Polyphenoloxidase is induced by methyljasmonate and *Meloidogyne javanica* in soybean roots but is not involved in resistance

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Summary – The role of the enzyme polyphenoloxidase (PPO) in the response of two soybean varieties, Hartwig (resistant) and Cristalina (susceptible), to *Meloidogyne javanica* was studied in plants where root systems were exposed to the known PPO inducer, methyljasmonate. Chlorogenic acid was the best substrate for root PPO. Treatment of both varieties with 100 and 400 μM methyljasmonate solutions induced a similar increase in enzyme activity 72 h after treatment. Inoculation of roots with second-stage juveniles (J2) induced PPO increase in cv. Cristalina but not in cv. Hartwig. Moreover, combined treatment of methyljasmonate and J2 inoculation enhanced PPO activity in both varieties. Two PPO cDNAs were isolated from the roots of the resistant variety 48 h after J2 inoculation, and Southern blot experiments in both varieties, using *GmPPOJH1* and *GmPPOJH2* cDNAs as probes, indicated that PPOs are represented by a multigene family in soybean. RT-PCR assays showed more *GmPPOJH1* transcripts in plants treated with methyljasmonate and infected with nematodes, where high PPO activity was also observed. Plants treated with methyljasmonate and infected with J2 showed a marked decrease of nematode population 35 days after inoculation. These findings suggest that methyljasmonate triggers a resistant response in soybean roots to *M. javanica* but PPO is not involved in the resistance process.

Keywords – *Glycine max*, jasmonic acid, *Meloidogyne*, phenolic compounds, resistance induction.

Polyphenoloxidases (PPOs) are enzymes responsible for two types of reaction: hydroxylation of monophenols to o-diphenols (EC 1.14.18.1) and oxidation of o-diphenols to their respective o-quinones (EC 1.10.3.2). PPOs are plastidial enzymes (Vaughn et al., 1988; Koussevitzky et al., 1998) and their activity is only expressed when they come in contact with their substrates, the phenols, which are mostly stored in the vacuole. PPO activity is typical of wounded or bruised plant tissues, and it has been frequently associated with defence mechanisms against herbivory. Proteins and quinones form a stable complex that decreases the N availability for insects feeding on plants (Felton et al., 1989; Mayer, 2006). Alternatively, quinones can form a complex with proteins of the digestive tract of insects disturbing nutrient absorption and interfering with insect growth.

*Meloidogyne* spp. are among the nematodes responsible for significant crop losses, causing problems in more than 2000 species of annual and perennial crops (Abad et al., 2003). *Meloidogyne incognita*, *M. javanica*, *M. exigua* and *M. hapla* are the most important root-knot nematodes and the first sign of their infection in compatible interactions is galling of invaded roots where several giant cells are induced by nematode feeding (Abad et al., 2003).

*Meloidogyne* spp. have evolved a very sophisticated mode of parasitism in that they are able to alter gene expression in specific host cells and to modify them into specialised feeding cells. Infective second-stage juveniles (J2) migrate in the soil and are attracted to root tips where they penetrate and migrate towards a suitable site in the host root. The J2 become sedentary and establish an intimate relationship with their host by induction and maintenance of specialised feeding cells from which they are completely dependent for their life cycle. During migration in the intercellular space, the middle lamella is separated probably as a mechanical consequence of...
the nematode stylet as well as a response to cell wall degrading enzymes from pharyngeal gland secretions (Hussey et al., 2002; Davis et al., 2004).

In contrast to insects and other microorganisms, only limited information concerning the association between PPO and nematode resistance is available. An association has been proposed between resistance to Pratylenchus and Radopholus, and PPO and phenols. Unlike Meloidogyne, Pratylenchus and Radopholus are migratory parasites and, during penetration, they destroy the root cells, causing the development of a brown coloration, typical of phenol oxidation by PPO (Trudgill, 1991; Fogain & Gowen, 1996; Plowright et al., 1996, 1998; Valette et al., 1998).

