Biological Control 51 (2009) 409–416

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Growth stimulation in bean (*Phaseolus vulgaris* L.) by *Trichoderma*

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ARTICLE INFO

Article history:
Received 14 March 2008
Accepted 29 July 2009
Available online 3 August 2009

Keywords:
Trichoderma
Phaseolus
Phosphate solubilization
Siderophore
Auxins
Endophyte

ABSTRACT

Trichoderma species are commonly used as biological control agents against phytopathogenic fungi and some strains are able to produce metabolites that enhance plant growth. In the current study we evaluated the production of potential growth-promoting metabolites, rhizosphere competence and endophytism for 101 isolates of *Trichoderma* from Colombia, and assessed the relationship of these factors to the enhancement of early stages of growth on bean seedlings. Twenty percent of these *Trichoderma* strains were able to produce soluble forms of phosphate from phosphoric rock. Only 8% of the assessed strains showed consistent ability to produce siderophores to convert ferric iron to soluble forms by chelation. Sixty percent of isolates produced indole-3-acetic acid (IAA) or auxin analogues. The production of any of these metabolites was a characteristic of specific strains, as the ability to produce these metabolites varied greatly within species. Moreover, the production of these substances did not correlate with enhanced growth on bean seedlings, measured as the combined increase in length of roots and aerial parts in the V3 stage of growth. Seven *Trichoderma* isolates significantly improved the growth of bean seedlings. However, metabolite production varied widely in these seven strains, and some isolates did not produce any of the assessed growth-promoting metabolites. Results indicated that growth was enhanced in the presence of rhizosphere competent and endophytic strains of *Trichoderma*, and these characteristics were strain-specific and not characteristic for species.

1. Introduction

The fungal genus *Trichoderma* (teleomorph *Hypocrea*—Ascomycetes: Hypocreales) includes species of economic importance for production of antibiotics and enzymes (Howell, 2003; Viestrus et al., 1996), degradation of xenobiotic compounds (Ezzi and Lynch, 2005; Zhou et al., 2007), biological control activity against fungi and nematodes (Brunner et al., 2005; Sahebani and Hadavi, 2008) and induction of systemic acquired resistance in plants by endophytism (Brunner et al., 2005; Hanson and Howell, 2004; Kubicek et al., 2001). *Trichoderma* species can improve plant growth and development (Chang et al., 1986; De Souza et al., 2008; Gravel et al., 2007; Windham et al., 1986). Growth stimulation is evidenced by increases in biomass, productivity, stress resistance and increased nutrient absorption. Increased crop productivity associated with the presence of *Trichoderma* has been observed in a broad range of species, such as carnation, chrysanthemum, *Tagetes*, petunia, cucumber, eggplant, pea, pepper, radish, tobacco, tomato, lettuce, carrot, corn, poppy, cotton, millet, bean, cocoa and ornamental grasses (Björkman et al., 1998; De Souza et al., 2008; Gravel et al., 2007; Harman et al., 2004; Inbar et al., 1994; Klefield and Chet, 1992; Mackenzie et al., 1995; Ousley et al., 1994; Resende et al., 2004; Windham et al., 1986; Yedidia et al., 1999).

Presumed mechanisms involved in the stimulation of plant growth by *Trichoderma* include interactions with plant roots similar to mycorhizae, in which *Trichoderma* penetrates and colonizes root tissues without eliciting specific defense responses against the colonizing strain (Yedidia et al., 1999). The plant benefits by the presence of *Trichoderma,* suggesting an interaction as avirulent symbionts (Howell et al. 2000; Harman et al., 2004; Yedidia et al.,1999, 2000). Yedidia et al. (2000) demonstrated that cucumber roots inoculated with *Trichoderma* showed activity of glucanases, chitinases, cellulases and peroxidases, evidence of the activation of plant defense mechanisms by *Trichoderma. Trichoderma* can also produce metabolites with activities analogous to plant hormones (Cutler et al., 1989, 1991). Harman (2006) reviewed the principal mechanisms by which *Trichoderma* may be an effective biocontrol agent, including interactions that can alter the transcriptome in the host plant, and induce host resistance against pathogens. Altomare et al. (1999) found that *T. harzianum*

