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Performance of a *Trichoderma harzianum* Bentonite–vermiculite Formulation Against Fusarium Wilt in Seedling Nursery Melon Plants

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Abstract. **Practical use of *Trichoderma harzianum* requires feasible formulated products. The objective of this study was to investigate the survival and effectiveness of a *T. harzianum* bentonite–vermiculite formulation against fusarium wilt and its growth-promotion effect on melon plants under nursery conditions compared with the incorporation of this agent as a nonformulated conidia suspension. The effectiveness of the application of *T. harzianum* to melon plants was related directly to its formulation, as the formulation had a clear influence on its survival on peat. The formulated form maintained the inoculation level of *T. harzianum* colony-forming units after 8 weeks, whereas the nonformulated form reduced it by two orders of magnitude. Plants treated with the bentonite–vermiculite formulation showed a higher shoot weight and higher resistance to fusarium wilt disease.**

One of the major limitations to the application of biological control agents (BCAs) such as *Trichoderma* species is the development of appropriately formulated products (Fravel, 2005). Although a wide range of formulations of the biofungicide *Trichoderma* sp. has been tested (Batta, 2004; Kolombet et al., 2008; Kücük and Kivanc, 2005; Zohar-Perez et al., 2003), they are focused on keeping the culture viable and active during storage. Some additives (protectants and carriers) have been used to increase BCA survival under adverse environmental conditions (Wraight et al., 2001). Some carriers include fine clay, peat, vermiculite, alginate, wheat, bran, talc, diatomaceous earth, and pasteurized soil (Boyetchko et al., 1998). We propose the use of bentonite–vermiculite as a solid substrate for both *T. harzianum* proliferation and as a carrier based on the consideration that both are harmless to the environment, inexpensive, and easily available.

A solid bentonite–vermiculite formulation based on *T. harzianum* (CECT 20714) and the use of this agent as a nonformulated conidia suspension (direct inoculation) were both tested for their potential use as inocula for controlling fusarium wilt and promoting melon plant growth under in vivo greenhouse nursery conditions.

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Materials and Methods

Melon (*Cucumis melo* L., cv. Giotto) seeds were used as the host plant. *T. harzianum* (CECT 20714) was isolated from agricultural soil. To prepare the nonformulated form, *T. harzianum* was grown in potato dextrose agar for 6 d at 28 °C. Sterile Ringer solution (10 mL) was added to the fungal cultures and the conidia were dislodged with a plastic rod to obtain a fungal suspension containing 10^8 conidia/mL. The formulated form was prepared by mixing 20 g of oat, 50 mL of bentonite, 100 mL of vermiculite (Table 1), and 60 mL of water. The blend was sterilized, inoculated with *T. harzianum*, and incubated at 28 °C for 8 d to reach the stationary phase, resulting in a fungal population of 10^9 conidia/g. *Fusarium oxysporum* f.sp. *melonis* was cultivated in potato dextrose broth at 28 °C on a shaker at 120 rpm for 5 d.

The experiment was performed in specific nursery plant polystyrene containers, with 10 cells, at Semilleros el Mirador (Murcia, Spain). The treatments were: 1) control treatment; 2) nonformulated *T. harzianum* treatment (*T. harzianum* inoculated directly as a liquid conidial suspension); and 3) bentonite–vermiculite *T. harzianum* formulated treatment (*T. harzianum* was inoculated in a bentonite–vermiculite solid formulation). The treatments were prepared by mixing sterilized commercial peat with the different form of inocula. *T. harzianum*, both formulated and nonformulated forms, was mixed with the peat reaching a population density of 10^6 conidia/g of peat. The nursery plant polystyrene containers were filled with the different treatments (10 g per cell). Each specific container was considered as a unit, and five replicates of each treatment were established. Ten melon seeds were sown (one seed per well) in the polystyrene container and covered with vermiculite. Seedlings were grown using standard nursery culture conditions, which included irrigation without any fertilizer. Four weeks after planting, *F. oxysporum* was inoculated into the peat at a concentration of 10^4 conidia/g. Eight weeks after planting, plants were harvested. Fresh and dry (105 °C, 5 h) shoot weights were recorded. Plant tissues were ground before chemical analysis. Phosphorus and potassium concentrations were determined after digestion in nitric-perchloric acid (2:1) for 2 h, phosphorus was determined by colorimetry (Murphy and Riley, 1962), and potassium by flame photometry (Schollenberger and Simon, 1945). Nitrogen concentration was determined by a modified Kjedahl method (Bremner and Mulvaney, 1982). The total foliar chlorophyll content was determined according to Arnon (Arnon, 1949).

