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Incorporation of *Brassica seed meal* soil amendment and wheat cultivation for control of *Macrophomina phaseolina* in strawberry

Mark Mazzola · Aaron Agostini · Michael F. Cohen

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Abstract *Macrophomina. phaseolina* is the cause of charcoal rot, a disease of emerging importance in strawberry production systems. Brassicaceae seed meals (SM) and prior cultivation of soils with wheat were evaluated for the capacity to suppress charcoal rot of strawberry and to determine the relative contribution of seed meal derived chemistry and soil biology in disease control. Brassicaceae seed meal amendments suppressed the abundance of *M. phaseolina* detected in soil systems, but optimal SM-induced pathogen suppression required a functional soil biology. Suppression of *M. phaseolina* was obtained with SM sourced from various Brassicaceae species and was not associated with a biologically active chemistry such as that generated by *Brassica juncea* SM amendment (e.g. allyl isothiocyanate). Disease control observed in natural soil was abolished when SM amended soils were pasteurized prior to infestation with *M. phaseolina*, suggesting a functional role of soil biology in disease suppression that was observed. Cultivation of soils with wheat prior to pathogen infestation resulted in a level of disease control superior to SM amendment, however no additive effect on disease suppression was observed with integration of the two treatments. In small scale field trials, SM amendment induced phytotoxicity was observed and may have contributed to a lack of apparent control of charcoal rot. In the same trials, significant weed control was achieved in response to SM amendment. Across trials conducted in controlled and field environments there has been a lack of consistent association between the effect of SM amendment or wheat cultivation on *M. phaseolina* soil density and resulting level of root infection. This suggests that the observed disease control may have a greater dependence upon microbial interactions that transpire in the rhizosphere than that which occurs in the bulk soil environment.

Keywords Suppressive soil · Charcoal rot · Strawberry · Soil-borne disease

M. Mazzola (✉)
USDA-ARS Tree Fruit Research Laboratory, 1104 N. Western Ave, Wenatchee, WA 98801, USA
e-mail: mark.mazzola@ars.usda.gov

A. Agostini · M. F. Cohen
Department of Biology, Sonoma State University, Rohnert Park, CA 94928, USA

Introduction

Intensive strawberry production in California, and worldwide, has relied on pre-plant soil fumigation to control key soilborne pathogens, weeds and pests. Methyl bromide and chloropicrin mixtures were the most common soil fumigant utilized in strawberry production systems, but its use is currently restricted under the Montreal Protocol. Alternative fumigant chemistries are available but limitations in terms of efficacy relative to methyl bromide continue to exist (Duniway 2002). Despite advances in fumigation technology that have led to a reduction in emissions, the long-term availability of these alternative fumigant chemistries is not certain. Continuing review and regulatory actions will

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potentially limit access to or use of these fumigants on a state-wide or regional basis. In addition, changes in fumigation chemistries and application methods have been associated with the emergence of important soil-borne diseases that incite plant collapse including charcoal rot caused by *Macrophomina phaseolina* (Zveibil and Freeman 2005; Koike 2008). Fumigation with 1,3-dichloropropene + chloropicrin was not consistently as effective as methyl bromide in decreasing viability of *M. phaseolina* sclerotia and it was suggested that the increased prevalence of the disease in certain locations may be related to the phase-out of methyl bromide use (Zveibil et al. 2012).

Current and potential restrictions on the use of soil fumigants has resulted in an examination of other potential pest control strategies in strawberry production systems (Daugovish et al. 2004; Subbarao et al. 2007; Samtani et al. 2011; Muramoto et al. 2014). Organic residue-based soil amendments, including various composts and green manures, have been evaluated for control of soil-borne diseases in strawberry production systems, but such an approach has failed to yield consistent disease suppression (Millner et al. 2004; Leandro et al. 2007). A major limitation to the effective use of composts and other organic amendments for disease control is the lack of knowledge concerning the mechanism(s) operative in eliciting pathogen suppression. In certain instances, composts may confer disease control and improved plant growth simply due to an enhanced overall level of biological activity (Chen et al. 1988) as the amendment serves as a significant growth substrate. In other instances, specific microorganisms resident to the compost are responsible for disease control (Hoitink et al. 1991) and in the absence of specific functional microbial populations disease control is unlikely to be realized. The limited scope of pathogen isolates or members of a pathogen complex examined in plant assays may also contribute to a level of optimism concerning compost mediated disease control that is not realized when examined under field conditions. Significant variability in compost capacity to suppress disease has been exhibited when multiple isolates of the same fungal pathogen species or anastomosis group were evaluated (Termorshuizen et al. 2007; Tewoldemedhin et al. 2015).

Soil incorporation of Brassicaceae plant residues has been viewed as a potential measure for the control of various plant pests including pathogens, insects and weeds. The efficacy of this management protocol for the control of soil-borne diseases in strawberry has been highly variable. Subbarao et al. (2007) demonstrated that rotations with broccoli and Brussels sprouts followed by postharvest residue incorporation reduced the number of *Verticillium dahliae* microsclerotia in soil resulting in reductions in Verticillium wilt incidence and increased strawberry fruit yield. These treatments did not alter densities of *Pythium* spp. in soil and overall the rotations were not as effective as methyl bromide + chloropicrin fumigation in terms of disease control and plant yields. Incorporation of cover crop residues of *Brassica juncea* or *Sinapis alba* had no effect on *V. dahliae* or *Fusarium* spp. soil densities and failed to suppress soil-borne diseases of tomato (Hartz et al. 2005). Similarly, Daugovish et al. (2004) reported that *B. juncea* and *S. alba* green manures failed to reduce *V. dahliae* microslerotia densities in strawberry field soils, but did suppress growth of *Phytophthora cactorum*. Mattner et al. (2008) reported that volatiles produced by a *Brassica rapa/Brassica napus* macerated crop suppressed the growth of six different soil-borne pathogens affecting strawberry and reduced survival of *P. cactorum* and *Cylindrocarpon destructans* in strawberry field soil even though the biofumigant crop did not produce detectable levels of isothiocyanates (ITCs) in soil. Seed meal of *B. juncea* was reported to reduce soil densities of *M. phaseolina* and *Fusarium oxysporum* when used in concert with irrigation in the high temperature environments found in an arid region of India (Lodha et al. 1997)

The vast majority of investigations concerning the use of Brassicaceae residues for pest control have been undertaken with the notion that efficacy is predominantly a function of biologically active glucosinolate hydrolysis chemistries. However, it has been demonstrated that mechanisms of a biological rather than chemical nature may have a prominent role in the disease and weed control achieved in response to these amendments (Cohen et al. 2005; Mazzola et al. 2007; Yulianti et al. 2007; Hoagland et al. 2008; Friberg et al. 2009; Motisi et al. 2009). The dominant mechanism functional in disease suppression may vary from pathogen to pathogen (Mazzola et al. 2007; Weerakoon et al. 2012) and host genotype can modulate disease control efficacy (Mazzola et al. 2009). For instance, in response to the application of certain Brassicaceae seed meals (SM), "biofumigation" (chemistry) initially exerts a biocidal effect on *Pythium* spp. whereas SM-induced amplification of specific elements of the resident soil microbial

community results in induction of plant systemic resistance to *Rhizoctonia solani* (Cohen et al. 2005; Mazzola et al. 2007). Failure to consider such factors has led to inconsistent results when attempting to utilize Brassicaceae residue amendments for the management of soilborne diseases.

