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Alfalfa (*Medicago sativa* L.) resistance to the root-lesion nematode, *Pratylenchus penetrans*: defense-response gene mRNA and isoflavonoid phytoalexin levels in roots

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Abstract

Alfalfa (*Medicago sativa*) varieties with antibiosis-based resistance to the root-lesion nematode (*Pratylenchus penetrans*), a migratory endoparasite of many crops, have been developed by recurrent selection. Individual plants from these varieties that support significantly lower nematode reproduction were identified for molecular and biochemical characterization of defense responses. Before nematode infection, RNA blot analysis revealed 1.3–1.8-fold higher phenylpropanoid pathway mRNA levels in roots of three resistant plants as compared to three susceptible alfalfa plants. The mRNAs encoded the first enzyme in the pathway (phenylalanine ammonia-lyase), the first in the pathway branch for flavonoid biosynthesis (chalcone synthase), a key enzyme in medicarpin biosynthesis (isoflavone reductase) and a key enzyme in the pathway branch for biosynthesis of lignin cell wall precursors (caffeic acid *O*-methyltransferase). After nematode infection, the mRNAs declined over 48 h in resistant roots but rose in susceptible plants during the first 12 h after-infection and then declined. Acidic β-1,3-glucanase mRNA levels were initially similar in both root types but accumulated more rapidly in resistant than in susceptible roots after nematode infection. Levels of a class I chitinase mRNA were similar in both root types. Histone H3.2 mRNA levels, initially 1.3-fold higher in resistant roots, declined over 6–12 h to levels found in susceptible roots and remained stable in both root types thereafter. Defense-response gene transcripts in roots of nematode-resistant and susceptible alfalfa plants thus differed both constitutively and in inductive responses to nematode infection. HPLC analysis of isoflavonoid-derived metabolites of the phenylpropanoid pathway revealed similar total constitutive levels, but varying relative proportions and types, in roots of the resistant and susceptible plants. Nematode infection had no effect on isoflavonoid levels. Constitutive levels of the phytoalexin medicarpin were highest in roots of the two most resistant plants. Medicarpin inhibited motility of *P. penetrans in vitro*.

Introduction

The root-lesion nematode (*Pratylenchus penetrans* [Cobb] Filipjev Stekhoven) is found in regions of temperate climate. This migratory endoparasite has a broad plant host range, is capable of severely damaging alfalfa (*Medicago sativa* L.) stands [30], and is a threat to other forage crops [53]. Nematode eggs are deposited within or on roots and juveniles pass through four molts in a life cycle requiring 2–3 months. Juveniles and adults freely enter and leave roots, feeding preferentially on root hairs but also attacking cortical tissue in lateral roots. As the nematodes migrate through root cells, stopping for rest or prolonged feeding periods, they kill contacted cells [62]. Damage extends to adjacent cells [55,

Proprietary names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

62] and brown, water-soaked lesions containing oxidized polyphenols and lignin-like substances form, suggesting induction of a plant defense response [55, 56].

Plant defense-responses to migratory ectoparasitic nematodes (Longidoridae and Trichodoridae) and particularly, sedentary endoparasitic nematodes in the genera *Globodera* and *Heterodera* (cyst) and *Meloidogyne* (root-knot), have historically attracted research interest [37, 47, 57, 60]. Early studies indicated that the defense-response involved chemical rather than nutritional or structural penetration barriers [24] and implicated specific plant enzyme systems such as peroxidases and the phenylpropanoid pathway [6, 61]. Edens and coworkers [12] reported accumulation of phenylpropanoid pathway enzyme mRNAs in resistant soybean roots infected with *Meloidogyne incognita* or *Heterodera glycines* but less or no accumulation in susceptible roots. Roots of resistant soybeans inoculated with *M. incognita* exhibited a hypersensitive response and accumulated the phytoalexin glyceollin, a product of the isoflavonoid branch of the phenylpropanoid pathway [25]. Glyceollin inhibited oxidative respiration and motility of *M. incognita in vitro* [26] and accumulated immediately adjacent to the head region of soybean cyst nematodes (*H. glycines*) in resistant but not susceptible soybean root tissues [21]. While other isoflavonoids have been associated with plant resistance to nematodes, the work with glyceollin provides the best evidence that phytoalexins, classically defined as antimicrobial defense compounds, may play a role in resistance to nematodes.

Little information exists on biochemical signalling mechanisms involved in plant interactions with nematodes. Because chitinases and glucanases function in resistance to fungal infections by catalyzing hydrolytic reactions leading to release of ento- and exo-elicitors of defense-responses [11], it is possible that they play similar roles in nematode resistance. Direct evidence for this hypothesis is lacking, but it is interesting that potato plants whose roots were infected with *Globodera* cyst nematodes had increased β-1,3-glucanase activity in leaves but not in roots [43]. This observation is perhaps indicative of the fact that chitinases and glucanases are typically encoded by multi-gene families whose expression is likely to be regulated by complex pathways involving developmental and tissue-specific factors as well as defense-response components.

Recent molecular studies have focused on identification of new or specific plant genes involved in interactions with sedentary nematodes. Examples of effects on specific genes include root-knot nematode-regulated expression of a tobacco gene encoding a membrane-bound transport protein critical to establishment of nematode feeding sites [39]. Also in tobacco, feeding of root-knot, but not cyst nematodes, induced expression of a gene encoding extensin, a major cell wall structural protein [36]. In tomato, root-knot nematodes induced expression of extensin genes as well as a putative osmoprotectant gene [58]. A gene associated with syncytial establishment in the potato-potato cyst nematode (*Globodera rostochiensis*) interaction has been isolated [20] as well as genes of unknown function induced in tomato roots by *M. incognita* [29]. Analysis of *Arabidopsis* and tobacco plants transformed with a reporter gene construct designed to allow identification of nearby activated promoters within the plant genome and subsequently infected with cyst and root-knot nematodes showed that expression of a large number of genes was altered during development of nematode feeding structures [17]. Most recently, a wild beet gene encoding a protein of unknown function but conferring resistance to the beet cyst nematode in cultivated sugar beets has been cloned with a strategy based on mapping of chromosomal breakage points [7].

While information concerning the biochemistry and molecular biology of plant defense responses to sedentary endoparasitic nematodes has accumulated [37], little effort has been devoted to studies with migratory endoparasitic nematodes, perhaps due to lack of resistant plant germplasm. However, work with migratory endoparasitic nematodes has also been made difficult because these highly mobile parasites do not establish long-term feeding sites, thus denying investigators easily identifiable areas of plant cells specifically responding to nematode infection. The availability of resistant alfalfa plants (Table 1), selected from germplasm with field resistance to *P. penetrans* [52], has provided an opportunity to initiate study of resistance to migratory endoparasitic nematodes at the molecular and biochemical levels. The resistance is quantitative and antibiosis-based in that it reduces or prevents nematode growth and development [40]. Growth chamber studies have shown that resistant plants support several-fold lower levels of nematode reproduction than do susceptible plants [2, 52].

