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Entomopathogen *Metarhizium anisopliae* Promotes the Early Development of Peanut Root

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Abstract

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The benefit of the entomopathogen *Metarhizium anisopliae* to early root development was evaluated. Two inoculating methods, conidia-suspension-drenching (T_1) and conidia-coating (T_2), were used when sowing peanut. The results showed that taproot length and lateral root number in T_1 significantly increased compared to the control (T_0) in days 4–10 after treatment, whereas no significant difference was found between T_2 and T_0. The fungal density by T_1 and T_2 fluctuated in the first 8 days, followed by a gradual decline. The ratio of the taproot length or lateral root number in T_1 and T_0 was significant relative to the fungal persistence. It suggested that *M. anisopliae* promotes peanut root development and should be considered as important factor in plant protection besides pest controls.

Keywords: entomopathogenic fungus; growth promoting; taproot; lateral root; pollution-free plant protection

The entomopathogenic fungus *Metarhizium anisopliae* is a natural enemy against a number of insect pests and has been extensively studied for biological control in plant protection (Zimmermann 1993; Roberts *et al.* 2004; Skinner *et al.* 2013; Behle *et al.* 2015). Recent studies have found that *Metarhizium* spp. not only depend on insect infestation to propagate but also survive as a saprophytic- and rhizosphere-competent fungi as well as plant endophytes. It has been added to a list of fungi that have bifunctional lifestyles as an entomopathogen as well as a plant growth promoter. Four species in *Metarhizium* genus were reported to persistently survive in rhizospheric and nonrhizosphere soils sampled from a long-term experimental farm cropping soybean, corn, or alfalfa (Kepler *et al.* 2015). Hu and St. Leger (2002) monitored the fate of a *M. anisopliae* isolate labelled green fluorescent protein (*gfp*) gene in a cabbage experimental plot, and found that the fungal densities in the rhizosphere were 10^5 propagules/g of bulk soil and after several months persisted at 10^3 propagules/g of bulk soil. It demonstrated that rhizospheric soils are a potential reservoir for *M. anisopliae*.

We previously applied *M. anisopliae* to control white grubs, *Holotrichia parallela* Motschulsky and *Holotrichia oblita* Fald, and monitored the fungal persistence in soil during the peanut growing. It showed that the fungal density rapidly declined in the early 30 days, followed by a gradual decline to a stable or slight increase throughout the remaining peanut growth period. Interestingly, the density in rhizosphere declined faster than that in bulk soil

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away from root in the first 30 days, although it had a much better recovery after 60 days (Liu *et al.* 2011, 2016). So it is necessary to understand the interaction between the fungus and the root, especially how the fungus influences the early development of peanut roots.

Roots are important parts of plants for colonisation, nutrient absorption, and the synthesis of physiologically active substances. Meanwhile, roots can interact intimately with environmental soil and microorganisms. Later development and physiological functions could be impacted by early root growth. The aim of the present study was to evaluate the effect of *M. anisopliae* application on the early development of peanut roots and on pest control in the field.

MATERIAL AND METHODS

Fungal strain and cultures. *Metarhizium anisopliae* strain IPPM010202 was originally isolated by our laboratory from an infected corpse of white grub. This strain was highly virulent against the larvae of scarabs, *Holotrichia parallela* (Coleoptera: Scarabaeidae) (Liu *et al.* 2011). The fungus was inoculated on PSAY (200 g potato juice, 20 g sucrose, 10 g yeast extract, and 18 g agar in 1000 ml) plates and incubated at 26°C for 14 days. The conidia were harvested by scraping the surface of the plates, dried with silica gel, and then stored at 4°C. The conidial culture was at a concentration of 3.125×10^{10} spores/g by microscopic counts with a hemocytometer, and its viability was 98.2% based on germination incubation at 26°C for 24 h on 2% sucrose medium.

