Allelopathic Effect of Glucosinolate-containing Plant Green Manure on Pythium sp. and Total Fungal Population in Soil

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Abstract. Two Brassicaceae (Iberis amara L. selection ISCI14 and Rapistrum rugosum All. selection ISCI4) and a Capparidacea (Cleome hassleriana L. selection ISCI2) possessing glucosinolates whose degradation products exhibit high fungitoxic activity in vitro were assayed as biocidal plants in a green manure simulation. The trials were carried out in pots, using aboveground fresh plant tissues incorporated at a realistic field rate into soil naturally infected by Pythium sp. The effect of these plant tissues on total fungal populations and Pythium sp. were compared with Crambe abyssinica H. cv. Mario, a Brassicaceae containing glucosinolates whose degradation products exhibit low fungitoxic activity in vitro, and a plant (Helianthus annuus L.) not containing glucosinolates. All green manure treatments induced increases in total fungi over a 10-week period, showing an enhanced microflora level compared with untreated soil. Pythium sp. was strongly suppressed by the C. hassleriana, I. amara, and R. rugosum selections, while sunflower and crambe treatments increased Pythium sp. in a manner similar to that observed for total fungal population. These findings indicate that the green manures assayed suppress Pythium sp. and also induced an increase in total soil microbial activity.

The production of several vegetable crops depends on the use of methyl bromide (MB) soil fumigation to control a wide array of soilborne fungi, nematodes, insects, and weeds. In accordance with the U.S. Clean Air Act, the use of MB as a fumigant will be banned in developed nations by 2005 (United Nations Environment Programme, 1992). This decision has prompted increased interest in the development of alternative strategies with a lower environmental impact to control soilborne pathogens.

One alternative approach might be provided by the well-known fungitoxic activity of enzymatic hydrolysis-derived products (DPs) of glucosinolates (GLs), natural compounds of the Brassicaceae (Walker et al., 1937). Their high biological activity has been observed against various fungi using parts of cruciferous plants (Charron and Sams, 1999), crude extracts (Mayton et al., 1996), cruciferous meal (Smolinska et al., 1997), and also purified GLs hydrolyzed by pure myrosinase (MYR) (Mari et al., 1993). The presence of GLs (Sang et al., 1984) and MYR in Brassicaceae plant organs suggests the possibility of amending soil with fungicidal compounds by using these plants as green manure. In healthy plants, GLs and MYR are located in different cell compartments and come into contact only after cell collapse, such as occurs following a pathogen attack or when plants are chopped up, with the consequent production of fungicidal GL-DPs (isothiocyanates, thiocyanates, and/or nitriles) in soil. Brassicaceae green manures have been shown to be active against Fusarium oxysporum f. sp. conglutinans (Ramirez-Villapudua and Munnecke, 1988), Verticillium dahliae (Subbarao and Hubbard, 1996), Aphanomyces euteiches (Chan and Close, 1987; Muehlchen et al., 1990), Thielaviopsis basicola (Adams, 1971), Pythium ultimum and Sclerotium rolfsii (Gamliel and Stapleton, 1993), and to strawberry root rot (Lazzeri et al., 1999). Many Brassicaceae used in these studies are commonly available Brassica species, such as cabbage (Brassica oleracea L. var. capitata L.), Indian mustard (B. juncea L.), yellow seed mustard (B. juncea L. ssp. trilocularis Olsson), and rapeseed (B. campestris L.), as well as other common Brassicaceae, like white mustard (Sinapis alba L.) and oil radish (Raphanus sativus L.).

Recent studies carried out in vitro with purified GLs and MYR on Fusarium culmorum have shown that the fungicidal activity of DPs varies according to the characteristics of the GL side chain. The DPs from glucoiberin, glucocheirolin, and glucoerucin (GLs with an extra S atom in their side chain [thiofunctionalized glucosinolates (GLThio)]) and from glucocapparin (methyl-glucosinolate) have shown significantly higher fungicidal activity than DPs obtained from aliphatic or hydroxy GLs (Manici et al., 1997). Again in vitro, these GL-DPs showed suppressive activity against some widespread soilborne fungi (Rhizoctonia solani, Pythium sp., and others) (Manici et al., 1999), supporting the hypothesis that these molecules can be used as natural biofumigants. The quality and quantity of GLs in cruciferous plant organs vary according to the genera, species, and, in many cases, the variety (Rosa et al., 1997). Therefore, the biocidal activity of a green manure may vary with the quality and quantity of the GL content of the green manure plant species (Smolinska and Horbowicz, 1999). In recent years, at the Research Institute for Industrial Crops of Bologna, some ecotypes of the Brassicaceae family (Iberis amara L., Rapistrum rugosum All.) and one of the Capparidaceae (Cleome hassleriana L.) were selected for their high content of GL whose DPs have exhibited strong activity in vitro.