Use of cover crops or green manures prior to establishment of a commercial crop has been employed extensively as a means to control soil-borne diseases. In a modified manner, control of Rhizoctonia root rot of apple was attained when replant orchard soils were pre-cropped with specific wheat genotypes prior to replanting apple (Mazzola and Gu 2002; Mazzola and Mullinix 2005). In this application, the wheat root system was shown to modify microbial community structure in a manner that led to disease suppression and the green plant matter was removed from the system rather than incorporated into the soil profile.

The current study was undertaken to examine the potential of Brassicaceae seed meal amendments and/or prior wheat cropping to suppress the density of *M. phaseolina* in soil, to determine the efficacy of these amendments in suppressing disease development and to assess the relative contribution of biological and chemical mechanisms in the resulting disease control response.

Materials and methods

Soils Assays were conducted in strawberry field soil from Santa Maria, CA. The soil was naturally infested with *M. phaseolina* and was collected in August 2009 and transported to the USDA-ARS laboratory in Wenatchee, WA. Alternatively, studies were conducted in field soils obtained from the Washington State University Columbia View research and demonstration site (CV soil) near Orondo, WA (N 47° 46′ 32″, W120° 1′ 16″) and were artificially infested with the *M. phaseolina* as described below.

Seed meals Brassicaceae seed meals were sourced from different plant species that yield different spectra of biologically active chemistries. Seed meals used in these studies were obtained from *Brassica juncea* cv. Pacific Gold which possessed a glucosinolate content of 176.3 μmol g^{-1}, *Brassica napus* cv. Athena with a glucosinolate level of 25.4 μmol g^{-1} and *Sinapis alba*, with a glucosinolate content of 170.8 μmol g^{-1}(Handiseni et al. 2011). The dominant glucosinolates detected were 2-propenyl, 3-butenyl and 4-OH-benzyl for *B. juncea, B. napus* and *S. alba* seed meals, respectively. The seed meals are pressed, dehydrated plant residues from the oil extraction process and have the appearance of irregular-shaped flakes. The seed meal was sized with 4-mm^2, 2-mm^2 and 1-mm^2 sieves and fine (<1 mm) particles were used for the following experiments. A single lot of the SM was used throughout this study.

M. phaseolina sensitivity to AITC Among the seed meals utilized, only *B. juncea* SM yields a biologically active volatile (allyl isothiocyanate, AITC) when incorporated into soil. AITC is commonly reported as an active disease control mechanism (Chung et al. 2002; Dhingra et al. 2004, Mari et al. 2002), therefore, the sensitivity of *M. phaseolina* to this compound was examined. *M. phaseolina* strain 07–3 was cultured on potato dextrose agar (PDA). Seed meal was incorporated into soil at a rate of 1.0% (*w*/w). Seed meal amended or non-treated soil (250 g) was placed in 0.946 L Mason jar with three replicate jars per soil treatment. Five 0.5 cm plugs from the margin of an active *M. phaseolina* culture were contained in an open Petri dish suspended 3 cm above the soil surface in the Mason jar which was then fastened with a lid containing a rubber septum. After 1, 2, 4, 6 and 8 h incubation one agar plug was removed from each replicate jar and plated onto water agar amended with ampicillin (100 μg ml^{-1}). Plates were incubated at 24 °C and hyphal growth was measured after 24 h. The headspace was sampled immediately prior to extraction of fungal plugs at each sampling time point and at 10 and 24 h by piercing the septum with a 3.8-cm-long 18-gauge needle and extracting a 1-ml vol which was analyzed by gas chromatography using the methods previously described (Mazzola et al. 2007).

Soil and plant assays

Inoculum production Oat grain inoculum was prepared using previously described methods (Wilkinson et al., 1985). Microsclerotia inoculum was prepared as follows: a 0.5 cm agar plug was excised from the growing margin of *M. phaseolina* 07–3 cultured on PDA and transferred to 15 ml potato-dextrose broth in a 9 cm dia

Petri dish. Plates were sealed with Parafilm and cultures were incubated for 4 wk. at 24 °C. The liquid medium was filtered through a double layer of cheese cloth and the mycelia and sclerotia were rinsed from the cheese cloth, transferred into a blender, and comminuted by blending for 2 min in 100 ml water. The resulting solution was poured into a 100 ml graduated cylinder and sclerotia were allowed to settle to the base. The supernatant and associated fungal hyphae were decanted, sclerotia were washed with sterile dH_2O, and the settling process was repeated two additional times. After the final wash, sclerotia were air-dried in a laminar flow hood, collected, and stored at 4 °C.

Effect of brassicaceae seed meals on suppression of M. phaseolina in soil Initial studies were conducted to assess the capacity of the three different Brassicaceae seed meals to suppress *M. phaseolina* in artificially infested pasteurized and non-pasteurized CV soil. Pasteurization was conducted by placing soil in heat-resistant bags and exposing to steam at 80 °C for 3 h. Soil was cooled overnight prior to repeating the steaming cycle. Soil was infested with ground *M. phaseolina* oat grain inoculum (particle size 0.25–0.5 mm diameter) at a rate of 0.03% (*w*/w). Seed meal was applied to soil at a rate of 0.3% (*w*/w), mixed thoroughly and decanted into Ray Leach Cone-tainers (size SC10 [21 cm long × 3.8 cm diameter], Stuewe & Sons, Tangent, OR) with 10 replicates per treatment including a no treatment control, arranged in a complete randomized design. Soils were incubated in a controlled environment growth chamber at 28 °C, were covered with plastic to retain moisture and were watered twice weekly. Soil samples were collected at two weeks after treatment. DNA was extracted from a 5 g soil sample and *M. phaseolina* was quantified by qPCR as described below.

Ensuing studies examined the effect of SM amendment on survival of *M. phaseolina* in soil and infection of strawberry. CV soil was infested with *M. phaseolina* at a rate of 2.5 microsclerotia g^{-1} soil and *B. juncea, B. napus* or *S. alba* SM was applied to soil at a rate of 0.3% (*w*/w). SM amended and non-treated (control) soils were decanted into 3.8 L pots with five replicates per treatment and soils were planted with strawberry (cv. Camarosa). Plants were incubated for an initial 6 weeks at 28 °C with a 16 h photoperiod, and watered (75 ml) on an alternating day basis. After an additional 2-week growth period at 32 °C, plants were harvested and a soil and root sample was collected from each pot. DNA was extracted from 5 g soil and 50 mg root samples, and pathogen density in soil and root samples were determined by real-time quantitative PCR as described below. The experiment was repeated.

