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Effects of entomopathogenic fungi used as plant inoculants on plant growth and pest control

Fernanda Canassa

Thesis presented to obtain the degree of Doctor in Science. Area: Entomology

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Bachelor in Biological Sciences

Effects of entomopathogenic fungi used as plant inoculants on plant growth and pest control

versão revisada de acordo com a resolução CoPGr 6018 de 2011

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"Work gives you meaning and purpose and life is empty without it."

Stephen Hawking

SUMMARY

RESUMO

Efeitos da utilização de fungos entomopatogênicos como inoculantes no crescimento de plantas e controle de pragas

Fungos entomopatogênicos dos gêneros *Metarhizium* e *Beauveria* são capazes de colonizar endofiticamente uma ampla variedade de espécies de plantas e conferir à estas, proteção contra artrópodes pragas; além de acelerar o seu desenvolvimento; e atuar como antagonistas de fitopatógenos. O objetivo geral deste projeto foi avaliar o potencial de fungos entomopatogênicos como inoculantes contra o ácaro rajado *Tetranychus urticae* e seus efeitos na promoção de crescimento de plantas. O efeito tri-trófico no consumo e comportamento alimentar do ácaro predador *Phytoseiulus persimilis* também foi estudado. A estratégia avaliada traz vários potenciais benefícios comparado ao uso exclusivo de fungos entomopatogênicos como agentes de controle biológico de contato, como o controle duplo de pragas e fitopatógenos; compatibilidade com outros inimigos naturais; menor exposição de propágulos às condições ambientais adversas, além de acelerar a emergência de sementes e o crescimento de plantas. Diante disso, os efeitos da inoculação de sementes usando dois isolados de *Metarhizium robertsii* e *Beauveria bassiana* foram avaliados na Universidade de Copenhagen, Dinamarca, na promoção de crescimento das plantas (biomassa e produção) e no crescimento populacional de *T. urticae* em um sistema modelo com plantas de feijão em casa-de-vegetação. Efeitos no comportamento alimentar de *P. persimilis* foram também estudados em condições de laboratório. No Brasil, estudos foram conduzidos na ESALQ/USP com plantas de morangueiro em casa-de-vegetação e em quatro áreas de produção comercial de morangueiro em Atibaia-SP e Senador Amaral-MG. Nos estudos em casa-de-vegetação, os efeitos de 15 isolados de *Metarhizium* spp., 5 de *B. bassiana* e 5 de *Cordyceps* (= *Isaria*) *fumosorosea* foram estudados, enquanto em área comercial um isolado de *Metarhizium* e *Beauveria* foram utilizados. Raízes de morangueiro foram inoculadas por imersão em suspensões fúngicas, e foram avaliados o crescimento populacional do ácaro rajado e o desenvolvimento das plantas, quantificando o comprimento de raiz, biomassa de raiz e de parte aérea, e massa de frutos de morango. Os resultados mostraram redução significativa na população de *T. urticae* e em geral melhor desenvolvimento das plantas nas duas culturas. A produção de vagens em plantas de feijão e de frutos de morango foram superiores nas plantas inoculadas em relação às não inoculadas. Não se observou diferenças na taxa de predação e comportamento alimentar do ácaro predador *P. persimilis* quando oferecidos *T. urticae* provenientes de plantas inoculadas e não inoculadas. Em campo foram observadas populações significativamente menores de *T. urticae* e menos sintomas de doenças nas plantas inoculadas com os fungos, comparado às plantas não inoculadas. Os resultados obtidos por este projeto trazem uma nova perspectiva do uso de *Metarhizium e Beauveria* como agentes protetores de plantas revelando que a utilização de fungos entomopatogênicos como inoculantes pode ser uma estratégia promissora.

Palavras-chave: Interações fungo-planta; *Phaseoulus vulgaris*; Morangueiro; Controle microbiano; *Tetranychus urticae*; Manejo Integrado de Pragas (MIP)

ABSTRACT

Effects of entomopathogenic fungi used as plant inoculants on plant growth and pest control

Entomopathogenic fungi (EPF) of the genera *Metarhizium* and *Beauveria* are able to endophytically colonize a wide variety of plant species, providing protection against arthropod pests; besides increasing the plant development; and act as phytopathogen antagonists. The main objective of the present project was to evaluate the potential of entomopathogenic fungi as plant inoculants against the two-spotted spider mite *Tetranychus urticae* and the effects on plant growth promotion. Tritrophic effects were also studied, by evaluating prey consumption and feeding behavior of the predatory mite *Phytoseiulus persimilis*. The evaluated strategy has several potential benefits compared to the sole use of EPF as contact biocontrol agents, as it may control both pests and phytopathogens; be compatible with other natural enemies; provide limited exposure of fungal propagules to adverse environmental conditions, and accelerate seed emergence and plant growth. Considering this, the effects of seed inoculation using two isolates of *Metarhizium robertsii* and *Beauveria bassiana* were evaluated at University of Copenhagen, Denmark, on plant development (i.e. biomass and yield) and *T. urticae* population growth in a model system with bean plants under greenhouse conditions. Effects on feeding performance of *P. persimilis* were also studied in laboratory conditions. In Brazil, inoculation studies with EPF were conducted at ESALQ/USP with strawberry plants in greenhouse conditions and in the field in four commercial production areas of strawberries in Atibaia-SP and Senador Amaral-MG. In greenhouse studies, the effects of 15 isolates of *Metarhizium* spp., 5 isolates of *B. bassiana* and 5 of *Cordyceps* (= *Isaria*) *fumosorosea* were studied, whereas in the commercial area one isolate of *Metarhizium* and *Beauveria* was used. Strawberry roots were inoculated by submersion in fungal suspensions, and the population growth of spider mites, while plants development was assessed by measuring root lengths, biomass of roots and leaves, and the strawberry fruit weight. The results showed a significant reduction in *T. urticae* population and in general better plant development in both crops. The production of string beans and strawberry fruits were higher in inoculated plants than in non-inoculated plants. There was no difference in predation rate and feeding behavior of the predator mite *P. persimilis* towards *T. urticae* from fungal inoculated and uninoculated plants. In the commercial strawberry production areas there were significantly lower populations of *T. urticae* and fewer symptoms of plant diseases on plants in the fungal treated beds compared to plants in untreated beds. The results of this project bring a new perspective on the use of *Metarhizium* and *Beauveria* as plant protecting agents revealing that the use of entomopathogenic fungi as plant inoculants may be a promising strategy.

Keywords: Fungus-plant interactions; *Phaseoulus vulgaris*; Strawberry; Microbial control; *Tetranychus urticae*; Integrated Pest Management (IPM).

RESUME

Effekter på afgrødevækst og skadedyrsbekæmpelse ved planteinokulering af insektpatogene svampe

Insektpatogene svampe indenfor slægterne *Metarhizium* og *Beauveria* kan kolonisere en række forskellige plantearter som endofytter, hvilket kan føre til forbedret beskyttelse mod skadedyr og sygdomme samt øgning af den inokulerede plantes vækst. Det primære formål med dette ph.d. projekt var at vurdere potentialet af at inokulere udvalgte afgrøder med insektpatogene svampe som bekæmpelsesstrategi mod spindemider *Tetranychus urticae* og som vækstfremmer. Tre-trofiske effekter blev også vurderet ved at undersøge byttefangst og søgeadfærd for rovmiden *Phytoseiulus persimilis*. Den anvendte strategi er fordelagtig i forhold til den traditionelle brug af insektpatogene svampe i biologisk bekæmpelse ved kontakt og infektion, da den har potentiale for at bekæmpe både skadedyr og sygdomme samtidig med at øge plantevæksten. Desuden kan strategien være kompatibel med brug af andre naturlige fjender, og de anvendte svampesporer vil være mindre eksponeret for skadelige miljøfaktorer. På denne baggrund blev først inokulering af bønnefrø med to isolater af svampene *Metarhizium robertsii* og *Beauveria bassiana* testet ved Københavns Universitet. Effekter på plantevækst og udbytte samt på populationsvækst af spindemider blev vurderet i væksthusforsøg. Desuden blev prædationsforsøg gennemført i laboratoriet med *P. persimilis* på materiale fra svampeinokulerede planter. Ved University of São Paulo blev rodinokulering med 25 insektpatogene svampeisolater testet på jordbærplanter i væksthus; 15 isolater af *Metarhizium* spp., 5 isolater af *B. bassiana* og 5 isolater af *Cordyceps* (= *Isaria*) *fumosorosea*. To af disse isolater blev også testet i kommercielle jordbærmarker. Jordbærplanternes rodsystemer blev inokuleret ved nedsænkning i sporesuspension og udplantet. Plantevækst og æglægning af spindemider blev undersøgt i væksthusforsøget, mens skadedyrangreb, populationsstørrelser af rovmider og forekomst af plantesygdomme blev undersøgt i feltforsøget. Resultaterne viste en signifikant reduktion af spindemidepopulationer og æglægning på svampeinokulerede planter både i bønne og jordbær. Produktionen af bønner og jordbær var højere på de svampeinokulerede planter. Rovmiden *P. persimilis* udviste ingen forskel i prædation på spindemider fra bønneplanter, som var svampeinokulerede og kontrolbehandlede. I feltforsøgene var der signifikant færre spindemider og mindre forekomst af plantesygdomme på jordbærplanterne i de svampebehandlede parceller i forhold til planter i kontrolparceller, mens populationerne af rovmider ikke var påvirket af svampebehandlingen. Resultaterne af dette ph.d. projekt peger i en ny retning for anvendelse af insektpatogene svampe til både bekæmpelse af skadegørere og forstærket afgrødevækst ved rodinokulering, som samtidig tyder på at være kompatibel med brug af andre naturlige fjender.

Nøgleord: Svampe-plante interaktioner; *Phaseoulus vulgaris*; Jordbær; Mikrobiologisk bekæmpelse; *Tetranychus urticae*; Integrated Pest Management (IPM)

1. INTRODUCTION

1.1. Strawberry crop

Strawberries are a popular commodity throughout the world whose production was approximately 9.2 million tons in 2016, with a yield of 22.690 kg/ha (FAOSTAT, 2018). China is the greatest producer whose production in 2016 was around 3.8 million tons. Brazil produced more than 3 thousand tons with a yield of 8.396 kg/ha (FAOSTAT, 2018).

The strawberry genotype currently cultivated is *Fragaria* x *ananassa* Duchesne (Rosales: Rosacea), originated from inbreeding between *Fragaria virginiana* Mill (from North America) and *Fragaria chiloensis* (Linnaeus) Duchesne (from Chile) (Hancock, 1990). Strawberry production has been widely exploited as a promising, growing and quite profitable market. Globally, strawberries are one of the grown fruits most widely distributed, due to their genotypic diversity and high environmental adaptability (Larson, 1994).

Nevertheless, strawberries have a huge complex of arthropod pests and plant diseases which limit yields and result in production losses (Solomon et al., 2001; Coll et al., 2007; Wilson and Tisdell, 2001). The main strawberry pest control strategy is still through the use of chemical pesticides which can lead to the development of resistance and impacts on populations of natural enemies; besides it may cause environmental contamination and the presence of toxic residues on fruits (Cavalcanti et al., 2010; Attia et al., 2013). It is therefore becoming increasingly important to develop innovative strategies that can be adopted in integrated pest management (IPM) to reduce the use of chemical pesticides.

1.2. Strawberry pests

The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) is considered one of the most important species of mite pests throughout the world, responsible for damage to more than 150 economically important host plants (Jeppson et al., 1975; De Moraes and Flechtmann, 2008); besides it is one of the main pests of strawberries worldwide (Klingen and Westrum, 2007). The spider mite feeding occurs mainly on the lower surface of leaves, which can reduce photosynthetic activity and

lead to an injection of phytotoxic substances (Attia et al., 2013), decreasing foliar and floral development, besides reducing the quality and quantity of fruits (Rhodes et al., 2006). The spider mites have a high capacity for population increase; the life cycle takes only 8 ± 12 days at 30ºC (Wermelinger et al., 1990). Considering that each female can lay an average of 90-110 eggs, the number of spider mites can increase very rapidly during the summer, with several generations in a year (Solomon et al., 2001).

Other mites are associated with strawberry crops in several countries and one species that has been currently considered harmful to strawberries is the cyclamen mite *Phytonemus pallidus* (Banks) (Acari: Tarsonemidae) (Ajila et al., 2018). The cyclamen mite infests young leaves, flowers and fruits, leading to a reduction in petioles development and, consequently, the fruits are reduced in size, and become brown or brittle and unfeasible for harvest (Smith and Goldsmith, 1936; Croft et al., 1998; Easterbrook et al., 2001; Tuovinen and Lindqvist, 2010).

Another serious pest of strawberries is the western flower thrips, *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) where damages by nymphs and adults feeding result in flower abortion, fruit bronzing, and fruit malformation causing therefore yield loss (Coll et al., 2007). The following species are also considered important insect pests of strawberries; the moths *Spodoptera* spp., *Helicoverpa* spp. (Lepidoptera: Noctuidae) and *Duponchelia fovealis* Zeller (Lepidoptera: Crambidae)*;* the beetle *Lobiopa insularis* Castelnau (Coleoptera: Nitidulidae)*;* the aphids *Chaetosiphon fragaefolli* Cockerell and *Aphis forbesi* Weed (Hemiptera: Aphididae) (Bernardi et al., 2015). The species *Neopamera bilobata* Say (Hemiptera: Rhyparochromidae) and the spotted wing drosophila, *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) have recently invaded and caused economic losses in the production of many strawberry fields in Brazil (Kuhn, 2014; Andreazza et al., 2016).

The high incidence of diseases is another problem faced by strawberry farmers, which can occur at various stages of the crop cycle, from the newly planted seedlings to the fruits at the final production stage, emphasizing the fungal diseases as the most important (Garrido et al., 2011).

1.3. Control strategies of strawberry pests

The main control strategy of strawberry pests is still through the use of chemical pesticides, mainly in conventional cropping systems (Van Leeuwen et al., 2015). The main active ingredients recently used in Brazil to control the major strawberry pests are pyrethroid, avermectin and tetranortriterpenoid (AGROFIT, 2018). The intensive use of these products in strawberry production led to the selection of resistant pest populations, mainly in spider mites, which has concerned the producers (Sato et al., 2005). Besides, the frequent use of chemical pesticides may also cause impacts on natural enemy populations, environmental contamination and the presence of toxic residues on fruits (Cavalcanti et al., 2010; Attia et al., 2013).

Biological control agents have been considered as a sustainable alternative to synthetic chemical pesticides. Eilenberg et al. (2001) defined biological control as “the use of living organisms to suppress the population density of a specific pest organism, making it less abundant or less damaging than it would otherwise be”. The commercially available biocontrol agents in Brazil recommended to be used in strawberries are predatory mites of the species *Neoseiulus californicus* (McGregor) and *Phytoseiulus macropilis* (Banks), both (Acari: Phytoseiidae), and the entomopathogenic fungus *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Hypocreales: Cordycipitaceae). These three biocontrol agents have already been used in organic farming systems of strawberry, mainly against the two-spotted spider mite.

Eilenberg and Hokkanen (2006) considered microbial control as the most viable alternative to chemicals such as entomopathogenic fungi, mainly Ascomycota species, known to have a wide host range (Castro et al., 2016). Around 80% of the diseases that occur in insects are caused by entomopathogenic fungi, which belong to about 90 genera and more than 700 species (Alves, 1998). In Brazil, more than 20 fungal genera naturally occur as infections in economically important insect pests and the most relevant are *Metarhizium*, *Beauveria*, *Cordyceps* (= *Isaria*), *Akanthomyces* (= *Lecanicillium*), *Aschersonia* and *Hirsutella* (Alves, 1998; Shah and Pell, 2003). Hence, entomopathogenic fungi are promising candidates that may be adopted in IPM programs to decrease the use of chemical pesticides.

1.4. Microbial control with entomopathogenic fungi

Entomopathogenic fungi enter their insect or arachnid hosts directly through the cuticle or by natural openings (Shah and Pell, 2003; Goettel et al., 2005; Charnley and Collins, 2007). The beginning of the invasion occurs with the adhesion of conidia or specialized fungal structures to the host cuticle; followed by germination, penetration into the host, modulation of cellular and humoral defenses, and fungal growth inside the hemocoel. Then, the death of the host is caused by nutrient depletion, invasion of tissues and organs, and asphyxia due to the development of the fungus in the respiratory system and/or the production of toxic metabolites. The sporulation of the fungus completes the life cycle, which occurs when hyphae emerge from the cadaver and produce conidiophores and conidia, allowing horizontal transmission (Alves, 1998; Goettel et al., 2005; Charnley and Collins, 2007).

Most of the commercially produced entomopathogenic fungi are primarily hypocrealean Ascomycetes, including species of *Beauveria*, *Metarhizium*, *Cordyceps*, *Akanthomyces* and *Hirsutella,* which are easily mass produced on artificial media (Faria and Wraight, 2007). These fungi are considered promising microbial control agents for implementation in IPM programs, however there are some aspects that limit their use, such as the non-consistent control effect of pests and the survival of the fungal propagules in the environment (Hajek and Delalibera, 2010). These aspects are greatly influenced by abiotic factors as temperature, UV light intensity, humidity and rainfall (Meyling and Eilenberg, 2007; Castro et al., 2013); and by biotic factors represented by the multitrophic interactions among plants, invertebrates and other microorganisms (Meyling and Eilenberg, 2007; Meyling and Hajek, 2010; Quesada-Moraga et al., 2014). Hence, it is important to understand these interactions, in order to optimize pest control by using entomopathogenic fungi.

1.5. Entomopathogenic fungi as plant associates and endophytes

Several studies have recently shown that entomopathogenic fungi may play additional roles beyond entomopathogenicity in terrestrial ecosystems by associating with plants, e.g. as endophytes (reviewed in Vega, 2008, 2018; Vega et al., 2009). Endophytes are considered to be fungi or bacteria that colonize inner parts of plant tissues without causing negative effects to their hosts (Carroll, 1988; Stone et al., 2004;

Sikora et al., 2007; Vega, 2008). The endophyte-host interaction can provide several advantages both to the microorganism, which benefits from protection, feeding and transmission in the plant, and also to the plant, which benefits from the growth promotion, reproduction and resistance to environmental changes (Saikkonen et al., 2004). The transmission of endophytic microorganisms can occur vertically through hyphae that grow in seeds (Saikkonen et al., 1998) and, after germinating, colonize the emerging plant, and horizontally from the surrounding environment by penetrating through openings, such as stomata, regions of roots emission and wounds (Hallmann et al., 1997). In addition, some endophytes are able to enter the plant tissue through the secretion of hydrolytic enzymes; others have specialized structures such as haustoria and apressoria, and some of them can directly cross the cell wall (Stone, 1987; Stone et al., 1994).

The fungal genera *Metarhizium* (Hypocreales: Clavicipitaceae) and *Beauveria* (Hypocreales: Cordycipitaceae) are considered as both entomopathogens and symbionts; they are able to cause mortality of economically important arthropod pests, and also colonize a wide variety of plant species (Vega, 2008, 2018; Ownley et al., 2010), leading to increased plant growth (Sasan and Bidochka, 2012; Jaber and Enkerli, 2016, 2017; Tall and Meyling, 2018), and protection of plants against pests and phythopathogens (Ownley et al., 2010; Jaber and Alananbeh, 2018; Jaber and Ownley, 2018). Several *Cordyceps* spp. (= *Isaria* spp.) have already been isolated as endophytes, but there is still limited knowledge about the establishment of species within this genus as endophytes and possible effects on plant growth and against herbivorous (Bills and Polishook, 1991; Giordano et al., 2009; Vega, 2008, 2018).

Although the mechanisms related to the negative effects caused by entomopathogenic fungi as endophytes still remain largely unknown, it has been suggested that they result from compounds produced by the plant or by the associated fungus (Vidal and Jaber, 2015; McKinnon et al., 2017). In the beginning of the invasion process, the endophytic fungi are recognized by the plant as potential invaders causing the plant to trigger immune responses and, consequently, synthesize specific regulatory elements, such as transcription factors which are related to resistance against herbivores (Brotman et al., 2013; McKinnon et al., 2017). Secondary plant metabolites have also been considered, such as terpenoids, which have anti-herbivore properties (Gershenzon and Croteau, 1991; Fürstenberg-Hägg et al., 2013; Vega, 2018). Another possible mechanism against herbivores is the production of fungal

secondary metabolites *in planta* (McKinnon et al., 2017; Jaber and Ownley, 2018), because entomopathogenic fungi are a primary source of bioactive secondary metabolites with antimicrobial, insecticidal and cytotoxic activities (Gibson et al., 2014). For instance, *B. bassiana* produces several insecticidal metabolites such as beauvericin, bassianolides, bassiacridin, bassianin, beauverolides, oosporein, bassianolone and others (reviewed in Ownley et al., 2010; Jaber and Ownley, 2018). Species within *Metarhizium* also produce insecticidal metabolites, such as destruxins and cytochalasins (Roberts, 1981). These toxins can be dissipated throughout the host plant, which allows the control of insects even without the presence of the endophyte in the attacked area, causing repellency; inducing weight loss, decreasing growth, development, and consequently, increasing the rate of pest mortality (Azevedo et al., 2000).

Regarding the effects of entomopathogenic fungi as endophytes on plant pathogens, few studies have been conducted compared to the effects on arthropod pests (Jaber and Alananbeh, 2018), but it is suggested that the mechanisms could also be related to the production of secondary metabolites by the associated fungus, i.e., antibiosis (Vidal and Jaber, 2015; McKinnon et al., 2017; Jaber and Alananbeh, 2018); or induced systemic resistance of plants (Brotman et al., 2013; McKinnon et al., 2017). Further, the plant pathogens can also be exposed to competition for space and nutrients with the endophyte inside the shared host plant (Jaber and Alananbeh, 2018; Jaber and Ownley, 2018).

