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Brassica napus seed meal soil amendment modifies microbial community structure, nitric oxide production and incidence of *Rhizoctonia* root rot

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Abstract

A low glucosinolate content (21.8 μmol g^{-1}) *Brassica napus* seed meal (RSM) applied to orchard soils altered communities of both pathogenic and saprophytic soil micro-organisms. RSM amendment reduced infection by native and introduced isolates of *Rhizoctonia* spp. and recovery of *Pratylenchus* spp. from apple roots. Root infection by *Rhizoctonia solani* AG-5 was also suppressed in split-root assays where a portion of the root system was cultivated in RSM-amended soils and the remainder grown in the presence of the pathogen but lacking RSM. *R. solani* hyphal growth was not inhibited by RSM amendment. Suppression of *Pratylenchus* was attained to an equivalent extent by amending soils with either RSM or soybean meal (SM) when applied to provide a similar N content. Thus, glucosinolate hydrolysis products did not appear to have a significant role in the suppression of *Rhizoctonia* spp. or *Pratylenchus* spp. obtained via RSM amendment. RSM amendment elevated populations of *Pythium* spp. and of ammonia-oxidizing bacteria that release nitric oxide but suppressed fluorescent pseudomonad numbers. *Streptomyces* spp. soil populations increased significantly in response to RSM but not SM amendment. The vast majority of *Streptomyces* spp. recovered from the apple rhizosphere produced nitric oxide and possessed a nitric oxide synthase homolog. We propose that transformations in the bacterial community structure are associated with the observed control of *Rhizoctonia* root rot, with NO production by soil bacteria potentially having a role in the induction of plant systemic resistance.
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Keywords: *Streptomyces*; Nitric oxide synthase; Biofumigation; Induced systemic resistance

1. Introduction

Numerous strategies have been investigated for the capacity to modify soil microbial communities in a manner that leads to improved growth through the suppression of soil-borne plant diseases. Crop management practices including crop rotation (Huber and Schneider, 1982), input system (organic versus conventional) (Workneh et al., 1993; van Bruggen, 1995), tillage and fertilization (Smiley, 1978), among others, will influence ecological processes that affect microbial communities involved in the suppression of soil-borne plant pathogens. These observations infer that, given knowledge of the operative biological mechanisms, the capacity exists to enhance or diminish the suppressive nature of a resident microbial community through timely application of the appropriate agronomic practices (Workneh and van Bruggen, 1994; Hoeper and Alabouvette, 1996; Pankhurst et al., 2002). As the induction of soil suppressiveness is often mediated through transformations in soil microbial communities over time (Liu and Baker, 1980; Larkin et al., 1993; Raaijmakers et al., 1997; Mazzola and Gu, 2002; Weller and Raaijmakers, 2002), there may be a significant opportunity to enhance the onset of the disease-suppressive state, a notable pre-requisite to the adoption of such a disease control strategy.

Organic soil amendments commonly have been used to provide for plant nutrition and control of soil-borne plant pathogens. In many instances, the active mechanism

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contributing to the disease-suppressive nature of a specific soil amendment has remained unknown and therefore has lacked widespread adoption. Composts have been used extensively in high value cropping systems, and it is believed that the control of certain pathogens, such as *Pythium* spp., is brought about through an overall increase in microbial activity, typically referred to as general suppression. However, among those soil systems examined, the microbial complex introduced through compost amendment has been less effective than the resident microbial community when such materials have been evaluated in natural disease-suppressive soils (Cotxarrera et al., 2002; Kowalchuk et al., 2003).

More recently, bioactive plant products, introduced either as a cover crop or soil amendment, have acquired significant use as a disease control measure in alternative cropping systems. Members of the plant family Brassicaceae, including *Brassica napus*, produce glucosinolates which upon hydrolysis yield biologically active compounds. As isothiocyanates have a broad spectrum of antimicrobial activity, investigators have focused on the use of these plants as a 'biofumigant', where incorporation of plant residue into soil ultimately results in the release of active hydrolysis products (Angus et al., 1994; Brown and Morra, 1997).

Certain evidence suggests that Brassicaceae plant residues may operate in the suppression of fungal pathogens via a different, as yet unidentified, mechanism. Reports exist of the effective use of *B. napus* residues to control soil-borne plant pathogens even though separate reports suggest that these plant residues yield isothiocyanates having relatively low antimicrobial activity (Manici et al., 1997). Likewise, the protective effect of Brassicaceae residues against soil-inhabiting fungal plant pathogens and insect pests was found to increase for weeks after isothiocyanates had been lost from the soil either by volatilization or degradation (Papavizas, 1966; Lewis and Papavizas, 1971). Mortality of the whitefringed weevil in soil amended with *Brassica juncea* seed meal did not directly correlate with the resulting concentration of isothiocyanate (Warton et al., 2003). Further support for an alternative functional mechanism comes from the finding that suppression of certain apple root pathogens and parasites, including *R. solani* and *Pratylenchus penetrans*, was obtained via the incorporation of *B. napus* (rapeseed) seed meal (RSM) irrespective of the glucosinolate content of the amendment (Mazzola et al., 2001).

We have hypothesized that oxidation of N in soil amendments to nitric oxide (NO) may be among the bacterial activities that contribute to disease suppression since NO is known to stimulate certain plant defense pathways (Delledonne et al., 1998; Durner et al., 1998). Four decades ago, Huber and co-workers noted a negative correlation between fungal root disease and the capacity of an amendment to stimulate nitrification (Huber et al., 1965). It is now known that nitrifying bacteria are the major source of NO from soils (Veldkamp and Keller, 1997; Harrison and Webb, 2001; Jousset et al., 2001). In the first step of nitrification, ammonia is oxidized to NO and nitrite (Whittaker et al., 2000; Jetten, 2001) by members of the autotrophic genera such as *Nitrosomonas* and *Nitrospira*, as well as by various heterotrophic nitrifiers (Gunner, 1963; Anderson et al., 1993). Another pathway for NO production, via NO synthase (NOS), has been found in a variety of Gram-positive bacteria (Cohen and Yamasaki, 2003). Of the two *Streptomyces* genomes sequenced to date, *Streptomyces avermitilis* (Omura et al., 2001) possesses a NOS homolog while *Streptomyces coelicolor* does not (Bentley et al., 2002). Recently, in the first demonstration of a function for NOS in a *Streptomyces* spp., Kers et al. (2004) reported a requirement for NOS activity in the nitration of a peptide phytotoxin by *Streptomyces turgidiscabies*.

Our aim was to determine the effect of RSM on composition and function of soil microbial communities, and to discern whether relationships existed between any observed transformations in resident microbial communities and the control of *R. solani*. Furthermore, studies were conducted to discern the function of added N in the suppression of *R. solani* and the influence of such amendments on the activity of nitrifying bacteria and NO concentrations in RSM-amended soils.