Integration of wheat cultivation and B. juncea SM amendment for control of M. phaseolina Plant bioassays were conducted in a strawberry field soil from Santa Maria, CA that was naturally infested with *M. phaseolina*. A high incidence of charcoal rot and resulting plant collapse was visible at the time of field soil collection in August 2009. Soil was treated with *B. juncea* SM at a rate of 0.3% (*w*/w) or was left untreated (control), and decanted into 3.8 L plastic pots with ten replicate pots per treatment. Soils were incubated on a greenhouse bench at 24° ± 3 °C and were watered (100 ml) twice weekly. After 4 weeks, five pots of each treatment (*B. juncea* SM and no treatment) were planted with a wheat (*Triticum aestivum*) seed blend (1:1) of 'Lewjain'/ 'Penawawa' with 10 g of seed per pot. Wheat was grown for four weeks at which time shoot growth was harvested. Soils were then mixed by hand and replanted with the same wheat seed blend. The second cropping of wheat was removed after four weeks. Strawberry (cv. Camarosa) plants grown in pasteurized potting mix for four weeks at a day/night temperature regime of 24/18 °C with a 12 h photoperiod. Strawberry plants were transferred to all pots (control, *B. juncea* SM, wheat, *B. juncea* SM + Wheat) and grown in environmental growth chambers at 32 °C with a 16 h photoperiod. Soil and root samples were collected at 0, 4, 8 and 20 weeks after planting soils with strawberry. Presence of *M. phaseolina* in soil, root and crown tissues was determined as described below. Population densities of culturable *Streptomyces* spp., fluorescent *Pseudomonas* spp., total fungi and total bacteria in soil were determined as previously described (Cohen and Mazzola 2006a). The experiment was conducted twice.

Soil treatment for induced suppression of M. phaseolina CV soil was placed in eight plastic flats (surface area of 0.16 m^2, 12 cm deep) and four of the flats were each planted with 20 g of pre-germinated wheat seed (cv. Louise). All soils were watered on an alternate day basis and incubated on a greenhouse bench for five weeks at 24 °C, at which time wheat shoots were excised and removed. Soils from four flats for the individual treatments were pooled and mixed. *B. juncea*

seed meal was applied to a portion of each soil, (0.3% *w*/w), and mixed thoroughly. At completion of the seed meal treatment, all soils were aerated for five weeks on the greenhouse bench at which time soils were infested with *M. phaseolina* at a rate of 2.5 microsclerotia g^{-1} soil. Strawberry (cv. Camarosa) plants prepared as described above were transferred to the treated and non-treated CV soils with one plant per pot and 10 replicates per treatment. Plants were grown for 8 wks in environmental growth chambers using a day/night temperature regime of 30/18 °C and 16 h photoperiod. Soil, root and crown samples were collected at harvest. The experiment was conducted three times.

Brassicaceae seed meal field trial evaluation Field trials were conducted during the 2009–2010 and 2010–2011 growing seasons in raised beds (4.1 m × 2.0 m × 0.23 m) at research plots near Sonoma State University, Rohnert Park, CA. Soil (Wright Loam, pH 7.4) treatments were distributed using a randomized split-split plot design with four replicates per treatment. During the 2009–10 growing season, *M. phaseolina* inoculum soil densities were amplified by cultivation of plots with strawberry (cv. Camarosa) and soybean (*Glycine max*), with seven plants of each species per plot. After four months, the plant shoot biomass was removed leaving the root matter in place. Ten days after plant removal (October 30, 2009), *B. juncea* SM was applied to treatment plots at a rate of 6.75 t ha^{-1} and supplemental nitrogen in the form of urea pellets was added to the non-amended soils at 5.05 kg ha^{-1} soil. SM and urea were incorporated to a soil depth of 15 cm using a rotary hoe. Plots were watered to surface saturation and immediately covered with clear 4 mm polyethylene mulch which was removed after 7 days. Total weed biomass was determined on a plot basis 43 days after SM application (prior to planting) and at termination of the experiment. Beds were covered with 4 mm black polyethylene mulch and plots were planted with certified virus free strawberry cv. Camarosa bare root crowns 46 days after soil treatment and the experiment was terminated 154 days post-planting. The experiment was repeated in the 2010–11 growing season except that the SM amendment consisted of a 1:1 formulation of *B. juncea/S. alba* SM and soils were planted to strawberry 28 days after SM application. Fruit production, runner biomass and plant mortality were monitored throughout the growing season. Effect of treatments on pathogen soil density and root infection were determined by real-time quantitative PCR analysis as described below.

DNA isolation and detection of M. phaseolina For controlled environment studies, DNA was isolated from all plants and a soil sample from each growth container (pot or cone-tainer). For field trials, DNA was extracted from two composite root and soil samples from each plot. DNA was extracted from approximately 50 mg strawberry root or crown tissue using the MoBio Ultraclean plant DNA isolation kit (MoBio Laboratories, Carlsbad, Ca) with two replicate extractions conducted for each sample. DNA was extracted from two 5 g soil samples per pot using the MoBIO Ultraclean Mega soil DNA isolation kit (MoBio Laboratories).

Real-time quantitative PCR (qPCR) analysis was used for the detection and quantification of *M. phaseolina* in soil and plant roots. Initial qPCR assays employed the species-specific primer set, MpKFI/MpKRI, reported by Babu et al. (2007) which targets the internal transcribed spacer (ITS) region. Repeated attempts at amplification using purified *M. phaseolina* 07–3 DNA as the target and the conditions and primers reported by Babu et al. (2007) failed to yield any discernible product. Upon sequencing of the ITS region from this isolate, and comparison to *M. phaseolina* sequences in GenBank, a one base-pair mismatch at the 5′ end of the published MpKRI primer sequence was noted. The MpKFI primer (Babu et al. 2007) was edited to the following: 5′-GCTCCGAA GCGAGGTGTATT-3′.

Quantitative PCR was performed in 10 μl volume reactions using a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA). A primer concentration of 20 pmol μl^{-1} was used in all reactions. The PCR reaction mixture for amplification of soil or strawberry root/crown DNA consisted of 5 μl of nanopure water, 3 μl of PCR Master mix SYBR® Green (Applied Biosystems), 1 μl of each primer (20 μM stock), and 1 μl of template DNA diluted 1:100 in H_2O. The standard curve was generated from dilution of purified DNA isolated from *M. phaseolina* strain 07–3 ranging from 1 ng μl^{-1} to 1 fg μl^{-1}. All reactions were performed in triplicate. The amplification protocol consisted of the following conditions: 95 °C 10 min, (95 °C 15 s-62 °C 1 min-72 °C 1 min) × 40 cycles, followed by a melt curve with an 0.3 °C s^{-1} increase in temperature from 60 °C to 95 °C.