Substrate samples were plated on potato dextrose agar (PDA) amended with 50 $mg \cdot L^{-1}$ rose bengal and 100 $mg \cdot L^{-1}$ streptomycin sulfate, to calculate the number of *T. harzianum* colony-forming units (CFUs). Komada medium (Komada, 1975) was used for quantitative isolation of *F. oxysporum*. Plates were incubated at 28 °C for 5 d. To assess *F. oxysporum* infection, stem segments (≈1.5 cm) above crowns were cut, surface-sterilized by soaking in 1% sodium hypochlorite, and rinsed with sterilized water. The segments were incubated on PDA at 28 °C for 6 d, and the appearance of *F. oxysporum* colonies was considered to be indicative of infected plants.

The data were subjected to analysis of variance using SPSS software (SPSS system for Windows, Version 15.0; SPSS Inc., Chicago, IL). The statistical significance of the results was analyzed by the performance of Tukey's multiple range test ($P \leq 0.05$).

Results and Discussion

Bentonite–vermiculite formulation treatment maintained the initial inoculation level of *T. harzianum* CFUs in the plant growth substrate after 8 weeks, whereas the nonformulated treatment reduced the initial inoculated level of *T. harzianum* CFUs by two orders of magnitude after 8 weeks (Fig. 1A). This low survival rate suggests the sensitivity of *T. harzianum* to environmental conditions, although seedling nurseries seem to provide

Table 1. Bentonite and vermiculite main physicochemical parameters.

Parameter	Bentonite	Vermiculite
pH	9.5	9.9
Electrical conductivity ($mS \cdot cm^{-1}$)	0.41	0.19
Total organic carbon (%)	0.84	0.84
Total nitrogen (%)	0.03	0.06
Total phosphorus (%)	0.1	0.02
Total potassium (%)	1.01	0.38

