

Resident bacteria, nitric oxide emission and particle size modulate the effect of *Brassica napus* seed meal on disease incited by *Rhizoctonia solani* and *Pythium* spp

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Abstract Amendment of orchard soil with low-glucosinolate *Brassica napus* (rape) seed meal (RSM) suppresses infection of apple roots by *Rhizoctonia solani* but increases incidence of *Pythium* spp. infection. Following incorporation of *Brassica* sp. seed meals, soils were monitored for changes in populations of selected saprophytic and plant pathogenic microorganisms. When conducted in pasteurized soil, which possessed high numbers of *Bacillus* spp. and lower than detectable numbers of *Streptomyces* spp., RSM amendment did not provide control of *R. solani*. Populations of streptomycetes in RSM-amended soil increased to stable levels >20-fold higher than in non-amended soil. Disease suppressiveness was restored to pasteurized RSM-amended soil by adding any of several *Streptomyces* strains. Maximal rates of nitrification in orchard soil, determined by nitric oxide emission, were observed within two weeks following RSM amendment and inhibition of nitrification via application of nitrapyrin abolished the capacity of RSM to sup-

press *R. solani* infection of apple roots when seedlings were planted one day after soil amendment. Apple seedling mortality and *Pythium* spp. root infection were highest for seedlings planted immediately following incorporation of *B. napus* cv. Athena RSM, particularly when meal was added in a flake rather than powder form. Lower infection frequencies were observed for seedlings planted four weeks after RSM incorporation, even for soil in which densities of culturable *Pythium* spp. had not declined. Our results demonstrate that suppression of *Rhizoctonia* root rot in response to RSM amendment requires the activity of the resident soil microbiota and that initial disease control is associated with the generation of nitric oxide through the process of nitrification.

Keywords Apple · Disease suppression · Nitrification · *Pythium* · *Rhizoctonia* root rot · *Streptomyces*

Introduction

Replant disease of apple is caused by complexes of root-pathogens, often including the fungus *Rhizoctonia solani* (Mazzola 1998), that become established in mature apple orchards and infect trees subsequently planted into the same soil. The disease induces substantial long-term productivity

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losses and is a major impediment to the establishment of economically viable orchards on sites previously planted to apple or related tree crops. Amendment of soil with *Brassica napus* (rape) seed meal (RSM) prior to planting apple can reduce replant disease symptoms (Mazzola and Mullinix 2005). Plants cultivated in RSM-treated soil show lower incidences of infection by *R. solani* and infestation by *Pratylenchus penetrans* (Cohen et al. 2005; Mazzola et al. 2001), and substantially higher productivity (Mazzola et al. 2001; Mazzola and Mullinix 2005) compared to plants in non-treated soils.

Typical of most organic amendments, addition of certain RSM varieties can stimulate increases in populations of *Pythium* spp. that are capable of causing disease, particularly in new plantings (Hendrix and Campbell 1973). This increase can be prevented by utilizing RSM containing sufficient amounts of glucosinolates to yield upon hydrolysis quantities of isothiocyanates toxic to *Pythium* spp. (Mazzola et al. 2001). However, breeding programs have developed several varieties of low-glucosinolate *B. napus* that now represent a major share of the RSM market. Although use of high glucosinolate content RSM is a potential alternative, glucosinolate content is influenced significantly by climatic conditions and can vary widely for the same plant variety. For instance, in our studies glucosinolate content for seed meal of the variety Dwarf Essex obtained from the same source over multiple years ranged from 20 to 113 $\mu\text{mol g}^{-1}$ (Mazzola et al. 2001; Mazzola and Mullinix 2005). Preventing proliferation of *Pythium* spp. after incorporating such RSM can be accomplished by application of the soil drench mefenoxam, the more active enantiomer contained in the racemic anti-oomycete agent metalaxyl. In cases of orchard renovation, amendment of soil with low-glucosinolate RSM in concert with a post-plant mefenoxam soil drench reduces replant disease symptoms, often to an extent equivalent to that obtained in response to chemical soil fumigation (Mazzola and Mullinix 2005). However, soil treatment with mefenoxam, though less onerous than broad-spectrum fumigants such as methyl bromide, is not compatible with organic management practices and is not a long-term

solution since oomycetes develop insensitivity to mefenoxam (Mazzola et al. 2002; Taylor et al. 2002). Thus, one goal of this study was to better define the conditions that contribute to *Pythium* spp. virulence in RSM-amended soil as a first step in the development of sustainable procedures for the management of disease inciting oomycetes in new plantings.