2. Materials and methods

2.1. Soils and soil treatments

Studies were conducted at or in soils collected from the Columbia View Experimental (CV) orchard, Orondo, WA, the Wenatchee Valley College-Auvil Research and Demonstration (WVC) orchard, East Wenatchee, WA and a commercial (GC) orchard, Manson, WA. Orchard history, soil properties, and composition of the pathogen complex inciting replant disease at these sites have been described (Mazzola, 1998, 1999). A composite soil sample was collected from each orchard for use in greenhouse studies. Samples were obtained by collecting soil within the root zone of 15 randomly selected trees at a depth of 10–30 cm and 1–2 m from the tree base. Additional studies were conducted in an uncultivated clay loam soil (pH 7.4; 6 mg kg^{-1} soluble N) obtained from a cleared forest patch in Okinawa, Japan.

The *B. napus* seed meal (Montana Vegetable Oils, Great Falls, MT) used in these studies possessed a low total glucosinolate content (21.8 μmol g^{-1}), and 6% total N. Soybean meal (SM) had 3.05% total N and was generated by grinding dried seed-containing soybean pods in a Waring blender.

In greenhouse trials, RSM was incorporated into soils at a rate of 0.5% (vol/vol). SM was added in a manner proportional to RSM amendment to obtain equivalent quantities of total N added. Treated and non-treated

(control) soils were placed in 3.8 l plastic pots, with four pots per treatment, and incubated on the greenhouse bench at 20±3 °C. In plant trials conducted at the CV and WVC orchards, RSM was applied in the autumn of 2001 at a rate of 8533 kg ha^{-1} and incorporated into the soil profile by rotovation. Telone C17 (DowElanco, Indianapolis, IN) was applied in September 2001 at a rate of 282 l ha^{-1}. The study was arranged in a randomized design, with four replicates of each treatment. As *B. napus* seed meal amendments stimulated populations of *Pythium* spp. resulting in apple seedling mortality and root rot (Mazzola, 2003), Ridomil Gold® (Syngenta Crop Protection, Greensboro, NC) was applied 2 weeks post-planting as a soil drench at the label rate in field trials.

A separate trial was established at the CV orchard to conduct an analysis of RSM-induced modification of soil microbial communities. Treatments were applied on 29 March 2003 as described above and included Telone C17, RSM and a non-treated control, with each treatment represented by four replicates in a randomized complete block design.

2.2. Soil sampling and microbial analysis

Soils incubated in the greenhouse were sampled at 2 week intervals beginning 2 weeks post-amendment for up to 18 weeks. Soils were collected from field plots established in 2001 immediately prior to planting in May 2002 and 5 months post-planting. For the field plots established at CV orchard in March 2003, microbial communities were monitored over 12 weeks with soil sampled every 2 weeks. Two separate 1 g sub-samples from each pot or plot were re-suspended individually in 10 ml sterile distilled water. Soil suspensions were vortexed for 60 s and serial dilutions were plated onto 1/50th-strength tripticase soy agar (TSA), King's Medium B^+ agar (Simon and Ridge, 1974), PSSM (Mazzola et al., 2001), and 1/5th-strength potato dextrose agar (PDA) for estimates of the numbers of total bacteria and *Streptomyces* spp., fluorescent pseudomonad, *Pythium* spp, and total fungi, respectively.

In vitro inhibition of *R. solani* hyphal growth by *Streptomyces* spp. isolates was assayed by spotting a suspension of *Streptomyces* spores or mycelia on opposite sides of a 9 cm dia plate containing 1/10th-strength nutrient broth yeast extract agar, 8 mm from the edge of the medium. After 48 h at 28 °C, a 3 mm dia agar plug from a *R. solani* AG5 strain 5–104 PDA culture was placed at the center of the plate. Plates were incubated for another 72 h and widths of inhibition zones between the margins of the *Streptomyces* colonies and fungal growth were measured. Assays for inhibition of *Bacillus mycoides* strain PVL2, an isolate recovered from a ground seed of *Phaseolus vulgaris* that had been surface-sterilized by treatment with hypochlorite, were conducted on 1/20th-strength TSA using a similar method except that the *B. mycoides* inoculum was streaked linearly to the edges of the *Streptomyces* colonies.

Streptomyces spp. were isolated from the roots of 'Gala'/M26 that had been established in RSM-amended soil at the CV orchard in June 2002 (Mazzola, 2003). Root sampling was conducted 18 months post-planting. Adherent soil on root samples was manually removed, and 0.5 g of root tissue placed in 10 ml of sterile water. Samples were vortexed and serial dilutions of the root rinse plated onto 1/50th-strength TSA. *Streptomyces* sp. strains were differentiated based on colony morphology, antibiosis profiles and NO production concentrations (described below). For some strains total DNA isolated with the MoBio (Carlsbad, CA) Microbial DNA Isolation Kit served as a template for amplification of a conserved region of the 16S rRNA gene following an established procedure (Gu and Mazzola, 2003). The amplified gene fragments were directly sequenced by use of a Dye Terminator Cycle Sequencing Quick Start Kit and a CEQ 8000, Genetic Analysis System capillary-based DNA sequencer (Beckman Coulter, Fullerton, CA). Resultant nucleotide sequences from the apple root isolate strain RR2 and strain RM9, isolated from dry RSM, were deposited in GeneBank under accession numbers, AY779272 and AY779271, respectively. BlastN analysis revealed a complete sequence match between the 16S rRNA gene gamma region of *Streptomyces lavendulae* strain IFO 12340 (Anzai and Watanabe, 1997) and that of strain RR2.

2.3. Plant assays

At the completion of incubation (see Section 2.1) greenhouse soils were planted with 12-week-old Gala apple seedlings and harvested at 12 weeks later. At harvest, seedling root systems were rinsed under a stream of tap water, and plant height, shoot weight, and root weight were determined. In the field, the CV and WVC orchard trials were replanted in May 2002 with M26 and M7 rootstocks, respectively.

Numbers of lesion nematode (*Pratylenchus* spp.) resident to apple roots were determined at harvest for greenhouse assays, and 5 months post-planting in field trials. Root samples were obtained from each pot (greenhouse) or from each of two trees in each of four blocks (field). A 0.5 g root sub-sample was placed into a 125-ml flask containing 80 ml sterile distilled water and shaken on a gyrotory shaker at 150 rev min^{-1} for 5 days. Nematodes were collected by passing the suspension twice through a 38 μm^{-2} sieve and backwashing into a counting dish, and *Pratylenchus* spp. were counted using a light microscope (40×).

