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RESEARCH PAPERS

The combined use of *Pochonia chlamydosporia* and plant defence activators - a potential sustainable control strategy for *Meloidogyne chitwoodi*

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Summary. Sustainable strategies are required for control of the root-knot nematode *Meloidogyne chitwoodi* to reduce dependence on toxic chemical pesticides. The efficacy of the nematophagous fungus *Pochonia chlamydosporia* in biocontrol could be enhanced by integration with control measures that reduce initial nematode infestations. The use of foliar sprays with plant defence activators can reduce the susceptibility of potato plants to *M. chitwoodi*. This study assessed effects of combined soil application of *P. chlamydosporia* with foliar sprays of benzothiadiazole (BTH) or *cis*-jasmone on infection of potatoes by *M. chitwoodi*. *Solanum tuberosum*, cv. Désirée plants were grown in soil mixed with 5000 chlamydospores g^{-1} of soil, sprayed twice with BTH or *cis*-jasmone and inoculated with 300 *M. chitwoodi* second-stage juveniles. Forty-five days after inoculation, nematode reproduction, numbers of colony-forming units of the fungus g^{-1} of soil and g^{-1} of root, and egg parasitism were assessed by standard techniques. Foliar sprays of BTH or *cis*-jasmone combined with the fungus reduced nematode reproduction ($P<0.05$, LSD). The presence of the fungus slightly increased the efficacy of *cis*-jasmone, as the number of eggs per egg mass was less in plants treated both with *cis*-jasmone and the fungus than in the plants treated only with the defence activator. The proportion of parasitized eggs was greater in the *cis*-jasmone treatment where rhizosphere colonisation was less, suggesting that *P. chlamydosporia* became a poorer rhizosphere coloniser but a more efficient nematode parasite. The addition of *P. chlamydosporia* to soil in combination with application of inducers of plant defence could be an alternative control strategy to be used against *M. chitwoodi* in potato.

Keywords: benzothiadiazole (BTH); *cis*-jasmone; root-knot nematodes; biological control.

Introduction

The root-knot nematode (RKN), *Meloidogyne chitwoodi* Golden O'Bannon Santo and Finley 1980, is an economically important damaging species of plant-parasitic nematodes that has been reported in Argentina, Belgium, Germany, the Netherlands, Mexico, Portugal and South Africa, and has been listed as a quarantine organism in Europe since 1998 (Conceição *et al*., 2009; OEPP/EPPO, 2009). This nematode has a wide host range, including economically important crops such as potato (*Solanum tuberosum* L.) and tomato (*S. lycopersicum* L.), and is tolerant of low soil temperatures (Santo *et al*., 1980; O'Bannon *et al*., 1982; Santo and O'Bannon, 1982; Ferris *et al*., 1993). Control measures, such as the use of nematicides, are less effective against *M. chitwoodi* than against other phytoparasitic nematodes, and combined nematicidal treatments are required to achieve control (Pinkerton *et al*., 1986; Santo and Wilson, 1990; Ingham *et al*., 2000). However, the potential negative impact of most chemical nematicides on

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non-target organisms and the environment led to a total ban or restricted use of these chemicals (Chitwood, 2003). Thus, the development of new control approaches that can be integrated in environmentally friendly pest management strategies is essential (Oka *et al*., 2000). Natural enemies of nematodes and the natural defence mechanisms in plants could both be used in sustainable and environmentally friendly management control strategies to reduce population densities of RKN.

Fungal antagonists are among the most promising biological control agents for nematodes (Stirling, 2011). *Pochonia chlamydosporia* (Goddard) Gams and Zare is a ubiquitous, facultative, nematophagous fungus with parasitic activity against eggs and sedentary females of economically important plant-parasitic nematodes. It has been associated with soils that suppress the multiplication of cyst nematode populations (Kerry and Crump, 1977; Kerry *et al*., 1982). The biocontrol potential of *P. chlamydosporia* has been widely studied in pots and field experiments (Tzortzakakis, 2000, 2007, 2009; De Leij *et al*., 1993; Bourne and Kerry, 1999; Atkins *et al*., 2003; Tzortzakakis and Petsas, 2003; Verdejo-Lucas *et al*., 2003; Tobin *et al*., 2008; Puertas and Hidalgo-Díaz, 2009).