Soybean leaves are reported to have a high constitutive PPO activity, thought to be poorly induced by mechanical damage or methyljasmonate (MJ) in the induction of PPO and nematode resistance. M. javanica infection, as well as the possible role of two PPO cDNAs were tested during the initial phase of nematode infection. This paper reports a study on the role of PPO in the response of two soybean varieties, Hartwig (resistant) and Cristalina (susceptible), to M. javanica infection. This nematode has caused losses in soybean cultivation in Brazil (Carneiro et al., 1999). PPO activity and expression of two PPO cDNAs were tested during the initial phase of nematode infection, as well as the possible role of methyljasmonate (MJ) in the induction of PPO and resistance to the nematode.

Materials and methods

PLANT MATERIAL

Seedlings of Glycine max (7-day-old) of the varieties Hartwig (resistant) and Cristalina (susceptible) were used in the experiments. They were obtained from seeds sterilised in 1% sodium hypochlorite for 2 min and germinated in autoclaved vermiculite (120°C, 1 bar, 30 min).

NEMATODES

J2 were obtained from tomato plants (Lycopersicon esculentum var. Rutgers) infected with M. javanica, kindly given by Dr Roberto Kasuhiro Kubo from the Biological Institute, Campinas, SP, Brazil. J2 were collected from egg masses placed in Petri dishes with distilled water for 1 week, then counted and used for the experiments.

PPO ACTIVITY

Proteins were extracted from liquid N2-powdered roots using 0.1 M Na-phosphate buffer, pH 7.0 (4°C), containing 0.1% Triton X-100 and polyvinylpolypyrrolidone (PVPP; 1:10 w/v), followed by centrifugation at 27 000 g for 15 min at 4°C. The supernatant was used for enzymatic assays. Protein content was determined using a Bio-Rad kit reagent (Bradford, 1976).

Experiments to determine the best PPO substrate were carried out with 7-day-old seedling roots, following the absorbance at 420 nm for 3-min period, at 15-s intervals, in 50 mM Na-phosphate buffer (pH 6.0) containing 0.1% sodium dodecyl sulphate (SDS), at 25°C. The substrates 4-methylcatechol (4MC), chlorogenic acid (5-caffeoylquinic acid – CGA), catechol, 3,4-dihydroxy-L-phenylalanine (DOPA), caffeic acid, protocatechuc acid, tyrosine, p-coumaric acid, syringaldazine, dopamine, ferulic acid, ascorbic acid and p-phenylenediamine were tested at a final concentration of 2 mM. Six replicates were used with each substrate.

In other experiments PPO activity was evaluated by O2 consumption using an oxymeter (Mazzafera & Robinson, 2000). The substrate was chlorogenic acid at 2 mM final concentration and the reaction was followed for 2 min.

PPO cDNA ISOLATION AND SOUTHERN BLOT ANALYSES

PPO cDNA isolation and cloning was done on cv. Hartwig, resistant to M. javanica. Total RNA was extracted (Rezaian & Krake, 1987) from 48 h infected roots and the first strand of cDNA synthesis was obtained with AMV reverse transcriptase and the universal primer B26 (Frohmann et al., 1998). The cDNA was used in RT-PCR reactions with PPO degenerated primers (Thygesen et al., 1995). The sense primers tested were: S3: 5'-GCCAATTCTT(T/C)(T/C)TTCCITT(T/C)(T/C)(A/C)G-3', S7: 5'-GCCAATTCAA(T/C)(T/C)(T/C)GA(T/C)(A/C)GIATGTGG-3', S8: 5'-GCCAATTCTCGATCCACCCITT(T/C)(T/C)(T/C)(T/C)(T/C)(T/C)(A/C)GIATGTGG-3', S9: 5'-GCCAATTCTTCIT(A/C)(T/C)(T/C)(T/C)(T/C)(T/C)(T/C)(A/C)GIATGTGG-3', S10: 5'-GCCAATTCTTCIT(C/T)(T/C)(T/C)(T/C)(T/C)(T/C)(T/C)(A/C)GIATGTGG-3'. The reverse primers were: R1: 5'-GCCCTGCAGCCACAT(T/G)(A/G)ITCACT(A/C)(T/G)TT-3', R2: 5'-GCCCTGCAGCT(C/T)TC(A/G)TC(A/G)TAGAA-3'.

