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Metarhizium anisopliae (Metschnikoff) Sorokin Promotes Growth and Has Endophytic Activity in Tomato Plants

García Julia Elena, Posadas Julieta Beatriz, Perticari Alejandro and Lecuona Roberto E.

Institute of Agricultural Microbiology and Zoology, National Institute of Agricultural Technology, IMYZA- INTA Castelar, Las cabañas y Los Reseros s/n. C.C. 25 (1712), Castelar, Buenos Aires, Argentina

Abstract: The present study aimed to evaluate the effect of the inoculation with *Metarhizium anisopliae* on the growth of tomato plants and to determine the endophytic activity of this fungus. The three isolates of *M. anisopliae* evaluated (Ma 8, Ma 10 and Ma 20) significantly increased plant height, root length, shoot and root dry weight when compared to the untreated control, although the response obtained depended on the isolate and the inoculation rate. In addition, the three isolates showed endophytic activity and were isolated from roots and shoots. Ma 8 was also found in leaves. This study reveals significant new data on the interaction between *M. anisopliae* and plants although there is a need for further research to understand the mechanisms through which *M. anisopliae* promotes plant growth.

Key words: Fungal endophyte · *Lycopersicom esculentum* · *Metarhizium anisopliae* · Plant growth promotion

INTRODUCTION

The indiscriminate use of pesticides and chemical fertilizers in agriculture has raised a number of ecological problems such as resistance development in plant pathogens and pests, environmental pollution and negative impacts on human health [1]. In recent years, there has been an increasing interest in the use of native and non-native beneficial microorganisms to improve plant health and productivity [2]. In this context, beneficial microorganisms are now integrated into a wide variety of growing systems as part of integrated pest and productivity management practices [3], which allow a significant reduction in the use of synthetic chemicals.

Entomopathogens are among the natural enemies of insect pests in agroecosystems. The entomopathogenic fungi *Beauveria bassiana* (Bals.-Criv.) and *Metarhizium anisopliae* (Metschn.) Sorokin are natural enemies of a wide range of insects and arachnids [4] and have been extensively studied for biological control [4-6]. Nevertheless, there is evidence in the literature indicating that these two fungi have the potential to engage in fungus-plant interactions. The pioneering studies were conducted by Bing and Lewis [7, 8], who reported that *B. bassiana* grows endophytically within the tissues of *Zea mays* after a foliar application and it provides suppression of *Ostrinia nubilalis*. Further studies [9] demonstrated that *B. bassiana* does not lose virulence towards the mentioned insect once it colonizes corn. Based on the above results, several authors have aimed at introducing *B. bassiana* as an endophyte and at examining the insect performance on colonized plants [10-12]. Introducing *B. bassiana* as an endophyte has led researchers to study other roles of this entomopathogenic fungus in plants such as antifungal activities [13, 14].

As regards *M. anisopliae*, it has been reported that it is capable of colonizing the plant rhizosphere. In field studies, the population of *M. anisopliae* persists better in the rhizosphere of cabbage plants than in the bulk soil [15]. Moreover, Bruck [16] reported that *M. anisopliae* colonizes the rhizosphere of *Picea abies* and demonstrated that plant roots inoculated with this entomopathogenic fungus are able to control *Otiorhynchus sulcatus* F. (black vine weevil). More recently, Meyling and Eilenberg [17] suggested that *B. bassiana* is associated only with insect hosts above ground, while *M. anisopliae* is associated exclusively with hosts on or below the soil surface in temperate agroecosystems. In a field study, corn seeds treated with *M. anisopliae* conidia resulted in significant increases

Corresponding Author: Agr. Eng. Julia Elena García, IMYZA. INTA Castelar, Las cabañas y Los Reseros s/n. C.C. 25 (1712), Castelar, Buenos Aires, Argentina., E-mail: jgarcia@cnia.inta.gov.ar.

in plant stand density and corn fresh weight [18]. These authors attributed these results to wireworm (*Agriotes obscurus* L.) control since there was no effect of *M. anisopliae* on the germination rate and root growth in laboratory experiments. It is also possible that *M. anisopliae* has other roles in plant protection [19]. In this way, Chul *et al*. [20] reported that *M. anisopliae* has antifungal activities *in vitro* against *Fusarium oxysporum, Botrytis cinerea* and *Alternaria solani*.