Pochonia chlamydosporia is a relatively poor competitor in soil, but seems able to proliferate in the rhizosphere by using nutrients released in root exudates (De Leij *et al*., 1993; Kerry *et al*., 1993; Bourne and Kerry, 2000). This fungus is predominantly confined to the rhizosphere although limited endophytic growth (within the roots) has been detected. Additionally, *P. chlamydosporia* may promote plant growth in the initial stages of root colonization, and elicit plant defence against endophytic colonization (Bordallo *et al*., 2002; Lopez-Llorca *et al*., 2002; Mácia-Vicente *et al*., 2009a,b). While rhizosphere colonization by the fungus is essential for nematode control, fungal abundance is not always correlated with parasitism of nematode eggs (Kerry and Hirsch, 2011). Although *P. chlamydosporia* was more abundant on nematode-infected than healthy roots (Bourne *et al*., 1996; Bourne and Kerry, 1999), this may be related to intricate interactions of nematode-host preference, root physiological status or changes in nutrient availability in the microenvironment of the rhizosphere around the egg masses (Atkins *et al.*, 2009; Kerry and Hirsch, 2011; Ward *et al.*, 2012). Furthermore, in the presence of hosts very susceptible to the nematode or large nematode population densities, RKN galls can often be large, with several females, and eggs may be deposited in egg masses inside the roots. Highly susceptible hosts therefore have negative effects on the efficacy of control by *P. chlamydosporia*, as egg masses inside the roots are protected from fungal attack and, in addition, the fungus does not prevent the initial infestation of roots by second-stage juvenile nematodes (J2) (Bailey *et al*., 2008). Therefore, biocontrol efficacy could be enhanced by combining its use with control measures that prevent or reduce initial infestations, such as crop rotation with poor hosts of the nematode (Bourne *et al*., 1996; Kerry and Bourne, 1996). While crop rotation has been the basis of control programmes worldwide, it is not an effective strategy against *M. chitwoodi* because cereal crops (wheat, oats, barley and maize), common in rotation with potato, are considered good hosts of this nematode (OEPP/EPPO, 2009).

An alternative means to control this pest is to target plant defence mechanisms. Plant defences are triggered by pathogen attack but can also be activated by exogenous application of compounds, such as salicylic acid (SA) and its synthetic mimics, like benzothiadiazole (BTH), or jasmonic acid (JA) and its derivatives, and by the non-protein amino acid β-aminobutyric acid (BABA) (Cohen, 2002; Gozzo, 2003; Lucas, 2010). The use of foliar sprays of BTH and JA and its derivatives can reduce plant-parasitic nematode invasion, development and reproduction (Owen *et al*., 2002; Chinnasri *et al*., 2003; Cooper *et al*., 2005; Collins *et al*., 2006; Curtis *et al*., 2009; Molinari and Baser, 2010; Berry *et al*., 2011; Fujimoto *et al*., 2011; Nahar *et al*., 2011; Pankaj *et al*., 2013, Vieira dos Santos *et al*., 2013a).

The application of plant defence activators that reduce the overall susceptibility of crop plants to root-feeding nematodes could be a key strategy for boosting the control potential of a biological control agent shown to be more efficient when associated with poor plant hosts. The aim of the present study was to assess the effects of combining soil application of the fungus *P. chlamydosporia* with foliar sprays of the activators of plant defence, BTH (Bion®, Syngenta) or *cis*-jasmone, on infection of potato by *M. chitwoodi*.

Materials and methods

Meloidogyne chitwoodi isolate

One *M. chitwoodi* population, from infected potato tubers collected in Porto, Portugal (Conceição

et al., 2009), was propagated on tomato plants cv. Easypeel, in 800 cm^3 pots containing sterilized sandy soil in the Nematology laboratory at the University of Coimbra.

Pochonia chlamydosporia isolate

Pochonia chlamydosporia isolate Pc2, obtained from *Globodera rostochiensis* eggs extracted from cysts from soil samples collected in a Portuguese potato field, was maintained on 1.7% corn meal agar (CMA, Oxoid), at 25°C in the Nematology laboratory at the University of Coimbra (Vieira dos Santos *et al*., 2013b). Production, using a barley:sand substrate (1:1), and extraction of chlamydospores were performed following the methodology described by De Leij and Kerry (1991). Chlamydospore viability and germination were assessed on sorbose agar with antibiotics (Abrantes *et al*., 2002).

Plant material and *Pochonia chlamydosporia* inoculation

Potato plants cv. Désirée were each grown from a single sprout in plastic containers with 800 cm^3 of autoclaved sandy soil, commonly used for growing potato plants in pots, and previously mixed thoroughly with 5000 chlamydospores g^{-1} of soil. Controls were made with non-inoculated soil. The pots were placed in a growth chamber at 23ºC and a 16 h light, 8 h dark illumination cycle, and watered every 2 d.