In addition, the ability of plant associated entomopathogenic fungi to promote plant growth has been related to the production of plant growth regulators by the fungi, for example, in a recent study it was shown that an isolate of *Metarhizium robertsii* Bisch., Rehner & Humber produces the plant growth regulator indole-3-acetic acid (IAA; an auxin), which promoted root growth in *Arabidopsis*, suggesting the importance of auxins in the ability of *M. robertsii* to stimulate plant growth (Liao et al., 2017). In the same study, it was also recorded that isolates of *Metarhizium anisopliae* (Metchinikoff) Sorokin, *Metarhizium brunneum* Petch and *B. bassiana* also produced IAA (Liao et al., 2017). Another insight is related to the involvement of entomopathogenic fungi in the transfer of nitrogen to plants (Vega, 2018), which was reported by Behie et al. (2012) and Behie and Bidochka (2014), whose studies showed that *Metarhizium* spp. may transfer insect-derived nitrogen to their plant hosts from a soil-borne insect, via fungal hyphae in an endophytic association, providing an evidence of a specific mechanism

that can promote plant growth. In exchange, the host plant provides photosynthetically fixed carbon to root-colonized *Metarhizium*, which increases the overall stability of this partnership (Behie et al., 2017).

1.6. The genus *Metarhizium*

Species of *Metarhizium* are entomopathogenic fungi with a cosmopolitan distribution (Jaronski, 2007), and they can be the most abundant entomopathogenic fungi in agricultural soils (Bidochka et al.,1998). In Brazil, the following species have been reported in different habitats: *M. anisopliae*, *M. robertsii*, *M. brunneum*, *M. acridum*, *M. pingshaense*, *M. lepidiotae*, *M. pemphigi*, *M. majus*, *M. blattodeae*, *M. flavoviride*, *M. brasiliense*, *Metarhizium* (= *Nomuraea*) *rileyi*, the new species *M. alvesii* (Lopes et al., 2018) and five indetermined species: *Metarhizium* sp. indet. 1, *Metarhizium* sp. indet. 2, *Metarhizium* sp. indet. 3, *Metarhizium* sp. indet. 4, *Metarhizium* sp. indet. 5 (Rocha et al., 2009, 2013; Lopes et al., 2013a, 2013b, 2014; Rezende, 2014; Rezende et al., 2015; Zanardo, 2015; Iwanicki, 2016; Castro, 2016; Lopes et al., 2018).

Metarhizium spp. can infect more than 200 species of insects and arachnids (Roberts and Hajek, 1992), besides acting as plant associates mainly by the colonization of the rhizosphere (Behie et al., 2012), or as soil saprophytes (Meyling and Eilenberg, 2007). The adhesion to insect and plant surfaces is related to the expression of two different proteins, MAD1 (*Metarhizium* Adhesin-protein 1) and MAD 2 (*Metarhizium* Adhesin-protein 2), which have been identified as being differently induced by insect cuticle and plant root exudate, respectively (Wang and St Leger, 2007; Wyrebek et al., 2013).

Furthermore, *Metarhizium* spp. are able to transfer nitrogen from infected insects in the soil to plants via mycelium in a tritrophic association between host insect, fungus and plant in the rhizosphere (Behie et al., 2012; Behie and Bidochka, 2013, 2014), resulting in an increase in the overall plant productivity. Besides, several studies have already shown successful experimental plant inoculations with *M. anisopliae*, *M. brunneum*, *M. robertsii* and other species (reviewed by Vega, 2018), with fungal establishment in different plant species (Sasan and Bidochka, 2012; Batta, 2013).

1.7. The genus *Beauveria*

The genus *Beauveria* presents various entomopathogenic species, with *B. bassiana* being the most notable (Zimmermann, 2007). The species *B. bassiana* has a worldwide distribution, and it has been found on infected insects from most orders both in temperate and tropical areas throughout the world (Zimmermann, 2007). In addition to being an entomopathogen, several strains of *B. bassiana* have been reported to colonize plant tissues and to become endophytic (Bing and Lewis, 1992; Vega, 2008). This species has been experimentally established as an endophyte in many important crops, such as corn, potato, cotton, tomato, sorghum, palm, banana, cocoa, poppy, coffee, pine and sugarcane (Vega, 2008; Brownbridge et al., 2012; Donga et al., 2018). *B. bassiana* can establish as an endophyte within all plant tissues (Behie et al., 2015), and is often reported causing negative effects on pest populations in the crops (McKinnon et al., 2017). Also, besides causing negative effects on arthropod pests, *B. bassiana* as a plant inoculant has also been reported to improve plant growth (Jaber and Enkerli, 2016, 2017; Tall and Meyling, 2018) leading to higher yields (Lopez and Sword, 2015; Gathage et al., 2016; Jaber and Araj, 2018).

In addition, this species has shown potential in the protection of plants against phytopathogens (Ownley et al., 2008), since different *B. bassiana* isolates were observed inhibiting *in vitro* and *in vivo* mycelial growth of several soil and phytopathogenic diseases, including *Fusarium* spp., *Botrytis cinerea*, *Rhizoctonia solani*, and others (Bark et al., 1996; Lee et al., 1999; Ownley et al., 2004, 2008, 2010; Jaber and Alananbeh, 2018; Jaber and Ownley, 2018).

1.8. The genus *Cordyceps* (= *Isaria*)

Recently, as a result of a phylogenetic framework, Kepler et al. (2017) proposed the new name *Cordyceps* for *Isaria* and yet recommended the rejection of *Isaria* to avoid further splitting of *Cordyceps*, in order to resolve conflicts between competing names for sexually and asexually typified generic names.

The species *Cordyceps fumosorosea* [formerly *Isaria fumosorosea* (Kepler et al., 2017)] (Wize) Kepler, B. Shrestha & Spatafora, comb. nov. (Hypocreales: Cordycipitaceae) is globally distributed and is related to a wide variety of hosts, being considered an important microbial control agent (Zimmermann, 2008). Among the

hosts of this species are included mites, and insect species within the orders Diptera, Hymenoptera, Lepidoptera, Coleoptera, Neuroptera, Hemiptera, Isoptera and Thysanoptera (Zimmermann, 2008).

Species of *Cordyceps* have already been reported as endophytes from American hornbeam trees (*Carpinus caroliniana* Walter) (Bills and Polishook, 1991), *Pinus sylvestris* L. (Giordano et al., 2009), coffee (Vega et al., 2008), and bean *Phaseolus vulgaris* L. (Fabales: Fabaceae) (Dash et al., 2018). However, as previously stated there is still limited knowledge about the establishment of *C. fumosorosea* as an endophyte and its potential effects on plant growth and against arthropod herbivores.

1.9. Potential of entomopathogenic fungi as plant inoculants and current knowledge gaps

Several studies have shown successful experimental plant inoculations by species within *Metarhizium* and by *B. bassiana* in different plant species (e.g. Sasan and Bidochka, 2012; Batta, 2013; Bamisile et al., 2018). In most of the reported studies, isolates of both taxa are often reported to cause negative effects on pest populations (McKinnon et al., 2017) and also to improve plant growth (Sasan and Bidochka, 2012; Jaber and Enkerli, 2016, 2017; Tall and Meyling, 2018). However, there are still various research needs and knowledge gaps which must be full-filled for the successful implementation of entomopathogenic fungi as plant inoculants into outdoor IPM programs to become a feasible alternative.

For example, although results of inoculation of bean seeds with entomopathogenic fungi, as *B. bassiana*, have already been reported to cause negative effects on *T. urticae* population growth and reproduction, besides causing increased bean plant growth and biomass (Dash et al., 2018), there are so far no reports of evaluation of plant inoculations with *Metarhizium* spp. towards *T. urticae*. Besides, we are also interested in prospecting our various indigenous isolates in order to hopefully develop a commercial product to be used as inoculant in Brazil. It is also important to highlight that the variability among entomopathogenic fungi isolates may cause different effects in both pest control and plant growth, but this aspect of inter- and intra-specific variability among fungal species has received limited attention.

Also, there is still limited knowledge of the combined use of beneficial fungi for plant protection. One of the only studies was the co-inoculation of wheat seeds with *M. brunneum* and the mycoparasitic fungus *Clonostachys rosea* (Link) Schroers et al. (Hypocreales: Bionectriaceae), for the protection of plants roots against both an insect and a plant pathogen (Keyser et al., 2016). Considering that *Metarhizium* and *Beauveria* usually exhibit differential localization in plant tissues, with *Metarhizium* spp. mainly being found in the root system and *B. bassiana* in all plant tissues (Behie et al., 2015), it is possible that any complimentary localization in crops could potentially provide additive effects against pests, representing an innovative strategy for incorporation in IPM programs aiming to control both below- and above-ground pests and hopefully improve plant growth and pest control to higher extent than the single fungal species.

The effects of entomopathogenic fungi as inoculants on arthropod natural enemies remains little explored, and current studies have mainly focused on effects on parasitoid species (Bixby-Brosi and Potter, 2012; Akutse et al., 2014; Jaber and Araj, 2018), with no studies reporting the effects on predators, including predatory mites (e.g. Seiedy et al., 2013; Dogan et al., 2017). Considering that all this knowledge is relevant to create a robust plant protection strategy, the current PhD research focused on investigating these overall questions.

The present research is based on evaluation of the indirect effects, i.e. plant-mediated by linking below-ground inoculation with above-ground protection, without evaluation of the entomopathogenic fungi for their direct pest infection capacity. The research experiments were conducted both in a model system of bean with seed treatment; and using root inoculation of strawberry plants, because this is an economically meaningful crop in both countries where the studies were carried out, Brazil and Denmark. Also, the inoculation of strawberry plants with entomopathogenic fungi has been reported just in temperate regions and these studies aimed at controlling soil-borne insect pests directly (Ansari and Butt, 2013; Klingen et al., 2015). No studies have evaluated the indirect effects in strawberries of fungal inoculations against *T. urticae*, particularly in the subtropical regions. Considering that there is a wide diversity of naturally occurring entomopathogenic fungal species in Brazil and the unexploited resource for plant protection that these local isolates represent, we investigated in our second study, the effects of strawberry root inoculations of a wide selection of 25 indigenous Brazilian isolates of *M. anisopliae*, *M. robertsii*, three

taxonomically unassigned lineages of *Metarhizium*, *B. bassiana* and *C. fumosorosea* on spider mite *T. urticae* oviposition and plant growth in greenhouse.

Furthermore, many of the studies reported in the literature have included sterile and highly controlled systems, i.e., sterilized seeds and soil, and in the current studies it was prioritized to replicate the natural conditions, in order to investigate how the inoculations could be feasible to practically apply the fungi to crops. In addition, most of the published studies were performed under controlled experimental conditions, and few studies have investigated the pest control potential of entomopathogenic fungi as inoculants under field conditions, and no field studies have evaluated effects against plant pathogens (Jaber and Ownley, 2018). For this reason, a focus was on evaluating the potential of the two selected isolates of *M. robertsii* and *B. bassiana*, which showed good results in the model system with bean plants and with strawberry plants in greenhouse, now as root inoculants of strawberry plants for above-ground pest management under field conditions.

1.10. Objectives and hypotheses

Considering the importance of developing strategies that increase the crop production with minimal environmental impact by inclusion of alternative control methods by farmers such as the use of biological control agents, the overall aim of this research was therefore to evaluate selected isolates of entomopathogenic fungi as inoculants in two crop plants for effects on pest control and plant productivity. The research focused on inoculation of fungi in the early stage of plant development in bean and strawberry by seed treatment and root dipping, respectively, and endpoint measurements were taken with focus on *T. urticae* population parameters while final plant biomass and yield were evaluated. Further, in one study the potential effects of the fungal inoculation on feeding behavior of predatory mites were assessed.

Thus, the first study of this thesis aimed to evaluate seed inoculations by two Brazilian isolates of *M. robertsii* and *B. bassiana* individually and in combinations in bean plants, *P. vulgaris*, as a model system, for evaluation of the effects on plant growth and spider mite populations. Besides, potential effects on the predator mite *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) towards spider mites from inoculated plants were also investigated. The hypotheses of this study were therefore:

I) spider mite population growth will be inhibited on fungal inoculated plants compared to control plants; II) plants inoculated with both *M. robertsii* and *B. bassiana* isolates individually and in combination will enhance the bean plant growth when compared to control plants; III) inoculation with the *M. robertsii* and *B. bassiana* isolates in combination on the same plant improves the plant growth and reduces the spider mite populations to higher extend than on plants inoculated with only a single fungal isolate; and IV) predatory mite predation rates on spider mites are unaffected by whether leaf substrate and spider mite originated from inoculated plants or from control plants.

In the second study, the objective was to evaluate the variability among Brazilian entomopathogenic fungal isolates in the potential as plant inoculants. This was done by testing root inoculations of strawberry plants with 25 indigenous Brazilian isolates representing *M. anisopliae*, *M. robertsii*, three taxonomically unassigned lineages of *Metarhizium*, as well as isolates of *B. bassiana* and *C. fumosorosea.* Response variables were spider mite *T. urticae* oviposition levels, plant growth and fruit yield assessed in greenhouse conditions. The hypotheses of this study were: I) spider mite oviposition will be reduced on fungal inoculated plants compared to control plants; II) the strawberry plants growth and yield will be enhanced on plants inoculated with the fungal isolates compared to control plants, but for both hypotheses it was expected that the responses would be variable depending on fungal isolate.

The third study aimed at extending the knowledge on the use of entomopathogenic fungi as root inoculants of strawberry plants and their effects on pests in field conditions. The objective was to evaluate the potential of two selected isolates of *M. robertsii* and *B. bassiana* as root inoculants of strawberry plants for above-ground pest management in four strawberry commercial fields, during two seasons in Brazil. The main hypothesis was that the inoculation would provide long-term control of *T. urticae* populations under field conditions without detrimental effects on natural predatory mite populations. In addition, it was expected that additional benefits would be observed on occurrence of selected insect pests and important strawberry foliar pathogens.

1.11. Obtained results and future perspectives

In the first study (Manuscript 1), bean plants inoculated with both *M. robertsii* isolate ESALQ 1622 and *B. bassiana* isolate ESALQ 3375 reduced *T. urticae* population growth. The inoculation of *M. robertsii* and *B. bassiana* isolates in combination on the same plant also reduced spider mite populations and improved plant growth as compared to control plants, but not to higher extend than plants inoculated with only a single fungal isolate. Although the experiments with the predatory mite *P. persimilis* were limited in scale, the data indicated that the feeding capacity and behavior on spider mites reared on fungal inoculated and control plants was similar. It can therefore be concluded that the *M. robertsii* and *B. bassiana* isolates used as bean seed inoculants are potential candidates for biological plant protection above-ground, with no short-term negative effects on feeding capacity of predators. As previously stated, Dash et al. (2018) also showed negative effects on *T. urticae* population growth and reproduction on bean plants whose seeds were inoculated with three isolates of *B. bassiana* (B12, B13, B16), and isolates of *C. fumosorosea* (isolate 17) and *Akanthomyces* (= *Lecanicillium*) *lecanii* (isolate L1), when compared to non-inoculated plants. They reported a significant reduction in spider mites development, adult longevity, female fecundity and increased bean plant heights and biomass, when reared on *B. bassiana* inoculated plants. However, this is the first study to report the effects of plant inoculations with *Metarhizium* spp. on *T. urticae*, the effects of the combined use of two different entomopathogenic fungi species in a same plant on pest control and plant growth, and also assessing the effects of entomopathogenic fungi as inoculants on a predatory mite species.

The second study (Manuscript 2) showed that inoculating roots of strawberry plants before planting with most of the 25 individual fungal isolates from *Metarhizium* spp., *B. bassiana* and *C. fumosorosea* reduced *T. urticae* oviposition. Also, the inoculations with some isolates of *Metarhizium* spp., *B. bassiana* and *C. fumosorosea* increased dry weight of roots and aerial plant part when compared to control non-inoculated plants. Eight of the 25 tested isolates provided higher strawberry yield, highlighting the importance to evaluate several different isolates in order to be able to select promising candidates, once the existing genetic variability among species and isolates can interfere in the efficacy, virulence and persistence in the environment of

an introduced isolate for biological control (Alves, 1998). It is though the first demonstration that root inoculations with entomopathogenic fungi can promote strawberry yield which is eventually a more important endpoint than simply biomass increase. This study is also the first to report negative effects of different species and isolates of entomopathogenic fungi inoculated by dipping roots in strawberries on spider mite oviposition. Indeed, most of the previous studies reported effects of plant inoculated entomopathogenic fungi on insects, belonging to the orders Lepidoptera, Hemiptera, Coleoptera, Diptera, Hymenoptera, Thysanoptera and Orthoptera, in descending order (Vega, 2018), while a single recent study focused on spider mites (Dash et al., 2018) without inclusion of *Metarhizium* spp. as inoculants.

In the field study (Manuscript 3), inoculation of strawberry roots with the isolates *M. robertsii* ESALQ 1622 and *B. bassiana* ESALQ 3375 resulted in reduced populations of *T. urticae* adults compared to non-inoculated plants over 180 days. The present results also showed that the fungal inoculated strawberry plants had reduced proportions of leaf damage by Coleopteran pests, while no effects on whiteflies and thrips in flowers were observed. Few studies have investigated the potential of plant inoculated entomopathogenic fungi as microbial control agents under natural field conditions (reviewed by Jaber and Ownley, 2018; Vega, 2018). For instance, field studies have been reported on the negative effects of the inoculation in bean plants, *P. vulgaris,* with *B. bassiana* against *Liriomyza* leafminers (Diptera: Agromyzidae) (Gathage et al., 2016); in *Sorghum bicolor* L. (Moench) (Poales: Poaceae) colonized by *B. bassiana, M. robertsii*, and *C. fumosorosea* against larvae of *Sesamia nonagrioides* (Lefebre) (Lepidoptera: Noctuidae) (Mantzoukas et al., 2015); and in cotton *Gossypium* spp. (Malvales: Malvaceae) after seeds have been treated with *B. bassiana* against *Aphis gossypii* Glover (Homoptera: Aphididae) (Castillo-Lopez et al., 2014).

Besides, the present research also demonstrated a significant reduction in the prevalence of the foliar plant pathogenic fungi *Mycosphaerella fragariae* and *Pestalotia longisetula* in strawberry plants inoculated with the *B. bassiana* and *M. robertsii* isolates. According to Jaber and Alananbeh (2018), there are few reports on the effects of entomopathogenic fungi as inoculants affecting plant pathogens and so far, no field studies have been carried out.

Further, populations of the predatory mite *N*. *californicus*, were not negatively affected by the fungal inoculations over the 180-day assessment period, and no adverse non-target effects should therefore be expected using this strategy. As previously mentioned, the published studies on effects of entomopathogenic fungi as inoculants on arthropod natural enemies have mainly focused on parasitoid species with no indication of negative effects. For example, according to Akutse et al. (2014), no differences were observed in the parasitism rates by two parasitoids of the leafminer *Liriomyza huidobrensis* Blanchard (Diptera: Agromyzidae) kept in bean plants (*Vicia faba* L. and *P. vulgaris*) whose seeds were inoculated with the endophytic fungi *Hypocrea lixii* Patouillard (syn. *Trichoderma lixii*) *and B. bassiana* under laboratory conditions. The compatibility between isolates of *B. bassiana* and *M. brunneum* as inoculants of sweet pepper plants and the aphid endoparasitoid *Aphidius colemani* Viereck (Hymenoptera: Braconidae) for *Myzus persicae* Sulzer (Homoptera: Aphididae) suppression under controlled greenhouse conditions was reported by Jaber and Araj (2018). No differences were observed in development time, percentage female, adult longevity and parasitism of *A. colemani* progenies among inoculated and control plants (Jaber and Araj, 2018). Finally, this is the first study to report the potential of plant inoculated entomopathogenic fungi on pests, natural enemies and plant diseases in strawberries under commercial cultivation regimes.

In the three studies, most of the isolates were able to colonize bean and strawberry plants, but to variable degrees. The isolates of *Metarhizium* spp. were mainly recovered from roots and soil, while the isolates of *B. bassiana* were mostly found in the leaves. Four of the five evaluated isolates of *C. fumosorosea* were recovered from roots and leaves, and all of them colonized the soil samples. It has already been reported that *B. bassiana* is a more extensive colonizer of foliar tissues than *Metarhizium* spp., which are commonly found in the rhizosphere of various plants (Ownley et al., 2008; Quesada-Moraga et al., 2009; Akello and Sikora, 2012; Akutse et al., 2013; Behie et al., 2015; Jaber and Araj, 2018). It is important to emphasize that the colonization evaluations represent only accessory studies, given that the contribution of this work lies on reporting the effects of the bean seed and strawberry root inoculations on plant growth and spider mite control, and this effect may not be directly linked with establishment of the fungi as endophytes, but can also be caused by systemic mechanisms as outlined in the item 1.5. This thesis advances the current scientific knowledge as it brings a new perspective on the use of entomopathogenic

fungi for increasing plant health in bean and strawberry production, with identification of promising isolates for increasing crop yield and for spider mite control as a first step, revealing that the use of entomopathogenic fungi as seed and root inoculants may be promising methods for future plant protection. Besides, the inoculation of bean and strawberry plants with entomopathogenic fungi through seed and root dipping, respectively, may be used in combination with predatory mites for control of *T. urticae* as part of an innovative biological control strategy to be implemented in IPM and organic production with additional effects against other insect pests and foliage diseases.