The aim of this study was to investigate the allelopathic effect in soil of GL-containing plant green manure. It remains to be clarified as to how much the suppressive effect of cruciferous amendments on soilborne pathogens is due to the fungitoxic effect of GL-DPs (Manici et al., 2000) or the stimulation of microbial activity (Gamliel and Stapleton, 1993; Mazzola, 2000). Therefore, we evaluated variations in Pythium sp. population, a pathogen very sensitive to GL-DPs (Manici et al., 1999), and those of the total fungal population, one of the several components of the soil microbial activity.

Materials and Methods

In 1997 and 1998, fresh plant tissues of Iberis amara selection sel. ISCI14 (iberis), Rapistrum rugosum sel. ISCI4 (rapistrum), and Cleome hassleriana sel. ISCI2 (cleome), with a high content of strongly active GL-DPs, were incorporated into a naturally infested soil in pots. Crambe abyssinica H. cv. Mario (crambe), a species containing GLs producing low fungitoxic DPs, and Helianthus annuus L. cv. Goloriasol (sunflower), a plant that does not contain GLs, were inserted in the experiments as nonbiocidal green manure controls.

Green manure crop production. In both years, the green manure crops were cultivated in the Po Valley (Budrio, Bologna, Italy; continental climate), in plots (15 m2) arranged in a randomized block design, with three replicates. The trials were performed on a silt loam soil (clay 26%, silt 54%, sand 20%) with a high content in available potassium (240 ppm) and average content in phosphate (35 ppm) and organic matter (1.8%). At sowing time, 80 kg ha⁻¹ of N (ammonium nitrate) and 100 kg ha⁻¹ of sulfur were applied. Biocidal plants were sown in the first week of March, with a

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density of ~250 seeds/m², in rows 28 cm wide for rapistrum and crambe and 14 cm wide for iberis and cleome. During cultivation, no irrigation water and pesticide treatments were applied. At full flowering, the fresh biomass yield (t ha⁻¹) of aboveground parts was evaluated and the dry matter (DM) content (%) was measured by oven-drying a plant sample at 105 °C overnight.

Glucosinolate and myrosinase determination. At full flowering, a sample of each GL-containing plant was weighed, immediately frozen at ~20 °C, and subsequently freeze-dried using an Edwards Minifast D1 freeze-drier (from ~40 to ~18 °C in 8 h with a vacuum of 10⁻¹ mbar). The freeze-dried materials were then homogenized with a mortar and their GL content was determined following the procedure proposed for rapeseed analysis in the Official Journal of the European Community (1990), with some minor modifications (Visentin et al., 1992). A Hewlett Packard high-pressure liquid chromatograph (HPLC) model 1090L equipped with a diode array detector and a 200 × 4.6 mm 5-µm column HP ODS Hypersil C18, was used. A CH₃CN/H₂O (1:22) gradient was used at the flow of 2 mL min⁻¹.

To confirm the presence of MYR in plant organs, the freeze-dried tissues were homogenized in water (1:2) with an Ultra-Turrax TP18/2K Ika Werk Staufen, Germany, in screw-capped vials and incubated in a water bath at 37 °C for 12 h. The mixtures were then filtered through paper (Whatman 1, England) and the GL-DPs were extracted with CHCl₃ (four extractions). The extracts were dried adding anhydrous Na₂SO₄, filtered on paper, concentrated under N₂ stream, and finally analyzed in GC-MS with a GCD Hewlett Packard model G1800A equipped with a 30 m × 0.25 mm capillary column HP-5 MS. The flow rate of the carrier gas (He) was 1 mL min⁻¹ and the sample (1 µL) was injected in the splitless mode. Column temperature was 40 °C at the start and 220 °C at the end of the analysis, with a rate of 10 °C min⁻¹. The injector and detector temperature were 260 °C. Ionization energy was 70 W and mass spectra were scanned in the 10–425 m/z range. Peaks of GL-DPs were identified using the NBS75K library.

