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Effects of individual and combined use of bio-fumigation-derived products on the viability of *Verticillium dahliae* microsclerotia in soil

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ABSTRACT

Verticillium dahliae is the causal agent of strawberry wilt. A microencapsulated terpene product containing cineole, camphor and borneol, digestate from anaerobic digestion, and BioFence™ derived from a mustard-based defatted seedmeal were tested for their suppressive activity against *V. dahliae*. First, naturally infested soil was amended with microencapsulated terpene, lavender waste pellet and BioFence™ (pellet) in a laboratory test to assess the efficacy against *V. dahliae*. Next, mini-field-plot experiments were conducted to evaluate the efficacy of individual and combined use of terpene, BioFence™ (liquid) and digestate against *V. dahliae*; sterile distilled water treatment and untreated control were also included. In the laboratory test, all treatments significantly reduced *V. dahliae* densities, with the control efficacy ranging from 27% (BioFence™) to 69% (lavender waste pellet). Although the lowest (1×) rate of terpene treatment resulted in a much lower control efficacy (35%) than the other two higher rates (3× – 55%; 9× – 53%), these differences were not statistically significant. In the field mini-plot trials, all treatments led to significant reductions in the *V. dahliae* density, with the efficacy ranging from 50% (digestate) to 78% (combined three-product treatment), irrespective of the initial wilt level. There were no significant differences in all comparisons of pairwise treatments except between digestate and combined three-product treatment. For the combined two or three-product treatments, the observed efficacy was significantly less than the expected efficacy on the assumption of Bliss independence. Furthermore, there were no significant differences between the observed efficacy of combined treatments and the best single component product efficacy. Although the observed efficacy for the combined three-product treatment was consistently higher than the best single component across replicate plots, such a difference was not statistically significant. The results indicate the value of these alternative treatments in practice but these are not likely to reduce *V. dahliae* inoculum sufficiently to eliminate the risk of strawberry wilt and question the value of combined treatments.

1. Introduction

Verticillium dahliae Kleb. is a cosmopolitan soilborne fungus causing wilt diseases on more than 400 plant species (Pegg and Brady, 2002; Klosterman et al., 2009). In the UK, it can result in 75% crop failure in susceptible cultivars of strawberry (*Fragaria* × *annanasa*), such as cv. Elsanta. The pathogen may persist in the soil for more than 10 years, in the absence of a host plant, as melanized microsclerotia (Pegg and Brady, 2002), the resting structure and primary inoculum of the pathogen in soil.

Control of Verticillium wilt is traditionally carried out by reducing the density of viable microsclerotia levels to below the threshold at which the crop losses are within an acceptable range by fumigating the soil (Butterfield et al., 1978; Klosterman et al., 2009). Incidence of Verticillium wilt of many hosts rises with increasing inoculum density in soil (Harris and Yang, 1996; Khan et al., 2000; Berbegal et al., 2007; Lopez-Escudero and Blanco-Lopez, 2007; Goud et al., 2011; Wei et al., 2015); this relationship can be satisfactorily described by logistic models (Wei et al., 2015) or negative experimental models (Berbegal et al., 2007). As cultivar tolerance/resistance to wilt disease increases so does the inoculum threshold values (Wei et al., 2015). Even as little as 1 CFU g^{-1} (colony forming unit per gram) of soil can lead to significant wilt symptoms on some crops, such as strawberry (Harris and Yang, 1996). Soil fumigation with methyl bromide and chloropicrin have been an indispensable tool for over 50 years because of their

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broad-spectrum and excellent efficacy (Martin, 2003). However, control of Verticillium wilt has become a major problem in UK horticulture since the withdrawal of methyl bromide under the Montreal Protocol. The remaining chemicals (chloropicrin, dazomet) are expensive and face an uncertain future due to legislation restricting their use.

Over the last two decades, there has been concerted effort in searching for alternative treatments (Martin, 2003; Matthiessen and Kirkegaard, 2006). One of the alternatives is the use of biofumigation, suppression of soilborne pests and diseases by volatile hydrolysis products, principally isothiocyanates (ITCs), released in soil after incorporation of plant tissues containing glucosinolates (Kirkegaard et al., 1992). Brassicaceae plants, including *Brassica* species, have been used in rotations or as green manures to control Verticillium wilt (Subbarao et al., 2007; Berbegal et al., 2008; Neubauer et al., 2014). The potential of 19 cultivars of *Brassica juncea*, *Rhaphanus sativus* and *Sinapis alba* as biofumigants was evaluated and only *B. juncea* shoot tissue was shown to reduce the viable microsclerotia significantly with efficacy from 69.3 to 81.3% in laboratory bioassay (in sterile quartz sand) (Neubauer et al., 2014). However, the efficacy in naturally infested soil was more variable and lower because of low release efficiencies of 2-propenyl ITC. Unlike biofumigation, rotations do not eradicate soil microsclerotia, but maintain the inoculum density below threshold levels (Klosterman et al., 2009). However, this method is not possible for most strawberry growers in the UK because of the shortage in the supply of productive land.