Meanwhile, there are several research questions that should be addressed in further studies, including:

- Studies to test the compatibility between the use of entomopathogenic fungi as inoculants and predatory mites in the whole plant for combined spider mite control;
- Laboratory and field studies for in depth understanding of the antagonism towards plant pathogens caused by entomopathogenic fungi as inoculants;
- Further studies to understand the mechanisms responsible for the negative effects caused by entomopathogenic fungi as plant inoculants on arthropod pests and plant growth promotion;
- Studies considering the context dependency, i.e. how the inoculation effects may be influenced by abiotic factors, such as the temperature, relative humidity, UV radiation, type of soil/substrate;
- Establish the implementation of this strategy in production systems and in IPM programs, and ensure the efficacy through the development of formulations, appropriate application technology, and extension to and training of the producers, in order to benefit the most from the potential of the entomopathogenic fungi as plant inoculants.

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2. EFFECTS OF BEAN SEED TREATMENT BY THE ENTOMOPATHOGENIC FUNGI *Metarhizium robertsii* AND *Beauveria bassiana* ON PLANT GROWTH, SPIDER MITE POPULATIONS AND BEHAVIOR OF PREDATORY MITES

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Abstract

The fungal genera *Metarhizium* and *Beauveria* are considered as both entomopathogens and endophytes; they are able to colonize a wide variety of plants and can cause increased plant growth and protect plants against pests. In view of the need for new biological methods for plant protection and how promising and little studied candidates entomopathogens are, the aim of this research was to evaluate the potential of two isolates of *Metarhizium robertsii* (ESALQ 1622) and *Beauveria bassiana* (ESALQ 3375) to suppress spider mite *Tetranychus urticae* population growth and ability to promote growth of bean plants *Phaseolus vulgaris* after seed treatment, in order to develop an innovative strategy by using these fungi as inoculants to improve both spider mites control and plant growth and yield. In addition, behavioral responses and predation rates of the predatory mite *Phytoseiulus persimilis* towards fungal treated plants and spider mites from these plants were also evaluated in leaf disc assays to assess potential conflicting effects of the fungal inoculations on overall pest control at higher trophic levels. Seed inoculations by the two isolates of *M. robertsii* and *B. bassiana* were done individually and in combinations to evaluate potential benefits of co-inoculants. The results showed a significant reduction in *T. urticae* populations and improved plant development when inoculated with *M. robertsii* and *B. bassiana* individually and in combination. The predatory mite *P. persimilis* showed no difference in the predation rate on *T. urticae* from treated and untreated plants even though the predators were most likely to feed on spider mites from fungal treated plants during the first half of the trial, and on spider mites from control plants during the remainder of the trial. Overall, the two fungal isolates have potential as seed inoculants to suppress spider mites in bean and the strategy appears to have no conflict with use of predatory mites. Co-inoculation of both fungal isolates showed no additional benefits compared to single isolate applications under the given test conditions.

Keywords: Endophytes; Biological control; *Tetranychus urticae*; Plant growth; *Phytoseiulus persimilis*

2.1. Introduction

The fungal genera *Metarhizium* (Hypocreales: Clavicipitaceae) and *Beauveria* (Hypocreales: Cordycipitaceae) are considered as both entomopathogens and endophytic symbionts of plants; i.e. besides causing mortality of economically important arthropod pests, these fungi are also able to colonize a wide variety of plant species (Vega, 2008, 2018; Ownley et al., 2010), causing increased plant growth (Sasan and Bidochka, 2012; Jaber and Enkerli, 2016, 2017; Tall and Meyling, 2018), and protection of plants against pests and phythopathogens (Ownley et al., 2010; Jaber and Alananbeh, 2018; Jaber and Ownley, 2018).

Studies have shown successful experimental plant inoculations by *Metarhizium anisopliae* (Metchinikoff) Sorokin and *Metarhizium robertsii* J.F. Bisch., Rehner &

Humber with fungal establishment in different plant species (Sasan and Bidochka, 2012; Batta, 2013; Bamisile et al., 2018). The species *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin has also been experimentally established as endophyte in many important crops, such as corn, potato, cotton, tomato, sorghum, palm, banana, cocoa, poppy, coffee, pine and sugarcane (Brownbridge et al., 2012; Bamisile et al., 2018; Donga et al., 2018), where it often is reported causing negative effects in pest populations feeding on the crop (McKinnon et al., 2017). For example, inoculation of bean seeds, *Phaseolus vulgaris* L. (Fabales: Fabaceae), by *B. bassiana* significantly reduced the growth and reproduction of the spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) (Dash et al., 2018); and *M. robertsii* established as an endophyte in stems and leaves of sorghum, *Sorghum bicolor* L. (Moench) (Poaceae), resulted in reduced infestation levels by the larvae of *Sesamia nonagrioides* (Lefebre) (Lepidoptera: Noctuidae) compared to the control and supressed tunneling by 87% (Mantzoukas et al., 2015).

Besides causing negative effects on arthropod pests, both *B. bassiana* and *Metarhizium* spp. as plant inoculants have also been reported to improve plant growth (Garcia et al., 2011; Sasan and Bidochka, 2012; Liao et al., 2014; Jaber and Enkerli, 2016, 2017; Tall and Meyling, 2018) leading to higher yields (Lopez and Sword, 2015; Gathage et al., 2016; Jaber and Araj, 2018). *Metarhizium* spp. are able to transfer nitrogen from infected insects in the soil to plants via mycelium-root connections in a tritrophic association between host insect, fungus and plant in the rhizosphere (Behie et al., 2012; Behie and Bidochka, 2013, 2014), resulting in an increase in the overall plant productivity. Likewise, Dash et al. (2018) found increased bean plant heights and biomass after seed inoculation with three strains of *B. bassiana*. Furthermore, the two fungal genera frequently exhibit differential localization in plant tissues with endophytic *Metarhizium* spp. being restricted almost exclusively to the root system while *B. bassiana* establishes as an endophyte within all plant tissues (Behie et al., 2015), indicating a potential for complimentary localization in crops and effects against pests.

There is limited knowledge of the combined use of beneficial fungi for plant protection. In a recent study, the co-inoculation of wheat seeds with *Metarhizium brunneum* Petch and the mycoparasitic fungus *Clonostachys rosea* (Link) Schroers et al. (Hypocreales: Bionectriaceae) allowed for the protection of plants roots against both an insect and a plant pathogen (Keyser et al., 2016). This approach is representing an innovative strategy, which should increase the interest in exploring combinations of

beneficial fungi, including entomopathogens, for incorporation into integrated pest management programs. However, effects of such combinations on arthropod natural enemies are also relevant in order to create a robust plant protection strategy. The interactions among endophytic fungal entomopathogens, arthropod pests and their natural enemies have been explored mainly with parasitoid species (Bixby-Brosi and Potter, 2012; Akutse et al., 2014; Jaber and Araj, 2018). Although there are several studies focusing on the direct interactions of *Metarhizium* spp. and *B. bassiana* on predators, including predatory mites (e.g. Seiedy et al., 2013; Dogan et al., 2017), there are so far no studies reporting the effects of entomopathogenic fungi as plant inoculants on predators.

In the present study, seed inoculations by two Brazilian isolates of *M. robertsii* and *B. bassiana* individually and in combinations were studied in bean plants, *P. vulgaris* as a model system. Effects on plant growth and populations of spider mites *T. urticae* feeding on inoculated plants were evaluated under greenhouse conditions. In addition, feeding responses of the predator mite *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) towards spider mites from inoculated plants were assessed to evaluate potential effects at higher trophic levels.

The hypotheses of this study were: I) spider mite population growth will be inhibited on fungal inoculated plants compared to control plants; II) besides reducing the population of spider mites, plants inoculated with both *M. robertsii* and *B. bassiana* isolates individually and in combination will enhance the bean plant growth when compared to control plants; III) inoculation with the *M. robertsii* and *B. bassiana* isolates in combination on the same plant improves the plant growth and reduces the spider mite populations to higher extend than on plants inoculated with only a single fungal isolate; and IV) predatory mite predation rates on spider mites are unaffected by whether leaf substrate and spider mite originated from inoculated plants or from control plants. The overall aim of this research is the development of a robust and innovative biological control strategy by combining predatory mites and entomopathogenic fungi against spider mites.

2.2. Material and Methods

2.2.1. Organisms

The entomopathogenic fungal isolates ESALQ 1622 of *M. robertsii* and ESALQ 3375 of *B. bassiana* were used for the experiments. The isolates were selected from the entomopathogen collection "Prof. Sérgio Batista Alves" in the "Laboratory of Pathology and Microbial Control of Insects" at Escola Superior de Agricultura “Luiz de Queiroz” – University of São Paulo (ESALQ/USP), Piracicaba, São Paulo, Brazil, where they are kept at -80°C. These two isolates showed positive results in the endophytic colonization capability of strawberry plants and as strawberry plants growth promoters (F. Canassa, unpublished). The isolate *M. robertsii* ESALQ 1622 was obtained from soil of a corn field in Sinop City – Mato Grosso State – Brazil and *B. bassiana* ESALQ 3375 originates from soil of a strawberry field in Senador Amaral City – Minas Gerais State – Brazil.

Seeds of bean, *Phaseolus vulgaris* L. variety Lasso, were obtained untreated from the company Olssons Frö AB, Helsingborg, Sweden, and stored at 4ºC. The seeds received fungal treatments (see 2.2.3.) and were planted in 3 L pots containing peat soil supplemented with 5% gravel (grid size: 1-3 mm), clay (grid size: 2-6 mm), limestone (pH: 5.5-6.5), special fertilizers (PG-Mix) and micronutrients (Krukväxtjord Lera & Kisel, Gröna linjen, Sweden) and kept in a greenhouse with weekly fertirrigation containing the following components: N - 170 ppm, P - 26 ppm, K - 222 ppm, Ca - 196 ppm, Mg - 29 ppm, S - 97 ppm, Fe - 1,49 ppm, Mn - 1,06 ppm, B - 0,23 ppm, Zn - 0,26 ppm, Cu - 0,09 ppm, Mo - 0,068 ppm. The *T. urticae* rearing was initiated with spider mites from the company EWH Bioproduction, Tappernøje, Denmark and the mites were kept on bean plants in laboratory cages at ambient light and temperature conditions. The continued rearing was ensured by the cutting of leaves with high infestation by spider mites and placing these leaves on new bean plants. The plants were replaced at regular intervals to ensure the quality of food provided.

2.2.2. Fungal suspensions

Cultures of the two isolates were prepared from stock cultures in Petri dishes (90 x 15 mm) containing 20 ml of Sabouraud Dextrose Agar (SDA; Sigma-Aldrich,

Darmstadt, Germany) and were kept in darkness at 23ºC for 14 days. Subsequently, conidia were harvested with a sterile spatula and suspended in sterile distilled water supplemented with 0.05% Triton X-100 (Sigma-Aldrich, Darmstadt, Germany), and then centrifuged (4R Centrifuge, IEC Centra, TermoFisher Scientific, Roskilde, Denmark) at 3.000 RPM (1900 g) for 3 min to remove hyphal fragments, conidial clumps and bits of agar. This procedure was repeated twice. Each suspension was then vortexed and conidial concentrations were estimated using a Fuchs-Rosenthal haemocytometer (Assistent, Sondheim von der Rhön, Germany). Conidial viability was checked by transferring 150 µl of the suspension onto SDA and counting conidia germination after 24 h at 24ºC. Suspensions were only used if germination rates were higher than 95%.

2.2.3. Inoculation of bean seeds in entomopathogenic fungi suspensions

The isolates *M. robertsii* ESALQ 1622 and *B. bassiana* ESALQ 3375 were used to inoculate bean seeds using suspensions at a concentration of 1 x 10^8 conidia ml^{-1} in distilled water + 0.05% Triton X-100. The following four treatments were prepared: A) isolate *M. robertsii* ESALQ 1622; B) isolate *B. bassiana* ESALQ 3375; C) isolate *M. robertsii* ESALQ 1622 in combination with isolate *B. bassiana* ESALQ 3375; D) Distilled water + 0.05% Triton X-100.

Fungal suspensions for each treatment were prepared as above and adjusted to 1 x 10^8 conidia ml^{-1}. For combined treatment C), individual suspensions were mixed creating a final concentration of 1 x 10^8 conidia ml^{-1} in a mixed suspension represented by 50% of each isolate. Subsequently, 10 bean seeds were inoculated by immersion in 10 ml of the treatment suspensions for 2 hours at 28ºC. Later, the seeds were left on filter paper in Petri dishes for 5 minutes to dry and then they were transferred to the greenhouse and planted individually in 3 L pots and covered with 1 cm of substrate. The plants were grown in a greenhouse during the experimental period at ± 28ºC, photophase 16 hr (1200 watt/6m^2). If the sunlight had higher intensity than 400 watts/m^2, the lamps were turned off.

2.2.4. Effects of *M. robertsii* and *B. bassiana* on population growth of the spider mite *T. urticae*

At 21 days after seed inoculation and planting, 10 spider mite females from the laboratory rearing were inoculated on a leaflet of the third trifoliate leaf (V4 phenological step) of each plant. After infestation, transparent plastic cylinders (60 cm high, 15 cm diameter) with fine mesh at the open top end (0.09 mm mesh size) were placed inside the rim of pots covering the aerial part of the plant and preventing the spread of spider mites to other plants. The spider mite populations were estimated by counting the number of spider mite adults on each plant daily for the first seven days and then 10 and 14 days after infestation, representing at least one mite generation as the life cycle of *T. urticae* takes around 8 days at 30ºC (Wermelinger et al., 1990; Solomon et al., 2001). A randomized block design was used with five replicate plants for each of the four treatments. The experiment was repeated on four occasions.

2.2.5. Effects of *M. robertsii* and *B. bassiana* on bean plant growth

Plant growth parameters were evaluated on bean plants used in the spider mite experiments (2.2.4., plants with spider mites) and also on plants used in the experiments with predatory mites (2.2.6., plants without spider mites). The height of plants was measured weekly with a ruler at 7, 14 and 21 days after seed inoculations. At the end of the evaluations of the spider mite experiment (2.2.4., 35 days after fungal inoculation, 14 days after spider mite release), plants were harvested and the length of roots and aerial part, number of leaves per plant, and number of string beans per plant were assessed. The fresh weight of roots and aerial part (stem and leaves) were weighed separately on an electronic balance to nearest 0.01 g (A&D model FA-2000, UK), then these same plant parts were placed inside paper bags and kept in a drying oven (Memmert model 600, Germany) at 60ºC for 3 days. After this, the roots and aerial plant parts (below and above ground dry biomass) were weighed on the same electronic balance.

2.2.6. Effects of *M. robertsii* and *B. bassiana* inoculated bean plants on behavior of the predatory mite *P. persimilis*

New bean seeds were inoculated by immersion in suspensions of *M. robertsii* ESALQ 1622, *B. bassiana* ESALQ 3375 and the combination of these both isolates as described under 2.2.3., and plants were grown for 21 days in the greenhouse at 28ºC. Then, leaf discs (30 mm diameter) were cut from a leaflet of the third trifoliate leaf (V4 phenological step) of inoculated and control plants. The leaf discs were distributed in pairs in Petri dishes (90 x 15 mm) containing 15 ml water agar (1.5%) with 10 mm between them, according to the following treatments: A) *M. robertsii* ESALQ 1622 leaf disc *versus* control leaf disc; B) *B. bassiana* ESALQ 3375 leaf disc *versus* control leaf disc; C) *M. robertsii* ESALQ 1622 in combination with *B. bassiana* ESALQ 3375 leaf disc *versus* control leaf disc. The position of inoculated and control leaf discs (left side or right side) were randomized in each replicate; 10 replicate arenas were prepared for each treatment and the bioassay was repeated four times.

Six *T. urticae* adult females from the rearing were transferred to each of the two leaf discs in the arena and one hour later a female predatory mite (*P. persimilis*), obtained from the company EWH Bioproduction, was released in the center of a bridge of Parafilm (20 x 20 mm) placed to connect the two leaf discs (Asalf et al., 2011). All the predatory mites had been starved individually in a plastic recipient with lid and moist filter paper in a climate room at 23°C, 16 h L: 8 h D and 70% RH for 24 h before the bioassay. The predatory mite was released onto the Parafilm bridge with opportunity to choose between the two leaf discs (from plants with and without fungal treatment). Immediately after the introduction of the predatory mite, its behavior was observed for 20 minutes in each arena and the time (in seconds) spent on the following behaviors was recorded: 1) searching for prey, 2) encountering prey, 3) feeding, 4) walking outside leaf, 5) walking on parafilm (Jacobsen et al., 2015).

The sequence of the evaluated treatments was randomized at each observation day, as well as the direction of the treated leaf discs (right and left). The evaluations were performed in a controlled climate room at 23°C with no lights coming from the sides (Jacobsen et al., 2015).

2.2.7. Predatory mite feeding capacity on fungal inoculated plants

The feeding capacity of predatory mites was also evaluated on single 30 mm leaf discs from fungal inoculated or non-inoculated plants. The experiment consisted of the following treatments: A) *M. robertsii* ESALQ 1622 leaf disc; B) *B. bassiana* ESALQ 3375 leaf disc; C) *M. robertsii* ESALQ 1622 + *B. bassiana* ESALQ 3375 leaf disc and D) Control (Distilled water + 0.05% Triton X-100) leaf disc; treatments were completely randomized with five replicates and the bioassay was repeated four times.

Leaf discs were cut from a leaflet of the experiment on spider mites population growth (2.2.4.), taking only one leaflet from each plant at the end of the spider mites experiment 35 days after inoculations and 14 days after release of spider mites. The leaf discs were cleaned with a brush and placed individually in the middle of Petri dishes (90 x 15 mm) containing 20 ml of 1.5% agar-water. Then, 10 spider mite adults were randomly collected from the same plant that the leaflet was removed from and released on the respective leaf disc. After 1 hour, one predatory mite adult, previously starved for 24 h as above, was released onto the same leaf disc. The Petri dishes were sealed and kept in an incubator at 28ºC and photophase 14 h for 24 h after which the number of spider mites consumed was assessed.

2.2.8. Evaluation of endophytic colonization level of *M. robertsii* and *B. bassiana* in bean plants

The bean plants inoculated with the different fungal treatments were collected and washed in distilled water for soil removal at 35 days after inoculation. Subsequently, the plant material was cut in fragments; the roots and stems of 5 cm and the leaves of 4 cm height x 1 cm length. These samples (roots, stems and leaves) were surface sterilized by immersion in 70% ethanol for 1 minute, 1% sodium hypochlorite for 2 minutes, 70% ethanol for 1 minute again and rinsed three times in sterile distilled water and dried on sterile filter paper. The efficacy of the sterilization was confirmed by plating 100 µl of the last rinsing water on SDA media (Parsa et al., 2013) and by imprinting each leaf section on SDA media before and after the sterilization (Greenfield et al., 2016).

The plant samples were then individually placed in Petri dishes (90 x 15 mm) containing 20 ml of SDA with 0.5 g/L of cycloheximide, 0.2 g/L of chloramphenicol, 0.5

g/L of Dodine (65%) and 0.01 g/L of Crystal Violet (Behie et al., 2015). The Petri dishes were incubated in darkness at 24°C for 15 days. After the incubation period, the fungal colonization rate, i.e., the number of colonies similar to *Metarhizium* or *Beauveria* that grew from the plant parts was evaluated visually by observation of fungal growth characteristic of the genera.

Suspensions prepared of the peat substrate where the plants had grown was also plated on the same selective media in the four following concentrations after serial dilution in distilled water + 0.05% Triton X-100: 1x10, $1x10^{-1}$, $1x10^{-2}$ and $1x10^{-3}$. The Petri dishes were incubated in darkness at 24°C for 15 days and the presence of colonies was quantified in each concentration after the incubation period.

2.2.9. Statistical analysis

Goodness-of-fit was assessed using half-normal plots with simulation envelopes (Moral et al., 2017). All analyses were carried out in R (R Core Team, 2018). Poisson generalized linear mixed models were fitted to the spider mite count data, with inclusion of experiment and block as nuisance factors, and a different quadratic polynomial per treatment over time, as well as random intercepts and slopes per each group of observations measured over time, given they are correlated. Likelihood-ratio (LR) tests were used to assess the significance of the fixed effects of the model and to compare treatments.

Linear mixed models (assuming a normal distribution for the error) were fitted to the plant height data, given their continuous nature. Poisson generalized linear mixed models were fitted to the number of leaves per plant at 7, 14 and 21 days after inoculation, given their discrete nature. For both types of models, we included in the linear predictor the effects of experiment and block as nuisance factors, and different intercepts and slopes per each treatment (i.e. an interaction between time and treatment). Because observations measured over time on the same experimental unit are correlated, we also included random intercepts and slopes per each group of observations, so as to take this correlation into account. LR tests were used to assess the significance of the fixed effects of the model and to compare treatments.

Linear models (assuming a normal distribution for the error) were fitted to the plant weight and length data at 35 days after inoculation (using a log transformation only for the root dry weight data to satisfy the assumptions of the model), including

experiment and block as nuisance factors, and the effects of treatment in the linear predictor. Multiple comparisons were obtained using Tukey's test at a confidence level of 95%.

Poisson generalized linear models were fitted to the count data (number of leaves and string beans), including the same effects in the linear predictor as for the continuous data. Because the string bean data presented overdispersion (Demétrio et al., 2014), i.e., variance greater than the mean, quasi-Poisson models were used to take this into account. Multiple comparisons were carried out by obtaining the 95% confidence intervals for the linear predictors.

For the behavior of predatory mites, multinomial models for correlated data were used. The correlated measures are due to the fact that the mites were observed over time. The association structure among the correlated multinomial responses is expressed via marginalized local odds ratios by Generalized Estimation Equations (Touloumis et al., 2013). Considering that the original data are sparse due to many zeros, categories were grouped in order to make possible the application of the method. Therefore, it was considered the responses searching for prey, encountering prey and walking outside leaf as one category of response (S/E/W) with two levels: control (x) and treatment (t). The category 5 (walking on parafilm) was fixed as reference category. In the linear predictor, the effects of treatment and experiment were included. Wald tests were used to assess the significance of the treatment effect.

