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Trichoderma spp. tolerance to *Brassica carinata* seed meal for a combined use in biofumigation

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Abstract

Biofumigation by *Brassicaceae* green manure or seed meal incorporation into soil is an ecological alternative to chemical fumigation against soil-borne pathogens, based on the release of glucosinolate-derived compounds. This study aimed at investigating the tolerance of the beneficial fungus *Trichoderma* to these compounds in view to combined utilization with *Brassica carinata* seed meal (BCSM). Forty isolates of *Trichoderma* spp. were tested *in vitro* for tolerance to toxic volatiles released by BCSM and in direct contact with the meal. They were found to be generally less sensitive than the assayed pathogens (*Pythium ultimum*, *Rhizoctonia solani*, *Fusarium oxysporum*), even if a fungistatic effect was observed at the highest dose (10 μmole of sinigrin). Most of them also were able to grow on BCSM and over the pathogens tested. A preliminary experiment of integrating BCSM with *Trichoderma* in soil was carried out under controlled conditions with the patho-system *P. ultimum*—sugar beet. BCSM incorporation increased pathogen population, but reduced disease incidence, probably due to indirect mechanisms. The greatest effect was achieved when BCSM was applied in combination with *Trichoderma*, regardless of meal ability to release isothiocyanate. These findings suggest that disease control can be improved by this integrated approach. This study also highlighted that a reduction of allyl-isothiocyanate concentration in soil could occur due to the activity of some *Trichoderma* isolates. This effect could protect resident or introduced *Trichoderma* isolates from depressing effects due to the biocidal compounds, but, on the other hand, could reduce the efficacy of biofumigation against target pathogens.

Keywords: *Trichoderma*; Antagonism; Biological control; *Brassica carinata*; Biofumigation; Green manure; Seed meal; Glucosinolate; Myrosinase; Sinigrin; Allyl-isothiocyanate; Soil-borne pathogens; *Pythium ultimum*; *Rhizoctonia solani*; *Fusarium oxysporum*

1. Introduction

In recent years, sustainable agricultural systems aimed at safeguarding the environment have gained more and more interest, and considerable efforts have been made to adopt strategies which reduce chemical inputs. Concerning crop protection, the diseases caused by soil-borne pathogens have always been difficult to control, even by chemicals. One reason could be the complicated ecosystem of the soil, where a number of interactions occur. Under favorable conditions such diseases spread rapidly, almost without any possibility of control, apart from methods with a high environmental impact like soil fumigations. Less aggressive alternatives are represented by biological methods but the awareness of their moderate effectiveness suggests combining some of them in a multiple integrated approach (Gamliel et al., 2000).

Trichoderma (class *Ascomycota*, ord. *Hypocreales*, fam. *Hypocreaceae*) are free-living beneficial fungi commonly found in soil, able to produce antibiotics and lytic enzymes (cellulase, hemicellulase, xylanase, chitinase) of industrial interest, useful for plant protection purposes in agriculture (Wong and Saddler, 1992; Tronsmo and Hjeljord, 1998; Nieves et al., 2004). Commercial products based on selected *Trichoderma* isolates are currently utilized in the biological control of many pathogenic fungi, from soil-borne to foliar pathogens (Monte, 2001). *Trichoderma* spp. are able to interact both in the plant rhizosphere and in the phyllosphere through multiple mechanisms, such

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as antagonism, competition for space and nutrients, mycoparasitism and release of antibiotics and lytic enzymes, which directly inhibit pathogen growth. In addition, indirect mechanisms, such as plant growth promotion and induction of systemic or localized resistance, have more recently been highlighted (Howell, 2003; Harman et al., 2004).

Biofumigation by means of *Brassicaceae* green manure or seed meal incorporation into soil is a promising, environmentally friendly alternative to chemical fumigation by methyl bromide for the control of soil-borne pathogens. This biological approach is based on the release of glucosinolate-derived toxic compounds, mediated by endogenous myrosinase (E.C. 3.2.1.147) from *Brassicaceae* disrupted tissues or seed meals, in the presence of water (Brown and Morra, 1997).

Glucosinolates are glucosidic compounds characteristic of *Brassicaceae*. They are important constituents of the defensive system together with the enzyme myrosinase. There are more than 120 glucosinolates in nature and frequently one predominates in a specific plant, tissue or seed (Fahey et al., 2001). Glucosinolates can be aliphatic, thiofunctionalized, aromatic or indolic, determining the biological activity of their hydrolysis derivative products (Lazzeri et al., 1993; Manici et al., 1997,1999).