Foliar sprays of potato plants

All plant activators were mixed with 0.1% non-ionic surfactant ethoxylated nonylphenol (EBV) (Tennants Distribution Ltd, UK) before foliar spray applications. Three-week-old potato plants were sprayed twice with a fine mist sprayer with a 7-d interval, 35 cm above the plant, at a rate of 1 mL per plant to just below runoff, with either 2.5 mM BTH (Bion®) or 1 mM *cis*-jasmone (Sigma, USA). These concentrations corresponded to *circa* 1.050 mg per plant (BTH) and 0.213 mg per plant (*cis*-jasmone). For each treatment, ten pots containing soil inoculated with *P. chlamydosporia* and five without fungal inoculum were sprayed and controls were treated with water or 0.1% EBV. To avoid contact of the plant activators with the soil and roots, the soil surface and the pots were covered with aluminium foil. Following each spray, the pots were kept separate for 2 d to avoid cross-contamination from volatiles. They were then returned to the growth chamber and the plants were watered every 2 d.

Nematode inoculation

Egg masses of *M. chitwoodi* were hand-picked from infected roots of tomato cv. Easypeel, and placed on a plastic sieve (10 μm mesh aperture) with tap water, in the dark, at room temperature. Freshly hatched J2 were collected and their number in the suspension was estimated by counting 1 mL replicates of the suspension using a stereomicroscope. Two d after the first spray, five plants per treatment with *P. chlamydosporia* or without were inoculated with 300 J2 per plant.

Experimental design

Foliar sprays of BTH, *cis*-jasmone, water or EBV were applied to potato plants grown in soil inoculated and non-inoculated with *P. chlamydosporia* in the presence and absence of *M. chitwoodi* (Table 1). The experiment was carried out in a growth chamber in a completely randomized design, with five replicates per treatment, over 67 d.

Meloidogyne chitwoodi reproduction

Nematode reproduction was assessed 45 d after inoculation with the nematode. The lengths and fresh weights of the plant shoots and roots were recorded. The numbers of galls and egg masses per root system were counted, and the final nematode population density (Pf) was estimated taking into account the number of eggs from each root system, extracted using 0.52% sodium hypochlorite (Hussey and Barker, 1973). Reproduction factor (Rf = Pf/Pi where Pi= 300 J2), the numbers of eggs per egg mass and eggs g^{-1} of root were also assessed for each plant.

Pochonia chlamydosporia proliferation and parasitism

The numbers of colony forming units (cfu) g^{-1} of soil and cfu g^{-1} of root were evaluated by dilution plating (De Leij and Kerry, 1991). Parasitism was assessed in eggs extracted from ten egg masses collected randomly from each root system. Egg masses were disrupted by maceration in sterilised distilled water to

release the eggs. These were placed on 0.8% technical agar (Sigma) with antibiotics (streptomycin sulfate, chloramphenicol and chlortetracycline, each at 50 mg L^{-1}). For each root system, three plates with 200 eggs were prepared. After 3 d of incubation at 25°C, the percentage of parasitized eggs was determined by examining 100 eggs per plate for signs of fungal colonization using an inverted microscope according to standard methods (Kerry and Crump, 1977).

Data analysis

Differences between treatments and the controls were compared by analysis of variance (ANOVA) using the General Linear Model command in SPSS (IBM® SPSS® Statistics 19, SPSS Inc., USA). Square root transformations were used to ensure a normal distribution and homogeneity of variance when required. Whenever the ANOVAs returned statistically significant effects ($P<0.05$), differences among treatments were further assessed using the LSD test at the 5% probability level.

Results

Effects of *Pochonia chlamydosporia* and plant activators against *Meloidogyne chitwoodi* on potato cv. Désirée

Although variable, shoot and root length, in the presence of both the nematode and the fungus, was greater overall than for the water control (Table 1). In plants treated with the defence activators, root weight was significantly greater in the presence of the nematode than in treated plants that were not inoculated with the nematode (Table 1). No evidence of phytotoxic effects associated with BTH or *cis*-jasmone application were detected.