In order to gain insight into the metabolic basis of alfalfa resistance to nematodes, we studied mRNA levels for enzymes involved in known resis-

Table 1. Numbers of *P. penetrans* extracted from roots and soil of resistant (0.42, 112, M31) and susceptible (B02, B07, B09) plants 12 weeks after inoculation. Values shown are means of six replicates of each plant inoculated with 300 nematodes two weeks after planting.

Clone	Roots	Soil	Total
042	174	882	1056
112	511	549	1060
M31	234	609	834
B02	1299	3378	4677
B07	2596	2567	5163
B09	1543	3614	5157

tance mechanisms in *P. penetrans*-resistant and susceptible alfalfa roots before and after nematode infection. In view of their known roles in elicitation of defense responses, we included mRNA levels for the hydrolytic enzymes acidic β-1,3-glucanase and a class I chitinase in our study. We also studied mRNA levels of phenylpropanoid pathway enzymes including those of the first in the pathway, phenylalanine ammonia-lyase (PAL), and a key enzyme, caffeic acid *O*-methyltransferase (COMT), involved in the lignin biosynthesis branch of the pathway. Strengthening of cell walls through lignification may play a role in response to pathogen attack. As indicators of a possible phytoalexin-based defense mechanism, we studied mRNA levels of chalcone synthase, an early and key enzyme in the isoflavonoid branch of the phenylpropanoid pathway, and of a later enzyme in the branch, isoflavone reductase, critical to synthesis of medicarpin, the major known phytoalexin in alfalfa. Lastly, we studied mRNA levels of histone H3.2 as a likely 'housekeeping' or constitutive gene whose expression would be independent of resistance and infection. Concentrations of flavonoids, including medicarpin, in resistant and susceptible roots were measured by HPLC before and after nematode infection. The effect of medicarpin on nematode motility *in vitro* was tested. This is the first reported study of plant defense-response gene transcript levels in relation to resistance to a migratory endoparasitic nematode.

Materials and methods

Alfalfa root culture

Alfalfa plants were derived from the susceptible cultivar Baker and two resistant germplasm, MNGRN-2 and MNGRN-4, which show superior performance in fields heavily infested with *P. penetrans* [1]. Randomly selected plants were evaluated for resistance (based on *P. penetrans* reproduction) in growth chamber assays [2, 52, unpublished data]. Shoot cuttings of resistant alfalfa plants M31, 042 and 112 and susceptible clones B02, B07 and B09 were rooted in moist vermicultite for 14–21 days under greenhouse conditions. Fertilizer with trace minerals (Peat-Lite Special 20-10-20; Peters Fertilizers) was applied initially and at 10 days. Rooted cuttings were removed from growth pans and vermiculite was released from the roots by gentle shaking. Roots were sectioned 3–5 cm above the growth tip and held on parafilm-sealed 1.5% agar Petri plates at room temperature.

Nematode inoculum

Nematode inoculum was obtained from a strain of *P. penetrans* originally isolated at Grand Rapids, MN, and maintained in axenic culture at the University of Minnesota on Iochief hybrid sweet corn (#436-381; Gurney Seed and Nursery Co., Yankton, SD) seedling roots grown on Gamborg's B5 medium without vitamins or plant growth regulators [16]. Nematodes were recovered from the medium into sterile water with the Cornell modified pie pan extraction method, concentrated by centrifugation at $500 \times g$ and resuspended in supernatant at 10 nematodes/μl. Alfalfa roots on agar plates were inoculated with 50 nematodes/cm root by pipetting the nematode solution along the length of the roots. Controls were mock-inoculated with supernatant. The plates were sealed with parafilm and held at room temperature for 0–48 h. The experiment was replicated 14 times and from 100–400 mg of roots (1–4 plates) were collected for each time point (up to 1.7 g at 0 h) with or without nematode inoculation.

RNA isolation

Root sections were recovered from agar plates at 0, 6, 12, 24 and 48 h after inoculation, quick-frozen in liquid nitrogen and held at -80 °C. Total RNA was recovered from the roots with the method of Chomczynski and Sacchi [9] and the commercial reagent TRIzol

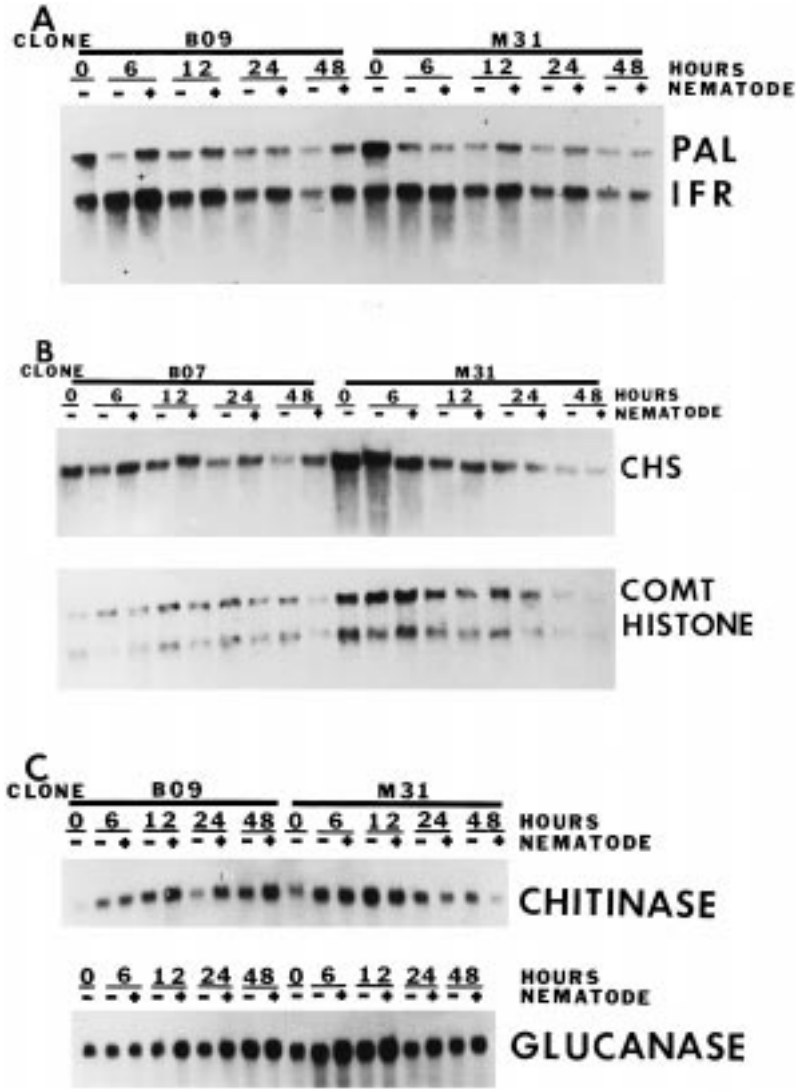

Figure 1. Representative northern blot analyses of PAL and IFR (A, top). CHS, COMT and histone H3.2 (B, middle) and chitinase and β-1,3-glucanase (C, bottom) mRNA levels in roots of susceptible alfalfa plants B07, and B09 and resistant plant M31. Control (−) and nematode-infected (+) roots were held on agar plates for 0–48 h after inoculation.