Quasi-binomial generalized linear models were fitted to the predation rate data, including experiment as a nuisance factor and treatment effects in the linear predictor. Multiple comparisons were carried out by obtaining the 95% confidence intervals for the linear predictors.

Binomial generalized linear models (McCullagh and Nelder, 1989) were fitted to the colonization data including the effects of experiment and block, and treatment. A colonization success was recorded when there was fungal growth by either of the strains. When no colonization could be detected for all observations in a specific treatment, i.e., the data consisted only of zeros, the observations in all plants of the treatment were not included in the analysis, given they did not contribute to the variability. Multiple comparisons were performed by obtaining the 95% confidence intervals for the linear predictors.

2.3. Results

2.3.1. Effects of *M. robertsii* and *B. bassiana* on population growth of the spider mite *T. urticae*

The plants whose seeds were inoculated with the three fungal treatments (*M. robertsii*, *B. bassiana* and the combination *B. bassiana* + *M. robertsii*) significantly reduced the spider mites population growth over the 14 days period compared to control treatment with distilled water and 0.05% Triton X - 100 (interaction between treatments and time: LR = 19.58, d.f. = 6, p = 0.0033) (Figure 1). There was no difference between population growth of spider mites on plants whose seeds had been inoculated with the combination of *M. robertsii* ESALQ 1622 and *B. bassiana* ESALQ 3375 in the same conidial suspensions compared to when these isolates were inoculated individually, i.e. there was no difference among the three fungal treatments (grouping treatments *M. robertsii*, *B. bassiana*, and *B. bassiana* + *M. robertsii*: LR = 20.25, d.f. = 6, p = 0.1146).

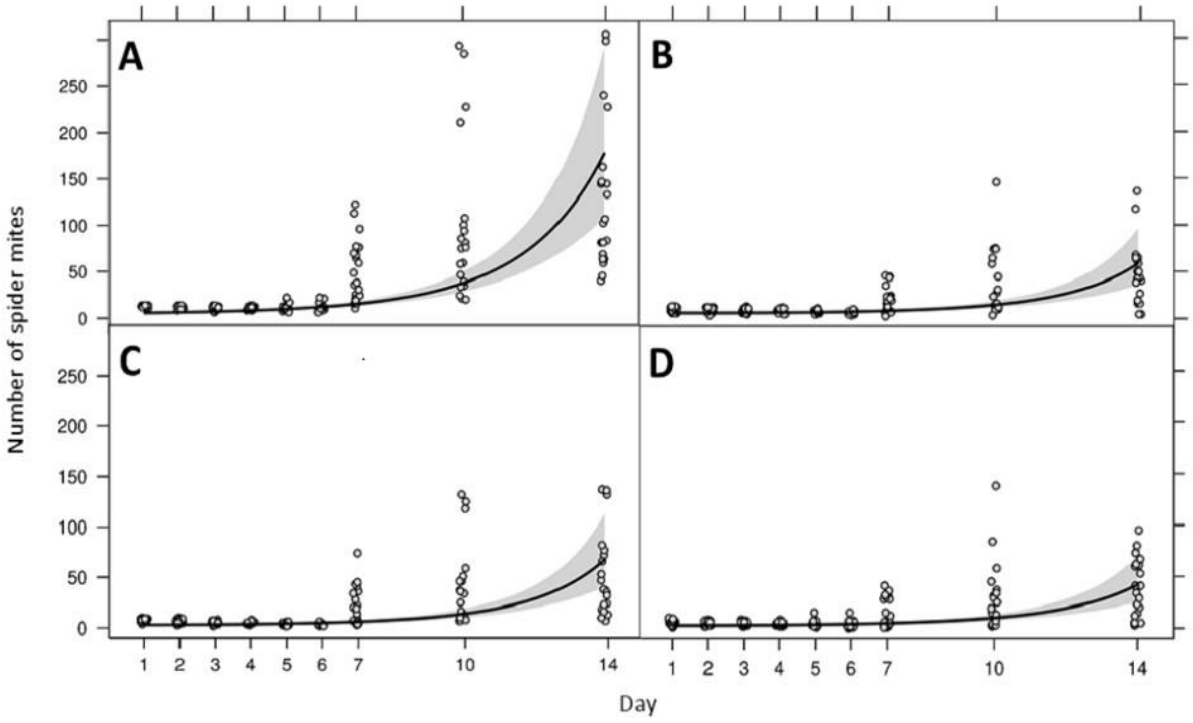

Figure 1. Number of spider mites (*Tetranychus urticae*) over time, observed from all four experiments, from 21 (day 1) to 35 (day 14) days after inoculations of bean seeds in fungal (1 x 10^8 conidia ml^{-1}) or control suspensions. A) 0.05% Triton X - 100 (control),

B) *Beauveria bassiana*, C) *Metarhizium robertsii* and D) *B. bassiana* + *M. robertsii*. The dots are the observations; the solid lines are the fitted curves and the gray areas represent 95% confidence intervals for the true development over time.

2.3.2. Effects of *M. robertsii* and *B. bassiana* on bean plant growth

The inoculation of bean seeds in conidial suspensions of *M. robertsii* and *B. bassiana* increased plant height as compared to control plants during the first 21 days of the experiment (interaction between treatments and time: LR = 21.38, d.f. = 3, $p < 0.0001$). However, there was no difference in the plant heights among the fungal treatments, i.e. *M. robertsii, B. bassiana* and *B. bassiana* + *M. robertsii* (LR = 8.40, d.f. = 4, p = 0.0781), and hence plants treated with the fungal suspensions differed from plants from the control treatment with 0.05% Triton-X (Figure 2) [common slope (SE) for *M. robertsii*, *B. bassiana*, and *B. bassiana* + *M. robertsii* = 1.5142 (0.0448); and slope (SE) for Triton-X (control) = 1.0687 (0.0531)]. At 7, 14 and 21 days after inoculation the following average plant heights ± SE were found, respectively: *M. robertsii* = 5.20 cm ± 0.53; 11.74 cm ± 0.63; 26.10 cm ± 1.65; *B. bassiana* = 6.28 cm ± 0.29; 12.86 cm ± 0.45; 27.09 cm ± 0.90; *B. bassiana* + *M. robertsii* = 6.25 cm ± 0.56; 12.90 cm ± 0.43; 29.05 cm ± 1.39; and Triton-X (control) = 2.68 cm ± 0.54; 8.40 cm ± 0.67; 16.73 cm ± 1.65.

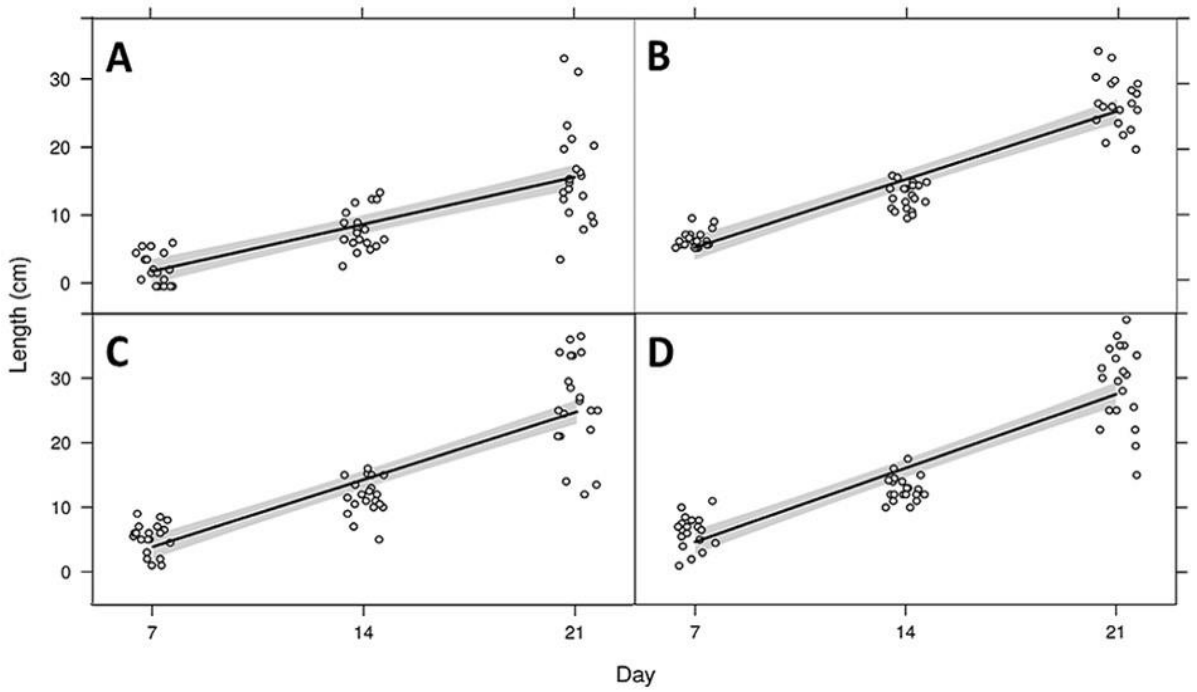

Figure 2. Length of bean plants measured at 7, 14 and 21 days after inoculations of bean seeds in fungal (1 x 10^8 conidia ml^{-1}) or control suspensions: A) 0.05% Triton-X (control), B) *Beauveria bassiana*, C) *Metarhizium robertsii* and D) *B. bassiana* + *M. robertsii*. The dots are the observations; the solid lines are the model predictions and the gray areas represent 95% confidence intervals for the true development over time.

The number of leaves at 7, 14 and 21 days after inoculation were not different over time (interaction between treatments and time: LR = 0.21, d.f. = 3, p = 0.9762). However, there were significant treatment (LR = 19.37, d.f. = 3, p < 0.0001) and time (LR = 881.16, d.f. = 1, p < 0.0001) effects. The number of leaves on plants of the three fungal treatments was statistically equal (grouping treatments *M. robertsii*, *B. bassiana*, and *B. bassiana* + *M. robertsii*: LR = 0.15, d.f. = 2, p = 0.9266), and the only difference was found for Triton-X (control); i.e., plants of the latter treatment developed a lower number of leaves at 21 days after inoculation (Figure 3). The following average number of leaves ± SE were obtained in the four treatments at 21 days: *M. robertsii* = 8.0 ± 0.41; *B. bassiana* = 8.0 ± 0.36; *B. bassiana* + *M. robertsii* = 8.0 ± 0.39; and Triton-X (control) = 5.0 ± 0.78.

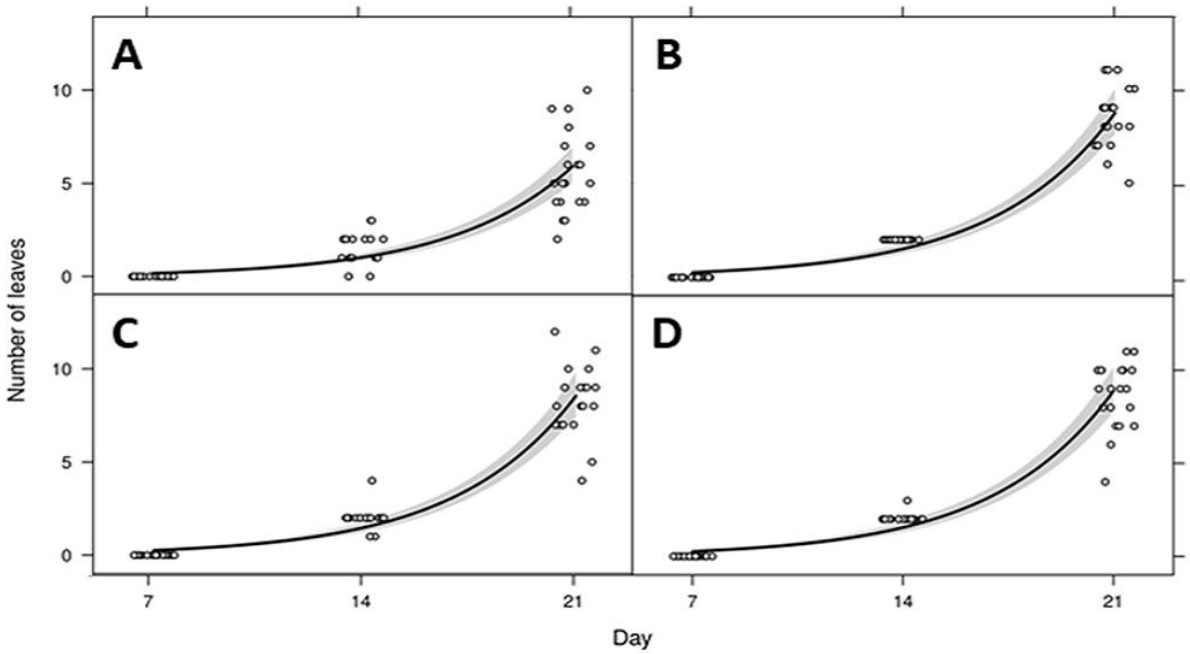

Figure 3. Number of leaves counted at 7, 14 and 21 days after inoculations of bean seeds in fungal (1 x 10^8 conidia ml^{-1}) or control suspensions: A) 0.05% Triton-X (control), B) *Beauveria bassiana*, C) *Metarhizium robertsii* and D) *B. bassiana* + *M. robertsii*. The dots are the observations; the solid lines are the fitted curves and the gray areas represent 95% confidence intervals for the true development over time.

At 35 days after the inoculations, there was significant effect of the treatment on all plant growth parameters. Beginning for the number of leaves, there was a significant treatment effect (deviance = 60.54, d.f. = 3, $p < 0.0001$). Comparing the treatments using the 95% confidence intervals for the linear predictors, it was found that the three fungal treatments were equal, and they all differed from the control plants. The mean numbers of leaves ± SE in the four treatments were: *M. robertsii* = 33.8 ± 1.79; *B. bassiana* = 34.9 ± 1.47; *B. bassiana* + *M. robertsii* = 36.8 ± 1.59; and Triton-X (control) = 24.3 ± 1.72.

The mean values of fresh and dry weight of roots and aerial part were significantly higher in all the fungal treated plants than in the control plants (Table 1). The lengths of roots and aerial parts were not different from control in the treatment with *B. bassiana*, while *M. robertsii* and *B. bassiana* + *M. robertsii* (*Bb* + *Mr*) treated plants had longer roots and aerial parts than control plants (Table 1).

Table 1. Means ± SE of plant growth response variables at 35 days after fungal inoculation with summaries of generalized linear models. All experimental plants were exposed to spider mites from day 21 to 35. Separate analyses were performed for each response variable.

Treatment[2]	Assessment[1]						
	Fresh weight Roots	Dry weight Roots	Fresh weight Aerial part	Dry weight Aerial part	Length of Roots	Length of Aerial part	Nº of string beans
B. bassiana	4.41 ± 0.33 a	0.54 ± 0.07 a	57.35 ± 2.58 a	5.23 ± 0.22 a	53.17 ± 3.18 ab	48.89 ± 1.78 ab	5.10 ± 1.32 a
M. robertsii	4.38 ± 0.26 a	0.46 ± 0.05 a	56.62 ± 2.38 a	5.16 ± 0.24 a	57.02 ± 3.59 a	52.35 ± 1.77 a	5.85 ± 1.45 a
Bb + *Mr*	5.32 ± 0.36 a	0.60 ± 0.08 a	59.89 ± 2.62 a	5.42 ± 0.28 a	59.62 ± 4.77 a	52.88 ± 2.18 a	6.15 ± 1.53 a
Triton – X	3.09 ± 0.30 b	0.29 ± 0.03 b	39.58 ± 3.44 b	3.75 ± 0.33 b	47.99 ± 2.56 b	43.92 ± 2.88 b	1.35 ± 0.63 b
F	9.58	15.64	18.59	10.86	4.94	5.47	13.52
d.f.	3, 57	3, 57	3, 57	3, 57	3, 57	3, 57	3, 57
P-value	<0.0001	<0.0001	<0.0001	<0.0001	0.0041	0.0022	<0.0001

[1]Data (mean ± SE) followed by different letters within a column are significantly different (GLM, followed by *post hoc* Tukey test, $P < 0.05$).

[2]Treatments included seed inoculations of the entomopathogenic fungal isolates *Beauveria bassiana* ESALQ 3375 (*B. bassiana*), *Metarhizium robertsii* ESALQ 1622 (*M. robertsii*), a combination of the two isolates (*Bb* + *Mr*), and control treatment with 0.05% Triton-X.

2.3.3. Effects of *M. robertsii* and *B. bassiana* inoculated bean plants on feeding behavior of the predatory mite *P. persimilis*

In the leaf disc experiments, seed treatment did not significantly affect the probabilities associated with the different behaviors of the predatory mites in time spent in each category of the grouped behaviors or "S/E/W" state (searching for prey, encountering prey and walking outside leaf) in the three fungal treatments (*M. robertsii, B. bassiana* or *B. bassiana* + *M. robertsii*) (Wald Statistic = 8.69, d.f. = 8, p-value = 0.3686) (Figure 4). The effect of time was significant (Wald Statistic = 38.32, d.f. = 4, p-value <0.0001). The probability of remaining on the parafilm decreased over time, as the predatory mites exhibited different behaviors. The probability of the "S/E/W" state increased over time for both fungal treated and control plant leaf discs (Figure 4). Also, the predatory mites were more likely to feed on spider mites from fungal treated plants than control plants until the middle of the experiment (600 seconds). During the second half of the observation period, the predatory mites were more likely to feed on spider mites from control plants than from fungal treated plants (600 to 1200 seconds) (Figure 4).

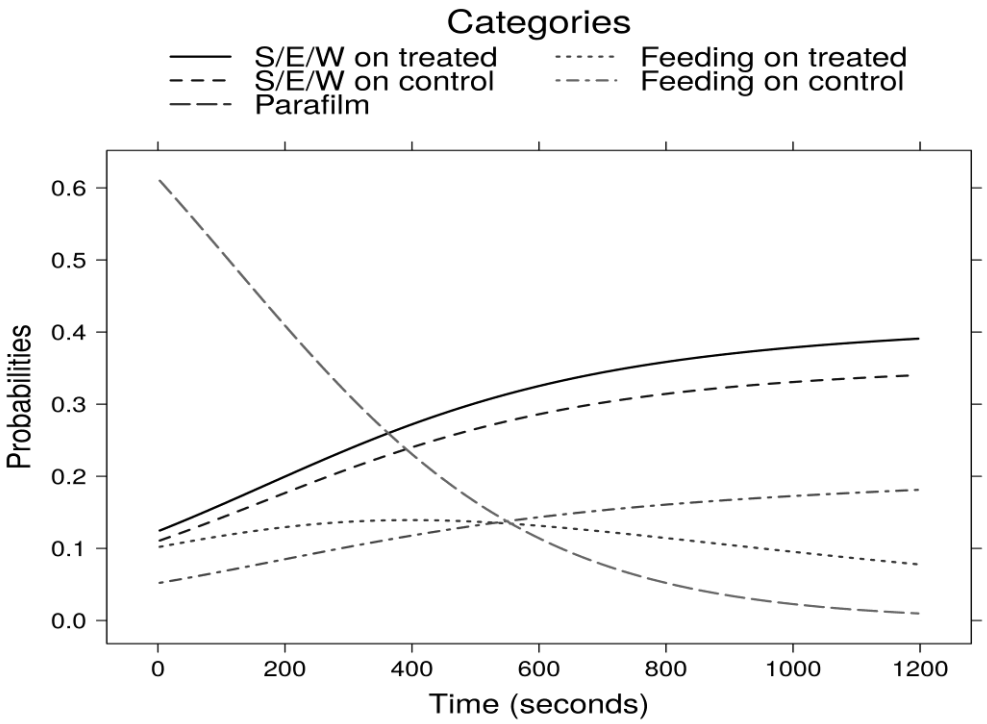

Figure 4. Probabilities of predatory mites exhibiting each different behavior over time, as predicted by the multinomial model. The grouped category S/E/W on treated plants means the time spent by *Phytoseiulus persimilis* searching for prey (S), encountering

prey (E) or walking outside leaf (W) on fungal inoculated plants (the three fungal treatments combined); and the grouped category S/E/W on control plants means the time spent by *P. persimilis* searching for prey (S), encountering prey (E) or walking outside leaf (W) in control non-inoculated plants; the category parafilm means the time spent by *P. persimilis* in the bridge of parafilm.

No differences were observed in the predation rate of *T. urticae* kept on leaf discs from inoculated and from control non-inoculated plants for *P. persimilis* ($F_{3,73}$ = 0.57, p = 0.6393). The mean proportion of the 10 presented spider mites that were consumed in 24 h (± SE) for the four treatments were: *M. robertsii* = 38% (± 5.4%); *B. bassiana* = 45% (± 6.5%); *B. bassiana* + *M. robertsii* = 40% (± 5.5%); and Triton-X (control) = 41% (+ 5.0%).

2.3.4. Evaluation of endophytic colonization level of *M. robertsii* and *B. bassiana* in bean plants

Both isolates of *M. robertsii* and *B. bassiana* became endophytic with relatively low colonization levels at 35 days after the inoculations of bean seeds (n=10 per treatment). In the single fungus treatments, the frequencies of occurrence in respective tissues of *B. bassiana* were 20% in roots, 30% in stems and 50% in leaves. For *M. robertsii*, 30% of roots were colonized, while stems and leaves were not found to be colonized by *Metarhizium*. In the combination of the two fungal isolates, *M. robertsii* was found to colonize 40% of the roots, while *B. bassiana* colonized 10% of the roots and 30% of the leaves. In all three fungal treatments, 20% of soil samples contained the fungi that were inoculated. None of the target fungi were recovered from the plant tissue or soil substrate in the control treatment. Occasionally, other unidentified fungi were cultivated from the plant tissues, but with no apparent relation to treatment.