Sowing peanut treatments with Metarhizium anisopliae. The testing peanut species (*Arachis hypogaea* L.) was the Chinese strain Luhua 11. The experiment was conducted in a homogeneous plot of 1.5 × 25.5 m in the testing field of the Cangzhou Academy of Agriculture and Forestry Science, Hebei, China. The plot was equally divided into 11 sections in parallel. Each section was 1.5 × 2.3 m. The sections at the ends were both left without treatment. The other 9 sections sequentially underwent three different treatments, with three repetitions each. The treatments were: (1) the conidia-suspension-drenching treatment (T_1): digging 35 seed pits by 5 × 7 arrangements in a section and sowing 3 seeds per pit. Next, weighing 1.00 g of conidia and preparing as a suspension in 3.5 l of sterile aqueous 0.05% Tween 80 solution. Then, drenching 100 ml of the suspension into each seed pit and drenching supplemental 400 ml of sterile water. The final content was 8.93×10^8 conidia per pit. (2) The conidia-coating treatment (T_2): picking 320 peanut seeds of the same size. Next, coating the seeds in 20 ml of preparation composed of polyvinyl and *M. anisopliae* conidia, gentle stirring for several minutes, followed by seeds removal and air-drying. Calculating the conidial content per coated seed as 7.46×10^6 conidia based on the concentration of the seed-coating preparation and surplus volume. Digging seed pits and sowing the same way as above. Drenching 500 ml of sterile water into each seed pit. (3) The untreated control (T_0): sowing untreated seeds into the seed pits and drenching 500 ml of sterile water into each seed pit the same way as described above.

Plant samples. Eighteen plants from six pits of each treatment were collected and cautiously dug out on days 2–5 after planting daily, on days 6–16 every other day. Distinguishable soil particles attached on the roots were removed. The seed germination rate, seedling emergence, taproot length, and lateral root number were measured and recorded.

Soil samples and detection of M. anisopliae persistence. While collecting plants, the sample plants were shaken gently to remove the root bulk soil until only the media tightly adhering to the seeds and roots remained, then the rhizosphere soil from each plant was swept carefully with a brush. The *Metarhizium* densities in rhizosphere and bulk soil were monitored by the detection of colony forming units (CFU) on a selective isolation medium (PDA supplemented with 0.2 g of chloramphenicol and 0.1 g of dodine in 1000 ml; Liu *et al.* 2011). The detection included the following steps: an aliquot of 1000 g of sample soil was added to 9 ml of sterile 0.5% Tween 80 solution and shaken for homogenisation. Then, serial dilutions were inoculated onto selective medium to determine an appropriate dilution multiple for each treatment. The dilution of each sample soil was inoculated and dispersed onto five repeat plates. The colonies on the plates were counted after 5 days of incubation at 26°C, and the CFU were calculated.

Statistical analysis. The data were analysed by SAS 9.0 software. A variance analysis was performed using one-way ANOVA. Significant differences between the mean values were identified using Duncan's Multiple Range Test, and Pearson's correlation analysis was used to analyse the relationship between the development of peanut root and the densities of *M. anisopliae* persistence.

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RESULTS

Effect of *M. anisopliae* on peanut root growth

Effect on seed germination. The sown seeds did not germinate in one day, a few radicles protruded in two days. On the 3rd day, more than half of the sown seeds germinated, and the germinating rates of the conidia-suspension-drenching treatment (T_1), conidia-coating treatment (T_2), and control (T_0) were 83.3, 77.8, and 77.8%, respectively. There were no significant differences between these values ($P > 0.05$). According to the amount of cotyledons breaching the surface 5 days after plantation, the germinating rates of the three treatments were greater than 95%. Compared to the control, the T_1 and T_2, inoculation treatments did not produce a significant difference in the seedling rate, which was greater than 95%. These results indicate that both drenching and coating for fungal application had no effect on the seed germination of peanut.

Effect on taproots and lateral roots. The development process of peanut roots with *Metarhizium* treatment appeared similar to that of the untreated control. The radicles broke through the seed epidermis and the taproots extended to 1–2 cm in two days. The lateral roots emerged in 3–4 days. The seedling leaves breached the surface on days 5–6. In days 7–16, the growth of the taproots and lateral roots maintained an upward trend. The secondary fibrous roots appeared later on.

The length of the taproots and the number of lateral roots were measured every two days for 16 days. The results showed that the peanut roots with or without *Metarhizium* grew continuously in their respective line with the dynamics equation by regression analysis (Table 1). A relatively fast growth occurred on days 2–4 and 10–12. The ANOVA showed that the conidia-suspension-drenching treatment (T_1) caused a greater increase in taproot length and lateral root number on days 4–10 compared to those of the untreated control ($P < 0.05$ for taproot; $P < 0.05$ for lateral root). There was no significant difference in the two measured values between the conidia-coating treatment (T_2) and the control throughout the 16-day test (Figure 1).

