Biological Control xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Entomopathogenic nematodes induce components of systemic resistance in plants: Biochemical and molecular evidence

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ARTICLE INFO

Article history:
Received 24 December 2008
Accepted 19 June 2009
Available online xxxx

Keywords:
Arobidopsis thaliana
Biological control
Catalase
Entomopathogenic nematodes
Guaiacol peroxidase
Hosta
Induced systemic resistance
Plant-parasitic nematodes
Pyrogallol peroxidase
Steinernema carpocapsae
Xenorhabdus nematophilus

ABSTRACT

Antagonism between entomopathogenic nematodes (EPNs) and plant-parasitic nematodes (PPNs) has been documented over the past two decades but its mechanism and ecological significance remain elusive. We investigated the effects of *Steinernema carpocapsae* and its symbiotic bacterium, *Xenorhabdus nematophila* applied to the potting medium on pyrogallol peroxidase (P-peroxidase), guaiacol peroxidase (G-peroxidase) and catalase activities in *Hosta* sp. and *Arabidopsis thaliana* leaves as components of induced systemic resistance. We found that P-peroxidase activity was significantly higher in the leaves from hosta plants treated with *S. carpocapsae* infective juveniles (IJs) and *S. carpocapsae* infected insect cadavers than in the leaves from the control plants 2 weeks after treatment. The G-peroxidase activity was significantly higher in *S. carpocapsae* infected cadaver and *X. nematophila* treatments 10 and 15 days after treatment (DAT) and in *S. carpocapsae* IJs treatment 5 and 15 DAT. The catalase activity in hosta leaves was significantly higher in *S. carpocapsae* infected cadaver and *X. nematophilus* treatments compared with the control 5 and 15 DAT and in *S. carpocapsae* IJs treatment 5 and 10 DAT. Further, the catalase activity in *A. thaliana* leaves was significantly higher in *S. carpocapsae* IJs treatment than in the control 7 DAT. We also determined the effects of *S. carpocapsae* infected cadavers and *S. carpocapsae* IJs on *PR1*-gene expression in transgenic *A. thaliana* leaves through GUS (β-glucuronidase) activity assay and found that the *PR1*-gene was expressed in leaves from all treatments except the control. Thus, we conclude that the EPNs and their symbiotic bacteria can induce systemic resistance in plants which may explain the elusive antagonistic effect of EPNs on PPNs.

1. Introduction

Antagonism between entomopathogenic nematodes (EPNs) and plant-parasitic nematodes (PPNs) has been documented since its discovery two decades ago (Bird and Bird, 1986; Ishibashi and Kondo, 1986) but there are no direct ecological links observed between the two nematode trophic groups (Lewis and Grewal, 2005). EPNs *Steinernema* and *Heterorhabditis* are lethal parasites of insects (Grewal et al., 2005) whereas PPNs feed on the plants. Both *Steinernema* and *Heterorhabditis* are associated with symbiotic bacteria, *Xenorhabdus* and *Photorhabdus*, respectively. The symbiotic bacteria are vectored into the insect haemocoel by the nematode infective juveniles (IJs). Toxins produced by the developing nematodes (Burman, 1982) and multiplying bacteria kill the host usually within 48 h. These bacteria also produce a plethora of metabolites, toxins and antibiotics with bactericidal, fungicidal and nematicidal properties (Hu et al., 1999; Grewal et al., 1999; Webster et al., 2002), which ensure conditions suitable for reproduction of EPNs.

Previous field trials have shown reduction in populations of PPNs following the applications of EPNs (see Lewis and Grewal (2005) for review). For example, *Heterorhabditis bacteriophora* Poinar reduced the population of *Tylenchorynchus* spp. and *Pratylenchus pratensis* Filipjev (Smitley et al., 1992), *Steinernema riobrave* Cabanillas, Poinar and Raulston, reduced the population of *Meloidogyne* sp., *Belonolaimus longicaudatus* Rau, and *Criconemiodes* sp. (Grewal et al., 1997), *Steinernema carpocapsae* Weiser, reduced the population of *Globodera rostochiensis* (Wollenweber) Behrens (Perry et al., 1998), *Trichodorus* sp. (Jagdale et al., 2002), and *Aphelenchoides fragariae* (Ritzema Bos) Christie (Jagdale and Grewal, 2008) and *S. feltiae* Filipjev/*S. glaseri* Steiner, suppressed population of *Meloidogyne javanica* Treub (Fallon et al., 2006). There have also been reports showing no effect of EPNs on certain PPN species (see Lewis and Grewal (2005) for review).

Several mechanisms have been proposed to explain the interaction between EPNs and PPNs. First, EPNs attracted to plant roots may be forcing PPNs away (Bird and Bird, 1986). Second, EPNs

