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Effect of Inoculation with the Arbuscular Mycorrhizal Fungus *Glomus Intraradices* on the Root-Knot Nematode *Meloidogyne Incognita* in Cucumber

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ABSTRACT

A pot experiment was carried out to investigate the tolerance of cucumber plants (*Cucumis sativus* L.) to root-knot nematode after inoculation with *Glomus intraradices*. Plants were inoculated with *G. intraradices* for four weeks and then transplanted in soil treated with *Meloidogyne incognita* for a further five weeks. The low phosphorus (P) loamy soil was amended with 50 and 100 mg P kg^{-1} soil. Mycorrhizal colonization increased shoot dry weight, shoot length, leaf numbers, root fresh weight and shoot P concentration, whereas nematode penetration and reproduction were significantly decreased. Similarly, P fertilization usually increased shoot growth and significantly decreased the number of galls and the number of egg masses and eggs per g root. Our results indicate that inoculation with *G. intraradices* and P fertilizer confer tolerance of cucumber plants to *M. incognita* by enhancing plant growth and by suppressing reproduction and/or galling of nematodes during the early stages of plant growth.

Keywords: Arbuscular mycorrhizal, *Cucumis sativus*, *Meloidogyne incognita*, phosphorus

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INTRODUCTION

Root-knot nematode disease is a worldwide problem, with annual losses caused by *Meloidogyne* spp. of more than US $100 billion globally (Sasser et al., 1987). Root-knot nematode is also one of the factors most limiting vegetable production in China and annual losses of more than US $30 billion are attributable to yield losses caused by root-knot nematodes (Duan and Wu, 2002). A three-year survey on the occurrence of root-knot diseases in vegetables was conducted from 2001–2003 in Shandong province, northeast China (Dong et al., 2004) which accounts for 17% of the total vegetable production in China (Chinese Ministry of Agriculture, 2006). Root-knot nematode disease was widespread in greenhouse vegetable systems and was observed in 67.6% of greenhouses investigated, with approximately 50% of plants infected. In China four species of root knot nematodes represent a significant threat to vegetable production, namely *M. incognita*, *M. arenaria*, *M. hapla,* and *M. javanica*, of which *M. incognita* is commonly found in many plant species.

Cucumber is one of the most economically important vegetables in China, and is grown extensively with a cultivated area in 2002 of 1.25 million ha, accounting for nearly 10% of the total vegetable cultivation area (Li, 2003). In recent years serious outbreaks of root-knot nematode disease have occurred in cucumber, particularly in greenhouse systems with continuous cropping. Research has shown that after four or more crops, the rate of occurrence was up to 95% (Dong et al., 2004), leading to fruit yield losses of 20–30% and no fruit productions in some cases (Peng, 1998).

Various physical, chemical, and agronomic measures have been proposed to control root-nematode disease (van der Putten et al., 2006). Chemical nematicides are most effective in inhibiting nematode infestations but most of the active ingredients are prohibited due to their detrimental effects on human health and the environment. Agronomic practices including crop rotations, fallow, and cover crops can also reduce disease incidence but these methods are difficult to practice under Chinese conditions due to the limited land area and intensive cultivation systems. Biological control techniques are preferable because of their limited effects on the environment and the land can be used continuously for economic production.

Both arbuscular mycorrhizal fungi (AMF) and root knot nematodes are indigenous soil organisms. Due to the beneficial effects of AMF on host plants under various biotic and abiotic conditions (Smith and Read, 1997), AMF are suggested to have the potential to serve as both biological fertilizers and biological control agents (Carling et al., 1996). Research has shown that AMF have interactions with root nematodes of many crop plant species including banana and *Hyoscyamus niger* (blacke henbane) with *M. incognita* (Jaizme-Vega et al., 1997; Pandey et al., 1999); Chrysanthemum with *Meloidogyne hapla* (Waceke et al., 2001); peanut with *M. arenaria* (Carling et al., 1996); Prunus rootstocks with *M. javanica* (Calvet et al., 2001); and olive planting stocks

with *Meloidogyne incognita* and *M. javanica* (Castillo et al., 2006). In most circumstances, inoculation with AMF can increase the tolerance of host plants to nematodes. The proportion of reduction of root-knot nematode numbers by AMF was found to be 33% on average (Hol and Cook, 2005). However, in the literature there are few reports on the relationship between AMF and root knot nematodes of cucumber. The aim of the present study was to investigate the interactions between AMF and *M. incognita* in cucumber, together with the effect of phosphorus (P) fertilizer because P nutrition is closely related to AMF-mediated effects on host plants.

