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside is further supported by the results obtained in the test using the semi-purified compounds.

In methyl jasmonate treated plants the estimated concentration of *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside reached 9.4 mg/g fresh weight (62.9 mg/g dry weight) in roots and 6.7 mg/g fresh weight (47.6 mg/g dry weight) in shoots. These were greater than the concentrations of the root (0.16 mg/ml) and shoot (0.47 mg/ml) extracts used in the bioassay as well as in the semipreparative HVPE (0.82 mg/ml) that were effective against nematodes. This indicates that the induced concentrations in the plant are sufficient to provide protection.

It is possible that induction and biosynthesis of the biologically active flavone-*C*-glycoside occurs in the roots because there was a lack of induction by the stem invading *D. dipsaci* in this study. However, Edwards *et al.* (1995) reported that infection with stem nematode in alfalfa systemically elicited the accumulation of phytoalexin in the roots. In contrast, the accumulation of flavonoid glucosides in the shoots but not in the roots was observed upon the infection with *D. dipsaci* in clover (Cook *et al.*, 1995). Similarly, the flavonoid, sakuranetin, and a related phenylpropanoid in the leaves of rice were induced by *D. angustus* infestation and were considered to be responsible for the resistance of some cultivars (Plowright *et al.*, 1996).

There was a systemic response of the induction of flavonoids by the root nematodes *H. avenae* and *P. neglectus* in this study similar to that reported for glyceollin by root-knot nematodes in soybean (Huang and Barker, 1991; Kaplan *et al.*, 1980a,b). Cook *et al.* (1995), on the other hand, showed a localised effect of the phytoalexin response in clover. This highlights the variation in defence response to nematodes among different plant species.

The protection provided by methyl jasmonate treatment of plants against the nematodes *H. avenae* and *P. neglectus*, adds support to the argument that the inducible flavonoids are involved in plant defence. In the absence of methyl jasmonate, nematodes were able to cause damage to the plant before sufficient flavone-*C*-glycoside accumulated. There was no obvious penalty associated with methyl jasmonate treatment, since shoot and root weights were similar to those of untreated controls. It is known in other plants that jasmonic acid induces genes involved in biosynthesis of phytoalexins (Choi *et al.*, 1994; Creelman *et al.*, 1992) and phenolics (Constabel and Ryan, 1998; Doares *et al.*, 1994; Farmer and Ryan, 1992; Stout *et al.*, 1994) that are involved in plant defence (Baldwin *et al.*, 1997; Creelman and Mullet, 1995; Creelman and Mullet, 1997; Turner *et al.*, 2002). While methyl jasmonate may have induced other compounds in oats that were not detected by our system, the induction of the flavone-*C*-glycoside in high concentrations and the direct inhibitory effect of this flavonoid on the nematode are sufficient to account for the protection observed.

This is the first report of a novel function and involvement of a flavone-*C*-glycoside, *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside, in plant defence of oats against nematodes extending the known range of function of flavonoids in plants (Harborne, 1999). Among cereals, oats has been identified to have useful levels of resistance to nematodes such as *Pratylenchus* and *Heterodera* (Hollaway, 2002). The relatively high constitutive levels of flavone-*C*-glycosides and elicitor-induced accumulation may have contributed to the degree of resistance in oats to some nematodes. More studies to relate the association of the flavone-*C*-glycoside in resistance to nematodes in oats are underway comparing the flavonoid content and nematode resistance of different cultivars of oats. Further study on levels of enzymes and genes involved in this possible resistance mechanism to nematodes in oats is a logical step.

In summary, the inducible flavone-*C*-glycoside, *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside, provided protection against the major cereal nematodes *Heterodera* and *Pratylenchus* in oats. This is the first report implicating the compound to plant defence against any pest or pathogen. The deliberate induction or breeding for enhanced constitutive production of defensive flavonoids in economically important plants may prove a useful approach for nematode population management.