2.4. Discussion

In this study, bean plants inoculated with both *M. robertsii* ESALQ 1622 and *B. bassiana* ESALQ 3375 reduced the *T. urticae* population growth, supporting the first hypothesis. The inoculation with the isolates of *M. robertsii* and *B. bassiana* in combination on the same plant also reduced the spider mite populations, but not to

higher extend than plants inoculated with only a single fungal isolate, thus not supporting our initial hypothesis. Besides, inoculating the fungi individually and combined equally improved the plant growth as compared to control plants. Although the experiments with predatory mites were limited in scale, the data indicated that *P. persimilis* had similar feeding capacity on spider mites reared on fungal inoculated and control plants. It was found that the predators were likely to spend marginally more time feeding on spider mites originating from the rearing when presented on leaf discs from non-inoculated plants than on leaf discs from fungal inoculated plants during the course of the behavioral observations. However, we conclude that the selected isolates of entomopathogenic fungi used as seed inoculants are potential candidates for biological plant protection above-ground and that the inoculation approach did not show any short-term detrimental effects on feeding capacity of predators in the plant canopy.

In a recent study, Dash et al. (2018) also reported negative effects on population growth and reproduction of *T. urticae* when they were kept on bean plants (*P. vulgaris*) grown from seeds inoculated by three isolates of *B. bassiana* (B12, B13, B16), and isolates of *Cordyceps* (= *Isaria) fumosorosea* (isolate 17) and *Akanthomyces* (=*Lecanicillium*) *lecanii* (isolate L1), compared to non-inoculated control plants. They reported a significant reduction in larval development, adult longevity and female fecundity of spider mites when reared on *B. bassiana* treated plants; in addition, increased bean plant heights and biomass were reported (Dash et al., 2018). Reduced insect herbivore population growth on fungal inoculated plants compared to control plants has also been reported by Gathage et al. (2016) who found lower infestation levels of *Liriomyza* leafminers (Diptera: Agromyzidae) in *P. vulgaris* plants endophytically colonized with *B. bassiana* isolate G1LU3 compared to control; besides lower numbers of pupae were also observed. Qayyum et al. (2015) reported a high mortality of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) when fed tomato plants colonized by *B. bassiana* isolate WG-40. Similarly, *B. bassiana* isolates ITCC 5408 and ITCC 6063 as endophytes reduced the stem weevil *Apion corchori* Marshall (Coleoptera: Curculionidae) in white jute (Biswas et al., 2013). Gurulingappa et al. (2010) reported a reduction of the population growth rate of *Chortoicetes terminifera* (Walker) (Orthoptera: Acrididae) nymphs when fed wheat leaves colonized by a *B. bassiana* strain. Furthermore, *B. bassiana* isolate G41 reduced larval survivorship of banana weevil, *Cosmopolites sordidus* Chevrolat (Coleoptera: Curculionidae), in

banana (Akello et al., 2008). Endophytic colonization by *B. bassiana* isolate 0007 significantly reduced damage caused by *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) (Cherry et al., 2004); and *B. bassiana* isolate ARSEF 3113 by *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) (Bing and Lewis, 1991), both in maize.

There are fewer reports of plant inoculations with *Metarhizium* spp. causing negative effects against arthropod pests. For example, Jaber and Araj (2018) reported that the inoculation of *M. brunneum* strain BIPESCO5 in sweet pepper (*Capsicum annuum* L.) by plant root drench resulted in fewer aphids, *Myzus persicae* Sulzer (Homoptera: Aphididae), including prolonged development time and reduced reproduction compared to aphid populations on control plants. The inoculations of *M. anisopliae* isolate ICIPE 20 in bean (*P. vulgaris*) by seed soaking reduced the bean stem maggot, *Ophiomyia phaseoli* Tryon (Diptera: Agromyzidae) (Mutune et al., 2016). The inoculation by spraying on leaves until runoff of *M. robertsii* (an isolate from click beetles) in sweet sorghum against the Mediterranean corn stalk borer, *Sesamia nonagrioides* Lefebre (Lepidoptera: Noctuidae), supressed tunneling by 87% and caused 100% mortality (Mantzoukas et al., 2015).

The mechanisms behind the negative effects caused by plant associated *B. bassiana* and *Metarhizium* spp. still remain largely unknown. However, based on the present study it is likely that the two fungal taxa have similar effects against spider mites, suggesting comparable mode of action. It is suggested that compounds produced by the plant or by the associated fungus is causing the reported sub-lethal negative effects (Vidal and Jaber, 2015; McKinnon et al., 2017). The plant colonization by inoculated fungi can at first be recognized by the plant as potential invaders leading to the triggering of immune responses with synthesis of specific regulatory elements, such as transcription factors involved in resistance against herbivores (Brotman et al., 2013; McKinnon et al., 2017). Induction of proteins related to plant defense or stress reponse in *Phoenix dactylifera* leaves colonized by *B. bassiana* has also been reported (Gomez-Vidal et al., 2009). Production of secondary plant metabolites may also be considered, for example, terpenoids have anti-herbivore properties (Gershenzon and Croteau, 1991; Fürstenberg-Hägg et al., 2013; Vega, 2018). It was reported by Shrivastava et al. (2015) that tomato plants endophytically colonized by *B. bassiana* showed higher levels of monoterpenes and sesquiterpenes compared to control plants and larvae of *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) feeding on fungal colonized plants had lower weight than those that had been feeding on control plants,

suggesting that the observed difference in the levels of terpenoids may be related to a defense response of fungus-inoculated plants.

Alternatively, the production of fungal secondary metabolites *in planta* could also be a possible mechanism for observed negative effects against herbivores (McKinnon et al., 2017; Jaber and Ownley, 2018), since fungal entomopathogens are a primary source of bioactive secondary metabolites with antimicrobial, insecticidal and cytotoxic activities (Gibson et al., 2014). Specifically, *B. bassiana* is able to produce a range of secondary metabolites such as beauvericin (Grove and Pople, 1980; Wang and Xu, 2012), bassianolides (Kanaoka et al., 1978), bassiacridin (Quesada-Moraga and Vey, 2004), bassianin, beauverolides, bassianolone and others (reviewed in Ownley et al., 2010; Jaber and Ownley, 2018). Such metabolites extracted *in vitro* from the mycelia of an endophytic isolate of *B. bassiana* (isolated from *Orthorhinus cylindrirostris* Fabricius (Coleoptera: Curculionidae) caused mortality and reduced reproduction of *Aphis gossypii* Glover (Hemiptera: Aphididae) (Gurulingappa et al., 2010, 2011). Similarly, Leckie et al. (2008) reported that larvae of *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) had delayed development, lower weight and higher mortality when fed on diets containing mycelia of a *B. bassiana* isolate compared to control larvae*,* and beauvericin was detected in the broth cultures added into the diet. *Metarhizium* spp. can also produce secondary metabolites, particularly destruxins (Roberts, 1981). Golo et al. (2014) detected destruxins in roots, stems and leaves of cowpea plants (*Vigna unguiculate*) inoculated with *M. robertsii* ARSEF 2575 at 12 days after seed inoculation. Ríos-Moreno et al. (2016) and Resquín-Romero et al. (2016) detected destruxin A in potato and tomato leaves, respectively, when endophytically colonized by a *M. brunneum* isolate. Similarly, Garrido-Jurado et al. (2017) detected destruxin A in melon leaves endophytically colonized by a *M. brunneum* isolate, and also in *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) nymphs that fed on the melon leaves*.* However, it is unknown if the reported destruxin levels in the plant tissues are sufficient to cause negative effects on arthropod herbivores. Non-entomopathogenic fungi are also reported to have negative effects against *T. urticae* based on defensive inductions in the plant (e.g. Pappas et al., 2018). Given the emerging knowledge of comparable effects on many different herbivores feeding on various plants colonized by variable taxa of entomopathogenic fungi, it seems relevant to focus future research on whether these fungi moderate the plant defense systems as has been reported from other beneficial microbes (e.g. Pineda et al., 2013).

In our study, the inoculation of bean seeds with suspensions of *M. robertsii* ESALQ 1622 and *B. bassiana* ESALQ 3375 improved plant growth mainly at 21 and 35 days after inoculation compared to control non-inoculated plants, including higher bean pod production, demonstrating that growth promotion effects were also evident during exposure to biotic stress by *T. urticae*. Entomopathogenic fungi have previously been reported to improve plant growth (e.g. Garcia et al., 2011; Sasan and Bidochka, 2012; Liao et al., 2014; Jaber and Enkerli, 2016, 2017) and reduce damage related to pest infestation and feeding, eventually leading to higher yields (Lopez and Sword, 2015; Gathage et al., 2016; Jaber and Araj, 2018). The incorporation of the fungal endophytes *Hypocrea lixii* Patouillard F3ST1 and *B. bassiana* G1LU3 in a *P. vulgaris* production system under field conditions improved the management of *Liriomyza* leafminers and increased significantly the crop yield (Gathage et al., 2016). Furthermore, Jaber and Araj (2018) also confirmed growth promotion by *B. bassiana* (commercial strain NATURALIS) and *M. brunneum* (commercial strain BIPESCO5) in sweet pepper plants while also reporting of negative effects on the development and fecundity of the aphid *Mizus persicae* (Sulzer) (Hemiptera: Aphididae). Consistent increase in plant growth during infestation with two successive *M. persicae* generations indicated ability of these fungi to promote growth under experimentally-imposed biotic stress (Jaber and Araj, 2018), as was also recorded in the present study.

Our results contradicted the third hypothesis; although the combination of *M. robertsii* ESALQ 1622 and *B. bassiana* ESALQ 3375 in the same conidia suspension reduced spider mite populations and improved the plant growth compared to control plants, the effects were not different than when plants were inoculated with only a single fungal isolate. We expected that the differential localization of *M. robertsii* and *B. bassiana* within the plant (Behie et al., 2015) could lead to complementarity, but the results rather indicate that the fungi are redundant although *B. bassiana* was the only fungus recovered from above-ground tissues. It has been shown that plants treated with combinations of beneficial microbes show limited additional effects on insect herbivores and plant growth than single species additions (Gadhave et al., 2016). For example, the endophytes *Rhizobium etli* and *Fusarium oxysporum* individually induced systemic resistance against *A. gossypii*, but inoculation by both microbes did not show a significant additive biocontrol effect compared to the individual treatments (Martinuz et al., 2012). Similarly, colonization of strawberries by two individual mycorrhizal species of *Glomus* spp. reduced the growth and survival of larvae of *Otiorhynchus*

sulcatus F. (Coleoptera: Curculionidae), however the combination of the two species did not lead to additional reduction (Gange, 2001).

In the present short-term leaf disc experiments, no differences were observed in the predation rates by the predatory mite *P. persimilis* on adults of *T. urticae* kept on leaves of inoculated and control non-inoculated plants. Furthermore, there was no treatment effect of fungal species on the four evaluated *P. persimilis* behaviors although the predatory mites were more likely to feed on spider mites from fungal treated plants to begin with and on spider mites from control plants since halfway through the observation period. The experiments were conducted using excised leaf discs which may potentially affect predator behavior. However, this approach is a widely used method for evaluation of mite behavior in experimental arenas (e.g. Gyuris et al., 2017; Wu et al., 2018). Other results may have been obtained using intact plants, thus further studies using *P. persimilis* on fungal inoculated and uninoculated plants are needed to evaluate effects at spider mite population level and on predator fitness to conclude on compatibility between seed inoculation of entomopathogenic fungi and release of *P. persimilis* for combined spider mite control. However, the present study does not provide any indication that the two types of beneficial organisms should not be combined.

Trophic interactions between two types of natural enemies and arthropod herbivores may vary depending on the biological attributes of the species and the type of plant where they occur (Kennedy, 2003). Akutse et al. (2014) studied the interactions among the leafminer *Liriomyza huidobrensis,* the endophytic fungi *Hypocrea lixii* and *B. bassiana* inoculated by soaking seeds, and two leafminer parasitoids under laboratory conditions; no differences were observed in the parasitism rates between inoculated and non-inoculated bean plants, and adult survival of both parasitoids were similar among treatments. Jaber and Araj (2018) reported the compatibility between *B. bassiana* and *M. brunneum* as inoculants of sweet pepper plants and the aphid endoparasitoid *Aphidius colemani* Viereck (Hymenoptera: Braconidae) for *M. persicae* suppression under controlled greenhouse conditions. Furthermore, it was reported by Schausberger et al. (2012) that mycorrhizal inoculated plants infested with *T. urticae* were more attractive than non-mycorrhizal plants to the spider mite predator, *P. persimilis*. It was suggested that this effect was mediated by the increased production of ß-ocimene and ß-caryophyllene, indicating that the predatory mites learned to recognize the plant response (Patiño-Ruiz and Schausberger, 2014) and show greater

oviposition rates on these plants resulting in enhanced *T. urticae* suppression (Hoffmann et al., 2011).

The two fungal isolates used in the present study, *M. robertsii* ESALQ 1622 and *B. bassiana* ESALQ 3375, were able to colonize the bean plants, with *M. robertsii* only being recovered in the roots and from soil, and *B. bassiana* recovered from soil and from the three different parts of *P. vulgaris*, both when combined and individually inoculated. Similar spatial segregation patterns of the fungal genera were reported by Behie et al. (2015) under laboratory and field conditions, where *M. robertsii* was restricted to the roots of haricot bean plants (*P. vulgaris*) while *B. bassiana* was found throughout the plant, indicating specific variation in the endophytic capacity of the recovered isolates to colonize different plant tissues. Likewise, Akello and Sikora (2012) reported that an isolate of *M. anisopliae* just colonized roots while a *B. bassiana* isolate endophytically colonized different plant parts of *Vicia faba* L. (Fabales: Fabaceae). Several studies have reported that *B. bassiana* can establish as an endophyte throughout the entire plant (reviewed by Jaber and Ownley, 2018). In contrast, Greenfield et al. (2016) found both *M. anisopliae* and *B. bassiana* colonizing only roots of cassava plants, but not stems and leaves. Jaber and Araj (2018) found both *M. brunneum* and *B. bassiana* to colonize the roots and stems of sweet pepper more frequently than leaves in two experiments, but *B. bassiana* colonized more leaves and stems in a second experiment than *M. brunneum*, which was mostly recovered from roots. However, the colonization of the two entomopathogenic fungi had similar negative effects on *M. persicae* development and fecundity (Jaber and Araj, 2018). According to Gathage et al. (2016) and other researchers, the differential colonization of *P. vulgaris* tissues did not necessarily affect the ability of endophytes to confer protection against *Liriomyza* leafminer flies indicating that the plant protection potential of the fungi is not dependent on ability to endophytically colonize the respective plant tissues.

The percentage of colonization in our study was limited when evaluated 35 days after inoculation. Akutse et al. (2013) also reported that despite poor colonization of different parts of *P. vulgaris*, two isolates of *B. bassiana* had negative effects on the number of pupae and emergence of *L. huidobrensis.* Isolates of *M. anisopliae* that could not be confirmed to colonize bean plants endophytically still resulted in reduced feeding, oviposition, pupation, and emergence of the bean stem maggot *Ophiomyia phaseoli* Tryon (Diptera: Agromyzidae) (Mutune et al., 2016). Differential colonization

rates of plants by fungal isolates could have various causes, such as innate characteristics of the fungal isolate (Posada et al., 2007); host plant genetics (Arnold and Lewis, 2005); leaf surface chemistry (Posada et al., 2007); and competition with other endophytes naturally occurring within plants (Posada et al., 2007; Schulz et al., 2015; Jaber and Enkerli, 2016).

The bean seed treatment by the entomopathogenic fungal isolates *M. robertsii* ESALQ 1622 and *B. bassiana* ESALQ 3375 in combination with application of the predatory mite *P. persimilis* are expected to contribute to reduced population growth of the two-spotted spider mite *T. urticae*, besides improving the vegetative and reproductive growth of *P. vulgaris* plants. The results bring a new perspective on the use of plant associated *Metarhizium* spp. and *B. bassiana*, revealing that the use of entomopathogenic fungi as seed inoculants may be a promising plant protection strategy.

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3. BENEFITS OF STRAWBERRY ROOT INOCULATIONS WITH ENTOMOPATHOGENIC FUNGI ON PLANT GROWTH AND YIELD AND REDUCTION OF TWO-SPOTTED SPIDER MITE OVIPOSITION VARIES WITH FUNGAL ISOLATE AND CROP CULTIVAR

Abstract

Root inoculations of crop plants by beneficial fungi constitute a promising strategy for growth promotion and control of above-ground pests and diseases. Here, strawberry roots (cultivar 'Albion' and 'Pircinque') were inoculated with 25 different Brazilian entomopathogenic fungal isolates of three genera and the effects on *Tetranychus urticae* oviposition and plant growth were evaluated in greenhouse experiments. Reductions in number of *T. urticae* eggs compared to control treatments were observed on both cultivars inoculated with almost all isolates. For the cultivar 'Albion', *Metarhizium anisopliae* (ESALQ 1604, ESALQ 1669), *M. robertsii* (ESALQ 1622, ESALQ 1635), *Metarhizium* sp. Indet. (ESALQ 1684), and *Beauveria bassiana* (ESALQ 3323) increased dry weight of roots and leaves, and fruit yield, while *M. robertsii* (ESALQ 1634), and *Metarhizium* sp. Indet. (ESALQ 1637) and (ESALQ 1636) improved fruit yield and dry weight of leaves, respectively. For the cultivar 'Pircinque', *M. anisopliae* (ESALQ 1669) was the only isolate observed to increase dry weight of roots. These results suggest that inoculation of strawberry roots with entomopathogenic fungi may be an innovative strategy for pest management above-ground, but effects depend to some extend on fungal strains. Further, such inoculations may also stimulate plant growth and strawberry production but the effects depend on crop cultivar.

Keywords: Biological control; *Tetranychus urticae*; Plant growth promotion; Phytobiome; Integrated Pest Management (IPM)

3.1. Introduction

Strawberry (*Fragaria* x *ananassa* Duch.; Rosales: Rosaceae) is an important commodity throughout the world and the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) is one of its major pests in many countries (Raworth, 1986; Garcia-Mari and Gonzalez-Zamora, 1999; Easterbrook et al., 2001; Solomon et al., 2001). This mite is also a pest of several other cultivated crops worldwide (Solomon et al., 2001; Greco et al., 2005). Control of *T. urticae* has been done mainly by the use of synthetic acaricides (Van Leeuwen et al., 2010; Attia et al., 2013), but *T. urticae* populations often reach damaging levels following pesticide treatments. This may be caused by pesticide side effects and the resulting reduction of natural enemies and also development of pesticide resistance (Van de Vrie et al., 1972; Cavalcanti et al.,

2010), which may again lead to an even higher pesticide application rate, the so-called pesticide-treadmill (Weddle et al., 2009). A higher pesticide application rate may further result in acaricide residues in the environment and in the edible product (Kumar et al., 2005). To reduce the use of synthetic chemical pesticides, alternatives such as biological control with entomopathogenic fungi are becoming increasingly important and may be implemented as viable alternative tools in integrated pest management (IPM) (Singh and Singh, 2017).

The entomopathogenic fungus *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Hypocreales: Cordycipitaceae) and species of *Metarhizium* Sorokin (Hypocreales: Clavicipitaceae) are able to infect and kill a range of insects, and they are also plant associated and able to colonize a wide variety of plant species as endophytes (Hajek and Meyling, 2018). These fungi-plant associations have been reported to cause plant growth promotion effects (Sasan and Bidochka, 2012; Jaber and Enkerli, 2016, 2017; Tall and Meyling, 2018) and reduction in populations of plant pests and diseases (Ownley et al., 2010; McKinnon et al., 2017; Jaber and Ownley, 2018). The *Isaria* species complex [proposed new name *Cordyceps* (Kepler et al., 2017)] have also been confirmed to be endophytes (Bills and Polishook, 1991; Vega et al., 2008; Giordano et al., 2009), but there is still limited knowledge about the experimental inoculation of plants with the entomopathogenic fungus *Cordyceps fumosorosea* [formerly *Isaria fumosorosea* (Kepler et al., 2017)] (Wize) Kepler, B. Shrestha & Spatafora, comb. nov. (Hypocreales: Cordycipitaceae) and the potential effects on pests and on plant growth. A wide diversity of naturally occurring entomopathogenic fungi have been documented in Brazil, including undescribed species within *Metarhizium* (Lopes et al., 2013; Rezende et al., 2015; Castro et al., 2018). These fungi represent an unexploited resource for plant protection, using local isolates that may be adapted to the local environmental conditions. Soil drench granulate or root dipping application of Met52® *Metarhizium brunneum* (reported as *M. anisopliae* (Metsch.) Sorokin) to strawberry against the soil living larvae of the black vine weevil *Otiorhyncus sulcatus* in a temperate region (UK) has been tested and suggested to be a potential strategy (Ansari and Butt, 2013). Further, the persistence of locally adapted isolates of *M. brunneum* Petch and *Beauveria pseudobassiana* Rehner & Humber applied as granulates close to strawberry roots were confirmed in studies in Norway (Klingen et al., 2015). However, none of these studies evaluated the potential of these fungi for improving plant productivity or controlling pests above-ground in strawberry.

It is well established that entomopathogenic fungal isolates vary considerably in virulence against insect hosts, both intra- and interspecifically (e.g. Luz et al., 1998; Valero-Jiménez et al., 2014). There are also reports of variable effects on plant pests, e.g. *Liriomyza huidobrensis* (Diptera: Agromyzidae) of isolates of various fungal genera established as endophytes in bean plants *Phaseolus vulgaris* L. (Fabaceae) (Akutse et al., 2013). Besides, Jaber and Ownley (2018) highlighted the importance of studying the differential expressions in fungal genotype-plant genotype interactions considering different environmental conditions, and also the selection of most adapted endophytic entomopathogenic fungi isolates to specific host plants and cultivars to their further development as biocontrol agents. To our knowledge, no studies have been reported on the potentially variable effect of root inoculations of different strawberry cultivars with different isolates of entomopathogenic fungi in the Hypocreales on plant productivity and above-ground plant pests.