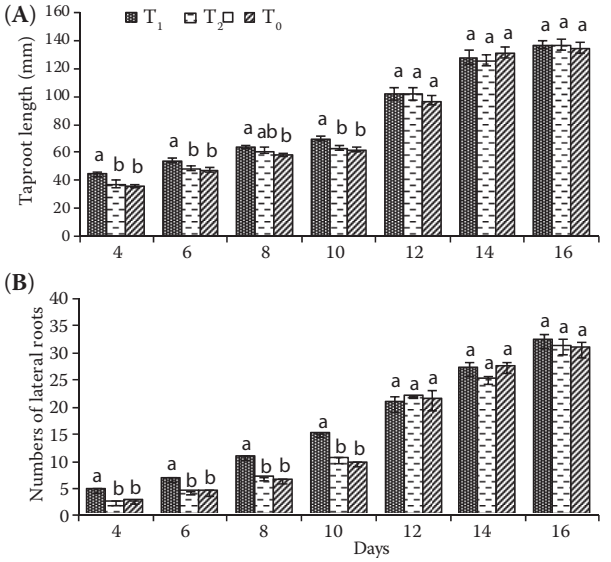

Figure 1. Taproots length (A) and lateral root number (B) of peanuts 16 days post sowing with *M. anisopliae* treatments

Different lowercase letters indicate significant differences in the three treatments on the same day, α = 0.05

Persistence of the *M. anisopliae* density in the peanut rhizosphere and bulk soil

Concomitantly with observing the peanut root growth, the *M. anisopliae* density was monitored in the peanut rhizosphere and bulk soil. The results showed that by the conidia-suspension-drenching treatment (T_1) the density of the applied fungus remained always significantly higher than by the conidia-coating treatment (T_2) ($P < 0.05$). At the rhizosphere, the density dynamics of both treatments showed slight fluctuations with no significant difference compared to the initial value in the first 8 days ($P < 0.05$) followed by a gradual decline. Finally the persistent densities were 48.4 and 40.1% of the initial populations, respectively (Figure 2).

Table 1. Equations of the increased rate for taproot length and for lateral root number

Treatments	Taproot length		Lateral root number	
	equation	R^2	equation	R^2
T_1	$y = 0.0397x^2 + 1.2426x - 0.5006$	0.9728	$y = 0.3595x^2 + 1.8881x + 1.8714$	0.9974
T_2	$y = 0.0734x^2 + 0.9116x - 0.1866$	0.9708	$y = 0.4393x^2 + 1.5464x - 0.4857$	0.9586
T_0	$y = 0.0833x^2 + 0.8487x - 0.3397$	0.9681	$y = 0.5643x^2 + 0.7214x + 0.3143$	0.9729

T_1 – conidia-suspension-drenching treatment; T_2 – conidia-coating treatment; T_0 – untreated control

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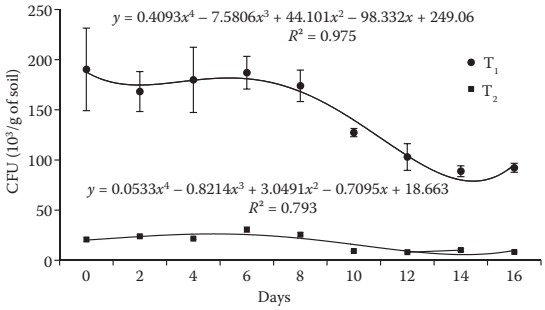

Figure 3. Persistent density of *M. anisopliae* in the peanut rhizosphere post sowing

In the first 8 days, the T_1 density fluctuated from +5.8 to –6.5%, with an average of 179.9 × 10^3 CFU, being slightly lower than initially; the T_2 density ranged from +24.9 to –15.5%, with an average of 24.5 × 10^3 CFU, being slightly higher than initially. A greater decrease appeared in successive 8–12 days. The final densities on day 16 were 92.1 × 10^3 CFU and 8.3 × 10^3 CFU for T_1 and T_2, respectively