MATERIALS AND METHODS

Cucumber (*Cucumis sativus* L. cv. 'Zhongnong16') seeds were selected because this cultivar is susceptible to root-knot nematode disease and is widely cultivated in north China. The seeds were firstly surface sterilized in a 10% (v/v) solution of hydrogen peroxide for 10 min, thoroughly washed with deionized water and then planted in a mixture of peat and vermiculite (1:1, v:v). The substrate was either mixed with inoculums of *Glomus intraradices* (BEG141) at a ratio of 9:1 (mass ratio) or remained uninoculated but received an equivalent amount of sterilized inoculums together with a filtrate (0.45 μm pore size) of unsterilized soil to provide a similar microflora except for the absence of the mycorrhizal fungus. The inoculums consisted of rhizosphere soil containing spores, hyphae, and mycorrhizal maize and white clover root fragments. After four weeks' growth, seedlings were transplanted into 1-L pots containing fumigated soil amended with one of two levels of P fertilizer. Each pot had one plant. Seedlings were irrigated daily with deionized water and with P-free Hoagland nutrient solution once a week. Each seedling was inoculated with 1200 (juvenile) nematodes one week after transplanting.

The soil used, collected from Daxing county in suburban Beijing, was a low-P sandy loam with the following properties: pH 8.44 (H_2O:soil 2.5:1, v:v), total organic matter 0.30%, total nitrogen (N) 0.08%, available P (Olsen-P) 7.72 mg kg^{-1}, and available potassium [K; ammonium acetate (NH_4OAc-K)] 33.6 mg kg^{-1}. Phosphorus was supplied as monopotassium phosphate (KH_2PO_4) at rates of 50 (P50) and 100 (P100) mg P kg^{-1} soil to represent low and optimal P supply levels respectively. N [as ammonium nitrate (NH_4NO_3)] and K [as potassium sulfate (K_2SO_4)] were added to the soil at a rate of 200 mg kg^{-1} as basal fertilizer. The soil was sieved to 2 mm and sterilized at 121°C for two hours.

The inoculums of *Glomus intraradices* (BEG141) was produced by growing maize and white clover as the host plants in a greenhouse for four months. Soil including spores, mycelia, and roots of maize and white clover were used as inoculums. The root-knot nematode, *M. incognita,* was kindly provided by Professor Zhiping Cao of the College of Resources and Environmental Sciences, China Agricultural University, Beijing. The nematode inoculums consisted of

a population which was grown on tomato plants (*Lycopersicon esculentum* cv. 'Hezuo908') from single-egg-mass culture and collected according to the method of Hussey and Barker (1973). Briefly the detached eggs from the roots were extracted in 1.5% sodium hypochlorite (NaClO). The eggs were transferred into deionized water by using a 25-μm sieve and stirred to stimulate the development of J2 juveniles. Afterwards the separation of eggs and juvenile nematodes followed the method of Baerman (Oostenbrink, 1960). The concentration of the suspension of nematodes was adjusted to 200 J mL^{-1}. When the cucumber plants were five weeks old, they were inoculated with 1200 (J2) nematodes per pot by injecting the suspension into 6 holes of 2-cm depth, which were located in a circle 2 cm from the base of the cucumber seedlings.

The experiment consisted of eight treatments: 1) uninoculated control in low P soil (Control P50); 2) uninoculated control in optimum P soil (Control P100); 3) *M. incognita*-inoculated plants in low P soil (*M. incognita* P50); 4) *M. incognita*-inoculated plants in optimum P soil (*M. incognita* P100); 5) *G. intraradices*-inoculated plants in low P soil (*G. intraradices* P100); 6) *G. intraradices*-inoculated plants in optimum P soil (*G. intraradices* P100); 7) *G. intraradices*- and *M. incognita*-inoculated plants in low P soil (*G. intraradices* + *M. incognita* P50); 8) *G. intraradices*- and *M. incognita*-inoculated plants in optimum high P soil (*G. intraradices* + *M. incognita* P100).

Each treatment had five replicates. The pots were arranged in a randomized complete block design in the greenhouse of China Agriculture University, Beijing. Seedlings were grown from 12 March to 20 May 2006, whereby under a temperature regime of 35°C/23°C (day/night) with a 16h/8h (light/dark) photoperiod and at 50–75% relative humidity.

