IMPORTANCE OF APPLICATION TIME AND INOCULUM DENSITY OF *FUSARIUM OXYSPORUM* 162 FOR BIOLOGICAL CONTROL OF *MELOIDOGYNE INCOGNITA* ON TOMATO

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ABSTRACT

Dababat, A. A. and R. A. Sikora. 2007. Importance of application time and inoculum density of *Fusarium oxysporum* 162 for biological control of *Meloidogyne incognita* on tomato. Nematropica 37:267-275.

The mutualistic endophyte *Fusarium oxysporum* 162 (FO162) was investigated for its activity against the root-knot nematode *Meloidogyne incognita*. This fungal strain has been shown to reduce *M. incognita* damage by inhibiting juvenile penetration and development when present in the root system of tomato (*Lycopersicon esculentum* Mill.) plants. The results showed that inoculation of tomato plants with FO162 at sowing in seedling trays led to significant reduction in both numbers of galls and egg masses compared to the non-treated controls. Dual inoculation of the fungus at sowing and transplanting resulted in slightly higher levels of biocontrol but was not significantly different when compared with single inoculation at sowing. Strain FO162 applied at 10^4 or 10^5 cfu/g soil at sowing led to a significant reduction in nematode penetration after transplanting. Treatment only at transplanting was not as consistent, causing a significant reduction in nematode penetration in only one of the two trials. Inoculating the seedlings with 10^5 cfu/g seedling substrate at sowing and with an additional 10^4 cfu/g soil at transplanting time gave the best nematode control in both trials in the second experiment.

Key words: biocontrol, endophyte, *Lycopersicon esculentum*, *Meloidogyne incognita*, root-knot nematode.

RESUMEN

Dababat, A. A. y R. A. Sikora. 2007. Importancia del Momento de Aplicación y la Densidad de Inóculo de *Fusarium oxysporum* 162 para el Control Biológico de *Meloidogyne incognita* en Tomate. Nematropica 37:267-275.

Investigamos la actividad del hongo mutualista endofítico *Fusarium oxysporum* 162 (FO162) contra el nematodo agallador *Meloidogyne incognita*. Se ha demostrado que esta cepa del hongo reduce el daño causado por *M. incognita* al inhibir la penetración y el desarrollo de juveniles cuando se encuentra presente en las raíces de tomate (*Lycopersicon esculentum* Mill.). Los resultados de este estudio indican que la inoculación de plantas de tomate con FO162 al momento de la siembra en bandejas causa reducción significativa en la cantidad de agallas y de masas de huevos. Se observó control levemente superior al hacer doble inoculación con el hongo, una en el momento de la siembra y otra al momento del transplante, pero esta diferencia no fue significativa al comparar con una sola inoculación al momento del transplante. La cepa FO162 aplicada en el momento de la siembra a razón de 10^4 ó 10^5 ufc/g suelo causó una reducción significativa en la penetración del nematodo después del transplante. El tratamiento sólo al transplante no fue tan consistente, y causó reducción significativa en la penetración del nematodo en sólo uno de dos ensayos. El mejor control de nematodos se observó cuando se inoularon plántulas con 10^5 ufc/g sustrato en el momento de la siembra y 10^4 ufc/g suelo al momento del transplante, en ambos ensayos del segundo experimento.

Palabras clave: control biológico, endófito, *Lycopersicon esculentum*, *Meloidogyne incognita*, nematodo agallador.

INTRODUCTION

Many different microorganisms have been used to directly treat field soil for the biological management of a range of plant parasitic nematodes (Sikora, 1992; Kerry, 2000; Hallmann *et al.*, 2001; Meyer and Roberts, 2002). The mechanisms of antagonism vary greatly and include predation, pathogenesis, competition, repellence and induced resistance (Jaffee and Zehr, 1985; Stirling, 1991; Hallmann *et al.*, 2001; Timper *et al.*, 2005). Interest has recently shifted to bacterial and fungal antagonists that reside endophytically in the endorhiza of the host plant (Pocasangre *et al.*, 2000; Sikora *et al.*, 2003). Endophytic microorganisms can easily be applied to seeds or transplants, thereby reducing the amount of inoculum and overall costs of nematode management compared with soil application (Harman, 1991; Sikora *et al.*, 2000; Elzein *et al.*, 2006).

