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The root endophytic fungus *Trichoderma atroviride* induces foliar herbivory resistance in maize plants

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ABSTRACT

Plant roots naturally associate with *Trichoderma* spp., which can promote plant health and nutrition. Despite that *Trichoderma* spp. are well-known biocontrol agents, information on their effects against foliar insect herbivory is limited. Here, we examined the effects of *T. atroviride* in providing maize (*Zea mays*) resistance against the insect herbivore *Spodoptera frugiperda*. Increased plant growth, reduced herbivory and altered insect feeding pattern were observed after maize inoculation with *T. atroviride*. Plant protection was correlated with increased emission of volatile terpenes and accumulation of jasmonic acid, an activator of defense responses against herbivory. Chemical analyses revealed that *T. atroviride* produced the volatiles 1-octen-3-ol and 6-pentyl-2*H*-pyran-2-one. Pharmacological tests showed that both compounds reduced the consumption of foliar tissue and altered the feeding pattern of *S. frugiperda* in a similar way to *T. atroviride*. These results provide new insight into the role of *T. atroviride* in plant health in terms of induction of resistance to insect herbivory and production of antifeedant secondary metabolites.

1. Introduction

The fall armyworm, *Spodoptera frugiperda*, is a serious pest of numerous crops including maize (Cruz et al., 1999), where it is responsable for severe yield losses every year in several countries (de Lange et al., 2014). Conventionally, *S. frugiperda* is managed with chemical insecticides, which have adverse impacts on human health and the environment, such as non-target effects on beneficial insects including pollinators and natural pest enemies (Pimentel, 1995). Fungi of the genus *Trichoderma* are common rhizosphere inhabitants (Druzhinina et al., 2011). They have been widely studied as biological control agents principally against plant pathogenic fungi, but also the activity against bacteria and nematodes is well documented (Harman et al., 2004; Reino et al., 2008; Martínez-Medina et al., 2017). Biocontrol of *Trichoderma* includes the activity of hydrolytic enzymes that degradate the cell wall of the target organism. Secondary metabolites as peptaibols and isoprenoids from *Trichoderma* have been involved in the biocontrol of both foliar and root pathogens (Velázquez-Robledo et al., 2011; Contreras-Cornejo et al., 2014). Particularly, the metabolite 6-pentyl-2*H*-pyran-2-one (6-PP), a pyrone from *Trichoderma* spp. have been shown to inhibit the growth of the fungal pathogens *Rhizoctonia solani*, *Fusarium oxysporum* and *Botrytis cinerea* (Reino et al., 2008). In the rhizosphere, plant roots recognize molecules derived from microorganisms as auxin-like metabolites or volatile organic compounds (VOCs), which in turn regulate developmental processes or activate defense responses effective against multiple agressors (Pozo et al., 2005; Garnica-Vergara et al., 2016; Hung et al., 2014). Plants respond to feeding by arthropod herbivores by producing a number of secondary compounds, including VOCs that are not only known to attract natural enemies of the herbivores, but they may also prime inducible defense responses in neighbouring plants, resulting in stronger and faster defense responses in plants exposed to those molecules (Dicke et al., 2009; Von Mèrey et al., 2011).

In the belowgrownd, *Trichoderma virens* releases a blend of VOCs that trigger defense responses dependent of jasmonic acid (JA) signaling pathway (Contreras-Cornejo et al., 2014). Similarly, chewing insects, such as caterpillars predominantly activate the JA-mediated pathway, whereas feeding phloem-sucking herbivores frequently activates the salicylic acid (SA) pathway (Rodriguez-Saona et al., 2010).

The molecular mechanisms activated in response to herbivore attack involve several key regulatory proteins (Pieterse and Dicke, 2009; Kim and Felton, 2013). In this sense, the transcription factor MYC2, a

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positive regulator for JA-responsive genes, has been shown to play a role in microbial induced systemic resistance (Pozo et al., 2008; Verhage et al., 2011; Carvalhais et al., 2015) and shapes plant defense responses against tissue-chewing caterpillars (Verhage et al., 2011).

Maize root colonization by *Trichoderma harzianum* and plant perception of fungal cellulose triggered defense responses dependent of JA involving the up-regulation of the protein MYC2 and such enhanced plant defense was effective against the foliar pathogen *Curvularia lunata* (Saravanakumar et al., 2016). Similarly, *T. virens* and *T. atroviride* activated defensive mechanisms to restrict aboveground the infection caused by the necrotrophic fungus *Botrytis cinerea*, and in the belowground, it is known that *T. harzianum* primes tomato defense responses against the root knot nematode *Meloidogyne incognita*; such enhanced plant immunity triggered by *Trichoderma* spp. is undulated, since time course studies have demonstrated that the expression of JA and SA-responsive genes change over time (Contreras-Cornejo et al., 2011; Martínez-Medina et al., 2017).

Soil microorganisms might play key roles in plant-insect above and belowground interactions because they have been shown to improve plant health against insect herbivory (Heil, 2011; Erb et al., 2012). Reports on the biocontrol of aboveground leaf feeding insects by *Trichoderma* are scarce because these fungi naturally interact belowground with other microorganisms and plant roots (Saravanakumar et al., 2017). However, it has been described that *Trichoderma* spp. can have some biocontrol effect against Lepidopteran insects since *Trichoderma* chitinases can attack under suitable conditions the insect cuticle and they can negativelly affect the peritrophic matrix of silkworms (Berini et al., 2016).

