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Multitrophic interactions between maize mycorrhizas, the root feeding insect *Phyllophaga vetula* and the entomopathogenic fungus *Beauveria bassiana*

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ABSTRACT

Root feeding larvae of *Phyllophaga vetula* causes severe damage in maize agroecosystems and their conventional management with chemical pesticides adversely affects the environment. Plant beneficial rhizosphere microorganisms such as mycorrhzial and entomopathogenic fungi (EPF) offer an alternative sustainable pest management strategy, but their successful integration in agroecosystems requires profound knowledge about their ecology. Here we explored interactions between a commercial isolate of the EPF *Beauveria bassiana* and indigenous maize mycorrhizas and their single and combined effects against root herbivory from the insect larvae *P. vetula.* Maize plants were grown in a greenhouse for seven weeks in pots with P limited soil with all possible combinations of the three main factors including native arbuscular mycorrhizal fungi (AMF) (without and with), EPF (without and with) and *P. vetula* (without and with).

Root herbivory by *P. vetula* reduced plant growth and nutrition, and in addition reduced AMF root colonization. In contrast, AMF improved plant growth and P nutrition, but also reduced the population density of *B. bassiana*. Inoculation with *B. bassiana* increased root dry weight and counteracted a decrease in shoot N content from *P. vetula* root herbivory, despite that only 5% of *P. vetula* larvae were infected with *B. bassiana*. Dual inoculation with AMF and *B. bassiana* increased plant growth and N shoot content allowing the plants to compensate for the damage caused by *P. vetula* herbivory. In conclusion our results show that combination of native AMF populations and the EPF *B. bassiana* induce tolerance in maize from root herbivory by *P. vetula* in terms of plant growth performance and nutrition.

1. Introduction

Maize is one of the world basic crops for human consumption and animal fodder (FAOSTAT, 2009). In Mexico, which is the origin of domestication of maize with a diverse genetic population (Sánchez et al., 2000), maize is covering around 36% of all arable land and is being cultivated by small holders and at industrial scale (Paliwal, 2001).

Root feeding insects of the white grub complex *Phyllophaga* spp. (Coleoptera: Melolonthidae) cause severe yield losses in maize agroecosystems in Mexico due to their high abundance, diversity and wide distribution (Jackson and Klein, 2006; Rodríguez del Bosque and Morón, 2010).

Conventionally chemical insecticides are used to manage root feeding insects, which however have strong environmental impacts contaminating soil and water ecosystems and adversely affect human health (Gómez-Arroyo et al., 2011). Development of insecticide resistance is another concern (Oerke, 2006), which causes new pest outbreaks increasing the amount of insecticides applied to agroecosystems. Also possible non-target effects of pesticides on plant beneficial

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insects such as pollinators and natural enemies of insect pests is an important environmental impact to consider (Desneux et al., 2007).

Biological control with natural enemies offers a sustainable pest management strategy (Hajek, 2004) including insect pathogens such as entomopathogenic fungi (EPF) (Vega et al., 2012). *Beauveria bassiana* (Clavicipitaceae, Hypocreales) is among the most applied EPF against arthropod pests in agricultural systems (De Faria and Wright, 2007) and is known as an efficient biocontrol agent against the white grub complex in maize roots (Carrillo-Benitez et al., 2013; Guzmán-Franco et al., 2012). Besides the before mentioned entomopathogenic traits in the soil ecosystem, *B. bassiana* is also known as a plant growth promoting root endophyte (Vega, 2008; Vidal and Jaber, 2015; McKinnon et al., 2017).

Maize roots also naturally form mycorrhizal associations with arbuscular mycorrhizal fungi (AMF), which are obligate biotrophic fungi known to improve plant growth and nutrition (Smith and Smith, 2011) and root health (Whipps, 2004; St-Arnaud and Vujanovic, 2007). Formation of arbuscular mycorrhiza has been shown to increase content of defense compounds and nutrients in roots of the host plant, which affect insect herbivore performance (Bennett et al., 2006; Vannette and Hunter, 2009; Currie et al., 2011).

Multitrophic interactions between plants, microorganisms and insects are important to consider when developing biocontrol strategies against pests (Gange, 2007; Gehring and Bennett, 2009). Also when developing such strategies it is important to consider the possible ecosystem impacts and factors influencing performance of the biocontrol agent when applied to agroecosystems (Ownley et al., 2010). However, information on interactions between EPF and other plant beneficial root associated microorganisms such as AMF is yet limited (Gualandi et al., 2014).

Here we explored interactions between a commercial isolate of the EPF *B. bassiana* and indigenous maize mycorrhizas and their single and combined effects against root herbivory from the insect larvae *P. vetula*. Our main hypothesis was that dual inoculation with *B. bassiana* and indigenous AMF would result in less damage from root herbivory by *P. vetula* than that from single inoculation with either AMF or *B. bassiana*; this due to their complementary biocontrol mode of action.

2. Materials and methods

2.1. Experimental design

The experiment was a greenhouse pot experiment with a complete randomized factorial design. Main factors examined included: 1) AMF (with and without), 2) *B. bassiana* (with and without) and 3) *P. vetula* (with and without). Each of the eight treatments had five replicates, giving a total of 40 experimental units.