GENERAL DISCUSSION

When challenged with pests and pathogens, plants exhibit complex responses that involve the activation of different signal cascades leading to the activation of the local and systemic defences. This study shows evidence that phytoecdysteroids are inducible in some plants other than spinach (Schmelz *et al.*, 1998). Flavone glycosides, known to be inducible in a wide range of plants were also induced oats. Plant tissue damage by nematodes and methyl jasmonate elicited the accumulation of these compounds. This is consistent with the substantial evidence of the local and systemic accumulation of defensive compounds by mechanical wounding of plants by feeding herbivores (Green and Ryan, 1973; Bergey *et al.*, 1996). Jasmonic acid involved in wound signalling due to herbivory or mechanical damage has been reported previously (Farmer and Ryan, 1990, 1992; Pearce *et al.*, 1991; Hildmann *et al.*, 1992; Rojo *et al.*, 1998, 1999; Park *et al.*, 2002), and exogenous application of jasmonic acid induces resistance to a broad range of herbivores (Baldwin, 1998; Omer *et al.*, 2000; Thaler *et al.*, 2002).

There are many examples of plant-pest interaction where a higher concentration of the defence compounds accumulate in resistant cultivars compared to susceptible. However, it is rarely the case that induction of defence compounds occurs only in incompatible interactions. A significant concentration of phytoallexins had been shown to accumulate in a compatible interaction (Mansfield, 1982), which is consistent with this study where a significant accumulation of potential defensive compounds was observed in compatible interactions. However, it is not sufficient to just demonstrate a greater accumulation of the defence compound. It is also necessary to show that the concentration accumulated is inhibitory to the nematode, which was demonstrated in this study with both ecdysteroids and flavone glycoside. Another factor affecting the induction of these compounds in plants was the initial nematode density. An inoculum density below 100 *Pratylenchus neglectus*/plant in this study was not sufficient to induce ecdysteroids. In

addition, induction of ecdysteroids occurs faster with the application of the elicitor, methyl jasmonate, than induction by nematode damage. Hence, induction of phytoecdysteroids is a function of amount of damage necessary to activate wound signal molecules to induce these compounds in plants and time from induction.

For any inducible defence mechanism to be effective, it has to occur at the right location, at the right time and to the extent necessary to inhibit development of the parasitic nematode involved. To be effective, accumulation must occur rapidly enough in the initial stages of infection. In many interactions, accumulation does occur more rapidly in incompatible than in compatible interaction (Weigand, 1986). Besides the inherent toxicity of the metabolite, the other factors that will determine whether a particular compound contributes effectively to the inactivation of the pest is the time taken to reach the critical concentration and be maintained at this level long enough. It was also apparent that to be effective, accumulation must occur rapidly to inhibit further invasion or development of the nematode.

In plant-pest interactions, it is possible that a number of inducible defence mechanisms or compounds may operate or are expressed concurrently. There is a problem in resolving the effects of any one defence response on the invading pest from the effects of any other that is operating simultaneously. Identifying the signalling events associated with individual mechanisms is difficult. Methyl jasmonate is recognised to induce various compounds that are involved in plant defence such as phytoalexins, phenolics and proteins. Implicating the induced phytoecdysteroids and flavone glycoside is not without an element of uncertainty, as other compounds may not have been detected by the system used in the study. The direct inhibitory effects of the ecdysteroids and flavone glycoside, *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside, on the nematodes are sufficient to account for the protection observed in the plant. The biological activity of these compounds using relevant bioassays strongly supports their involvement in plant defence against nematodes.

This was further confirmed during the direct exposure of the nematodes in induced plants wherein the phytoecdysteroids and flavone glycoside provided protection against the major parasitic nematodes in plants. The plant defence involved imparts tolerance to the major nematodes by reducing their damaging impact on yield and resistance by reducing the nematode capacity to reproduce, an obvious consequence of the effects observed. This is the first study implicating these compounds as plant defense against parasitic nematodes and may reveal a more general resistance mechanism in plants against multiple arthropod pest species.