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doi:10.1016/j.biocontrol.2009.06.009

applied to soil for insect control may stimulate the build-up of general nematode antagonists and predators (Ishibashi and Choi, 1991). Third, EPN infected cadavers filled with both symbiotic bacteria and various life stages of nematodes can release high concentrations of ammonia (Grewal et al., 1999; Shapiro et al., 2000; De Nardo et al., 2006) and other allelochemicals, which can be toxic to PPNs (McInerney et al., 1991; Grewal et al., 1999; Molina et al., 2007). The secondary metabolite, 3,5-dihydroxy-4-isopropulstilbene from nematode infected cadavers inhibited egg hatch of the PPN *Meloidogyne incognita* (Kofoid and White) Chitwood, and caused significant mortality of two fungal-feeding nematodes, *Aphelenchoides rhytium* Massey, and *Bursaphelenchus* spp (Hu et al., 1999). Indeed, both *Xenorhabdus* and *Photorhabdus* bacteria produce large amounts of ammonium in culture, which have been shown to be toxic to second stage juveniles of *M. incognita* (Hu et al., 1999; Grewal et al., 1999). *S. riobrave* and *S. feltiae*/*S. glaseri* applied in the infected host cadavers significantly reduced the number of egg masses of *Meloidogyne partityla* Kleynhans (Shapiro et al., 2006) and *M. javanica* (Fallon et al., 2006), respectively. Applications of even dead IJs have been shown to reduce populations of several species of plant-parasitic nematodes (Jagdale et al., 2002; Molina et al., 2007). Jagdale and Grewal (2008) found that the soil application of insect cadavers infected with EPNs and their symbiotic bacteria suppressed population of the foliar nematode *A. fragariae* in the rhizosphere. In addition they found that the cadaver treatments significantly reduced the multiplication of *A. fragariae* in the leaves even though there was no direct contact between *A. fragariae*, and EPNs and their symbiotic bacteria. Based on these observations, Jagdale and Grewal (2008) suggested that EPNs and their symbiotic bacteria may induce systemic resistance in plants against the plant-parasitic nematodes.

Induced systemic resistance (ISR) is a plant defense mechanism, which is known to be triggered by exogenous application of virulent or avirulent pathogens, plant growth promoting rhizobacteria (PGPR), and various chemical elucidators including salicylic acid (SA), jasmonic acid (JA), benzothiadiazole-7-carbothioc acid S-methyl ester (BTH), 2,6-dichloro isonicotinic acid (DCINA) and β-aminobutyric acid (BABA) (Kuć, 2001; Oka and Cohen, 2001; Ramamoorthy et al., 2001; Van Loon, 2007). Induced systemic resistance against root-knot nematode *Meloidogyne hapla* Chitwood, and pine wood nematode *Bursaphelenchus xylophilus* Steiner and Buhrer, was reported in tomato and pine, respectively by pre-inoculation of avirulent strains of *M. incognita* (Ogallo and McClure, 1996) and *B. xylophilus* (Kosaka et al., 2001), respectively. Application of BABA and PGPR also protected several crop plants systemically against root-knot and cyst nematodes (Hasky-Gűnther et al., 1998; Ramamoorthy et al., 2001; Oka and Cohen, 2001). Induction of resistance against many pathogens has also been correlated with the synthesis and accumulation of SA, JA, pathogenesis-related proteins, and enzymes such as catalase and peroxidase in different plant species (Ramamoorthy et al., 2001; Yu et al., 1999). Therefore, we hypothesized that EPNs and their symbiotic bacteria would induce components of systemic resistance in plants rendering them less suitable for PPN infection and reproduction.

We studied the effects of *S. carpocapsae* IJs, *S. carpocapsae* infected *Galleria mellonella* L. cadavers and *Xenorhabdus nematophilus* Poinar and Thomas (symbiotic bacteria associated with *S. carpocapsae*) on activities of two H_2O_2-scavenging enzymes, catalase and peroxidase as components of induced resistance in hosta (*Hosta* sp.) and *Arabidopsis thaliana* L. plants. We used JA and SA as positive controls as these compounds have been shown to activate the defense genes in plants. We also used transgenic *A. thaliana* to further confirm the activation of plant resistant protein (*PR1*) promoter by EPNs and their symbiotic bacteria through the use of β-glucuronidase (*GUS*) reporter gene.

2. Materials and methods

2.1. Sources of nematodes and symbiotic bacteria

S. carpocapsae: All strain IJs were produced in the last instar wax moth *G. mellonella* at 25 °C using methods described by Kaya and Stock (1997). EPN infected *G. mellonella* cadavers (hereafter termed as 'cadavers') were obtained by infecting each last instar *G. mellonella* larva with approximately 100 *S. carpocapsae* IJs. The symbiotic bacteria *X. nematophilus* were isolated from surface sterilized *S. carpocapsae* IJs and smeared onto triphenyltetrazolium chloride plates and incubated in the dark at 28 °C for 24 h (Kaya and Stock, 1997). The bacteria were identified by their cell and colony morphology following Boemare (2002). Individual Phase I colonies were then sub-cultured in 500 ml flasks containing 250 ml of Luria–Bertani broth (Kaya and Stock, 1997; Boemare, 2002) up to a concentration of 10^8 cells/ml in an incubator shaker at 200 rpm for 48 h in the dark at 28 °C. Nutrient broth (250 ml) (Kaya and Stock, 1997; Boemare, 2002) containing 16×10^{-10} bacteria cells were mixed in 1 L of water immediately prior to application.

2.2. Protein determination

Total protein in hosta and *A. thaliana* leaves was determined using bovine serum albumin as a standard (Lowry et al., 1951). The activities of pyrogallol peroxidase (P-peroxidase, EC 1.11.1.7), guaiacol peroxidase (G-peroxidase, EC 1.11.1.7) and catalase (EC 1.11.1.6) enzymes measured (see below) in the leaf samples were expressed as units/min/mg of protein.