The development of *M. anisopliae* to be applied in agriculture as a biological control agent against a wide range of insects has been widely researched; however, no data are currently available about the effect of this fungal entomopathogen on plant growth. The aim of the present study was thus to evaluate the effect of the inoculation with *M. anisopliae* on the growth of tomato plants and to determine the endophytic activity of this fungus.

MATERIALS AND METHODS

Effect of *M. anisopliae* on Plant Growth: *Fungal isolates and cultures:* Three *M. anisopliae* (Ma) isolates were used in the experiment: Ma 8, Ma 10 and Ma 20. These isolates are deposited at the collection of the Laboratory of Entomopathogenic fungi of the Institute of Agricultural Microbiology and Zoology of the National Institute of Agricultural Technology (INTA), Argentina. Ma 8 and Ma 10 had been previously isolated from soil samples from Santa Fe province, Argentina and Ma 20, from the ant *Acromyrmex lundi* (Hymenoptera: Formicidae) in Castelar, Buenos Aires, Argentina. *M. anisopliae* isolates were cultured on rice in polypropylene bags. An aliquot of 90 g of rice was placed in each bag with the addition of 45 mL of distilled water. The bags were sealed, autoclaved for 20 min at 121°C and then inoculated. The inocula were produced on potato-dextrose agar (PDA) Oxoid with chloramphenicol (0.5 g L^{-1}) and 2 cm^2 of culture medium was used for inoculating each bag containing rice. After inoculation, the bags were homogenized and incubated at 27 ±1°C and a 16:8 photoperiod. After fungal growth, conidia were collected by sieving and suspended in sterile aqueous 0.05% Tween 80 solution. The conidial concentration was quantified in an improved Neubauer chamber and adjusted to 1.7 x 10^8 conidia mL^{-1}.

Tomato Plants and Growth Conditions: Tomato seeds (*Lycopersicom esculentum* Mill. Hybrid var. 8625 from Abbot and Cobb inc.), treated with Thiram, were sown in 100 cm^3 nursery cones filled with sterile vermiculite. The cones were placed in a growth chamber belonging to Laboratory of Plant Growth Promoting Bacteria of the Institute of Agricultural Microbiology and Zoology of the National Institute of Agricultural Technology (INTA), Argentina, at 24°C. Plants were watered on demand with sterile rain water and each plant received 3 mL of Hoagland's nutrient solution 21 days after sowing.

Inoculation: The conidial suspension was applied directly to the substrate, close to the base of the shoot of 14-day-old seedlings. Tomato seedlings were treated with three inoculation rates of each *M. anisopliae* isolate. The rates evaluated were $8x10^7$, 5 $x10^8$ and $1x10^9$ conidia per plant and an untreated control was also included.

Experimental Design: The experiment was run as a Randomized Complete Block with nine replications per block and one seedling per replication. Seedlings were grown for 28 days in a gnotobiotic system, then removed from the containers and the following parameters measured: plant height, root length, shoot and root dry weight. Data were analyzed by ANOVA followed by Duncan´s test ($p \leq 0.05$) using the Infostat statistical package.

Recovery of *M. anisopliae*: The recovery of *M. anisopliae* from the rhizosphere, the substrate and as an endophyte in roots, shoots and leaves was evaluated by culture methods in plants inoculated with $1x10^9$ conidia of *M. anisopliae* $plant^{-1}$ 21 days post-inoculation and in untreated plants. The selective medium used was PDA with 0.6 g L^{-1} of CTAB (Hexadecyltrimethylammonium bromide) [21]. The results were expressed as a percentage of positive plates, considering a positive sample when at least one colony of *M. anisopliae* grew on it. A test based on Fisher´s exact test (Infostat statistical package) was performed for the comparison of two proportions to determine if the difference between the proportions each of the inoculated treatments and the proportion of the untreated control was significantly different from zero. The test was done for each recovery trial (rhizosphere, substrate and endophytic growth in roots, shoots and leaves).

Colonization of the Rhizosphere and the Substrate: Two harvested roots were shaken to obtain the substrate adhering to them. An aliquot of 1 g of this

substrate was placed into a test tube containing 9 mL of sterile 0.05% Tween 80 solution and 0.1 mL of this solution and from serial dilutions was plated onto Petri dishes containing the selective medium. Ten grams of the substrate from the bottom of the container was placed into a 250-mL Erlenmeyer flask containing 90 mL of 0.05 Tween 80 solution, shaken in an orbital shaker at 150 rpm for 20 min and serial dilutions were plated onto the selective media mentioned above. Eight plates were sown for each recovery trial (rhizosphere and substrate) and for each treatment (untreated control and inoculated treatments with the different isolates).

