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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.
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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

Table 1
Matrix of isolation, ability to grow over *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium ultimum*, (as class) and on *Brassica carinata* defatted seed meal, measured as lag phase length (*d*) and growth speed (mm d^{-1}), of the 40 *Trichoderma* isolates assayed

Trichoderma isolate	Isolation matrix	Growth over pathogens[a]			B. carinata seed meal colonisation	
		F. oxysporum	*R. solani*	*P. ultimum*	Lag phase (*d*)	Growth speed (mm d^{-1})[b]
Lt35	Sugar beet soil	0	2	2	4	12.9
X44	Spontaneous plant	2+	2+	2	4	12.2
Regione	Unknown	2	0	2	4	11.9
S2	Peat	2	2	2	6	11.6
Lt36	Sugar beet soil	0	0	2	6	10.9
B13	Sugar beet rhizosphere	1+	2+	2	4	10.8
Lt31	Sugar beet soil	2+	2+	2	4	10.8
N2	*Brassica nigra* rhizosphere	2+	1+	2	4	10.4
N3	*Brassica nigra* rhizosphere	2+	2+	2	8	10.0
B51	Sugar beet rhizosphere	2+	2+	2	4	9.8
Ba10	Sugar beet soil	0	2	2	5	9.8
3B8	Sugar beet soil	2	2	2	4	9.6
B25	Sugar beet rhizosphere	1+	2+	2	4	9.5
Ba24	Sugar beet soil	2	0	2	6	9.1
J21	*Brassica juncea* rhizosphere	2+	2+	2	4	8.3
J23	*Brassica juncea* rhizosphere	1+	2+	2	5	8.0
N1	*Brassica nigra* rhizosphere	2+	1+	2	4	7.9
K15	Spontaneous plant	0	2	2	6	7.8
J15	*Brassica juncea* rhizosphere	2+	2+	2	4	7.7
X11	Spontaneous plant	1+	1+	2	4	7.6
B41	Sugar beet rhizosphere	2+	2+	2	4	7.5
J14	*Brassica juncea* rhizosphere	2+	2+	2	4	7.5
Ba13	Sugar beet soil	2	2	2	4	7.3
J33	*Brassica juncea* rhizosphere	2+	2+	2	5	6.7
Ba9	Sugar beet soil	0	2	2	6	6.3
3B24	Sugar beet soil	2	2	2	6	6.3
Ba18	Sugar beet soil	2	2	2	6	6.0
J56	*Brassica juncea* rhizosphere	1+	1+	2	5	6.0
ISCI86/6	Sugar beet soil	2	2	2	4	5.9
Bf2	Sugar beet leaf	0	2	2	6	5.5
P8	Potato rhizosphere	0	2	2	6	5.0
J27	*Brassica juncea* rhizosphere	1+	2+	2	9	5.0
P5	Potato rhizosphere	1	2	2	5	4.0
Ba12	Sugar beet soil	2	2	2	4	3.6
Ba15	Sugar beet soil	0	0	0	4	3.4
B57	Sugar beet rhizosphere	1	0	2	5	3.3
Ba8	Sugar beet soil	0	2	2	6	2.3
Lt1/05	Sugar beet soil	2+	2+	2	6	2.1
Ba19	Sugar beet soil	0	0	0	f.e.	0
Ba21	Sugar beet soil	2	2	2	f.e.	0

[a] 0, 1, 2: none, incomplete or complete growth over the pathogen colony, respectively; +: heavy browning of the pathogen colony (*F. oxysporum*) or the contact band (*R. solani*); f.e.: fungicidal effect.
[b] Mean values were separated by Fisher's protected least significant difference (±1.939) test for $p \leqslant 0.05$ after ANOVA.

plus N2 (one and two plugs, respectively) in the presence of BCSM (66 mg), in comparison to BCSM alone.