(Gibco-BRL, Gaithersburg, MD). To remove contaminating polysaccharides the RNA pellets were reprecipitated from 4M LiCl followed by chloroform extraction and ethanol precipitation. The final RNA pellets were resuspended in diethylpyrocarbonate-treated water and quantitated by UV spectroscopy.

cDNA probes

Digoxigenin-labelled double-stranded cDNA probes were prepared by random-primed Klenow reactions according to the manufacturer's protocol (Boehringer Mannheim, Indianapolis, IN). DNA inserts from recombinant plasmids containing cDNA clones of *M. sativa* genes were released by restriction enzyme digestion, purified twice by agarose gel electrophoresis, and used as templates in labelling reactions. The cDNA probes represented the following alfalfa genes: phenylalanine ammonia-lyase (PAL) [18], isoflavone reductase (IFR) [41], chalcone synthase (CHS2) [23], *S*-adenosyl-L-methionine:caffeic acid 3-*0*-methyltransferase (COMT) [19], β-1,3-glucanase [31], histone H3.2 [27, 59] and a class I chitinase (Purevin and Samac, unpublished).

Northern blot analysis

Five μg aliquots of total RNA were electrophoresed on agarose/formaldehyde gels by standard methods [45], transferred onto Hybond N^+ nitrocellulose membrane (Amersham, Arlington, IL) by rapid downward alkaline blotting [35] and fixed by baking at 80 °C under vacuum for 2 h. The blotted RNAs were hybridized with digoxigenin-labelled cDNA probes and exposed to Kodak X-OMAT AR film as described by Engler-Blum [14]. Exposures were adjusted to remain within the ca. 16-fold linear response range of the film and relative mRNA levels were quantitated by scanning densitometry using an Ambis radioanalytic image analyzer (Ambis, San Diego, CA). For each autoradiogram representing a replicate experiment with a given probe, the highest band density was assigned a value of 1 and all other band densities were converted to decimal proportions of 1. Replicates ranged from 6 for chitinase to 14 for IFR and the data was averaged as decimal proportions over replicates. Because the overall patterns of mRNA accumulation were consistent among the 3 susceptible plants and among the 3 resistant plants and data were pooled for plotting as resistant versus susceptible plants.

Root isoflavonoid analysis

Root samples were harvested, weighed, and stored at −70 °C until analysis. Samples weighing from 0.4 to 1.6 g were ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was mixed with 3.5 ml of 80% acetonitrile in 1 mM acetic acid, filtered to remove particulates, and rinsed with 0.5 ml 80% acetonitrile. Samples were evaporated under nitrogen at 45 °C and stored at −70 °C. For HPLC analysis, samples were resuspended in 60 μl 100% acetonitrile. Compounds were separated with a Waters HPLC system using a Novapak C18 reversed-phase column, 30 cm × 3.6 mm diameter with 4 μm beads (Waters Corporation, Milford, MA). The mobile phase was 20% acetonitrile in 1 mM acetic acid, increasing to 60% over 55 min and then to 98% over 5 min, with a flow rate of 0.8 ml/min. The effluent was monitored for absorbance at 210 nm with a UV detector, and isoflavones were quantified as peak area units mV/s) by Milennium Chromatography software (Waters Corporation, Milford, MA) with pure medicarpin and coumestrol as standards for these compounds. Medicarpin was kindly provided by David Gustine and coumestrol was from Eastman Kodak, Rochester, NY.

Nematode motility assay

HPLC-purified medicarpin (a gift from Dr C. P. Vance, University of Minnesota) was resuspended in 95% ethanol and mixed with 8 ml molten water-agar at final concentrations of 10, 20, 40 and 80 μg/ml. Two 35 mm Petri plates were poured with 4 ml aliquots of each medicarpin/agar solution. All plates, including 4 water-agar controls, contained 1.2% ethanol. A 2 cm long sterile maize seedling root (see above) was placed at an edge of each plate as a nematode attractant. After 5 days growth in darkness at room temperature, the roots were approximately the same length but were covered with numerous root hairs. A 12 μl aliquot of inoculum containing 300 nematodes was pipetted onto each plate 2 cm from the root. Each inoculum spread over a circular area 8 mm in diameter. At 24 h after inoculation the number of visible motile and non-motile nematodes was determined by light microscopy. Invisible nematodes were assumed to have entered the roots.

Results

Northern blot analyses

No significant differences were found in levels of total RNA recovered from susceptible as compared to resistant alfalfa roots or from control as compared to nematode-infected roots. However, total RNA per mg root declined from 0.40 to 0.27 μg by 6 h after infection and linearly thereafter to 0.17 μg at 48 h after infection. Analysis of electrophoresed RNA integrity by ethidium bromide staining showed that the RNA was largely intact and was used to verify equivalence of loading in each lane before production of northern blots (examples shown in Figure 1). We were unsuccessful in attempts to use heterologous actin cDNA probes to monitor alfalfa actin mRNA transcripts as a putative 'constitutive' gene control with which to normalize our data for the other transcripts studied. Furthermore, we found that transcripts of histone H3.2, our second choice as a constitutive control, were not independent of resistance or nematode infection (see below). Because of these results and caution indicated in use of 'constitutive' transcripts or rRNA as controls to normalize autoradiographic data [49], we rigorously replicated the experiments from 6 to 14 times, depending on the probe, and then pooled data from replicates for graphical analysis (Figure 2 and 3)

as an alternative means of normalizing for lane loading differences and increasing confidence in our results.

At 0 h after infection, histone H3.2 mRNA levels were 1.3-fold higher in resistant than in susceptible roots (Figure 2A). At 6–12 h, mRNA levels in control and nematode-infected resistant roots had declined but were marginally higher than levels in comparable susceptible roots in which they had not shown a similar pattern of decline. Thereafter, the mRNA levels were similar and remained relatively stable in both root types with or without nematode challenge.

