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Amendment of soils with fresh and post-extraction lavender (*Lavandula angustifolia*) and lavandin (*Lavandula* × *intermedia*) reduce inoculum of *Verticillium dahliae* and inhibit wilt in strawberry

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ABSTRACT

Soils that were naturally infested with high levels of *Verticillium dahliae*, the causal agent of wilt disease in strawberry, were amended with fresh and waste lavender, fresh and waste lavandin, hydrosols generated during essential oil production, BioFence™ (a mustard-based defatted seedmeal pellet), or water in microcosms to estimate their efficacy against microslecrotia of the pathogen. Single chemicals and mixtures of the chemicals detected from the substrata were also effective in microcosms. The mixtures were more effective than were the individual chemicals. Microplot evaluation of fresh and waste lavender and lavandin was also made in comparison to BioFence™ and water controls. Lavandin waste was compared to BioFence™ and untreated controls at three sites in field plots that were subsequently planted to strawberry. Disease incidence and severity were measured over time in the field. All of the *Lavandula*-based materials could be associated with large reductions in the numbers of viable of microsclerotia recovered in all but one experiment with greatest effect in microcosms and smaller effects in microplots and field plots, as could BioFence™ pellets. Due to the high levels of inoculum found at all field sites the reduction in pathogen inoculum density was not necessarily associated with a corresponding reduction in disease incidence, nor with severity as determined by yield. The monoterpenoids associated with the *Lavandula* spp. are of lower volatility than the isothiocyanates associated with crucifer decomposition and were detected for more than one week after materials were incorporated in soil. This suggests both differences in mode of action and the possibility of combining either the chemicals or the materials that produce them in order to further enhance efficacy. Several non-target effects were considered: numerosity and diversity of bacterial and fungal populations; infection by arbuscular mycorrhizal fungi; and functional diversity of soil microflora. No persistent non-salient effects were detected.

1. Introduction

With the loss of methyl bromide and other fumigants, strawberry (*Fragaria* × *annanasa*) production has come under increasing threat of losses due to wilt caused by *Verticillium dahliae* Kleb. Biofumigation using decay products of green manures is one approach that has been investigated as showing potential for management of the disease (Matthiessen and Kierkegaard, 2006), as has breeding for resistance in the host (Wilhelm, 1981). Microbially-mediated biological control has also been observed as having beneficial effects (Martin and Bull, 2002; Müller et al., 2004). In addition to disease inhibition, additions of organic wastes have positive effects on soil structure, nutrient availability and inoculum viability (Yohalem et al., unpublished data). Disease threat from *V. dahliae* is most frequently assessed by culturing infested soils and determination of colony forming units of the pathogen per gram of soil. Disease incidence and severity in the crop are also used to determine treatment efficacy.

Earlier work (Yohalem and Hall, 2009) has shown that fresh English lavender (*Lavandula angustifolia* P. Mill.) could reduce inoculum to below the detection limit of the method of Harris et al. (1993). However, the use of fresh lavender is not an economically viable approach, nor is lavender production consistent with strawberry agronomy. Spike lavender, *L. stoechas*, has also been shown to reduce populations of *V. dahliae* (Mwanza and Blanco-López, 2001; López-Escudero et al., 2007). Lavandin (*Lavandula* × *intermedia* Emeric ex Loisel), a sterile hybrid of *L. angustifolia* and *L. latifolia*, is grown more abundantly and is more prolific than *L. angustifolia*. Its chemical profile is similar to, but distinct from that of lavender (Moon et al., 2007). Both plants are most commonly grown for the essential oils they produce and the post-extraction waste material is most frequently burned or composted. Also produced during the extraction of lavender and lavandin oils are lower value aqueous hydrosols of different concentrations depending

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on the methods used in the extraction process (primarily varying with whether or not the water used in distilling the oil is recycled through the distillation apparatus or collected as run-off). Fresh and waste lavandin materials all contain monoterpenoid volatile components, albeit in different ratios. Monoterpenes are reported to act upon membrane integrity, being either directly lethal to microorganisms or rendering them susceptible to physical, chemical or biological attack (Mourey and Canillac, 2002; Trombetta et al., 2005).

We report here on microcosm, microplot and field experiments on the chemistry and effects of English lavender, lavandin and their wastes on viability of microsclerotia and on disease expression in susceptible cultivars of strawberry, as well as on the detection of their volatile components from microcosms and from field experiments. They are compared to a *Brassica*-based seedmeal which has demonstrated efficacy against the pathogen (Yohalem and Hall, 2009; Yohalem et al. Unpublished data). We also examine non-target effects of soil amendment including mycorrhizal colonization, functional diversity and phylogenetic diversity in order to determine if there are positive or negative effects on populations salient to strawberry production.