Data analysis Data were analyzed using SigmaPlot 12.2 (Systat Software Inc., San Jose, CA). Soil population data were transformed to log10 values. Data were subjected to one way analysis of variance and mean separation was performed using the Student-Newman-Keuls test in which $P < 0.05$ was considered significant. In instances where data failed the Shapiro-Wilk test for normality, data were analyzed using the Kruskal-Wallis test on ranks.

Results

M. phaseolina sensitivity to AITC The addition of *B. juncea* SM in a fine or coarse particle form to soil resulted in generation of detectable quantities of AITC within 1 h post-SM application (Fig. 1). Maximum AITC concentration in the head space was detected at 7 h and 8 h post-amendment for soils treated with fine particle and coarse particle SM, respectively. During the initial 8 h post-amendment period, quantity of AITC detected was higher from soils treated with fine particle SM than coarse particle SM. With the exception of the 1 h exposure to AITC emitted from coarse particle amended soils, *M. phaseolina* growth on PDA after removal from the AITC environment was diminished relative to growth of a culture that was not exposed to AITC (Fig. 1). *M. phaseolina* growth from colonized agar plugs was consistently observed when plated on fresh PDA after incubation in the AITC environment, irrespective of the exposure period.

Seed meal amendment effects on M. phaseolina soil density and plant infection In soil, the effect of seed meal amendments on quantity of *M. phaseolina* detected was irregular and varied with seed meal type. In CV soil artificially infested with *M. phaseolina*, all SM treatments significantly ($P < 0.001$) suppressed the density of the pathogen detected by qPCR relative to the no treatment control at the two week sampling point (Fig. 2a). The quantity of *M. phaseolina* DNA detected did not differ significantly among seed meal treatments. When the same assay was conducted in pasteurized CV soil, the quantity of *M. phaseolina* detected in SM amended soil increased at least three orders of magnitude relative to the corresponding treatment in the non-pasteurized CV soil (Fig. 2b). In pasteurized soil, the density of the pathogen was significantly ($P < 0.001$) higher in all seed meal amended soils relative to the no treatment control, and was higher in *S. alba* SM amended soil than soil treated with either *B. juncea* or *B. napus* SM.

When duration of the incubation period was extended to eight weeks, SM soil amendments exhibited an inconsistent and diminished capacity to suppress the density of *M. phaseolina* in artificially infested soil (Figs. 3a and 4a). In general, there was no consistent and significant effect of SM amendment on quantity of *M. phaseolina* DNA detected in soil at the end of the 8 wk. growth period. However, strawberry root infection

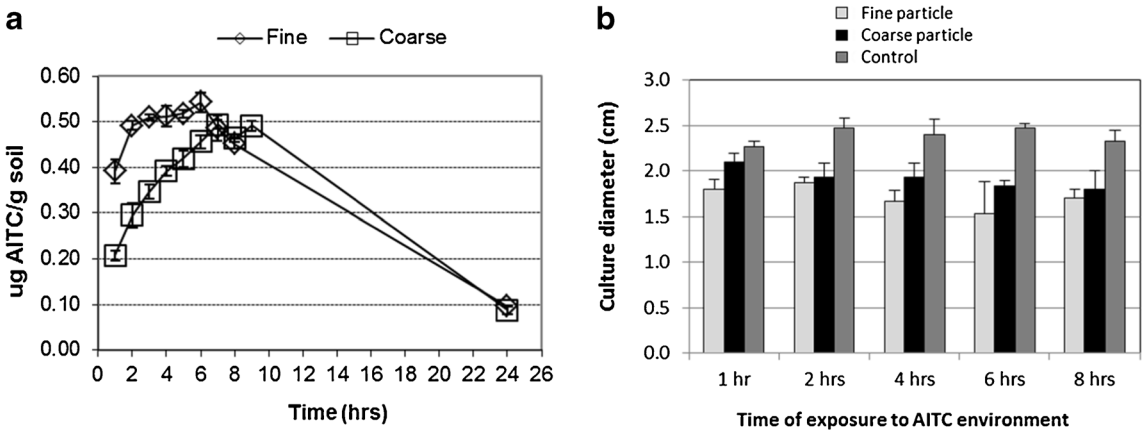

Fig. 1 Emission of allyl isothiocyanate (AITC) from soil amended with coarse or fine particles of *Brassica juncea* cv Pacific Gold seed meal (*left panel*) and hyphal growth of *Macrophomina phaseolina* on fresh potato dextrose agar after exposure to the emitted AITC for the denoted time period (*right panel*)

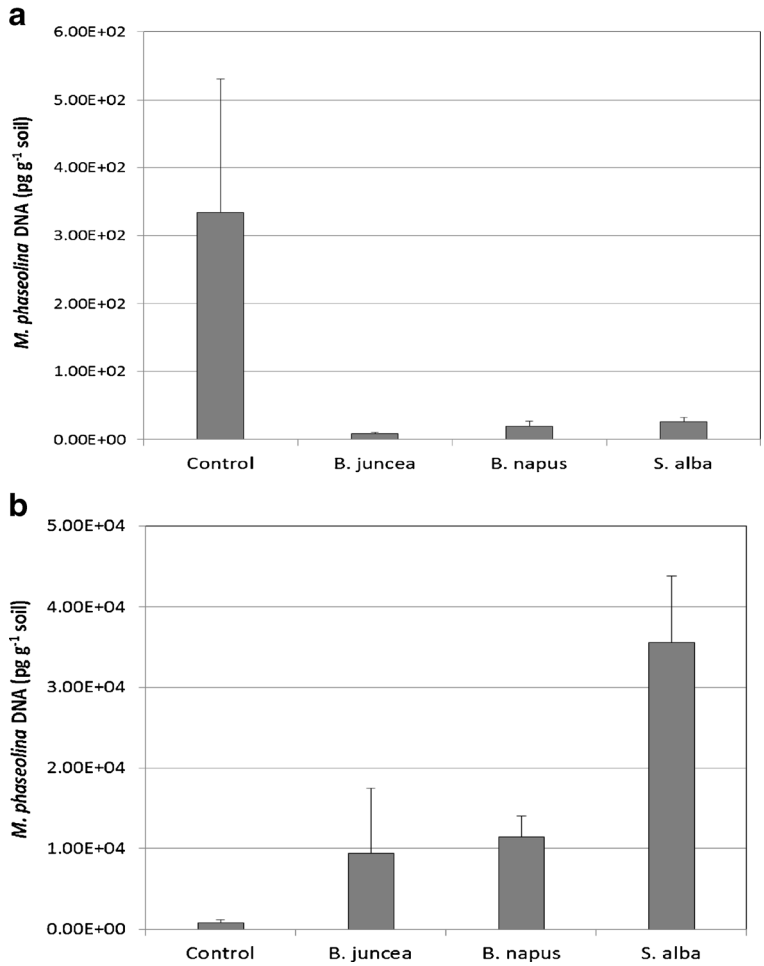

Fig. 2 Quantity of *Macrophomina phaseolina* DNA detected in natural (**a**) and pasteurized (**b**) soil as determined by real-time quantitative PCR after two weeks incubation at 28 °C. Soil was infested with the pathogen at a rate of 0.03% (*w*/w) after pasteurization and prior to seed meal application at a rate of 0.3% (*w*/w). Bars represent one standard deviation of the mean. Treatments: *B. juncea* = *Brassica juncea* cv. Pacific Gold; *B. napus* = *Brassica napus* cv. Athena; *S. alba* = *Sinapis alba* cv. IdaGold

by *M. phaseolina* was consistently suppressed by all three SMs even in instances where pathogen soil density was not altered by the amendment (Figs. 3b and 4b).