Many fungi have been shown to grow endophytically in the endorhiza (Hussey and Roncadori, 1982; Sikora, 1992; Waceke *et al.*, 2001; Sikora *et al.*, 2003). One of the predominant non-pathogenic fungi found in roots of many plant species is *Fusarium oxysporum* (Alabouvette *et al.*, 1998). With the exception of forma specialis of *F. oxysporum*, which are important pathogens on many crops (Alabouvette *et al.*, 1998; Larkin and Fravel, 1999), the vast majority of *F. oxysporum* detected in roots are non-pathogenic saprophytes, facultatively colonizing the root (Sikora, 1992). Non-pathogenic strains of *F. oxysporum* have been used effectively for the control of Fusarium wilt of tomato in the field (Kroon *et al.*, 1991; Lemanceau and Alabouvette, 1991; Alabouvette *et al.*, 1993).

Non-pathogenic strains of *F. oxysporum* also have been shown to reduce root-knot nematode (*Meloidogyne incognita*) infection of tomato (*Lycopersicon esculentum* Mill.) (Hallmann and Sikora, 1994a; 1994b; Sikora *et al.*, 2003; Dababat and Sikora, 2007a, b). For example, inoculation of tomato seedlings with the *F. oxysporum* strain 162 reduced gall formation of tomato caused by *M. incognita* up to 75%, and enhanced root growth significantly (Sikora *et al.*, 2003). Non-pathogenic strains of *F. oxysporum* have also been shown to reduce population densities of lesion nematodes on maize (Kimenju *et al.*, 1998), and burrowing nematodes on banana (Niere *et al.*, 1998; Pocasangre, 2000; zum Felde *et al.*, 2005; Vu *et al.*, 2006).

Banana tissue culture plantlets transplanted into substrate pre-inoculated with a mutualistic *F. oxysporum* and challenged with *Radopholus similis* for 5 weeks had 50% lower nematode densities in their roots compared with the untreated control plants (Sikora *et al.*, 2000; Vu, 2005). Sikora and Pocasangre (2004) obtained an 86% reduction of *R. similis* densities in plantain suckers following inoculation with non-pathogenic *F. oxysporum*. Several authors have reported *R. similis* control using the endophytic fungi under greenhouse and field conditions (Schuster *et al.*, 1995; Niere *et al.*, 1998; Niere, 2001; zum Felde *et al.*, 2005).

Variation in the level of control is influenced by both abiotic and biotic factors affecting the colonization and growth of the antagonist in the endorhiza. Lack of standardized inoculum production and techniques, and improper application technology could also result in inconsistent performance. Control efficiency toward the wilt pathogen *F. oxysporum* f. sp. *lycopersici* on tomato, following pre-inoculation with non-pathogenic, *F. oxysporum* strains depends on the ratio of the population densities of these fungi in the soil. High levels of the non-pathogenic fusarium are required for biocontrol efficiency (Alabouvette *et al.*, 1993; Minuto *et al.*, 1995; Duijff *et al.*, 1999). There-

fore, determination of optimum inoculum densities can lead to more effective endophytic colonization of the roots and thereby to a higher level of biological control.

Studies on the optimum inoculum density of non-pathogenic *F. oxysporum* required for the management of nematodes or pathogens on vegetables are still lacking. Most researchers used only one inoculum density (Kroon *et al.*, 1991; Mandeel and Baker, 1991; Alabouvette *et al.*, 1993; Hallmann and Sikora, 1994b; Pocasangre *et al.*, 2000). In addition, the methods of application vary greatly, and include surface drenching of the seedlings, soil incorporation, and root ball dipping (Hallmann and Sikora, 1994a; Olivain and Alabouvette, 1999). The objectives of this study were to determine the most efficient forms of application for the mutualistic endophyte *Fusarium oxysporum* 162 (FO162) for effective nematode control.

MATERIALS AND METHODS

Root-knot nematodes (*Meloidogyne incognita*) were cultured on tomato plants cv. 'Furore'. Eggs were extracted from galled tomato roots as described by Hussey and Barker (1973). Nematode eggs were collected on a 25-µm mesh sieve and transferred to tap water and agitated for 7 to 10 days to induce juvenile hatching. Freshly hatched second stage juveniles (J2) were separated from eggs using a modified Baerman technique (Barker, 1985), and were used as nematode inoculum.

