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ORIGINAL RESEARCH

Carbon source-dependent antifungal and nematicidal volatiles derived during anaerobic soil disinfestation

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Abstract Anaerobic soil disinfestation (ASD) has been shown to be effective in the control of a wide range of soil–borne plant pathogens but has not been examined as a means for disease control in perennial fruit crops such as apple. Since ASD has demonstrated a broad spectrum of biological activity, it may be well suited as an alternative to current fumigation–based control of apple replant disease (ARD) which is caused by a diverse pathogen complex. The efficacy of ASD for control of ARD pathogens was evaluated in growth chamber experiments using soils from two orchard sites having a history of the disease. Suppression of *Pratylenchus penetrans* apple root densities was dependent upon carbon source utilized during the ASD process. Volatiles emitted during the anaerobic phase from soils treated with ethanol, grass residues, or *Brassica juncea* seed meal as the carbon input effectively retarded growth of *Rhizoctonia solani* AG–5, *Pythium ultimum* and *Fusarium oxysporum*. Each carbon amendment generated a unique volatile profile produced in the treated orchard soil during ASD. Allyl isothiocyanate (AITC) and dimethyl trisulphide (DMTS) were emitted from *B. juncea* SM treated soils whereas the latter and 2–ethyl–1–hexanol were detected in soils treated with grass residues. When assayed individually using pure standards, Decanal, DMTS, and AITC retarded in vitro growth of all three fungal/oomycete pathogens. Nonanal was inhibitory toward only *P. ultimum* and *R. solani* AG–5, whereas 2–ethyl–1–hexanol only suppressed growth of *P. ultimum*. AITC and DMTS caused significantly higher mortality of *P. penetrans* compared to other tested volatiles. These findings demonstrate that carbon source–dependent volatile chemistries contribute significantly but not exclusively to suppression of certain ARD pathogens during the ASD process.

Keywords · Anaerobic soil disinfestation · Apple replant disease · Antifungal volatiles · Nematicidal volatiles

Introduction

Replant disease refers to the difficulty in establishing plants in soil previously planted to the same or closely related species. In apple, the disease has been attributed to the action of a site specific, multi–kingdom, pathogenic and parasitic biological complex. In Washington State, this includes the fungal components *Cylindrocarpon* spp., *Rhizoctonia solani* anastomosis group (AG)–5 and AG–6, multiple species of the oomycetes *Phytophthora*, and *Pythium* spp., as well as the lesion nematode *Pratylenchus penetrans* (Mazzola 1998; Mazzola and Mullinix 2005; Mazzola

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and Brown 2010). Conventional methods of apple replant disease (ARD) management have been challenged by a number of factors. Pre–plant soil fumigation with methyl bromide, or in the long run, alternative fumigants such as chloropicrin, 1, 3–dichloropropene and methyl isothiocyanate, is not sustainable (Momma et al. 2006). These broad–spectrum biocides require commercially licensed application, are costly and are potentially hazardous to agricultural workers and the environment (Mazzola and Manici 2012). Due to a growing organic apple industry, non–chemical based management practices would relieve growers from the requirement of temporary withdrawal of fumigated replant sites from organic production (Mazzola and Manici 2012). Moreover, it has been observed that fumigated orchard soils lack resilience in limiting re–infestation by soil–borne pathogens (Mazzola and Strauss 2013) and thus may not provide optimal productivity over the long–term.

In the pursuit of viable non–fumigant ARD control alternatives, effective and durable methods have been sought which utilize diverse attributes of rhizosphere microorganisms that govern disease suppression via a multiplicity of mechanisms. The early observation of soil–borne pathogen suppression under anaerobic conditions by Cook and Baker (1983) was later advanced as a potential method of soil–borne disease management in the Netherlands (Blok et al. 2000) and Japan (Shinmura 2000) for asparagus and onion. In these studies, the process of anaerobic soil disinfestation (ASD) was examined as a plausible means to attain soil–borne disease control through multiple mechanisms of action. ASD is stimulated by soil reduction which is brought about by the introduction of a labile organic material followed by flooding of soil through irrigation, and covering the soil surface with a gas impermeable plastic film. The rate of organic matter application to soil has varied widely from as little as 1 to 2 t ha^{-1} (Momma 2008) to as much as 30 t ha^{-1} (Messiha et al. 2007). The effectiveness of ASD when utilizing wheat bran or molasses as the carbon input has been demonstrated across a range of pathogens including fungi, bacteria and nematodes (Blok et al. 2000; Shinmura 2000; 2004; Takeuchi 2004).

Attributes of the ASD process proposed to contribute to disease suppression include toxic by–products of anaerobic decomposition (e.g. acetic, butyric, propionic acids), production of volatile compounds, biocontrol by anaerobic soil microorganisms, or oxygen depletion (Momma et al. 2006; Momma 2008; Katase et al. 2009). Emission of volatile compounds from soil can be used as a characteristic for determining the presence of microorganisms that proliferate under certain conditions that yield disease suppression. Numerous factors may influence the range of volatile organic compounds (VOCs) produced in a soil environment. VOCs resulting from microbial activity may be affected by soil–specific community composition and physiological state of the microorganisms (Insam and Seewald 2010), factors which themselves are influenced by numerous environmental parameters including soil moisture, texture, temperature and pH, oxygen availability, and substrate quality (Stotzky and Schenck 1976; Wheatley et al. 1996, 1997; Asensio et al. 2007; McNeal and Herbert 2009; Insam and Seewald 2010). A greater diversity and higher level of microbial derived volatile production is observed under anaerobic compared to aerobic soil conditions (Stotzky and Schenk 1976). A preponderance of culturable soil microorganisms has been found to produce VOCs (Linton and Wright 1993). This phenomenon has been demonstrated with bacterial genera such as *Streptomyces* (Schöller et al. 2002), *Pseudomonas*, *Serratia* and *Enterobacter* (Schöller et al. 1997) and fungi within the genus *Penicillium* (Larsen and Frisvad 1995; Schnüner et al. 1999).

Volatiles have also been implicated in the disease control capabilities of diverse microbial biocontrol agents. Fernando et al. (2005) found that benzothiole, cyclohexanol, n–decanal, dimethyltrisulfide, 2–ethyl–1–hexanol, and nonanal were present among volatiles produced by bacteria isolated from canola and soybean plants which completely suppressed *Sclerotinia sclerotiorum* mycelial growth and sclerotia formation. Fiddaman and Rossall (1993) reported production of volatile compound(s) by *Bacillus subtilis* which were inhibitory toward *R. solani* AG–4 and *Pythium ultimum*. However, the nematicidal activity (NA) of volatile organic compounds has not been examined widely. In a study that examined VOC production by 200 isolates of soil bacteria, 149 (74.5 %) and 165 (82.5 %) were found to exhibit greater than 20 % NA against the free–living nematode *Panagrellus redivivus* and the pinewood nematode *Bursaphelenchus xylophilus*, respectively (Gu et al. 2007).