The objectives of the present study were therefore to evaluate the effect of root inoculations of two strawberry cultivars with 25 Brazilian isolates of different taxa on *T. urticae* oviposition rate and on strawberry plant biomass production and fruit yield under greenhouse conditions. The effects of the isolates were compared to well-established microbial inoculants used for plant growth promotion in Brazil. The hypotheses were that strawberry root inoculation with entomopathogenic fungal isolates from different species would cause: I) reduction in spider mite oviposition rates, II) an increase in the strawberry plants growth and fruit yield, III) and that these effects will be variable among fungal isolates, species and strawberry cultivar. The overall aim of the present research was to identify promising candidate fungal isolates for pest management in strawberry production in Brazil.

3.2. Material and Methods

3.2.1. Organisms used in the experiments

3.2.1.1. Fungal isolates

Twenty-five isolates of entomopathogenic fungi from different Brazilian biomes and crops were evaluated and are described in Table 1. Five of the isolates were of *Metarhizium robertsii* Bisch., Rehner & Humber, five isolates of *M. anisopliae*, and five

isolates belonging to three separate taxonomically unassigned lineages of *Metarhizium* which do not cluster within the currently recognized limits of the described *Metarhizium* species; hence the taxonomies of these isolates are referred to as *Metarhizium* sp. Indet. 1, 2 and 4 (Rezende et al., 2015). Five isolates of *B. bassiana* and five isolates of *C. fumosorosea* were also included. Among the tested isolates, the following are active ingredients of registered commercial biological control products in Brazil: *M. anisopliae* ESALQ 1604 (Biotech G®, BIOTECH), *B. bassiana* ESALQ PL63 (Boveril® WP, Koppert) and *C. fumosorosea* ESALQ 1296 (Challenger® SC, Koppert).

Isolates are kept at -80°C in the entomopathogens collection "Prof. Sérgio Batista Alves" in the "Laboratory of Pathology and Microbial Control of Insects" at Escola Superior de Agricultura “Luiz de Queiroz” – University of São Paulo (ESALQ/USP), Piracicaba, São Paulo, Brazil.

Table 1. Description of entomopathogenic fungal isolates used in the experiments.

Isolate[a]	Fungal species	Origin	Location in Brazil City - State[b]
ESALQ 43	*Metarhizium anisopliae*	Hemiptera: Cercopidae	Fleixeiras del Estado - AL
ESALQ 1604	*Metarhizium anisopliae*	Hemiptera: Cercopidae	Unknown
ESALQ 1641	*Metarhizium anisopliae*	Hemiptera: Cercopidae	Boca da Mata - AL
ESALQ 1610	*Metarhizium anisopliae*	Banana soil	Sinop - MT
ESALQ 1669	*Metarhizium anisopliae*	Sugarcane soil	Iracemápolis - SP
ESALQ 1622	*Metarhizium robertsii*	Corn soil	Sinop - MT
ESALQ 1629	*Metarhizium robertsii*	Corn soil	Sinop - MT
ESALQ 1618	*Metarhizium robertsii*	Forest soil	Bagé - RS
ESALQ 1634	*Metarhizium robertsii*	Forest soil	Delmiro Gouveia - AL
ESALQ 1635	*Metarhizium robertsii*	Forest soil	Delmiro Gouveia - AL
ESALQ 1608	*Metarhizium* sp. Indet. 1*	Savanna Soil	Rio Verde - GO
ESALQ 1637	*Metarhizium* sp. Indet. 1*	Savanna Soil	Rio Verde - GO
ESALQ 1638	*Metarhizium* sp. Indet. 1*	Savanna Soil	Rio Verde - GO
ESALQ 1636	*Metarhizium* sp. Indet. 2*	Savanna Soil	Sinop - MT
ESALQ 1684	*Metarhizium* sp. Indet. 4*	Sugarcane rhizosphere	Iracemápolis - SP
ESALQ PL63	*Beauveria bassiana*	Hymenoptera: Formicidae	Piracicaba - SP
ESALQ 1451	*Beauveria bassiana*	Coleoptera: Curculionidae	Piracicaba - SP
ESALQ 1587	*Beauveria bassiana*	Strawberry soil	Inconfidentes - MG
ESALQ 3323	*Beauveria bassiana*	Strawberry soil	Inconfidentes - MG
ESALQ 3375	*Beauveria bassiana*	Strawberry soil	Senador Amaral - MG
ESALQ 1296	*Cordyceps fumosorosea*	Hemiptera: Aleyrodidae	Jaboticabal - SP
ESALQ 1709	*Cordyceps fumosorosea*	Strawberry soil	Cambuí - MG
ESALQ 3692	*Cordyceps fumosorosea*	Strawberry soil	Inconfidentes - MG
ESALQ 3693	*Cordyceps fumosorosea*	Strawberry rhizosphere	Inconfidentes - MG
ESALQ 3703	*Cordyceps fumosorosea*	Strawberry rhizosphere	Inconfidentes - MG

[a]Isolates (identified to species level by molecular techniques) from the entomopathogen collection of the "Laboratory of Pathology and Microbial Control of Insects" at Escola Superior de Agricultura “Luiz de Queiroz” – University of São Paulo (ESALQ/USP), Piracicaba, São Paulo, Brazil.

[b]Brazilian states abbreviations: AL: Alagoas, MT: Mato Grosso, SP: São Paulo, RS: Rio Grande do Sul, GO: Goiás.

*Isolates belonging to three separate taxonomically unassigned lineages of *Metarhizium* (Rezende et al., 2015).

3.2.1.2. *Tetranychus urticae* cultures

A colony of *T. urticae* was originally obtained from the Department of Acarology at ESALQ/USP, Piracicaba, São Paulo, Brazil, which was kept on Jack bean plants, *Canavalia ensiformis* L. DC (Fabaceae) in laboratory cages at 28ºC and 12h photophase. The spider mites were then transferred to strawberry plants (cultivar

'Albion' and 'Pircinque') and reared in laboratory cages at ambient light and temperature conditions, in the Laboratory of Pathology and Microbial Control of Insects at ESALQ/USP.

3.2.2. Fungal suspensions

Each fungal isolate was retrieved from the -80ºC culture collection and plated onto Petri dishes (90 x 15 mm) containing 20 ml Potato Dextrose Agar (PDA; Merck, Darmstadt, Germany) and kept at 26ºC and 12h photophase for 10 days. Subsequently, conidia were harvested by adding 10 ml sterile 0.05% Tween 80® to the culture and scraping off with a sterile spatula. Conidial concentrations were adjusted to 1 x 10^8 conidia ml^{-1} by using a Neubauer hemocytometer (Merck, Darmstadt, Germany). Then, 10 ml of each suspension was inoculated with a pipette into individual 250 ml Schott bottles containing 50 g autoclaved (121ºC, 20 min) parboiled rice and incubated at 26ºC and 12h photophase for 10 days, in order to multiply and obtain the required amount of conidia for the experiments.

Prior to use in the experiment, the conidial viability was checked by preparing a conidial suspension by adding 1 g of rice with sporulating fungi from the Schott bottle to 10 ml sterile 0.05% Tween 80® and diluting it to 10^{-3}. Then, 150 µl of this conidial suspension was transferred to PDA and the percentage of conidia germination was evaluated after 24 h according to Oliveira et al. (2015). Suspensions were only used if germination rates were higher than 95%.

Conidia from each isolate was then harvested by adding 150 ml sterile distilled water and 0.05% Tween 80® into the Schott bottles with rice and fungi. The resulting conidial suspension was then sieved through a sterile sieve and poured into a sterile 250 ml Erlenmeyer flask and conidial concentrations were adjusted to 1 x 10^8 conidia ml^{-1} by using a Neubauer hemocytometer (Merck, Darmstadt, Germany).

Two reference treatments with products that are reported to have growth promotion abilities to other crops were included. This was the isolate ESALQ 1306 of *Trichoderma harzianum* (active ingredient of the commercial product Trichodermil® SC 1306, Koppert, Brazil) and the inoculum was prepared in the same way as for the other entomopathogenic fungal isolates tested. The other product was Nemix® FMC that contains 1.6 x 10^{10} colony forming units (CFUs)/g *Bacillus subtilis* + 1.6 x 10^{10} CFUs/g

Bacillus licheniformis at a dose of 1g/100ml which was the concentration used here. The control treatment consisted of sterile distilled water with 0.05% Tween® 80.

3.2.3. Root inoculation and experimental set up of strawberry cultivar 'Albion'

Strawberry plants, *Fragaria* x *ananassa* cultivar 'Albion' (University of California, 2006), were obtained at 2-4 leaf stage from the nursery "Irmãos Baptistella", Itatiba, São Paulo, Brazil.

Roots of individual strawberry plant were immersed for two min in 30 ml for each treatment. Plants were then immediately transplanted individually into 2 L pots containing 50% of surface soil, 40% of substrate Tropstrato V-9 Mix (Vida Verde, Mogi Mirim, São Paulo, Brazil) and 10% of medium texture sand (250 mm). The remaining suspensions of each treatment after root dipping were poured over the soil substrate of each respective strawberry plant. Treated strawberry plants were grown in a greenhouse (22º42'45.918''N, 47º37'36.811''W) for 180 days at 28±2ºC and 12 h L: 12 h D, with biweekly fertilization, switching between the fertilizers Master 13.40.13 (13% N – 40% P – 13% K), Buschle & Lepper, Santa Catarina, Brazil and Master 3.11.38 (3% N – 11% P – 38% K) Valagro, Atessa, Italy. The fertilizer Brexil (10% S, 1.5% Mg, 2% B, 5% Mn, 0.5% Mo, 6% Zn), Buschle & Lepper, Santa Catarina, Brazil was also used once a month.

Pots with treated strawberry plants were arranged as a randomized block design with ten replicate plants for each of the 28 treatments. The experiment was repeated twice, one time from July 2016 to January 2017, and other time from March to October 2017.

3.2.3.1. Evaluation of effect on *T. urticae* oviposition

Sixty days after inoculation of strawberry roots, one *T. urticae* female from the laboratory rearing was placed on a randomly selected leaflet of each of five strawberry plants per treatment while the other five plants per treatment were not infested with *T. urticae*. After infestation, the leaflet with one *T. urticae* female was covered with a clip cage (4.5 cm high, 3.8 cm diameter) with fine mesh at the open top end (0.09 mm mesh size) preventing the spread of *T. urticae* to other parts of the plant. Seven days after the infestation with *T. urticae* females, each infested leaflet was detached and the

number of eggs and post-embryonic immatures under the clip cage was counted under a 10X stereomicroscope (Optech, München, Germany).

3.2.3.2. Evaluation of effect on inoculated strawberry plant growth and fruit yield

Plant growth parameters and production of fruits were also evaluated for all strawberry plants inoculated with either of the 25 isolates of entomopathogenic fungi, two reference treatments (*T. harzianum* ESALQ 1306 and Nemix® FMC) and control (0.05% Tween® 80). All ripe strawberries were collected and weighed weekly at the beginning of fruit bearing, for a total of 20 harvest events. At the end of the evaluations (180 days after inoculation), all plants were uprooted and washed in tap water to remove soil and plants were separated into above and below ground parts.

Length of roots per plant was then measured and fresh weights of roots and aerial part (stem and leaves) were recorded on an electronic balance to nearest 0.5 g (Bel, Mark 5200 model, Brazil). Roots and aerial plant parts were then placed inside separate paper bags and kept in a drying oven (Marconi, MA033 model, Brazil) at 60ºC for 3 days, and below and above ground dry weight biomass were recorded.

3.2.4. Root inoculation and experimental set up of strawberry cultivar 'Pircinque'

The second greenhouse experiment was conducted from January to July 2018, with the strawberry cultivar 'Pircinque' (PIR 04.228.5, Italy, 2010). Seedlings at the 2-4 leaf stage were obtained from the nursery "Irmãos Baptistella". The same 25 isolates, the reference treatment with *T. harzianum* and control (0.05% Tween® 80) were the same as described for 'Albion', but instead of using Nemix, the product Quartzo® FMC (1.0×10^{11} CFUs/g *Bacillus subtilis* + 1.0×10^{11} CFUs/g *Bacillus licheniformis*) was used as reference treatment in this experiment, because Nemix was no longer available. The experimental set up and substrates were also the same reported for 'Albion' cultivar.

Also, in this experiment with 'Pircinque', the effect on number of *T. urticae* eggs and post-embryonic immatures was evaluated twice, first at 60 days and then at 120 days after root inoculation, in order to evaluate if effects of the fungal inoculations were also evident after 120 days. Hence infestation of plants with adult *T. urticae* females in clip cages on random leaf of strawberry plants were conducted 60 and 120 days after

inoculation. The effect on plant growth parameters was evaluated as described for ‘Albion’.

3.2.5. Evaluation of occurrence of entomopathogenic fungi in strawberry plants and soil samples

The occurrence of isolates of *Metarhizium* spp*., Beauveria bassiana* and *Cordyceps fumosorosea* was evaluated in five strawberry plants and soil samples after 180 days of inoculations of each treatment in all experiments. Three root fragments (5 cm) and three sections of leaves (4 cm x 1 cm) were cut from each plant. These root fragments and leaf sections were surface sterilized by immersion in 70% ethanol for 1 min, 1% sodium hypochlorite for 2 min, one more time in 70% ethanol for 1 min and then rinsed three times in sterile distilled water and air dried on sterile filter paper for 1 min. The efficacy of the sterilization was confirmed by plating 100 µl of the last rinsing water onto PDA (Parsa et al., 2013). Subsequently, root fragments (three) and leaf sections (three) were placed in individual Petri dishes (90 x 15 mm) with 20 ml of a selective media containing PDA with 0.5 $g.L^{-1}$ of cycloheximide, 0.2 $g.L^{-1}$ of chloramphenicol, 0.5 $g.L^{-1}$ of Dodine (65%) and 0.01 $g.L^{-1}$ of Crystal Violet (Behie et al., 2015). Root and leaf samples were then gently pressed with the cut edge into the agar and incubated at 26°C and 12h photophase for 15 days before observation of fungal growth and morphological identification of fungal genera, according to Humber (2012). Soil samples from the soil adjacent to strawberry plant roots from pots were also plated on the selective media described above. Five samples from de same pots used to plant fragments were performed for each treatment. This was done by adding 1 g of soil sample from each pot to 10 ml of sterile water with 0.05% Tween 80®. The suspension was then vigorously vortexed for 30 s and four consecutive ten-fold serial dilutions in distilled water + 0.05% Tween 80® were prepared. Petri dishes (90 x 15 mm) with the selective agar media were divided into four equal quarters by marking the bottom of the Petri dish with a permanent marker and 100 µl of each of the four dilutions was pipetted onto each quarter. Petri dishes were then incubated at 26°C and 12 h photophase for 15 days and the presence of entomopathogenic fungi was detected according to fungal growth in each plate. The frequency of occurrence was estimated as the number of plant fragments or soil samples with entomopathogenic fungi in relation to the total number of samples and expressed in percentages.

3.2.6. Statistical analysis

To account for overdispersion on count data, we fitted negative binomial generalized linear models to the sum of number of eggs and number of post-embryonic immature *T. urticae* per clip cage in the experiment with the cultivar 'Albion', using the linear predictor (Demétrio et al., 2014):

$$\log(\mu) = \text{Exp} + \text{Bl [Exp]} + \text{Treat} + \text{Treat : Exp}$$

where Exp, Bl [Exp], Treat and Treat : Exp are the effects of experiment, block within experiments, treatment and interaction treatment by experiment, respectively. Multiple comparisons were performed using the Scott Knott test at a 95% confidence level (Bony et al., 2001).

Generalized additive models (GAMLSS) (Stasinopoulos and Rigby, 2007) were fitted to the length of roots (Y), using normal distribution for the response variable ($Y \sim N(\mu, \sigma^2)$) and modelling the location and scale parameters to account for mean differences and heterogeneity of variance, that is:

$$\mu = \text{Exp} + \text{Bl [Exp]} + \text{Treat} + \text{Treat : Exp} \quad (1)$$

and:

$$\log \sigma^2 = \text{Treat : Exp}.$$

A classical linear model with normal distribution was fitted to the weight of fruits and the linear predictor given by (1), while an inverse Gaussian model was fitted to dry weight of roots and dry weight of leaves, using the same linear predictor (1). Significance was assessed using F tests. Multiple comparisons were performed using the Scott Knott test at a 95% confidence level, to group treatments that represented similar results.

For the cultivar 'Pircinque', quasi-Poisson models were fitted to the count data (sum of number of eggs and number of post-embryonic immatures of *T. urticae* per clipcage at 60 and 120 days after inoculation) to account for overdispersion (Demétrio et al., 2014) with linear predictor given by:

$$\log(\mu) = \text{Block} + \text{Treat}.$$

Multiple comparisons were carried out by obtaining the 95% confidence intervals for the linear predictors. Classical linear models with normal distribution were fitted to the length of roots, dry weight of roots and aerial part and weight of fruits, using the linear predictor

$$\mu = \text{Block} + \text{Treat}.$$

Multiple comparisons were performed using the Scott Knott test at a 95% confidence level.

3.3. Results

3.3.1. Effect of inoculated strawberry plants on number of eggs and post-embryonic immatures of *T. urticae* (cultivar 'Albion')

Root inoculation of strawberry plants with all fungal isolates and the bacterial mix (Nemix®) significantly affected the number of *T. urticae* eggs and post-embryonic immatures produced over a period of 7 days in clip cages 60 days after inoculation (60 DAI) (Deviance = 203.57, d.f. = 27, p<0.0001). All *Metarhizium* spp. and *B. bassiana* isolates and four of the five *C. fumosorosea* isolates significantly reduced the number of *T. urticae* eggs and post-embryonic immatures compared to the control treatment and to the *T. harzianum* (active ingredient of Trichodermil®) and Nemix® (*B. subtilis* + *B. licheniformis*) treatments (Figure 1).

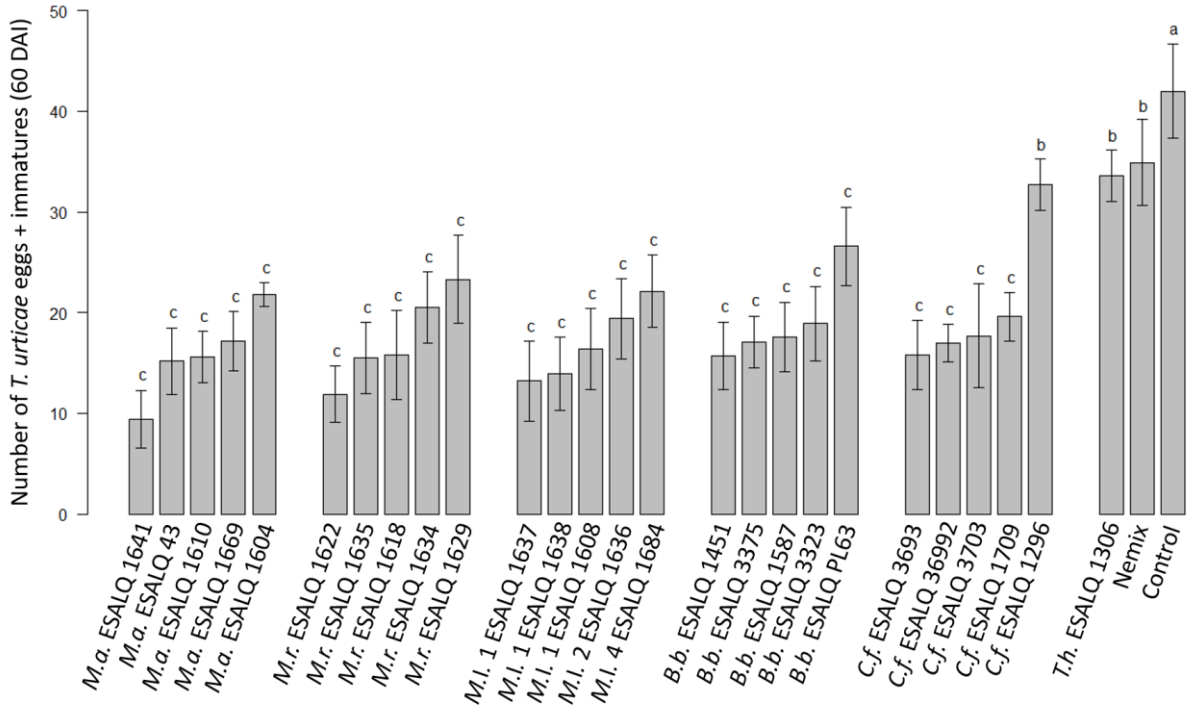

Figure 1. Effect of strawberry (cultivar 'Albion') root inoculation on number of eggs + post-embryonic immatures of *Tetranychus urticae* produced per female after 7 days in clip cages. Plants had been root inoculated for 60 days with entomopathogenic fungal

isolates, *Trichoderma harzianum* ESALQ 1306 (active ingredient of the commercial product Trichodermil®, Koppert, Brazil), Nemix® FMC (commercial product with *Bacillus subtilis* + *Bacillus licheniformis*) and sterile distilled water with 0.05% Tween® 80 (Control). *M.a*.= *Metarhizium anisopliae*; *M.r*.= *M. robertsii*; *M.*I. = *Metarhizium* sp. Indet.; *B.b*.= *Beauveria bassiana*; *C.f*.= *Cordyceps fumosorosea*; or with *T.h.* = *Trichoderma harzianum* ESALQ 1306. Different letters denote significant statistical differences (p = 0.05).

3.3.2. Effects on inoculated strawberry plant growth and fruit yield (cultivar 'Albion')

Significant effect of treatments on plant growth parameters (root length, dry weight of roots and leaves) and fruit yield (weight of fruits) were observed (Table 2).

