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Colonization of the rhizosphere, rhizoplane and endorhiza of garlic (*Allium sativum* L.) by strains of *Trichoderma harzianum* and their capacity to control allium white-rot under field conditions

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Abstract

Five strains (C4, C13, C17, C30, C44) of *Trichoderma harzianum* that efficiently antagonise *Sclerotium cepivorum* in vitro, were used in central México to inoculate field grown garlic (*Allium sativum* L.). Endorhiza, rhizosphere and soil colonization were evaluated at different times and at various root and soil depths. Experiments were conducted in both the 2000 and 2001 crop cycles to evaluate the capacity of five strains as biological control agents (BCA) against *S. cepivorum.* Significant differences were observed among the strains in terms of their ecological behavior. Strain C4 showed the best endorhizal and rhizosphere colonization. Strain C44, besides being a good rhizosphere colonizer, dispersed widely in the soil. In terms of all three variables, strain C30 had poor colonization ability, whereas the strains C4, C17 and C44 were best able to control white rot in the field. Of these, strain C4 showed the best performance over 2 years when it was applied as a pre-colonized substrate at planting time. High early endorhizal colonization was positively correlated with the BCA capacity of the strains. These data suggest that the ecological behavior of *Trichoderma* strains should be included as a criterion for strain selection for biological control purposes.

Keywords: Ecological behavior; Biological control; AFLP; Mycoparasitism

1. Introduction

Globally, soil-borne plant diseases are a serious constraint on agricultural production and there is an ever greater demand for practical, economical and environmentally safe management options to control root diseases (Campbell and Neher, 1996). Garlic (*Allium sativum* L.) is an important crop in México, particularly in the Bajío region. As in many parts of the world, the main limitation to garlic production is the disease known as white rot caused by *Sclerotium cepivorum* Berk (Crowe, 1995). For producers, the most viable management strategy includes the use of methyl bromide which exacts high economic and environmental costs and whose use since January 2005 is restricted to 15 agricultural sectors (US Environmental Protection Agency, 2005). *Trichoderma* has been successfully used as a means of biological control of *Fusarium, Pythium, Rhizoctonia, Sclerotinia,* and *Sclerotium* species in various crops (de Oliveira et al., 1984; Papavizas, 1985; Chet, 1987). Although, its commercial use presents some difficulties, it is a practical alternative to the use of synthetic chemicals for management of soil-borne plant diseases (Sivan and Chet, 1992; Cook, 1993). Ecological studies of *Trichoderma* as a biological control agent (BCA) indicate that this fungus is not a good competitor in soil and thus needs a source of edaphic organic nutrients (Papavizas, 1982). Similarly, conidial suspension formulations have a restricted gradient of dispersion in soil, which

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limits their use in controlling damping-off diseases (Baker, 1987). One way to achieve a better soil dispersion by *Trichoderma* is to apply a mixture of wheat bran and peat moss as substrate (Sivan and Chet, 1992; Kay and Stewart, 1994a, b).

The main problem in field ecological studies lies in devising ways of distinguishing introduced *Trichoderma* strains from the native population because this fungi is ubiquitous. Strategies to overcome this limitation include soil sterilization (Chao et al., 1986), use of fungicide resistant mutants (Abd-El Moity et al., 1982; Sivan and Harman, 1991) and reporter genes such as those encoding the green fluorescent protein or β-glucoronidase (Green and Jensen, 1995; Bae and Knudsen, 2000). Sterile soil is not representative of natural conditions however, and the use of antibiotic or fungicide-resistant mutants needs additional studies to verify that the antagonistic ability is retained. Furthermore, the release of transgenic micro-organisms in the field needs aditional risk studies (Spadaro and Gullino, 2005). The purpose of this study was to investigate the correlation between ecological behavior of *Trichoderma harzianum* (*Hypocrea lixii*) strains belonging to genetically disperse groups and their antagonistic ability against *Sclerotium cepivorum* under field conditions.

