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The impact of biological pesticides on arbuscular mycorrhizal fungi

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ABSTRACT

Arbuscular mycorrhizal (AM) fungi have a key role for plant nutrition in organic farming systems where crop protection relies on biopesticides. Although these are considered safe, their effects on non-target organisms, such as AM fungi, are not known and should be evaluated. A pot and a field experiment were employed to investigate the impact of biological pesticides (azadirachtin, spinosad, pyrethrum and terpens) on exogenous AM fungal inoculum (pots) and on indigenous AM fungi (field). The synthetic fungicide carbendazim and non-pesticide treated controls with or without mycorrhizal inoculation were also included. Plant growth and root colonization were measured 20 and 40 days post inoculation (dpi) in the pot experiment, or 40 and 90 dpi in the field study. Pesticide effects on the structure of the intraradical AM fungal community were determined via DGGE and cloning. Spinosad, pyrethrum and terpenes did not affect the colonization ability and the structure of the AM fungal community. On the contrary, pot application of azadirachtin resulted in a selective inhibition of the *Glomus etunicatum* strain of the inoculum. DGGE analysis showed that the field application of azadirachtin induced significant and persistent shifts in the AM fungal community. Carbendazim completely hampered mycorrhizal colonization in pots, compared to its field application which had a transitory effect on the colonization ability and the community structure of indigenous AM fungi. Our study provides first evidence for the effects of biological pesticides on the diversity of AM fungi.

1. Introduction

Arbuscular mycorrhizal (AM) fungi are beneficial soil microorganisms that live symbiotically in association with the vast majority of plant species (Helgason and Fitter, 2005). Their benefits to plant fitness and resistance against diverse stresses have been documented extensively (Smith and Read, 1997). However, their practical use in conventional agriculture is impaired by agronomic practices such as the application of synthetic fertilizers and pesticides. Previous studies have shown a range of effects of synthetic pesticides on AM fungi. Fungicides benomyl and carbendazim significantly inhibited the ability of AM fungi to colonize plants and P uptake (Schweiger and Jakobsen, 1997), while chloroneb and aldicarb stimulated or had no effect respectively (Spokes et al., 1981). The vast majority of such studies have used plant growth, colonization or P uptake as endpoints of the potential toxicity of pesticides on AM fungi, whereas no studies have investigated pesticides impact on the diversity and community structure of AM fungi. Our knowledge of community ecology has been significantly advanced during the last decade with the introduction of molecular methods which revealed a large unknown diversity of AM fungi (Rosendahl, 2008). The use of such molecular tools in combination with existing conventional methods could substantially advance our knowledge on the deleterious effects of pesticides on the diversity and function of non-target key soil microorganisms like AM fungi.

In contrast to conventional agriculture, in organic farming AM fungi have a key role in promoting soil fertility and increasing crop production (Gosling et al., 2006). In such systems crop protection relies on the use of pesticides of biological origin like plant and microbial extracts (EEC 2092/91). These pesticides are generally considered environmentally safe due to their biological origin and fast decomposition rates (Isman, 2006). However there is limited information on their effects on non-target soil microorganisms like AM fungi, and their compatibility with them should be evaluated.

Azadirachtin is a botanical insecticide/nematicide which is produced by the neem tree (*Azadirachta indica* Juss) (Schmutterer, 1990). It is generally considered of low persistence in the soil with half-life values ranging from 7 to 21 days (Stark and Walter, 1995). Soil microbes play a key role in its degradation (Thoeming

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et al., 2006). Previous studies have indicated that azadirachtin may possess fungicidal activity (Akça et al., 2005), and when applied at doses 10× the recommended could inhibit the culturable fraction of nitrifying bacteria and fungi (Gopal et al., 2007). More recently, Spyrou et al., (2009) first showed via culture-independent methods (phospholipid fatty acid analysis) that azadirachtin did not alter the structure of the soil microbial community when applied at the recommended dose rates.

Spinosad is a bacterially-derived insecticide which was only recently given authorization for use in organic farming (Cleveland et al., 2002). It is generally microbially degraded (Hale and Portwood, 1996), with half-life values ranging from 2 to 8 days (Thompson et al., 2002). Pyrethrum is a botanical insecticide extracted from dried flowers of *Chrysanthemum cinerariaefolium* (McLaughlin, 1973). Although it is used in organic agriculture for many years there are only limited information regarding its environmental fate and toxicity on terrestrial ecosystems. Taiwo and Oso (1997) observed a decrease in the diversity of soil fungi after application of pyrethrum. Terpenes are the main components of the essential oil of plants and several of them are known to possess insecticidal (Isman, 2006) and nematicidal activities (Ntalli et al., 2011). Currently, mixtures of plant-derived terpenes are under evaluation for use as nematicides in Europe. Several previous studies have shown that their release in soil could stimulate co-metabolism of recalcitrant organic pollutants by soil microorganisms (Suttinun et al., 2009), whereas little is known regarding their potential effect on AM fungi when applied as formulated crop protection products.

Thus the aim of this study was to assess the effects of biological pesticides commonly used in organic farming on the ability of AM fungi to colonize plant roots and on the structure of their intraradical community at both laboratory and field scale level.