From the same samples, infection of apple roots by *Rhizoctonia* spp. was assessed. After root washing, 10 root segments (0.5–1.0 cm) from each seedling or 20 root segments from each rootstock were plated onto 1.5% water agar amended with ampicillin (100 $\mu g\ ml^{-1}$). Agar plates were incubated at room temperature and examined using a light microscope (100×) at 48 and 72 h for hyphal growth of *Rhizoctonia* spp.

2.4. Influence of seed meal amendment on R. solani apple root infection

Inoculum of *R. solani* AG5 strain 5–104 was prepared as colonized oat grains (Mazzola et al., 1996). Soils were amended with RSM or SM 1 week prior to infesting soils with the pathogen by placing 14 oat grains at a depth of 8 cm in each pot. Soils were kept for an additional 1 week on a greenhouse bench and each pot was subsequently planted with five 8-week-old Gala apple seedlings. Each treatment consisted of four replicates with five seedlings each. At harvest, plants were processed as described above and relative root infection frequency by the introduced pathogen was determined by plating 10 root segments from each seedling onto 1.5% water agar and examining root segments for hyphal growth indicative of *R. solani* after 48 h incubation at room temperature. Numbers of total culturable bacteria, fluorescent *Pseudomonas* spp., *Streptomyces* spp., *Pythium* spp. and total fungi in soils were determined immediately prior to planting and at harvest as described above.

An alternative evaluation system employed a split-root growth assay. The plant growth apparatus included two neighboring conical tubes (21 cm×4 cm top dia), with one tube possessing non-treated soil infested with one *R. solani* AG-5 strain 5–104-colonized oat kernel at a depth of 8-cm, and the adjacent tube containing the same soil amended with 0.5% (vol/vol) RSM without addition of the pathogen. The control treatment included the same planting design but without addition of RSM. Gala seedlings (11 weeks old) were planted with one-half of the root system placed into the *R. solani* infested soil and the remainder of the root system planted into non-infested RSM-amended soil through a 6-mm-dia hole in the tubes. Plants were incubated in the growth chamber with a 16 h photoperiod and a 24/18 °C day/night temperature regime, and were harvested after 3 weeks. At harvest, plants were processed as described above to assess seedling growth and infection of apple roots by *R. solani*.

2.5. Effect of RSM on R. solani growth and N-oxidizing soil micro-organisms

A hyphal emergence assay was established to determine the effect of RSM amendment on *R. solani* growth in soil. *R. solani* AG-5 strain 5–104-colonized oat grains were placed into sets of 20 test tubes (1 grain per tube) and covered to a 4 cm depth with CV soil that had been amended 4 weeks earlier with 0.5% RSM, or 1.15 mg NH_4Cl g soil^{-1} or no amendment. After 6 days at 24 °C, tubes were examined under a dissecting microscope for emergence of *R. solani* hyphae from the soil surface.

Based on the high N content of amendments utilized in these studies, findings from split-root plant assays and assessment of microbial communities, further studies were conducted to examine the effect of RSM amendment on NO producing capacities of resident *Streptomyces* spp., stimulation of nitrifying bacteria and NO concentrations in soil. Isolates of *Streptomyces* were recovered from non-treated and RSM-amended soils during the conduct of microbial analyses described above. Individual isolates recovered on 1/50th-strength TSA were streaked onto yeast extract-malt extract agar in 9-cm dia Petri plates, sealed with parafilm and incubated at 25 °C. Commencing at 48 h, NO was sampled at 48 h intervals from the Petri plate head space by puncturing the parafilm with a 1-mm dia needle connected to a chemiluminescence-based NO analyzer (Sievers NOA-280i; Boulder, CO). Non-inoculated agar medium contained up to 4.3 ng NO l^{-1}. Selected strains were removed from culture and tested for NO production by fluorometric reaction with diaminofluorescein-FM diacetate (DAF-FM DA) as described by Cohen and Yamasaki (2003). Following a 3 min incubation, cells were rinsed with 50 mM phosphate buffer (pH 7.4) to remove extracellular DAF-FM DA. Intracellular NO was imaged as fluorescence by use of a Bio-Rad (Hercules, CA) Radiance 2100 Confocal and Multi-Photon Imaging System.

Strains were also analyzed for the presence of a NOS gene homolog by Southern hybridization of NcoI-, BsaI- or PstI-digested total DNA to a probe generated from PCR amplification of a 359-bp internal region of the *S. avermitilis* NOS gene (forward primer: 5′-ACGAGCAGCT-CATCCGGTA-3′; reverse primer: 5′-GCCGATCTCGG-TACCCATGT-3′). PCR fragments were labeled during the amplification process with digoxigenin (Roche, Basel, Switzerland). Reaction conditions consisted of an initial 5 min incubation at 94 °C and 5 cycles of 1 min at 94 °C, 1.5 min at 50 °C and 1 min at 72 °C followed by another 35 cycles, identical except for shortening the incubation time at 50 °C to 1 min.

The chemiluminescence-based NO analyzer system was utilized to determine NO concentrations in soil. Just prior to the onset of the experiment, 4 l Shimajiri soil was sieved (2 mm) and one half (2 l) amended with 0.5% (vol/vol) RSM and the other half not amended. To inhibit nitrification 1 l of each of the above soils was treated by incorporating 360 mg dicyandiamide (Sigma, St Louis, MO) kg dry soil^{-1} (Tenuta and Lazarovits, 2002). The soils were then divided equally (0.5 l) into duplicate cut-off and inverted polyethylene terephthalate bottles having a 150 cm^2 surface area. Periodic measurements were made by inserting a 1-mm dia needle to a soil depth of 38 mm at three randomly chosen locations within each container and the average NO concentration value was recorded.

Numbers of autotrophic nitrifiers in soils were estimated by the most probable number method (Alexander, 1982). Soils were serially diluted in ATCC *Nitrosomonas* medium 221 (http://www.atcc.org/mediapdfs/221.pdf) prepared without cresol red. Tubes were incubated at 28 °C and after 6–8 weeks 0.5 ml samples were removed and combined with sulfanilamide and *N*-napthylethylenediamine dihydrochloride to colormetrically test for

the presence of nitrite (Sakihama et al., 2002). After a 20 min incubation, the presence of nitrate was assayed by adding a few grains of Zn and monitoring the solution for an increase in color.

2.6. Data analysis

Percent data were transformed to arcsine-square root values prior to analysis. Microbial soil population data were transformed to $\log_{10}$ values prior to analysis. Data were subjected to analysis of variance and means separation using the Tukey test. Statistical analyses were conducted using Sigma-Stat, version 2.0 (SPSS, San Rafael, CA).