The goal of the current study was to determine the pathogen and disease–suppressive nature of volatiles generated during the anaerobic phase of ASD. The specific objectives were to determine the effect of

carbon source input on generation of antifungal, anti–oomycete and nematicidal volatiles during the anaerobic phase of ASD, to identify the volatile compounds and to discern the activity of selected ASD derived volatiles against elements of the biological complex that incites ARD.

Materials and methods

Orchard soils

Soils used in this study were obtained from three orchard sites; a certified–organic commercial orchard (SMR) near Chelan, WA, USA (latitude 47° 85′ 76 N, longitude 120° 20′ 60″ W), a conventionally managed commercial (GC) orchard located near Manson, WA (latitude 47° 53′ 05″ N, longitude 120° 09′ 30″ W) and the Washington State University Sunrise (SR) Research Orchard (Rock Island, WA; latitude 47° 31″97′ N, longitude 120° 07′ 30″ W). Apple (*Malus domestica* Borkh.) cv Gala on M.26 rootstock was previously planted at SMR orchard (Mazzola and Brown, 2010). The GC orchard was established in 1991 on orchard ground previously planted to apple and was not fumigated prior to replanting 'Golden Delicious' on M.7 rootstock. Soils from SR orchard were obtained from the rhizosphere of 'Gala'on M.9 rootstock that had been planted in 2010. The dominant soil type at SMR and GC orchards is Chelan gravelly sandy loam and the dominant soil type at SR orchard is a Pogue fine sandy loam. Soil pH at the SMR, GC and SR orchard sites were 4.8, 7.6 and 6.8 and possessed an organic matter content of 4.2 %, 3.2 % and 1.2 % respectively. The ARD pathogen complex at SMR orchard includes binucleate *Rhizoctonia* AG–G and AG–I, *P. ultimum*, *Pythium sylvaticum*, *Pythium irregulare*, *Pythium rostratifingens*, *Phytophthora cactorum*, *Cylindrocarpon destructans* and *P. penetrans* (Mazzola and Brown 2010). At the GC orchard, ARD results primarily from the activity of P. penetrans acting in concert with a fungal/oomycete complex composed of *C. destructans*, various species of *Pythium*, and *R. solani* AG–5 (Mazzola 1998; Mazzola and Mullinix 2005; Mazzola et al. 2009; Mazzola and Brown 2010). The ARD pathogen complex at the SR orchard is dominated by *P. ultimum*, *P. sylvaticum*, multinucleate *Rhizoctonia* spp. *Cylindrocarpon* spp., and *P. penetrans* (Mazzola, unpublished data). Soils were collected from the root zone of established trees at a soil depth of 10–30 cm during summer and autumn of 2012. Soils from different orchards were separately placed in 5–l closed containers, and transported to the USDA–ARS Tree Fruit Research Laboratory in Wenatchee, WA. Soils from the root zone of different trees from the same orchard collected during the same season were mixed in a 16–l closed bin; large roots were removed by hand and soil were well mixed in a cement mixture (Kobalt®, Mooresville, NC) for 3–5 min prior to use to obtain a representative sample from the orchard.

Substrate inputs used during ASD

Powdered rice bran (RB) was added to SMR orchard soil at a rate of 12.3 mg ml^{-1} (11.1 t ha^{-1}). The amendment rate was reduced to 5.5 mg ml^{-1} (4.9 t ha^{-1}) for trials conducted in GC and SR soils as a result of the observation that *Pythium* spp. densities were elevated at the higher rice bran application rate. *Brassica juncea* cv Pacific Gold seed meal (SM) was applied to all orchard soils at a rate of 5.5 mg ml^{-1} (4.9 t ha^{-1}). This seed meal possessed a glucosinolate content of 178 μmol g^{-1} (Handiseni et al. 2012). The SM was passed through a 1 mm standard sieve prior to use to obtain particles with a diameter <1 mm. Freshly cut orchard grass residues (GR; Dactylis glomerata L.) were obtained from the Columbia View Experimental (CV) Orchard located 19 km north of East Wenatchee, WA. Grass residues were air dried in the lab overnight and stored in the cold room at 4 °C in black polyethylene bags until use. Grass was applied to soil at a rate of 44.3 mg ml^{-1} (40.0 t ha^{-1}) for SMR soil and a rate of 22.1 mg ml^{-1} (20.0 t ha^{-1}) for GC and SR soils. Composted steer manure (CM) used in the experiments consisted of 30 % steer manure and 70 % composted plant residue and was applied at 12.3 mg ml^{-1} (11.1 t ha^{-1}). Prior to use, composted steer manure was sieved through 1 mm standard sieve to obtain particles<1 mm. Each carbon amendment was analyzed for nutrient content and C: N ratio (Table 1; Soiltest Farm Consultants, Inc., Moses Lake, WA). Ethanol (ET; ethyl alcohol 200 proof; Pharmco–AAper, Shelby Drive, KY) was diluted to 10 % concentration using distilled water.

Plant material

Plant bioassays were conducted using 'Gala' apple seedlings. Apples were harvested in September 2008 from an eleven–year–old planting of 'Gala'/M26 at the CV

Table 1 Content of selected minerals and C:N ratio of carbon inputs used in the conduct of anaerobic soil disinfestation

[a]Carbon amendment	Element							C:N ratio
	Percent					Amount/ mg kg^{-1}		
	P	K	S	Ca	Mg	B	Zn	
CM	0.72	2.20	0.45	2.86	0.84	15	184	11:1
GR	0.24	1.73	0.18	0.44	0.17	6	28	19:1
RB	1.53	1.60	0.18	0.10	0.79	10	59	19:1
SM	0.59	0.70	0.93	0.82	0.32	21	38	16:1

[a] CM=composted steer manure; GR=orchard grass residues; RB=rice bran; SM=Brassica juncea seed meal

orchard and stored in a cold room at 0–1 °C. Seeds were extracted from fruit, rinsed with tap water and soaked for 2–3 min in sodium hypochlorite (1.6 mol dm^{-3}; Homelife, Prairie, MN) solution followed by extended water rinsing. Seeds were air–dried and stored in the cold room at 0-1 °C. For stratification, seeds were moistened, dusted with captan 50WP, placed in an enclosed bag and incubated at 4 °C for 6–8 weeks. Approximately 100 stratified seeds were sown in plastic planting trays filled with pasteurized potting mix soil (McConkey, Sumner, WA), soil was moistened with tap water and trays were wrapped in plastic film to retain moisture. Trays were transferred to an incubator and plants were grown under a 12 h photoperiod at a constant 24ºC. The plastic wrap was removed upon seedling contact and plants were watered every other day thereafter. Seedlings were 5–weeks–old at the time of use in assays.