In contrast to *Pythium* spp., factors other than glucosinolate hydrolysis products appear to function in the control of *R. solani* observed in response to RSM soil amendment. RSM-amended soil retains the capacity to suppress Rhizoctonia root rot of apple for at least months after incorporation into soil, long after isothiocyanates from glucosinolate hydrolysis have dissipated, and amending soil with low-glucosinolate RSM is just as effective as high-glucosinolate RSM in suppressing *R. solani* infection of apple roots (Mazzola et al. 2001). Furthermore, growth of *R. solani* is not inhibited in RSM-amended soil, which implies that the mechanism of suppression is not based on antibiosis per se (Cohen et al. 2005). In fact, RSM-mediated protection of apple seedlings against *R. solani* appears to be systemic; exposure of a portion of a root system to RSM-amended soil lowers *R. solani* infections in adjacent roots of the same plant growing in untreated soil (Cohen et al. 2005).

Bacteria are known to influence plant systemic resistance (van Loon et al. 1998) and RSM dramatically alters the structure of the resident soil microbial community (Cohen et al. 2005; Mazzola et al. 2001). Following application of *B. napus* cv. Dwarf Essex RSM, numbers of resident *Streptomyces* spp. in soil increase by approximately 100-fold and are maintained at levels over 20-fold higher than found in non-amended soil for at least 16 weeks (Cohen et al. 2005). In contrast to *Streptomyces*, following an initial upsurge, substantial declines in fluorescent pseudomonad populations to levels below those found in non-treated control soil have been observed in RSM-amended soils in field (Mazzola et al. 2001) and greenhouse studies (Cohen et al. 2005), making it unlikely that this group of organisms has a role in RSM-induced disease control.

As a group the *Streptomyces* produce an abundance of compounds with anti-microbial

and plant-stimulatory properties (Challis and Hopwood 2003; Igarashi et al. 2002). Although suppression of plant disease by *Streptomyces* spp. can in some cases be attributed to antibiosis (Rothrock and Gottlieb 1984), effects on plant physiology can also have a primary role (Cohen et al. 2005; Shimizu et al. 2001; Tokala et al. 2002). Compared to bulk soil, *Streptomyces* spp. recovered from roots of 'Gala'/M26 apple possessed a lower proportion of isolates that demonstrated in vitro antagonism toward *R. solani* but a higher proportion of isolates that produced nitric oxide (NO) via nitric oxide synthase (Cohen et al. 2005). NO is a gaseous free radical known to activate plant systemic defenses (Cohen et al. 2005; Neill et al. 2003), however, the role, if any, of NO production by *Streptomyces* spp. in RSM-mediated protection of the plant host is unknown. The vast preponderance of NO produced in RSM-amended soil is derived not from NO synthase activity but from the activity of nitrifying bacteria that oxidize the ammonium released from incorporated RSM (Cohen et al. 2005). A positive correlation between the capacity of an organic amendment to stimulate nitrification and to suppress disease was noted long ago by Huber et al. (1965). We have hypothesized that the influence of NO on plant defenses might provide an explanation for the influence of nitrification activity on plant susceptibility to disease (Cohen et al. 2006).

The present report is part of an overall program designed to investigate amendment of orchard soils with *Brassica* spp. seed meals as an alternative to chemical fumigation for controlling replant disease. Specific goals were to assess the role of native soil microbial communities in low glucosinolate content RSM-mediated control of *R. solani*, the importance of initial NO emission in the expression of disease control, and the capacity of native *Streptomyces* for replicating disease control realized through RSM amendment. As seed meal obtained from processing facilities varies widely from a flake to a powdered form, the impact of seed meal amendment on inoculum potential of *Pythium* spp. and resulting infection of apple seedling roots was examined.

Materials and methods

Plant residues

Brassica napus cv. Athena (ATH) seed meal flakes (5.8% N, 1.2% P, 1.3% K, 0.6% S) was kindly provided by Jack Brown of the University of Idaho, Moscow. *B. napus* ATH was bred to have a low glucosinolate content as determined by HPLC-MS analysis (Borek and Morra 2005), and contains 25.5 μmol total glucosinolates g^{-1} seed meal. The mineral (5.6% N, 1.2% P, 1.4% K, 0.9% S) and glucosinolate content (21.8 μmol g^{-1}) is similar to that of the *B. napus* cv. Dwarf Essex (DE) seed meal utilized in our previous studies (Cohen and Mazzola 2005; Mazzola et al. 2001).

Soils

Soils from the Columbia View Experimental (CV) orchard, Orondo, WA, and the Wenatchee Valley College-Auvil Research and Demonstration orchard (WVC-A), East Wenatchee, WA, were used in these studies. Orchard history, soil properties, soil collection procedures and composition of the pathogen complex inciting replant disease at these sites have been described (Mazzola 1998, 1999).