Levels of β-1,3-glucanase and chitinase mRNA at 0 h after infection were similar in resistant and susceptible roots (Figure 2B and C). Within 6 h, glucanase mRNA levels rose 1.7- and 2.6-fold in control and nematode-infected resistant roots, respectively. The mRNA levels displayed a similar profile of increase in comparable susceptible roots but rose to levels only about half that of the resistant roots. Subsequently, mRNA levels were stable in control resistant roots and declined slightly in nematode-infected resistant roots. In susceptible roots, mRNA levels continued to rise but more rapidly and to a higher level in nematode-infected than in control roots. The rise in mRNA levels was notably faster in nematode-infected roots of both root types than in controls. In contrast, chitinase mRNA levels rose about 4-fold during the first 12 h in both root types with or without nematode-infection. Subsequently, chitinase mRNA levels slowly declined in resistant roots and remained stable in susceptible roots.

At 0 h after infection, mRNA levels of PAL, CHS, IFR and COMT were 1.8-, 1.7-, 1.6- and 1.6-fold higher, respectively, in roots of resistant than in susceptible plants (Figure 3A, B, C and D). With the exception of a small increase in IFR mRNA 6 h after nematode infection of resistant roots, the phenylpropanoid pathway mRNAs exhibited a profile of steady decline over time in both control and infected resistant roots, with levels remaining slightly higher in infected than in control roots. In contrast, the mRNA levels rose within 6 h in infected susceptible roots but declined in control susceptible roots with the exception of IFR. The mRNA increase was most striking in the case of PAL, rising 0.4-fold in susceptible roots in the first 12 h after infection and exceeding levels in comparable nematode-infected resistant roots by 0.6-fold. The increase in infected susceptible root mRNA was least striking in the case of COMT which displayed a slow and weak response compared to the other three mRNA species. Levels of CHS and IFR

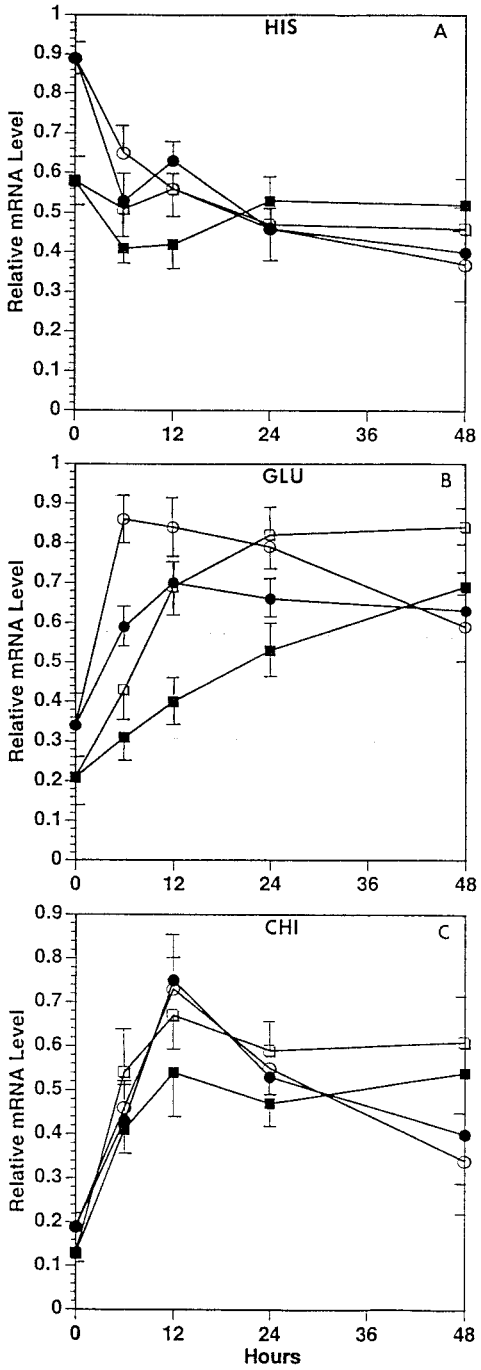

Figure 2. Relative mRNA levels of histone H3.2 (HIS, A), β-1,3-glucanase (GLU, B) and chitinase (CHI, C) in susceptible (box symbols) and resistant (circle symbols) alfalfa plants over a 48 h time-course. Control mRNA levels are indicated by filled symbols and nematode-infected mRNA levels by open symbols. Data points represent means (standard error bars indicated) of 6–10 replicate experiments.

mRNAs in infected susceptible roots displayed intermediate increases which were most apparent 6 h after infection. Lastly, we noted that increased levels of PAL and IFR mRNAs in infected versus control susceptible roots were better sustained at longer time points than were those of CHS and, particularly, COMT.

Root isoflavonoid levels

Because mRNA levels of phenylpropanoid pathway enzymes involved in synthesis of medicarpin (CHS and IFR) were constitutively higher in roots of resistant as compared to susceptible plants, we suspected that medicarpin, and precursor or closely related isoflavonoids, might also be constitutively higher in resistant as compared to susceptible roots. HPLC analysis of root extracts revealed the presence of medicarpin (R_t = 37.9 min), but not the similar isoflavans vestitol and sativan, in all root samples (Table 2). Resistant plants M31 and 042 contained significant more medicarpin than susceptible plants B02, and B09, but all 3 susceptible plants contained more medicarpin than resistant plant 112. Root isoflavonoid levels were not affected by infection with *P. penetrans* over 48 h (data not shown).

The root extracts also contained small quantities of two unidentified flavonoids similar in structure to medicarpin (compound A; R_t = 21.1 min) and daidzein (compound B; R_t = 43.5 min), as well as coumestrol (R_t = 25.5 min) and large quantities of the medicarpin precursor formononetin (data not shown; R_t = 31.7 min). The identity of the known compounds was confirmed by PDA analysis combined with spectral analysis of each fraction using the Integrity LC-MS and Millenium v2.21 (Waters Corporation, Milford, MA). Compounds A and B, were unstable and exhibited several peaks upon further PDA and MS analysis. Results of MS analysis revealed a minor impurity peak in the medicarpin fraction, whose UV and MS spectra matched that of one of the degradation products in each of peaks A and B. The molecular weight of this compound was 254, with the formula $C_{15}H_{10}O_4$, and a structure similar to that of daidzein except for the location of a hydroxyl group.

The proportion of isoflavonoids in conjugated forms in intact tissues was not determined. Demalonylation of conjugates may have occurred under the acidic conditions used in the HPLC system. Cleavage of glycosidic bonds or demalonylation may also have occurred either completely or partially during the extraction procedure, during the infection process, or as a result of enzyme activity in intact tissues. To ensure conversion of any undetected conjugated forms to their aglycones, sample extracts were digested with B-glucosidase following the method of Edwards *et al.* [13]. HPLC chromatograms of enzymatically treated samples revealed no increases in quantities of isoflavone aglycones, nor corresponding decreases in peaks likely to represent conjugated forms.