Nematology
The RT-PCRs were carried out according to the following conditions: two cycles at 94°C/2 min; 37°C/2 min; slope up to 72°C in 2 min and maintained a further 2 min at the same temperature; 28 cycles at 94°C/2 min, 56°C/2 min and 72°C/2 min; 72°C/7 min. The reaction products were separated on 1% agarose gels, purified from the gel using extraction kit (DNA Gel Extraction Kit, Millipore, Watford, UK) and then inserted in the pGEM-T Easy vector (Promega pGEM-T Easy Vector Systems, Promega, Madison, WI, USA) to be cloned further in Escherichia coli DH5α by electroporation. The cloned cDNAs were sequenced in both strands. The cDNA sequences were aligned and compared among themselves using the Align program (Pearson et al., 1997). They were also compared with the database of the National Center for Biotechnology Information (NCBI – http://www.ncbi.nlm.nih.gov/).

For the Southern blot assays, total DNA was extracted from leaves (Guillemaut & Maréchal-Drouard, 1992), digested with Dral or EcoRI and probed with whole cloned cDNAs labeled with [32P]dCTP (Moore, 1987). The Cyclone Image Analyser device (Packard) was used to check for hybridisations. This assay was repeated twice with similar results.

**PPO INDUCTION BY METHYLJASMONATE**

Soybean seeds from the varieties Cristalina and Hartwig were germinated in 120 ml glass tubes (two in each tube) containing a mixture of soil and sand (1:1, v/v). Tubes and substrate were previously autoclaved (120°C, 1 bar, 2 h). Seven-day-old seedlings were watered with 100 ml of aqueous solution with 0 (control), 100 and 400 µM of MJ, respectively. The roots were then washed with running water and used for PPO analyses after 6, 12, 24, 48 and 72 h of MJ application. Quantification of PPO activity was carried out by using an oxymeter. Ten replicates were used in each treatment.

**PPO INDUCTION BY METHYLJASMONATE AND NEMATODE INFECTION**

In this assay, 7-day-old soybean seedlings growing in 120 ml glass tubes were watered with 100 µM MJ solution and 24 h later were inoculated with 20000 J2. Control plants were only inoculated with nematodes. Roots were harvested at the time of inoculation and 24 and 48 h after inoculation. PPO activity was measured with an oxymeter and the expression of two isolated genes was studied by semi-quantitative RT-PCR, where the annealing time was reduced to 1 min and the number of cycles was reduced to 20. The reaction products were separated on 0.8% agarose gels and photographed under UV light. Relative intensity values of the separated bands were obtained with the software Quantity One of the GelDoc device (Bio-Rad Laboratories, Hercules, CA, USA). Ten seedlings were used in each treatment: eight of them were used for PPO activity and two in the RT-PCR reactions.

Seedlings treated with MJ were also inoculated with 1000 J2 for determination of the reproduction index. After 10 days they were transferred from the tubes to 3 dm³ plastic pots containing a sterile mixture of soil and sand (1:1, v/v), where they were kept up to 35 days from inoculation, when the roots were extracted and eggs + J2 counted (Coolen & D’Herde, 1972). Ten replicates of each treatment were evaluated here.

**STATISTICS**

The data were compared by analysis of variance (ANOVA) and differences between means were detected using the Tukey test (P < 0.05).