Impact of pasteurization on *B. napus* seed meal-induced suppression of *R. solani*

B. napus ATH seed meal was provided from the seed pressing facility as irregularly-shaped brittle flakes having an average diameter of approximately 4 mm and a thickness of 0.5 mm. A powdered form of this RSM was obtained by grinding flakes in a blender and utilizing the material that passed through a 1 mm² metal mesh. Pasteurized powder RSM was prepared by placing seed meal in a heat-resistant bag and exposing it to steam at 102°C for 3 h. The meal was cooled overnight prior to repeating the steaming cycle, and the RSM was subsequently dried in an oven overnight at 80°C prior to incorporation into soil. This pasteurized RSM was used in studies to examine the potential that RSM derived microorganisms contributed to observed disease control.

Native CV orchard soil was amended with pasteurized or non-pasteurized RSM at a rate of 0.5% (vol/vol) ($0.67 \text{ mg ml soil}^{-1}$), and soils were mixed thoroughly by hand. Soils were incubated at room temperature for 7 weeks and then decanted into conical tubes (21 cm length \times 4 cm top diameter). A single oat-grain colonized with *R. solani* AG-5 strain 5-104 (Mazzola et al. 1996), an isolate originally recovered from the roots of a Gala/M26 apple tree (Mazzola 1997), was buried to a depth 3-cm below the lowest apple seedling root. Eight-week-old Gala apple seedlings, raised in pasteurized potting mix as previously described (Mazzola 1998), were planted into soils with one seedling per tube and 10 seedlings per soil treatment in a randomized design. Plants were incubated in a growth chamber using a 16 h photoperiod and a 24/18°C day/night temperature regime and watered (10 ml) every other day. After two weeks, plants were harvested and roots were washed under a stream of tap water to remove adhering soil. *R. solani* infection frequency was determined by plating 20 root segments (0.5–1.0 cm length) on 1.5% water agar amended with ampicillin ($100 \mu\text{g ml}^{-1}$) and monitoring emergence of hyphal growth using a light microscope (100 \times).

Immediately prior to planting, soil samples were collected for measurement of *Streptomyces* spp. populations, numbers of aerobic endospores and soil dry weight. For the estimation of *Streptomyces* numbers, soil (1 g) was suspended in sterile water and serial dilutions were plated in triplicate on 1.5% agar containing 1/50th-strength tripticase soy broth (TSA). After 72 h incubation at room temperature, colonies exhibiting a growth characteristic representative of members belonging to this genus, were subjected to microscopic examination (100 \times) for confirmation of identity. For enumerating aerobic endospores (Williams et al. 1952), vegetative cells were killed and endospores activated by boiling the dilution tubes for 15 min prior to plating in triplicate onto 1/10th-strength TSA. Plates were incubated at 24°C and monitored for the appearance of colonies over the course of six days.

Impact of soil pasteurization on RSM induced disease suppression

CV orchard soil was amended with RSM as described above. Soil was incubated at room temperature for 7 weeks to allow for proliferation of resident *Streptomyces* spp. and passage of the peak period of NO emission. Soil was pasteurized using the same procedure described for the pasteurization of seed meal but with omission of the overnight drying treatment. Infestation of soil with *R. solani* AG-5 strain 5-104, plant growth conditions, and disease assessment was conducted as described above.

Impact of NO inhibition on RSM-induced disease control

CV and WVC orchard soils were amended with *B. napus* ATH seed meal at a rate of 0.5% (vol/vol) as described above. Nitrapyrin, was immediately added to a portion of the seed meal treated soil to inhibit nitrification. To ensure uniform dispersal, a 20 mg nitrapyrin ml^{-1} methanol solution was first combined with talc at a rate of 0.5 ml g^{-1} . The methanol was evaporated by allowing the mixture to stand for 15 min at room temperature and the talc suspension was then mixed into soil to attain a final concentration of 10 mg nitrapyrin l soil^{-1} .

0.5 l of soil representing each treatment was placed into individual quart-sized mason jars. Just prior to measurement, jar tops were fastened with lids containing a serum-stopper pierced with a 2.54 cm long 18 gauge needle as a vent and a 3.8 cm 18 gauge needle connected to a chemiluminescence-based NO analyzer (Sievers NOA-280i, Boulder, CO) set to an intake of 240 ml air min^{-1} . NO concentration values were recorded upon achieving signal level stabilization, usually within 5 min.

Soils were immediately placed into growth tubes without the extended incubation period cited above and infested with *R. solani* AG-5 as described above, with each treatment represented by 10 Gala seedlings. Preparation of seedlings, plant growth conditions and assessment of *R. solani* root infection were conducted as stated previously.

Impact of individual *Streptomyces* isolates on incidence of *R. solani* root infection

Numbers of *Streptomyces* in orchard soils have consistently increased in response to *B. napus* seed meal amendment, both in field and greenhouse studies (Cohen and Mazzola 2005; Mazzola et al. 2001). In contrast, numbers of fluorescent pseudomonads in these same soils typically decrease over time, and at the time of planting *Streptomyces* spp. characteristically represent roughly 10% of the total culturable bacteria population recovered from RSM amended soils. Therefore, studies were conducted to assess whether *Streptomyces* spp. isolates recovered from orchard soils possessed the capacity to provide control of Rhizoctonia root rot of apple, and if so, whether disease control was elicited in a fashion corresponding with the apparent induction of plant resistance which has been described in the case of RSM amendment (Cohen and Mazzola 2005).