AITC concentration was evaluated by sampling the Petri dish atmosphere after 30 and 100 h by a gastight syringe through a little hole previously made in the cover and sealed with tape. Analyses were carried out using a Varian GL-3800 gas-chromatograph equipped with a J & W DB 23 column (15 m, ID 0.25 mm, film 0.25 μm), FID detector. The analytical conditions were: injector temperature: 200 °C; detector temperature: 260 °C; flow: 8 ml min^{-1} of nitrogen as carrier; column oven temperature: 50 °C; manual injection of 200 μl of sample in splitless mode. Quantity was determined by a calibration curve defined using pure AITC (Fluka). Analyses were repeated twice.

2.3. Ability to colonize active BCSM

A system was set up in order to provide a combined assay both for volatiles and residual effects of BCSM on the growth of *Trichoderma* and the pathogens *P. ultimum*, *R. solani* and *F. oxysporum*. A PDA-plug of an actively growing colony of the fungi was put in the center of a 90 mm-Petri dish on solidified water agar, three replicates. BCSM (200 mg, ground at 0.5 mm) was then evenly distributed on the entire surface of the dish, also covering the mycelium plug. Dishes were closed but not sealed by parafilm®M to avoid excessively stressed conditions, due to the large amount of the meal necessary to cover the whole dish surface. Additional water was not necessary to activate sinigrin enzymatic hydrolysis, due to

the fact that BCSM was moistened by substrate humidity. Controls on water agar were included.

Colony growth was then observed, evaluating the lag phase length and the growth speed over a 10-day period at 22 °C as already described. The experiment was repeated to confirm the results.

Trichoderma and pathogen growth was also assayed on BCSM after myrosinase inactivation by heating BCSM at 120 °C for 20 min, in comparison to control on water agar, three replicates. The diameter elongation was recorded daily for 6 days at the same conditions as above. The experiment was repeated to confirm the results.

2.4. Ability to grow over soil-borne pathogens

Each *Trichoderma* isolate was tested *in vitro* for its ability to grow over *P. ultimum*, *R. solani* and *F. oxysporum* colony in dual culture. One mycelial disc (5 mm) of *Trichoderma* and of the pathogen were put together in a PDA Petri dish, 4 cm apart, two replicates (dishes). After a two week incubation at 22 °C, the ability to completely or incompletely grow over the pathogen colony was observed and scored according to Ortiz and Orduz (2000), with some modifications: 0, no growth over the pathogen colony; 1, partial growth over the pathogen colony; 2, growth over the entire pathogen colony. The experiment was repeated to confirm the results.

2.5. Soil experiment

Aluminium trays (10 cm × 10 cm × 5 cm height) were filled with 250 g of a loamy-clay soil cultivated with sugar beet for years without rotation. Soil was artificially inoculated with a pathogenic strain of *P. ultimum*, isolated from diseased sugar beet plantlets grown in the same soil. The *inoculum* was prepared mincing the contents of two Petri dishes (90 mm diam.), containing 48 h old sporified colonies of the pathogen on PDA, and stirring for 20 min in 150 ml of water. Five millilitre of this suspension were then added to 75 ml of water and used to wet each tray. Soil was thoroughly mixed, trays were then covered with a plastic film and incubated at 19 °C for 14 days. The final *inoculum* concentration corresponded to about 5×10^3 c.f.u. g^{-1} of soil, assessed by plating 1 ml of the water suspension of soil (1:100 w/v) on a semi-selective medium (PARP) containing Pimaricin, Ampicillin, Rifampicin, PCNB, corn meal agar (Kannwischer and Mitchell, 1978). The colonies were counted after 48 h-incubation at 19 °C, three replicates.

The following treatments were compared: inoculated control (C); *Trichoderma* (T); active BCSM (A); not active BCSM (NA); *Trichoderma* + active BCSM (T + A); *Trichoderma* + not active BCSM (T + NA), with two replicates (trays) per treatment.

For "T" treatments, *Trichoderma* isolate N2 was chosen among those that were able to grow in direct contact with the active meal, showing the least fungistatic effect (4d) and the maximum growth rate (>10 mm d^{-1}). *Trichoderma* was added as a spore suspension (25 ml per tray) obtained by washing, with 2% malt extract solution, 1-week old sporified colonies grown on PDA in Petri dishes (4×10^7 spores ml^{-1}). The trays were incubated for 14 days at 19 °C. At this time *inoculum* concentration corresponded to about 6×10^5 c.f.u. g^{-1} of soil, assessed by plating 1 ml of the water suspension of soil (1:10000 w/v) on a *Trichoderma* selective medium (TSM), three replicates (Elad et al., 1981). This *Trichoderma* treatment was performed 14 days after *P. ultimum* inoculation.