**Results**

**SUBSTRATE SPECIFICITY**

There is a lack of information about the best substrate for soybean PPO, so an assay was carried out to test different substrates. For both varieties, CGA was the best substrate (variation of 0.23 absorbance units mg⁻¹ protein min⁻¹) but some activity was also observed with 4MC and DOPA, representing approximately 40% and 10% of the CGA activity, respectively. No other phenol was oxidised by soybean root PPO. The results were similar for both soybean varieties. In the light of these results, CGA was chosen as the substrate for detection of the PPO activity.

**ISOLATION OF PPO CDNAS**

To isolate PPO cDNA, RNA was extracted from roots of the resistant cv. Hartwig 48 h after nematode inoculation. Using different combinations of degenerated primers it was possible to isolate and clone two PPO cDNAs. The primers S9 and R1 amplified the cDNA GmPPOJH1 (EF158427), whose nucleotide sequence gave 93% similarity with Ipomoea batatas (AJ309175) and 89% with Vicia faba (Z11702). The deduced amino-acid sequence for GmPPOJH1 showed 70% similarity with PPO of Populus balsamifera × Populus deltoides
Deduced amino-acid sequences from GmPPOJH1 (JH1) and GmPPOJH2 (JH2). Residues in boxes indicate the two copper (A and B) sites of PPO.

(Southern blot) GmPPOJH1 and GmPPOJH2 cDNAs were used to produce [32P]dCTP-labelled probes and used in Southern blot assays (Fig. 2). The GmPPOJH1 probe identified up to five genes coding for PPO in Cristalina and at least two in Hartwig. Using GmPPOJH2 as probe, three genes for both soybean varieties were identified. The presence of a fragment in the upper part of the membrane that hybridised with the GmPPOJH1 probe is probably uncut DNA.

PPO induction by methyljasmonate

Using CGA as a substrate, PPO activity was analysed in the root of soybean seedlings that received aqueous solutions containing 100 and 400 µM MJ, respectively. The analyses started 6 h after the treatments and lasted up to 72 h. A slight increase in PPO activity was detected at 20 h for both varieties (Fig. 3). In cv. Cristalina, enzyme activity peaked at 48 h, showing a steady state at 72 h. In cv. Hartwig the steady level was detected between 20 and 48 h with a sharp increase at 72 h. No difference in PPO activity between the two MJ concentrations in both cvs Cristalina and Hartwig was recorded (Fig. 3).

PPO induction by methyljasmonate and nematode infection

In this experiment, seedlings received a 100 µM MJ aqueous solution and 24 h later were inoculated with J2. PPO activity was determined soon after nematode inoculation, i.e., 24 h after exposure to MJ, and then after 48 and 72 h (Fig. 4A, B). In agreement with the
Polyphenoloxidase and root-knot nematode resistance in soybean

Fig. 2. Southern blots of total DNA from leaves of soybean cvs Hartwig and Cristalina treated with the restriction enzymes DraI and EcoRI. GmPPOJH1 (A) and GmPPOJH2 (B) cDNAs were labelled with [32P]dCTP and used as probes. Arrows indicate PPO genes.

Fig. 3. Time-course of PPO activity in roots of 7-day-old soybean seedlings treated with methyljasmonate (MJ). Seedlings were watered with 100 ml of aqueous solution containing 0 (control), 100 and 400 μM of MJ, respectively. Data are means of ten replicates and asterisks indicate statistical difference (5% Tukey) from the control 72 h after MJ treatments. Bars show standard deviation of the mean.

results shown in Figure 3, in this experiment both varieties showed an increase of PPO activity 72 h after 100 μM MJ treatment. In cv. Cristalina (Fig. 4A) there was also an increase of PPO in the treatment inoculated with J2 and where MJ was combined with nematode inoculation. Curiously, the combination of J2 and MJ was not additive (Fig. 4A). Cultivar Hartwig also showed an increase of PPO 72 h after MJ treatment and when MJ was combined with J2 inoculation, but not with J2 inoculation (Fig. 4B).