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1049-9644/$ - see front matter © 2009 Published by Elsevier Inc.
doi:10.1016/j.biocontrol.2009.07.018

strain T22 could produce soluble forms of manganese, metallic zinc and calcium phosphate *in vitro*, and also that the fungus produced metabolites that reduced ferric iron (III) to the ferrous form readily assimilated by plants. Studies by Jalal et al. (1986, 1987) showed that *T. virens* produces the siderophores mono- and di-hydroxamates as mechanisms of Fe III chelation. According to these investigators, the conversion of metal oxides to soluble forms by *Trichoderma* spp. involved chelation and reduction, both mechanisms also implicated in the control of plant pathogens and components of growth stimulation and biocontrol (Anusuya and Jayarajan, 1998; Harman, 2006; Woo et al., 2006).

Growth stimulation studies involving Trichoderma have usually included only relatively few strains (Altomare et al., 1999; Gravel et al., 2007; MacKenzie et al., 1995; Yedidia et al.,1999, 2000). In this study, 101 *Trichoderma* strains representing nine species were evaluated for their ability to stimulate the early stages of growth in bean seedlings. Potential growth-promoting metabolites were assayed *in vitro* for each of these strains, and data examined for correlates with growth enhancement. The metabolites evaluated were (i) phosphate solubilizing agents, (ii) siderophores capable of converting ferric iron to soluble forms, and (iii) indole-3-acetic acid (IAA) and auxin analogues. Phosphate solubilizing agents can stimulate plant growth where phosphates are limiting, especially in acid soils in which phosphorus is rapidly immobilized after addition to the soil as a soluble fertilizer becoming unavailable for assimilation by plants (Kim et al., 1997; Vásquez et al., 2000; Whitelaw et al., 1999; Yedidia et al., 2001). Siderophores can promote rhizosphere colonization and plant growth in synergy with other substances, a mechanism also suggested in plant growth enhancement by pseudomonads (Anke et al., 1991; Lugtenberg et al., 2001; Sharma et al., 2003). Finally, IAA and its analogues have been shown to positively effect root growth and morphology (de Brito and Gagné, 1995; Gravel et al., 2007; Patten and Glick, 2002).

2. Materials and methods

2.1. Trichoderma strains

Trichoderma isolates from the Microorganism Culture Collection of Unidad de Biotecnología y Control Biológico of the Corporación para Investigaciones Biológicas (CIB, Medellín) were used in this study (Table 1). These 101 strains were identified from sequences of the internal transcribed spacer regions (ITS 1 and 2) of the ribosomal RNA repeat, and of a 0.9 kb fragment at the 5′ end of the translation elongation factor 1-α gene (eEF1a1) (Hoyos et al. 2009). Conidial inoculum for our studies was obtained from cultures on potato dextrose agar (PDA) by flooding with sterile water. Inoculum concentrations (conidia/ml) were determined from counts in Neubauer chambers and standardized as appropriate for the analyses.

2.2. Biochemical characterization

The qualitative determinations of phosphate solubilization, siderophore and auxin production were replicated six times for each *Trichoderma* strain.

2.2.1. Phosphate solubilization (PS)

Three qualitative procedures were used to screen for phosphate solubilizing (PS+) *Trichoderma* strains. The first procedure used tribasic calcium phosphate as a phosphate source, with bromocresol purple (0.1 g/L) included in the media as a pH indicator for acidification (Vásquez et al., 2000). After 48 h incubation at 25 °C, PS+ isolates turned the media from purple to yellow in zones of acidification. The second method employs the same media without bromocresol purple, with the presence of cleared zones where the calcium phosphate is consumed indicative of a positive response (Cattelan, 1999). The third method incorporated slow degrading phosphoric rock in the growth media (Kim et al., 1997). PS+ *Trichoderma* isolates developed clear zones in the media after 7d incubation at 26 °C. For each procedure plates were inoculated with 100 µl of a solution of 1×10^5 conidia/ml and incubated at the temperature indicated for each protocol. An isolate of *Mortierella* sp. was employed as a positive control (Osorio and Habte, 2001).