For "A" treatment BCSM was incorporated into soil 14 days after *Trichoderma* inoculation, at the dose of 0.5 g per tray. For "NA" treatments, BCSM was previously autoclaved at 120 °C for 20 min to inactivate myrosinase and prevent the release of glucosinolate hydrolysis products.

The whole experiment was carried out at 19 °C in the dark, covering the trays with a plastic film after the treatments and restoring the water losses over time.

The effect of the treatments on *P. ultimum* and *Trichoderma* populations was assessed by sampling 2 g of soil from each tray just before BCSM incorporation and on the 3rd, 7th, 15th and 31st day. Samples were suspended in 200 ml of water and stirred for 10 min. One ml of soil suspension (1:100 w/v and 1:1000 w/v) was then plated on PARP medium to assess the effect on *P. ultimum*, in Petri dishes, three replicates per each tray. The same suspension was further diluted (1:10000 w/v) and 1 ml was plated on TSM, three replicates, to assess the effect on *Trichoderma.* The c.f.u. g^{-1} of soil were evaluated, after 48 h incubation at 19 °C for *P. ultimum* and 5 days at 25 °C for *Trichoderma*. The experiment was repeated.

A bioassay was carried out on the same trays, sowing sugar beet seeds not coated with fungicide (cv Dardo) 21 days after meal incorporation (25 seeds × two replicates) under controlled conditions (25 °C, 100% RH). The seed germinability was higher than 90%. Disease incidence was evaluated counting the number of alive and dead plants from the 6th (emergence) up to the 10th day after sowing. The experiment was repeated, sowing sugar beet seeds in the same trays, having checked that the *inoculum* level of *P. ultimum* was comparable to that existing before the first sowing.

2.6. Statistical methods

Data were submitted to analysis of variance after checking error variance homogeneity. Means were separated by Fisher's protected least significant difference test at $p \leqslant 0.05$ significance level, using Statgraphics plus 5.1 statistical program (StatPoint Inc., Herndon, Virginia, USA).

3. Results

3.1. In vitro tolerance to volatile biocidal compounds from BCSM

Visible hyphal extension for all *Trichoderma* and pathogen isolates was observed after 24 h in the absence of the

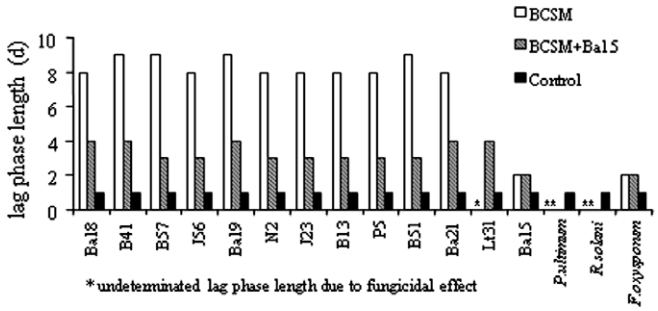

Fig. 1. Lag phase length (*d*) of 13 *Trichoderma* isolates, *Pythium ultimum*, *Rhizoctonia solani* and *Fusarium oxysporum* cultured on PDA at 22 °C in the presence of 66 mg of wetted *Brassica carinata* seed meal (BCSM) and BCSM + Ba15 *Trichoderma* isolate, in comparison to control. Dishes were opened on the 7th day to remove toxic volatiles.

meal. The dose of 33 mg (5 μmole of sinigrin) of BCSM allowed the growth of all the *Trichoderma* isolates assayed (data not shown) whereas the dose of 66 mg (10 μmole of sinigrin) was more discriminating. Out of the 40 isolates tested, only Ba15 (*T. koningii*) showed tolerance to the highest dose of volatiles released by wetted BCSM. In fact it showed only a moderate growth delay with a lag phase of 48 h in comparison with 24 h of the control (Fig. 1).