Medicarpin and nematode motility

The differences among resistant and susceptible alfalfa roots in levels of phenylpropanoid pathway enzyme transcripts and of the phytoalexin product of the pathway, medicarpin, prompted us to consider the potential effects of medicarpin on *P. penetrans*. Because the limited quantity of available medicarpin precluded extensive experiments and the literature contained several reports of phytoalexin effects on nematode motility, we decided to use our medicarpin to investigate its effect on nematode motility. Medicarpin inhibited nematode motility in a concentration-dependent manner (Figure 4). An average of 18 non-motile and 38 motile nematodes were observed on control plates with no medicarpin (ca. 240, or 85%, of the nematodes per plate were therefore assumed to have migrated to and penetrated the roots). As medicarpin concentration increased from 10 to 80 μg/ml, non-motile nematodes/plate increased from 25 to 128 while motile nematodes/plate decreased from 43 to 6. Linear regression analysis of non-motile nematodes (R^2 = 0.9606) suggested an ED_{50} range of 55–75 μg/ml.

Discussion

We have studied alfalfa clones expressing a multigenic antibiosis-based resistance that prevents growth and development of *P. penetrans*, presumably through several enzymatic pathways [2, 52]. Based on general knowledge of plant resistance, we predicted that resistant alfalfa roots might have higher levels, relative to susceptible roots, of defense-response gene transcripts for phenylpropanoid pathway enzymes and wound-response proteins, but similar levels of transcripts for proteins such as actin or histones. We were surprised to find that levels of histone H3.2 mRNA were not independent of resistance or nematode infection.

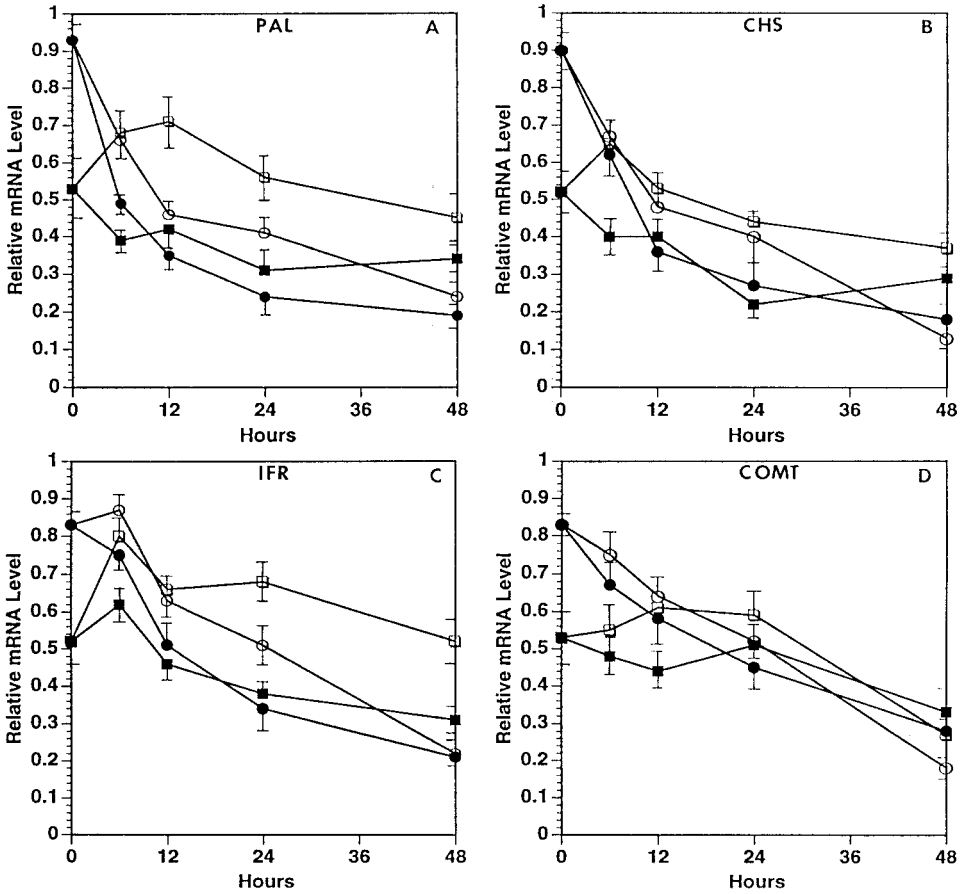

Figure 3. Relative mRNA levels of phenylalanine ammonia-lyase (PAL, A), chalcone synthase (CHS, B), isoflavone reductase (IFR, C) and caffeic acid *O*-methyltransferase (COMT, D) in susceptible (box symbols) and resistant (circle symbols) alfalfa plants over a 48 h time-course. Control mRNA levels are indicated by filled symbols and nematode-infected mRNA levels by open symbols. Data points represent means (standard error bars indicated) of 8–14 replicate experiments.

Table 2. Constitutive content of medicarpin and other flavonoid compounds in roots of alfalfa plants differing in resistance to *Pratylenchus penetrans*.

Plant	Resistance to *P. penetrans*[a]	Isoflavonoid compound (μg/g fresh weight)			
		medicarpin	compound A	compound B	coumestrol
M31	R	7.72 a[b]	0.39 ab	2.51 a	2.68 a
042	R	6.32 ab	0.25 bc	4.17 a	2.45 a
B07	S	6.14 ab	0.17 bc	4.38 a	2.76 a
B09	S	4.37 bc	0.19 bc	2.80 a	1.97 a
B02	S	3.52 c	0.67 a	3.58 a	2.42 a
112	R	2.25 c	0.00 c	3.42 a	2.08 a

[a]R, resistant; S, susceptible.
[b]Values are means of 3 replications. Within a column, values with the same letter are not significantly different, $P = 0.05$, according to Duncan's multiple range test.

Because alfalfa histone H3 variants are differentially expressed during environmental stress [27] and histones may influence plant defense-responses by altering chromatin structure near defense-response genes [5], we do not discount their potential significance in expression of alfalfa defense-response genes.

We found higher constitutive levels of phenylpropanoid pathway transcripts for PAL, CHS, IFR and COMT in roots of resistant than in those of susceptible alfalfa clones. The steady decline over 48 h of phenylpropanoid pathway transcript levels in control and nematode-infected resistant roots contrasted with the pattern of initial accumulation followed by gradual decline of these transcripts in nematode-infected susceptible roots. Our results also contrast with those of Edens and coworkers [12] who studied phenylpropanoid pathway mRNA levels in soybean roots infected with the sedentary endoparasitic nematodes. *H. glycines* and *M. incognita*. They found little difference in constitutive levels of phenylpropanoid pathway mRNAs and enzyme activities in nematode-resistant relative to susceptible soybean lines. Four days after nematode infection, phenylpropanoid pathway mRNA levels and enzyme activities increased in resistant more than in susceptible roots and that of PAL actually declined in susceptible lines. Phenylpropanoid pathway transcript levels also fluctuate differentially in *M. incognita*-infected resistant and susceptible alfalfa roots (C. Potenza and C. Sengupta-Gopalan, personal communication), rising within 12 h of infection in resistant roots but to lower levels and only after 24–36 h in susceptible roots. The rapid accumulation of phenylpropanoid pathway transcripts in *P. penetrans*-infected susceptible alfalfa roots versus the slower response in legume roots infected with sedentary endoparasitic nematodes may be due to differences in nematode behavior. Sedentary endoparasitic nematodes migrate intercellularly within roots, causing limited damage, and cease movement after establishing a feeding site dependent on significant local alterations in host cell structure and function. In contrast, *P. penetrans* migrates intracellularly, killing contacted cells and damaging adjacent cells during rest and feeding periods [55, 62], presumably leading to rapid transcriptional induction of defense-response genes. However, experimental protocols and factors inherent to the host plants may also play roles in the observed differences in legume responses to sedentary and migratory endoparasitic nematode infection and additional data will be required to resolve the issue.