3. Results

3.1. Effect of seed meal amendments on microbial communities

Micro-organisms resident to orchard soils responded differentially to the application of RSM. Invariably, numbers of total culturable bacteria increased one to two log units within 2 weeks after amendment (Figs. 1(A) and 2(A)). Total numbers of bacteria in RSM-amended soils subsequently decreased slowly or remained static but were always significantly larger than bacterial numbers in the untreated soils. SM amendment induced a response in total bacterial numbers which was similar to that observed in RSM-amended soils (Fig. 1(A)).

RSM and SM amendments elicited differential effects on fluorescent *Pseudomonas* spp. and *Streptomyces* spp. communities resident in orchard soils. After a brief expansion following incorporation of RSM, numbers of fluorescent pseudomonads declined to one to two orders of magnitude less than that detected in non-treated soils in both greenhouse and field trials (Figs. 1(B) and 2(B)). Fluorescent *Pseudomonas* spp. numbers also exhibited an initial positive response to SM amendment, which was followed by a more gradual decline over time and commonly were maintained in significantly larger numbers than in non-treated soils throughout the period of observation (Fig. 1(B)). *Streptomyces* spp. populations increased significantly in response to RSM amendment in both field and greenhouse trials. In greenhouse trials conducted in WVC soil, *Streptomyces* spp. numbers had increased within 2 weeks post-amendment from approximately 10^5 to 6×10^6 cfu g^{-1} soil, and did not decline significantly over the next 16 weeks in RSM-amended soils (Fig. 1(C)). SM amendment resulted in an initial increase in *Streptomyces* spp. populations,

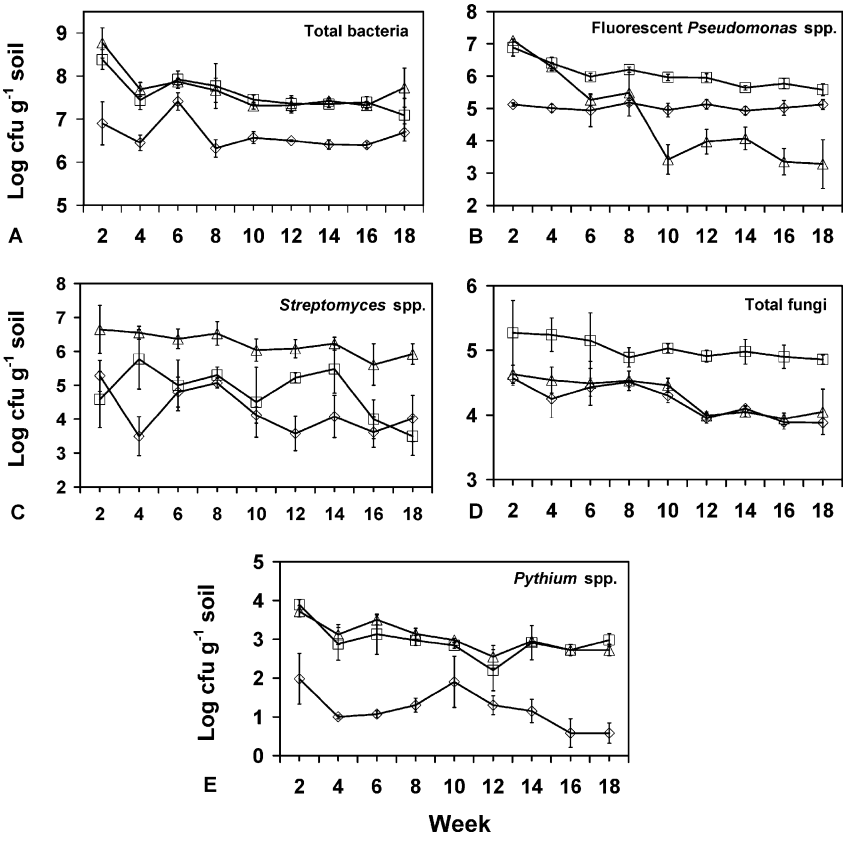

Fig. 1. Effect of soil amendments (◇, control; △, rapeseed seed meal; □, soybean seed meal) on populations of total culturable bacteria (A), fluorescent *Pseudomonas* spp. (B), *Streptomyces* spp. (C), total fungi (D) and *Pythium* spp. (E) recovered from Wenatchee Valley College orchard soil incubated in the greenhouse at 20±3 °C. Soils were sampled and microbial populations were estimated as described in Section 2.2.

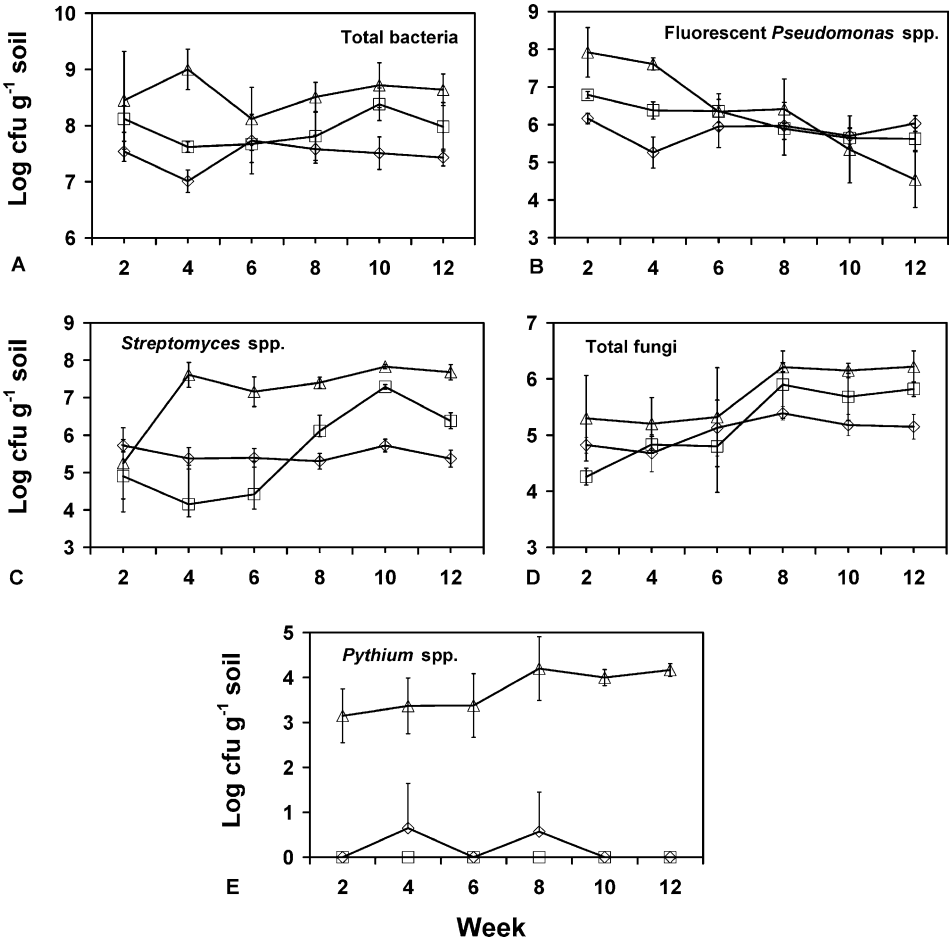

Fig. 2. Effect of soil treatments (◇, control; △, rapeseed seed meal; □, Telone C17) on populations of total culturable bacteria (A), fluorescent *Pseudomonas* spp. (B), *Streptomyces* spp. (C), total fungi (D) and *Pythium* spp. (E) recovered from Columbia View Experimental orchard soils. Fumigation and seed meal amendment were applied on 29 March 2003. Soils were sampled and microbial populations were estimated as described in Section 2.2.