Wheat cultivation, *B. juncea* SM amendment and the combination of these two treatments each suppressed pathogen density and strawberry root infection in assays conducted in Santa Maria strawberry field soil naturally infested with *M. phaseolina* (Fig. 5). All treatments suppressed root infection in a similar manner relative to the control. However, strawberry shoot and root biomass production was significantly greater for the wheat or wheat/*B. juncea* SM treatments, relative to the *B. juncea* SM alone treatment (Table 1).

Treatment effects on soil microbiology Diverse changes in soil densities of broad microbial groups were detected in response to treatments applied to the Santa Maria soil. As would be expected, the addition of substrate to the soil, in the form of SM amendment or wheat cropping, resulted in an overall increase in microbial densities (Table 2). Particular responses in soil microbial densities followed trends associated with increased plant growth and reduced *M. phaseolina* root infection. For instance, total soil fungal populations followed a pattern inverse to the trends observed with regard to plant root infection by *M. phaseolina*. At four weeks after planting, fungal density was not significantly different between the no treatment control and SM-amended soils. At the same time point, fungal populations were significantly ($P < 0.001$) greater in wheat cropped soils; 1.62×10^6 and 1.81×10^6 cfu g^{-1} soil for wheat and *B. juncea* SM + wheat treatments, respectively. The density of total fungi in the control soil did not exceed 6.79×10^4 cfu g^{-1} soil at any sampling point in this study, and by 20 weeks fungal densities were significantly greater for all treated soils relative to the control,

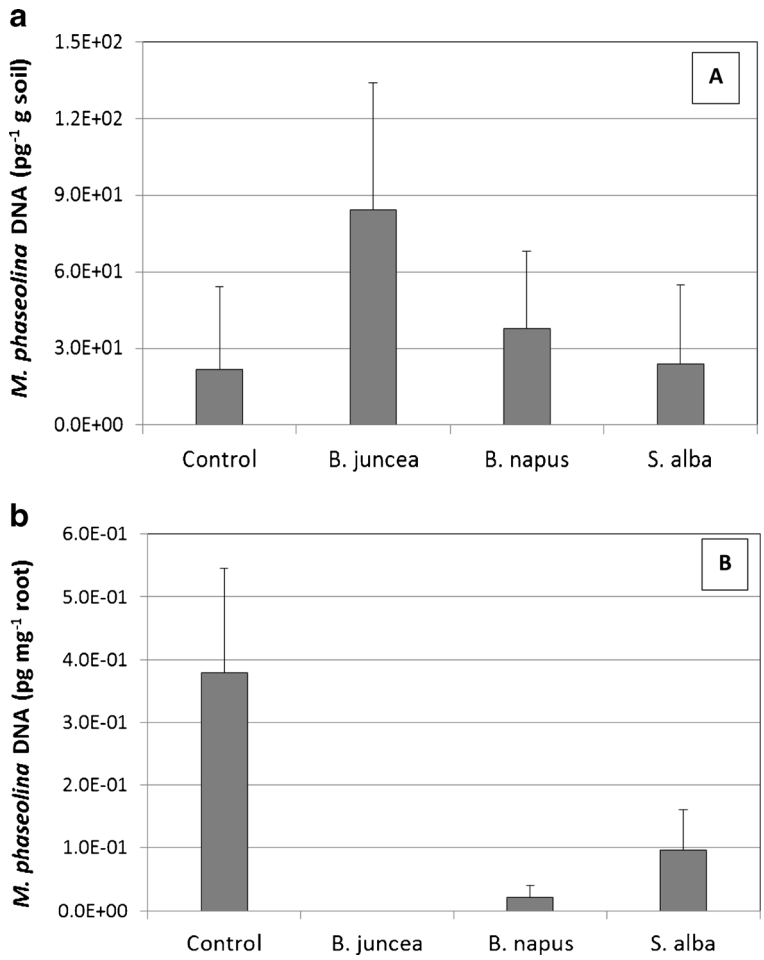

Fig. 3 Trial 1 evaluating the effect of seed meal amendments on quantity of *Macrophomina phaseolina* DNA detected in soil (**a**) and strawberry roots (**b**). Soil was artificially infested with the pathogen at a rate of 2.5 microsclerotia per gram soil. Seed meal amendments were applied at a rate of 0.3% (*w*/w) and pathogen DNA concentration was determined after eight weeks post-planting. Assays employed a 28 °C incubation temperature for six weeks followed by anadditional two weeks incubation at 32 °C. Bars represent one standard deviation of the mean. Treatments: *B. juncea* = *Brassica juncea* cv. Pacific Gold; *B. napus* = *Brassica napus* cv. Athena; *S. alba* = *Sinapis alba* cv. IdaGold

and were higher in wheat treated soils than in the *B. juncea* SM amended soil. Fluorescent *Pseudomonas* spp. soil populations increased by an order of magnitude in response to all soil treatments 4 weeks after planting. Thereafter, although fluorescent *Pseudomonas* spp. density was stable at 10^5 in the no treatment control, a gradual decline in population of approximately one order of magnitude was observed for all other treatments at harvest (20 weeks post-planting). Significant sustained increases in *Streptomyces* spp. soil densities were limited to those treatments that included *B. juncea* SM application. Total soil bacterial populations increased in a similar fashion for all treatments relative to the no-treatment control.