A strong fungistatic effect was observed for all the other isolates, revealed by longer lag phases (the time period needed before colonies show measurable growth): eight isolates only began to grow on the 7th day (P8, Ba12, Lt1/05, Ba24, ISCI86/6, K15, Lt36 and Bf2) while the majority of the isolates were only able to grow 1 or 2 days after dish opening on the 7th day (data not shown). A fungicidal effect was observed for three isolates, namely 3B24, Ba13 and Lt31 which were still unable to grow even 2 days after removal of the parafilm®M, as also seen for *R. solani* and *P. ultimum* (Fig. 1). On the contrary, *F. oxysporum* tolerated BCSM volatile exposure, showing a moderate fungistatic effect, (48 h lag phase) (Fig. 1).

Ba15 seemed to favor the growth of some other *Trichoderma* isolates when grown together in the same Petri dish in the presence of wetted BCSM. To assess this effect, a dedicated experiment with the same protocol as above was set up on 13 *Trichoderma* isolates chosen among those which had revealed fungistatic (the majority) or fungicidal (Lt31) effects when exposed to wetted BCSM and thus cultured in Ba15 absence/presence (Fig. 1). A similar test was carried out to verify this effect on *P. ultimum*, *R. solani* and *F. oxysporum*. The results reported in Fig. 1 show a considerable reduction of the lag phase length in the presence of Ba15 for those isolates on which a fungistatic effect had been observed, and overcoming of the fungicidal effect for Lt31. The same effect was not observed on *P. ultimum* and *R. solani*, which were still killed by toxic volatiles even in the presence of Ba15, while the *F. oxysporum* lag phase was not affected by the presence/absence of Ba15 (Fig. 1).

3.2. Determination of AITC in Petri dish headspace

The concentration of AITC released by wetted BCSM in the presence of actively growing Ba15 isolate underwent a progressive reduction to undetectable levels, as shown in Table 2. A lesser decrease in concentration over time was also recorded for the control and for the dishes containing N2 plugs (Table 2).

The previously observed behavior of *Trichoderma* growth was confirmed, namely Ba15 tolerance and fungistatic effect on N2 up to the 4th day. In addition, Ba15 showed a greater mean colony area in the Petri dishes containing three plugs than in those with one plug (Table 2).

3.3. Ability to colonize BCSM

The pathogens were unable to grow on active BCSM (data not shown), while *Trichoderma* isolates showed variable responses. The lag phase varied from 4 to 9 days and among those which showed the shortest lag phase (4d), only six isolates (Lt35, X44, Regione, B13, Lt31 and N2) grew more than 10 mm per day (Table 1).

Some *Trichoderma* isolates showed a red colored reaction after just a few days, probably due to a toxic effect, while a fungicidal effect was observed on two of them, Ba19 and Ba21. A red coloration of the plug was also observed for *R. solani*.

The assay performed on inactivated BCSM showed that *Trichoderma* isolates were mostly stimulated by BCSM, albeit at variable levels. Fig. 2 reports the growth variation

Table 2
Number of *Trichoderma* agar-plugs per dish, mean colony area (cm^2 per plug) and allyl-isothiocyanate (AITC) concentration ($mg\,l^{-1}$) in Petri dishes containing wetted *Brassica carinata* seed meal (66 mg) and Ba15 or N2 *Trichoderma* isolates or the combination of the two, in comparison to control (wetted meal only), after 30 and 100 h at 22 °C

Thesis	Plugs per dish (*n*)		Colony area (cm^2 per plug)				AITC ($mg\,l^{-1}$)[a]	
	Ba15	N2	30 h		100 h		30 h	100 h
			Ba15	N2	Ba15	N2		
Control	—	—	—	—	—	—	0.168a	0.070a
Ba15	3	0	1.77	—	15.90	—	0.050d	0.000c
Ba15 + N2	1	2	0.38	0.20	7.07	0.20	0.071c	0.000c
N2	0	3	—	0.20	—	0.20	0.128b	0.048b

[a] Means in columns followed by the same letter are not significantly different ($p \leqslant 0.05$) according to Fisher's protected least significant difference test.