2.3. Effect of S. carpocapsae infected cadavers and IJs applied to the potting medium on peroxidase and catalase activities in hosta leaves

2.3.1. Activities of P-peroxidase and catalase

An experiment was conducted in the growth chamber to compare the effects of *S. carpocapsae* infected *G. mellonella* cadavers and SA (15 mM) with that of water (control) on the peroxidase activity in the hosta leaves. Nine plastic pots (346 cm^2 surface area) with dormant hosta (var. Patriot) crowns were obtained from a commercial nursery in Perry, Ohio and transferred into a growth chamber where they were allowed to grow to 5-6-leaf stage at 22 ± 1 °C, 16 h photoperiod and 70% relative humidity. For cadaver treatment, 100 *S. carpocapsae* infected cadavers (ca. 25 g wet weight) were mixed into the potting medium around each plant at 4–5 cm depth. For SA treatment, 200 ml of SA solution was directly sprayed on each plant using a spray bottle until run off into the pots. Similarly, control plants were sprayed with 200 ml of tap water as a control treatment. Treated pots were arranged in a randomized block design (RBD) with 3 replications. One week after treatment (WAT), three leaves were randomly collected from each plant, then chopped together and 50 mg sub-sample of leaf tissue was homogenized in 500 μl of 0.1 M phosphate buffer (pH 6.0). The homogenates were centrifuged at 14,000*g* for 20 min at 4 °C and the supernatants (enzyme extracts) were used to determine peroxidase activity. Sigma protocol (Sigma chemical Co. St. Lois, MO) was followed to assess P-peroxidase activity by measuring the formation of purpurogallin from pyrogallol substrate (hereafter called "P-peroxidase" activity) at 25 ± 1 °C. The reaction mixture contained 328 μl of 0.1 M potassium phosphate buffer (pH 6.0), 164 μl of 0.147 M hydrogen peroxide solution, 328 μl of 5% (w/v) of pyrogallol solution and 2130 μl of water. The reaction mixture was equilibrated at 20 °C using Spectronic® Genesys 2 spectrophotometer (Spectronic Instruments Inc., Rochester, New York) and the initial absorbance was monitored until it reached steady baseline with the blank at 420 nm. Immediately, 50 μl aliquots of the enzyme extract were transferred into

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each cuvet, 50 μl of ddH_2O in a blank cuvet and increase in absorbance at the maximum linear rate was recorded at 420 nm. This experiment was repeated as descried above except that 17,000 IJs of *S. carpocapsae* were drench applied in each pot ($n = 3$) as an additional treatment and the P-peroxidase activity in the leaves was recorded one and two WAT.

Another follow up experiment was conducted in the growth chamber. Treatments included: (i) a single cadaver, (ii) five cadavers, (iii) SA, (iv) 17,000 *S. carpocapsae* IJs and (v) tap water. All the treatments were applied as described above except that 1-L of each SA solution, IJ suspension or tap water was drench applied in each pot. P-peroxidase and catalase activities in hosta leaves were determined from 100 mg leaf tissue as described in Worthington Enzyme Manual (Worthington Biochemical Corp. Freehold, NJ) at two WAT. Briefly, leaf tissue was homogenized in 4000 μl of 0.05 M phosphate buffer (pH 7), the homogenates were centrifuged at 14,000*g* for 20 min at 4 °C and supernatants were used to determine catalase activity. The reaction mixture contained 1.9 ml of water and 1.0 ml of 0.059 M hydrogen peroxide substrate prepared in 0.05 M phosphate buffer (pH 7) was incubated for 5 min at 25°C using spectrophotometer. After achieving equilibrium at 25 °C, reaction was initiated by adding 100 μl of enzyme extract in each cuvet and 100 μl of ddH_2O in the blank cuvet. Decrease in absorbance was recorded at 240 nm for 2–3 min.

2.3.2. Activities of G-peroxidase and catalase

The above growth chamber experiment was again repeated except that the single *S. carpocapsae* infected cadaver treatment was replaced with symbiotic bacteria *X. nematophilus* and JA (180 μM) was included as an additional treatment. There were 6 replications for each treatment, and the peroxidase activity was determined using guaiacol substrate (hereafter called "G-peroxidase" activity) as the hydrogen donor. Also, 1-L of each of JA solution or bacterial suspension (16×10^{-10} cells) was drench applied in each pot. G-peroxidase activity in hosta leaves was recorded at 5, 10 and 15 days after treatment (DAT) following Hammerschmidt et al. (1982). Briefly, 100 mg wet tissue was homogenized in 1200 μl of 0.1 M phosphate buffer (pH 6.2) and the homogenate was centrifuged at 12,000*g* for 35 min at 4 °C and supernatant was used for determination of enzyme activity. Fresh 50 ml reaction mixture was prepared in 20 mM sodium phosphate buffer (pH 6.2) by adjusting its concentration to 18 mM of guaiacol and 100 mM of hydrogen peroxide. Then in each cuvet, 2.95 ml of reaction mixture was transferred and reaction was initiated by adding 50 μl of enzyme extract and 50 μl of ddH_2O in the blank cuvet. The increase in absorbance at the maximum linear rate was recorded by monitoring the formation of tetraguaiacol at 470 nm. The activity of catalase was also determined from 100 mg hosta leaf tissue 5, 10 and 15 DAT as described above except that the tissue was homogenized in 1200 μl of 0.05 M phosphate buffer (pH 7) to reduced dilution of enzyme.