ASD treatment

Orchard soils were placed in plastic pots 900 g each (diameter :12–cm; volume: 1179.9 ml; McConkey Co.). For the pasteurized control (PC), moistened soil was placed in a plastic bag and heated overnight in an oven at 85 C on two successive days. Non–pasteurized soil without addition of a carbon substrate was used as the no–amendment control (C). After application of the individual carbon inputs at the rates noted above, soils were watered to field capacity for all treatments with the exception of the ethanol treatment. For the latter, water volume required to attain field capacity was adjusted appropriately to account for the 100 ml of 10 % ethanol solution added to each pot (8. 47 μl ml^{-1}). All pots were sealed in a double layer of two gas impermeable transparent Saranex bags (17.8 × 20.3 cm, Bitran Series "S" bags, Com–Pac International Carbondale, IL). Each treatment consisted of three replicates and pots were arranged in a complete randomized design (CRD) inside environmental growth chambers. Assays were conducted using a day/night temperature regime of 24/18 °C with a 12 h photoperiod over a period of two weeks. At completion of the anaerobic phase, pots were removed from the growth chambers, bags were opened and oxidation reduction potential (ORP) was measured at 5 cm soil depth using an ORP meter (Oakton® pH 6 m, Acorn series). A soil sample was collected using a sterile cork–borer and pH was determined (Beckman Instruments Inc. Fullerton, CA) in a 1:1 soil–water solution (Burt 1996).

Plant assay

At completion of the ASD process, soils were aerated for two weeks while maintaining the soil moisture by watering 50.0 ml to each pot every other day. Two, five–week–old apple seedlings were transplanted into each pot and grown for five weeks in environmental growth chambers under the conditions noted above. At harvest, plant roots were washed to remove soil particles and seedling shoot length and weight were measured. A representative sample of twenty root segments (0.5-1.0 cm in length) per seedling were plated on a *Pythium* semi selective medium (PSSM; Mazzola et al. 2001). Emergence of mycelial growth from root segments was monitored daily to determine percent root colonization by *Pythium* spp. For each replicate pot, *P. penetrans* were extracted from a 0.5 g root sample according to previously described methods (Mazzola 1998).

Antifungal/nematicidal volatile assays

The production of antifungal and nematicidal volatiles during the ASD process was assessed in GC and SR orchard soil, respectively. Soils were amended with the carbon sources and at rates as mentioned above. For the antifungal assay, a 3-mm diameter mycelial plug excised from one–week–old cultures of *R. solani* AG–5, *F. oxysporum*, and *P. ultimum* grown on potato dextrose agar (PDA) were inoculated in the centre of 5.2–cm diameter petri plates containing 1/10th–strength PDA

amended with rifampicin (10 μg ml^{-1}; GoldBio, St Louis, MO). The three independent petri plates for each fungus/oomycete were placed on the surface of amended, irrigated soils and ASD was applied as described above. Pots were arranged in a CRD inside an environmental growth chamber with five replicates per treatment and incubated at the temperature and light regimes as described above. External controls (EXC) for each pathogen prepared and cultured on the same medium were arranged inside the same growth chamber outside the sealed bags containing ASD treated pots and mycelial growth was monitored daily. When mycelial growth of *P. ultimum*, the fastest growing among the three pathogens, reached the edge of the external control petri plate, cultures were removed from the bags and colony radius was obtained at four perpendicular points for each sample. The experiment was carried out in two trials. Cultures were observed microscopically (100 ×) for morphological changes of the mycelia.

Pratylenchus penetrans was reared for use in nematicidal assays as follows. Five, 5–week–old Gala apple seedlings were planted into 900.0 g of SR soil in 10 pots and grown for 5 weeks inside an environmental growth chamber using the temperature and light regimes as described previously. Nematodes were extracted from seedling roots and 200 μl of the extraction containing 50-100 *P. penetrans* was applied to water agar amended with kanamycin (75 μg ml^{-1}; Sigma, St. Louis, MO). Three plates were placed on the surface of the amended, irrigated soil in triplicate pots and bagged. Pots were arranged in a CRD inside an environmental growth chamber and incubated at temperature and light regimes as noted above. After two days of incubation, pots were removed from the bags and nematodes were observed microscopically at 40× (Olympus BH2 series system). Numbers of living and dead *P. penetrans*\ were recorded. Nematode mortality was determined by an absence of movement for extended period or observation of internal disintegration.

Identification of volatiles generated during ASD

ASD was applied to GC soil using the carbon sources at rates mentioned above and incubated for one week. A 400-g soil sample from each treatment was removed and placed in a sealable 1 gal glass jar. Samples were equilibrated for 30 min by using purified air passed through a Tenax® TA 60-80 mesh filter (9 cm length × 4 cm diameter). An air sample of 300 ml was collected on a trap consisting of silanized glass tube (11.5 cm by 6 mm o.d.) loosely packed with Tenax® TA porous polymer (60-80 mesh). A flow rate of 100 ml/min through the Tenax® trap was maintained during volatile collection.

Volatile analysis was conducted using a Hewlett–Packard 5890A Gas Chromatograph (GC) equipped with a DB–5 ms column (30 m length × 0.25 mm i.d., 0.25 μm thick film) and attached to a Hewlett–Packard 5971A mass selective detector. Volatile samples were introduced into the GC with a Gerstel Thermal Desorption System Autosampler and Thermal Desorption System with the run parameters of initial temperature 20 °C; delay time 0.50 min; initial time 0.50 min; ramp rate 60 °C/min, end temperature 250 °C; hold time 3.00 min; desorption mode splitless. Cooled injection system parameters were: initial temperature 130 °C, equilibrium time 0.50 min, initial time 0.0 min with cryo cooling, split mode: solvent venting, splitless time: 2.00 min, purge time: 0.00 min, ramp rate 10.0 °C/s, end temperature 250 °C, hold time 2.00 min and runtime: 29.50 min. Helium was used as the carrier gas and the flow rate was maintained at 1 ml/min. Column temperatures were programmed from 35 °C for 5 min to 250 °C at 10 °C/min ramp rate and then held at 250 °C for 3 min. All treatments except ethanol were analyzed using the same method whereas ethanol treatment was analyzed using a 4.0 min solvent delay. The mass spectra of the unknown compounds were compared with those in the National Institute of Standards and Technology MS Search 2.0 d (Apr 26, 2005). The identity of selected compounds was confirmed by comparison of spectra to those of pure standards. Standards were prepared as follows: A 1: 5000 dilution of each of 2–ethyl–1–hexanol (Sigma–Aldrich Chemical Co. Inc., St. Louis, MO, USA), allyl isothiocyanate (Sigma–Aldrich), dimethyl trisulphide (Alfa Aeser, Ward Hill, MA, USA), nonanal (Sigma–Aldrich), or decanal (Sigma) was carried out in hexanes (HPLC grade; Fisher Scientific, Fair Lawn, NJ, USA). A subsample of 0.5 μl was injected onto Tenax® in the column and immediately flushed with liquid N_2. Standards were run using the same method. The five compounds were quantified by integrating the peak area for each treatment. Amount of each selected volatile generated from soil amended with the individual carbon inputs was quantified by integrating the area of the peaks.