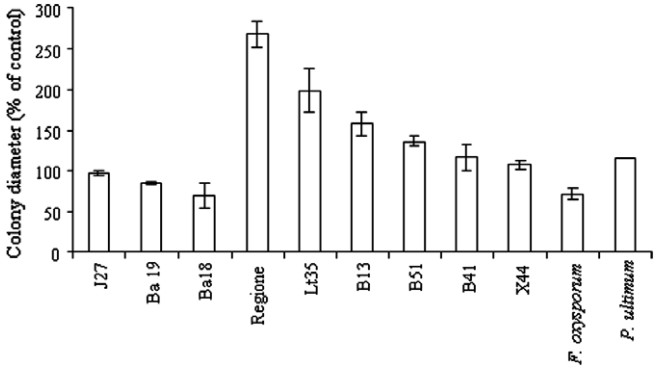

Fig. 2. Colony growth variation of some representative *Trichoderma* isolates cultured on *Brassica carinata* seed meal as well as of *Fusarium oxysporum* (after 3 days) and *Pythium ultimum*, (after 2 days) at 22 °C, expressed as % of control on water agar. Vertical bars represent the mean ± s.d.

as percentage of control on water agar of the most representative ones as well as the opposite behavior of *P. ultimum* and *F. oxysporum*, the first one being highly stimulated and the second one being somewhat inhibited.

3.4. Ability to grow over soil-borne pathogens

The dual growth method showed the differential ability to grow over *R. solani* and *F. oxysporum*. Most isolates could grow and sporulate over the pathogens, even if not always completely. Some of them provoked a heavy darkening of the *F. oxysporum* colony or the appearance of a band in the contact zone as in the case of *R. solani* (Table 1).

With regard to three isolates not able to grow over *F. oxysporum* (namely Ba9, K15 and P8), a distinct "inhibition band" (area of absent growth for both organisms) was found between the colonies under dual growth.

All the isolates were able to grow and sporulate over *P. ultimum*, except for Ba19 and Ba15 which also remained confined by this pathogen.

3.5. Soil experiment

The population of *Trichoderma* in soil remained stable in the time course regardless of the seed meal addition, with a number of c.f.u. g^{-1} of soil over 1×10^5 (data not shown). Fig. 3 shows the trends for the *P. ultimum* population throughout the experiment, before and after the different treatments. The pathogen population decreased 2 days after the active meal treatment (A) occurred on day 1, but then rapidly increased up to values significantly higher than the untreated control (C). In two weeks the pathogen population reached maximum values and tended to become stable. The same occurred in the presence of *Trichoderma* (T + A). The addition of not active meal also increased pathogen c.f.u. per g^{-1} of soil regardless of *Trichoderma* presence (NA, T + NA), but at a lesser extent to active meal.

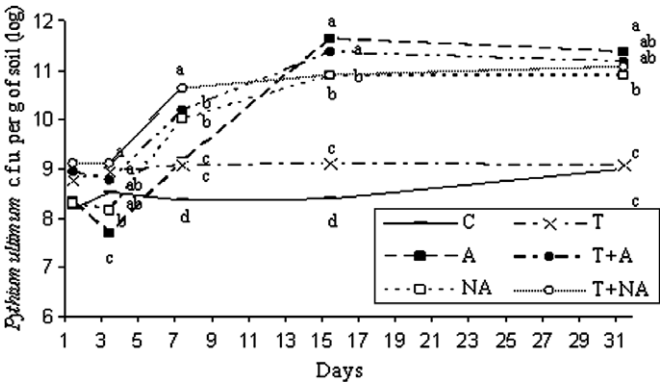

Fig. 3. Trends of *Pythium ultimum* population (log c.f.u. g^{-1} of soil) after the different treatments until the 31st day. BCSM was incorporated on day 1. C, untreated control; A, active *Brassica carinata* seed meal; NA, not active *B. carinata* seed meal; T, *Trichoderma* isolate N2; T + A, *Trichoderma* N2 + active *B. carinata* seed meal; T + NA, *Trichoderma* N2 + not active *B. carinata* seed meal. Values labeled with the same letter do not differ for $p \leqslant 0.05$ (Fisher's protected least significant difference test).