Root colonization by beneficial fungi results in plant protection against agricultural pests. For example, onion (*Allium cepa* cv. Red Creole) plants colonized by *Trichoderma* spp. had significantly lower feeding punctures by *Thrips tabaci* (Muvea et al., 2014). *Trichoderma longibrachiatum* associated with tomato roots altered the profile of plant host VOCs resulting in improved attractiveness to the aphid parasitoid *Aphidus ervi* and the aphid predador *Macrolophus pygmaeus* (Battaglia et al., 2013). Importantly, plant tolerance to biotic stress has been correlated with the presence of fungal secondary metabolites and this warrats further research (Aly et al., 2010).

In general terms, little has been reported as to the role of fungal signals in regulating plant growth and resistance against herbivory. Considering that *Trichoderma* in association with plant roots can regulate the protective defenses against multiple agressors, we examined whether induced systemic resistance triggered by the fungus can serve to reduce an aboveground insect attack.

In this work, we studied the interaction between *T. atroviride*, maize plants, and *S. frugiperda*. We particularly asked the following questions: i) Can *T. atroviride* protect maize plants against herbivory? ii) Are there changes in the emission of VOCs and accumulation of JA in the plant when it is associated with *T. atroviride* or when attacked by *S. frugiperda*? iii) Can volatile metabolites produced by the fungus reduce insect attack? and finally iv) Is there a correlation between the possible changes in the accumulation of JA and fungal volatile metabolites and the levels of herbivory? To answer these questions, both chemical and pharmacological aproaches were employed.

2. Materials and methods

2.1. Plant material and growth conditions

Maize (*Zea mays* L.) seeds from a Mexican land race (Chalqueño) were used in this work. Seeds were surface sterilized with 50% (v/v) household bleach (6% NaOCl) for 10 min and 70% (v/v) ethanol for 10 min. After five washes in distilled water, seeds were germinated in darkness at 25 ± 1 °C, 60 ± 5% of relative humidity (RH) by 48 h and each maize embryo was surgically extracted. Next, embryos were sterilized with 50% (v/v) bleach for 2 min and placed into glass tubes (24 × 149 mm) containing ~10 ml of sterilized and solidified 1.0× MS medium (Murashige and Skoog basal salts mixture, catalog number M5524: Sigma, St. Louis). Tubes were placed vertically at a 60° angle to allow root growth on the agar, and unimpeded aerial growth of the cotyledon and leaves. Plants were grown in cabinets at 25 ± 1 °C with a photoperiod of 16 h of light/8 h dark, RH of 60 ± 5% and a light intensity of 100 μmol m^2 s^{-1}.

2.2. Fungal growth and plant inoculation experiments

Trichoderma atroviride wild type strain IMI 206040 was used in this work. *T. atroviride* was evaluated *in vitro* to test its possible effects on *S. frugiperda* performance and if it confers tolerance or resistance against the insect in maize plants. Fungal densities of 10^6 spores suspended in ~5 μl of sterile water were inoculated on the surface of solidified agar (MS 1.0×) of tubes containing 14-days-old maize plants (1 plant per tube; $n = 30$ per treatment) and fungal growth was allowed for 5 days. Tubes were arranged in a completely randomized design and placed under the growth conditions described above. For plant growth experiments, a group of five plants per treatment were recovered from the interaction medium, and excised at the root/shoot junction, and weighed on an analytical scale.

2.3. Herbivory assays

Spodoptera frugiperda (Lepidoptera: Noctuidae) was used as a model insect herbivore. Eggs collected from laboratory-mated adults were used to maintain the colony. Larvae of *S. frugiperda* were reared on an agar-based optimal semi-synthetic diet before herbivory (Poitout and Bues, 1974). Four days after fungal inoculation, 18-days-old maize plants (shoot length ~7.5 ± 2.0 cm) were infested with two larvae in the third instar. After 24 h of treatment with the insect, the percentage of larvae feeding on the tissue and the number of leaves exhibiting wounds were evaluated by visual inspections. The number of wounds was determined by counting the bites present in the leaves ($n = 30$). Herbivory was determined as follow: leaves with wounds were excised at the junction zone and scaned. In these images, the removed area was filled to mimic leaves without herbivory and to calculate the portion of consumed tissue and expressed as percent herbivory. The proportion of leaf area (cm^2) removed was measured using the Sigmascan Pro 5® software applied on the scaned images ($n = 30$).

The same procedures were followed when herbivory bioassays were performed with synthetic compounds 1-octen-3-ol (cat. number O5284-25G; 98%) and 6-PP (cat. number W369608; ≥96% purity) both obtained from Sigma-Aldrich. In these experiments compounds were applied on a cotton piece inserted in each tube. Then, 0.3 mg of each compound were applied per tube 24 h after herbivory to mimic the condition of the belowground root-fungus interaction after insect damage and other 0.3 mg of each compound were added in the moment of infestation with the larvae to reach a final concentration of 0.6 mg 1-octen-3-ol was diluted in 500 μl of water and 6-PP in the same volume of a mixture of ethanol in water (1.5 μl per 498.5 μl). Hereafter, tubes were sealed with parafilm and maintained in the growth chamber until further analysis. Maize plants treated with water and water + ethanol were used as control treatment. Also, bioassays to determine the effect of fungal metabolites on the feeding performance of the larvae were employed. First, a fungal density of 10^6 spores suspended in ~5 μl of sterile water was inoculated on a side of plastic Petri plates with a center partition that contained solid medium MS 1.0×. After 4-days of fungal growth five *S. frugiperda* larvae of third instar were placed on the opposite side of the plate ($n = 50$). Insects were allowed to feed on 0.2 g of semi-synthetic diet for 24 h and the percentage of feeding larvae was determined by visual inspections at the end of this period of time. The same procedure was followed when the solvent (ethanol; control treatment), and increasing concentrations (0.06, 0.6, and 6 mg) of 1-octen-3-ol or 6-PP were added on the semi-synthetic diet placed