2.4. Effect of S. carpocapsae infected cadavers and IJs applied to the potting medium on G-peroxidase and catalase activities in A. thaliana leaves

The effects of five *S. carpocapsae* infected cadavers, 3500 *S. carpocapsae* IJs, 90 μM of JA and water (control) on G-peroxidase and catalase activities in the wild type *A. thaliana* was evaluated in the growth chamber. *A. thaliana* seedlings were grown on Murashige and Skoog salt and vitamin mix media (Gibco™, Grand Island, NY) in 9 cm diam petri dishes following standard protocols (Huitema et al., 2003) for 4–5 days. These seedlings were then transplanted in plastic pots (73.90 cm^2 surface area) filled with germinating media, Fafard (BFG supply Co. Burton, OH) and allowed to establish for one week at environmental conditions stated above. Eighty milliliters of JA solution or IJ suspension and 80 ml of tap water (control) was drench applied to each pot or 5 cadavers were buried in the growing medium at equidistance around the plant. The pots were arranged in a RBD with 6 replications. The G-peroxidase activity in *A. thaliana* leaves was determined one WAT as described above except that 75 mg leaf tissue was homogenized in 200 μl of buffer and the reaction was initiated by adding 150 μl of enzyme extract in each cuvet and 150 μl of ddH_2O in the blank cuvet. The activity of catalase in *A. thaliana* leaves was also determined one WAT as described above except that only 25 mg wet leaf tissue was homogenized in 300 μl of 0.05 M phosphate buffer (pH 7). In this test, we only used 75 and 25 mg of leaf tissue to determine G-peroxidase and catalase activities, respectively, because it was difficult to get a separate 100 mg leaf tissue sample for each enzyme assay from small *A. thaliana* plants.

2.5. Effect of S. carpocapsae infected cadavers, IJs, SA and JA applied to the potting medium on activation of defense response genes in A. thaliana

An experiment was conducted on transgenic *A. thaliana* (*PR1*) plants to compare the effects of *S. carpocapsae* infected cadavers and IJs with SA and JA on the induction of systemic acquired resistance in a growth chamber at environmental conditions stated above. The expression of plant resistant protein (*PR1*) promoter to the β-glucuronidase (GUS) reporter gene in transgenic *A. thaliana* plants was observed following histochemical staining method described by Huitema et al. (2003). Seedlings of transgenic *A. thaliana* were grown as described above and 10 days after germination, single seedling was transplanted in a plastic pot (95 cm^2 surface area) filled with germinating media, Fafard. Seven days after transplanting, treatments including 2000 *S. carpocapsae* IJs (rate equivalent to 2.5×10^9 IJs/ha), 5 *S. carpocapsae* infected cadavers, 100 ml of 10 mM SA, 100 ml of 90 μM JA and 100 ml of water (control) were applied in each pot without disturbing the plants. The treated pots were arranged in a RBD with 5 replications. After 72 h, each plant was carefully uprooted and transferred to a plastic centrifuge tube (50 ml capacity) containing GUS staining solution (5 mM NaHP04—pH 7.2, 0.5% Triton X—100, 1 mM X—Gluc). Plants were completely submerged in GUS solution and transferred under vacuum to remove the trapped air in the leaves. After 1 h, tubes were removed from the vacuum, capped and incubated with agitation at 37 °C for 48 h. During this process, extra care was taken to avoid injury to the plants. After incubation, staining solution was replaced with 70% ethanol to remove the chlorophyll. Then ethanol solution in these tubes was replaced with fresh 70% ethanol at every 12 h until chlorophyll was completely removed from the leaves. Leaves from all the treatments were then recorded for the presence (+) or absence (−) of blue color, which is an indication of GUS activity.

2.6. Statistical analyses

Data on both peroxidase and catalase activities were transformed using the function log + 1 to correct for heterogeneity of variances and non-normality and were subjected to analysis of variance (ANOVA) using General Linear Models Procedure (SAS Institute, 1998). Significant differences between treatments were determined using Tukey's multiple range tests at $P < 0.05$. Data on changes in peroxidase and catalase activities over time were compared using Tukey's mixed repeated measures analysis (Littell et al., 2002).

3. Results

3.1. Effect of S. carpocapsae infected cadavers and IJs applied to the potting medium on peroxidase and catalase activities in hosta leaves

3.1.1. Activities of P-peroxidase and catalase

Application of *S. carpocapsae* IJs, *S. carpocapsae infected* cadavers and SA influenced the activity of P-peroxidase enzyme in excised

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hosta leaves (Fig. 1A–C). When compared with the control, the activity of P-peroxidase significantly ($P \leqslant 0.005$) increased in both the cadaver and SA treatments one (Fig. 1A) and two (Fig. 1B) weeks after treatment (WAT) whereas in the *S. carpocapsae* IJ treatment only two WAT (Fig. 1B). Application of even one cadaver in the potting medium showed significantly ($P \leqslant 0.005$) higher activity of P-peroxidase in the hosta leaves than the control one WAT (Fig. 1C).

Application of *S. carpocapsae* infected cadavers, *S. carpocapsae* IJs, and SA also influenced the activity of catalase enzyme in excised hosta leaves (Fig. 1D). When compared with the control, application of 5 cadavers to the potting medium significantly ($P \leqslant 0.005$) enhanced the activity of catalase but application of *S. carpocapsae* IJs and SA significantly ($P \leqslant 0.005$) reduced catalase activity in excised hosta leaves one WAT (Fig. 1D).