Green manures of other plant species, including Sudan grass, oat, sweetcorn, rye, buckwheat, and Austrian winter pea, have been shown to reduce the inoculum density and wilt severity with varying degrees of success (Wiggins and Kinkel, 2005; Ochiai et al., 2007). However, the mechanisms are still not well understood. oil amendments with animal manures can also reduce the density of microsclerotia (Conn et al., 2005), with the efficacy varying with soil and environmental conditions (Conn and Lazarovits, 2000). *Lavandula stoechas*, fresh and waste *Lavandula angustifolia* (English lavender) and *L.* × *intermedia* cv. Grosso (lavandin) are effective as biofumigants against wilt (López-Escudero et al., 2007; Yohalem and Passey, 2011). However, growing lavender does not fit well with commercial strawberry production in the UK, in addition to the lack of productive land.

Anaerobic soil disinfestation (ASD), which requires neither high temperature nor long term incubation, incorporates organic material, irrigation and mulching. ASD has been explored intensively over the past decade for its effects against soilborne pathogens (Goud et al., 2004; Momma, 2008; van Overbeek et al., 2014). Easily decomposable organic materials used for ASD, such as waste products from agriculture or horticulture, encourage rapid soil microbial growth and respiration, leading to depletion of available soil oxygen and creation of anaerobic conditions. In contrast, digestate, a by-product from anaerobic digestion (AD) in a biogas plant, has not been evaluated yet for its potential effects on soilborne pathogens. AD is a process in which organic matter breaks down naturally in the absence of oxygen to produce biogas and digestate. Biogas is a useful source of renewable energy, whilst digestate is usually described as a soil conditioner, and a valuable biofertiliser (Lukehurst, 2003). Plant fungal diseases can be inhibited or killed during mesophilic digestion (Haraldsson, 2008; Zetterstrom, 2008).

This paper reports results from both laboratory and field studies investigating the effect of alternative products derived from biofumigation and AD on the density of *V. dahliae* inoculum in soil. We first carried out a small laboratory study to determine the efficacy of microencapsulated terpene, pelletised lavender waste and a pelletised Brassica seedmeal product. This was done to determine the efficacy of the microencapsulated terpene product relative to lavender waste and whether its efficiency was sufficiently high enough to justify further field trials. Based on results from the initial laboratory study, we conducted field trials to evaluate the efficacy of individual and combined use of the microencapsulated terpene product, a *Brassica* seedmeal product, and digestate on the density of *V. dahliae* inoculum. All three products were in the liquid formulation for standardised application into soil.

2. Material and methods

2.1. Products tested

An experimental product of microencapsulated terpenes was manufactured by Eden Research (Witney, Oxfordshire, England) using its patented technology; this product contained 9.9% cineole, 3.3% camphor and 3.3% borneol – these terpenes were identified as key compounds in lavender waste responsible for the observed biocidal effect against *V. dahliae* (Yohalem and Passey, 2011). The rate of using this product was recommended by the manufacturers based on other terpene products manufactured by the company (Edmonds of Eden Research – pers. com.). Before use, this product was diluted with water to a ratio of 1:20 (product:water, ca. 4.8%). BioFence™, a pellet formulation of defatted seedmeal of *Brassica carinata*, was obtained from Tozer seeds (Cobham, Surrey, England) and used in the laboratory test with the maximum permissible rate under the UK NVZ (nitrate vulnerable zones) regulations given the estimated N concentration in BioFence™. In the field test, a liquid formulation of BioFence™ was used for the ease of application. Lavender waste was collected from a commercial farm after the oil had been extracted and pelletised by a commercial company; the rate of application was determined as for BioFence™. Liquid digestate was obtained from St Nicholas Court Farms (Birchington, Kent, UK); feedstock was 5% chicken manure, 5% fruit waste, 75% maize silage, 15% grass silage; retention time in the digesters is roughly 90 days. The rate of use was determined based on the approximated N concentration and NVZ regulations.

2.2. Quantifying V. dahliae inoculum density

The density of *V. dahliae* inoculum in each soil sample was estimated using a well-established wet-sieving and plating method (Harris et al., 1993) with several modifications. In short, soil samples were first air-dried for 3 weeks to kill conidia and the mycelial fragments of *V. dahliae* before sieving (2 mm mesh). A sample of 10 g of soil was placed into a screw-cap bottle (200 mL) and distilled water was added to a volume of 40 mL. The bottle was shaken vigorously for 1 h on a reciprocating shaker (Edmund Buhler, 7400 Tubingen Shaker-SM 25, Germany) at 175 rpm to break-down soil clumps. Then the suspension was washed through nested 160 and 20 μm sieves with tap water. The material on the 20 μm sieve was recovered into the original bottle and made up to 20 mL with distilled water. Aliquots of 1 mL soil suspension were transferred individually with a pipette to each of 20 Petri dishes (9-cm diameter) of semi-selective medium. The suspension was stirred while withdrawing each aliquot. The semi-selective 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 with 1 M KOH prior to addition of the agar. After autoclaving, the basal medium was held at 55 °C and 10% by volume sterile filtered antibiotic solution added (containing 6 mg streptomycin, 6 mg chloramphenicol, 6 mg chlortetracycline, 0.6 mg biotin per 10 mL water). Soil plates were incubated at 22 °C for 4 weeks, before soil particles were washed away with tap water. The plates were scanned for