Efficacy of B. juncea SM or wheat cultivation on induced suppression of M. phaseolina The same soil treatments noted above were applied to CV soil prior to pathogen introduction to examine their potential to provide long-term suppression of *M. phaseolina*. Soils cropped to wheat or amended with *B. juncea* SM prior to pathogen infestation exhibited some level of reduction in subsequent strawberry crown and root infection by *M. phaseolina*. Wheat cultivation of soil prior to pathogen infestation was the only treatment that reduced the quantity of *M. phaseolina* DNA detected in strawberry crown tissue across all three independent experiments, and corresponded with relative plant survival among treatments (Table 3). Plant mortality at the end of the growth period ranged from 60 to 100% for strawberry grown in the non-treated control soil. Plants grown in *B. juncea* SM and *B. juncea* SM/wheat treated soils had mortality rates ranging from 0 to 100%, while mortality ranged from 0 to 20% for the wheat alone treatment. Quantity of pathogen DNA detected in soil by qPCR did not differ among treatments and did not correspond with resulting crown infestation or plant mortality. A similar trend was observed for quantity of

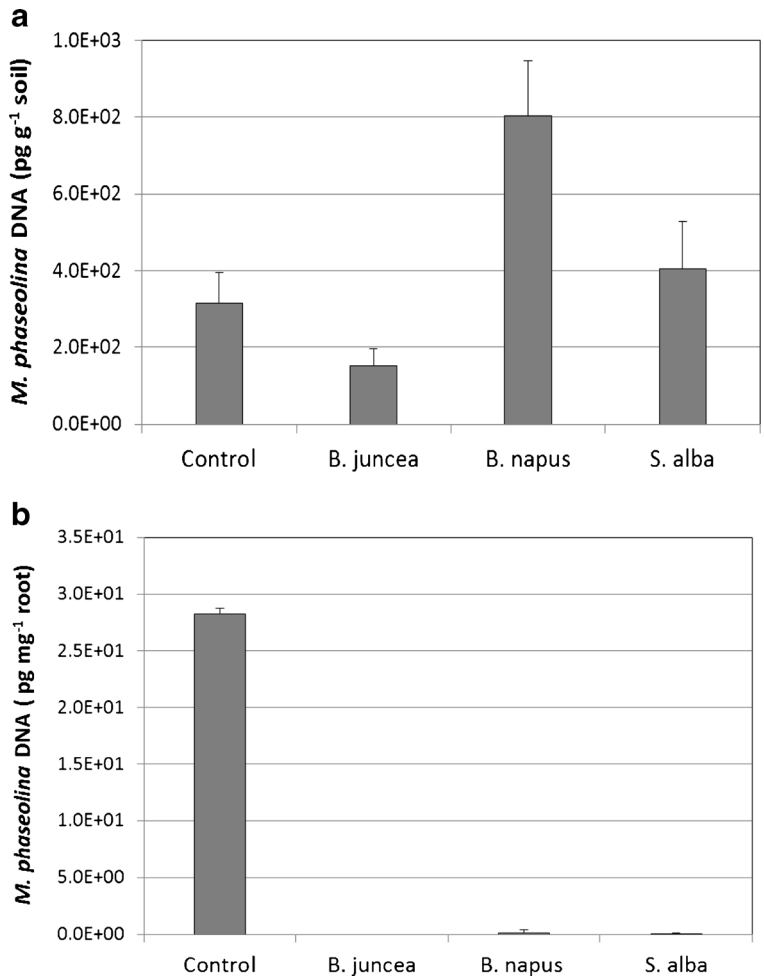

Fig. 4 Trial 2 evaluating the effect of seed meal amendments on quantity of *Macrophomina phaseolina* DNA detected in soil (**a**) and strawberry roots (**b**). Soil was artificially infested with the pathogen at a rate of 2.5 microsclerotia per gram soil. Seed meal amendments were applied at a rate of 0.3% (*w*/w) and pathogen DNA concentration was determined after eight weeks post-planting. Assays employed a 28 °C incubation temperature for six weeks followed by an additional two weeks incubation at 32 °C. Bars represent one standard deviation of the mean. Treatments: *B. juncea* = *Brassica juncea* cv. Pacific Gold; *B. napus* = *Brassica napus* cv. Athena; *S. alba* = *Sinapis alba* cv. IdaGold

pathogen DNA detected in roots; although quantity of *M. phaseolina* DNA in roots of strawberry was lower for the SM treatment relative to the no treatment control in experiment 2, there was no corresponding reduction in quantity of the pathogen detected in crown tissue or plant mortality.

Field trial Brassicaceae SM amendment significantly ($P < 0.05$) reduced weed biomass production irrespective of the presence or absence of *M. phaseolina* (Fig. 6). The SM treatment had no significant effect on strawberry plant biomass, total number of fruit produced or total fruit biomass when plants were cultivated in *M. phaseolina* infested soil (data not shown). In fact, when cultivated in *M. phaseolina*-infested soil strawberry plants exhibited a trend towards reduced productivity in SM treated soil relative to non-treated soil though these differences were not statistically ($P > 0.05$) different. In the absence of *M. phaseolina*, biomass production in control plots treated with SM was significantly lower than that in control plots that did not receive the SM amendment. Likewise, in the absence of the pathogen, plant death in SM treated plots was approximately 16% while no plant death was observed in the control plots. In the pathogen infested plots, plant death in SM treated plots was 22% and again no plant death was observed in the pathogen-infested control plots.

Discussion

Brassica SM amendments were found to provide incomplete suppression of disease incited by *M. phaseolina* on strawberry. Disease control, in terms