Green manure simulation. Trials were carried out using a silty clay loam soil (39% clay, 49% silt, 12% sand), series Medicina, fine, mixed, mesic Vertic Ustochrepts (Soil Taxonomy, 1994), naturally infested with *Pythium* sp., collected in May 1997 and 1998 in Cesena (eastern Po Valley) from nonfumigated fields, which had been cultivated with strawberry for several years. The field was fallow in 1997. In autumn 1997, burley green manure was planted, followed by strawberry in summer 1998. Soil (pH 8.2) was collected at random from the top 20 cm, partially dried at room temperature for 3 d, and then stored at 8 to 10 °C for 4 d. Before treatment, soil was sieved through a 5-mm mesh screen and homogenized by hand.

Intact aboveground plant tissues were collected at full flowering time, chopped in a razor blender, and immediately incorporated into the soil. Roots were not included, due to difficulty in obtaining complete grinding during plant chopping, which could result in lower GL-DP yields. Plant biomass was applied using a realistic field rate, which was based on the biomass obtained in the field during 1997 (Table 1), without considering roots. Sunflower and crambe, also collected at full flowering time, chopped in a 1-mm mesh screen and homogenized by hand.

Table 1. Glucosinolate content of fresh matter of biocidal selections used in green manure simulation.

| Biocidal selections | Glucosinolates | Biomass incorporated into the soil (g kg⁻¹ of soil) | Glucosinolates added to soil (µmol kg⁻¹ of soil) |
|---------------------|----------------|-----------------------------------------------|-----------------------------------------------|
|                      | Content (µmol g⁻¹ of FM) | Main¹ | Main² | (µmol kg⁻¹ of soil) | DM² | (mg kg⁻¹ of soil) |
| 1997                |                           |       |       |                      |     |                    |
| *Iberis amara* ISCI14 | 4.5 ± 0.4             | IBE 98% | 40 | 7.4 | 0.18 ± 0.02 | 83 ± 8  |
| *Rapistrum rugosum* ISCI14 | 6.1 ± 0.4 | GCH 95% | 60 | 11.1 | 0.37 ± 0.02 | 173 ± 10 |
| *Crambe abyssinica* cv. *Mario* | 4.1 ± 0.4 | E-PRO 90% | 60 | 15.1 | 0.24 ± 0.03 | 103 ± 12 |
| *Cleome hassleriana* ISCI2 | 5.4 ± 0.8 | GCA 90% | 40 | 8.0 | 0.22 ± 0.04 | 79 ± 13 |
| *Helianthus annuus* L. | Absent |       | 60 | 10.8 | --- | --- |
| 1998                |                           |       |       |                      |     |                    |
| *Iberis amara* ISCI14 | 4.5 ± 0.4             | IBE 98% | 40 | 6.8 | 0.18 ± 0.02 | 83 ± 8  |
| *Rapistrum rugosum* ISCI14 | 6.2 ± 0.4 | GCH 95% | 60 | 11.7 | 0.37 ± 0.02 | 173 ± 10 |
| *Crambe abyssinica* cv. *Mario* | 7.0 ± 0.5 | E-PRO 85% | 60 | 14.8 | 0.42 ± 0.03 | 179 ± 12 |
| *Cleome hassleriana* ISCI2 | 8.9 ± 0.6 | GCA 81% | 40 | 8.5 | 0.35 ± 0.03 | 131 ± 10 |
| *Helianthus annuus* L. | Absent |       | 60 | 10.6 | --- | --- |

¹IBE = Glucoberin; GCH = Glucocheiroin; E-PRO = Epi-progoitrin; GCA = Glucocapparin.  
²Dry matter.  
³Mean ± SD.