Endophytic Activity: Two tomato plants per treatment were washed with running tap water and surface-disinfected by submersing them in 70% ethanol for 15 s, followed by 20 min in 5% sodium hypochlorite and rinsing 10 times in sterile distilled water. To determine the effectiveness of the disinfecting process to remove the epiphyte microorganisms, the final rinse water was plated onto the same media [22, 23]. Plants were dried on sterile paper towels and tissues from leaves, shoots and roots were cut in about 4 mm^2 sections with a sterile scalpel and about 10 of them were placed on Petri dishes containing the selective medium. Eight plates were sown for each tissue (roots, shoots and leaves) and for each treatment (untreated control and inoculated treatments). To confirm that colonies were *M. anisopliae,* some colonies considered as positive results were randomly selected from a number of different plates and transferred to PDA [16], PDA with 0.5 g L^{-1} cloranphenicol and malt extract agar. All the colonies transferred were allowed to sporulate and then identified as *M. anisopliae* based on their morphological characteristics [24].

RESULTS

Effect of *M. anisopliae* on Plant Growth: ANOVA demonstrated significant effects for plant height ($p<0.0001$), root length ($p=0.0002$), shoot ($p<0.0001$) and root ($p<0.0001$) dry weight. Although the three isolates significantly increased the aerial parameters, the response obtained depended on the isolate and the inoculation rate. In plant height, the greatest increase was obtained with the treatments with Ma 8 and Ma 10, both with the highest inoculation rate ($1x10^9$ conidia $plant^{-1}$). The average increase obtained with these treatments was 48%. Shoot dry weight increased by 332% with the best isolate-rate combination, which was Ma 8 - $1x10^9$ conidia $plant^{-1}$. Inoculation with the lowest rate ($8x10^7$ conidia per plant) combined with any of the isolates did not significantly increase this parameter. The response in root parameters also varied according to the isolate and the inoculation rate tested. Isolate Ma 20 did not produce significant increases in root parameters. Plants inoculated with Ma 8- $1x10^9$ and $5x10^8$ conidia $plant^{-1}$ and Ma 10 - $5x10^8$ conidia $plant^{-1}$ presented significant longer roots as compared to untreated control plants (Table 1). A significant increase in root dry weight was obtained only through inoculation with the greatest and intermediate inoculation rates ($1x10^9$ and $5x10^8$ conidia $plant^{-1}$) of Ma 8 and Ma 10 isolates. The root dry weight of plants inoculated with these treatments was, in average, 205% higher than untreated control plants.

Recovery of *M. anisopliae*: The three isolates were recovered from the rhizosphere as well as from the substrate from the treated plants (Table 2, 3), whereas no positive plate was obtained from the untreated control.

Table 1: Growth parameters of 28-day-old tomato seedlings according to the isolate of *M. anisopliae* (Ma) used. Different letters within each column indicate significant differences (Duncan´s test; $p \leq 0.05$)

Treatment	Height (cm)		Shoot dry weight (g)		Root Length (cm)		Root dry weight (g)	
Untreated control	4.44	e	0.0087	e	12.52	d	0.0034	e
Ma 8-8 x 10^7	5.17	d	0.0122	de	13.65	cd	0.0054	de
Ma 10-8 x 10^7	5.19	d	0.0118	de	14.17	bcd	0.0049	de
Ma 20-8 x 10^7	5.06	d	0.0113	de	13.39	d	0.0048	de
Ma 8-5 x 10^8	5.68	c	0.0233	c	15.59	abc	0.0072	cd
Ma 10-5 x 10^8	6.19	b	0.0324	b	15.98	ab	0.0146	a
Ma 20-5 x 10^8	5.46	cd	0.0153	d	13.85	cd	0.0041	de
Ma 8-1 x 10^9	6.62	a	0.0376	a	16.30	a	0.0108	ab
Ma 10-1 x 10^9	6.55	a	0.0300	b	13.88	cd	0.0090	bc
Ma 20-1 x 10^9	5.89	bc	0.0214	c	13.64	cd	0.0048	de

Table 2: Percentage of positive plates with *M.anisopliae* isolates Ma 8, Ma 10 and Ma 20 recovered from 21-day inoculated tomato plants

Type of recovery trial	Ma8	Ma 10	Ma 20
Rhizosphere	100	100	100
Substrate	100	100	100
Roots	83	0	50
Shoots	100	100	100
Leaves	33	0	0

Percentage of positive plates on the total number of samples (n=8)
Considered positive when at least one colony of *M. anisopliae* grew in any of the samples

Table 3: Probability that the observed differences between proportions of the untreated control and each inoculated treatment was greater than zero (P values)

Tissue	Ma 8	Ma 10	Ma 20
Roots	0.007*	0.09	0.03*
Shoots	0.01*	0.001*	0.003*
Leaves	0.09	0.5	0.5

Values denoted with (*) mean that the two proportions differ significantly ($\alpha \leq 0.05$)

The effectiveness of the disinfecting process in the recovery as endophytic was confirmed because the rinse water did not yield any fungi.