2.2.2. Ferric siderophore production (Sid)

Two qualitative methods were used to test *Trichoderma* isolates for siderophore production and the conversion of ferric ions (Fe III) to soluble forms (Fe II) by chelation. In the first method (Alexander and Zuberer, 1991) agar media containing chrome azurol S was inoculated with *Trichoderma,* and colonies exhibiting a pink halo after 5 days incubation (28 °C) were considered positive for siderophore production. In the second method 8-hydroxyquinoline (50 mg/L) was incorporated in malt extract agar (De Brito Alvarez and Gagné, 1995), and *Trichoderma* strains capable of growth on this media were considered positive for reduction of ferric iron (Fe III) to soluble ferrous forms (Sid+). Plates were inoculated with 100 µl of a solution of 1×10^5 conidia/ml and incubated at the temperature indicated in each protocol. *Pseudomonas aeruginosa* ATCC 27853 was used as a positive control.

2.2.3. Production of indole acetic acid (IAA) or analogues

A modification of the qualitative procedure for assessing production of IAA and analogues described by de Brito and Gagné (1995) was employed, with and without the addition of 5 mM tryptophan. Agar plates were inoculated with 100 µl of a solution of 1×10^5 conidia/ml of *Trichoderma* and covered with an 82-mm nitrocellulose membrane disk (RPN82D, Amersham, Buckinghamshire, UK). After 7 days incubation at 25 °C, the membrane disks were removed and overlaid on Whatman No. 2 filter paper saturated with Salkowski reagent at room temperature. After five minutes strains producing IAA or analogues were identified by a characteristic pink or red colored halo in the membrane, and strains producing other indoles produced a yellow to yellow–brown pigment. *Agrobacterium tumefasciens* (AT 650) (Unidad de Biotecnología Vegetal-CIB) was used as a positive control.

2.3. Evaluation of plant growth

Seeds of *Phaseolus vulgaris* L. variety "Cargamanto mocho®" (Semillas and Semillas, Medellín, Colombia) were disinfected in 70% ethanol for 2 min, followed by 2.0% NaOCl for 2 min, thoroughly washed and pre-germinated in sterile distilled water. Inoculum of *Trichoderma* (10^7 conidia/ml) was prepared from 7 day old cultures on PDA, comparable to inoculum concentrations used for growth stimulation experiments under greenhouse conditions by Yedidia et al. (1999) (*i.e.* 10^7 to 10^8 cfu/g soil). Radicles of bean seedlings in V1 stage (Fernández et al., 1982) were inoculated by immersion in the conidial solution under aseptic conditions for 30 min. Control plants were treated with sterile distilled water.

The inoculated bean seedlings were planted in 5 g sterile soil (Andisol A-horizon soil, pH 5.8, 26.4% organic matter, 55 mg/kg N, 3 mg/kg P, 162 mg/kg Fe, 3 mg/kg Mn, 14 mg/kg Cu, 2 mg/kg Zn, 10 mmol/kg Ca, 20 mmol/kg Mg, 1.9 mmol/kg K) using a fully randomized plot design. For every *Trichoderma* strain, 10 inoculated plants were grown at 26 °C, 60% relative humidity, with a circadian cycle of 14 h light 10 h dark. After 6 days 50% of the seedlings had reached the V3 state, and taproot length (LR), length of the aerial part (LAP—located between neck root and the shoot of the apical meristem including stem and leaves), and dry weight (DW) were measured.