In plants sprayed with BTH or *cis*-jasmone, the numbers of galls, egg masses and eggs g^{-1} of root were significantly smaller ($P<0.05$) than in the controls and were not affected by the fungus (Table 2). No significant differences were found between plant defence activators. However, nematode reproduction was significantly affected by the foliar spray of BTH in the presence or absence of the fungus when

Table 1. Mean shoot and root lengths and weights of potato, *Solanum tuberosum*, cv. Désirée, grown in soil with *Pochonia chlamydosporia* (Pc), 45 d after inoculation with 300 *Meloidogyne chitwoodi* (Mc) second-stage juveniles, and treated with foliar sprays of: 2.5 mM benzothiadiazole (BTH); 1 mM *cis*-jasmone (Cis-J); 0.1% ethoxylated nonylphenol (EBV); or water (H_2O)[a].

Treatment	Shoot				Root			
	Length (cm)		Weight (g)		Length (cm)		Weight (g)	
BTH+Pc[b]+Mc	18.2 ± 1.7	ab	3.2 ± 0.7	cd	27.8 ± 1.8	ab	6.1 ± 1.1	b
BTH+Pc[b]	23.7 ± 2.9	bcd	4.2 ± 0.5	de	27.8 ± 4.0	ab	2.8 ± 0.7	a
BTH+Mc	28.8 ± 6.3	d	2.2 ± 0.4	abc	28.4 ± 0.6	ab	7.8 ± 0.5	c
Cis-J+Pc[b]+Mc	23.6 ± 0.6	bcd	4.2 ± 0.2	de	33.2 ± 3.4	bc	6.6 ± 0.3	bc
Cis-J+Pc[b]	26.7 ± 2.7	cd	1.8 ± 0.1	ab	30.2 ± 1.1	abc	3.5 ± 0.7	a
Cis-J+Mc	17.8 ± 0.8	ab	3.6 ± 0.3	de	28.6 ± 1.2	ab	6.9 ± 0.7	bc
EBV+Pc[b]+Mc	20.8 ± 0.5	bc	4.4 ± 0.3	e	34.8 ± 3.0	bc	2.1 ± 0.3	a
EBV+Pc[b]	24.8 ± 0.4	cd	1.8 ± 0.3	ab	31.5 ± 1.5	abc	3.0 ± 0.7	a
EBV+Mc	22.3 ± 0.7	bcd	1.4 ± 0.2	a	25.4 ± 1.3	a	2.2 ± 0.4	a
H_2O+Pc[b]+Mc	15.5 ± 1.0	a	3.4 ± 0.5	de	27.0 ± 0.2	a	1.9 ± 0.3	a
H_2O+Pc[b]	18.3 ± 2.0	ab	1.6 ± 0.3	a	29.7 ± 1.5	abc	2.8 ± 0.2	a
H_2O+Mc	28.2 ± 1.9	d	3.0 ± 0.6	bcd	29.6 ± 2.3	abc	2.6 ± 0.9	a

[a] Values are means of five replicates ± standard errors. Values followed by the same letter within a column are not significantly different according to LSD test ($P<0.05$).
[b] 5000 chlamydospores g^{-1} of soil.

Table 2. Mean numbers of galls, egg masses and eggs g^{-1} of root, eggs per egg mass and reproduction factor (Rf) in potato, *Solanum tuberosum*, cv. Désirée, grown in soil with or without *Pochonia chlamydosporia* (Pc) and treated with foliar sprays of 2.5 mM benzothiadiazole (BTH), 1 mM *cis*-jasmone (Cis-J), 0.1% ethoxylated nonylphenol (EBV), or water (H_2O), 45 d after inoculation with 300 *Meloidogyne chitwoodi* second stage juveniles (J2)[a].

Treatment	Galls g^{-1} of root (No.)		Egg masses g^{-1} of root (No.)		Eggs g^{-1} of root (No.)		Eggs per egg mass(No.)		Rf[c]	
BTH+Pc[b]	5.0 ± 0.4	a	5.2 ± 0.4	ab	413.0±8.9	a	81.8±7.7	ab	7.0±0.8	a
BTH	4.1 ± 0.9	a	3.8 ± 0.8	a	317.9±65.2	a	90.5±16.7	ab	7.7±1.2	a
Cis-J+Pc[b]	9.2 ± 1.2	a	9.2 ± 1.2	b	481.7±214.8	a	47.7±14.8	a	11.3±5.7	ab
Cis-J	13.5 ± 2.5	a	10.7 ± 2.0	b	991.7±127.7	b	99.1±12.3	bc	20.8±2.0	c
EBV+Pc[b]	28.8 ± 5.3	b	28.6 ± 5.2	c	2496.3±349.3	c	93.0±15.3	ab	17.4±2.3	bc
EBV	40.2 ± 3.1	c	40.4 ± 3.8	de	2414.9±82.5	c	61.3±5.6	ab	17.5±2.5	bc
H_2O+Pc[b]	34.8 ± 5.5	bc	34.9 ± 5.3	cd	4760.1±1156.3	c	145.1±39.4	c	32.2±9.9	c
H_2O	59.4 ± 4.2	d	50.3 ± 6.2	e	3283.8±583.5	c	69.2±22.3	ab	27.3±13.6	c