Tomato cv. 'Hellfrucht Frühstamm', which is susceptible to *M. incognita* and to pathogenic isolates of the fungus *F. oxysporum*, was used. Plants were fertilized with a slow release formulation of 2 g per liter of water (N: P: K = 14:10:14) and watered as needed.

The endophytic *F. oxysporum* 162 strain was initially isolated from field grown tomato cv. 'Moneymaker' by Hallmann and Sikora (1994a). *Fusarium oxysporum* 162 was cultured for 2 weeks in an incubator at 24°C on potato dextrose agar containing 150 mg/L streptomycin and 150 mg/L chloramphenicol. The mycelium and conidia were scraped from the media and suspended in 100 ml tap water. Spores were separated from the mycelium by sieving the content through a 50 µm sieve and/or three layers of cheese cloth. The concentration of spores was calculated using a Thomas hemacytometer (Marienfeld, Germany) and adjusted to the desired concentration by dilution.

Experiment I. Application Time

The experiment consisted of the following five treatments: 1) untreated control, 2) *M. incognita* at transplanting, 3) FO162 at sowing, 4) FO162 at sowing + *M. incognita* at transplanting, and 5) FO162 at sowing and at transplanting + *M. incognita* at transplanting. Each treatment consisted of 10 replicates arranged in a randomized complete block design. The experiment was conducted twice.

Tomato seeds were sown at one seed/plug into 2 commercial 70-plug seedling trays, with each plug measuring 4 × 4.5 × 2.5 cm. One of the two trays was treated with FO162 and the other was left untreated. Each plug of the treated tray was inoculated one week after sowing with 1 ml of a tap water suspension of FO162 containing 1.5×10^7 spores/ ml. The fungal inoculum was gently pipetted into 3 holes made with a plastic rod around the germinated seed. Plugs of the untreated tray were treated with tap water. The 5th treatment received a second dose of FO162 at transplanting, when one ml of tap water containing 1.5×10^7 cfu was pipetted into three holes around the seedling stem base.

Seedlings were kept for 5 weeks in the greenhouse and then transplanted into pots (11 × 14 × 9.5 cm) containing 800 g sterilized sand: soil mixture (2:1, v/v). Immediately after transplanting, 3 ml of tap water with or without 1000 second-stage juveniles of *M. incognita* were pipetted into 3 holes around the plant. Plants were grown in a greenhouse at 22°C ± 5 with 16 hr of supplemental artificial light per day.

The experiment was terminated eight weeks after nematode inoculation (14 weeks after seed sowing). The roots were removed and washed free of soil. Root and shoot fresh weight, as well as plant height were measured. Roots were then stained with 0.015% Phloxine B for 20 min, washed with tap water to remove extra stain, and the numbers of galls and egg masses were recorded (Shurtleff and Averre, 2000).

The level of FO162 endophytic root colonization was determined in 12 root segments. Each root segment was 0.5 cm long, cut from roots with similar diameters and placed onto PDA plates after surface sterilization in a 1.5% solution of NaOCl for three min. This was followed by three rinses in sterilized water. Successful re-isolation was confirmed when the growth characteristics of the outgrowing fungi corresponded to those of FO162 as described by Hallmann and Sikora (1994a).

Experiment II. Inoculum Density

The experiment consisted of 10 treatments listed in Table 1, and was set up similar to experiment I, except that 2 FO162 inoculation densities used were 10^4 or 10^5 cfu/g seedling substrate. The fungal solution was inoculated as described in experiment I.

The seedlings were kept for 5 weeks in the greenhouse and then transplanted into pots (7 × 7 × 8 cm) containing 400 g sterilized sand: field soil mixture (2:1, v/v). One day after transplanting, the same procedures for nematode inoculation were followed as described in experiment I. At this time, specific treatments received an additional dose of FO162 by applying 10^4 or 10^5 cfu/g soil as described in experi-

Table 1. List of treatments used in Experiment II.