2. Materials and methods

2.1. Pot experiment

A factorial experimental design was followed for the pot experiment including two sampling dates (20 and 40 days), four biological (azadirachtin, pyrethrum, spinosad, terpenes) and one synthetic pesticide (carbendazim) and two non-pesticide treated controls which were either inoculated or not inoculated with a known AM fungal inoculum, whose composition is described below. Carbendazim was included since it is known to have detrimental effects on AM fungi (Schweiger et al., 2001). There were five replicates per treatment and sampling time summing to a total of 70 pots (3 L).

Appropriate amounts of a 1:1 mixture of sand and soil (sandy, pH 7.81, electrical conductivity 0.017 mmhos cm^{-1}, organic matter content 8.8 g kg^{-1}, P-Olsen 2 mg g^{-1}, K 215 mg g^{-1}, Mg 265 mg g^{-1}) used as plant growing substrate were autoclaved and left to equilibrate for 24 h. Subsequently the substrate was divided into seven equal amounts (35 kg). The first five bulk samples were treated with 50 ml of appropriate aqueous solutions of the pesticides resulting in the application of the recommended pesticide doses for pest control (Table 1). The other two bulk samples received the same amount of water without pesticide to serve as non-pesticide treated controls. From the biological pesticides selected, azadirachtin (Thoeming et al., 2006; Lynn et al., 2010) and terpenes (Ntalli et al., 2011) are used as soil applied pesticides for the control of nematodes, while spinosad has been also applied as soil drench for the control of leaf-mining insects in horticultural crops (Weintraub and Mujica, 2006). On the contrary pyrethrum is a foliar insecticide which was considered in our study as soil applied for reasons of uniformity. For pyrethrum, azadirachtin and terpenes their recommended mode of application includes successive low-dose applications on 15- or 30-day intervals. For those chemicals, the experimental application rate was calculated as the sum of all proposed applications per season. This way a worst-case exposure scenario for AM fungi was used where the whole pesticide amount was applied at once assuming no dissipation between subsequent applications. After pesticide application, the moisture content of the substrate was adjusted to 45% of water holding capacity by addition of sterilized dH_2O. The substrate was hand-mixed and stored in aerated plastic bags at 4 °C overnight. Subsequently, the treated substrate was distributed in 3 L pots filling the lower and upper quarter, while a mixture of inoculum and treated substrate (400 g and 17.5 kg respectively) was distributed in the middle. One of the two sets of the non-pesticide treated samples received the same amount of substrate without inoculum to serve as non-pesticide treated, non-mycorrhizal inoculated controls. Three week old pepper plants (*Capsicum annum* L. cv Ozho) (kindly supplied by AgriPlant A.S. and checked for absence of mycorrhizal colonization) were then transplanted in all pots and placed randomly in the growth chamber at 22 °C using a 16 h light–8 h night period. The plants were watered as needed and 30 ml of a 10% Hoagland solution (Hoagland and Arnon, 1938) was applied twice weekly. The plants were harvested 20 and 40 days post inoculation (dpi) by cutting the shoots at the soil line. The plant height was recorded, roots were washed free of soil, and three weighted portions were removed and used for further analysis. The first root portion was utilized for estimation of mycorrhizal colonization, the second was stored at −20 °C for DNA extraction, and the third was used for determination of the dry mass of roots. Root and shoot dry mass were determined by oven drying at 60 °C for 48 h.

Table 1
Commercial pesticide formulations, application rates and soil concentrations used in the current study.

Pesticide	Formulation (g L^{-1})	Recommended Doses (L of formulation ha^{-1})	Intended soil concentrations (mg kg^{-1} soil dry weight)[b]
Spinosad	LASER® SC 480	8.1 (1)[a]	2.00
Pyrethrum	PIRESAN® EC 18.6	1.2 (3)	0.23
Azadirachtin	NEEMAZAL® EC 10	6.0 (9)	2.79
Terpenes	Under Development Formulation EC 300	12.0 (4)	7.00
Carbendazim	OCCIDOR® 500	2.85 (1)	2.00

[a] The numbers in brackets indicate the number of applications proposed for effective nematode control.
[b] The intended concentrations of the pesticides in soil were calculated assuming an overall soil bulk density of 1.3 mg L^{-1} following pesticide diffusion in the top 15 cm of the surface soil.

2.2. Field experiment

A field-scale experiment was also established using the same treatments described in the pot study. The field experiment was conducted from May to August 2009 which is a common growing season for peppers in inland areas in central Greece. A block experimental design with three 28 m^2 (17.5 × 1.6 m) blocks separated by concrete pavement were established. Distances of the pepper plants within and between rows were 40 cm and 90 cm respectively. Each block comprised of two rows, one for every sampling date (at 40 and 90 dpi), with randomization of treatments in each block. For every treatment in each block there were five plants in each row. Two days before transplanting the soil (loam – 49% sand, 34% silt, 17% clay, pH 8.1, P-Olsen 79 mg g^{-1}) was tilled, leveled and irrigated at water holding capacity. Next day, all