2. Methods

2.1. Microcosms

2.1.1. Lavender, lavandin and post-distillation plant materials

Experiments with four replicates were conducted to evaluate the efficacy of lavender (*L. angustifolia* cv. Maillette), lavandin (*L.* × *intermedia* cv. Grosso) and waste materials that had been extracted for their essential oils from both. These were compared to unamended treatments in 200 g microcosms using naturally infested Marlow Series typical paleo-argillic silt loam. For each microcosm 10 g of the material was cut into small pieces between 0.5 and 1.0 cm in length with scissors, briefly ground in a pestle and mortar and mixed with 190 g dry weight of soil moistened with 25 ml water in 350 ml glass jars (Down et al., 2004). Each material was evaluated for microscelerotial population numbers by the wet-sieve method of Harris et al. (1993) after 28 d incubation. In brief, 10 g of air-dried sieved (2 mm mesh) soil were shaken for 1 h at 175 rpm and the 20–160 μm fraction obtained. This fraction was resuspended in 20 ml sterile water and distributed over the surfaces of 20 Petri dishes (9 cm, single vent) into which a selective medium has been poured. The medium contained 2 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4{\cdot}7H_2O$, 2 g PGA, 1 ml tergitol NPX (Sigma, UK) and 15 g agar (Fluka, UK) per litre water, adjusted to pH 6.4 prior to addition of the agar. After autoclaving, 1% by volume sterile filtered antibiotic solution was added (0.06 g streptomycin, 0.06 g chloramphenicol, 0.06 g chlortetracycline, 6 mg biotin). Aliquots (1 ml) of the soil suspension were poured and spread on the surface of the medium and incubated for 28 d at 22 °C. After washing the soil from the surface of the plate, the microsclerotia were enumerated under a dissecting microscope. The method gives a detection threshold of 0.1 colony forming unit g^{-1}.

Analysis of variance (ANOVA) was performed and contrasts were calculated to separate treatment effects (Genstat 9.1, UK). Contrasts were calculated for: the comparison of source materials (lavender vs. lavandin); for waste materials versus fresh; and for their interaction. A further experiment comparing effects of fresh lavandin and untreated controls after 28 d and 56 d incubations for lavandin and analyzed by repeated measures. Evaluation of results was made at $\alpha = 0.05$.

Elemental nutrient analysis of lavandin waste was accomplished in the following manner: initial drying and grinding of the sample as described in Faithfull (1986); followed by a wet digest process for all elements with the exception of boron. This latter process is based on the digestion process in Faithfull (1986) [Method 43 for Nitrogen in Plant Material]: a subsample of the ground material was subjected to a wet micro-Kjeldahl type digestion process to destroy all organic matter for subsequent ICP-AES emission spectroscopic analysis of key elements in the plant material, and for the separate analysis of nitrogen by Fiastar 5000 Flow Injection Analysis (Foss, UK) instrument with the same digest. A separate dry ashing of the powdered sample was undertaken to determine boron content.

Volatile chemicals were detected from head spaces above the soils in microcosms. They were sampled by solid phase microextraction using a polydimethylsiloxane fibre (75 μm; Supelco, UK) inserted through the cover of the jar for 5 min. Samples were taken at intervals up to 14 d. The volatiles were analyzed by gas chromatography coupled to mass spectrometry (GC–MS) on a HP6890 GC and HP5973 Mass Selective Detector (Agilent) fitted with a fused silica capillary GC column (30 m × 0.25 mm i.d.) coated with SPB5 (250 μm; Supelco). The fibre was desorbed in the injector (220 °C) for 0.75 min, and the GC oven temperature programmed at 50 °C for 2 min then increased at 6 °C min^{-1} to 240 °C. Carrier gas was helium (1 ml min^{-1}). Data were captured and processed with the Chemstation software.

2.1.2. Evaluation of individual synthetic compounds

Ten of the chemicals detected in head spaces above soils amended with candidate biofumigant materials were evaluated for their effects on *V. dahliae* microsclerotial populations in comparison with water amended controls. The chemicals were dimethyldisulphide, dipropyldisulphide, dimethyltrisulphide, allyl isothiocyanate (ITC), phenyl ITC, phenethyl ITC, α- and β-pinene (1:1), 1,8-cineole, camphor and borneol. All chemicals were obtained from SigmaAldrich (Gillingham, Dorset, UK) except for dimethyltrisulphide (Oxford Chemicals, Billingham, Teeside, UK; now Frutarom (UK) Ltd.). A sterile filter paper (1 cm × 1 cm) placed at the bottom of a glass sample tube (70 mm × 25 mm diameter) was treated with 1 μl of a chemical and covered with 30 g dry weight of naturally heavily infested Fyfield Series 2, a typical argillic brown earth moistened with 2.5 ml of water. The tube was covered with polythene sheet and incubated at 20 °C as above. There were four replicates of each treatment and the control in a completely randomized design and samples were taken for estimation of *V. dahliae* microsclerotial populations at 14 d and 28 d.

2.1.3. Evaluation of mixtures of synthetic compounds

Two synthetic mixtures were prepared to simulate the chemical profiles detected from the Brassicaceous materials and *L. angustifolia*, respectively. The former was made up of dimethylsulphide (1 μl), dimethyldisulphide (2 μl), allyl ITC (2 μl) and dimethyltrisulphide (1 μl). The latter contained α-pinene (1 μl), β-pinene (1 μl), 3-carene (1 μl), 1,8-cineole (10 μl), camphor (4 μl) and borneol (2 μl). Each mixture was applied to a filter paper, covered with moistened Fyfield Series 2 soil naturally infested with *V. dahliae* and incubated as above. The two mixtures of chemicals were compared with soil amended with 1.5 g BioFence™ and soil with water only. There were four replicates of each treatment and the control in a completely randomized design and estimations of *V. dahliae* microsclerotial populations were made after 14 d and 28 d.

2.1.4. Lavender and lavandin hydrosols

In separate experiments, lavandin waste (10 g $microcosm^{-1}$) that had been stored for 7 months prior to initiation of the experiment, three lavandin hydrosols, two lavender hydrosols and unamended water controls (23 ml $microcosm^{-1}$) were compared in three randomized complete blocks. To each microcosm, 23 ml of hydrosol was added to 200 g soil in a 360 ml glass jar, as above.