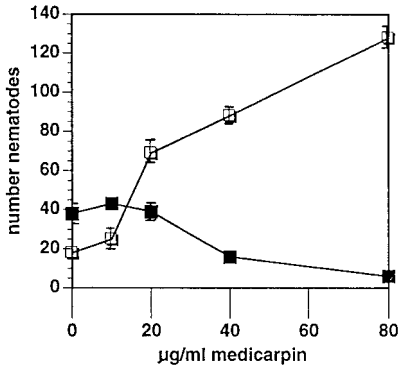

Figure 4. Effect of medicarpin on *P. penetrans* motility. Open symbols represent non-motile nematodes, closed symbols represent motile nematodes. Standard error bars are indicated.

The conjugates medicarpin-3-*0*-glucoside-6-*0*-malonate and formononetin 7-*0*-glucoside-6-*0*-glucoside are commonly found in roots and cell cultures of alfalfa [13, 28, 54] and have been associated with fungal defense responses in alfalfa [38, 51]. Edwards *et al.* [13] reported systemic induction of isoflavonoid conjugate accumulation in alfalfa roots when shoots were inoculated with the stem nematode *Ditylenchus dipsaci*. Conjugates of the isoflavonoids medicarpin, formononetin (a central intermediate in the biosynthesis of medicarpin) and coumestrol accumulated to higher levels in the root tissue of resistant alfalfa seedlings when the bud and cotyledons were infected with the stem nematode. Accumulation of the conjugates was not associated with the presence of nematodes in the root. Although it was not clear that the isoflavonoids or their conjugates have a role in the resistance of plants to the stem nematode, the increase in isoflavonoid conjugates occurred in response to a signal from the infected shoots, and was correlated with resistance. Although we found no similar induction of alfalfa root isoflavonoid levels by *P. penetrans* infection, constitutive medicarpin levels in 2 of 3 resistant plants were significantly higher than levels in 2 of 3 susceptible plants.

Medicarpin has been implicated in alfalfa resistance to fungal infection [4]. Elevated phenylpropanoid pathway enzyme transcripts and medicarpin levels in *P. penetrans*-resistant alfalfa roots may form part of a constitutive nematode defense response. The isoflavonoid phytoalexin, coumestan, has been associated with resistance of lima bean roots to *P. scribneri* and *in vitro* inhibition of nematode motility [44]. Another isoflavonoid, glyceollin, has been implicated in soybean resistance to *M. incognita* [12, 25] and

H. glycines [21]. Glyceollin inhibits *in vitro* oxidative respiration and motility of *M. incognita* [26] and extracts of necrotic alfalfa roots inhibit oxidative respiration of *P. penetrans* [8]. We found that medicarpin inhibits *in vitro* motility of *P. penetrans* and at concentrations closely resembling those reported for similar effects by glycoellin and coumestan on other nematodes. Interestingly, *M. incognita* penetrate resistant alfalfa roots but cease movement and fail to establish a feeding site whereas they move freely and establish feeding sites in susceptible roots [42]. Taken together, the results imply a possible role for medicarpin in alfalfa nematode resistance. The high concentrations of medicarpin which we and other investigators have found to be effective against nematodes *in vitro* are obviously not characteristic of total root extracts but might be present in localized cell populations in intact tissues. We must also emphasize that in the case of alfalfa and *P. penetrans* the genetic heterogeneity of the resistant and susceptible plants we studied and the antibiosis-based and quantitative nature of the resistance to *P. penetrans* implies variability in the importance of any given resistance factor among resistant plants.

The rapid increase in β-1,3-glucanase and chitinase mRNA in susceptible and resistant roots with or without nematode-infection suggests a general wounding response, particularly in the case of chitinase. Tissue damage by nematodes could conceivably trigger a wound response but it is difficult to visualize a specific role for chitinase in a defense-response because chitin is not present in nematode cuticles or plant cell walls. However, the rapid and high level of glucanase transcript accumulation in nematode-infected resistant as compared to susceptible roots was consistent with glucanase expression patterns in other plant-pathogen systems in which the susceptible response is slower and weaker than the resistant response [22]. Rahimi and colleagues [43] reported increased β-1,3-glucanase activity in leaves but not in roots of potato plants infected with *Globodera* cyst nematodes. With the caveat that we have measured changes in mRNA levels versus their direct measurements of enzyme activity, the dichotomy in the plant response to migratory versus sedentary nematode infection resembles the phenylpropanoid pathway mRNA level responses discussed above.

The hydrolytic glucanases occur as multiple isoforms with poorly defined roles in normal plant development but also function in fungal resistance by hydrolyzing hyphal cells walls [32, 46], releasing glucan exo-elicitors that amplify the plant defense-response synergistically with endoelicitors derived from plant cell wall degradation [11, 48]. Conceivably, glucanase activity might play a similar role in alfalfa resistance to *P. penetrans*. Nematode cuticle outer surface coats consist of complex mixtures of glycoproteins and carbohydrates [3, 15, 50] which might provide substrates for glucanases and related glycosidases leading to release of exo-elicitors. The rapid increase in alfalfa glucanase mRNA levels following nematode infection suggests an early event in a potential cascade of induced defense-responses. In addition, the likely release of previously synthesized vacuolar glucanases from cells ruptured by nematode migration and feeding ideally positions glucanases for a possible defense-response amplifier role. Potential consequences of such a rapid amplifier/elicitor response include induction of isoflavonoid phytoalexin production [10, 33] and, more speculatively, initiation of a programmed cell death resistance mechanism [34] consistent with the hypersensitive response of legume roots infected with *Pratylenchus* nematodes [25, 26, 44, 55, 62]. The decline in phenylpropanoid pathway mRNA levels in *P. penetrans*- resistant alfalfa roots might then be a consequence of both direct cellular destruction by nematodes and death of adjacent cells.