CV orchard soil was amended with RSM and then immediately pasteurized as described above prior to the introduction of individual isolates of *Streptomyces*. These included strains SCV22, CVR44, CR2 and RR2 previously recovered from CV soil, RSM-amended CV soil, and roots of Gala/M26 apple planted in non-amended and RSM-amended CV soil respectively (Cohen and Mazzola 2005), and *Streptomyces griseoviridis* originally isolated from peat (Lahdenpera et al. 1991). Isolates were characterized for the capacity to produce NO and to suppress the growth of *R. solani* AG-5 in vitro. For determination of NO production, individual isolates were cultured on 1/10th-strength TSA in 5 cm diameter Petri plates. After 48 h incubation at room temperature, Petri plates were sealed with parafilm and incubated overnight at room temperature. NO concentration in the head space of the Petri plate was determined by piercing the parafilm seal with an 18 gauge needle connected to the chemiluminescence-based NO analyzer. In vitro inhibition of *R. solani* hyphal growth by *Streptomyces* isolates was determined as previously described (Cohen et al. 2005).

For the *Streptomyces* recovered from CV soil habitats, isolates were cultured on 1/10th-strength

TSA medium and spores were scraped from the surface of an 8-cm diameter confluent growth of the bacterium. Spores were mixed into 5 g sterile talc, with the spore suspension, or talc only for the control, mixed into soil at 1 g l⁻¹. An additional positive control consisted of RSM amendment into native CV orchard soil. For enrichment of soil with *S. griseoviridis*, Mycostop powder (Ag-Bio Inc., Westminster, CO) was added in place of the talc suspension. After 3 weeks incubation at room temperature, the numbers of *Streptomyces* in the respective soils were determined and the soil was diluted to 10% (wt/wt) with newly pasteurized non-RSM amended soil, resulting in a final density of approximately 5 × 10⁸ cfu g soil⁻¹. After a further 4 days incubation, soils were placed into conical tubes, infested with *R. solani* AG-5, planted with Gala apple seedlings and incubated in a controlled environment growth chamber as described above.

Alternatively, assays were conducted using a split-root plant growth design (Cohen et al. 2005). In this system approximately one-half of the seedling root was established in pasteurized soil not treated with a *Streptomyces* isolate but which had been infested with oat grain inoculum of *R. solani* AG-5 strain 5-104. The remainder of the root system was established in native soil amended with RSM (positive control), pasteurized soil with no amendment (negative control) or soil that had been inoculated with an isolate of *Streptomyces*. In this system, each treatment was represented by seven replicates. After two weeks, *R. solani* root infection frequency was determined for the component of the root system established in pathogen infested soil by plating 20 root segments from each plant on ampicillin (100 µg ml⁻¹) amended 1.5% water agar and monitoring hyphal emergence as described above.

Impact of seed meal particle size on stimulation of resident *Pythium* spp. and control of *R. solani*

Studies were conducted to assess the impact of seed meal particle size on resident *Pythium* spp. populations and disease development. The “flake” preparation of RSM used in these experiments consisted of material that was

retained following sieving on a 2 mm² metal mesh (particles 2–4 mm² in size). The powder form of RSM was prepared as described above. Flake or powder RSM was incorporated into CV or WVC orchard soil at a rate of 0.5% (vol/vol). Immediately after RSM amendment, soils were decanted into conical tubes and planted with Gala seedlings. Plants were grown under the previously described conditions and harvested after seven days. Alternatively, after RSM amendment soils were incubated at room temperature for four weeks prior to planting with Gala seedlings and plants were harvested after 16 days. A soil sample was collected at plant harvest and *Pythium* numbers were determined by plating serial dilutions of a soil suspension on PSSM agar (Mazzola et al. 2001). Seedling root systems were washed under a stream of tap water and 20 root segments from each plant were plated onto this same medium. Agar plates were examined at 24 h intervals over three days and numbers of *Pythium* colonies or colonized root segments were recorded.

The effect of *B. napus* ATH seed meal particle size on suppression of root infection by the introduced pathogen *R. solani* AG-5 isolate 5-104 was determined. Assays were conducted as described above.

Data analysis

Statistical analyses were conducted using Sigma-Stat, version 2.0 (SPSS, San Rafael, CA). Percent root infection data were transformed to arcsine-square root values prior to analysis of variance and means separation using the Student–Newman–Keuls method. Microbial soil population data were transformed to log₁₀ values and subjected to analysis of variance and means separation using the Tukey test.