3.1.2. Activities of G-peroxidase and catalase

Application of *S. carpocapsae* IJs, *S. carpocapsae* infected cadavers, *X. nematophilus*, SA and JA influenced the activities of both G-peroxidase and catalase in the intact hosta leaves (Fig. 2). Applications of cadavers, *X. nematophilus*, JA and SA showed significantly ($P \leqslant 0.005$) higher activity of catalase than the control 5 and 15 DAT and *S. carpocapsae* IJs 5 and 10 DAT (Fig. 2). Application of SA and JA showed significantly ($P \leqslant 0.005$) higher G-peroxidase activity than the control throughout the experiment but cadavers and *X. nematophila* showed significantly ($P \leqslant 0.005$) higher activity of G-peroxidase than the control 10 and 15 DAT and *S. carpocapsae* IJs 5 and 15 DAT (Fig. 2).

3.2. Effect of S. carpocapsae infected cadavers and IJs applied to the potting medium on G-peroxidase and catalase activities in A. thaliana leaves

When compared with the control, applications of *S. carpocapsae* IJs and JA significantly ($P \leqslant 0.005$) enhanced the activities of catalase but not G-peroxidase in *A. thaliana* leaves (Fig. 3). Application of *S. carpocapsae* infected cadavers showed no significant influence

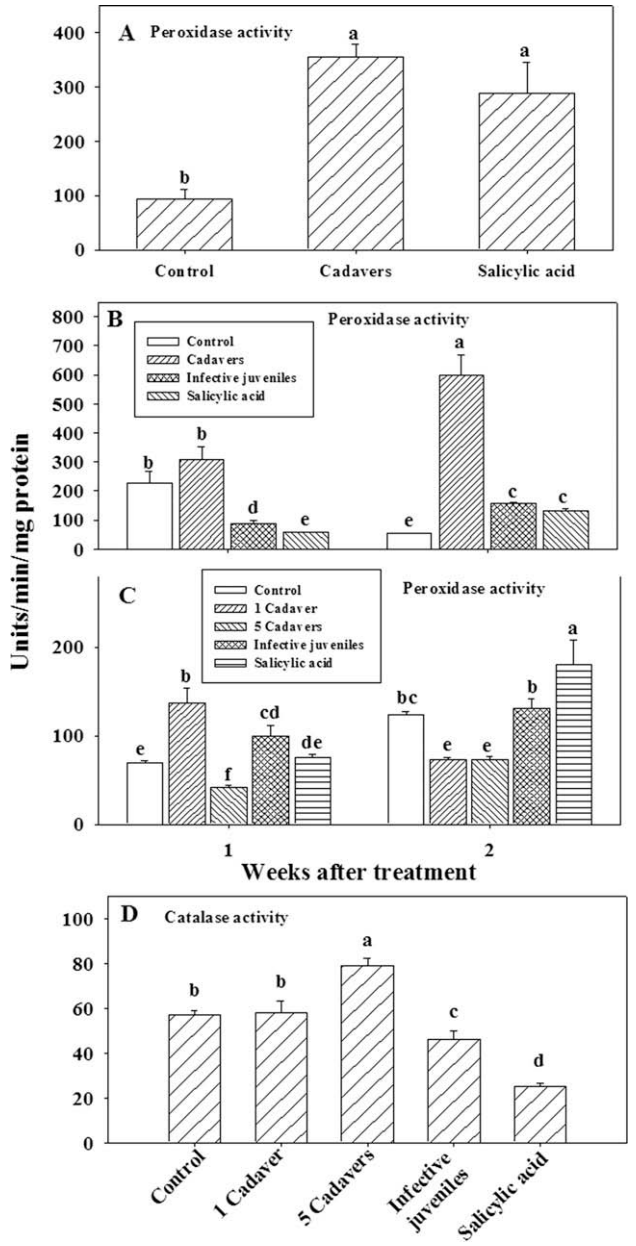

Fig. 1. Effect of *Steinernema carpocapsae* infective juveniles, *Steinernema carpocapsae* infected wax moth (*Galleria mellonella*) cadavers and salicylic acid on the activities of both P-peroxidase (A–C) and catalase (D) enzymes in the intact hosta leaves. (A and D): Bars (mean ± SE) with same letter(s) are not significantly different according to Tukey's multiple range test ($P < 0.05$). (B and C): Bars (mean ± SE) in the same and between the weeks with same letter(s) are not significantly different according to Tukey's mixed repeated measure procedure ($P < 0.05$).