In this experiment, the roots were collected 72 h after MJ treatment and the expression of GmPPOJH1 and GmPPOJH2 was verified by semi-quantitative RT-PCR (Fig. 4C). Increase in PPO expression was observed only with a probe prepared with GmPPOJH1 cDNA. While
the highest PPO expression was detected in cv. Cristalina roots treated with MJ combined with J2 inoculation, treatment with MJ only caused the highest expression in cv. Hartwig. Curiously, the expression of PPO (Fig. 4C) in cv. Cristalina did not match with the corresponding PPO activity (Fig. 4A), i.e., while 100 µM MJ + J2 treatment caused the highest GmPPOJH1 expression, the corresponding PPO activity was the lowest compared with the other two treatments. In cv. Hartwig, the highest PPO expression (Fig. 4C) was observed with 100 µM MJ and, except for the J2 treatment, PPO expression in cv. Hartwig agreed with enzyme activity (Fig. 4B). Although speculative, the discrepancies between GmPPOJH1 expression and PPO activity might be explained by the fact that enzyme activity resulted from other PPO genes which were also expressed but were not isolated here. The Southern blot experiments showed that PPO in soybean is a multi-gene family.

In the same assay, some seedlings treated or not with MJ were inoculated with 1000 J2 and eggs + J2 were quantified after 35 days (Fig. 5). Cultivar Hartwig is not immune to nematodes (Asmus, 2001) and this explains...
why some nematodes were able to reproduce. In seedlings of both varieties, the previous exposure to MJ led to a lower number of eggs + J2. In order to exclude the possibility that MJ might have acted directly on J2 before root penetration, J2 were kept in 100 µM MJ solution for 48 h, and did not show any loss of motility compared with those left in water.

Discussion

The great interest in PPO in plants has always been connected to the brown colour formation in tissue, mainly when browning develops during food processing or storage (Mayer, 2006). The physiological role of PPO is still unknown, although it has been suggested to play a role against insect herbivory (Mayer, 2006). Other reports showed a possible relationship between PPO and resistance to diseases (Bashan et al., 1985; Goy et al., 1992; Ray & Hammerschmidt, 1998). Recently, it has been shown that antisense downregulation of PPO results in enhanced susceptibility of tomato to Pseudomonas syringae. These findings seem to suggest a critical role for PPO-catalysed phenolic oxidation in limiting disease development (Thipyapong et al., 2004).

Reports evaluating PPO in plant roots infected with nematodes are scarce and, in most cases, the enzyme activity was carried out very late after nematode inoculation (Mazzafra et al., 1989; Sivakumar & Sivagami, 1995; Plowright et al., 1996). Therefore, the reported data indicate secondary effects in plant/pathogen interactions rather than the true role of PPO in plant response during nematode establishment.

Recently, the activities of phenylalanine ammonia-lyase, peroxidase and PPO were analysed in banana roots of resistant and susceptible cultivars before and 1, 3 and 7 days after inoculation with the burrowing nematode Radopholus similis (Wuys et al., 2006). The constitutive activities of these three enzymes were lower in the resistant than in the susceptible cultivar and an increase of phenylalanine ammonia-lyase during nematode infection was only observed in the resistant cultivar. The authors concluded that the increase of peroxidase and PPO activities could explain resistance in the resistant cultivar.

Hypersensitive reaction has been reported in resistant tomato plants infected by M. incognita. Necrosis developed in cells close to the proximal region of the nematode, which died or left the root (Williamson, 1998). The hypersensitive reaction developed only where the feeding site would be formed but not during nematode migration through the root. Migration and establishment of the J2 would take place about 12 h after inoculation, a time that coincided with the highest increase in phenylalanine ammonia-lyase activity (Williamson, 1998). A hypersensitive reaction was also observed during Heterodera glycines infection in Arabidopsis thaliana, which is not usually host to this nematode but sometimes feeding sites may be formed. Intense browning of cells injured by nematode migration as well as necrosis in the developing syncytia was reported (Grundler et al., 1997).