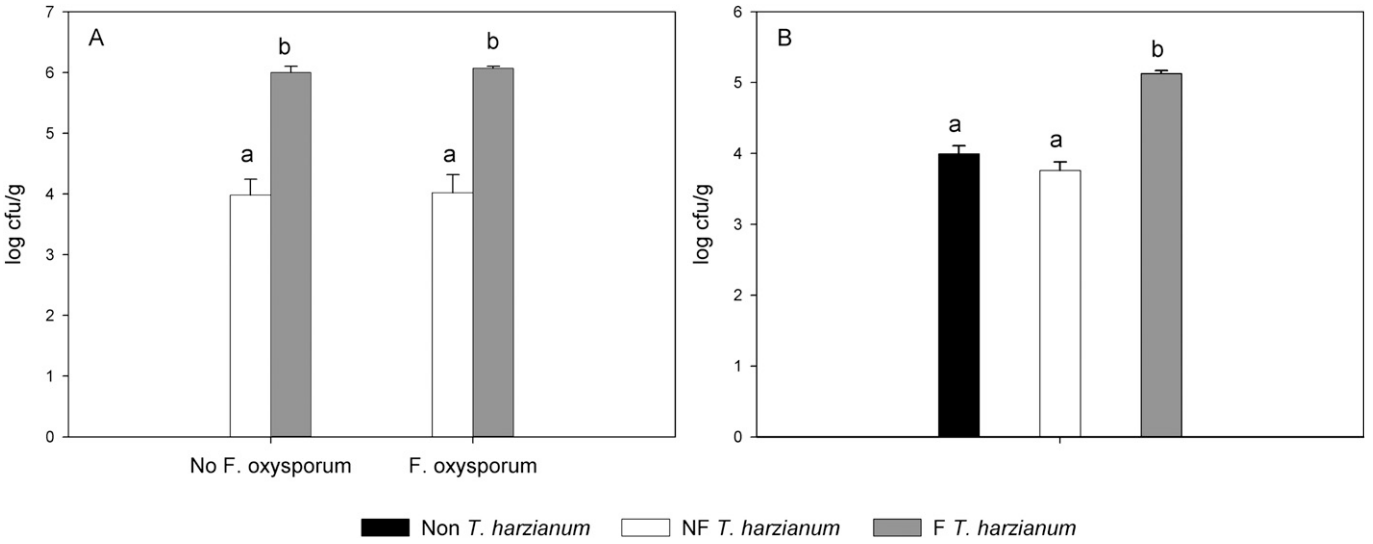

Fig. 1. *T. harzianum* and *F. oxysporum* population. (**A**) *T. harzianum* population inoculated as a nonformulated (NF) or formulated (F) form, 8 weeks after inoculation, in the presence or absence of *F. oxysporum*. (**B**) *F. oxysporum* population, 4 weeks after inoculation, in the presence or absence of *T. harzianum* inoculated as a nonformulated or formulated form (log colony-forming units per gram of substrate). Bars indicate SD. Values with the same letter represent no significant difference between treatments according to Tukey's multiple range test ($P \leq 0.05$; n = 5).

an optimal environment for application of BCAs. The reduction of *T. harzianum* population in a nonformulated treatment can be reasonably assumed to be attributed, in general, to a lack of protection against several factors, including sunlight, temperature, humidity, leaf surface exudates, and competitors (Jones and Burges, 1998). The bentonite–vermiculite formulation was found to be a suitable medium for the persistence and survival of *T. harzianum* in a nursery substrate. *T. harzianum* immobilization in solid beds by a clay alginate matrix results in greater survival in the face of ultraviolet irradiation than with a free cell suspension (Zohar-Perez et al., 2003). In addition, bentonite might offer protection against biological factors through the induction of the formation of microniches (Heynen et al., 1988) and formation of a physical barrier between the inoculant microorganisms and the surrounding environment (Heijnen et al., 1993). Our results indicate a clear positive effect of the solid carrier, in the bentonite–vermiculite formulation treatment, on *T. harzianum* viability in melon seedlings after peat inoculation in greenhouse nursery conditions. Similar results have been observed by Bernal-Vicente et al. (2009) who observed a decrease in the number of *T. harzianum* CFUs when inoculated as a conidia suspension. However, our results contrast with a previous report in which inoculation of a solid formulation of *Trichoderma atroviride* C52 decreased the *Trichoderma* population in the rhizosphere (McLean et al., 2005).

Plants treated with the bentonite–vermiculite formulation of *T. harzianum* showed a significant increase in fresh and dry weights and chlorophyll content compared with the nonformulated *T. harzianum* and with the control plants (Table 2). This biostimulant activity has been studied widely for different isolates of *Trichoderma* sp. (Björkman et al., 1998; Harman and Björkman, 1998; Harman et al., 2004; Ousley et al., 1994; Rabeendran et al., 2000). Several mechanisms by which *Trichoderma* stimulate plant development have been suggested such as the production of growth-promoting metabolites and an increase in the uptake of nutrients by the roots as a result of plant–*Trichoderma* interaction (Altomare et al., 1999; Windham et al., 1986; Yedidia et al., 2001). These effects have important economic implications in terms of shortening the plant growth period and the time in the seedling nurseries, thereby increasing production capacity. The formulated *T. harzianum* form also affected nitrogen (N), phosphorus (P), and potassium (K) contents of plants. A significant increase was observed in plant P content. On the other hand, plant N content decreased, whereas K content was slightly but not significantly lower in the formulated form of *T. harzianum* (Table 2). This decrease in N content could have been the result of competition for N between the plants and *T. harzianum* (Hodge et al., 2000; Kaye and Hart, 1997), although this decreased N uptake produced no adverse effect on plant growth.