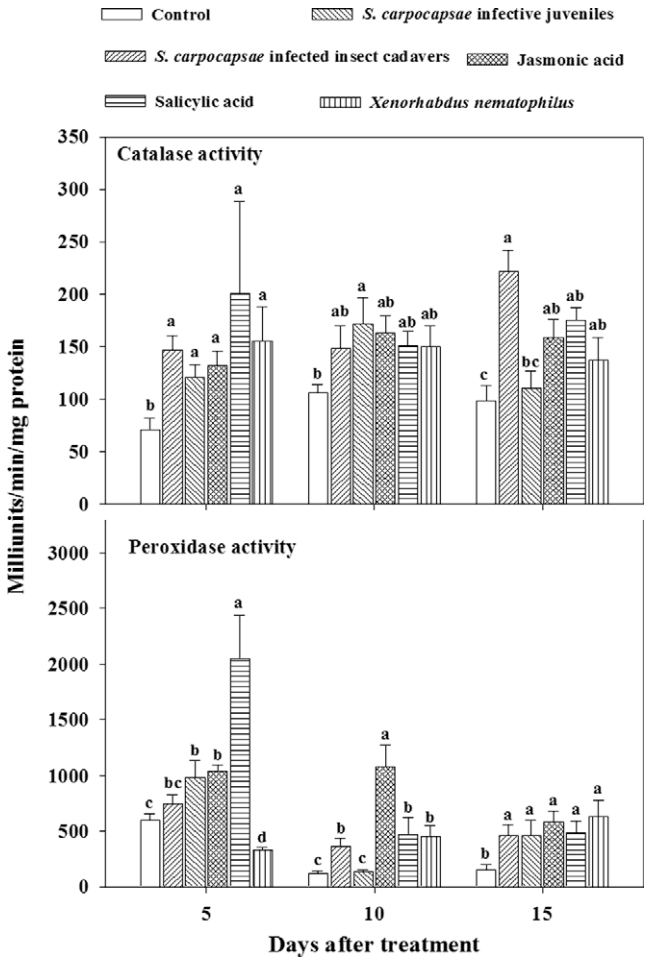

Fig. 2. Effect of *Steinernema carpocapsae* infective juveniles, *Steinernema carpocapsae* infected wax moth (*Galleria mellonella*) cadavers, jasmonic acid, salicylic acid and symbiotic bacteria, *Xenorhabdus nematophilus* on the activities of both catalase and G-peroxidase enzymes in the intact hosta leaves. Bars (mean ± SE) in the same and between the days with same letter(s) are not significantly different according to Tukey's mixed repeated measure procedure ($P < 0.05$).

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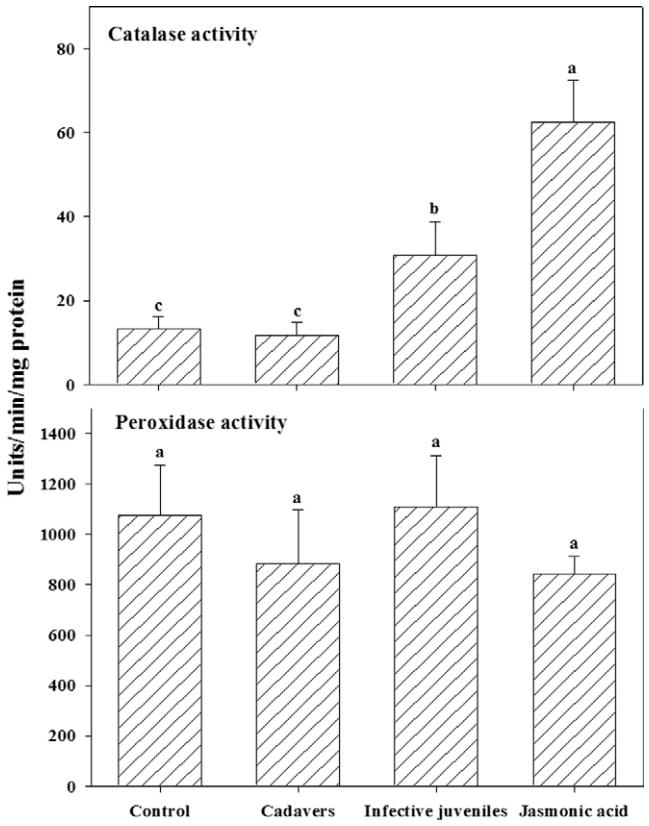

Fig. 3. Effect of *Steinernema carpocapsae* infective juveniles, *Steinernema carpocapsae* infected wax moth (*Galleria mellonella*) cadavers and jasmonic acid on the activities of both catalase and G-peroxidase enzymes in the intact *Arobidopsis thaliana* leaves. Bars (mean ± SE) with same letter(s) are not significantly different according to Tukey's multiple range test ($P < 0.05$).

on the activities of either G-peroxidase or catalase in *A. thaliana* leaves (Fig. 3).

3.3. Effect of S. carpocapsae infected cadavers, IJs, SA and JA applied to the potting medium on activation of defense response genes in A. thaliana

With the exception of the control, all treatments including *S. carpocapsae* IJs, *S. carpocapsae* infected cadavers, SA and JA showed a high level of systemic *PR1*-gene expression as indicated by the blue color GUS staining in leaves of *A. thaliana* (Fig. 4).

4. Discussion

The results of this study support the hypothesis that EPNs and their symbiotic bacteria induce components of systemic resistance in plants. We found that the EPNs and their symbiotic bacteria when applied in the rhizosphere of hosta plants systemically enhanced the activities of H_2O_2-scavenging enzymes, catalase and peroxidase in the leaves as a component of ISR. EPNs are not pathogenic to plants but are shown to be attracted to roots (Bird and Bird, 1986) and accumulate in rhizosphere. Root-colonizing PGPR can induce systemic resistance against diverse pathogens (Ramamoorthy et al., 2001; Van Loon, 2007) and this ISR was correlated with the synthesis and accumulation of several defense molecules including PR proteins (Maurhofer et al., 1994) and H_2O_2-scavenging enzymes (Nandakumar, 1998) in plants. For example, ISR was reported in *Pseudomonas fluorescens* Migula, treated tobacco plants against tobacco necrosis virus and was correlated with accumulation of PR proteins, β-1,3-glucanases and endochitinases (Maurhofer et al., 1994). Induction of isoenzymes of both peroxidase and catalase specific to ISR were also reported in PGPR-treated rice plants infected with rice sheath blight fungus, *Rhizoctonia solani* Kuehn (Nandakumar, 1998). Application of PGPR also protected several crops systemically against root-knot and cyst nematodes by altering root exudates that affected attraction and penetration of nematodes (Ramamoorthy et al., 2001). *Agrobacterium radiobactor* Beijerinck and van Delden strain G12 and *Bacillus sphaericus* Meyer and Neide strain B43 induced systemic resistance against potato cyst nematode *Globodera pallida* Stone, in potatoes (Hasky-Gűnther et al., 1998). However, this study is the first to report ISR with the application of insect-killing biocontrol nematodes that have no apparent direct relationship with the plants.