Treatment[z]	Inoculation at sowing (cfu/g seedling substrate)	Inoculation at transplanting (cfu/g soil)	*M. incognita* (1000 J2)
1	10^4	—	+
2	10^4	10^4	+
3	10^4	10^5	+
4	10^5	—	+
5	10^5	10^4	+
6	10^5	10^5	+
7	—	10^4	+
8	—	10^5	+
9	—	—	+
10	—	—	—

[z]Treatment numbers will be used further in the results figures.

ment I. Plants were grown in a greenhouse at 18°C ± 2 and 28°C ± 5 for trial 1 and trial 2, respectively.

Two weeks after nematode inoculation, the roots were separated from shoots, washed with tap water to free soil, blotted on tissue paper, and weighed. To determine nematode penetration, the root system was then stained in a 0.1% acid fuchsin solution, washed in tap water and macerated twice at high speed in water in a blender for 10 s. The nematodes in two 10 ml aliquots were counted and the total number of nematodes per root system was calculated.

Data were analyzed according to standard analysis of variance procedures with the SPSS program. The Tukey Test was used for mean comparison.

RESULTS

Experiment I. Application Time

Fungal inoculation only at sowing, as well as dual inoculation at sowing and at transplanting, led to reductions in the number of galls and egg masses per plant as compared to the control ($P \leq 0.05$, Fig. 1). No difference was observed between single inoculation at sowing as compared to dual inoculation at sowing and at transplanting.

Similarly, in the second trial, gall numbers were significantly reduced by the dual inoculation treatment at sowing and transplanting as well as by single treatment at sowing when compared to the control (Fig. 2).

Experiment II. Inoculum Density

Treatment with either dose of FO162 at sowing led to a significant reduction of up to 75% in nematode penetration in trial I (Fig. 3) and up to 48% in trial II (Fig. 4). Fungal inoculation only at transplanting caused a significant reduction of up to 36%

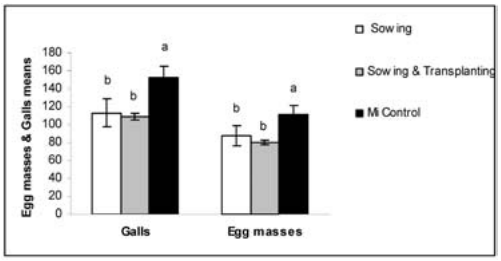

Fig. 1. Effect of *Fusarium oxysporum* strain 162 application time on the number of galls and egg masses formed by *Meloidogyne incognita* on tomato in the first trial. Means with the same letter are not significantly different based on Tukey Test ($P \leq 0.05$; n = 10).

in nematode penetration in the first trial ($P \leq 0.05$, Fig. 3). However, in the second trial, FO162 treatments were not different from FO162 non-inoculated control.

Inoculation at both sowing and transplanting at both doses gave significantly higher levels of biocontrol when compared to the control. However, there were no significant differences between the doses used in the repeated inoculation treatments at both sowing and transplanting times (Fig. 3). In the second trial, the level of control was more variable, which led to a lack of significant levels of control in some treatments.

Repeated inoculations of the seedlings with 10^5 cfu/g of FO162 of seedling sub-

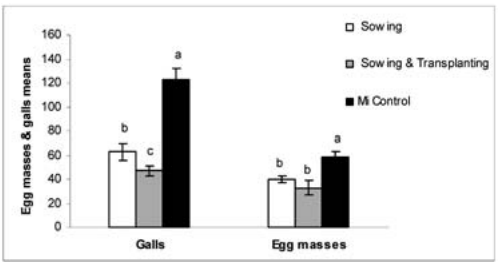

Fig. 2. Effect of *Fusarium oxysporum* strain 162 application times on the number of galls and egg masses formed by *Meloidogyne incognita* on tomato in the second trial. Means with the same letter are not significantly different based on Tukey Test ($P \leq 0.05$; n = 10).

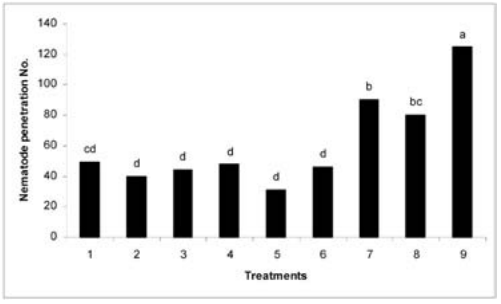

Fig. 3. Effect of *Fusarium oxysporum* strain 162 inoculation time and density on *Meloidogyne incognita* penetration per gram root of tomato plants, 2 weeks after nematode inoculation in the first trial. Treatments numbers are described in Table 1. Bars with the different letters are significantly different based on Tukey Test ($P \leq 0.05$; n = 10).