In conclusion, we have obtained preliminary evidence for involvement of several known plant defense-response genes in alfalfa resistance to *P. penetrans*. Nematode resistant plants have higher constitutive levels of transcripts for key enzymes involved in biosynthesis of isoflavonoid phytoalexins, which are known to play a role in fungal resistance and are implicated in resistance to both sedentary and migratory nematodes. Levels of these transcripts fell in nematode-infected resistant plants but were induced over 12 h before falling in infected susceptible plants, in contrast to the transcript induction in resistant plants observed in similar studies with sedentary endoparasitic nematodes. The validity of phenylpropanoid pathway enzyme transcript suppression in *P. penetrans*-infected resistant alfalfa plants is supported by the simultaneous induction of β-1,3-glucanase transcripts in a resistant versus susceptiple host pattern typical of many other plant/pathogen systems. Our data have allowed formulation of hypotheses useful in the design of further studies and development of the system as a model for study of resistance to migratory nematodes.

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References

1. Barnes DK, Thies JA, Rabas DL, Nelson DL, Smith DM: Registration of two alfalfa germplasms with field resistance to the root lesion nematode. Crop Sci 30: 751–752 (1990).
2. Basigalup DH: Resistance to *Pratylenchus penetrans* in alfalfa: mode of inheritance and effects on biological nitrogen fixation. M.S. thesis, Dept. of Agronomy, University of Minnesota (1990).
3. Bird AF, Bird J: The Structure of Nematodes, 2nd ed., pp. 44–74. Academic Press, San Diego, CA (1991).
4. Blount JW, Dixon RA, Paiva NL: Stress responses in alfalfa (*Medicago sativa* L.) XVI. Antifungal activity of medicarpin and its biosynthetic precursors; implications for the genetic manipulation of stress metabolites. Physiol Mol Plant Path 141: 333–349 (1992).
5. Brosch G, Ransom R, Lechner T, Walton JD, Loidl P: Inhibition of maize deacetylases by HC toxin, the host-selective toxin of *Cochliobolus carbonum*. Plant Cell 7: 1941–1950 (1995).
6. Brueske CH: Phenylalanine ammonia lyase activity in tomato roots infected and resistant to the root-knot nematode, *Meloidogyne incognita*. Physiol Plant Path 16: 409–414 (1980).
7. Cai, D, Kleine M, Kifle S, Harloff H-J, Sandal NN, Marcker KA, Klein-Lankhorst RM, Salentijn EMJ, Lange W, Stiekma WJ, Wyss U, Grundler FMW, Jung C: Positional cloning of a gene for nematode resistance in sugar beet. Science 275: 832–834 (1997).
8. Chang LM: The repellent effect of necrotic tissues on the nematode *Pratylenchus penetrans* (Cobb, 1917), Filipjev and Schurmans Stekhoven, 1941. M.S. thesis, University of Massachusetts (1969).
9. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium isothyiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159 (1987).
10. Davis KR, Darvill AG, Albersheim P: Several biotic and abiotic elicitors act synergistically in the induction of phytoalexin accumulation in soybean. Plant Mol Biol 6: 23–32 (1986).
11. Ebel J, Cosio EG: Elicitors of plant defense responses. Int Rev Cytol 148: 1–31 (1994).
12. Edens RM, Anand SC, Bolla RI: Enzymes of phenylpropanoid pathway in soybean infected with *Meloidogyne incognita* or *Heterodera glycines*. J Nematol 27: 292–303 (1995).
13. Edwards R, Mizen KA, Cook R: Isoflavonoid conjugate accumulation in the roots of lucerne (*Medicago sativa*) seedlings following infection by the stem nematode (*Ditylenchus dispaci*). Nematologica 41: 51–66 (1995).
14. Engler-Blum G, Meier M, Frank J, Muller GA: Reduction of background problems in nonradioactive northern and southern blot analyses enables higher sensitivity than ^{32}P-based hybridizations. Anal Biochem 210: 235–244 (1993).
15. Forrest JMS, Robertson WM: Characterization and localization of saccharides on the head region of four populations of the potato cyst nematode *Globodera rostochiensis* and *G. pallida*. J Nematol 18: 23–26 (1986).
16. Gamborg OL, Miller RA, Ojima K: Nutritional requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151–158 (1968).
17. Goddijn OJM, Lindsey K, van der Lee FM, Klap JC, Sijmons PC: Differential gene expression in nematode-induced feeding structures of transgenic plants harbouring promoter-*gusA* fusion constructs. Plant J 4: 863–873 (1993).
18. Gowri G, Paiva NL, Dixon RA: Stress responses in alfalfa (*Medicago sativa* L.) 12. Sequence analysis of phenylalanine ammonia-lyase (PAL) cDNA clones and appearance of PAL transcripts in elicitor-treated cell cultures and developing plants. Plant Mol Biol 17: 415–429 (1991).
19. Gowri G, Bugos RC, Campbell WH, Maxwell CA, Dixon RA: Stress responses in alfalfa (*Medicago sativa* L.) X. Molecular cloning and expression of S-adenosyl-L-Methionine:caffeic acid 3-*O*-methyltransferase, a key enzyme of lignin biosynthesis. Plant Physiol 97: 7–14 (1991).
20. Gurr SJ, McPherson MJ, Scollan C, Atkinson HJ, Bowles DJ: Gene expression in nematode-infected plant roots. Mol Gen Genet 226: 361–366 (1991).
21. Huang JS, Barker KR: Glyceollin I in soybean-cyst nematode interactions. Plant Physiol 96: 1302–1307 (1991).
22. Joosten MHAJ, de Wit PJGM: Identification of several pathogenesis-related proteins in tomato leaves inoculated with *Cladosporium fulvum* (syn. *Fulvia fulva*) as 1,3-β-glucanases and chitinases. Plant Physiol 89: 945–951 (1989).
23. Junghans H, Dalkin K, Dixon RA: Stress responses in alfalfa (*Medicago sativa* L.) 15. Characterization and expression patterns of members of a subset of the chalcone synthase multigene family. Plant Mol Biol 22: 239–253 (1993).
24. Kaplan DT, Keen NT: Mechanisms conferring plant incompatibility to nematodes. Revue Nematol 3: 123–134 (1980).
25. Kaplan DT, Keen NT, Thomason IJ: Association of glyceollin with the incompatible response of soybean roots to *Meloidogyne incognita*. Physiol Plant Pathol 16: 309–318 (1980).
26. Kaplan DT, Keen NT, Thomason IJ: Studies on the mode of action of glyceollin in soybean incompatibility to the root knot nematode, *Meloidogyne incognita*. Physiol Plant Path 16: 319–325 (1980).
27. Kapros T, Bogre L, Nemeth K, Bako L, Gyorgyey J, Wu SC, Dudits D: Differential expression of histone H3 variants during cell cycle and somatic embryogenesis. Plant Physiol 98: 621–625 (1991).
28. Kessmann H, Edwards R, Geno PW, Dixon RA: Stress responses in alfalfa (*Medicago sativa* L) V. Constitutive and