however this increase was transient and was significantly less than that attained in response to RSM amendment. In trials conducted at the CV orchard, *Streptomyces* spp. numbers increased from 5.2×10^5 to 4.1×10^7 cfu g^{-1} soil within 4 weeks of RSM application, which were maintained throughout the subsequent 8 weeks (Fig. 2(C)). *Streptomyces* spp. numbers were not significantly altered initially by Telone C17 soil fumigation, but subsequently increased until at 10 weeks post-fumigation *Streptomyces* spp. populations were significantly higher than in control soils.

SM amendment resulted in an increase in total fungal populations recovered from orchard soils, and populations remained static in significantly higher numbers than the residents in non-treated or RSM-amended soils (Fig. 1(D)). Fungal communities resident in orchard soils responded variably to RSM amendment. In WVC soil, changes in total fungal populations were not detected in response to RSM amendment during the 18 week incubation. At the CV orchard, no apparent response was detected over the initial 6 weeks, but by week 8 total fungal populations in RSM-amended soils were significantly greater than that recovered from non-treated soil, and this difference was maintained through week 12. Two weeks after fumigation, total fungal populations recovered from treated soil were significantly lower than non-treated CV orchard soil (Fig. 2(D)). Thereafter, fungal populations recovered in fumigated soil and were significantly greater than the control by week 12. Incorporation of either RSM or SM resulted in a consistent increase in populations of *Pythium* spp. resident to orchard soils, which were maintained throughout the 12–18 week observation period (Figs. 1(E) and 2(E)). *Pythium* spp. were not detected at any sampling period in fumigated soil.

3.2. NOS^+ *Streptomyces spp. and N-oxidation in RSM-amended soils*

Isolates of *Streptomyces* spp. were assayed for production of NO using a chemiluminescence-based NO analyzer. Both root habitat and RSM amendment were significantly correlated with a higher proportion of NO-producing *Streptomyces* spp. strains (Table 1). For selected strains, production or lack of production of NO was verified by staining with the NO-specific dye DAF-FM DA followed by confocal microscopic imaging (Fig. 3). Production of NO

Table 1
In vitro antagonism toward *Rhizoctonia solani* and *Bacillus mycoides*, and NO production by *Streptomyces* spp. recovered from apple roots and orchard soils

Source	RSM amendment	Number of isolates	Antibiosis of *R. solani*		Antibiosis of *B. mycoides*		NO production	
			% inhibiting strains	Inhibition zone (mm)	% inhibiting strains	Inhibition zone (mm)	% NO-producers	Maximal NO (parts 10^{-9})
Soil	−	46	26.1a	1.96ab	43.5bc	2.85a	78.3b	44.8a
Soil	+	53	71.7b	3.26b	24.5b	2.19a	92.5a	102.8b
Root	−	20	10.0a	0.5a	45.0ac	2.75a	95.0a	56.7ab
Root	+	31	16.1a	1.0a	67.7a	3.55a	96.8a	62.6ab

Values in the same column followed by the same letter are not significantly ($P > 0.05$) different based on Tukey tests of ANOVA comparisons for mm and parts 10^{-9} values and chi squared tests of the ratios used to calculate the % values.

could be visualized across long stretches of hyphae, as can be seen in the confocal micrograph of *Streptomyces* sp. strain RR2 isolated from M26 roots planted in RSM-amended CV soil (Fig. 3). The percentage of *Streptomyces* strains antagonistic to *R. solani* was significantly lower in the rhizosphere of roots compared to bulk soil (Table 1).

The numbers of autotrophic nitrifying bacteria had increased significantly following amendment of CV soil with 0.5% RSM; soil populations shifted from a baseline of 2.5×10^4 MPN g^{-1} to 3.0, 1.6 and 0.54×10^5 MPN g^{-1} at 7, 9 and 18 weeks after incorporation of RSM, respectively. The increase in bacterial nitrification was also evident in the considerable rise in soil NO production effected by RSM amendment (Fig. 4). Dicyandiamide completely suppressed the RSM-mediated stimulation of soil NO production to a concentration below that of non-amended soil, aside from a first-week increase in NO concentration that was most likely due to incomplete dissolution of the nitrification inhibitor within the soil (Fig. 4). Dicyandiamide treatment of non-amended soil lowered the baseline soil NO concentration from an average of approximately 10 to 6.5 ng l^{-1} (data not shown).

The possibility that the RSM-induced increase in soil NO concentrations resulted from higher numbers of *Streptomyces* spp. was ruled out by the observations that soil supplementation with dicyandiamide had no significant effect on RSM-mediated amplification of *Streptomyces* spp. populations and that NO-production by cultured isolates was not inhibited in the presence of 360 mg dicyandiamide l^{-1} in the culture medium (data not shown).