Analysis of antifungal/nematicidal activity of organic volatile compounds

Among the spectrum of volatile compounds identified by GC–MS comparison to known standards, five compounds were examined for antifungal/oomycete and nematicidal activity. A 3–mm diameter mycelial plug from the edge of an actively growing culture of *F. oxysporum*, *P. ultimum*, or *R. solani* AG–5 was placed onto the centre of a 1/10th–strength PDA plate amended with rifampicin (100 μg ml^{-1}). A 7.5 cm diameter filter paper disk was attached to the petri plate lid and 100 μl of a 1: 2000 dilution of a test compound in ethanol was pipetted onto the filter paper, the lid of the petri plate was attached and plates were immediately sealed with a double layer of parafilm. Five replicates of each pathogen/test compound were prepared and arranged in a CRD in a fume hood at room temperature (20-22ºC). Cultures were incubated for 24 h at which time the mycelial plug was transferred to fresh 1/10th–strength PDA. Cultures were incubated for an additional 24 h at which time mean colony radii was determined. The experiment was conducted in two trials. The range of effective concentration for the selected compounds was determined in a subsequent experiment that employed amounts of 6.67 μg ml^{-1}, 66.67 μg ml^{-1}, 666.67 μg $ml^{-1}$and 3333.33 μg ml^{-1}dissolved in hexanes with five replicates for each treatment.

Nematicidal assays were conducted in divided petri plates containing three sections with 5 ml of water agar (WA) added to two sections of each plate. A suspension of *P. penetrans* (150 μl), reared as described above, consisting of 50-100 nematodes was added to each WA section and presence of nematodes was confirmed microscopically. A 100 μl sample of an individual test compound, including 2-ethyl–1-hexanol, allyl isothiocyanate, dimethyl trisulphide, nonanal, or decanal, diluted 1:2000 in ethanol was placed in the remaining section of the divided plate and immediately sealed with parafilm. Plates were incubated in the fume hood for 3 h, at which time nematode mortality was determined by microscopic examination at 40×.

Data analysis

All data were analyzed using the SAS (SAS Institute Inc., Cary, NC, USA) statistical package using one way ANOVA with general linear model for continuous data and logistic regression for percentage data. In logistic regression analysis, number of apple seedling root segments infected by *Pythium* spp. and number of dead *P. penetrans* killed were considered 'event' and total number of root segments and total number of P. penetrans were considered 'non-event', respectively (Lottes et al. 1996). In the SAS proc logistic procedure, three asymptotically equivalent Chi–Square tests called likelihood ratio test, Score Chi–Square test and Wald Chi–Square test, test against the null hypothesis that at least one of the predictor's regression coefficients is not equal to zero in the model. When data were not normal or variances were not homogeneous for continuous data, transformation and Friedman's test to conduct ANOVA using ranks were used accordingly. Mean separation was based on the Fisher's Least Significant Difference method or with Dunnet's comparisons with the control treatment. Dunnett's comparison compared means between treatments and a single control. This method which is restricted to test only comparisons to the single control mean was used to achieve greater power (Dunnett 1955). Results of the Dunnett's test have been reported as the differences of means of the compared treatment and the control.

Results

Soil pH, and soil redox potential

Application of ASD induced a lowering of pH in both GC and SR orchard soils when conducted using RB, SM, or GR and in GC soil with ET as the carbon input (Table 2). ASD did not induce further acidification in SMR orchard soil. Average ORP values at the termination of the ASD treatment were lower in RB, SM, GR or ET–based ASD treatments compared to soils in which ASD was conducted using CM, pasteurized control and no amendment control treatments in GC soil (Table 3).

ASD effects on seedling growth and recovery of ARD pathogens

Carbon input used during ASD had significant effects on growth of apple seedlings in replant orchard soils. Soil pasteurization and ASD conducted with either ET or GR as the carbon input and the PC treatment resulted in significantly ($P<0.05$) greater mean seedling weight difference compared to the no–treatment control in SMR orchard soil (Table 4). In GC soil, PC treatment

Table 2 Effect of anaerobic soil disinfestation conducted with different carbon inputs on soil pH at the GC, SR and SMR orchards as determined at completion of a two week treatment period

Carbon amendment[a]	Orchard soil pH		
	GC[b]	SR	SMR
RB	5.51	5.99	4.48
SM	5.94	6.21	4.55
ET	5.82	7.03	4.85
GR	5.80	6.35	4.66
CM	7.06	7.25	5.39
PC	6.14	7.16	4.44
C	6.32	6.78	4.47

[a] RB=rice bran, 11.1 t ha^{-1} for SMR and 4.9 t ha^{-1} for GC and SR; SM=Brassica juncea seed meal, 4.9 t ha^{-1}; ET=10 % ethanol, 8.9 kl ha^{-1}; GR=orchard grass residues 20.0 t ha^{-1} for GC and SR soils and 40.0 t ha^{-1} for SMR soil; CM=composted steer manure, 11.1 t ha^{-1}; PC=pasteurized control; C=no-amendment control

[b] GC is a conventionally managed commercial orchard located near Manson, WA; SR is the Washington State University research orchard in Rock Island, WA; SMR is a certified-organic commercial orchard in Chelan, WA

yielded significantly higher seedling weight among all treatments but ASD had no significant effect on seedling biomass compared to the control regardless of carbon input utilized (data not shown). In SMR orchard soil, mean shoot length difference was significantly higher ($P<0.05$) for ASD treatments utilizing ET as the carbon input and the PC, compared to the no–amendment control (Table 4). In GC orchard soil, only pasteurization resulted in a significant mean shoot length difference compared to the no–amendment control (data not shown). Overall, a low incidence of apple seedling root infection by *Pythium* spp. was observed in SMR soil. Regression coefficients of *Pythium* spp. percent root infection for all treatments except CM, were not equal to zero in reference to the no–amendment control treatment ($P<0.05$) (Fig. 1). Hence, SM, GR and ET treatments effectively controlled *Pythium* spp. root infection of the seedling roots as PC treatment while RB treatment exacerbated it compared to the no–amendment control. CM treatment was not effective in controlling pathogen infection. In GC soil, all carbon inputs except ET significantly reduced *Pythium* root infection relative to the no–amendment control (data not shown). *Pratylenchus penetrans* root density was comparable and significantly reduced relative to the control by all ASD treatments, with the exception of the CM treatment, in both GC (Fig. 2) and SMR (data not shown) soil.

