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Beauveria bassiana: An entomopathogenic fungus alleviates Fe chlorosis symptoms in plants grown on calcareous substrates

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ABSTRACT

Entomopathogenic fungi are known due to their ability to kill insects and besides they can play other roles, such as promoting plant growth or improving nutrient uptake. The main aim of this work was to assess the potential of *Beauveria bassiana* strain EABb 04/01-Tip for improving iron (Fe) nutrition in plants grown on calcareous substrates. Plants of a dicot (tomato) and a monocot (wheat) were pot-grown on an artificial substrate consisting of 0–240 g kg^{-1} Fe oxide-coated sand (FOCS), 250 g kg^{-1} calcium carbonate sand and quartz sand. The plants were subjected to two different treatments, namely seed dressing, which involved inoculating seeds with a conidial suspension of *B. bassiana* before sowing, and a control without inoculation. Leaf chlorophyll concentrations (SPAD) and Fe uptake by plants were correlated with the FOCS content of the substrate, therefore the experimental design was appropriate to see different levels of Fe chlorosis symptoms. *B. bassiana* was able to colonise both tomato and wheat plants without a negative effect on plant height, plant dry weight, root development in tomato or grain production in wheat. In addition, *B. bassiana* alleviated Fe chlorosis symptoms (described for first time) in both crops during early growing stages (<50 days after sowing) but the intensity of the effect depended on the plant species and available Fe on substrate. Nutritional alterations in K due to fungal application were detected in tomato and wheat plants.

1. Introduction

Endophytic fungi are microorganisms that can live in a host plant without causing any symptoms of disease. Plant–endophyte symbiotic relationships occur in the vast majority of ecosystems where plants need fungi to withstand adverse factors (Rodriguez et al., 2004) such as biotic (herbivory and pathogens) and abiotic (e.g. high temperatures, salt, drought, low content of nutrients in soil, heavy metals) stresses (Akello et al., 2009; Arnold et al., 2003; Ownley et al., 2010; Redman et al., 2002; Saikkonen et al., 2013; Vega et al., 2009; Waller et al., 2005).

Ascomycetes, which constitute one of the most important group of endophytic fungi, have been found on a wide range of host plants (Ownley et al., 2010; Quesada-Moraga et al., 2014; Vega et al., 2009). The entomopathogenic fungi in this division are used as biopesticides because of their ability to kill insects by producing secondary metabolites that are of industrial and agricultural interest (Aly et al., 2011; Schulz et al., 2002; Zhang et al., 2006). However, they can play other not fully understood roles such as promoting plant growth (Liao et al., 2014; Maag et al., 2013; Sasan and Bidochka, 2012; Vega et al., 2009) or even improving plant nutrition (García-López et al., 2013).

Beauveria bassiana is an entomopathogenic fungus naturally present in soils that can infect insects, mites and ticks, and hence possesses a great potential for pest management (Meyling and Eilenberg, 2007). Some authors have shown *B. bassiana* to endophytically colonize crops such as opium, tomato, wheat, corn and sorghum (Gurulingappa et al., 2010; Quesada-Moraga et al., 2006; Tefera and Vidal, 2009), besides protecting crops against pest and disease (Lozano-Tovar et al., 2013). Also, this fungus has been found to contribute to rock and mineral bioweathering, and to alter the availability of metals such as Fe, Cu and Ag when it is directly applied to a metallic surface (Joseph et al., 2012).

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There are seemingly no literature references to the effect of *B. bassiana* on plant nutrition; however, experiments with other fungi used as biocontrol agents such as *Trichoderma* have provided evidence for increased nutrient (Fe) uptake by plants (de Santiago et al., 2009). Therefore, applying *B. bassiana* to crops grown on soils with low bio-availability of some nutrients might provide a twofold benefit: plant protection (well known) and improve nutrient uptake.

Calcareous soils span 30% of all arable land in the world and typically have a high calcium carbonate ($CaCO_3$) content and a pH of 7.5–8.5. Iron bioavailability at these pH levels is very low and results in deficiencies such as Fe chlorosis—a major problem in plants grown on calcareous soils (Tagliavini and Rombolà, 2001). The main symptoms of Fe chlorosis are interveinal yellowing of young leaves and diminished plant growth, because plants are unable to synthetize chlorophyll under these conditions. Necrotic spots can replace chlorosis and cause leaves to die or even decrease production yield and quality. Because Fe bioavailability depends on the specific surface area and crystallinity of Fe oxides (Schwertmann, 1991), it is increased by the presence in soils of non-crystalline Fe oxides such as ferrihydrite, wich has a high specific surface area (Diaz et al., 2009).

Although some of the above-described experiments, in which *B. bassiana* was used to protect crops against pests [*Bactrocera oleae* (Gmelin.) (Diptera; Tephritidae)] and disease (*Verticillium dahliae*, *Phytophthora megasperma* and *Phytophthora inundata*), were conducted on calcareous soils, not much is known about the effect of this endophytic fungus on plant growth or nutrition. Gatarayiha et al. (2010) studied the interactions between *B. bassiana* and potassium (K) silicate fertilization, and observed a higher efficiency of the fungus used to control two spotted spider when K fertilization was applied to inoculated dicot and monocot plants (cucumber, eggplant, bean and maize). However, there is a lack of information on the effect that *B. bassiana* causes the plant in absence of insects.

The objectives of this work were (i) to assess the colonization of leaf, stem and root of tomato (dicot) and wheat (monocot) plants after being inoculated with *B. bassiana* strain EABb 04/01-Tip (via seed) and (ii) to examine the effect of this entomopathogenic fungus on Fe nutrition and plant growth in tomato and wheat plants grown on artificial calcareous substrates.