No difference in the root length among treatments 180 days after inoculation was observed. However, dry root weight was significantly higher for four *M. anisopliae* isolates (ESALQ 1604, ESALQ 1641, ESALQ 1610 and ESALQ 1669); three *M. robertsii* isolates (ESALQ 1622, ESALQ 1635 and ESALQ 1618); *Metarhizium* sp. Indet. 1 (ESALQ 1608), *Metarhizium* sp. Indet. 2 (ESALQ 1636), *Metarhizium* sp. Indet. 4 (ESALQ 1684), *B. bassiana* (ESALQ 3323), *C. fumosorosea* (ESALQ 1709) and *T. harzianum* (ESALQ 1306 active ingredient of Trichodermil®) compared to Nemix® (*B. subtilis* + *B. licheniformis*) and the control.

Dry leaf weight was significantly higher for *M. anisopliae* (ESALQ 1604 and ESALQ 1669), *M. robertsii* (ESALQ 1622 and ESALQ 1635), *Metarhizium* sp. Indet. 1 (ESALQ 1637), *Metarhizium* sp. Indet. 4 (ESALQ 1684), *B. bassiana* (ESALQ 3323) compared to *T. harzianum* (ESALQ 1306, the active ingredient of Trichodermil®), Nemix® (*B. subtilis* + *B. licheniformis*) and the control.

Fruit yield was significantly higher for two isolates of *M. anisopliae* (ESALQ 1604 and ESALQ 1669), three isolates of *M. robertsii* (ESALQ 1622, ESALQ 1634 and ESALQ 1635), *Metarhizium* sp. Indet. 2 (ESALQ 1636), two isolates of *B. bassiana* (ESALQ 3323 and ESALQ PL63), and for *T. harzianum* (ESALQ 1306, the active ingredient of Trichodermil®) compared to Nemix® (*B. subtilis* + *B. licheniformis*) and the control.

Table 2. Effects of root inoculation of strawberry (cultivar 'Albion') with 25 different entomopathogenic fungal isolates, *Trichoderma harzianum* ESALQ 1306 (active ingredient of Trichodermil®), Nemix® (commercial product with *Bacillus subtilis* + *Bacillus licheniformis*) and sterile distilled water with 0.05% Tween® 80 (Control) on mean (±SE) values of root length, dry weight of roots and leaves 180 days after inoculation, and on cumulated weight of fruits per plant during (20 harvests). Separate analyses were performed for each response variable.

Isolate*	Assessment[1]			
	Root length (cm)	Dry root weight (g)	Dry leaf weight (g)	Fruit yield (g)
M.a. ESALQ 43	27.2 ± 1.47	1.4 ± 0.07 b	3.4 ± 0.32 b	69.5 ± 7.88 b
M.a. ESALQ 1604	28.2 ± 1.38	1.7 ± 0.15 a	4.5 ± 0.51 a	107.3 ± 12.58 a
M.a. ESALQ 1641	27.3 ± 2.44	1.6 ± 0.09 a	3.5 ± 0.37 b	71.7 ± 7.25 b
M.a. ESALQ 1610	28.0 ± 1.32	1.7 ± 0.09 a	3.7 ± 0.46 b	83.4 ± 8.11 b
M.a. ESALQ 1669	30.2 ± 1.13	2.0 ± 0.15 a	4.3 ± 0.67 a	93.8 ± 6.46 a
M.r. ESALQ 1622	30.9 ± 1.39	1.7 ± 0.14 a	4.3 ± 0.36 a	93.2 ± 8.93 a
M.r. ESALQ 1629	29.3 ± 1.62	1.4 ± 0.06 b	3.5 ± 0.33 b	81.5 ± 7.19 b
M.r. ESALQ 1618	27.3 ± 1.21	1.5 ± 0.13 a	3.8 ± 0.34 b	82.5 ± 8.71 b
M.r. ESALQ 1634	26.6 ± 1.73	1.3 ± 0.06 b	3.7 ± 0.42 b	99.9 ± 9.78 a
M.r. ESALQ 1635	29.6 ± 1.29	1.5 ± 0.15 a	4.4 ± 0.26 a	104.7 ± 12.46 a
M. I. 1 ESALQ 1608	24.9 ± 1.81	1.5 ± 0.14 a	3.6 ± 0.25 b	72.9 ± 10.06 b
M. I. 1 ESALQ 1637	26.7 ± 1.37	1.2 ± 0.05 b	4.1 ± 0.39 a	83.0 ± 5.02 b
M. I. 1 ESALQ 1638	30.6 ± 1.52	1.2 ± 0.04 b	3.2 ± 0.26 b	84.7 ± 4.55 b
M. I. 2 ESALQ 1636	26.6 ± 1.34	1.7 ± 0.12 a	3.8 ± 0.20 b	95.3 ± 4.80 a
M. I. 4 ESALQ 1684	26.9 ± 1.24	1.7 ± 0.17 a	4.2 ± 0.57 a	77.9 ± 10.47 b
B.b. ESALQ PL63	29.5 ± 1.70	1.4 ± 0.11 b	3.6 ± 0.24 b	99.5 ± 9.34 a
B.b. ESALQ 1451	30.9 ± 1.33	1.4 ± 0.08 b	2.9 ± 0.18 b	65.0 ± 4.98 b
B.b. ESALQ 1587	28.3 ± 1.30	1.2 ± 0.04 b	3.6 ± 0.36 b	74.8 ± 7.53 b
B.b. ESALQ 3323	27.9 ± 1.39	1.7 ± 0.12 a	3.9 ± 0.41 a	108.8 ± 7.61 a
B.b. ESALQ 3375	31.3 ± 1.55	1.3 ± 0.06 b	3.7 ± 0.36 b	82.8 ± 6.92 b
C.f. ESALQ 1296	27.2 ± 1.74	1.3 ± 0.03 b	2.9 ± 0.16 b	83.6 ± 5.32 b
C.f. ESALQ 1709	30.5 ± 1.03	1.8 ± 0.18 a	3.4 ± 0.25 b	83.1 ± 6.22 b
C.f. ESALQ 3692	26.8 ± 0.98	1.3 ± 0.04 b	3.5 ± 0.34 b	87.2 ± 12.23 b
C.f. ESALQ 3693	29.0 ± 0.86	1.3 ± 0.05 b	3.5 ± 0.26 b	71.9 ± 8.17 b
C.f. ESALQ 3703	29.0 ± 1.65	1.3 ± 0.10 b	3.5 ± 0.35 b	86.4 ± 6.76 b
T.h. ESALQ 1306	28.2 ± 1.51	1.5 ± 0.09 a	3.4 ± 0.33 b	92.4 ± 6.07 a
Nemix®	29.5 ± 2.09	1.3 ± 0.05 b	3.6 ± 0.26 b	86.8 ± 11.87 b
Control	25.6 ± 1.60	1.3 ± 0.07 b	3.3 ± 0.37 b	61.9 ± 6.61 b
Test statistic	LR=29.69, d.f.=27	$F_{27,531} = 4.68$	$F_{27,531} = 2.83$	$F_{27,531} = 2.07$
p-value	$P = 0.3280$	$P < 0.0001$	$P < 0.0001$	$P = 0.0014$

[1]Data (mean ± SE) followed by different letters within a column are significantly different (GLM, by *post hoc* Scott Knott test, $P < 0.05$).

**M.a.*= *Metarhizium anisopliae*; *M.r.*= *M. robertsii*; *M.*I.= *Metarhizium* sp. Indet.; *B.b.*= *Beauveria bassiana*; *C.f.*= *Cordyceps fumosorosea*, *T.h.* = *Trichoderma harzianum*; Nemix® = commercial product with *Bacillus subtilis* + *Bacillus licheniformis*; Control = sterile distilled water with 0.05% Tween® 80.

3.3.3. Effect of inoculated strawberry plants on number of eggs and post-embryonic immatures of *T. urticae* (cultivar 'Pircinque')

Root inoculation with entomopathogenic fungi significantly influenced number of *T. urticae* eggs and post-embryonic immatures at 60 days after inoculation (Deviance = 415.77, d.f. = 27, p = 0.0001; Figure 2) and at 120 days after inoculation (Deviance = 186.55, d.f. = 27, p = 0.0356; Figure 3). More specifically, plants inoculated with all 15 *Metarhizium* spp. isolates and five *B. bassiana* and *C. fumosorosea* isolates (except for the isolate ESALQ 3703 60 days after inoculation) had significantly lower number of *T. urticae* eggs and post-embryonic immatures both 60 and 120 days after inoculation (Figure 2 and Figure 3, respectively).

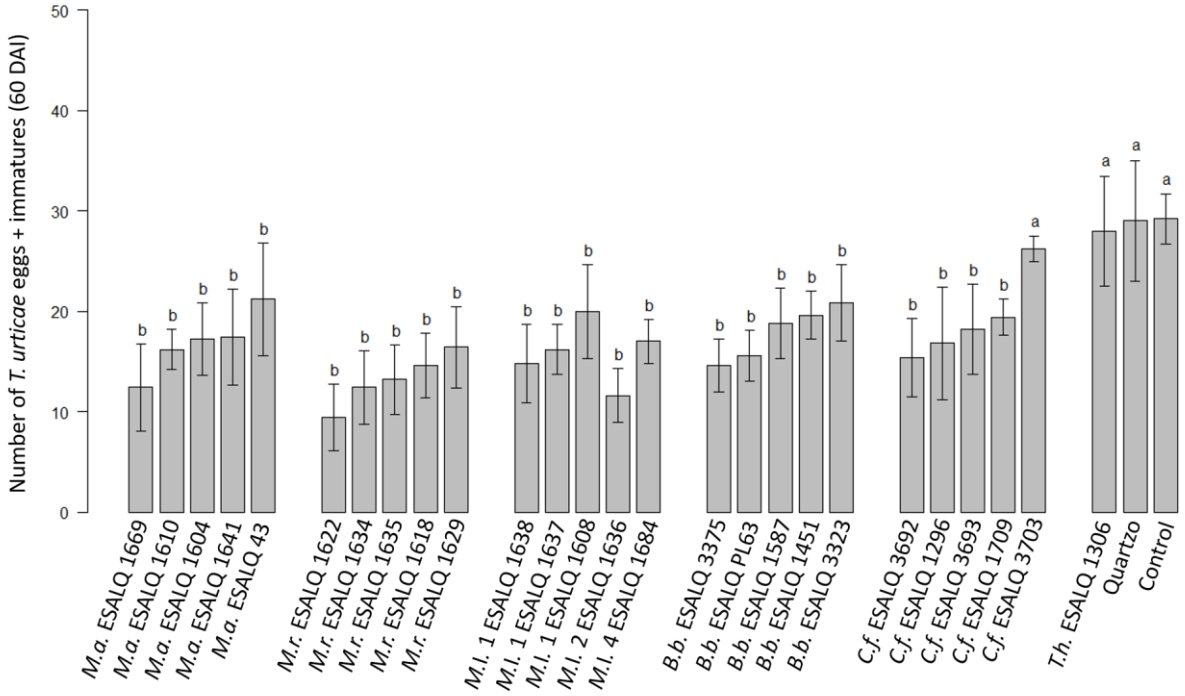

Figure 2. Effect of strawberry (cultivar 'Pircinque') root inoculation on number of eggs + post-embryonic immatures of *Tetranychus urticae* produced per female after 7 days in clip cages. Plants had been root inoculated for 60 days with entomopathogenic fungal isolates, *Trichoderma harzianum* ESALQ 1306 (active ingredient of the commercial product Trichodermil®, Koppert, Brazil), Quartzo® FMC (commercial product with *Bacillus subtilis* + *Bacillus licheniformis*) and sterile distilled water with 0.05% Tween® 80 (Control). *M.a.*= *Metarhizium anisopliae*; *M.r.*= *M. robertsii*; *M.*I.= *Metarhizium* sp. Indet.; *B.b.*= *Beauveria bassiana*; *C.f.*= *Cordyceps fumosorosea*; or

with *T.h. = Trichoderma harzianum* ESALQ 1306. Different letters denote significant statistical differences (p = 0.05).

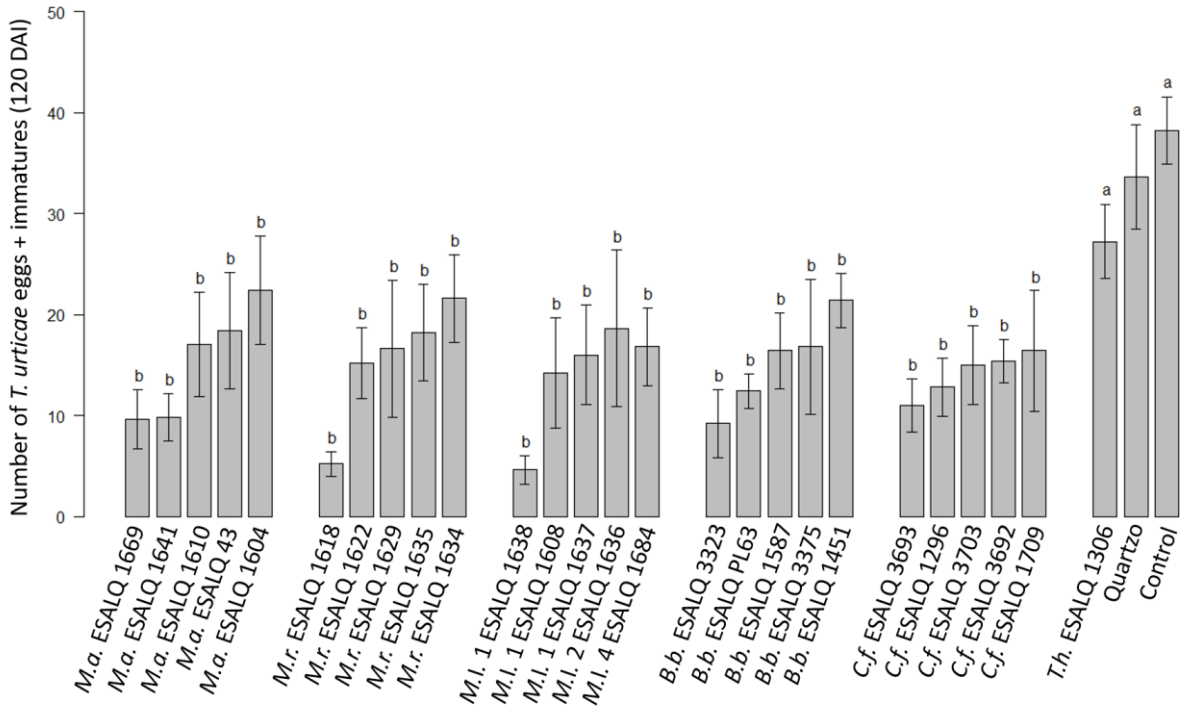

Figure 3. Effect of strawberry (cultivar 'Pircinque') root inoculation on number of eggs + post-embryonic immatures of *Tetranychus urticae* produced per female after 7 days in clip cages. Plants had been root inoculated for 120 days with entomopathogenic fungal isolates, *Trichoderma harzianum* ESALQ 1306 (active ingredient of the commercial product Trichodermil®, Koppert, Brazil), Quartzo® FMC (commercial product with *Bacillus subtilis* + *Bacillus licheniformis*) and sterile distilled water with 0.05% Tween® 80 (Control). *M.a.*= *Metarhizium anisopliae*; *M.r.*= *M. robertsii*; *M.*I.= *Metarhizium* sp. Indet.; *B.b.*= *Beauveria bassiana*; *C.f.*= *Cordyceps fumosorosea*; or with *T.h. = Trichoderma harzianum* ESALQ 1306. Different letters denote significant statistical differences (p = 0.05).

3.3.4. Effect on inoculated strawberry plant growth and fruit yield (cultivar 'Pircinque')

Inoculation of strawberry roots (cultivar 'Pircinque') with 25 entomopathogenic fungal isolates showed no significant difference in root length, dry leaf weight or fruit

yield between fungal treated plants and control plants. Dry root weight was, however, significantly higher for *M. anisopliae* ESALQ 1669 (Table 3).

Table 3. Effects of root inoculation of strawberry (cultivar 'Pircinque') with 25 different entomopathogenic fungal isolates, *Trichoderma harzianum* ESALQ 1306 (active ingredient of Trichodermil®), Quartzo® (commercial product with *Bacillus subtilis* + *Bacillus licheniformis*) and sterile distilled water with 0.05% Tween® 80 (Control) on mean (±SE) values of root length, dry weight of roots and leaves 180 days after inoculation, and on cumulated weight of fruits per plant during (20 harvests). Separate analyses were performed for each response variable.

Isolate*	Assessment[1]			
	Roots lenght (cm)	Dry root weight (g)	Dry leaf weight (g)	Fruit yield (g)
M.a. ESALQ 43	49.5 ± 4.05	3.4 ± 0.42 b	3.3 ± 0.29	44.3 ± 3.93
M.a. ESALQ 1604	45.8 ± 4.05	2.6 ± 0.32 b	2.8 ± 0.41	40.9 ± 2.10
M.a. ESALQ 1641	43.0 ± 3.99	2.5 ± 0.21 b	2.8 ± 0.28	37.9 ± 4.32
M.a. ESALQ 1610	48.0 ± 3.62	2.3 ± 0.37 b	2.5 ± 0.26	35.3 ± 2.93
M.a. ESALQ 1669	39.0 ± 2.58	5.1 ± 0.45 a	4.2 ± 0.25	35.5 ± 6.03
M.r. ESALQ 1622	43.6 ± 3.10	2.9 ± 0.30 b	3.2 ± 0.24	46.6 + 2.81
M.r. ESALQ 1629	45.2 ± 2.15	3.5 ± 0.54 b	3.2 ± 0.27	39.0 ± 3.01
M.r. ESALQ 1618	43.3 ± 3.45	3.7 ± 0.51 b	3.7 ± 0.34	39.3 ± 2.75
M.r. ESALQ 1634	43.8 ± 3.63	2.5 ± 0.25 b	2.8 ± 0.27	44.8 ± 3.09
M.r. ESALQ 1635	42.0 ± 4.36	2.7 ± 0.29 b	3.1 ± 0.33	36.6 ± 3.00
M. I. 1 ESALQ 1608	46.1 ± 4.11	3.1 ± 0.40 b	3.3 ± 0.31	41.9 ± 3.57
M. I. 1 ESALQ 1637	44.1 ± 2.21	3.1 ± 0.18 b	3.6 ± 0.15	36.4 ± 4.73
M. I. 1 ESALQ 1638	41.5 ± 2.66	2.8 ± 0.47 b	3.0 ± 0.45	41.9 ± 4.51
M. I. 2 ESALQ 1636	45.2 ± 2.38	3.3 ± 0.39 b	3.5 ± 0.41	38.9 ± 5.33
M. I. 4 ESALQ 1684	46.7 ± 3.17	2.9 ± 0.28 b	3.5 ± 0.19	41.5 ± 4.13
B.b. ESALQ PL63	39.9 ± 1.37	2.8 ± 0.48 b	3.2 ± 0.29	47.3 ± 2.12
B.b. ESALQ 1451	44.0 ± 3.81	2.6 ± 0.36 b	3.3 ± 0.30	35.9 ± 2.80
B.b. ESALQ 1587	44.5 ± 3.30	2.3 ± 0.24 b	2.6 ± 0.30	33.8 ± 2.54
B.b. ESALQ 3323	41.7 ± 3.20	2.9 ± 0.36 b	3.3 ± 0.22	41.6 ± 2.96
B.b. ESALQ 3375	42.4 ± 1.87	3.2 ± 0.49 b	3.5 ± 0.46	43.3 ± 3.79
C.f. ESALQ 1296	41.2 ± 3.04	2.8 ± 0.32 b	3.2 ± 0.40	38.8 ± 3.57
C.f. ESALQ 1709	40.1 ± 3.16	2.8 ± 0.39 b	3.4 ± 0.34	41.0 ± 2.86
C.f. ESALQ 3692	45.9 ± 3.06	2.6 ± 0.25 b	3.1 ± 0.21	41.1 ± 3.47
C.f. ESALQ 3693	45.5 ± 3.21	3.4 ± 0.61 b	3.6 ± 0.40	35.6 ± 3.14
C.f. ESALQ 3703	44.3 ± 1.17	3.4 ± 0.53 b	3.6 ± 0.27	35.5 ± 4.37
T.h. ESALQ 1306	43.4 ± 2.35	3.0 ± 0.12 b	3.6 ± 0.34	33.6 ± 4.36
Quartzo®	47.8 ± 4.21	2.6 ± 0.39 b	3.0 ± 0.21	47.2 ± 3.35
Control	38.1 ± 3.01	2.4 ± 0.33 b	2.6 ± 0.23	37.5 ± 3.74
Test statistic	$F_{27,243} = 0.75$	$F_{27,243} = 2.03$	$F_{27,243} = 1.52$	$F_{27,243} = 1.33$
p-value	$P = 0.8104$	$P = 0.0027$	$P = 0.0524$	$P = 0.1307$

[1]Data (mean ± SE) followed by different letters within a column are significantly different (GLM, by *post hoc* Scott Knott test, $P < 0.05$).

*M.a.= *Metarhizium anisopliae*; *M.r.= *M. robertsii*; *M.*I. = *Metarhizium* sp. Indet.; *B.b.= *Beauveria bassiana*; *C.f.= *Cordyceps fumosorosea*; *T.h. = *Trichoderma harzianum*; Quartzo® = commercial product with *Bacillus subtilis* + *Bacillus licheniformis*); Control = sterile distilled water with 0.05% Tween® 80.

3.3.5. Occurrence of entomopathogenic fungi in strawberry plants and soil samples

The frequencies of occurrence (%) of the entomopathogenic fungal treatments in samples of root, leaf and soil of strawberry plants ('Albion' and 'Pircinque') 180 days after inoculation, are presented in Table 4.