Plants treated with the formulated form of *T. harzianum* were more resistant to fusarium wilt in the nursery. Nontreated plants and plants treated with the nonformulated *T. harzianum* form showed a higher disease incidence, which was reduced significantly only in the plants treated with the formulated *T. harzianum* form (Table 3).

Furthermore, diseased plants, treated with the bentonite–vermiculite formulation of *T. harzianum*, showed a lower decrease in fresh weight and chlorophyll content (13% and 8% in fresh weight and chlorophyll content, respectively, with respect to noninfected plants) (Tables 2 and 3) when compared with diseased nontreated plants and diseased plants treated with the nonformulated *T. harzianum*.

The presence of *F. oxysporum* did not affect the *T. harzianum* population inoculated in either the formulated or nonformulated form (Fig. 1A); however, a significant increase in the pathogen population was found resulting from the presence of *T. harzianum* inoculated as the formulated form, but not in the nonformulated form (Fig. 1B). There are a number of reports of the biocontrol capacity

Table 2. Fresh and dry shoot weight, chlorophyll content, and nitrogen, phosphorus, and potassium content in melon plants inoculated with *T. harzianum* as a nonformulated (NF) and formulated (F) form 8 weeks after planting.

	Fresh wt (g)	Dry wt (g)	Chlorophyll ($g \cdot kg^{-1}$)	Nitrogen ($g \cdot kg^{-1}$)	Phosphorous ($g \cdot kg^{-1}$)	Potassium ($g \cdot kg^{-1}$)
Control	5.61 ± 0.71 a	0.65 ± 0.08 a	0.31 ± 0.07 a	19.6 ± 0.12 b	5.7 ± 0.10 a	23.3 ± 0.38 a
N.F. *T. harzianum*	5.72 ± 0.68 a	0.66 ± 0.08 a	0.35 ± 0.02 a	19.2 ± 0.05 b	5.6 ± 0.04 a	24.9 ± 0.20 a
F. *T. harzianum*	7.13 ± 0.42 b	0.79 ± 0.05 b	0.56 ± 0.10 b	16.4 ± 0.06 a	6.9 ± 0.03 b	19.4 ± 0.13 a

Data are means ± SD of five replicates. Values in the same row or column with the same letters represent no significant difference between treatments according to Tukey's multiple range test ($P \leq 0.05$).

Table 3. Disease incidence, fresh shoot weight, and chlorophyll content in melon plants inoculated with *T. harzianum* as a nonformulated (NF) and a formulated (F) 4 weeks after pathogen inoculation.

	Disease incidence (%)	Fresh wt (g)	Chlorophyll ($g \cdot kg^{-1}$)
Control	83.3 a	4.15 ± 0.40 a	0.22 ± 0.09 a
N.F. *T. harzianum*	76.6 a	4.38 ± 0.17 a	0.24 ± 0.13 a
F. *T. harzianum*	46.0 b	6.17 ± 0.73 b	0.51 ± 0.07 b

Data are means ± SD of five replicates. Values in the same row or column with the same letters represent no significant difference between treatments according to Tukey's multiple range test ($P \leq 0.05$).

of *Trichoderma* species (Howell, 2003) and various mechanisms of biocontrol have been reported such as mycoparasitism, antibiotic production, competition, or induction of plant defense (Harman et al., 2004). Because we observed an increase in the pathogen population, other mechanisms such as an induction of plant defense rather than a direct interaction of the BCA and the pathogen could explain the suppression of the disease development by *T. harzianum* (De Meyer et al., 1998).

In conclusion, the effectiveness of the application of *T. harzianum* to melon plants under seedling nursery conditions was related directly to its formulation, because the formulation had a clear influence on the survival of this antagonistic fungus on peat. A commercial bentonite–vermiculite formulation based on *T. harzianum* strain CECT 20714 could be effective in greenhouse nurseries with a double objective: 1) to enhance plant growth; and 2) to reduce the incidence of fusarium wilt in melon plants.

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