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Pythium sp. Three plates of selective media were spread with 0.5 mL of 10⁻¹ and 10⁻² soil dilutions according to the modified Jeffers and Martin (1986) method (commeal amended with 5 mg L⁻¹ Pimaricin, 250 mg L⁻¹ Ampicillin, 10 mg Rifampicin, 100 mg of PCNB, and 1 g L⁻¹ Ox-gall). After an incubation time of 24 h at 18 °C in the dark, the soil dilution was washed from plate surfaces under running water, and then *Pythium* colonies were counted. To record the relative *Pythium* sp. frequency, all germinating propagules with typical *Pythium* morphology on PARP+Ox-gall were transferred on PDA + 200 mg L⁻¹ streptomycin sulfate. *Pythium* colonies with common macroscopic and microscopic characters were clustered; the representative isolates of each cluster were identified on the basis of the Waterhouse key (1967) and description (1968). *Pythium* identification was performed, in one case (*Pythium deliensis Meuris*), and confirmed, in another case (*Pythium ultimum Trow. var. ultimum*), by the Centraalbureau voor Schimmelcultures (Baarn, The Netherlands).

*Pythium* inoculum was reported as colony forming units per gram (CFU g⁻¹) of air-dried soil.

Total fungal population: One milliliter of 10⁻² and 10⁻³ soil dilutions was mixed with 49 mL of water agar + 3 g L⁻¹ Ox-gall and 200 mg L⁻¹ streptomycin sulfate, distributed on five (9-cm-diameter) petri dishes, and incubated for 48 h at 24 ± 2 °C under natural light. Colonies were counted by visual observation and expressed as CFU g⁻¹ air-dried soil. Fungal isolates with different morphologies were transferred to PDA + 200 mg L⁻¹ streptomycin sulfate. Colonies were identified on the basis of macroscopic and microscopic morphological characteristics using the relevant taxo-
nomic keys (Nelson et al., 1983; Samson and van Reenen-Hoekstra, 1988; Sutton, 1980). Total fungal population was reported as CFU g⁻¹ of air-dried soil.

Statistical analysis. Data were subjected to one-way analysis of variance and to mean separation by least significant differences (LSD) test at P ≤ 0.05 significance level, using the Statgraphic Plus Program, version 2 (Manugistique, Rockville, Md.).

Results

Agro-technological plant characterization. All plant selections showed good adaptation to spring sowing in the Po Valley pedoclimatic conditions, with sufficient vegetative growth and abundant flowering. The cycle length from sowing to full flowering varied from 78 to 85 d in 1997 and from 95 to 99 d in 1998.

In 1997, at full flowering the biomass yield of aboveground parts alone was 2.7 ± 0.3 kg m⁻² fresh matter (FM) for iberis and cleome and 4.2 ± 0.9 kg m⁻² FM for rapistrum (Table 1). On the basis of these data and with the aim of reproducing in pots the field green manure conditions, the plant biomass incorporated into pots in the two trial years was 40 g kg⁻¹ of soil for iberis and cleome and 60 g kg⁻¹ of soil for rapistrum, crambe, and sunflower (Table 1).

The aboveground tissues of iberis and rapistrum in both years contained, respectively, 4.5 μmol g⁻¹ FM of glucobetin (3-methylsulfinylpropyl-glucosinolate) and >6.1 μmol g⁻¹ FM of glucocochetin (3-methylsulfinyl-propyl-glucosinolate). The GL content of crambe and cleome varied from year to year. Crambe GL content was 4.1 in 1997 and 7.0 μmol g⁻¹ FM in 1998. In both years, it included >85% of Epi-progoitrin (2-hydroxybut-3-enyl-glucosinolate) and 15% of glucocochelin (but-3-enyl-glucosinolate). Cleome GL content was 5.4 in 1997 and 8.9 μmol g⁻¹ FM in 1998. In both years, it contained >85% glucocapparin (methyl-glucosinolate) and 15% glucocleomin (2-hydroxy-2-methylbutyl-glucosinolate) (Table 1).

Endogenous MYR was able to completely hydrolyze GLs, as confirmed by the complete absence of GLs in plant extracts homogenized for 12 h in a water bath at 37 °C (data not shown). Iberis and rapistrum GLs produced primarily the corresponding isothiocyanate while crambe tissues produced primarily vinyl-oxazolidine-2-thione. Cleome produced a mixture of methyl-isothiocyanate and 5-methyl-5-ethyl-oxazolidine-thione.

Effect of green manure on total fungal population. In the two trial years, all five fresh tissue treatments increased the total fungal population in amended soil, compared with untreated soil.