2. Materials and methods

2.1. Substrate preparation

In order to simulate the typical conditions of calcareous soils, quartz sand and calcium carbonate sand were sieved and particles 0.2–0.5 mm in size selected for optimum hydraulic performance. The resulting fractions were washed 6 times with a large volume of tap water containing Na_2CO_3 at pH 9.5 (quartz sand) or plain water (calcium carbonate sand) to disperse clay and impurities. Both types of sand were also washed several times with de-ionized water to remove salts before drying in an oven at 40 °C. The preparation procedure was repeated once. The specific surface area as measured by N_2 adsorption (BET method, Brunauer et al., 1938) of the quartz sand was 0.14 $m^2\,g^{-1}$ and that of the calcium carbonate sand 0.27 $m^2\,g^{-1}$. Citrate/bicarbonate/dithionite-extractable Fe, used as a proxy for the total content in Fe oxides (Fe_d, Mehra and Jackson, 1960), accounted for 40 $mg\,kg^{-1}$ in quartz sand and 120 $mg\,kg^{-1}$ for calcium carbonate sand. Available P (Olsen et al., 1954) was less than 1 $mg\,kg^{-1}$ in both types of sand. Part of the quartz sand was coated with Fe oxides (ferrihydrite, called FOCS) as detailed in Rahmatullah (2000). FOCS comprised 380 $mg\,kg^{-1}$ Fe_d and 210 $mg\,kg^{-1}$ acid oxalate-extractable Fe (Fe_{ox}, Schwertmann, 1964). The latter is a measure of poorly crystalline Fe oxides (Reyes and Torrent, 1997), which constitute the main source of Fe for plants in calcareous media.

2.2. Plant material, culture, preparation of the conidial suspension and treatments

Five substrates containing variable proportions of FOCS (viz., 20, 40, 80, 120 and 240 $g\,kg^{-1}$), one part of calcium carbonate (250 $g\,kg^{-1}$) and quartz sand to complete 100% (730, 710, 670, 630 and 510 $g\,kg^{-1}$, respectively) were prepared. A positive control in the form of a substrate inducing no Fe chlorosis and consisting of 100% quartz sand (0 $g\,kg^{-1}$ FOCS) was also studied. Cylindrical PVC pots 12 cm high and 4.5 cm in diameter with a drainage hole in the bottom were filled with 250 g of the previous combinations following sterilization by heating twice at 121 °C for 20 min before sowing the crops.

Seeds of tomato (*Lycopersicon esculentum* Mill. cv. Marmande-Cuarenteno, commercial seeds) and wheat (*Triticum aestivum* L. cv. Chinese Spring, kindly provided by Dr. Pilar Prieto from Institute for Sustainable Agriculture of Córdoba, Spain) were immersed in a 6% H_2O_2 solution for 10 min and then gently washed with de-ionized water. After pre-germination at 25 °C in the dark for 2 days, the seeds were subjected to two different treatments. One, called the "control", involved immersing a part of the seeds in sterile, conidium-free de-ionized water containing Tween 80 (0.1% v/v) under shaking at 180 rpm for 4 h. The other one, called "seed dressing", was performed under the same conditions but used a *B. bassiana* suspension containing 1×10^8 conidia mL^{-1} to inoculate the remaining seeds. This fungal strain was routinely grown on slants of malt agar (MA; Biocult, Madrid, Spain) at 25 °C in the dark and stored at 4 °C. Fungus cultures were grown at 25 °C in the dark on malt agar containing 25 g malt agar and 7.5 g agar for 2 weeks, after which conidia were collected by scraping the surface of the culture with a sterile camel hairbrush into a 100 mL glass beaker containing 50 mL of sterile distilled water plus Tween 80 (0.1% v/v). The conidial suspension was stirred, filtered, adjusted to 1×10^8 conidia mL^{-1} and used to inoculate the seeds as described. A different fresh culture was prepared for each crop.

The conidial suspension was prepared by using *B. bassiana* strain EABb 04/01-Tip isolated from a dead *Iraella luteipes* larva. This strain, which had previously exhibited an endophytic behaviour upon inoculation to opium plants is deposited in the CRAF Entomopathogenic Fungi Collection of the University of Cordoba and the Spanish Collection of Culture Types (CECT) of the University of Valencia (CECT 20,744, accession number). A fresh conidial suspension was prepared for each crop.

Once treated, the seeds were dried under sterile conditions and 3 seeds sown in each pot. The pot experiment was performed in a growth chamber under the following conditions: 12 h day^{-1}, 250 $\mu mol\,m^{-2}\,s^{-1}$, 21 °C and 70% relative humidity. Seven days after sowing (DAS), all plants except one in each pot were cut off and the pots weighed and watered daily to keep soil moisture near field capacity. Also, each crop and pot were irrigated with a modified Hoagland solution on a weekly basis (5–10 mL each week depending on the extent of plant growth). The Fe-free modified Hoagland nutrient solution applied to the plants grown on the FOCS-containing substrates consisted of $Ca(NO_3)_2{\cdot}4H_2O$ (5 mM), KNO_3 (5 mM), $MgSO_4$ (2 mM), K Cl (0.1 μM), $Ca(H_2PO_4)_2{\cdot}H_2O$ (0.3 μM), H_3BO_3 (50 μM), $MnSO_4{\cdot}H_2O$ (4 μM), $ZnSO_4{\cdot}7H_2O$ (4 μM), $CuSO_4{\cdot}5H_2O$ (0.1 μM) and Na_2MoO_4 (6 μM). The solution was enriched with Fe (20 μM) for application to the pots containing no FOCS. This rate had previously proved effective to prevent Fe chlorosis.