Several cases showing efficacy against multiple plant pathogens by biofumigation are reported in the literature (Brown and Morra, 1997; Kirkegaard et al., 1996; Muelchen et al., 1990; Olivier et al., 1999; Smolinska and Horbowicz, 1999; Larkin and Griffin, 2007). However, additional studies are needed to understand the complex interactions occurring in soil, which in some cases negatively affect the entire system potentiality (Rosa and Rodrigues, 1999; Morra, 2004).

One point which still needs to be elucidated concerns the effects of the biocidal compounds derived from glucosinolate degradation, mainly isothiocyanates, on the beneficial soil microflora naturally occurring or artificially introduced as biological control agents. In particular, unclear effects of rapeseed meal on *Trichoderma* have been reported, suggesting incorporating the meal prior to *Trichoderma* since the direct combination seemed not to be compatible (Dandurand et al., 2000).

Seed meal incorporation into the soil also involves an enrichment in carbon source that may alter or even stimulate the resident or introduced microflora, both beneficial and pathogenic (Mazzola et al., 2002). More recently, disease control was even related to functional mechanisms other than biofumigation, but occurring as a consequence of green manures or seed meal incorporation, involving stimulation of resident streptomycetes or actinomycetes (Wiggins and Kinkel, 2005; Mazzola et al., 2007).

This study aimed at exploring the feasibility of a combined incorporation of *Trichoderma* with *Brassica carinata* Braun defatted seed meal (BCSM), in order to increase disease control efficacy through an integrated biological approach. The tolerance of 40 different isolates of the beneficial fungus *Trichoderma* was therefore investigated with respect to volatile biocidal compounds released by commercial BCSM. The same *Trichoderma* isolates were also screened *in vitro* for their ability to grow both over some soil-borne pathogens and on BCSM. A selected *Trichoderma* isolate was then used in combination with BCSM application in a soil artificially inoculated with a pathogenic strain of *Pythium ultimum* and sown with sugar beet, under controlled conditions.

2. Materials and methods

2.1. In vitro tolerance to volatile biocidal compounds from BCSM

Commercial defatted BCSM (Biofence, Triumph Italia S.p.A, Livorno, Italy) with a high biofumigating potentiality, containing mainly sinigrin as the glucosinolate at 151 μmole g^{-1} and active myrosinase, was utilized to test the *in vitro* tolerance of 40 *Trichoderma* isolates to biocidal volatile compounds, mainly allyl-isothiocyanate (AITC), released upon meal wetting. BCSM was ground and sieved at 0.5 mm before use.

The analysis of the glucosinolate content was performed by HPLC analysis of desulfo-derivatives (ISO 9167-1, 1992).

Trichoderma isolates of different origin (Table 1) were grown on potato dextrose agar (PDA), then one mycelium-agar plug (Ø 5 mm) from an actively growing colony was transferred to 90 mm four-sector Petri dishes on PDA, three different isolates for each dish, three replicates. BCSM was confined in the remaining sector to avoid direct contact with *Trichoderma*. The doses of 33 and 66 mg of meal, corresponding to 5 and 10 μmole of sinigrin, respectively, were chosen on the basis of previous experiments from the literature (Sanchi et al., 2005). The dishes were sealed with parafilm®M immediately after wetting the meal with 75 or 150 μl of distilled water, to avoid losing volatile compounds. Controls without meal were included. Dishes were incubated at 22 °C for 10 days, but the parafilm®M was removed and dishes aerated on the 7th day. Fungitoxic or fungistatic effects were recorded as well as lag phase length (the time needed for the mycelium to start growth) and growth speed (mean colony diameter elongation per day, excluding the lag phase).

The same protocol was used for testing the meal effect on pathogenic isolates of *P. ultimum*, *Rhizoctonia solani* and *Fusarium oxysporum* isolated in our laboratory from diseased sugar beet rhizosphere.

2.2. Determination of AITC in Petri dish headspace

Following the same protocol as above, a specific test on selected *Trichoderma* isolates, Ba15 and N2, was set up to follow the variation of AITC concentration in the Petri dish atmosphere over time. The test was done by culturing Ba15 alone (three plugs), N2 alone (three plugs) and Ba15