Table 3 Oxidation reduction potential (ORP) value in GC orchard soil at completion of the two week anaerobic solid is infestation incubation period

Carbon amendment	Measured ORP value/mV
RB[a]	-202
SM	-206
ET	-63
GR	-210
CM	75
PC	58
C	310

[a] RB=rice bran, 4.9 t ha^{-1}; SM=Brassica juncea seed meal, 4.9 ha^{-1}; ET=10 % ethanol, 8.9 kl ha^{-1}; GR=orchard grass residues, 20.0 t ha^{-1}; CM=composted steer manure, 11.1 t ha^{-1}; C=no-amendment control; PC=pasteurized control

Table 4 Effect of different carbon amendments used in anaerobic soil disinfestation (ASD) on shoot length and seedling weight of 'Gala' apple seedlings cultivated in SMR orchard soil.

Treatment comparison[a]	Difference between means[b]	
	Shoot length[c]	Seedling weight[d]
PC-C	13.39*	2.6852*
ET-C	6.607*	1.0699*
GR-C	8.52*	0.998
SM-C	3.68	0.0161
RB-C	4.607	0.15557
CM-C	-0.62	-0.5512

[a] ET=ethanol, 8.9 kl ha^{-1}; GR=orchard grass residues, 40.0 t ha^{-1}; SM=B. juncea seed meal 4.9 t ha^{-1}; PC=pasteurized control; RB=rice bran, 11.1 t ha^{-1}; CM=composed steer manure 11.1 t ha^{-1}; C=no-amendment control

[b] Comparisons significant at P=0.05 level using the Dunnett's test are indicated by *

[c] Values represent differences between means of the shoot length (cm) of the respective treatment and the no-amendment control treatment across all replicates and with n=5

[d] Values represent differences between seedling weight means (g) of the respective treatment and the no-amendment control treatment mean across all replicates, with n=5

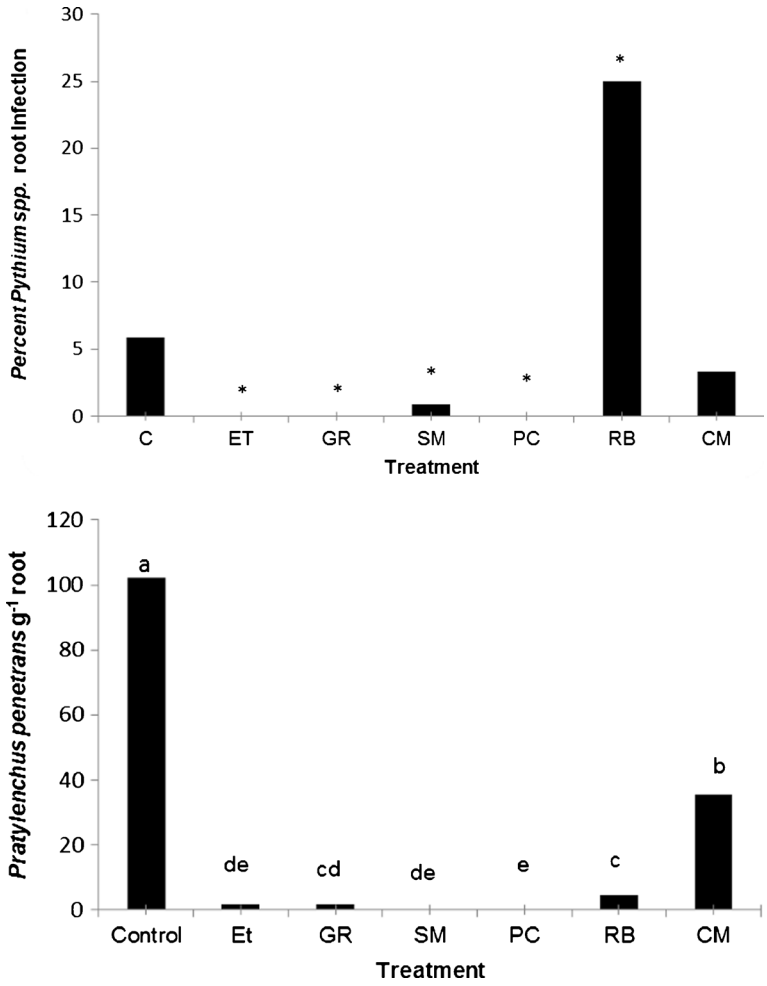

Fig. 1 Effect of different carbon amendments used in ASD on percent *Pythium* spp. infection (top panel) and *Pratylenchus penetrans* densities (bottom panel) in roots of 'Gala' apple seedlings. Soil treatments included ASD conducted with carbon input of 10 % ethanol (ET; 8.9 kl ha^{-1}), grass residues (GR; 40.0 t ha^{-1}), *Brassica juncea* seed meal (SM; 4.9 t ha^{-1}), rice bran (RB; 9.9 t ha^{-1}) or composted steer manure (CM; 11 t ha^{-1}), pasteurized control (PC) and no amendment control (C). For *Pythium* spp. root infection values, treatments indicated with (*) are significantly different from the C reference treatment as determined by logistic regression with Chi-Square as the test statistic. For P. *penetrans* densities, data were subjected to one-way ANOVA and columns designated with the same letter are not significantly different (P=0.05) based upon mean separation using Fisher LSD as the test statistic

Antifungal/oomycete and nematicidal effects of volatiles generated during ASD

Volatiles emitted during ASD treatment from GC soil when amended with ET, RB, GR or SM significantly suppressed growth of *P. ultimum*, *F. oxysporum* and *R. solani* AG–5 in the initial trial (Table 5). Among all treatments, ASD conducted using SM yielded the greatest reduction in mean colony radius of all three pathogens. In the second trial, ET, SM and GR treatments significantly reduced ($P<0.05$) growth of all three pathogens compared to the C, PC, EXC, CM and RB treatments (data not shown). Volatiles generated in ET, GR and SM ASD treatments induced hyphal degradation of *R. solani* AG–5 and *P. ultimum* as determined by microscopic examination. In the nematicidal assay, regression coefficients of *P. penetrans* percent mortality for all carbon inputs except CM and no–amendment control were not equal to zero in reference to the PC treatment ($P<0.05$) (Table 6) indicating that volatiles generated during the anaerobic phase of incubation when ET, GR, RB and SM have an effect on nematode mortality.