Here, we analysed PPO activity in two soybeans susceptible and resistant to M. javanica very early after nematode inoculation. CGA was the best substrate for PPO detection, although a good enzyme activity was also observed when 4MC and DOPA were used as substrates. PPO is known as an enzyme with low specificity to several ortho-diphenols and CGA has been reported as a good substrate for a series of plants, since it is a phenol typically found in plants (Constabel et al., 1996).

By assaying PPO activity and analysing the Gm-PPOJH1 expression, it can be assumed that MJ induces PPO in roots of both tested soybean varieties. The same results were observed when MJ application was combined with nematode infection. However, the susceptible cv. Cristalina when infected with nematodes showed an increase of PPO activity not observed in the resistant cv. Hartwig. This is the first evidence that PPO might be not involved in the resistance response of plants to nematode infection. It is interesting to note that the increase in PPO

**Fig. 5.** Eggs and J2 production in roots of soybean seedlings exposed or not to 100 µM MJ and inoculated 24 h later with Meloidogyne javanica. Evaluation was carried out 35 days after nematode inoculation. Data are means of ten replicates and asterisks indicate statistical difference (5% Tukey) from the controls.
was higher 48 h after nematode inoculation, when the J2 has reached the vascular cylinder and the feeding site starts to develop.

Induction of PPO activity by MJ was demonstrated in several plants, but not all respond to treatment with this hormone (Constabel & Ryan, 1998). Thus it is not possible to affirm that PPO induction by MJ is universal in plants (Mayer, 2006). In soybean, the PPO constitutive activity is high in leaves and there is only a slight response to MJ treatment when compared to other plants (Constabel & Ryan, 1998). Jasmonic acid production in plants is related to the octadecanoid wound-signalling pathway and several pathogenesis-related proteins are induced by this hormone (Pozo et al., 2004). Moreover, the induced jasmonate pathway is also regulated by other signals including cell wall-derived oligosaccharides and ethylene, making the resistance process highly complex (Howe, 2004).

In jasmonate induction by cell wall-derived oligosaccharides the response varies according to the size of the oligosaccharide (Basse et al., 1992). In the nematode pharyngeal gland cells, several cell wall-modifying enzymes, which may be involved in degradation of pectic polysaccharides, cellulose and hemicellulose of host cells, were found (Davis et al., 2004). However, pathogenesis-related proteins such as chitinase, glucanase and P14, which are usually associated with pathogen resistance, were not detected in tomato infected with M. javanica (Oka et al., 1997). On the other hand, in Heterodera avenae-infected roots of barley and wheat, several changes were observed after 4 days of infection. These protein changes were attributed to the migration of H. avenae nematodes that destroy cells and cause necrosis in the roots (Oka et al., 1997).

Therefore, one may speculate that fragments released by the action of these enzymes might induce release of MJ in cv. Cristalina, and the absence of specific receptors might explain the lack of response in cv. Hartwig. However, it is known that in resistant plants nematodes can invade the roots but cannot induce any feeding site, so they die or leave the root. In studies where microelectrodes were used to record electrophysiological signals from roots during nematode infection, applications of exoenzymes in order to mimic nematode secretions caused a similar membrane depolarisation pattern (variation in the membrane potential) in the roots of potato resistant and susceptible to Globodera rostochiensis, but differed from patterns obtained during nematode invasion or mechanical stimulation. Calcium was suggested to have a role in the signalling events that occur during nematode invasion (Sheridan et al., 2004).

Although we could not establish a relationship between resistance and PPO, it is clear that MJ treatment induces a marked decrease of nematode infection in soybean plants (Fig. 5). Jasmonic acid, exogenously applied to leaves, has also been reported to induce resistance to M. javanica in tomato plants (Cooper et al., 2005).

In conclusion, our results show that, despite the increase of PPO activity and its gene expression in soybean plants during M. javanica infection, this enzyme is not related to resistance. Nevertheless, MJ application might trigger other genes that confer resistance to root-knot nematodes.

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