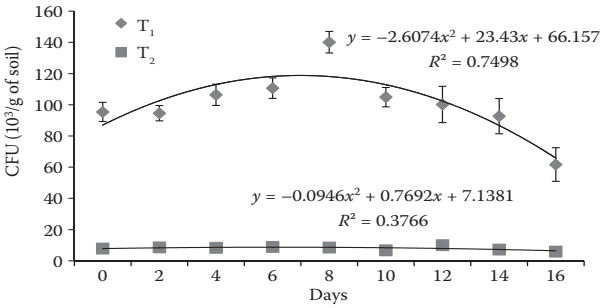

Figure 4. Persistent density of *M. anisopliae* in the peanut root bulk soil post sowing

The persistent densities of T_1 inoculation showed a steady-rise-decline routing, from the initial 95.4 × 10^3 CFU to 140.1 × 10^3 CFU peak on day 8, then declining to final 61.7 × 10^3 CFU on day 16. The T_2 density maintained a low level (5.9~10. 0 × 10^3 CFU)

In the bulk soil of the two treatments, the fungal densities at each sampling were always significantly lower than those at the rhizosphere ($P < 0.01$). The dynamics in T_1 showed a significant increase peak eight days after peanut sowing and fungal inoculation ($P < 0.01$), and then a gradual decline to 44.0% of the peak value. The fungal density in T_2 kept a low level throughout the experimental period (Figure 3).

Relationship between the *M. anisopliae* density and the peanut root growth

The above analysis showed that the taproots length and the lateral root number of the conidia-suspension-drenching treatment (T_1) were higher than those of the untreated control in days 4–10. The correlation analysis showed no significant correlation between the taproot length or the lateral root number and the quantities of *M. anisopliae* persistence based on direct measurements. Nevertheless, the ratios of the taproot length or lateral root number in T_1 and those in the untreated control (T_0) were significantly related to the CFU of *M. anisopliae* 4–16 days after sowing ($P = 0.0216$, $R = 0.8273$ for taproot length; $P = 0.0032$, $R = 0.9213$ for lateral root) (Figure 4). This result suggests that a certain density of *M. anisopliae* due to a high inoculating density could stimulate the development of peanut roots.

DISCUSSION

In fact, certain insect pathogenic fungi play multiple roles in ecological systems of the field. In some

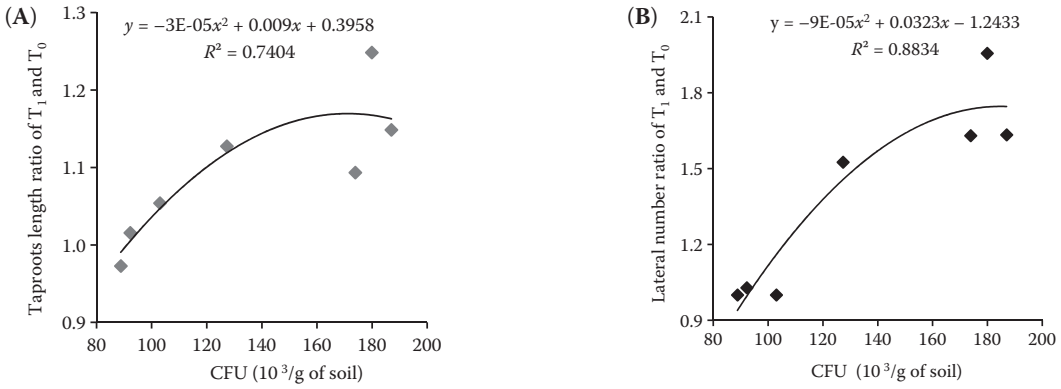

Figure 5. Relationship between the *M. anisopliae* density and the root increment in treatment T_1: (**A**) relationship between the *M. anisopliae* density and the taproots length ratio of T_1 and T_0 and (**B**) relationship between the *M. anisopliae* density and the lateral root number ratio of T_1 and T_0