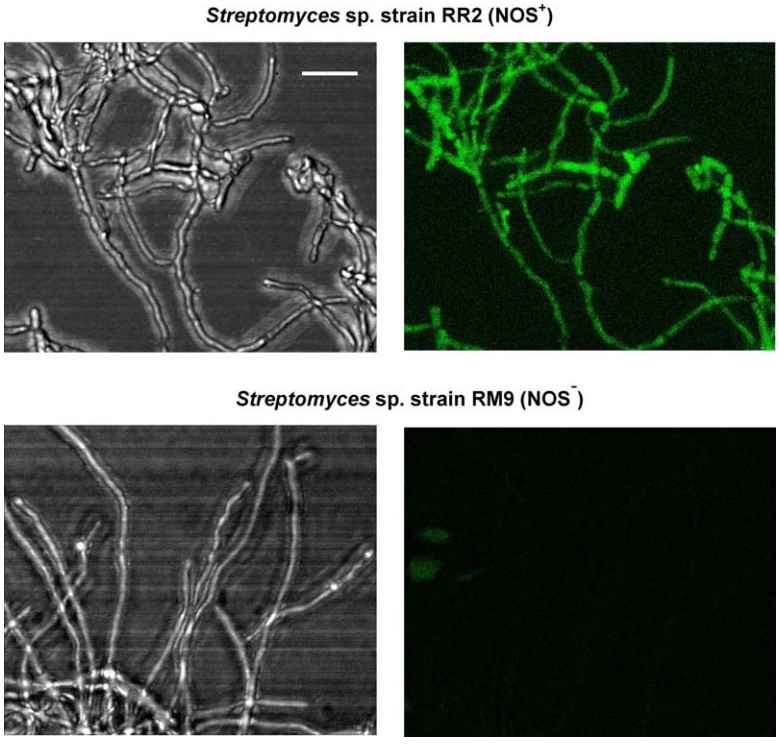

Fig. 3. Detection of NO production from *Streptomyces* hyphae by fluorometric reaction with DAF-FM DA. *Top*, Light micrograph and confocal image of *Streptomyces* sp. strain RR2, which also showed NO production on an agar plate assay (maximum value 550 ng NO l^{-1}) and the presence of NOS gene-hybridizing fragments by Southern analysis (not shown). *Bottom*, Lack of fluorescence from strain RM9 (isolated from dry RSM), which did not show NO production on the agar plate assay (maximum value 2.3 ng NO l^{-1}) or the presence of a NOS gene-hybridizing fragment. Bar, 10 μm.

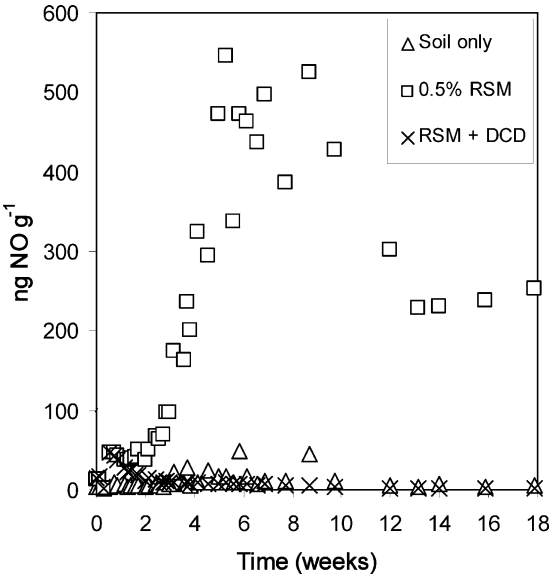

Fig. 4. NO concentrations in soils without amendment (control) and in the presence of 0.5% RSM or 0.5% RSM with 360 mg dicyandiamide (DCD) kg^{-1} soil.

3.3. Effect of seed meal amendments on control of *R. solani* and *Pratylenchus spp.*

RSM amendment significantly enhanced growth of apple in orchard soils, and the growth response could be attributed, in part, to the control of introduced as well as resident pathogens and parasites. In plant assays conducted in GC orchard soil, the application of either RSM or SM prior to planting resulted in significantly lower numbers of *Pratylenchus* spp. recovered from Gala seedling roots at harvest (Table 2). In the field, RSM amendment prior to planting had a similar influence on recovery of the lesion nematode from apple roots at the end of the initial growing season. RSM amendment was as effective as Telone C17 fumigation in reducing numbers of *Pratylenchus* recovered from M26 and M7 rootstocks at the CV and WVC orchards, respectively.

Table 2
Effect of soil treatments on recovery of *Pratylenchus* spp. from roots of apple grown in replant orchard soils

Orchard[a]			
Treatment[b]	WVC	CV	GC
Control	236a[c]	89a	110a
RSM	56b	3b	29b
SM	ND[d]	ND	9b
Telone C17	47b	12b	ND

[a] Orchard designations: WVC, Wenatchee Valley College, E. Wenatchee; CV, Columbia View Experimental Orchard, Orondo; GC, commercial orchard, Manson. WVC and CV trials were conducted in the orchard using M7 and M26 rootstock, respectively. GC trial was conducted in the greenhouse and employed Gala seedlings. Populations were assessed 3 and 5 months post-planting for greenhouse and field trials, respectively.
[b] RSM, rapeseed seed meal; SM, Soybean seed meal; RSM was incorporated into soils at a rate of 0.5% (vol/vol). SM was added in a manner proportional to RSM amendment to obtain equivalent quantities of total N added.
[c] Values are numbers of *Pratylenchus* g^{-1} root. Means in a column followed by the same letter are not significantly ($P=0.05$) different based on the Tukey test.
[d] Treatment was not evaluated.

Table 3
Isolation frequency (%) of *Rhizoctonia* spp. from roots of Gala seedlings cropped in Wenatchee Valley College orchard soil artificially infested with *R. solani* AG-5

Treatment[a]	Experiment-1	Experiment-2
Control	21.5a[b]	25.3a
RSM	8.3b	4.1b
SM	14.0ab	11.7ab

[a] RSM, rapeseed seed meal; SM, Soybean seed meal; RSM was incorporated into soils at a rate of 0.5% (vol/vol). SM was added in a manner proportional to RSM amendment to obtain equivalent quantities of total N added. Assessment of root infection was conducted at seedling harvest, 12 weeks post-planting.
[b] Means in a column followed by the same letter are not significantly ($P=0.05$) different based on the Tukey test. Each treatment consisted of four replicates with five seedlings each. Ten root segments were randomly excised from each seedling and plated on ampicillin (100 μg ml^{-1})-amended water agar. Fungal growth was examined after 48 h incubation at 20–24 °C.

RSM, but not SM, was effective in controlling root infection incited by *Rhizoctonia* spp. In two independent experiments, when Gala seedlings were grown in soils artificially infested with *R. solani* AG-5 isolate 5–104, recovery of *Rhizoctonia* spp. from apple roots was significantly reduced from a frequency of greater than 20% for the non-treated soils to less than 10% for seedlings grown in RSM-amended soil (Table 3). In both studies, pre-plant amendment of soil with SM did not significantly diminish *Rhizoctonia* root infection frequencies relative to the non-treated control. The suppression of *Rhizoctonia* root infection by RSM, but not SM, soil amendment was also evident based upon seedling growth data (Table 4). Root and shoot biomass of Gala seedlings grown in RSM-amended soils was significantly greater than seedlings grown in the non-treated control or SM-amended soils. As was observed in studies to assess the effect of the amendments on microbial community composition, RSM stimulated total culturable bacteria, and by seedling harvest had significantly depressed fluorescent *Pseudomonas* spp. numbers (Fig. 5). At harvest, numbers of

Table 4
Effect of soil amendments on growth of 'Gala' seedlings in *R. solani* AG-5 infested WVC-A orchard soil

Treatment[a]	Root weight (g)	Shoot weight (g)	Shoot length (cm)
Control	0.36a[b]	0.88a	8.2a
RSM	0.73b	1.93b	11.9b
SM	0.38a	1.21ab	8.4a

[a] RSM, rapeseed seed meal; SM, Soybean seed meal.
[b] Means in a column followed by the same letter are not significantly ($P=0.05$) different based on the Tukey test. Each treatment consisted of four replicates with five seedlings each.

