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Liquid culture production of microsclerotia and submerged conidia by *Trichoderma harzianum* active against damping-off disease caused by *Rhizoctonia solani*☆

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ABSTRACT

Media and culturing protocols were identified that supported the formation of submerged conidia and microsclerotia (MS) by *Trichoderma harzianum* Rifai strain T-22 using liquid culture fermentation. Liquid media with a higher carbon concentration (36 g L^{-1}) promoted MS formation at all C:N ratios tested. Hyphae aggregated to form MS after 2 d growth and after 7 d MS were fully melanized. This is the first report of MS formation by *T. harzianum* or any species of *Trichoderma*. Furthermore, submerged conidia formation was induced by liquid culture media, but yields, desiccation tolerance, and storage stability varied with C:N ratio and carbon rate. Air-dried MS granules (<4 % moisture) retained excellent shelf life under cool and unrefrigerated storage conditions with no loss in conidial production. A low-cost complex nitrogen source based on cottonseed flour effectively supported high MS yields. Amending potting mix with dried MS formulations reduced or eliminated damping-off of melon seedlings caused by *Rhizoctonia solani*. Together, the results provide insights into the liquid culture production, stabilization process, and bioefficacy of the hitherto unreported MS of *T. harzianum* as a potential biofungicide for use in integrated management programs against soilborne diseases.

Introduction

Biological control agents are gaining more attention in recent years owing to their potential to minimize or replace synthetic chemical pesticides in main-stream agriculture. The genus *Trichoderma* is a well-known, cosmopolitan soil fungus that has been widely explored as an antagonist of numerous plant pathogenic fungi in agriculture (Howell

☆ "Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by Embrapa or by USDA."

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2003; Harman 2006). Isolates of *Trichoderma* species can be successful in plant disease control due to directly antagonizing pathogen activity and/or inducing host resistance responses (Harman 2000). Furthermore, *Trichoderma*'s function as a plant growth promoter has been reported for some strains after establishment as a non-strict plant symbiont by colonizing the rhizosphere (Harman & Kubicek 1998; Harman 2000; Harman *et al.* 2004). Multitude modes of action for *Trichoderma* strains employed as biocontrol agents are claimed to be: a) rhizosphere competence by colonizing the soil and/or parts of the plant or by competition for nutrients; b) mycoparasitism by producing a wide variety of cell wall degrading enzymes against pathogens; c) antibiosis via production of antimicrobial compounds (volatiles and non-volatiles) that can kill the pathogens; d) growth promotion by improving plant development, and e) induction of systemic defensive responses in plants (Harman & Kubicek 1998; Howell 2003; Harman 2006).

The majority of *Trichoderma*-based biopesticides consists primarily on aerial conidia that are produced using solid substrate fermentation on moistened grains (Bettiol 2011; Woo *et al.* 2014). This process takes weeks for production and drying, which increases production costs (Pandey *et al.* 2008; Ramanujam *et al.* 2010). The production of fungal conidia on moistened grains suffers from numerous constraints including high labour costs, poor quality control, long fermentation times, environmental concerns for workers, and difficulties in scale-up. Liquid culture production methods have been investigated and focused on the production of submerged conidia and chlamydospores of *Trichoderma* (Lewis & Papavizas 1983; Papavizas *et al.* 1984; Tabachnik 1989; Harman *et al.* 1991; Jin *et al.* 1996; Sriram *et al.* 2011). Although liquid fermentation technology has been adopted by some biopesticide companies around the globe for production of submerged conidia, no reports on yields, fermentation time, production costs, and comparison with aerial conidia in terms of bioefficacy are provided. Formulation studies have focused on stabilization processes for *Trichoderma* biomass, aerial conidia, and chlamydospores that provided adequate storage stability and bioefficacy (Lewis & Papavizas 1985; Jin & Custis 2010; Yonsel & Batum 2010; Sriram *et al.* 2011). Despite these attempts to produce *Trichoderma* in liquid culture, low yields, long fermentation times, and poor desiccation tolerance and storage stability have impaired the large-scale adoption of this production methodology by industry.