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plants growth promotion by *Metarhizium* has been documented. For example, tomato plants treated with *M. anisopliae* showed significantly greater plant height, root length, and shoot and root dry weight than the untreated control, although the obtaining response depended on the isolate and the inoculation rate (García *et al.* 2011). Growth promotion by *M. anisopliae* was also detected in soybean, and the inoculated plants performed better than did the untreated control under salt stress (Khan *et al.* 2011). Sasan and Bidochka (2012) reported that *Metarhizium robertsii* could significantly promote the root length and number of lateral roots in switch grass (*Panicum virgatum*) and haricot beans (*Phaseolus vulgaris*). This fungus could also significantly improve the yield of corn and peanut (Kabaluk & Ericsson 2007; Li *et al.* 2011). Many other fungi also play diverse roles in the field. *Beauveria bassiana* strain 11-98 could not only infect insects, but also cause the endophytic colonisation of tomato and cotton seedlings and protect against plant pathogenic *Rhizoctonia solani* and *Pythium myriotylum* (Ownley *et al.* 2008, 2010). *Trichoderma harzianum* strain T22, known to control plant-pathogenic fungus, could make maize roots deeper and more robust with a greater surface area compared with the untreated control (Harman *et al.* 2004). *Aureobasidium pullulans* and *Paraconiothyrium sporulosum* were not only antagonistic to the tomato pathogen *R. solani*, but also exhibited tomato growth promotion (Miles *et al.* 2012). *Piriformospora indica* was found in endophytically colonized roots and could promote the root growth of a number of plant species, including maize, tobacco, and parsley (Varma *et al.* 1999). Therefore, when these species are used as biological insecticides, interactions with plants and soil factors should be considered.

The mechanism of plant growth promotion by insect pathogen remains unknown. Several hypotheses and previous studies have provided incomprehensive explanations. The interaction between fungi and plants is beneficial to plants, through improving the ability of the plant to tolerate unfavourable conditions (Hesse *et al.* 2003; Rodríguez *et al.* 2008), protecting the plant from diseases that can damage it (Flori *et al.* 1993; Ownley *et al.* 2008), warding off insect pests (Cherry *et al.* 1999, 2004; Powell *et al.* 2007), supplying nutrients to the plant through the transfer of nitrogen and the uptake of phosphorus and other minerals (Usuki & Narisawa 2007; Behie *et al.* 2012).

In this study, the conidia-suspension-drenching treatment (T_1) could promote the development of peanut taproots and lateral roots 4–10 days after inoculation, but this promotion did not appear in the conidia-coating treatment (T_2). Comparing the two ways of inoculation with the development process of peanut root, the fungal conidia drenched in T_1 could maintain a certain level of density in the surrounding soil of the seed and root, whereas the conidia adhering on the seed epidermis in T_2 would be partially rinsed off and maintained in soil, but most would be carried to the soil surface with the germ growth. This result means that there were fewer conidia around the radicles and developing roots in T_2. According to the detected CFU data, the rhizosphere density of *M. anisopliae* in T_2 was 9.2-fold lower than that in T_1 on inoculating day and gradually decreased during the 16 days of testing. Therefore, a low *Metarhizium* density could not play a role in promoting root growth.

The interaction between *M. anisopliae* and peanut root may be reflected in the fungal persistent density at rhizosphere and the development of peanut root. In this study, both at the T_1 or the T_2 inoculation, *Metarhizium* densities in rhizosphere were always significantly higher than those in bulk soil, even if the density trended on a downward line in the 8^{th} to 16^{th} days after detection. It suggested that the rhizosphere may provide a microenvironment beneficial to *Metarhizium* vitality. The microenvironment may contain more carbon released from root due to plant growth and metabolism (Hu & St. Leger 2002; Bruck 2005). Meanwhile, this study suggests that *M. a isopliae* inoculated by conidia-suspension-drenching or conidia-coating had no effect on the germination of peanut seeds, similarly to the results by Kabaluk and Ericsson (2007) in corn and by Diniz *et al.* (2009) in sweet pepper. The process of seed germination contains a series of orderly physiological and biochemical reactions and morphological changes that are regulated by genetics. The seed epidermis is a protective barrier against external interference. The fungus *M. anisopliae* in soil or adhered onto seeds was unable to participate in these reactions in the interior of seed. Additionally, a majority of conidia were dormant before being applied to the soil and required a short time to revive. *M. anisopliae* did not significantly affect the germination of peanut seeds.

In conclusion, this study revealed that *M. anisopliae* is a fungus with plant-root-promoting properties related to the density level in the vicinity of the root. The conidia-suspension-drenching inoculation was

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more conducive to plant growth than the conidia-coating inoculation. Therefore, *M. anisopliae* should be regarded as a fungus that can be used in multiple roles, ranging from warding off insect pests to promoting plant growth.

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