In 1997, total fungal population in the untreated soil did not vary significantly from the level observed in the previous year after 10 weeks. Three weeks after green manure treatments (Fig. 2A), total fungal population in the cleome treatment had increased to levels significantly higher than the other treatments, which, compared with untreated soil, had >200%. After 6 weeks, the fungal population remained >100,000 CFU g⁻¹ dry soil with no differences among the treatments (Fig. 2B). After 10 weeks, crambe and cleome showed a total fungal population significantly higher than iberis and sunflower, although this was not significantly different from rapistrum treatment (Fig. 2C). Even at the last sampling time, than sunflower and crambe (Fig. 1A). After 6 weeks, the total fungal population after iberis, rapistrum, and cleome treatments was very similar, maintaining values significantly higher than crambe and sunflower treatments (Fig. 1B). After 10 weeks, cleome generated the highest value, followed by rapistrum and iberis, while sunflower and crambe generated a significantly lower value (Fig. 1C). Even at this last sampling time, all green manure treatments increased total fungal population from 200% (sunflower and crambe) to 600% (cleome), compared with untreated soil.

In 1998, the total fungal population in untreated soil was higher than in 1997 and decreased strongly from the first (90,000 CFU g⁻¹ dry soil) to last sampling time (21,000 CFU g⁻¹ of dry soil) (Fig. 2A and C), reaching the level observed in the previous year after 10 weeks. Three weeks after green manure treatments (Fig. 2A), total fungal population in the cleome treatment had increased to levels significantly higher than the other treatments, which, compared with untreated soil, had >200%. After 6 weeks, the fungal population remained >100,000 CFU g⁻¹ dry soil with no differences among the treatments (Fig. 2B). After 10 weeks, crambe and cleome showed a total fungal population significantly higher than iberis and sunflower, although this was not significantly different from rapistrum treatment (Fig. 2C). Even at the last sampling time,
In both trial years, 60% to 80% of fungi recovered from soil were *Fusarium oxysporum* Schlecht., *Fusarium solani* (Mart.), *Fusarium equiseti* (Corda) Sacc., *Myrothecium verrucaria* (Albert. & Schweii.: Fries) Ditmar, *Macrocystis hiemalis* Wehmer, *Staphylotrichum coccosporum* J.A. Mayer & Nicot. In addition, a small number of the genera *Aphanomyces*, *Aspergiillus*, *Mortierella*, *Pestalotia*, *Rhizopus*, *Trichoderma*, and a sterile fungus were detected.

**Effect of green manure on Pythium sp.** In untreated soil, the *Pythium* sp. population gradually decreased in both trial years: in 1997 from a starting level of 305 to 22 CFU·g⁻¹ dry soil after 10 weeks (Fig. 3A and C), and in 1998 from 238 to 48 CFU·g⁻¹ dry soil (Fig. 4A and C).

In 1997, at all sampling times, the *Pythium* population with sunflower soil treatment was higher than with all other soil treatments. Even crambe induced a strong increase in the *Pythium* population, but significantly lower than sunflower, whereas cleome, iberis, and rapistrum strongly suppressed *Pythium* sp. (Fig. 3A–C). In detail, 3 weeks after green manure, sunflower and crambe increased *Pythium* to 2200 and 1800 CFU·g⁻¹ dry soil, respectively, while the three biocidal plant selections, cleome in particular, reduced *Pythium* population to a level lower than that of untreated soil. Six weeks after green manure, sunflower and crambe treatments showed an unchanged inoculum level as compared with the first sampling time. Iberis and cleome suppressed *Pythium* population at a level significantly lower than rapistrum, which, however, maintained *Pythium* population at ≈600 CFU·g⁻¹ dry soil (Fig. 3B). Ten weeks after green manure, *Pythium* population decreased strongly with all treatments even if there was still a significantly higher inoculum level with sunflower and crambe. Crambe was not statistically different from rapistrum, while iberis and cleome were again the most effective, suppressing *Pythium* to values <40 CFU·g⁻¹ dry soil (Fig. 3C).

Even in 1998, at all sampling times, sunflower and crambe increased the *Pythium* population to levels significantly higher than the three biocidal selections (Fig. 4A–C). Three and 6 weeks after green manure, in fact, sunflower and crambe increased *Pythium* population to values >3000 CFU·g⁻¹ dry soil without significant differences. As in 1997, iberis, rapistrum, and cleome decreased *Pythium* populations to levels lower or similar to that of untreated soil, showing no significant differences among them at three subsequent sampling times (Fig. 4A–C). After 10 weeks, the inoculum decrease with sunflower and crambe treatments was not as strong as in the first year.