Unlike arthropods, no study has demonstrated the effective control of plant parasitic nematodes by ecdysteroids in plants. The demonstration that putative ecdysteroids occur in nematodes, coupled with the possibility that these compounds may be essential regulators or hormones in their development provides opportunity to exploit ecdysteroids for control of nematodes. One advantage of pursuing this control strategy is that it may be less likely for nematodes to become resistant to a phytochemical acting at the same site and manner as one of their hormones.

Rees and Mendis (1984) suggested the following points of interference with the putative ecdysteroid system of nematodes for exploitation in control: a) inhibition of synthesis, b) inactivation of the ecdysteroids, c) the development of anti-hormones to interfere with the hormone action and d) application of potent ecdysteroid analogues to elevate hormonal activity at an inappropriate stage of the parasite development. However, the direct exposure to the actual hormone as might be the case with 20E in plants was not suggested. Nevertheless, with these points to consider, the predicted defensive role for phytoecdysteroids based on their known physiological or biochemical activities in nematodes, is only feasible if it can be clearly shown that the level of ecdysteroid occurring in plants can have an immediate detrimental effect on the parasite during invasion or development in plants. This was clearly demonstrated in the study.

It is also important to know if nematodes absorb, detoxify or efficiently excrete ecdysteroids because this will determine their sensitivity to or tolerance of phytoecdysteroids and flavone glycosides. Furthermore, the effects of different ecdysteroids and flavonoids on nematodes should be investigated since plants have a variety of these compounds and not all of them are likely to affect all nematodes to the same degree. In insects, cyasterone, inokosterone and ponasterone are more effective at disrupting insect development and reproduction than the common major ecdysteroids used in the study. Only relatively few plants have evolved to produce these detrimental compounds in arthropods (Jones and Firn, 1978). In an incompatible nematode-plant relationship, the nematodes may have the ability to recognise and avoid the phytoecdysteroid and flavonoid produced by a particular plant. This will give selective advantage to the nematodes not affected by the phytoecdysteroid present in that plant. Studies discussed earlier showed that nematode species differ in their sensitivity to ecdysteroids.

The study attempted to detect ecdysteroids in plant parasitic nematodes. Whether nematodes biosynthesize ecdysteroids from cholesterol or obtain them from their diet or host is unclear but the occurrence of ecdysteroids in animal and free-living nematodes has been established. Information on the presence and significance of ecdysteroids in plant parasitic nematodes would be valuable for development of novel control strategies. The major phytoecdysteroid, 20-hydroxyecdysone, was not detected in the plant parasitic nematodes tested. However, three compounds were identified by LC-MS and UV-Vis with properties similar to previously described ecdysteroids. Plant parasitic nematodes may have evolved moulting hormones that are less abundant than the main ecdysteroids in plants to avoid plant defence strategies. However, this is not evidence that 20-hydroxyecdysone is not essential nor detrimental at certain concentrations to plant parasitic nematodes as 20,26-dihydroxyecdysone and 20,26-dihydroxyecdysone 22-acetate are

conjugated 20-hydroxyecdysone.

To advance from the perceived potential of the phytoecdysteroids and flavone glycoside as defense compounds, to their application in field conditions and ultimately to their deployment as a management strategy against nematodes, will involve considerable further investigation. One of the proposed methods for the delivery of the active compounds because of its practicality is by green manure. In the study, the use of induced spinach as green manure suppressed invasion of the nematodes. Although the direct involvement of phytoecdysteroids was not established, the compounds may have contributed to the effects because the concentration in the amendment had been shown to be biologically active. Further work is necessary to examine the most effective rate, the timing of application, economically feasible plant source and other components essential for an effective green manure.