Identification of volatile chemistries generated during ASD

Analysis of volatiles by GC/MS demonstrated that each ASD treatment produced a unique spectrum of volatile compounds during the anaerobic phase (Online Resource 1). SM treatment generated a volatile profile consisting of isothiocyanates, alcohols, organic acids, organic sulphides and esters. GR yielded organic sulphides, ketones, organic acids, and hydrocarbons. RB treated soils emitted a spectrum of volatiles consisting of organic acids, alcohols, and esters. Volatiles generated in the ET treatment were dominated

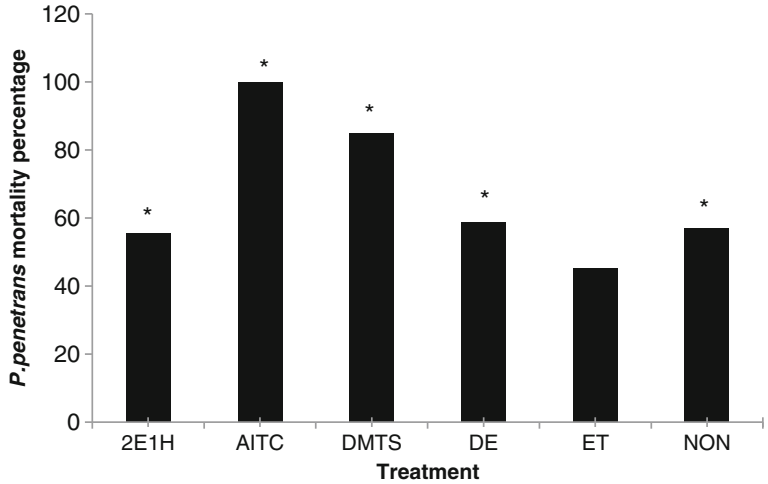

Fig. 2 Effect of pure volatile compounds on mortality of *Pratylenchus penetrans* after three hr of incubation. A volume of 100 µl of a 1:2000 dilutions of 2-ethyl-1-hexanol, nonanal, decanal, dimethyl trisulphide, and allyl isothiocyanate in ethanol were used in the in vitro nematicidal assay. 2E1H=2-ethyl-1-hexanol; AITC=allyl isothiocyanate; DMTS=dimethyl trisulphide; DE=decanal; ET=ethanol; NON=nonanal. Values represent mean percent mortality across all replicates, with n=5. Treatments indicated with (*) are significantly different from the ET solvent reference treatment as determined by logistic regression with Chi-Square as the test statistic

by esters. ASD conducted with CM as the carbon input did not generate volatile profiles that differed from the no amendment control. The identity of allyl isothiocyanate (AITC), dimethyltrisulphide (DMTS), 2–ethyl–1–hexanol (2E1H), nonanal (NON) and decanal (DE) which were initially classified by GC–MS was verified by comparing to the retention times of the standard volatiles. Volatile production during ASD when conducted with each carbon treatment was quantified by integrating the area under the peaks of the respective mass spectrum. Different carbon compounds yielded

Table 5 Effect of carbon amendment on activity of volatile compounds generated in GC orchard soil during the anaerobic phase of anaerobic soil disinfestation on colony growth of *Rhizoctonia solani* AG-5, *Pythium ultimum* and *Fusarium oxysporum*

Treatment comparison[a]	Difference between mean colony diameter[b]		
	F. oxysporum	P. ultimum	R. solani AG-5
PC-C	0.4000	0.000	0.0000
ET-C	-10.4000*[c]	-12.600*	-21.6000*
EXC-C	-3.8000*	0.000	-1.0000
GR-C	-12.0000*	-23.800*	-23.4000*
SM-C	-14.4000*	-24.000*	-24.0000*
RB-C	-9.0000*	-20.400*	-23.0000*
CM-C	0.4000	0.000	0.0000

[a] ET=ethanol, 8.9 kl ha^{-1} ; GR=orchard grass residues, 20.0 t ha^{-1} ; SM=B. juncea seed meal 4.9 t ha^{-1} ; PC=pasteurized control; RB=rice bran, 4.9 t ha^{-1} ; CM=composed steer manure 11.1 t ha^{-1} ; C=no-amendment control

[b] Values represent differences between mean colony radius (mm) of the respective treatment and the no-amendment control across all replicates, with n=5

[c] Comparisons significant at P=0.05 level using the Dunnett's test are indicated by *

Table 6 Logistic regression analysis of effect of volatile compounds produced during anaerobic soil disinfestation conducted in SR orchard soil with different carbon inputs on percent mortality of *Pratylenchus penetrans*

Odds Ratio Estimates				
Effect[a]	Point Estimate	95 % Wald		Pr>ChiSq
		Confidence	Limits	
C vs PC	1.929	1.453	2.561	<0.0001
CM vs PC	1.199	0.888	1.619	0.2354
ET vs PC	17.991	13.704	23.619	<0.0001
GR vs PC	69.706	50.683	95.871	<0.0001
RB vs PC	1.555	1.147	2.109	0.0045
SM vs PC	11.149	8.535	14.564	<0.0001

[a] C=No amendment control; ET=ethanol, 8.9 kl ha^{-1} ; GR=grass clippings, 20.0 t ha^{-1} ; SM=B. juncea seed meal 4.9 t ha^{-1} ; PC=pasteurized control; RB=rice bran, 4.9 t ha^{-1} ; CM=composed steer manure, 11.1 t ha^{-1} with nine replicates per treatment

different amounts of selected volatiles including a unique volatile profile from soil when specific amendment is used (Table 7).

Antifungal/oomycete and nematicidal activity of selected volatiles

The five primary compounds identified by GC–MS were examined for the capacity to inhibit the target pathogens. In the initial trial, in vitro growth of *P. ultimum* was significantly suppressed by all test volatile compounds compared to the control. All compounds except 2E1H significantly reduced colony growth of *R. solani* AG–5 (data not shown). Only DE, DMTS and AITC significantly limited mycelial growth of *F. oxysporum*. In the second trial only AITC significantly retarded growth all three pathogens compared to the controls ($P<0.05$) (Table 8). DE and DMTS were only effective in limiting growth of R. solani AG–5 and *P. ultimum* whereas 2E1H was only effective against *P. ultimum*. Regression coefficients for *P. penetrans* percent mortality of all volatile compounds was greater than zero in reference to ET solvent treatment ($P <0. 05$) (Fig. 2) demonstrating their activity against this nematode.