The *Pythium* population was represented by two main clusters, one *P. deliense* and the other *P. ultimum* var. *ultimum*. Those two phytopathogenic species were the only ones isolated from soil samples in this 2-year trial.

**Discussion**

In recent years, interest has increased in the use of cover crops to control soilborne pathogens (Elmer and LaMondia, 1999; Nunez, 1999). On the other hand, a well-known biological effect of amending soil with fresh plant tissues is the rapid increase in soil microbial biomass (Ploetz et al., 1985), of which fungi are commonly the largest component (Anderson and Domsch, 1975), and represent an important soil fertility parameter (Lynch and Panting, 1980).

In this study, two phytopathogenic *Pythium* species, *P. ultimum* and *P. deliense*, were isolated from amended and nonamended soil samples. *Pythium ultimum*, one of the most widespread agents of damping-off and root rot in annual and perennial crops, has also been reported to be an agent of strawberry black root rot (Watanabe et al., 1997). *Pythium deliense* is a less common *Pythium* species and is reported as an agent of damping-off of tomato seedling and many other crops.
good level of GLs (lar effect even though it provided populations. Crambe green manure had a similar effect in field soil. 

Sunflower green manure, included in the trials as a plant that does not contain GLs, confirmed the rapid response of pathogenic and nonpathogenic saprophytic fungi to the availability of a fresh organic substrate. At the same time, the suppression of Pythium by the ISCI biocidal selections was clearly evident. This effect was probably due to GL-DPs released during plant chopping, whose fungitoxic effect widely overcame the stimulant effect of organic matter amendment. The increase in total fungal population and contemporary decrease in Pythium, observed with GL containing plant treatments, may be due to the high sensitivity of Pythium to GL-DPs and to the low sensitivity of Deuteromycetes, the most common of the fungi recovered (Manici et al., 1997, 1999).

Crambe green manure clearly increased the Pythium population in a manner similar to that of sunflower. This suggests that plants containing GLs producing DPs with low fungitoxic activity can generate an increase in Pythium populations similar to that of conventional green manure. On the contrary, the ISCI selections reduced Pythium to values lower than untreated soil, with a clear fungitoxic effect, confirming that the biofumigant effect in soil depends not only on the amount of GLs, but above all on the fungitoxicity of their DPs.

This study in potted, naturally infected soil was planned to simulate green manure in the open field, even if the controlled laboratory conditions probably improved both the fresh plant tissue biofumigant effect and the organic matter decomposition process. Fresh tissues were in fact incorporated into the soil in a partially closed system (pots) that, probably, reduced the GL-DPs loss compared with the open field. Furthermore, the fine plant tissue grinding and the soil watering generated a rapid GLs hydrolysis, improving the fungitoxic effect of biocidal green manure, enhancing the response of Pythium and total fungi to fresh biomass addition. Therefore, the changes in Pythium sp. and total fungal populations in our experiments were probably faster than in the field conditions. In addition, the silty clay loam soil from east Po valley, a vegetable-growing area of northern Italy, was probably aerated more in pots than under field conditions, thus further enhancing fresh biomass decomposition. For this reason, in soils with a similar texture, green manure of plants selected for GLs content may be useful in low input or organic farming horticulture, where green manures are commonly adopted. In fact, the suppression of the Pythium population in soil observed during the first weeks after fresh tissue incorporation into the soil, could prevent the rapid increase of Pythium, thus reducing the period in which this pathogen can cause damping-off or root rot of seedlings and young plants.

Pythium survives as a saprophyte in competition with other microorganisms (Chen et al., 1988) and so the strong increase in soil fungal populations may represent an addi-
Conclusions

The suppressive activity toward Pythium spp. and, at the same time, the increase in total fungal population after rapistrum, iberis, and cleome green manure suggest additional benefits to the well-known effects of incorporating organic matter into the soil.

These preliminary results, together with the interest in new, ecologically sustainable methods for the control of soilborne fungi, both in organic agriculture as well as an alternative to MB in conventional agriculture, open interesting, practical prospects for the use of biocidal green manure even under field conditions.

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