Identification of compounds which specifically interfere with the mode of action of ecdysteroids in plant parasitic nematodes is a promising mode of managing the pest while reducing the effects on other animal groups and lowering environmental hazards. The compound in lucerne shoot which inhibited the action of 20E in the *D. melanogaster* B_{II} cell bioassay may be a potential antagonistic compound that could interfere in the action of ecdysteroids. The effectiveness of such strategy has recently been demonstrated by the identification of non-steroidal bisaclhydrazines as agonists of ecdysteroid receptor complex and the development of two of the compounds as selective and successful insect control agents (Dinan *et al.*, 1999).

Ecdysteroids have limited potential as pesticides because they are too polar and chemically complex, which are undesirable properties for pesticides (Dinan, 1998). However, these compounds can be harnessed as lead compounds for study and formulation of analogues that are non-steroidal molecules, which fit the pharmacophore hypothesis and may lead to development of novel pesticides (Dinan *et al.*, 1999).

An alternative approach to crop protection based on ecdysteroids and flavonoids is to modify ecdysteroid and flavonoid levels in crops to enhance protection against nematodes or even including a broader spectrum of pests. Modification of the activity of genes, which regulate ecdysteroid biosynthesis, could be achieved by breeding or genetic modification. Application of an elicitor like methyl jasmonate would be another strategy to elevate ecdysteroid levels.

In summary, exploiting the ecdysteroid and flavonoid system of plants has potential to provide novel nematode control, and is worth pursuing considering the imperative for a healthier environment and more sustainable agriculture.

APPENDICES

A.1 FORMULATION OF UNIVERSITY OF CALIFORNIA (U. C.) MIX

The potting mix was concocted at the Plant Growth Research Center (University of Adelaide).

400 l coarse washed sand sterilized at 100°C for 30 min

300 l peatmoss (Team Peat, Canada) added and mixed at 80°C

Mixture is cooled and the following fertilizers are added:

Calcium hydroxide	700 g
Calcium carbonate	480 g
Nitrophoska (15:4:12)	600 g
Total nitrogen ($5\%NH_4$, $4\%NO_3$,$1\%NH_2$, 5% IBDU)	15%
Total phosphorus	3.9%
Potassium sulphate	12.4%
Magnesium Carbonate	1.25%
Dicalcium phosphate	3.4%
Sulphate	5.3%
Iron oxide	0.3%
Copper oxide	0.0002%
Zinc oxide	0.007%
Calcium borate	0.01%
Molybdenum oxide	0.0003%

A.2 ANALYSIS OF TAILEM BEND SAND

The analysis was calculated based on an oven dry weight basis.

Sand content	
Course (%)	88
Fine (%)	11
Clay	1.20
Silt	0.40
pH	7.10
Total Carbon (%)	0.03
Total Nitrogen (%)	<0.005
Exchangeable cations (Cmol/kg)	
Ca	0.75
Mg	0.42
K	0.07
Na	0.19
Cu (mg/kg)	0.10
Fe (mg/kg)	1.80
Mn (mg/kg)	0.70
Zn (mg/kg)	0.50

A.3 HOAGLAND SOLUTION

This nutrient solution was modified from Hoagland and Arnon (1950).

Macronutrients	g/liter
$Ca(NO_3)_2 \cdot 4H_2O$	0.236
KNO_3	0.101
NH_4NO_3	0.016
$MgSO_4 \cdot 7H_2O$	0.493
KH_2PO_4	0.068
FeNa-EDTA	0.046
Micronutrients	mg/liter
H_3BO_3	3.090
$MnCl_2 \cdot 4H_2O$	1.979
$ZnSO_4 \cdot 7H_2O$	0.288
$CuSO_4 \cdot 5H_2O$	0.062
$Na_2MoO_4 \cdot 2H_2O$	0.097

A.4 FORMULATION OF OSMOCOTE PLUS ®

OsmocotePlus® (Australia) is a controlled release fertilizer (8-9 months release at 21°C).

Element	%
N	16
P	3.5
K	10
S	1.2
Mg	1.2
Fe	0.15
Mn	0.06
Cu	0.05
Mo	0.02
B	0.02
Zn	0.02
Ca	2

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