Effective concentrations of selected volatiles for antifungal/oomycete activity

The range of effective concentration of the selected volatiles in inhibiting mycelial growth of target pathogens was determined using pure volatile compounds. Colony growth suppression by NON was constant at amounts higher than 10.0 mg for all three pathogens (Table 9). The minimum effective amounts for DMTS were between 1.0 mg–10.0 mg for *F. oxysporum* and 0.1 mg–1.0 mg for *P. ultimum* and *R. solani* AG–5. Colony growth suppression was constant at DE rates higher than 10.0 mg for *F. oxysporum* and 1.0 mg for *P. ultimum* and *R. solani* AG–5. Effective range of 2E1H was between 1.0 mg–10.0 mg for *F. oxysporum*, *P. ultimum*, and

Table 8 Effect of pure volatile compounds on in vitro mycelial growth of *Fusarium oxysporum Pythium ultimum* and *Rhizoctonia solani* AG-5

Volatile compound[a]	Volatile concentration in the headspace (μg ml^{-1})[b]	Mean colony radius[c]		
		F. oxysporum	P. ultimum	R. solani AG-5
SDW	3.0	3.4ab	11.3a	4.2a
EXC	-	3.5a	11.0a	3.45b
2E1H	2.7	3.4ab	2.4ed	2.95cb
ET	2.63	3.5a	5.55b	2.85cb
NON	2.75	3.3ab	4.55cb	2.8cb
DE	2.77	3.6a	3.95 cd	2.3 cd
DMTS	4.0	3.0b	1.65e	1.6d
AITC	3.4	0.0c	0.0f	0.65e

[a] SDW=Sterile distilled water; EXC=External control; 2E1H=2-ethyl-1-hexanol; ET=ethanol; NON=nonanal; DE=decanal; DMTS=dimethyl trisulphide; AITC=allyl isothiocyanate

[b] An aliquot of 100 μl of a 1: 2000 dilution of a test compound in ethanol was pipetted onto a Petri plate (diameter: 5.2 cm, height: 1.4 cm), filled to ½ depth with agar media

[c] Each test organism was placed on 1/10th-strength potato dextrose agar amended with rifampicin (10 μg ml^{-1}) and 100 μl of a 1:2000 dilution of an individual test compound was pipetted onto sterile filter paper in an enclosed petri dish. Values represent mean colony radius (mm) across all replicates with n=5. Data were analyzed using SAS PROC GLM method . Means within a column designated with the same letter are not significantly different ($P>0.05$)

Table 7 Quantities of selected volatiles evolved from GC orchard soil amended with different carbon inputs

Carbon input[a]	Amount of volatile compounds evolved (pg g^{-1} of soil ml^{-1} of headspace)				
	AITC[b]	DE	DMTS	2E1H	NON
Brassica juncea seed meal	0.4000	0.0040	0.1600	0.0130	0.0080
Composted steer manure	ND	0.0028	0.0005	0.0029	0.0042
Ethanol	ND	0.0058	0.0001	0.0068	0.0039
Orchard grass	ND	0.0028	0.1740	0.0342	0.0045
Rice bran	ND	0.0026	0.0042	0.0142	0.0007

[a] Carbon input rates: *B. juncea* seed meal, 4.9 t ha^{-1} ; orchard grass residues, 20.0 t ha^{-1} ; rice bran 4.9 t ha^{-1} , ethanol 8.9 kl ha^{-1}

[b] AITC=allyl isothiocyanate; DE=decanal; DMTS=dimethyl trisulphide; 2E1H=2-ethyl-1-hexanol; NON=nonanal; ND=None-detected

R. solani AG–5. At 50 mg, DMTS and nonanal were fungicidal for all three pathogens while DE and 2E1H were only fungistatic for Fusarium oxysporum even at this highest concentration. Growth of all test organisms was completely suppressed by AITC at a concentration less than 0.05 mg (data not shown).

Discussion

Overall, the performance of ASD using ET or GR as the carbon input appears to have superior potential for control of a complex of soil–borne pathogens affecting apple. This conclusion is based upon the relative improvement in plant growth, reduction in root infection by *Pythium* spp. and infestation by *P. penetrans*, and anti–biological activity of volatiles generated during ASD, which were more efficacious with ET and GR compared to the control or other carbon amendments. Application of ethanol as a carbon source in ASD under field conditions has been explored against root knot nematode, *Fusarium oxysporum* f. sp. cucumerinum and *Ralstonia solanacearum* (Kobara et al. 2007; Uematus et al. 2007). Ethanol has been suggested to be more feasible ASD amendment relative to other solid organic material as it is nitrogen–free hence being able to be applied with irrigational tubes, it can penetrate deeply and uniformly into the soil (Momma et al. 2010). Although ASD using SM provided pathogen suppression comparable to ET and GR treatments in general, seedling growth as a result of this treatment was inferior. This outcome most likely was a result of phytotoxic effects which have been experienced even in field trials conducted with grafted trees (Mazzola 2011). It is likely that a lengthened aerobic period of incubation prior to planting and/or optimization of the level of SM amendment would alleviate phytotoxic effects observed as demonstrated in previous studies (Handiseni et al., 2013; Mazzola and Strauss, 2013).

Previously, it was reported that efficacious ASD treatments resulted in reduced soil pH and ORP values, accumulation of acetate and butyrate, and emission of noticeable odours from treated soil (Momma 2005; 2006). Similarly, in this study, ASD conducted with all effective carbon amendments, but not the ineffective CM amendment, yielded a drop in soil pH and ORP values, generated an unpleasant odour from soils and produced a characteristic volatile profile that differentiated it from the control or pasteurized control treatments. Unlike GC and SR soils, SMR soil which inherently has a low pH did not exhibit a further reduction of pH in response to ASD treatment. The high organic matter of SMR orchard soil may demonstrate an example of the buffering effects of soil organic matter which limits acute soil acidification due to its high ion exchange capacity (Bohn et al. 1985).

Based on results from root infection assays, it is unlikely that the poor seedling growth in response to ASD using SM, GR, or ET in GC soil was not associated with biotic disease but may have resulted from phytotoxicity caused by the amendments. The efficacy

Table 9 Determination of effective range of select volatile compounds produced during anaerobic soil disinfestationon *in vitro* growth of *Fusarium oxysporum*, *Pythium ultimum* and *Rhizoctonia solani* AG-5

Headspace concentration of volatile compound (μg ml^{-1})[a]		Mean colony radius[b]		
		F. oxysporum	P. ultimum	R. solani AG-5
Nonanal	0.0	10.3b	24.0a	20.6a
	6.7	10.9a	24.0a	19.0b
	66.7	9.3c	16.5b	14.4c
	666.7	1.1d	0c	0d
	3333.3	0e	0c	0d
2-ethyl-1-hexanol	0.0	10.0a	23.5a	19.6a
	6.7	10.1a	23.5a	19.0b
	66.7	7.9b	5.7b	13.0c
	666.7	0c	0.4c	5.5d
	3333.3	0c	0c	5.5d
Decanal	0.0	9.3a	20.6b	5.7a
	6.7	8.7a	17.9a	3.1a
	66.7	9.2b	18.8b	5.4a
	666.7	1.6c	0.2c	0.2b
	3333.3	0d	0c	0b
Dimethyl trisulphide	0.0	10.2a	23.4b	8.1a
	6.7	8.9b	24.0a	4.9b
	66.7	3.1c	2.8c	0c
	666.7	0d	0d	0c
	3333.3	0d	0d	0c

[a] Volatile compounds were applied at amounts of 0.1 mg, 1.0 mg, 10.0 mg and 50.0 mg onto the filter papers inside petri plates and colony growth of each fungus/oomycete was measured after 48 h incubation