At harvest shoot length, leaf number, shoot dry weight, root fresh weight, and shoot P concentration were determined. Fresh roots were divided into three parts. One part of the roots was used to record the egg masses, a second part to count the eggs and J_2, and the remainder was to determine the proportion of root length colonized by the AMF. Egg masses were stained in 0.015% phloxine B for 20 min, rinsed in sterilized distilled water and afterwards counted under a stereomicroscope. The *M. incognita* eggs were recorded according to the method of Hussey and Barker (1973). Briefly, fresh roots were cut into 1–2 cm root segments which were placed in 100 ml water with 10 mL 5.25% NaClO, mixed for 3 min and sieved. The eggs were then transferred to a 150-mL beaker and concentrated to a 25-mL suspension. One mL of the suspension was used to count the number of eggs. The number of galls on the roots was recorded using an arithmometer. The percentage of root length colonized by AMF was determined following the method of Baker and Gowen (1996). Briefly, the roots were placed in 0.2% sodium hydroxide (NaOH) at 90°C for 20 min and rinsed with water. The cleared roots were immersed in 1% hydrochloric acid (HCl) for 5 min, washed thoroughly with water and placed in the staining solution (a mixture of 500 mL lactic acid, 250 ml glycerol, 250 mL water and 1g of acid fuchsin) at 90°C for 5 min, then decolorized with

acid fuchin-free staining solution. The percentage of root length colonized by AMF was determined using the grid line intersect method under a stereoscopic microscope (Giovannetti and Mosse, 1980). Tissue P concentrations were measured by the vanadomolybdate method (Colwell, 1965) using a Shimadzu UV-120-02 spectrophotometer (Shimadzu, Japan).

Data were tested by three-way or two-way analysis of variance using the SAS statistical software package (Version 6.12, SAS Institute, Cary, NC, USA). Pairs of mean values were compared by calculation of least significant difference (LSD) at the 5% level.

RESULTS

AMF Root Colonization

No AM colonization was observed on the roots of non-mycorrhizal plants (data not shown). Roots of inoculated plants were extensively mycorrhizal and the mean proportion of root length colonized ranged from 56 to 64% (Figure 1). Root colonization rate at P100 was significantly lower than at P50 when inoculated with *G. intraradices* but there was no significant difference between P50 and P100 when inoculated with both *G. intraradices* and *M. incognita*. At P50, compared to sole inoculation with *G. intraradices*, combined inoculation with *G. intraradices* and *M. incognita* significantly decreased root infection rate ($P < 0.05$).

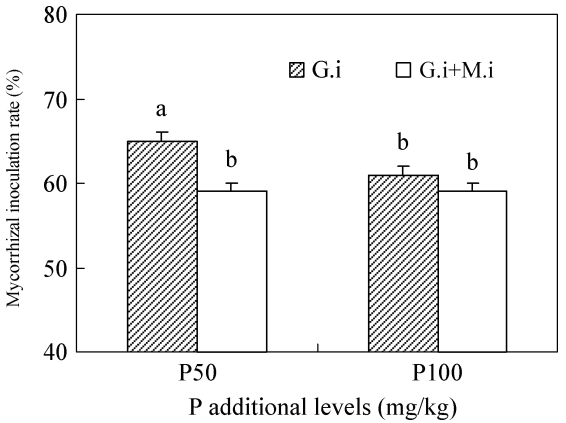

Figure 1. Effect of inoculation with *M. incognita* on the mycorrhizal colonization rate at two P addition levels of 50 mg P (P50) and 100 mg P (P100) kg^{-1} soil. Data are the mean values of five replicates. Vertical bars represent the standard errors (SE). Within columns, treatments with the same letter are not significantly different by LSD at the 5% level.