2. Materials and methods

2.1. Strains

An initial group of thirty-nine *Trichoderma* isolates was obtained from garlic fields in the Bajío region of central México, in areas with a history of epidemics of garlic white rot. They were subjected to AFLP, DNA fingerprinting to assess their similarity, and 11 genetically diverse groups were obtained (unpublished data). Five of these strains, belonging to genetically disperse groups, were selected using the criterion of their in vitro growth rate at pH 7 and 20 °C. This was necessary because the optimal temperature for growth of *S. cepivorum* is 16–18 °C and because the predominant pH in the Bajío soils is close to neutral. Additionally, an in vitro antagonism assay against *S. cepivorum* strain 2fd11 at 16 °C was used with a 1–5 modified scale (Bell et al., 1994; Kay and Stewart, 1994a) where: (1) *S. cepivorum* overgrows *T. harzianum* and stops its growth; (2) both fungi grow and their mycelia overlap, but remain clearly distinguishable; (3) both, *T. harzianum* and *S. cepivorum* colonize 50% of the plate and stop growing; (4) *T. harzianum* overgrows *S. cepivorum*, growing on 75% of the plate surface; and (5) complete inhibition of *S. cepivorum* with fast and complete overgrowth by *T. harzianum*, impeding sclerotia formation. Strains were stored in 14% (v/v) glycerol at −72 °C.

2.2. Species identification

The species to which these selected *Trichoderma* isolates belong were determined based on the analysis of the ITS1 DNAr region. Briefly, DNA from the five *Trichoderma* strains was obtained using the protocol described by Rader and Broda (1985) and the internal transcribed spacer ITS1 was amplified by PCR using primers ITS1 and ITS2 (White et al., 1990). The product obtained from each strain was sequenced and compared with *Trichoderma*-type sequences deposited in the NCBI Genbank.

2.3. Preparation of Trichoderma inoculum

T. harzianum. strains were plated on potato dextrose agar (PDA) medium, incubated for 5 days at room temperature (24 ± 4 °C) with a 12 h photoperiod. Conidia were harvested by pipetting 10 ml of distilled sterile water over the mycelial growth, which was rubbed with an aluminum bar to separate the conidia. The suspensions were transferred into 100 ml sterile glass containers with 50 ml of sterile distilled water. Conidia concentrations were determined in 50 µl aliquots using a Neubauer Chamber® (Hausser scientific, Horsham, PA, USA). The substrate for the inoculum formulation consisted of a mixture of wheat bran and peat moss (Sunshine®, Sun Gro Horticulture, Bellevue, WA, USA) 1:1 v/v. The substrate was sterilized three times in an autoclave (1 h each time) over three consecutive days at 121 °C. Approximately 2×10^4 conidia g^{-1} of substrate were applied and adjusted to 50% humidity on a dry weight basis. The inoculated substrate was incubated at room temperature (25 ± 3 °C) for 10 days.

2.4. Field assays for ecological behavior

Two field experiments were conducted in a Vertisol pelic soil (Spaargaren, 1994) at the Bajío region in Guanajuato state, central México: one in the 2000–2001 garlic crop cycle near the city of Irapuato (20° 42′N, 101° 20′W) and the second in the 2001–2002 cycle near the city of Cortazar (20° 30′N and 101° 00′W). The *T. harzianum* inoculum was applied just before planting at a rate of 25 g m^{-1} in a band form (5 cm wide). Garlic clove-seed was placed over the applied substrate, and covered with about 4 cm of soil. Treatments consisted of each of five *T. harzianum* strains (C4, C13, C17, C30 and C44) and an uninoculated control (CTR) in the 2000–2001 cycle. In 2001–2002 cycle two additional treatments were used: the first (C4csus) consisted of immersion of the garlic seed into a suspension of the C4 strain conidia (2×10^4 conidia ml^{-1}) before planting; the second (SUS) consisted of the application of sterile wheat bran-peat substrate at the rate of 25 g m^{-1} of plants. The experimental area was irrigated by rows. Garlic cultivar ''Jaspeado'' was grown at a density of 35×10^4 plants ha^{-1} in 80-cm wide beds containing two rows of plants separated by 25 cm. The experimental design was an array of randomized blocks (six or eight treatments) with four replications and five sub-plots per replicate. Each replicate consisted of an area of 2 × 5 m with plants in two rows (area of 0.8 × 0.5 m with 14 plants) in the middle of each sub-plot.