elicitor-induced accumulation of isoflavonoid conjugates in cell suspension cultures. Plant Physiol 94: 227–232 (1990).
29. Lambert K, Williamson V: cDNA library construction using PCR. Soc Nematol Mol Biol Newsl 4: 10–11 (1992).
30. Leath KT, Erwin DC, Griffin GD: Diseases and nematodes. In: Hansen AA, Barnes DK, Hill Jr RR (eds) Alfalfa and Alfalfa Improvement. American Society of Agronomy, Madison, WI,. Monograph 29, pp. 621–670 (1988).
31. Maher EA, Lamb CJ, Dixon RA: Stress responses in alfalfa (*Medicago sativa* L.) XVII. Identification of multiple hydrolases and molecular characterization of an acidic glucanase. Physiol Mol Plant Path 43: 329–342 (1993).
32. Mauch F, Mauch-Mani B, Boller T: Antifungal hydrolases in pea tissue II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol 88: 936–942 (1988).
33. Miller KJ, Hadley JA, Gustine FL: Cyclic β-1,6-1,3-glucans of *Bradyrhizobium japonicum* USDA 110 elicit isoflavonoid production in the soybean (*Glycine max*) host. Plant Physiol 104: 917–923 (1994).
34. Mittler R, Lam E: Sacrifice in the face of foes: Pathogen-induced programmed cell death in plants. Trends Microbiol 10: 10–15 (1996).
35. Munch S: Nonradioactive northern blots in 1.5 days and with substantially increased sensitivity through 'alkaline blotting'. Biochemica 11: 29–31 (1994).
36. Niebel A, De Almeida Engler J, Tire C, Engler G, Van Montagu M, Gheysen G: Induction patterns of an extensin gene in tobacco upon nematode infection. Plant Cell 5: 1697–1710 (1993).
37. Niebel A, Gheysen G, Van Montagu M: Plant-cyst nematode and plant-root-knot nematode interactions. Parasitol Today 10: 424–430 (1994).
38. O'Neill NR: Defense expression in protected tissues of *Medicago sativa* is enhanced during compatible interactions with *Colletotrichum trifolii*. Phytopathology 86: 1045–1050 (1996).
39. Opperman CH, Taylor CG, Conkling MA: Root-knot nematode-directed expression of a plant root-specific gene. Science 263: 221–223 (1994).
40. Painter RH: The mechanisms of resistance. In Insect Resistance in Crop Plants, pp. 23–83. MacMillan, New York (1951).
41. Paiva NL, Edwards R, Sun Y, Hrazdina G, Dixon RA: Stress responses in alfalfa (*Medicago sativa* L.). 11. Molecular cloning and expression of isoflavone reductase, a key enzyme of isoflavonoid phytoalexin biosynthesis. Plant Mol Biol 17: 653–667 (1991).
42. Potenza CL, Thomas SH, Higgins EA, Sengupta-Gopalan C: Early root response to *Meloidogyne incognita* in resistant and susceptible alfalfa cultivars. J Nematol 28: 475–484 (1996).
43. Rahimi S, Perry RN, Wright DJ: Identification of pathogenesis-related proteins induced in leaves of potato plants infected with potato cyst nematodes, *Globodera* species. Physiol Mol Plant Path 49: 49–59 (1996).
44. Rich JR, Keen NT, Thomason: Association of coumestans with the hypersensitivity of lima bean roots to *Pratylenchus scribneri*. Physiol Plant Path 10: 105–116 (1977).
45. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, NY (1989).
46. Sela-Buurlage MB, Ponstein As, Bres-Vloemans SA, Melchers LS, van den Elzen PJM, Cornelissen BJC: Only specific tobacco (*Nicotiana tabacum*) chitinases and β-1,3-glucanases exhibit antifungal activity. Plant Physiol 101: 857–863 (1993).
47. Sijmons PC: Plant-nematode interactions. Plant Mol Biol 23: 17–931 (1993).
48. Smith CJ: Accumulation of phytoalexins: defense mechanism and stimulus response. New Phytol 132: 1–45 (1996).
49. Spanakis E: Problems related to the interpretation of autoradiographic data on gene expression using common constitutive transcripts as controls. Nucl Acids Res 21: 3809–3819 (1993).
50. Spiegel Y, Cohn E, Spiegel S: Characterization of sialyl and galactosyl residues on the body wall of different plant parasitic nematodes. J Nematol 14: 33–39 (1982).
51. Stevenson PC, Turner HC, Haware MP: Phytoalexin accumulation in the roots of chickpea (*Cicer arietinum* L.) seedlings associated with resistance of fusarium wilt (*Fusarium oxysporum* f.sp. *ciceri*). Physiol Mol Plant Path 50: 167–178 (1997).
52. Thies JA, Basigalup D, Barnes DK: Inheritance of resistance to *Pratylenchus penetrans* in alfalfa. J Nematol 26: 452–459 (1994).
53. Thies JA, Petersen AD, Barnes DK: Host suitability of forage grasses and legumes for root-lesion nematode *Pratylenchus penetrans*. Crop Sci 35: 1647–1651 (1995).
54. Tiller SA, Parry AD Edwards R: Changes in the accumulation of flavonoid and isoflavonoid conjugates associated with plant age and nodulation in alfalfa (*Medicago sativa*). Physiol Plant 91: 27–36 (1994).
55. Townshend JL, Stobbs L, Carter R: Ultrastructural pathology of cells affected by *Praylenchus penetrans* in alfalfa roots. J Nematol 21: 530–539 (1989).
56. Townshend JL, Stobbs L: Histopathology and histochemistry of lesions caused by *Pratylenchus penetrans* in roots of forage legumes. Can J Plant Path 3: 123–186 (1981).
57. Trudgill DL: Resistance to and tolerance of plant parasitic nematodes. Annu Rev Phytopath 29: 167–192 (1991).
58. Van der Eycken W, Engler J, Inzé D, Van Montagu M, Gheysen G: A molecular study of root-knot nematode-induced feeding sites. Plant J 9: 45–54 (1996).
59. Wu SC, Grorgyey J, Dudits D: Polyadenylated H3 histone transcripts and H3 histone variants in alfalfa. Nucl Acids Res 17: 3057–3063 (1989).
60. Wyss U: Ectoparasitic root nematodes: Feeding behavior and plant cell responses. In: Zuckerman BM, Rohde RA (eds) Plant Parasitic Nematodes, pp. 211–220. Academic Press, New York (1981).
61. Zacheo G, Bleve-Zacheo T, Lamberti F: Role of peroxide and superoxide dismutase in resistant and susceptible tomato cultivars infested by *Meloidogyne incognita*. Nematol Mediterr 10: 75–80 (1982).
62. Zunke, U: Observations on the invasion and endoparasitic behavior of the root lesion nematode *Pratylenchus penetrans*. J Nematol 22: 309–320 (1990).