[a] Values are means of five replicates ± standard errors. Values followed by the same letter within a column are not significantly different according to LSD test (P<0.05).
[b] 5000 chlamydospores g^{-1} of soil.
[c] Rf= final population (Pf)/300 J2 (Pi).

compared to the controls. For the *cis*-jasmone treatment this effect was only statistically significant when foliar sprays were combined with the fungus (Table 2).

The numbers of cfu of *P. chlamydosporia* g^{-1} of soil were significantly greater in the nematode-inoculated plants than in potato plants not inoculated with nematodes (P<0.001), and no differences were detected among treatments (Figure 1a). Similar results were observed for cfu of *P. chlamydosporia* g^{-1} of root (Figure 1b). The percentage of parasitized eggs increased in all treatments (>32%), being significantly greater in plants treated with *cis*-jasmone (>52%) when compared to the controls (Figure 1c).

Discussion

The potential of *P. chlamydosporia* as a biological control agent combined with the use of plant defence activators against the RKN *M. chitwoodi* was investigated for the first time. It has already been demonstrated, in previous experiments we have conducted, that foliar sprays of BTH or *cis*-jasmone, using the concentrations applied in this study, are effective for reducing the susceptibility of potato plants to *M. chitwoodi* (Vieira dos Santos *et al*., 2013a). In the present study, these treatments reduced the numbers of egg masses g^{-1} root and eggs per egg mass, but the reproduction factor was only significantly affected by BTH and *cis*-jasmone treatments when combined with the fungus (Table 2). The efficacy of foliar sprays of BTH in reducing plant-parasitic nematode density, including *M. chitwoodi*, on potato under field conditions has also been reported by Collins *et al*. (2006). Moreover, application of commercial formulations of BTH (Bion® and Actigard®) to glasshouse-grown grape, sugarcane, cowpea and soybean plants had significant effects on *Meloidogyne* spp. by reducing nematode infection and reproduction, slowing nematode development and decreasing egg deposition (Chinnasri *et al*., 2003; Owen *et al*., 2002; Berry *et al*, 2011).

Pochonia chlamydosporia in soil was sparse in the absence of the nematode; once in the presence of the nematode, the fungus was able to proliferate above the initial inoculation rate (>15000 chlamydospores g^{-1} of soil). Fungal proliferation in soil is not related to the presence of RKN (Mauchline *et al*., 2002). However, the germination of chlamydospores inoculated in soil could be triggered by nutrients leaking from the roots (De Leij *et al*., 1993, Mauchline *et al*., 2002). Similarly, rhizosphere colonization increased in the