Pre-inoculation of avirulent pathogens can also induce systemic resistance in host plants against subsequent attack by virulent pathogens. For example, an avirulent strain of *Pyricularia oryzae* Cavara and non-rice pathogen *Bipolaris sorokiniana* (Sacc.) Shoemaker when pre-inoculated on rice leaves induced systemic resistance against pathogenic *P. oryzae* strain (Manandhar et al., 1999). Pre-inoculated avirulent strains of root-knot nematode *M. incognita* (Ogallo and McClure, 1996) and *B. xylophilus* (Kosaka et al., 2001) also induced systemic resistance against *M. hapla* and pine wood nematode *B. xylophilus* in tomato and pine plants, respectively. Similarly, avirulent *M. incognita* populations induced systemic resistance against virulent populations of *M. arenaria* infecting harmony grape rootstocks (McKenry and Anwar, 2007). Since the application IJs of EPNs in aqueous suspension or in host cadavers have been shown to reduce the population of several species of PPNs in the soil (Smitley et al., 1992; Grewal et al., 1997; Perry et al., 1998; Jagdale et al., 2002; Fallon et al., 2002, 2006; Shapiro et al., 2006; Jagdale and Grewal, 2008), it is possible that plants activate their defenses against PPNs when in contact with EPNs and their symbiotic bacteria in a similar manner as avirulent strains.

In addition to live pathogenic or non-pathogenic microorganisms (bacteria, fungi viruses and nematodes), metabolites produced by both live and dead (heat-killed) microorganisms have been shown to induce systemic resistance against plant pathogens and PPNs (Hasky-Gűnther et al., 1998; Madi and Katan, 1998). In the present study, we found that the nematode infected cadavers when applied in the rhizosphere of hosta plants enhanced the activities of catalase and peroxidases in the leaves suggesting that metabolites released by EPNs and their symbiotic bacteria may have been involved in ISR via accumulation of catalase and peroxidase. Metabolites of *Penicillium janczewskii* when infiltrated into leaves of melon (*Cucumis melo* L.) or cotton (*Gossypium barbadense* L.) induced systemic resistance against *R. solani* and reduced its infection in lower parts of the stem (Madi and Katan, 1998). This ISR was associated with increased activity of peroxidase in melon and cotton (Madi and Katan, 1998). Toxic effects of compounds produced by EPNs and their symbiotic bacteria (*Xenorhabdus* spp. and *Photorhabdus* spp.) have been reported against several species of PPNs (Grewal et al., 1999; Hu et al., 1999; Samaliev et al., 2000). According to Hasky-Gűnther et al. (1998), exopolysaccarides and lipopolysaccarides expressed on surface of heat-killed rhizobacteria *Agrobacterium radiobacter* were not destroyed by heat treatment and may be involved in ISR. Interestingly, heat-killed IJs of *S. carpocapsae* reduced population of several species of PPNs in the rhizosphere of *Buxus* spp (Jagdale et al., 2002). This suggests that the compounds produced and released by live/dead EPNs, their symbiotic bacteria or nematode infected host cadavers may be involved in ISR against PPNs.

We found that the soil application of EPNs, JA and SA (not tested on *A. thaliana*) enhanced the activities of peroxidase in hosta but not in *A. thaliana* plants. Van Loon (2007) reported that the appli-

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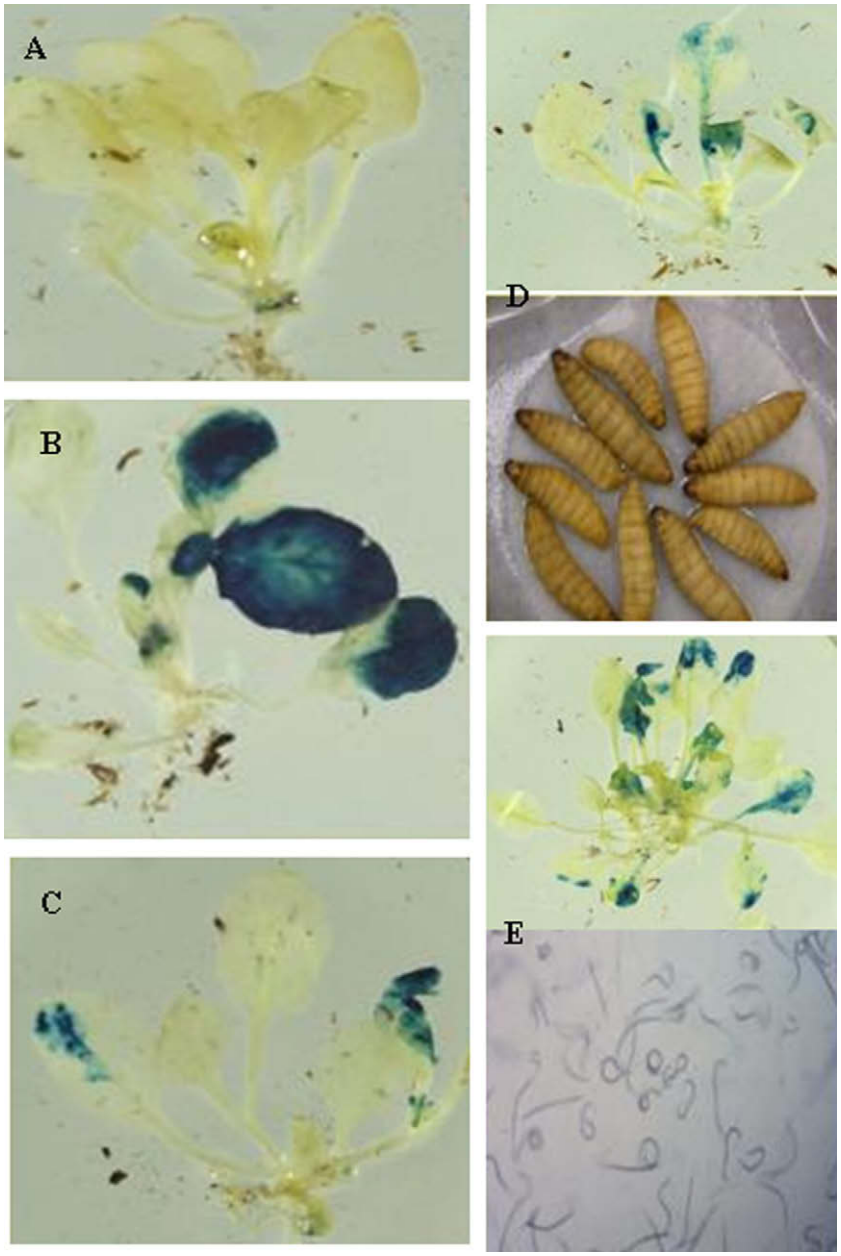