On the other hand, *Trichoderma* treatment (T) did not exert any reducing effect on the pathogen population, which slightly increased on the 7th day but again reached the value of the untreated control at the end of the experiment.

The effects of the treatments on the sugar beet plantlets are reported in Fig. 4. Sugar beet seedlings started to emerge on the 6th day from sowing, but rapidly began to die in the untreated control (C) with 70% of disease incidence on the 7th day. All the treatments produced a protective effect, clearly evident soon after emergence although at different degrees. On the 10th day from sowing, amending the soil with active BCSM (A) or with the spore suspension of *Trichoderma* (T) reduced the disease incidence by about

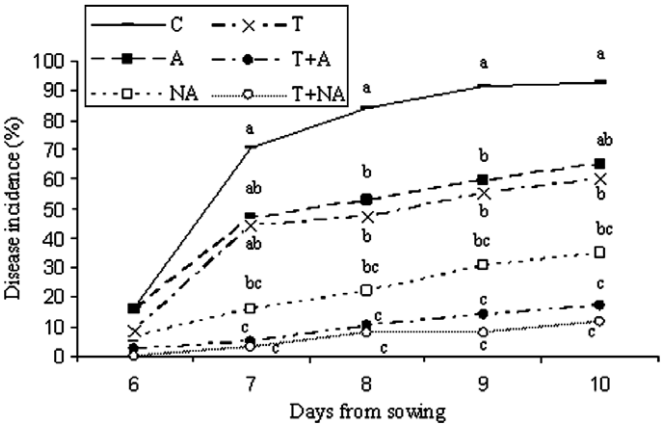

Fig. 4. Trends of mortality of sugar beet plantlets after the different treatments from the 6th up to the 10th day from sowing. C, untreated control; A, active *Brassica carinata* seed meal; NA, not active *B. carinata* seed meal; T, *Trichoderma* isolate N2; T + A, *Trichoderma* N2 + active *B. carinata* seed meal; T + NA, *Trichoderma* N2 + not active *B. carinata* seed meal. Values labeled with the same letter do not differ for $p \leqslant 0.05$ (Fisher's protected least significant difference test after arcsin transformation).

30% compared to the untreated control, which reached a value of more than 90% (C). A further statistically significant decrease (by 60%) was achieved by not active meal addition (NA).

The best results were achieved when *Trichoderma* and meal incorporation were combined. This approach exerted a clear protective effect, reducing the disease incidence up to values of 10–15%, independently of the activity of the meal (T + A, T + NA) (Fig. 4).

4. Discussion

This study reports the results of the effect on BCSM towards a number of *Trichoderma* isolates and three soil-borne pathogens, not only by assaying the volatiles in a sealed environment under strictly *in vitro* conditions, but also through a more direct assay which also comprised contact residual effects, testing the BCSM colonization ability in unsealed Petri dishes. The results obtained made it possible to select a *Trichoderma* isolate to be utilized in combination with BCSM in a preliminary soil experiment for the control of sugar beet damping off caused by *P. ultimum*, under controlled conditions.

4.1. In vitro tests

The findings obtained from testing up to 40 *Trichoderma* isolates suggest that the beneficial fungus is less sensitive to the toxic volatiles released by the meal than the tested pathogens, even if the last ones were only tested in mycelial form. *R. solani* or *P. ultimum* can in fact survive in soil as sclerotia or oospores, respectively. Recent studies have demonstrated a higher tolerance to isothiocyanates of *R. solani* hyphae originating from sclerotia in comparison to hyphae from PDA plugs (Yulianti et al., 2006).

Higher pathogen sensitivity was also recently demonstrated by Larkin and Griffin (2007), operating with chopped fresh *Brassica* tissues, while Sanchi et al. (2005) showed a greater tolerance by the commercial *Trichoderma* isolate T39 at a BCSM dose corresponding to 5 μmole of sinigrin, in comparison to *Sclerotinia sclerotiorum* and *S. minor*.