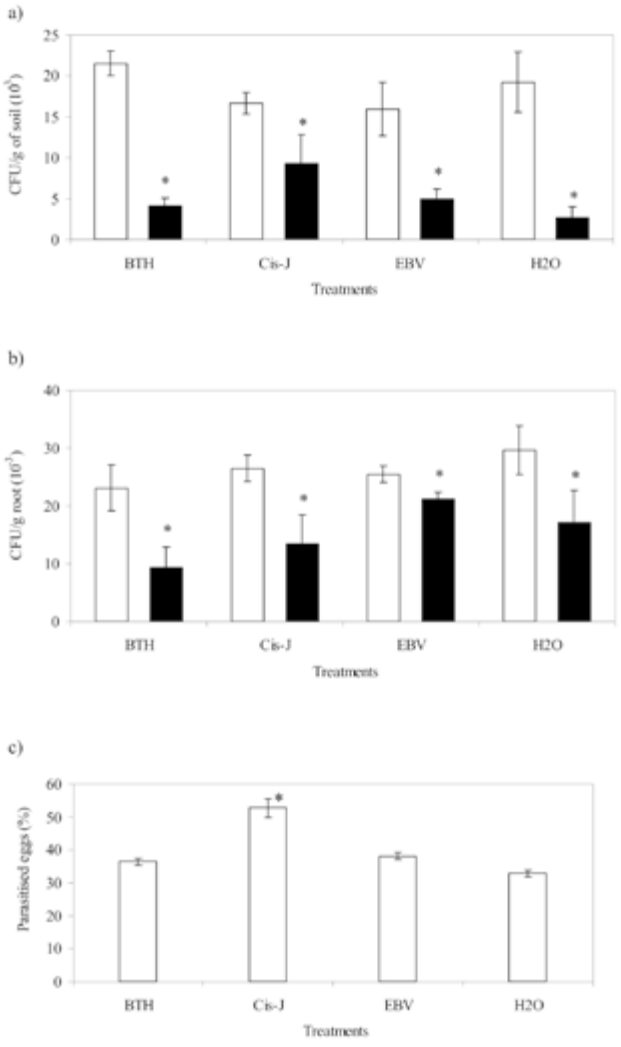

Figure 1. Mean numbers of colony forming units (cfu) of *Pochonia chlamydosporia* in soil (a) and roots (b) of potato, *Solanum tuberosum*, cv. Désirée and (c) egg parasitism. Treatments: foliar sprays of: 2.5 mM benzothiadiazole (BTH); 1 mM *cis*-jasmone (Cis-J); 0.1% ethoxylated nonylphenol (EBV); or water (H_2O). (□) inoculated with 300 *Meloidogyne chitwoodi* second-stage juveniles and (■) without the nematode. Bars represent standard error of means. *Significantly different according to Fisher LSD test (P<0.001).

presence of the nematode, which may be related to changes in root exudates due to nematode infection. Root exudates are thought to be the nutrient source used by the fungus to proliferate in the rhizosphere (De Leij *et al*., 1993; Kerry *et al*., 1993; Bourne and Kerry, 2000).

The numbers of *P. chlamydosporia* cfu g^{-1} of root decreased in plants treated with the plant defence activators in comparison with controls in the absence of the nematodes, implying that activation of induced plant defences may have influenced rhizosphere colonization by the fungus. Fungal growth within root tissues induces cell wall modifications, including papillae and appositions, along with the presence of compounds associated with plant resistance. Nevertheless, this elicitation of plant defence mechanisms does not affect fungal growth (Bordallo *et al*., 2002; Mácia-Vicente *et al*., 2009a).

The percentage of parasitized eggs was greater following the *cis*-jasmone treatment, where the number of cfu g^{-1} of root was less than in the untreated plants. This suggests that, under these conditions, *P. chlamydosporia* was a poor rhizosphere coloniser but an efficient nematode parasite. When *P. chlamydosporia* comes into contact with nematode egg masses in the rhizosphere, the fungus switches its saprophytic behavior to a parasitic phase. The factors that affect this switch are not well understood and, although nematode host preference at the infra-specific level may be involved, nutrients either released by plants or available in nematode eggs may also play important roles (Kerry, 2000; Morton *et al*., 2004; Esteves *et al*., 2009). It has been shown that readily metabolised sources of carbon and nitrogen and pH influenced the production of VCP1, an important enzyme in the initial parasitism stages (Ward *et al*., 2012).

Furthermore, the percentage of parasitized eggs increased in plants treated with *cis*-jasmone comparing to BTH-treated plants or the controls (Figure 1c). This suggests that the JA defence pathway may increase the efficacy of *P. chlamydosporia* as a biological control agent.

The potential of *P. chlamydosporia* isolates as biological control agents against RKN is greatly influenced by a number of traits, including their ability to establish in bulk soil and in the rhizosphere, and to parasitize nematode eggs (Abrantes *et al*., 2002). Such traits did not seem to be adversely affected by the application of plant defence activators. Therefore, we suggest that a strategy based on inducing plant defence mechanisms and the use of the nematophagous fungus *P. chlamydosporia* could be a potential alternative for the management of *M. chitwoodi*. The experiments described in the present study were conducted in controlled conditions and in sterilized soil, but to understand the intri-

cate interactions between plants, nematodes and the fungus under the effect of these plant defence activators, further *in vitro* experiments should be conducted. These will be important for testing the putative involvement of the JA defence pathway in the saprophytic/parasitic switch of *P. chlamydosporia*. Furthermore, both abiotic and biotic factors that influence the performance and efficacy of *P. chlamydosporia*, and the effects of inducing plant defence pathways on the complex and diverse naturally occurring interactions between plants and their associated soil microbial diversity, should also be evaluated in field studies.

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