Fig. 4. Effects of control (A), salicylic acid (B), jasmonic acid (C), *Steinernema carpocapsae* infected wax moth (*Galleria mellonella*) cadavers (D) and *S. carpocapsae* infective juveniles (E), on the activation of defense response genes through GUS (β-glucuronidase) activity in *Arobidopsis thaliana* leaves.

cation of PGPR, *P. fluorescens* WCS374 strain activated IRS in bean and tomato plants but not in carnation, radish and rice plants, and suggested that PGPR mediated ISR was plant specific. Several previous studies have also demonstrated that the application of avirulent pathogens enhance accumulation of signaling molecules such as SA (Malamy et al., 1990), jasmonate (Penninckx et al., 1996) and ethylene (Boller, 1991) that coordinate the activation of defense genes in plants. When applied exogenously these molecules also induced systemic resistance against several pathogens (Boller, 1991; Cohen et al., 1993) through expression of PR proteins including peroxidase (Ward et al., 1991; Fernandes et al., 2006). The enhanced activities of peroxidase have been observed in SA treated cowpea (*Vigna unguiculata* L.) (Fernandes et al., 2006) and JA treated broadleaf dock (*Rumex obtusifolius* L.) (Moore et al., 2003). This increased peroxidase activity was correlated with the enhanced plant defense against pathogen attack. In this study, the catalase activities influenced by *S. carpocapsae* IJs or SA treatments in the hosta leaves varied between experiments, but we found overall increase in catalase activities in both hosta and *A. thaliana* plants when treated with EPNs, SA and JA. These findings are in agreement with previous findings, which demonstrated that the treatments with SA (Dorey et al., 1998; Yu et al., 1999; Clarke et al., 2002), JA (Clarke et al., 2002) and PGPR (Nafie, 2003) enhanced the activities of catalase, which may be involved in the induction of plant defense responses in different plant species. This suggests that the EPNs, like chemical regulators SA and JA, may be involved in induction of general defensive compounds in plants against infection by PPNs and by other bacterial and fungal plant pathogens.

In this study, we also used transgenic *A. thaliana* to further confirm the involvement of EPNs in ISR via activation of *PR1* promoter using β-glucuronidase (GUS) reporter gene and found that EPNs,

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EPN infected cadavers, and chemical elicitors including SA and JA applied to rhizosphere caused a high level of systemic *PR1*-gene expression in *A. thaliana* leaves. Park and Kloepper (2000) reported that the application of avirulent rhizobacteria and SA activated *PR-1a* gene in transgenic tobacco plants expressing GUS reporter gene fused to the promoter region of *PR-1a*. Similarly, Mitter et al. (1998) demonstrated that the inoculation of leaves with incompatible tobacco mosaic virus or treatment of seedlings and mature leaves with JA strongly activated *A. thaliana* plant defensin gene *PDF1.2* in transgenic tobacco expressing *uidA* reporter gene encoding GUS gene fused to the promoter region of *PDF1.2*. This is the first study that demonstrates that the EPNs systematically express defense gene (*PR1*) in plants using GUS reporter gene.

We conclude that EPNs and their symbiotic bacteria induce defense mechanisms in plants in a manner similar to virulent pathogens. We found that EPNs and their symbiotic bacteria are capable of systemically activating the production of key defense enzymes in hosta and *A. thaliana* plants and expressing a plant resistant protein promoter *PR1*-gene in *A. thaliana* leaves. This induced plant defense may be contributing to reduced reproduction of PPNs on plants treated with EPNs or their symbiotic bacteria and we suggest this as a new mechanism of interaction between EPNs and PPNs.

Acknowledgments

We thank Ms. Judith Smith for helping to set up growth chamber. We also thank Klyn Nurseries Inc., Perry, Lake County, OH, for providing hosta plants. Financial support from American Hosta Society, Horticulture Research Institute, and Ohio Agriculture and development Center is acknowledged.

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