Commercially, *Trichoderma harzianum* Rifai strain T-22 is one of the most used active ingredients for biocontrol of root diseases in the USA (Woo *et al.* 2014). In 2011, there were five registered commercial products based on *Trichoderma*-aerial conidia in Brazil with several multinational companies working on developing *Trichoderma* products that are currently applied to more than 3 million ha per year (Bettiol 2011). To meet the biopesticide market expectations and promote *Trichoderma*'s widespread use in agriculture, an efficient liquid culture production technology must be developed that yields a high quality *Trichoderma*-based product.

Liquid fermentation technology has the potential to support high yields of stable, effective *Trichoderma* propagules produced under rigorous control quality and ensuring a consistent and uniform product. Although submerged conidia, mycelia, and chlamydospores of *T. harzianum* can be produced using liquid fermentation, these fungal forms are often produced in low yield, lack storage stability, or persist poorly in soil. While previous studies with other biocontrol fungi have shown that microsclerotia (MS) of *Colletotrichum truncatum*, *Mycoleptodiscus terrestris*, and *Metarhizium brunneum* could be rapidly produced in liquid culture, there are no reports of sclerotia formation in the genus *Trichoderma* (Jackson & Schisler 1995; Shearer & Jackson 2006; Jackson & Jaronski 2009, 2012; Behle & Jackson 2014). Fungal MS are preferable propagules for application in soil since they are overwintering, resistant fungal structures with the intrinsic ability to survive stress conditions, such as desiccation and soil fungistasis (i.e., competition with other soil microorganisms).

In the present study, we assessed the impact of various nutritional environments on the filamentous growth and morphogenetic differentiation of cultures of *T. harzianum*. Using baffled flasks, cultures of *T. harzianum* were grown in liquid media containing various carbon concentrations, carbon-to-nitrogen ratios, and nitrogen sources with measurements of biomass accumulation, propagule formation, and propagule yield during culturing. Propagules of *T. harzianum* produced under these various culture conditions included submerged conidia and MS. Submerged conidia and MS were air-dried to evaluate desiccation tolerance and stored as dry formulations to assess storage stability. Bioassays were conducted with air-dried MS against damping off disease in melon incited by *Rhizoctonia solani*.

Materials and methods

Culture maintenance

Trichoderma harzianum Rifai strain T-22 (ATCC 20847; Rootshield®, BioWorks, Geneva, NY) was used throughout this study. Pure cultures of *T. harzianum* were isolated from serial dilutions of Rootshield® and grown on potato dextrose agar (PDA, Difco®) at 25 ± 1 °C for at least 7 d. Single colonies were purified by re-isolation on PDA and a single hyphal tip was isolated and grown on PDA. The sporulated colony arising from this hyphal tip was used as a stock culture of *T. harzianum* T-22 and was cut into 1 mm^2 pieces, placed in cryovials containing 1 mL of a sterile solution of 10 % (v/v) glycerol (Fisher Scientific, Pittsburgh, PA, USA) prepared with double deionized water, and stored at −80 °C.

For liquid culture studies, conidial inocula were obtained by inoculating PDA plates with a conidial suspension from the frozen stock cultures and growing the cultures at 25 ± 1 °C for 2–3 weeks. Conidial suspensions were obtained from sporulated agar plates by rinsing plates with 10 mL of a sterile solution containing 0.04 % polyoxyethylene sorbitan mono-oleate (Tween 80, Sigma®).

Shake-flask culturing and media evaluation

Growth and propagule formation by *Trichoderma harzianum* was assessed in liquid media containing different carbon concentrations, carbon-to-nitrogen (C:N) ratios, and nitrogen sources using a semi-defined liquid medium composed of