Pythium spp. in RSM (5.75×10^2) and SM (3.12×10^2) were significantly higher ($P<0.001$) than that recovered from non-treated (3.8×10^1) WVC orchard soil. The reduced recovery of *R. solani* AG-5 from seedling roots in response to RSM amendment relative to the control or SM amendment was associated with enhanced recovery of *Streptomyces* spp. (Fig. 5(C)).

RSM soil amendment significantly ($P<0.05$) reduced apple root infection by native *Rhizoctonia* spp. at two field sites. For M26 rootstock grown in non-treated soil at CV orchard, *Rhizoctonia* spp were recovered from 16.2% of root segments but only 5.6% of root segments from the same rootstock grown in RSM-amended soils. At WVC orchard, infection of M7 rootstock by *Rhizoctonia* spp. was reduced from 13.1% in non-treated soil to 4.2% in RSM-amended soils. *Rhizoctonia* spp. were recovered from apple rootstocks grown in Telone C17 fumigated soil at a frequency of 3.8 and 5.7% at the CV and WVC orchards, respectively.

Ramification of *R. solani* mycelia through soil was not inhibited by the presence of RSM even though RSM-amended soil had considerably higher numbers of *Streptomyces* spp. and the community possessed a significantly higher proportion of strains antagonistic to *R. solani* (Table 1). Rates of emergence through 4-cm columns of soil that had been amended with 0.5% RSM, or 1.15 mg NH_4Cl g soil^{-1} (an amount of N equivalent to that in 0.5% RSM), or no amendment were nearly identical after a 6 day incubation (40–45% emergence).

3.4. Split-root assays

As RSM amendment did not limit hyphal growth of *R. solani* AG-5 in soil, split-root assays were conducted to assess whether induction of host resistance contributed to disease control. In an initial experiment, when a portion of the Gala seedling root system was established in RSM-amended soil, roots grown in the spatially-separated *R. solani*-infested soil were infected at a frequency of 10%. In the same system but absent the RSM amendment, *R. solani* infection frequency was 22%. These values were

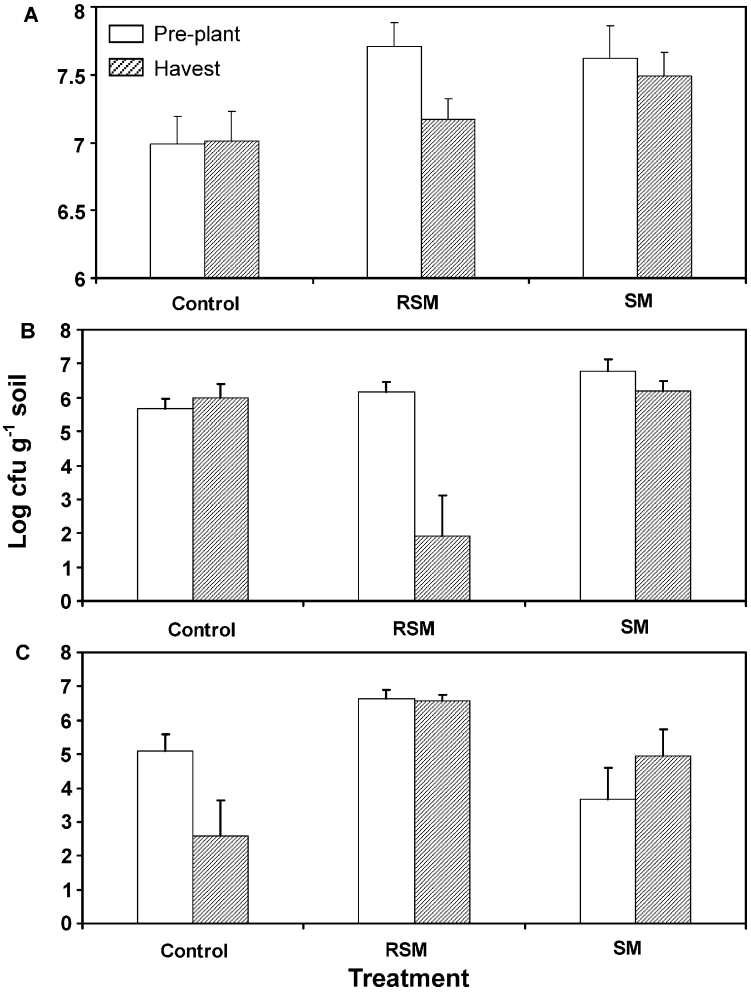

Fig. 5. Effect of rapeseed seed meal (RSM) and soybean seed meal (SM) amendment on populations of total bacteria (A), fluorescent *Pseudomonas* spp. (B) and *Streptomyces* spp. (C) recovered from WVC orchard soils infested with *Rhizoctonia solani* AG-5 strain 5–104. Soils were sampled 2 weeks post-amendment, immediately prior to planting soils with 8-week-old Gala apple seedlings (pre-plant) and at harvest (12 weeks post-planting).

not significantly different ($P=0.072$). In a subsequent experiment using the same plant growth system, *R. solani* root infection frequency for seedlings established with a portion of the root system growing in RSM-amended soil root infection frequency was significantly ($P<0.001$) less than the control; 36.2 and 63.6%, respectively.

4. Discussion

4.1. Effect of low glucosinolate content RSM on disease development

The application of organic amendments for the control of soil-borne plant diseases has been repeatedly explored as an environmentally sensible alternative to the use of synthetic pesticides. The widespread adoption of such an approach has been limited by variability in efficacy of such treatments, which in turn has defied resolution due to a general absence of knowledge concerning the mechanisms of disease suppression imparted by these amendments. Although the efficacy of Brassicaceae plant residues for control of soil-borne pathogens and parasites has typically been attributed to the toxicity of glucosinolate hydrolysis products (Angus et al., 1994; Smolinska et al., 1997; Brown and Morra, 1997) the timing of maximal-suppressive activity and the chemical composition of a variety of these materials including the *B. napus* seed meal we used in this study suggests an alternative functional mechanism.