Results

Impact of pasteurization on RSM-induced control of *R. solani*

Pasteurization of RSM prior to use as a soil amendment had no impact on the suppression of root infection by *R. solani* AG-5 in native orchard

soil. Roots of Gala seedlings grown in non-treated CV orchard soil were infected at a frequency of 45.4%, which was significantly ($P < 0.001$) higher than for seedlings grown in soils amended with pasteurized (20.0%) or non-pasteurized (24.6%) seed meal.

Pasteurization of CV orchard soil that had previously been amended with RSM abolished the capacity of this amendment to suppress apple root infection by an introduced isolate of *R. solani* AG-5. Roots of Gala seedlings established in pasteurized RSM-amended soil were infected at a frequency of 94%, which was significantly ($P < 0.001$) higher than non-amended pasteurized soil where root infection frequency was 45%. In native CV orchard soil, RSM significantly ($P = 0.02$) reduced seedling root infection by *R. solani* AG-5 from 60% in the non-amended control to 38% in amended soil. At the time of planting numbers of endospore cfu in CV soil that had been amended with RSM prior to pasteurization of soil had reached 2.0×10^7 cfu g⁻¹ soil compared to 1.0×10^5 cfu g⁻¹ in RSM-amended non-pasteurized soil. In the same respective soils, *Streptomyces* spp. populations were below the limit of detection ($< 10^3$ cfu g⁻¹) in RSM-amended pasteurized soil but were 7.2×10^8 cfu g⁻¹ in RSM-amended non-pasteurized soil.

Nitric oxide production and its impact on RSM-induced disease control

Emission of NO from CV soil amended with RSM in either the powder or flake form peaked within 8–12 days post-application and attained rates of release that were 2–3 × 10²-fold greater than that from non-amended soil (Fig. 1). NO production rates gradually diminished over time but even at 18 weeks after incorporation were 4.5 ± 0.5 and 4.1 ± 1.7 nmol NO h⁻¹ l soil⁻¹ from powder and flake amended soils, respectively, compared to 1.0 ± 0.2 nmol NO h⁻¹ l soil⁻¹ from control soil. NO was emitted in a similar pattern from RSM powder-amended WVC soil with a peak release rate of 156.6 ± 0.8 nmol NO h⁻¹ l soil⁻¹ measured 9 days post-amendment. As expected, the nitrification inhibitor nitrapyrin substantially lowered rates of NO production in CV (Fig. 1, inset) and WVC soils (not shown).

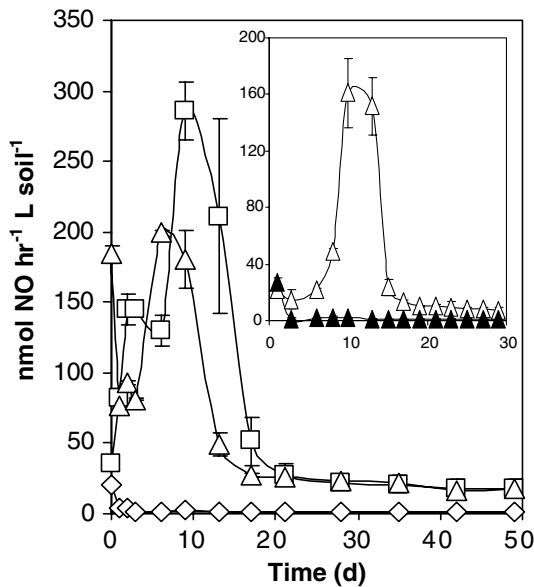


Fig. 1 NO emission from CV orchard soil without amendment (\diamond) and amended with flaked (\square) or powdered (Δ) *B. napus* var. Athena seed meal, and, inset, from CV soil amended with powdered seed meal in the presence (\blacktriangle) or absence (Δ) of nitrapyrin

When Gala seedlings were established in *R. solani* infested soil within 24 h post-RSM amendment, treatment of soil with the nitrification inhibitor nitrapyrin significantly ($P < 0.05$) increased infection of Gala seedling roots in CV and WVC soils (Table 1). The presence of nitrapyrin had no effect on RSM-induced amplification of *Streptomyces* populations, which at the time of planting had reached 5×10^7 and 4×10^7 cfu g^{-1} in RSM-amended CV soils with

Table 1 Effect of nitrapyrin on *Rhizoctonia solani* AG-5 infection of Gala seedling roots planted into soil 24 h after incorporating 0.5% (vol/vol) *B. napus* cv. Athena seed meal

Treatment	<i>R. solani</i> infection frequency (%) ^a	
	CV soil	WVC soil
Non-treated	30.5a	71.5c
RSM	20.5a	34.5a
RSM + Nitrapyrin	68.5b	54.5b

^aValues followed by different letters are significantly different ($P < 0.05$). For all treatments $n = 10$. Oat grain inoculum of *R. solani* AG-5 was prepared as previously described (Mazzola et al. 1996)

and without nitrapyrin, respectively, compared to 4×10^5 cfu g^{-1} in non-treated soil.