[b] Values represent mean colony radius (mm) across all replicates, with n=5. Means for a given test compound/organism in the same column followed by the same letter are not significantly different ($P>0.05$) based on Friedman's non-parametric test

of ASD treatments may be somewhat dependent on soil characteristics, especially initial organic matter content, as well as retention or exclusion of phytotoxic effects in treated soils. Similar effects have been observed in the application of Brassicaceae seed meal to different orchard soils (Mazzola 2011). In field trials, springtime SM applications made four weeks prior to planting were highly successful in controlling disease at the SMR orchard, but the same spring applications caused phytotoxicity and extensive tree mortality at the SR orchard. Seed meal applications made in the autumn six months prior to planting at the SR orchard abolished phytotoxic effects and provided fumigant levels of replant disease control (Mazzola 2011). In the current study, compared to GC soil, the SMR soil seemed to be less prone to retention of potential phytotoxic effects after ASD. Therefore, post–ASD treatment, a prolonged period of aeration may be needed prior to replanting GC soil. When cowpea, sun hemp, pearl millet and sorghum–sudangrass were used as carbon sources for ASD, crop performance measures were not affected by issues of phytotoxicity (Butler et al. 2012). However, it has been shown that there is great diversity in relative sensitivity of crop species to potential phytotoxic chemistries generated in response to organic amendments or ASD (Mazzola 2011; Handiseni 2012).

The use of soil amendments having a narrower (lower) C: N ratio has been associated with a greater capacity of the amendment to suppress plant parasitic nematodes (Oka et al. 2007; Rodriguez–Kabana et al. 1987). C: N ratios of CM, GR, RB and SM amendments were 11:1, 19:1, 19:1 and 16:1 respectively. In the current study, ASD conducted using GR provided suppression of *P. penetrans* while the CM treatment possessing a lower C:N ratio did not. In contrast, when ASD was applied using composted poultry litter, the treatment effectively reduced tomato root density of Meloidogyne incognita and root gall ratings (Butler et al. 2012). Amendment–dependent soil chemical parameters also appear to play a specific role in nematicidal activity. The pH in microsites surrounding wheat bran particles and nematodes was considered to be a more critical parameter in determining nematicidal activity of ASD than the overall soil pH (Katase et al 2009). It has also been found that the pH of nitrification microsites was considerably lower than the bulk soil pH (Strong et al. 1997). As shown by Katase et al. (2009), localized pH declines may be sufficient to cause nematicidal activity through generation of acidic volatile compounds from the carbon amendments used in this study. The three ASD carbon inputs (ET, GR and SM) providing the greatest control of *P. penetrans* also yielded the most active spectrum of nematicidal volatiles suggesting the important role of these chemistries in determining the efficacy of ASD for control of lesion nematode in orchard systems. Considering nematicidal activity of pure volatile compounds, DMTS was effective at a concentration of 0.996 μg ml^{-1} whereas AITC was inhibitory toward *P. penetrans* at a concentration of 0.845 μg ml^{-1} in this study after a 3 h exposure period. Further experiments are needed to estimate the time course required for generation of LC_{50} levels of the volatiles used in this study.

The antifungal/antioomycete nature of ASD–derived volatiles may be attributed to activity of a specific volatile (e.g. AITC) or due to synergistic activity of a unique volatile profile. Due to the complexity of volatile profiles generated in response to the different carbon inputs, only selected volatile compounds were chosen for further examination based on their previously reported antifungal activity. SM generated its characteristic AITC which has been widely investigated for its effectiveness against fungal and oomycete pathogens, including *R. solani* and *Pythium* spp. (Manici et al. 2000; Yulianti et al. 2006; Mazzola et al. 2007). It would be interesting to explore whether *B. juncea* seed meal provides superior disease control performance upon utilization as a carbon source in ASD relative to its reported efficacy through the generation of AITC when used as a soil amendment under aerobic soil conditions (Mazzola et al. 2007). The relative efficacy of Brassicaceae seed meal amendment for control of soil–borne pathogens under aerobic and anaerobic conditions has not been examined. Although there are no reports of the generation of sulphide compounds in response to ASD conducted using grass residues, multiple sulphide compounds were identified in this study by GC/MS analysis of volatile compounds emitted from grass residue amended ASD soils. McNeal and Herbert (2009) detected dimethyl disulfide and dimethyl trisulphide produced by *Streptomyces* spp. in a microcosm experiment with increased water additions.

Among the five ASD–derived compounds examined in this study, only DMTS and AITC demonstrated consistent activity across all three pathogens tested in two independent trials. The antifungal nature of organic volatiles is a well–studied phenomenon. Decanal, dimethyl trisulphide, 2–ethyl–1–hexanol and nonanal that originated primarily from *Pseudomonas* spp. isolates completely inhibited Sclerotinia sclerotiorum mycelial

growth or sclerotia formation in an in vitro assay (Fernando et al. 2005). Although 2–ethyl–1–hexanol did not show significant antifungal activity in this study, allyl alcohols have inhibited carpogenic germination of sclerotia of *S. sclerotiorum* (Huang et al. 1997). Other than sclerotial growth inhibition, allyl alcohols have enhanced sclerotial colonization by *Trichoderma* spp. (Huang et al. 1997). This compound was also reported to contribute toward increasing populations of beneficial bacteria such as *P. fluorescens* and *P. putida* (Domsch 1959); Altman and Lawlor 1966). Even though decanal and nonanal did not limit growth of fungi examined in this study, these two compounds were contained in essential oil of *Hibiscus cannabinus,* and were determined to be responsible for the fungitoxic activity of the material toward three *Colletotrichum* spp. (Kobaisy et al. 2001). A primary focus in studies of microbial volatile production is the identification of volatile chemistries and their source organisms as a prelude to use as novel agents for pathogen control (Bitas et al. 2013). Metabolic engineering of plants or plant–associated microbes to produce volatile organic compounds with fumigant activity is an attractive alternative to traditional chemical control of pests (Bitas et al. 2013). Some of the organic solvents such as ketones, ethers, alcohols, aldehydes, and carboxylic acids have been assessed for soil fumigant activity (Kobara et al. 2007). Dimethyl disulphide; one of the compounds identified from treated soil with *B. juncea* seed meal and orchard grass has already been registered as a soil fumigant (Owens, 2010).

This study was focused on determination of ASD efficacy for the suppression of apple root pathogens using carbon inputs consisting of a range of chemical characteristics. Based on the potential for pathogen suppression in response to ASD conducted with certain of these organic substrates, one possible mechanism of disease control, i.e. the antifungal/oomycete and nematicidal nature of volatile compounds, was assessed. ASD conducted with ET, GR or SM as the carbon input showed potential for the control of pathogenic fungi/oomycete and parasitic nematodes via a volatile chemistry–driven mechanism. Development of this method as a profitable disease management protocol awaits further clarification of optimal carbon input rates and evaluation under field conditions.

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