Suppression of *Pratylenchus* spp. observed in response to RSM amendment, though consistent with the known suppressive effects of Brassicaceae residues on plant parasitic nematodes, is unlikely due to the toxicity of isothiocyanates released from glucosinolate hydrolysis. Phenylethyl glucosinolate was associated with the reduced infestation of canola by *Pratylenchus* spp., and its hydrolysis products caused mortality of *Globodera rostochiensis* juveniles and inhibited egg hatching of *Heterodera glycines* (Serra et al., 2002). Phenylethyl glucosinolate was not present in the *B. napus* seed meal we used in this study. In addition, when applied at a similar total N content, SM was as effective as RSM in reducing numbers of *Pratylenchus* spp. recovered from the roots of Gala apple seedlings. In concert with our previous observation that RSM amendment suppressed lesion nematode numbers irrespective of glucosinolate content (Mazzola et al., 2001), these findings support our contention that RSM glucosinolate hydrolysis products do not have a significant role in the suppression of *Pratylenchus* spp.

Certain high N amendments are known to have nematicidal or nematistatic properties (Rodriguez-Kabana, 1986; Kaplan and Noe, 1993; Koenning et al., 2003). Under appropriate pH conditions, the addition of nitrogenous amendments results in elevated concentrations of NH_3 in soils (Oka and Pivonia, 2002; Tenuta and Lazarovits, 2002, 2004). The nematicidal activity of NH_3 has been repeatedly demonstrated and the use of organic and inorganic N sources have been used for nematode control (Rodríguez-Kábana et al., 1982; Rodriguez-Kabana et al., 1987; Oka and Pivonia, 2003). Alternatively, control of plant parasitic nematodes through application of certain high N amendments such as animal manure may result from a stimulation of soil micro-organisms that are antagonistic toward nematodes (Rodriguez-Kabana et al., 1987; Kaplan et al., 1992). Given that incorporation of either RSM or SM at equivalent N concentrations resulted in similar reductions in numbers of *Pratylenchus* spp. recovered from apple roots, either of the potential processes cited above is a more plausible mechanism contributing to the observed nematode suppression than is the activity of glucosinolate hydrolysis products.

Based on qualitative and quantitative attributes of the *B. napus* seed meal glucosinolate composition, as suggested for the suppression of *Pratylenchus* spp., it is unlikely that glucosinolate hydrolysis products contributed to the control of *R. solani*. Hydrolysis products derived from the dominant glucosinolate (2-OH-3-butenyl) contained in the seed meal employed in this study lack inhibitory activity toward *R. solani* (Manici et al., 2000). Likewise, suppression of apple root infection by *Rhizoctonia* spp. resident to orchard soils via the application of RSM was obtained in a previous study irrespective of seed meal glucosinolate content (Mazzola et al., 2001).

4.2. Influence of RSM on resident Streptomyces spp.

Streptomyces spp. exhibited a one to two order of magnitude increase within 2 weeks of RSM-amendment, and these elevated numbers were maintained throughout our study. This was observed consistently both in greenhouse and field trials. RSM itself contains very low diversity and numbers ($\sim 5\times 10^2$ cfu g^{-1}) of *Streptomyces* spp., and we have not recovered any apparent RSM-derived strains from apple roots planted in RSM-amended soils. These observations indicate that, rather than serving as a source, RSM amendment enhances communities of *Streptomyces* spp. resident to soils.

A role for *Streptomyces* spp. in the suppression of *Rhizoctonia* spp. has been suggested by Singh and Mehrotra (1980), Tahvonen (1982), Rothrock and Gottlieb (1984), Mazzola (1999), and Mazzola and Gu (2000). The disease-suppressive effects of *Streptomyces* spp. are generally attributed to their release of antibiotics and other secondary metabolites. RSM amendment appeared to selectively enhance bulk soil communities of *Streptomyces* spp. that produce antibiotics active against *R. solani*. However, such *Streptomyces* spp. isolates comprised a minority of the community recovered from apple roots, the likely point of microbial interaction and plant protection. In addition, the degree of in vitro antagonism toward *R. solani* expressed by isolates recovered from the apple rhizosphere was negligible relative to those isolates recovered from bulk

soil. In contrast, roots showed no bias against colonization by *B. mycoides*-antagonistic *Streptomyces* strains, and, at least in the case of roots in RSM-amended soil, showed a significant enrichment for these strains. The relative scarcity of strains producing *R. solani*-active compounds in the *Streptomyces* spp. community from the apple rhizosphere may be due to a generalized toxicity of many of these antibiotics against eukaryotes. As hyphal growth of *R. solani* in bulk soil was not inhibited by RSM amendment, it is presumed that antibiosis does not play a significant role in the observed disease suppression.

4.3. Potential involvement of Streptomyces spp. in RSM-induced disease control

Results from the split-root assays we used suggests that suppression of *Rhizoctonia* root rot of apple observed in response to *B. napus* seed meal amendment is a plant-mediated phenomenon. Certain rhizosphere-inhabiting bacteria exhibit the capacity to stimulate plant defense responses conferring resistance to a variety of plant pathogens (van Loon et al., 1998). Among those examined in this study, *Streptomyces* spp. resident to orchard soils were the sole microbial group which was recovered from control, RSM- and SM-amended soils in a differential manner which corresponded to the degree of disease control observed in greenhouse trials. While these data are associative and not conclusive in nature, the high proportion of NO producers among the isolates recovered from plant roots is suggestive of their plausible role in the induction of plant defense responses either through a direct effect of NO on the plant host or indirectly through a putative function of NO in the synthesis of plant-active compounds.

Based on our results, NO production by NOS-containing bacteria, including certain *Streptomyces* spp., is apparently a relatively minor source of NO in the environment, probably since it functions solely in non-essential processes such as stress gene regulation (Cohen and Yamasaki, 2003) and secondary metabolite synthesis (Kers et al., 2004). Our finding that nitrifying bacteria are overwhelmingly responsible for NO production in RSM-amended soil should not be surprising considering that these organisms must oxidize approximately 35 mol of ammonia to assimilate 1 mol of CO_2 (Atlas and Bartha, 1993) and is in accord with other studies that have demonstrated the primacy of nitrification in the production of NO in soils. However, the presence of root-colonizing NOS^+ *Streptomyces* spp. may have physiological relevance owing to the site of NO release to the plant host. Most *Streptomyces* spp. isolates recovered from the roots of apple grown in RSM-amended soils produced NO when assayed in vitro and were shown to produce NO along the length of the hyphal filaments. NO has a role in a variety of plant functions, such as stomatal movement, root-growth promotion and plant defense (Neill et al., 2003). NO, whether from endogenous or exogenous sources, can prime plant defenses by stimulating the production of phenylpropanoids including salycilic acid (Delledonne et al., 1998; Durner et al., 1998). Based on our findings, and the known ability of NO to activate plant defense genes, we believe that a possible role for bacterial-derived NO in disease suppression warrants further investigation in this and other plant systems.

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