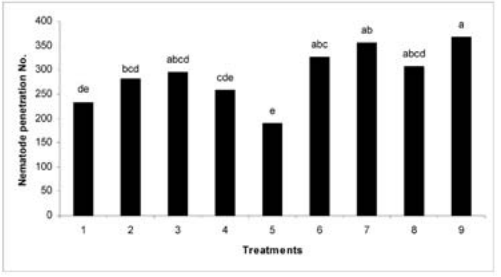

Fig. 4. Effect of *Fusarium oxysporum* 162 inoculation time and density on *Meloidogyne incognita* penetration per gram root of tomato plants, 2 weeks after nematode inoculation in the first trial. Treatments numbers are described in Table 1. Bars with the different letters are significantly different based on Tukey Test ($P \leq 0.05$; n = 10).

strate at sowing followed by 10^4 cfu/g soil at transplanting resulted in the highest reductions in nematode penetration over the controls in both tests (treatment 5, Figs. 3 and 4).

DISCUSSION

Experiment I. Application Time

The results from experiment I on the importance of inoculation time on the level of biocontrol showed that *M. incognita* infection measured as the number of galls per plant was significantly reduced when FO162 was applied at sowing or at sowing and again at transplanting. Multiple applications of *F. oxysporum* generally led to slightly higher levels of control though not always significantly different, when compared to single applications. Important is the fact that a single treatment is sufficient for good biocontrol. Hallmann and Sikora (1994a, b), Sankaranarayanan *et al.* (2002) and Diedhiou *et al.* (2003) showed that strains of non-pathogenic *F. oxysporum* reduced root-knot nematode infestation by preventing juveniles from invading the roots and by interfering with juvenile development within the root tissue. The reduction in the number of egg masses in both trials was probably also caused by a delay in penetration of the root due to repellent activity of FO162 on the juveniles (Dababat and Sikora, 2007b; Sikora *et al.,* 2007).

Experiment II. Inoculum Density

The level of biocontrol was not improved significantly by increasing the dose from 10^4 to 10^5. The higher level of nematode penetration in the second trial was attributed to higher temperatures in the greenhouse during the summer (28°C ± 5), compared to the first trial, which was carried out in winter at cooler temperatures (18°C ± 2).

Inoculation at transplanting provided lower levels of root-knot nematode control when compared to treatment at sowing. This was most likely caused by low levels of endophyte colonization in the 24 hr period between FO162 and nematode inoculation. The level of non-pathogenic *F. oxysporum* colonization in the rhizosphere of tomato needed for suppression of *Fusarium* wilt is *Fusarium* density dependent (Alabouvette *et al.*, 1993). The ratio of non-pathogenic *F. oxysporum* Fo47 to

pathogenic *F. oxysporum* f. sp. *lini* strain Foln3GUS must be 100:1 for suppression to be significant (Duijff *et al.*, 1999). However, such a relationship has not been observed with the biocontrol of nematodes with fungi or bacteria (Hallmann and Sikora, 1994a, b; Vu *et al.*, 2006) and testing more than 2 inoculation densities in the future could support this finding.

Dual inoculation did not significantly increase the overall level of nematode control over that attained by a single inoculation of FO162 at sowing. This demonstrates that the level of endophyte colonization required for control is already obtained when the antagonist is introduced 5 weeks before plant exposure to the nematode. Treatment at transplanting does not allow good colonization or nematode control, and adds to overall cost of the treatment, therefore it is not recommended.

Further optimization of inoculum production and improvement in formulation of FO162 could improve control and reduce the costs for nematode management. For example, Elzein *et al.* (2006) suggested that coating sorghum seeds with *Fusarium oxysporum* (Foxy 2) for control of the root parasitic weed Striga (*Striga hermonthica*), appears to be an attractive option for minimizing the amount of inoculum required. Finding isolates of *F. oxysporum* that colonize the roots quickly and at lower inoculum levels could also reduce treatment costs.

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