2.2. Experimental set-up

Agricultural soil was collected at the experimental field station of Universidad de Chapingo, Campus Morelia, Michoacán, Mexico. The soil texture was clayish consisting of 53.2% clay, 27.3% silt and 19.5% sand and the chemical characteristics of the soil were: organic matter content (2.7%), inorganic nitrogen (23.2 mg g^{-1}), available phosphorus (5.8 mg g^{-1}) and pH (H_2O) 7.3. The soil was mixed with quartz sand (1:1, w/w) and sterilized twice by autoclaving (15 lbs, 120 °C) during one hour. Full basic mineral fertilization except P was applied to each pot corresponding to the following amounts (mg kg^{-1} soil): K_2SO_4 (75), $CaCl_2{\cdot}2H_2O$ (75), $CuSO_4{\cdot}5H_2O$ (2.1), $ZnSO_4{\cdot}7H_2O$ (5.4), $MnSO_4{\cdot}H_2O$ (10.5), $CoSO_4{\cdot}7H_2O$ (0.39), $MgSO_4{\cdot}7H_2O$ (45), $Na_2MoO_4{\cdot}2H_2O$ (0.18). One liter pots were filled with 800 g of the soil:sand mix placed in a plastic bag to avoid leaching of nutrients from the pots after watering during the plant growth period.

In the AMF treatment 25% of the sterile soil:sand mix was replaced with non-sterilized soil:sand mix prior sowing. The agricultural soil used is known to harbor indigenous communities of AMF, which has previously been confirmed in other experiments using the same soil as AMF inoculum providing well established AM fungus root colonization (J. Larsen, unpublished results). In order to reintroduce soil microorganisms other than AMF in the non-AMF treatments all pots received 10 mL AMF inoculum filtrate prepared by sieving a suspension of 100 g of unsterilized soil in 1 L distilled water through a nylon mesh (20 µm).

The commercial powder-based product (Bea-sin, Agrobionsa) was used as *B. bassiana* inoculum. Before sowing 10^7 spores g^{-1} dry soil were added to the corresponding treatments with *B. bassiana*. To facilitate homogenous mixing of the spores into the soil the powder was first mixed carefully into approximately 100 g of soil:sand mix, which was subsequently mixed into the remaining soil:sand mix in each pot. Prior inoculation standard quality control of the inoculum revealed no contamination from other microorganisms and showed 83% viability of the conidia. The quality control was performed by counting and visual inspection of plates from 10-fold serial dilutions on potato dextrose agar (PDA) plates.

Three seeds of the maize hybrid DK-2042® (Dekalb) were sown in each pot, but were thinned to one uniform seedling in each pot after seedling emergence seven days after sowing.

Three weeks after sowing two larvae of *P. vetula* (third instar) were applied to each pot according to the respective treatments. The larvae were individually emptied out on the soil surface from their container without touching them to avoid damage. All larvae managed to enter the soil after no more than 15 min exposed on the soil surface.

Previously larvae were collected from maize roots in a small holder conventional maize field in Zacapu, Michoacan, Mexico. After the field collection, larvae were stored individually in 20 mL plastic containers filled with damp peat moss, fed with carrot and maintained at room temperature (15–25 °C) during 40 days. Only active feeding and pathogen symptomless larvae were selected for the experiment. Larvae were identified to species level based on the presence of *palidia* in the last abdominal segment (*raster*) and anal aperture morphology (Morón, 1983; Aragón and Morón, 2004). The specimens identified as *P. vetula* (Horn) were selected, as it was the most abundant species and also considered an important root pest in the region (Nájera-Rincón et al., 2003).

2.3. Plant growth conditions, harvest and analyses

Plants were grown under greenhouse conditions and watered by weight to 70% of the water holding capacity on a daily basis. Greenhouse temperature was approximately 15 °C at night and 25–30 °C during the daytime throughout the seven weeks period of the experiment. Nitrogen was supplied weekly (25 mg) in terms of a NH_4NO_3 solution with a total of 125 mg N per plant during the growth period.

At harvest seven weeks after sowing larvae were removed from the soil and examined visually for vitality and symptoms of fungal infection and weighed. Afterwards, the whole soil from each pot was individually mixed and 4 g subsample was taken and stored at 4 °C for one day before plating on EPF semi-selective media for measurement of *B. bassiana* soil population density.

Roots were gently washed free of growth substrate and the shoot separated from the root. Dry weights of shoots and roots were obtained after drying at 70 °C for 48 h. Dried maize shoots and roots were ground and sieved with a mesh No 40 (0.425 mm) for further analysis of P and N.

Samples were wet digested prior colorimetric analysis. First, a mixed digester (1 g $CuSO_4$; 10 g K_2SO_4) was added to 0.25 g of the plant tissue sample in a 75 mL glass tube, then 3 mL of H_2O_2 (30% v/v) was added to carry out the oxidation reaction and finally, 7 mL of sulfuric acid (H_2SO_4) was added. After 24 h, when the digestion was complete, the samples were placed in a digestor block to gradually increase the temperature (50 °C every 20 min) to 375 °C for 3 h. The