Effect of P Fertilizer and *G. intraradices* and/or *M. incognita* Inoculation on Growth and Shoot P Concentrations

Inoculation with *G. intraradices* significantly enhanced shoot growth and the growth promoting effect of the mycorrhizal fungus was greater at P50 than at P100 (Table 1). Shoot length, numbers of leaves and shoot dry weight of *G. intraradices*-inoculated plants were significantly higher than in the controls. Shoot length and shoot dry weight of mycorrhizal plants at P50 were higher than that of non-mycorrhizal plants at P100 but were comparable to mycorrhizal plants at P100. P addition increased the shoot growth of non-mycorrhizal plants at P50 and the effect was smaller in mycorrhizal plants. Shoot growth indexes were not significantly affected by inoculation with *M. incognita* but were influenced by the interaction between P supply level and inoculation with *G. intraradices*. Shoot growth of nematode-inoculated non-mycorrhizal plants decreased when compared to the controls without nematodes or mycorrhizal at both P supply levels. Root fresh weight was also significantly increased by inoculation with *G. intraradices* but was not affected by P supply level or inoculation with nematodes. Similarly, shoot P concentration of *G. intraradices*-inoculated plants was also significantly higher than in the controls and there was no significant effect of P addition or inoculation with nematodes on shoot P concentrations (Table 1).

Effect of P Fertilizer and Inoculation with *G. intraradices* and/or *M. incognita* on Galling and Reproduction of Nematodes on Roots

The number of galls on cucumber roots was significantly reduced by *G. intraradices* inoculation but not by P addition and the extent of the reduction of the gall numbers was 53% (Figure 2). Inoculation with *G. intraradices* significantly decreased egg masses (Figure 3A), number of eggs (Figure 2B), and number of J_2 (Figure 2C) g^{-1} root. At P50 the percentage reduction values were 63, 52, and 34% for these parameters and the corresponding values at P100 were 46, 58, and 48%. The number of egg masses and number of eggs g^{-1} root at P100 were significantly lower than at P50, while the number of J_2 g^{-1} root was not affected by P addition level. The proportions of reduction in the number of egg masses g^{-1} were 63% for control plants and 15% for *G. intraradices*-inoculated plants and the corresponding values for the number of eggs g^{-1} root were 78 and 58%.

DISCUSSION

Plant growth and crop yield can be greatly reduced by root-knot nematodes in many crops (Sasser et al., 1987). In the present experiment, although the

Table 1

Effects of P addition and inoculation with *G. intraradices* and/or *M. incognita (Mi)* on the mean biomass and shoot P concentration of cucumber plantlets

P supply level (mg kg $^{-1}$)	Inoculation with *G. intraradices*	Inoculation with *M. incognita*	Shoot length (cm)	Number of leaves	Shoot dry weight (g pot$^{-1)}$	Root fresh weight (g pot^{-1})	Shoot P concentration (%)
50	−AMF	− *Mi*	9.25	6	0.30	0.68	0.14
		+ *Mi*	6.05	7	0.25	0.58	0.17
	G. intraradices	− *Mi*	45.03	13	1.72	1.63	0.29
		+ *Mi*	61.00	15	2.15	2.58	0.23
100	−AMF	− *Mi*	24.30	10	0.79	1.48	0.16
		+ *Mi*	17.55	8	0.48	2.63	0.20
	G. intraradices	− *Mi*	50.85	13	1.97	2.03	0.25
		+ *Mi*	60.55	14	2.53	3.24	0.27
Significance[a] due to: P supply level			*	*	*	NS	NS
Inoculation of AMF			***	***	***	***	***
Inoculation of Mi			NS	NS	NS	NS	NS
P supply level × inoculation of AMF			NS	NS	NS	NS	NS
Inoculation of AMF×inoculation of Mi			NS	NS	NS	*	NS
P supply level × inoculation of Mi			*	*	*	NS	NS
P supply level × inoculation of AMF × inoculation of Mi			NS	NS	NS	NS	NS

[a]By analysis of variance: ***, $P < 0.001$; **, $P < 0.01$; *, NS, not significant.

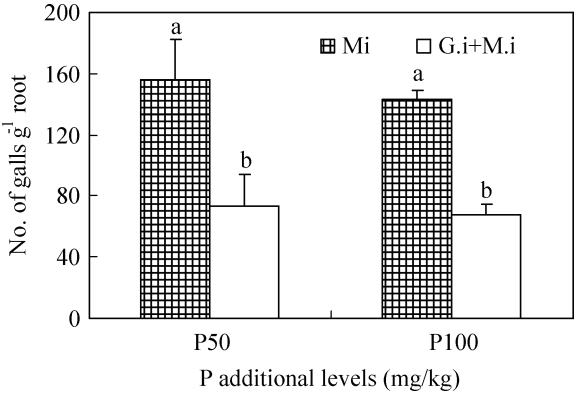

Figure 2. Effects of the two P addition levels of 50 mg P (P50) and 100 mg P (P100) kg^{-1} soil and of inoculation with *G. intraradices* on the number of galls g^{-1} root. The gall number g^{-1} root was dependent on AMF inoculation ($F = 26.24$; $P = 0.006$) and independent of phosphorus level ($F = 0.41$; $P = 0.536$) and there was no interaction between these two variables ($F = 0.05$; $P = 0.831$) by two-way analysis of variance. Data are the mean values of five replicates. Vertical bars represent the standard error (SE). Treatment columns with different letters are significantly different by LSD at the 5% level.