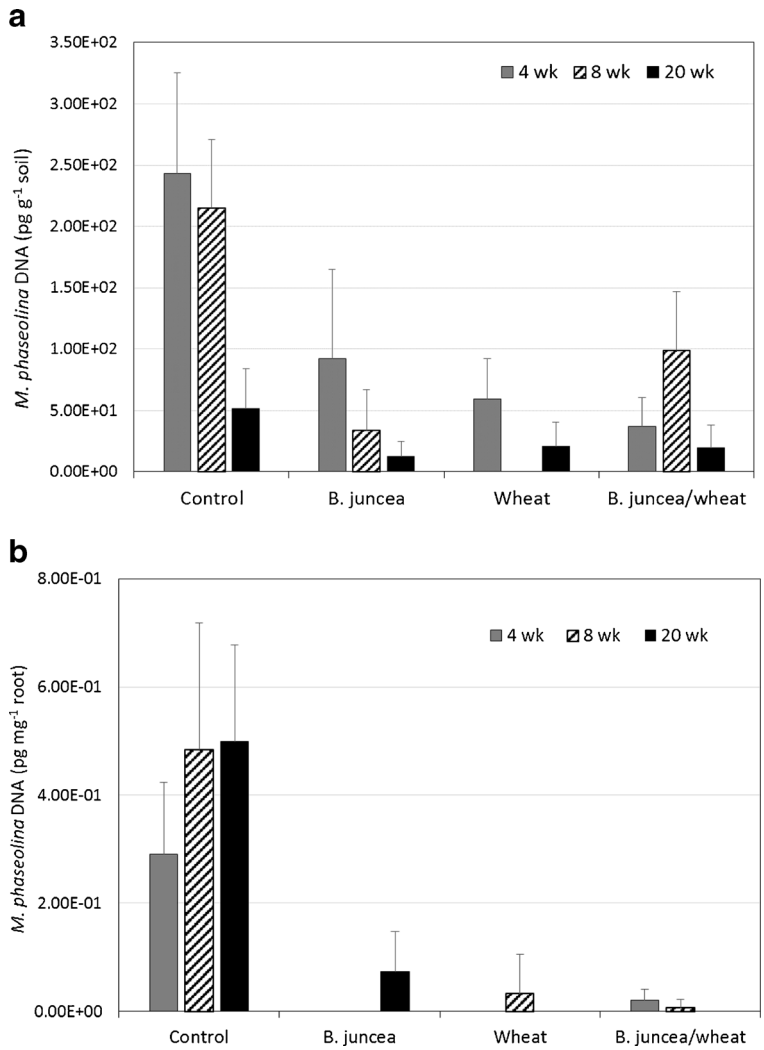

Fig. 5 Influence of prior wheat cultivation or *Brassica juncea* seed meal amendment on density of *Macrophomina phaseolina* DNA detected in soil (**a**) or strawberry roots (**b**). Assays were conducted in a naturally infested strawberry field soil obtained from Santa Maria, CA with plants grown at 32 °C with a 16 h photoperiod. Root and soil samples were collected at 4, 8 and 20 weeks after planting. Error bars indicate one standard deviation of the mean

of limiting quantity of the pathogen detected in strawberry roots, exhibited significant variation over the course of all experiments, but *B. juncea* SM appeared to provide the most consistent level of disease suppression among the three seed meals tested. Disease control attained through SM application may be conferred via multiple mechanisms, including the activity of the resident soil biology (Cohen and Mazzola 2006b; Mazzola et al. 2007). The potential contribution of soil biology in seed meal derived control of *M. phaseolina* is supported by the finding that *B. napus* SM demonstrated capacity to suppress pathogen density or root infection in both naturally and artificially infested soils. The *B. napus* SM used in this study contained very low glucosinolate levels and the chemical hydrolysis products derived from the dominant glucosinolate contained in this SM, 3-butenyl glucosinolate, are not known to have significant antifungal activity (Manici et al. 1997). Seed meal

Table 1 Strawberry biomass as affected by treatment of a Santa Maria, CA field soil naturally infested with *Macrophomina phaseolina*

Treatment[y]	Root biomass (g DW)	Shoot biomass (g DW)
Control	1.33a[x]	2.36a
B. juncea SM	2.57ab	3.71a
Wheat	3.51b	5.69b
B. juncea SM/Wheat	4.13b	5.11b

[y] *B. juncea* SM = *Brassica juncea* seed meal amendment

[x] Means in the same column followed by the same letter are not significantly ($P > 0.05$) different

Table 2 Effect of treatments on density of selected microbial groups in Santa Maria, CA field soil as determined at the respective sampling times after planting strawberry

Treatment[y]	Total bacteria	Fluorescent *Pseudomonas* spp.	Total Fungi	*Streptomyces* spp.
4 weeks				
Control	3.27E+07a[x]	1.05E + 05a	5.42E + 04a	9.48E + 05a
B. juncea SM	8.71E + 07b	1.88E + 06b	2.32E + 05ab	3.78E + 07c
Wheat	9.31E + 07b	4.09E + 06c	1.62E + 06b	4.58E + 06b
B. juncea SM+ wheat	1.45E + 08c	4.09E + 06c	1.81E + 06b	2.53E + 07bc
8 weeks				
Control	3.03E + 07a	3.33E + 05a	6.79E + 04a	2.56E + 06a
B. juncea SM	1.23E + 08b	1.47E + 06ab	4.46E + 05a	3.47E + 07b
Wheat	9.56E + 07b	1.09E + 06ab	2.11E + 06b	5.31E + 06a
B. juncea SM+ wheat	1.03E + 08b	3.29E + 06b	2.12E + 06b	2.91E + 07b
20 weeks				
Control	3.25E + 07a	1.24E + 05a	5.31E + 04a	3.36E + 05a
B. juncea SM	7.64E + 07b	5.85E + 05ab	2.71E + 05b	1.20E + 07c
Wheat	6.46E + 07bc	1.17E + 06b	8.18E + 05c	4.94E + 05a
B. juncea SM+ wheat	9.47E + 07c	9.81E + 05b	9.54E + 05d	7.58E + 06b

[y] *B. juncea* SM = *Brassica juncea* seed meal amendment

[x] For a given sampling date, means in the same column followed by the same letter are not significantly ($P > 0.05$) different

amendment was found to provide disease control when assays were conducted in natural (unaltered) soils, but disease suppression was abolished for all SM treatments when assays were conducted in pasteurized soil. Although *M. phaseolina* was inhibited by volatile allyl isothiocyanate generated in response to *B. juncea* SM soil amendment, active fungal growth was observed once AITC had been withdrawn from the system. This observation indicates that AITC is fungistatic and not fungicidal towards *M. phaseolina*. Similarly, Mattner et al. (2008) found in laboratory assays that volatiles generated from a mixture of *Brassica rapa*/*B. napus* root and shoot tissues killed a variety of strawberry soil-borne pathogens but only reduced the growth of *Fusarium oxysporum*. It is plausible that other materials that generate ITCs, including chemical fumigants, would yield a similar transitory stasis rather than toxicity toward *M. phaseolina*. In total, these findings indicate that Brassicaceae SM amendments altered soil biology in some manner to incur suppression of disease incited by *M. phaseolina*. Such a finding is not limited to this patho-system as specific transformations in soil or rhizosphere microbiology previously were shown to have a critical role in the suppression of other diseases incited by soil-borne pathogens (Mazzola et al., 2001; Cohen and Mazzola 2006b, Yulianti et al. 2007; Friberg et al. 2009; Weerakoon et al. 2012).

Prior wheat cultivation of a naturally infested strawberry field soil demonstrated greater efficacy than *B. juncea* SM amendment in suppressing soil density of *M. phaseolina* and limiting subsequent infection of strawberry. Although cropping of soils with wheat was shown to induce soil suppressiveness toward *Rhizoctonia solani* (Mazzola and Gu 2002; Mazzola and Mullinix 2005), the limited data generated to date suggest a different functional mechanism driving the suppression of *M. phaseolina*. Quantitative and qualitative changes in the fluorescent *Pseudomonas* spp. population in response to wheat cropping sequences was reported to contribute to suppression of apple root infection by *R. solani* (Mazzola and Gu 2002; Gu and Mazzola 2003). Amplification of fluorescent *Pseudomonas* spp. soil density was similar in seed meal amended and wheat cultivated soil; treatments that yielded distinctly different levels of disease suppression. In contrast, significant differences were observed among these soils in total fungal populations, with significantly higher densities observed in all treatments that included wheat cropping relative to the control or *B. juncea* SM amended soil at 8 and 20 weeks post-treatment.