The different fungistatic effects observed in this study at a double dose (10 μmole of sinigrin) suggest that, once toxic compounds have disappeared, *Trichoderma* inhabiting or introduced in the soil could be able to start growing, albeit with different lag times. The assay carried out in direct contact with active BCSM, to test an overall effect of wetted BCSM, did in fact reveal good tolerance towards the biocidal compounds and ability to grow rapidly for six out of the 40 isolates, as shown by a moderate delay in the growth (4d) and greater growth speed (>10 mm per day) (Table 1). However, since stress reactions such as red coloration and fungicidal effect were shown for some *Trichoderma* isolates, both as a consequence of volatile exposure and of direct contact with the meal, it cannot be completely excluded that meal incorporation into the soil could negatively affect *Trichoderma* population dynamics.

The assay performed on autoclaved BCSM, which prevented myrosinase from hydrolyzing sinigrin and giving the corresponding isothiocyanate, revealed that most of the tested *Trichoderma* were even stimulated by the meal over water agar in comparison to control on solely water agar (Fig. 2).

The peculiar behavior of the Ba15 isolate, which did not show any ability to grow over the pathogens but was able to reduce AITC concentration, thus decreasing the lag time of other *Trichoderma* isolates, suggests that a similar effect could occur in soil after BCSM incorporation. Ba15 activity seems to be linked to the ability to actively reduce AITC concentration. In fact no significant linear correlation was found between the total mycelial area (Ba15 + N2) and AITC concentration, either at 30 or 100 h ($R^2 = 0.58$ and 0.42, respectively), which would have accounted for a generic adsorption of the molecule by the mycelium. In addition, no growth stimulation was observed on other *Trichoderma* isolates when cultured in the same Petri dish in the absence of the meal. Further investigations are needed to elucidate the mechanisms at the basis of the reduction observed. This effect could be linked to the emission of volatile compounds able to biochemically interact with the AITC released by the meal or to the ability to metabolize it inside the mycelium, lowering the biocidal compound to a concentration not toxic for *Trichoderma*, but still lethal for the tested pathogens (*R. solani* and *P. ultimum*). This favorable situation observed *in vitro* could become dangerous in soil, where the reduction of volatile toxins by similar strains of fungi could decrease the effectiveness of biofumigation against target pathogens. The effectiveness of this technique in field conditions does in fact vary to a certain extent. One of the causes has been ascribed to possible negative interactions with resident micro-organisms. Low values or quick decreases of glucosinolates and their hydrolysis derived products in the soil after biofumigation have been reported (Borek et al., 1995). The phenomenon has frequently been attributed to the micro-organism action without clear recognition of the possible causes (Leoni et al., 2004; Rumberger and Marschner, 2003; Smelt et al., 1989; Warton et al., 2003).

The slow decrease in AITC concentration observed over time in the control dishes and in the dishes containing N2 plugs could be explained as a consequence of an interaction of the AITC with the sulfidryl groups, disulfide bonds and amino groups of protein and amino-acid residue of the meal and the fungus mycelium (Kojima and Oawa, 1971; Banks et al., 1986).

Further studies are needed to verify whether this mechanism of reduction of AITC concentration by Ba15 or similar *Trichoderma* spp. is also active in soil and whether it is able to affect biofumigation potential.

With regard to the pathogens, *F. oxysporum* behaved in a different way to the more sensitive *R. solani* and *P. ultimum*, well tolerating volatile compounds at the highest

meal dose (66 mg) as well as Ba15. This lower sensitivity was already reported by some authors (Manici et al., 1999; Larkin and Griffin, 2007). When kept in direct contact with active wetted BCSM, a fungicidal effect was instead observed for *F. oxysporum*, while most *Trichoderma* isolates were able to grow, again confirming their superior ability to survive and grow in the presence of high doses of BCSM.

On the other hand, the effects observed on the pathogens in the assay on inactivated BCSM suggest that under field conditions, where BCSM obviously can not be as effective as in a Petri dish, once AITC has disappeared, meal could represent a stimulating factor for *P. ultimum* rather than *F. oxysporum* which appeared not to take advantage of the presence of the meal (Fig. 2). Stimulation of *Pythium* populations in soil was already reported in experiments with seed meal incorporation of low glucosinolate canola and *Brassica napus* (Mazzola et al., 2001, 2007; Cohen and Mazzola, 2006).