cucumber cultivar used was susceptible to *M. incognita* infestation, no significant growth retardation was observed in plants treated with nematodes regardless of P supply level or mycorrhizal inoculation, and shoot growth of *M. incognita*-inoculated plants tended to be slightly enhanced (Table 1). Reduced plant growth due to the infection with nematodes has been commonly observed (Heald et al., 1989; Carling et al.,1996; Habte et al., 1999; Siddiqui and Akhtar, 2007) but an absence of host growth effects has also been reported in olive plant stocks irrespective of the nematodes or AMF with which the plants were inoculated (Castillo et al., 2006). In the present experiment cucumber seedlings were pre-inoculated and then harvested after inoculation of *M. incognita* for five weeks, and *M. incognita* would be expected to have the potential to cause damage to plants with the prolonged treatment with the nematodes, as shown also by the high gall numbers on the roots of *M. incognita*-inoculated control plants (Figure 2).

G. intraradices significantly enhanced shoot growth of cucumber plants at both P supply levels studied (Table 1) and the effect was more pronounced when plants were treated with *M. incognita*, indicating that *G. intraradices* has the potential to confer enhanced tolerance to root-knot nematode infestation to cucumber. The number of root galls and the numbers of egg masses, eggs, and J2 in mycorrhizal plants were significantly lower than in non-mycorrhizal controls (Figures 2, 3A, 3B, and 3C). The significantly lower root galling and reproduction of nematodes clearly indicate a suppressive effect of *G. intraradices*

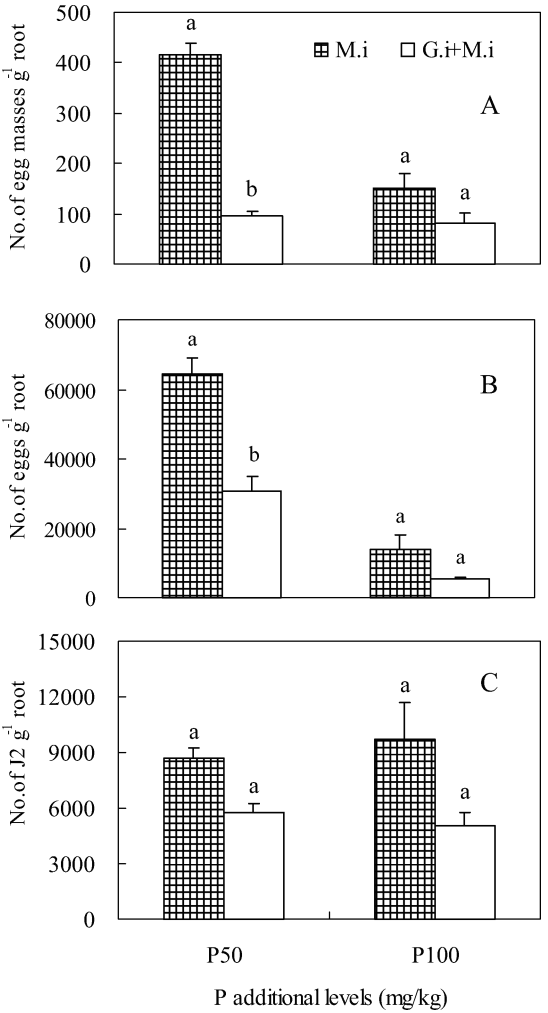

Figure 3. Effects of different P addition levels of 50 mg P (P50) and 100 mg P (P100) kg^{-1} soil and of inoculation with *G. intraradices* on the reproduction of egg masses g^{-1} root. A) The number of egg masses g^{-1} root was dependent on phosphorus level ($F = 29.86$; $P = 0.0004$) and AMF inoculation ($F = 58.31$; $P < 0.001$) and there was a significant interaction between these two variables ($F = 24.03$; $P = 0.0008$) by two-way analysis of variance. B) The number of eggs g^{-1} root was dependent on phosphorus level ($F = 34.16$; $P = 0.0002$) and AMF inoculation ($F = 10.52$; $P = 0.010$) and there was no interaction between these two variables ($F = 3.92$; $P = 0.079$) by two-way analysis of variance. C) The number of eggs g^{-1} root was dependent on AMF inoculation ($F = 13.95$; $P = 0.0047$) and independent of phosphorus level ($F = 0.03$; $P = 0.878$) and there was no interaction between these two variables ($F = 0.75$; $P = 0.408$) by two-way analysis of variance. Vertical bars represent the standard errors (SE).