Impact of *Streptomyces* isolates on *Rhizoctonia* root rot of apple

A series of experiments were conducted to determine whether addition of various *Streptomyces* sp. strains into pasteurized RSM-amended CV soil could restore soil suppressiveness toward *R. solani*. An initial experiment tested the ability of Mycostop, a commercial preparation of *Streptomyces griseovirdis*, to restore disease suppressiveness. The bacterium was established in pasteurized RSM-amended CV soil at an initial population of 2×10^6 cfu g^{-1} , which increased to 9×10^8 cfu g^{-1} soil by the time of planting at 16 days post-application. Roots of Gala seedlings planted into this soil that had simultaneously been infested with *R. solani* AG-5 were infected at a frequency of $28.0 \pm 10.1\%$, significantly lower ($P < 0.01$) than an equivalently treated soil that did not receive Mycostop, $65.7 \pm 4.8\%$. In culture, *S. griseovirdis* emitted NO at a high rate and produced a diffusible antibiotic that strongly inhibited mycelial growth of *R. solani*.

Streptomyces strains having the four possible combinations of two traits, NO production and in vitro antibiosis toward *R. solani*, were found to protect against disease development in a manner that did not show a relationship with the presence or absence of either trait (Table 2). In addition, all *Streptomyces* isolates applied individually in single-container or split-root assays provided significant protection against infection of apple seedling roots by *R. solani* (Table 2). In both assay systems, the level of disease control attained through inoculation of soil with a *Streptomyces* isolate was similar to that achieved through amendment of soil with *B. napus* ATH seed meal. The evidence of systemic protection supported by the findings from these split-root assays is similar to our previously reported observation on the effect of low-glucosinolate *B. napus* DE seed meal amendment in experiments using the same design (Cohen et al. 2005).

Table 2 *Rhizoctonia solani* AG-5 infection frequency for Gala seedling roots established in soils amended with seed meal of *Brassica napus* cv. Athena or individual *Streptomyces* sp. strains

CV soil treatment	Inoculated <i>Streptomyces</i> ^a			<i>R. solani</i> infection frequency (%) ^b	
	Strain	Inhibition zone (mm)	Maximal NO production (ppb)	Single container	Split-root container
0.5% Athena RSM	None added	n.a.	n.a.	13.3ab	22.1a
Steam pasteurized	None added	n.a.	n.a.	38.9c	47.1c
	CR2	0	n.d.	7.2a	17.1a
	SCV22	10	n.d.	17.2ab	25.7ab
	RR2	0	501	15.0ab	32.1b
	CVR44	10	69	25.7b	28.6ab

n.a., not applicable; n.d., not detected

^aZones of *Rhizoctonia solani* AG-5 growth inhibition were determined as in Cohen et al. (2005)

^bValues followed by the same letter are not significantly different ($P > 0.05$)

Impact of *B. napus* seed meal particle size on disease development

The physical characteristics of the *B. napus* seed meal had little demonstrable impact on the general transformation of the resident soil microbial community. One week after treatment, the numbers of fungal cfu were $1 \times 10^5 \text{ g}^{-1}$ in both RSM flake and powder amended soils. The density of *Streptomyces* spp. in the soil during the duration of root exposure to *R. solani* was higher in the RSM powder-amended soil ($3.23 \times 10^7 \text{ cfu g}^{-1}$) and RSM flake-amended soil ($1.06 \times 10^7 \text{ cfu g}^{-1}$) compared to that in non-treated soil ($1.26 \times 10^6 \text{ cfu g}^{-1}$). Also, both powder- and flake-amended soils exhibited an approximate 10-fold increase in the numbers of heat-resistant endospores (9.39 and $10.3 \times 10^4 \text{ cfu g}^{-1}$, respectively), an indicator of *Bacillus* spp. density, relative to non-treated soil ($1.20 \times 10^4 \text{ cfu g}^{-1}$).

The size of RSM particles used in the amendment of soils did not alter suppression of *R. solani* AG-5 infection of apple seedling roots when planting was conducted simultaneously with pathogen infestation of soil. *R. solani* infection of Gala seedling roots grown in powder (15.4%) or flake (15.8%) RSM-amended soils was significantly less ($P < 0.05$) than that in non-treated CV soil (48.0%).

Seed meal particle size did have a significant impact on the numbers and activity of resident *Pythium* spp. in both CV and WVC orchard soil.

One week following incorporation of the flake form of RSM into soil, the number of culturable *Pythium* spp. increased significantly in both soils. At the same sampling period, amendment with the powder form of RSM had resulted in a significant increase of *Pythium* populations in CV but not WVC soil, and for both soils flake amendment resulted in higher numbers than powdered seed meal (Table 3). Likewise, the severity of *Pythium* infection in CV or WVC-A soil following amendment with flakes was notably higher than when amended with the powder form of RSM (Table 3). When planting was delayed until four weeks post-seed meal amendment, root infection frequency and seedling mortality both appeared to diminish when planting was delayed until four weeks post-seed meal amendment. This was observed even though numbers of *Pythium* spp. remained above 2.4×10^4 and $2.7 \times 10^3 \text{ cfu g soil}^{-1}$ in CV and WVC soils, respectively (Table 3).