The general effect of stimulation on most of the *Trichoderma* isolates tested confirms once again the possibility of competing with the pathogens for meal colonization.

These findings suggested good possibilities for a combined use of *Trichoderma* as a biological control agent and the biofumigation technique through BCSM incorporation into the soil, and a preliminary soil experiment was thus carried out under controlled conditions. Regarding the selection of an isolate to be used in combination with the meal, we had to exclude Ba15, which tolerated the higher dose (10 μmole) of toxic volatiles from the meal very well, but presented other negative features. In fact Ba15 grew very slowly in direct contact with active BCSM, could not grow over any of the pathogens and was able to reduce AITC concentration, with possible detrimental effects on biofumigation effectiveness in practice.

The assays performed among the 40 *Trichoderma* isolates highlighted other candidate isolates which at the same time showed features of high tolerance to biocidal compounds at the dose of 5 μmole of sinigrin, even if a fungistatic effect was observed at the double dose. Some of them were also able to grow rapidly on BCSM, even being stimulated in some cases, and to grow over the considered pathogens. Among these isolates, N2 was chosen for a preliminary experiment in soil in combination with BCSM incorporation to control sugar beet damping off caused by *P. ultimum*.

4.2. Soil test

Apart from an initial slight effect, BCSM incorporation, regardless of the addition of *Trichoderma*, was ineffective in controlling *P. ultimum* population in soil, even if the meal contained a high level of one of the most active glucosinolates, i.e. sinigrin. A positive effect, similar to what was observed by other authors with *Brassica juncea*, rich in sinigrin, was instead expected (Mazzola et al., 2007). On the other hand the observed increase in *P. ultimum* population following meal application is consistent with experiments reported in the literature, with low glucosinolate canola or *B. napus* seed meal application (Mazzola et al., 2001; Cohen and Mazzola, 2006). In our case the stimulation of *P. ultimum* population seems to be closely linked to the amount of added organic matter, since even the incorporation of not active BCSM exerted an increase of the pathogen population.

The solely *Trichoderma* application, on the other hand, did not alter the pathogen population, but this beneficial fungus remained at stable level of *inoculum* over time, showing a good ability to survive in the conditions of the experiment.

The bioassay with sugar beet gave surprising results. Notwithstanding the increase in pathogen population, which caused a rapid damping off of the seedlings in the untreated control soon after emergence, all the treatments exerted a more or less marked degree of protection.

The effect of BCSM incorporation seems to be independent of its biofumigating potential, since disease incidence was not statistically different following active or not active BCSM applications. Indirect mechanisms, such as the stimulation of the resident beneficial microbial community, could thus be kept in mind (Mazzola et al., 2001, 2007). In addition, a visible white punctiform florescence was always observed on the soil surface already on the 2nd day after BCSM incorporation in all the experiments. Microscopic observations revealed cell aggregates similar to those of actinomycetes.

BCSM incorporation reduced the disease incidence at the same extent as solely *Trichoderma*, but when the approach was integrated, the effectiveness was maximised.

The most effective treatments were in fact represented by BCSM applied in the presence of *Trichoderma*, again irrespectively of meal activity, with a disease reduction to the level of only 10–15% in comparison with more than 90% of the control.

The additional protective effect exerted by *Trichoderma* could be due to the ability to colonize the sugar beet rhizosphere, as it was checked at the end of the experiment by plating surface sterilized rootlets of some healthy sugar beets on TSM. In fact, after a few days of incubation a large amount of *Trichoderma* mycelium was observed spreading from the entire rootlet surface. Even if competition through rhizosphere competence may not be among the principal mechanisms that drive biological control, it is considered a valuable adjunct (Howell, 2003). Recent discoveries indicate that some root colonizing strains are also able to produce compounds that induce in plants localized or systemic resistance responses to a variety of plant pathogens (Harman et al., 2004).

The findings of this study could be explained in terms of an additive effect of the two methods of biological control, which made the approach truly effective.

Further investigations are needed to evaluate the effect of this integrated approach with different *Brassica* or non *Brassica* meals, on other patho-systems and finally in field conditions.

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