The three isolates showed endophytic activity (Table 2). The comparisons between the two proportions (untreated control and inoculated treatement) for each type of tissue revealed that the three isolates were recovered from shoot and Ma 8 and Ma 20 also from roots. Although Ma 8 was found in the 33% of the plates of the leaf trial, the p value was not significant. No positive plates were found in untreated control in any of the recovery trials (Table 3).

DISCUSSION

There are not many published reports about the effects of *M. anisopliae* used as a plant-growth promoting microorganism. Kabaluk and Ericsson [18] reported that the germination rate of corn was not increased when seeds were treated with *M. anisopliae* in a laboratory experiment. More recently, Diniz *et al*. [25] studied the effect of *M. anisopliae* on the germination percentage of seeds, emergence rate, shoot and root dry matter and height of 30-day-old sweet pepper seedlings and observed that *M. anisopliae* did not cause a significant increase in the variables studied, when compared to the control. However, this study reveals that inoculation with *M. anisopliae* produces growth-promoting effects on 28-day-old tomato plants.

It is important to point out that the isolates which produced the greatest effect on plant growth (Ma 8 and Ma 10) were both isolated from the soils, whereas Ma 20, previously isolated from ants, did not produce effects on root parameters and the effects on the aerial part of the plant were significantly lower. Thus, the results indicate that the three *M. anisopliae* isolates used in this study have different capabilities for promoting the growth of tomato plants. As regards the inoculation rate, the lowest one ($8x10^7$ conidia $plant^{-1}$) was not enough to produce increases in shoot dry weight or root parameters.

The plant *M. anisopliae* interaction documented is that of the ability of *M. anisopliae* to colonize the plant rhizosphere [15, 16, 19]. According to this, in our experiment, *M. anisopliae* was recovered from the rhizospheric substrate as well as from the bottom of the pot, which suggests some degree of vertical movement either through colonization or via percolating water. This is in agreement with the results by Hu and St. Leger [15], who reported that *M. anisopliae* was isolated at a soil depth of 10 cm from plants in which the inocula were applied on the soil surface.

Although several fungal entomopathogens have been recovered as endophytes in various plants after inoculation with different techniques [9, 14, 22, 26, 27], to our knowledge, this is the first successful isolation of *M. anisopliae* as an endophyte. Several factors may have contributed to success in this finding. Our hypothesis is that the growth stage and the plant species used were appropriate since root colonization by *M. anisopliae* is plant-stage dependent (Stefan Jaronski, Personal communication). Furthermore, the inhibitor CTAB added to the selective media may have overcome the slow grow of *M. anisopliae* and may have thus allowed it to compete with other microorganisms [21].

Another important finding is that *M. anisopliae* was isolated from tomato plants at locations distant from the point of inoculation. Thus, *M. anisopliae* was capable of moving within the plant. Studies have shown that when *B. bassiana* is injected into the corn stem, it colonizes it and moves within the plant [7, 8]. These authors have argued that the movement within corn plants is due to the passive movement of *B. bassiana* within the xylem, since they observed fungal hyphae within the vascular elements.

Some endophytes have been reported to have beneficial effects on host plants, such as increased resistance against plant pathogens [13, 14], insect pests [10, 11, 12] and nematodes [28, 29]. Based on above reports, the ability to colonize internal tissues may

provide *M. anisopliae* with the potential for exerting other roles in the plant. Further studies should focus on selecting an entomopathogenic fungal isolate as a potential bio-tool in plant management and should aim at evaluating insect performance on colonized plants.

In conclusion, this study revealed significant new data of the interaction between *M. anisopliae* and plants but there is a need for further research to understand the mechanisms through which *M. anisopliae* promotes plant growth and the impact of *M. anisopliae* on plant physiology and other biological factors such as stress tolerance, uptake of nutrients and water.

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