to *M. incognita* in cucumber. Similarly Castillo et al. (2006) reported that *G. intraradices* suppressed *M. incognita* or *M. javanica* in olive plants by reducing the severity of root galling by 6.3–36.8% as well as reproduction of both nematode species by 11.8–35.7%. Habte et al. (1999) found that *G. intraradices* adversely influenced both the number of nematodes and the number of eggs of *M. incognita* in red clover although inoculation with this fungus produced less growth than *G. aggregatum* or *G. mosseae*.

Enhanced P nutrition is often closely linked with the AMF-mediated effect (Smith and Read, 1997). However, plant P nutrition is not fully relevant to the number of galls and the reproduction of *M. incognita* on the roots of cucumber plants in the present experiment. At P50 the numbers of galls (Figure. 2), egg masses (Figure 3A), and J2 (Figure 3C) in *G. intraradices*-inoculated plants were significantly lower than in non-mycorrhizal plants at P100 but P concentration in mycorrhizal plants at P50 were higher than in non-mycorrhizal plants at P100 (Table 1). In addition, although shoot P concentrations in dual-inoculated plants were comparable at P50 and P100 (Table 1), the numbers of eggs were significantly lower at P100 than at P50 (Figure 3B). Our results indicate the involvement of mechanisms other than P nutrition in the AMF-nematode interaction (Smith 1987, 1988). Hol and Cook (2005) summarized the possible mechanisms by which AMF may affect nematodes, namely changes in root exudates (Ryan et al, 2000), reduced penetration by *Meloigogyne* on AMF (Mahanta and Phukan, 2000), and competition for photosynthates and space (Hussey and Roncadori, 1982). Changes in root morphology, histopathological changes, physiological, and biochemical changes, and changes in host nutrition and water use have also been suggested (Siddiqui and Mahmood, 1995; Siddiqui and Akhtar, 2007). Li et al. (2006) showed that the root defense response of *G. versiforme* against *M. incognita* in grapevine included transcriptional activation of the class III chitinase gene VCH3. Clearly, as pointed by Hol and Cook (2005), more work is required to elucidate the actual processes by which AMF interact with nematodes.

Supplementary P fertilizer significantly inhibited the number of egg masses and eggs g^{-1} root as well as galling numbers g^{-1} root, and the effect was more pronounced when plants were not inoculated with *G. intraradices* (Figures 3A and 3B). Our results are in contrast to those of Carling et al (1996), but partly consist with Smith et al. (1986). Carling et al. (1996) found that *M. arenaria* reproduction on peanut increased with AMF development and P fertilizer. However, these authors pointed out that their system was unusual due to the observed simultaneous increased tolerance and susceptibility based on nematode reproduction. Smith et al. (1986) showed that *M. incognita* reproduction was stimulated by P when *M. incognita* inoculums was added at planting, but was similar to reproduction in the low-P non-mycorrhizal cotton plants when *M. incognita* addition was delayed by 28 days. Increased reproduction of root-knot nematodes was also observed on bean (Zambolin and Oliveira, 1986) and tomato (Thomson-Cason et al., 1983). The stimulatory effect is likely

attributable to increased root growth and/or P availability in the soil. Taken together, the difficulty in explaining the mechanisms by which AMF-mediated P and P fertility affect nematode reproduction indicates that the tripartite system of AMF, P, and nematodes needs further investigation.

In conclusion, our results indicate that pre-inoculation with AMF and P fertilizer increase the growth of cucumber plants and thus enhance the tolerance of the host plant to *M. incognita*. Due to the complexity of the AMF-P-nematode interactions, more fungal and nematode species should be tested and the mechanisms need to be further elucidated. Furthermore, because nurse planting before transplanting is a common practice in Chinese greenhouse cucumber production, inoculation of appropriate AMF prior to transplanting has practical implications for the suppression of infestation by *M. incognita* on plant roots.

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