Table 3 Survival of strawberry and detection of pathogen DNA when plants were grown in soil artificially infested with *Macrophomina phaseolina* at 2.5 microsclerotia g^{-1} soil 5 weeks following application of 0.3% *B. juncea* SM and/or 5 wks pre-cultivation with wheat

Treatment[y]	% Mortality	*M. phaseolina* DNA soil (pg g^{-1})	*M. phaseolina* DNA root (pg $mg^{-1)}$	*M. phaseolina* DNA crown (pg mg^{-1})
Experiment 1				
Control	60	2.31	0.03	299.7b
B. juncea SM	0	0	0	9.7a
Wheat	0	0.40	0.04	5.88a
Wheat/*B. juncea* SM	30	1.56	0	71.1ab
Experiment 2				
Control	100%	48.2	60.1b[x]	1217.3bc
B. juncea SM	100%	10.0	4.85a	243.5ab
Wheat	20%	7.3	1.01a	82.2a
Wheat/*B. juncea* SM	100%	8.7	12.7ab	1903.1c
Experiment 3				
Control	70%	57.1	38.4b	108.5b
B. juncea SM	60%	50.0	19.8ab	52.1a
Wheat	0%	25.1	4.9a	1.6a
Wheat/*B. juncea* SM	0%	58.1	4.3a	0.17a

[y] *B. juncea* SM = *Brassica juncea* seed meal amendment

[x] For a given experiment, for DNA quantities, means in the same column followed by the same letter are not significantly ($P > 0.05$) different

Wheat cropped soils also exhibited the greatest capacity to induce development of a disease suppressive soil system. In experiments where pathogen introduction was conducted after application of soil treatments, a reduction in quantity of *M. phaseolina* DNA detected in strawberry crown tissue was attained even in instances where pathogen soil densities were not diminished by a treatment. This finding suggests that disease control may have a greater dependence upon microbial interactions that transpire in the rhizosphere than that which occurs in the bulk soil environment.

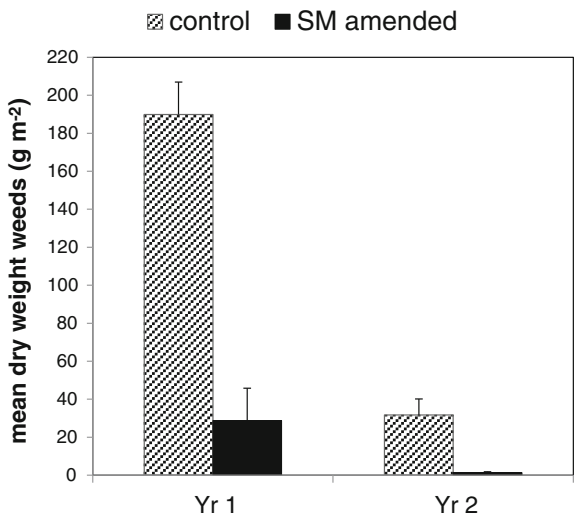

Fig. 6 Effect of Brassicaceae seed meal soil amendment on total weed biomass harvested from strawberry field trials conducted at Rohnert Park, CA. Bars represent one standard deviation of the mean

Results from the 2010–2011 field trial conducted at Rohnert Park, CA demonstrate the need for a more complete understanding of how environmental parameters (e.g. soil properties) influence the efficacy of Brassicaceae seed meal amendments for pest suppression in strawberry production systems. There has been little attention given to garnering insight into how management practices or soil characteristics will influence overall productivity of the cropping system, or provide desired outcomes such as disease or weed control, in response to Brassicaceae SM amendments. There are reports of inconsistent pest control in response to mustard seed meal amendments, and certainly that was observed in the current study. Understanding of the nature of biological mechanisms contributing to SM-induced disease suppression is necessary to optimize use of this approach. For instance, peak AITC concentration, and not total quantity of AITC generated, determined functional composition of the post-seed meal

amendment soil fungal community resulting that contributed to Pythium root rot control in apple (Weerakoon et al. 2012). Thus, factors influencing peak AITC generation, including SM glucosinolate content, particle size, soil organic matter content (Mazzola and Zhao, 2010; Mazzola et al. 2015), among others, must be considered in the use of SM for soil-borne disease suppression. These same factors, in addition to composition of the weed seed bank, will influence the efficacy of SM amendments as a weed control measure (Handiseni et al. 2011).

Similarly, much as is the case in using soil fumigants, care must be taken in the identification of appropriate plant back periods between SM application and establishment of strawberry. This also will be affected by several attributes of the production system including soil characteristics and SM formulation. In the initial field trial conducted in 2009–2010, *B. juncea* SM was applied independently using a 46 day plant back period and no symptoms of phytotoxicity were observed in strawberry. In the 2010–2011 field trial, results obtained in soils that were not infested with the pathogen indicated that phytotoxicity of the SM amendment may have contributed to this plant response. In this trial, *B. juncea* SM was used in concert with *S. alba* SM and the 28 day plant-back period was not sufficient to avoid damage to strawberry. *S. alba* SM exhibits greater herbicidal activity toward various broad leaf plants and its active chemistry (4-hydroxyl benzyl ionic thiocyanate) demonstrates greater persistence in soil systems than does the most prominent active chemistry, AITC, resulting from of *B. juncea* SM amendment (Handiseni et al. 2011). In apple, a six week plant back period was sufficient to avoid potential *B. juncea/S. alba* seed meal-induced phytotoxicity in a high organic matter (4.2%) sandy loam orchard soil but use of the same plant back period resulted in 21% tree mortality at an orchard possessing a sandy soil containing 1.7% organic matter (Mazzola et al. 2015). Attention to such detail is a notable characteristic when utilizing chemical fumigants for soil-borne pest control with extensive knowledge concerning the movement and persistence of such chemistries, and the effect of soil properties on these attributes, well documented over many decades. Similar information is required to effectively utilize Brassicaceae SMs as a pest control measure.

In other production systems, wheat cultivation of soil and *Brassica* SM amendments have provided fumigant levels of soil-borne disease control (Mazzola and Mullinix 2005; Mazzola et al. 2015). However, when evaluated in the strawberry production system SM soil amendment provided only partial control of crown and root infection by *M. phaseolina*. Although both treatments induced soil suppressiveness toward *M. phaseolina*, neither treatment was capable of abolishing subsequent plant infection, and in certain experiments the SM treatment did not substantially reduce the level of disease development relative to no treatment. Prior wheat cultivation was superior to *B. juncea* SM amendment for both control of disease in a naturally infested soil and in the induced-suppression of *M. phaseolina* crown infection and subsequent plant death when the pathogen was introduced post-soil treatment. The efficacy of wheat cultivation for control of Rhizoctonia root rot in apple was previously shown to be realized in a wheat-cultivar specific manner (Mazzola and Gu 2002), and was associated with specific changes in elements of the soil microbial community (Gu and Mazzola 2003). It is not known whether a similar response would be functioning in the strawberry system or whether wheat varieties adapted to the California cropping environment would yield a similar response. Further evaluation of this system will be conducted to address these important questions.

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