The preponderance of *Pythium* isolates recovered from WVC-A soil were *Pythium ultimum* and *Pythium sylvaticum* (data not shown), which are highly virulent toward apple, whereas the population from CV soil is dominated by isolates of *Pythium heterothallicum* and *P. intermedium* (aff. *P. attrantheridium* Allain-Boulé & Lévésque), species which express a lower level of virulence toward apple (Mazzola et al. 2002). Accordingly, seedling root infection and mortality were generally more severe in WVC-A compared to CV soil even though for equivalent treatments

Table 3 Impact of *Brassica napus* cv Athena seed meal particle size on *Pythium* spp. densities in soil and infection of Gala apple seedling roots by resident populations

Week ^a	Treatment	CV soil			WVC-A soil		
		cfu/g DW soil ($\times 10^2$) ^b	% Root infection ^c	Mortality	cfu/g DW soil ($\times 10^2$)	% Root infection ^c	Mortality
1	Control	0.38 \pm 0.24a	7.5a	0/10	0.80 \pm 0.16a	11.0a	0/10
	Athena Flakes	159.5 \pm 30.8c	95.5c	5/10	97.6 \pm 16.0b	94.0b	9/10
	Athena Powder	89.8 \pm 16.6b	73.0b	0/10	24.1 \pm 6.9a	94.0b	4/10
4	Control	4.0 \pm 0.3a	27.5a	0/10	0.54 \pm 0.15a	40.5a	0/10
	Athena Flakes	443.0 \pm 112.0c	72.5c	0/12	27.6 \pm 3.3b	50.8a	0/12
	Athena Powder	241.0 \pm 40.5b	55.4b	0/12	27.2 \pm 7.2b	45.5a	0/12

^aTime after incorporation of 0.5% (vol/vol) seed meal amendment and initial watering of soils

^bNumbers of *Pythium* spp. colony forming units per gram dry weight of soil. Values are mean \pm standard error and those within a column for a given week followed by the same letter are not significantly different

^cValues within a column for a given week followed by the same letter are not significantly different

the numbers of culturable *Pythium* spp. were lower in WVC-A soil (Table 3).

Discussion

Apple seedlings planted into soil amended with *Brassica napus* cv. Athena seed meal exhibited lower root infection by an introduced isolate of *Rhizoctonia solani* AG-5 in a manner similar to that observed in response to *B. napus* cv. Dwarf Essex seed meal (Cohen et al. 2005; Mazzola et al. 2001). Consistent with our previous reports on amendment with low-glucosinolate RSM, incorporation of *B. napus* ATH seed meal increased the incidence of apple root infection by *Pythium* spp. resident to orchard soils. Our work further demonstrates that the long-term protection against *R. solani* infection conferred by RSM amendment is primarily a result of biological transformations occurring in soil, most likely increases in resident *Streptomyces* spp. populations.

Microorganisms elicit disease control realized in response to *B. napus* seed meal amendment

Pasteurization of *B. napus* seed meal did not alter its capacity to suppress infection of apple roots by *R. solani*. In addition, suppression of apple root infection by an introduced isolate of *R. solani*

AG-5, which was repeatedly observed when assays were conducted in native soils, was eliminated if such assays were conducted in RSM-amended soils that had been pasteurized prior to introduction of the pathogen. These findings demonstrate that a biological element resident to native orchard soil systems is responsible for the *B. napus* seed meal induced control of *R. solani*.

Bacillus spp. and *Streptomyces* spp. increased in response to *B. napus* ATH seed meal amendment. Both genera possess members that are known to confer protection against plant infection by fungal pathogens (Weller et al. 2002). However, soil pasteurization following meal amendment reduced *Streptomyces* spp. to below the limit of detection but resulted in higher numbers of *Bacillus* spp., presumably due to the reduced number of competitors faced by cells arising from germinated endospores. In spite of the higher numbers of *Bacillus* spp., this soil was unable to suppress *R. solani* infection, suggesting that *Bacillus* spp. do not contribute significantly to the observed seed meal-induced disease suppression. In the absence of the putative biological protectant, the presence of RSM amendment in the pasteurized soil actually stimulated infection of apple roots by the introduced *R. solani* strain, presumably by serving as an energy source for the fungus. These observations, in addition to our previous findings (Cohen and Mazzola 2005;

Mazzola et al. 2001) provide further evidence that the specific glucosinolate hydrolysis products emanating from the degradation of *B. napus* seed meal do not contribute to the suppression of disease incited by *R. solani*.

The preferential survival of *Streptomyces* spp. in *B. napus* seed meal amended soils relative to other bacteria make these bacteria a suitable target of investigation as a biological mechanism contributing to the observed disease suppression. The enhanced survival of *Streptomyces* spp. in *B. napus* seed meal-amended soils may bear some relation to the differential susceptibility of these organisms to feeding by protozoa (Cohen and Mazzola 2005). The most common protozoan found in the amended soils, an amoeba-flagellate identified as *Naegleria americana*, show no capacity to utilize *Streptomyces* spp. as food in culture but readily consumed most strains of fluorescent *Pseudomonas* spp. at a significantly higher rate (Cohen and Mazzola 2005). However, evidence suggests that additional features of this system have a role, and the preferential utilization of *B. napus* seed meal by *Streptomyces* spp. in the soil may be such a factor. The extensive repertoire of extracellular enzymes produced by *Streptomyces* spp. permits *B. napus* seed meal to serve as high-yield feedstock for commercial production of *Streptomyces* spp. (Brabban and Edwards 1996).

The general capacity of *Streptomyces* spp. isolates recovered from apple roots or soil to provide control of Rhizoctonia root rot also argues for a role of these bacteria in the observed disease control. All four individual isolates protected apple against root infection by *R. solani* AG-5, and split-root experiments indicate that, similar to *B. napus* seed meal-amended soil (Cohen et al. 2005), the *Streptomyces*-enriched soils confer plant systemic protection against *R. solani*. However, disease control was not associated with the relative capacity of this limited number of strains to produce NO or antibiotics active against *R. solani* in vitro. Elimination of or support for either of these attributes as having a role in the observed disease control cannot be expressed based upon these data. A variety of plausible mechanisms for the observed protective effect

elicited by these bacteria remain to be investigated (van Loon et al. 1998; Weller et al. 2002).

We have previously reported that nitrification activity is responsible for the substantial increases in NO production observed in soils amended with RSM (Cohen et al. 2005). The previously reported peak in NO production from *B. napus* DE seed meal-amended forest soil was similar in amplitude to that observed from *B. napus* ATH-seed meal amended CV and WVC soils but occurred after four weeks of incubation (Cohen et al. 2005). The difference in timing may be a consequence of yearly ammonium nitrate fertilization having elevated the capacity of the microbial community resident to the orchard soils to more quickly nitrify ammonia released during seed meal degradation in comparison to the same community inhabiting the forest soil, which had not received prior fertilization. The *B. napus* seed meal-induced increase in NO production was prevented by adding the nitrification inhibitors dicyandiamide (Cohen et al. 2005) or nitrapyrin (Fig. 1, inset). The negative influence of nitrapyrin treatment on the capacity of RSM to provide control of *R. solani* root infection is consistent with observations in some other cropping systems (Huber et al. 1965). Although this may suggest some role for NO emissions in the observed disease control, the duration and potential mechanisms of action, including the loss of putative plant defense-inducing NO, requires further investigation.

Control of *Pythium*

A diversity of *Pythium* spp. are associated with apple and have the capacity to limit plant development (Mazzola et al. 2002). Though virulence of *Pythium* spp. toward non-seedling plants in some instances has been reported as generally low (Hendrix and Campbell 1973), infections do limit root development and in apple have on at least one occasion been cited as a causal agent of the potentially devastating disease, collar rot (Jeffers et al. 1982). Thus, a reduction in *Pythium* spp. disease pressure will be of benefit to the successful establishment of young apple trees in orchard soils.

Powdering of *B. napus* ATH seed meal prior to soil application reduced the incidence of *Pythium* infection compared to flakes, but more significant suppression of *Pythium* spp. infectivity would be required for practical use in orchards. This may be accomplished by treatment of amended soil with mefenoxam (Mazzola and Mullinix 2005). Although, these compounds are marketed specifically for the suppression of oomycetes, the spectrum of activity is more widespread across rhizosphere fungal communities (Girlanda et al. 2001). Moreover, resistance quickly develops among oomycetes in an environment regularly subject to mefenoxam treatment (Mazzola et al. 2002). The isothiocyanate derived from the 2-propenyl glucosinolate of *Brassica juncea* has been shown to inhibit *Pythium irregulare* in vitro at a concentration of 0.5 mg ml⁻¹ (Manici et al. 1997). Likewise, preliminary data revealed that the use of seed meal derived from *B. juncea* cv. Pacific Gold in combination with RSM can suppress the stimulation of *Pythium* populations consistently observed in response to application of RSM alone (Cohen and Mazzola unpublished data). Such a seed meal mixture may be a feasible organic alternative to a post-plant application of mefenoxam as a means for suppression of *Pythium* spp. while retaining effective control of *R. solani* when attempting to re-establish apple on sites previously planted to this or related tree fruit crops.

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