The first repetition with the cultivar 'Albion', resulted in low colonization rates of roots and leaves for all treatments but all soil samples resulted in recovery of fungal treatments, except for one *B. bassiana* isolate (ESALQ 3323). In the second repetition with the cultivar 'Albion', the frequencies of occurrence were higher than in the first. Among the 15 *Metarhizium* spp. tested, just three were not recovered from roots and four from leaves. Three *B. bassiana* treatments were found in roots and two in leaves, but just two *C. fumosorosea* were recovered from roots, and none from leaves. All treatments except two *B. bassiana* were recovered from soil samples, and several treatments gave a 100% recovery from soil 180 days after inoculation, most of them belonging to *Metarhizium* spp.

In the experiment with cultivar 'Pircinque' most of the fungal isolates were recovered in root samples, except from two of *B. bassiana* and two of *C. fumosorosea* treatments. Only two *B. bassiana*, three *C. fumosorosea* and none of the *Metarhizium* treatments resulted in recovery of fungal isolates from leaves. Almost all treatments resulted in recovery of fungi with similar morphology as the treatment from soil samples.

None of the root, leaf or soil samples from the control showed isolation of entomopathogenic fungi of genera similar to the inoculated fungi. Occasionally, however, other unidentified fungi were found on selective media from the surface sterilized plant tissues and soil.

Table 4. Frequencies of occurrence (%) of entomopathogenic fungi in samples of root, leaf and soil of strawberry plants 180 days after inoculation, in the two experiments with the cultivar ‘Albion’, and the single experiment with the cultivar ‘Pircinque’.

Treatments*	Experiment - cultivar ‘Albion’						Experiment - cultivar ‘Pircinque’		
	First repetition			Second repetition			Single experiment		
	Root (%)	Leaf (%)	Soil (%)	Root (%)	Leaf (%)	Soil (%)	Root (%)	Leaf (%)	Soil (%)
M.a. ESALQ 43	20	-	60	60	40	20	40	-	-
M.a. ESALQ 1604	40	-	80	-	-	40	40	-	40
M.a. ESALQ 1641	-	-	60	60	20	100	40	-	60
M.a. ESALQ 1610	-	-	60	60	20	100	20	-	40
M.a. ESALQ 1669	-	-	40	20	-	100	20	-	40
M.r. ESALQ 1622	-	-	60	60	40	100	40	-	40
M.r. ESALQ 1629	20	-	40	40	20	100	100	-	20
M.r. ESALQ 1618	40	-	100	100	60	100	60	-	-
M.r. ESALQ 1634	-	-	40	80	60	80	20	-	-
M.r. ESALQ 1635	-	-	100	40	20	80	20	-	60
M. I. 1 ESALQ 1608	20	-	60	40	40	100	40	-	20
M. I. 1 ESALQ 1637	-	20	60	20	20	100	80	-	40
M. I. 1 ESALQ 1638	-	-	80	-	20	80	80	-	80
M. I. 2 ESALQ 1636	-	-	80	-	-	60	40	-	20
M. I. 4 ESALQ 1684	20	20	40	40	-	80	40	-	40
B.b. ESALQ PL63	-	-	20	-	-	-	-	-	20
B.b. ESALQ 1451	-	20	20	-	-	40	-	-	-
B.b. ESALQ 1587	-	20	20	20	-	20	20	40	40
B.b. ESALQ 3323	-	20	-	60	60	-	20	-	40
B.b. ESALQ 3375	-	40	20	20	20	100	40	40	40
C.f. ESALQ 1296	-	-	40	20	-	100	-	-	20
C.f. ESALQ 1709	-	-	40	-	-	20	20	20	-
C.f. ESALQ 3692	20	20	60	-	-	100	40	20	-
C.f. ESALQ 3693	20	-	100	40	-	40	20	20	40
C.f. ESALQ 3703	-	-	80	-	-	100	-	-	80
H_2O + Tween 80	-	-	-	-	-	-	-	-	-

**M.a.= Metarhizium anisopliae*; *M.r.= M. robertsii*; *M.*I. = *Metarhizium* sp. Indet.; *B.b.= Beauveria bassiana* and *C.f.= Cordyceps fumosorosea*.

3.4. Discussion

This is the first study to evaluate the effects on above-ground pest control (*T. urticae*), plant growth promotion and strawberry yield when inoculating strawberry roots with a large range of isolates (25) of different species of entomopathogenic fungi. Reductions in number of *T. urticae* eggs were observed for plants inoculated with almost all isolates. Further, plants inoculated with some of the entomopathogenic fungal isolates resulted in increased plant growth and fruit yield, but only of one of the two tested strawberry cultivars. Overall, the isolates that showed the most consistent effects on *T. urticae* oviposition and increased plant growth and fruit yield were: *M. robertsii* ESALQ 1622 and ESALQ 1635; *M. anisopliae* ESALQ 1604 and ESALQ 1669, *B. bassiana* ESALQ 3323 and *C. fumosorosea* ESALQ 1709. These isolates should therefore be considered as promising candidates for plant health improvement in strawberry production in Brazil.

Reductions in oviposition of *T. urticae* females were observed in plants inoculated with almost all isolates of *Metarhizium* spp., *B. bassiana* and *C. fumosorosea* than plants inoculated with *T. harzianum* ESALQ 1306, the commercial products with *Bacillus subtilis* + *Bacillus licheniformis* and control (sterile distilled water with 0.05% Tween® 80) after 60 and 120 days of root inoculation. Recently, Canassa et al. (2019) reported similar results, i.e., seed inoculation of bean plants, *P. vulgaris*, with the isolates *M. robertsii* (ESALQ 1622) and *B. bassiana* (ESALQ 3375) significantly reduced the population growth of *T. urticae*, and improved length of roots, fresh and dry weight of roots and aerial part, and also yield (number of string beans), compared to control plants. The same isolate of *M. robertsii* (ESALQ 1622) tested in the present study with strawberry plants was one of the most promising isolates when considering *T. urticae* control, plant growth and fruit yield, while *B. bassiana* isolate ESALQ 3375 reduced *T. urticae* oviposition, but showed no effects on strawberry plants growth nor fruit yield. Furthermore, Dash et al. (2018) reported a significant reduction in larval development, adult longevity and female fecundity of *T. urticae* that fed on bean plants inoculated with *B. bassiana* (B12, B13, B16), one isolate of *C. fumosorosea* (= *I. fumosorosea*) (isolate 17) and one of *Akanthomyces* (= *Lecanicillium*) *lecanii* (Zimm.) Spatafora, Kepler & B. Shrestha (isolate L1). They also found increased bean plant height and fresh shoot and root weight for all inoculated plants (Dash et al., 2018). This association between fungal entomopathogens and

plants may thus affect the spider mite development by several potential mechanisms, such as feeding deterrence, antibiotic effects, production of fungal secondary metabolites or induction of systemic plant resistance (Vega, 2008, 2018; Lopez and Sword, 2015; Jaber and Ownley, 2018).

The inter and intraspecific variability of entomopathogenic fungi in virulence against pests has been widely reported and the variations associated to fungal morphophysiological characteristics and, geographical origin (Rehner and Buckley, 2005; Kope et al., 2006; Kryukov et al. 2010). For example, Kryukov et al. (2010) studied 35 isolates of *B. bassiana* obtained from several insect taxa and reported that isolates obtained within a small area or even at the same site from conspecific insects differed significantly in virulence. Valero-Jiménez et al. (2014) analysed the effects of natural variation within 29 isolates of *B. bassiana* from different parts of the world on adult female mosquitoes, *Anopheles coluzzii* Coetzee & Wilkerson (Diptera: Culicidae) survival, for possible exploitation for malaria control. In laboratory, several phenotypic characteristics of the fungal isolates related to virulence were studied and the authors reported that all isolates killed the mosquitoes at different rates among the isolates, with significant variation in virulence (Valero-Jiménez et al., 2014). Conversely, the variability of the indirect effect of entomopathogenic fungi used as endophytes against pests has not been investigated.

In the present study, the negative effects of strawberry plants inoculated with different isolates of entomopathogenic fungi against *T. urticae* varied among fungal species and isolates. The need for screening a large range of native isolates most adapted to a specific host plant, cultivar and to local environmental conditions in order to identify the most promising endophytic isolates has already been suggested by Jaber and Ownley (2018). These authors recommended that efforts should focus on the potential effects of interactions in fungal isolates with plant genotype taking into account the differential expressions under different environmental conditions.

Our results have also demonstrated that the inoculation of strawberry roots with isolates of *Metarhizium* spp., *B. bassiana* and *C. fumosorosea* resulted in increased plant biomass and fruit yield compared to the commercial microbial products with growth promotion abilities. A number of studies previously reported positive effects of plant inoculated entomopathogenic fungi on plant growth parameters (Gurulingappa, et al., 2010; Sasan and Bidochka, 2012; Jaber and Enkerli, 2016; Dash et al. 2018; Donga et al 2018; Jaber and Araj, 2018; Sánchez-Rodríguez et al., 2018; Canassa et

al. 2019). Growth promotion observed in plants colonized by fungal entomopathogens might be attributed to the production of organic acids, phytohormones or siderophores which can change the bioavailability of several nutrients (Khan et al., 2012; Krasnoff et al., 2014; Jirakkakul et al., 2015). Furthermore, studies on endophytic fungus-plant interactions revealed that the positive effects could also be due to fixation of nutrients from insect, plant, soil and microbes (Berg, 2009; Behie et al., 2012; Behie et al., 2017). In our study, however, not all isolates had the same potential to provide these effects in the two different strawberry cultivars (‘Albion’ and ‘Pircinque’). Interestingly, the positive effects of fungal inoculations on biomass and fruit yield were observed mostly in the cultivar ‘Albion’, the cultivar presenting higher yield compared to the cultivar ‘Pircinque’. Further studies are needed to elucidate the mechanisms of the interactions of fungal isolate and plant cultivar. Biological traits exhibited by certain fungal isolates with their plants host and their environment could be related to these results (Card et al, 2016). Regarding plant cultivars, it has already been reported that different strawberry cultivars showed significant differences in quality and yield (Tonin et al., 2017), highlighting the importance to test specific responses of different cultivars, in order to draw conclusions on growth promotion potential.

The ability of several species of fungal entomopathogens to establish associations with plants could be affecting the plant growth promotion (Jaber and Enkerli, 2017). In this study it was observed that the tested entomopathogenic fungi were recorded at different frequencies of colonization and in different parts of the strawberry plants. *Metarhizium* isolates were mostly recovered from roots and soil samples close to the roots, while *Beauveria* was mostly recovered from leaf tissues. Only few treatments of *C. fumosorosea* were recovered from roots and leaves, but almost always found in the soil samples close to the roots. In several studies *B. bassiana* has been shown to more commonly colonize foliar tissues than *Metarhizium* spp., which are mainly reported as rhizosphere colonizers (Klingen et al., 2002; Ownley et al., 2008; Quesada-Moraga et al., 2009; Akello and Sikora, 2012; Akutse et al., 2013; Behie et al., 2015; Klingen et al., 2015; Jaber and Araj, 2018). Behie et al. (2015) also reported *M. robertsii* restricted to the roots of haricot bean plants, while *B. bassiana* was found throughout the plant under both laboratory (experimentally infected seeds) and field conditions (natural occurrence), indicating specific variation in the endophytic capacity of these species to colonize different plant tissues (Behie et al., 2015). There is still limited knowledge about the effects of *C. fumosorosea* as an endophyte on plant

growth, but Kwaśna and Szewczyk (2016) reported reduced length and dry weight of oak (*Quercus robur*) stems and roots after two years of *C. fumosorosea* soil inoculation (isolate not mentioned).

The associations of entomopathogenic fungi with strawberry plants and in the soil were detected 180 days after root inoculation, so these associations seem to form long-term and could be related to the effects on the growth of strawberry plants and the mite control. Jaber and Ownley (2018) indicated that the persistence of fungal colonization within plants can be further improved by repeated application of the microbial agent through foliar spray or soil drench. The endophytic colonization of plants by *B. bassiana* was reported for 47–49 days post inoculation in cassava (Greenfield et al., 2016), three months in jute (Biswas et al., 2013), eight months in coffee (Posada et al., 2007) and nine months in radiata pine (Brownbridge et al., 2012). The present results demonstrated the persistence of isolates of *Metarhizium* spp., *B. bassiana* and *C. fumosorosea* six months post-inoculations under greenhouse conditions.

The inoculation of strawberry roots with selected isolates of *Metarhizium* spp., *B. bassiana* and *C. fumosorosea* can result in reduced oviposition of the two-spotted spider mite *T. urticae* and improved growth and yield of strawberry plants. However, not all isolates have the same potential to provide these effects, emphasizing natural variation in these traits in a comparable manner to variability in virulence against hosts. The selection of native isolates adapted to local environmental conditions and different cultivars can, therefore, enable the identification of promising candidates for the development of a new biological strategy for pest control which may also stimulate plant growth and yield.

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4. ROOT INOCULATION OF STRAWBERRY WITH THE ENTOMOPATHOGENIC FUNGI *Metarhizium robertsii* AND *Beauveria bassiana* REDUCE INCIDENCE OF ARTHROPOD PESTS AND PLANT DISEASES IN THE FIELD

Abstract

The effect of the inoculation of strawberry roots with two Brazilian entomopathogenic fungal isolates, *Metarhizium robertsii* (ESALQ 1622) and *Beauveria bassiana* (ESALQ 3375), on naturally occurring arthropod pests and plant diseases were investigated in four commercial strawberry fields during two growing seasons in Brazil. Three locations in São Paulo State represented open field production while strawberries were grown in low tunnels at the fourth location in Minas Gerais State. Population responses of selected predatory mites to the fungal treatments were also assessed. Root inoculation of strawberry plants by the fungal isolates resulted in significantly fewer two-spotted spider mites, *Tetranychus urticae* Koch, adults compared to control plants at all four locations. The mean cumulative numbers (±SE) of *T. urticae* per leaflet were: *B. bassiana* (206.5±51.48), *M. robertsii* (225.6±59.32) and control (534.1±115.55) at the three open field locations, while corresponding numbers at the location with tunnels were: *B. bassiana* (107.7±26.85), *M. robertsii* (79.7±10.02) and control (207.4±49.90). In addition, plants treated with *B. bassiana* ESALQ 3375 experienced 50% fewer leaves damaged by Coleoptera compared to controls, while there were no effects on populations of whiteflies and thrips observed in different treatments in any of the fields. Further, lower proportions of leaflets with symptoms of the foliar pathogens *Mycosphaerella fragariae* and *Pestalotia longisetula* were observed in the *M. robertsii* (4.6% and 1.3%) and *B. bassiana* (6.1% and 1.3%) treated plots compared to control plots (9.8% and 3.7%). The densities of naturally occurring predatory mites were unaffected by the fungal inoculations of strawberry plants. Both *Metarhizium* and *Beauveria* were isolated from strawberry leaf tissues and from soil samples 180 days after inoculation. Our results suggest that both isolates of *M. robertsii* and *B. bassiana* may be used as root inoculants of strawberry plants to protect against foliar pests, particularly spider mites, and against foliar diseases without harmful effects on natural populations of beneficial predatory mites.

Keywords: Microbial control; Plant-microbe interactions; *Tetranychus urticae*; Phytobiome; Integrated pest management (IPM)

4.1. Introduction

Strawberry is an important crop throughout the world and in 2016 approximately 9.2 million tons of fruits were produced worldwide, with yield of 22.690 kg/ha (FAOSTAT, 2018). Cultivated strawberry, *Fragaria* x *ananassa* (Duch; Rosales: Rosaceae), is attacked by a large complex of arthropod pests and plant diseases that may reduce yield (Solomon et al., 2001). The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), is an important pest of many crops throughout the

world (Greco et al., 2005), including strawberries (Raworth, 1986; Easterbrook et al., 2001; Solomon et al., 2001). *Tetranychus urticae* feeds mainly on the underside of leaves and this feeding may lead to reduced photosynthesis and increased transpiration as well as injection of phytotoxic substances (Sances et al., 1979, 1982; Attia et al., 2013). Feeding damage decreases foliar and floral development, causing reductions in quality and quantity of fruits (Rhodes et al., 2006). High incidence of plant pathogens, especially fungal pathogens, is another challenge faced by strawberry farmers. Pathogens cause problems throughout the crop cycle, from the newly planted seedlings to the final fruit producing stage (Garrido et al., 2011).

The main pest control strategy in strawberries throughout the world is the use of synthetic chemical pesticides (Solomon et al., 2001; Garrido et al., 2011). Dependency of these chemicals for pest control is associated with undesirable effects to the environment and human health (e.g. Attia et al., 2013; Barzman et al., 2015; Czaja et al., 2015). Outbreaks of *T. urticae* are often observed following some pesticide treatments (Klingen and Westrum, 2007; Van Leeuwen et al., 2009, 2010) due to the emergence of pest resistance to the particular pesticides and destruction of populations of the pests' natural enemies (Solomon et al., 2001; Sato et al., 2005). Biological control is considered a sustainable alternative to synthetic chemical pesticides for control of arthropod pests by use of invertebrate predators, parasitoids and microbial control agents (Garcia et al., 1988; Eilenberg et al., 2001). Except from the application of predatory phytoseiid mites for control of *T. urticae*, biological control is not widely used in strawberry production, and more development of macro- and microbial control agents and application strategies is therefore necessary (Solomon et al., 2001).

Entomopathogenic fungi within the order Hypocreales are used in microbial control and many species are known to have a quite wide host range (Goettel et al., 1990; Rehner, 2005). *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Cordycipitaceae) and several species of *Metarhizium* (Clavicipitaceae) have been considered as promising microbial control agents in strawberries (Sabbahi et al., 2008; Castro et al., 2018) that may be implemented in programs for integrated pest management (IPM) (Hajek and Delalibera, 2010). There are, however, constraints in the use of entomopathogenic fungi as biological control agents such as non-consistent effects against pests, short survival time of the fungal propagules in the environment, quality of commercial products, shelf life and costs (Lacey et al., 2015). These aspects

are influenced by abiotic factors such as temperature, light intensity, humidity and rainfall (Meyling and Eilenberg, 2007; Castro et al., 2013) and by biotic factors such as multitrophic interactions with plants, invertebrates, other microorganisms and plant pathogens (Klingen and Haukeland, 2006; Meyling and Eilenberg, 2007; Meyling and Hajek, 2010). In order to optimize pest control by entomopathogenic fungi, it is important to understand how these factors and their interactions affect the efficacy of the microbial control agent in question.

Recent studies have reported that entomopathogenic fungi in the Hypocreales, mainly *Metarhizium* spp. and *Beauveria* spp., may also interact with plants as endophytes (Vega, 2008, 2018; Vega et al., 2009; Greenfield et al., 2016). Endophytic fungi are able to colonize the internal tissues of a host plant and cause no apparent negative effect to the plant (Carroll, 1988; Stone et al., 2004; Vega, 2008). This relationship between entomopathogenic fungi and their host plant may protect the plant against arthropod pests and plant diseases (Bing and Lewis, 1991; Ownley et al., 2010; Jaber and Ownley, 2018). Furthermore, endophytic fungi are protected inside the plant tissues from the effect of ambient abiotic factors (Vega, 2008, 2018) and the challenge of short survival time of fungal propagule in the environment due to abiotic factors may therefore be reduced. The mechanisms responsible for any plant protection capacity of plant associated entomopathogenic fungi against arthropod pests and plant pathogens remains uncertain (Vidal and Jaber, 2015; McKinnon et al., 2017).

Most of the published studies on entomopathogenic fungi as plant inoculants were carried out under controlled experimental conditions, and so far, few studies have investigated the pest control potential of entomopathogenic fungi as inoculants of plants under field conditions while no field studies have evaluated effects against plant pathogens (Jaber and Ownley, 2018). Field studies have been carried out with inoculation of bean plants, *Phaseolus vulgaris* L. (Fabales: Fabaceae) with *B. bassiana* against *Liriomyza* leafminers (Diptera: Agromyzidae) (Gathage et al., 2016); in *Sorghum bicolor* L. (Moench) (Poales: Poaceae) colonized by *B. bassiana* and *Metarhizium robertsii* Bisch., Rehner & Humber (Mantzoukas et al., 2015); and in cotton *Gossypium* spp. (Malvales: Malvaceae) where seeds were treated with *B. bassiana* against *Aphis gossypii* Glover (Homoptera: Aphididae) (Castillo-Lopez et al., 2014). These recent studies report significant effects against foliar arthropod pests under field conditions suggesting that implementation of entomopathogenic fungi as

plant inoculants into outdoor IPM programs has major potential (Lacey et al., 2015; Jaber and Ownley, 2018).

The aim of the present study was to evaluate the potential of two selected isolates of entomopathogenic fungi as root inoculants of strawberry plants for above-ground pest management under field conditions in Brazil. The fungal isolates represented the species *M. robertsii* and *B. bassiana*, respectively, and were selected based on the ability to induce growth promotion in strawberries and to reduce *T. urticae* populations in greenhouse experiments (Canassa et al., under review; in prep.). The effects on natural predatory mite populations were also assessed to evaluate the effect of the fungal inoculation strategy on natural enemies of *T. urticae* in the strawberry foliage. Further, prevalence of insect pests and important strawberry foliar pathogens were also monitored.

4.2. Material and Methods

4.2.1. Fungal isolates

Based on earlier efficacy studies (F. Canassa, unpubl.), the entomopathogenic fungal isolates *M. robertsii* ESALQ 1622 and *B. bassiana* ESALQ 3375 were selected. Isolates were kept at -80°C in the entomopathogen collection "Prof. Sérgio Batista Alves" in the "Laboratory of Pathology and Microbial Control of Insects" at Escola Superior de Agricultura “Luiz de Queiroz” – University of São Paulo (ESALQ/USP), Piracicaba, São Paulo, Brazil. The isolate *M. robertsii* ESALQ 1622 was originated from soil of a corn field in Sinop (11°51'47"S; 55°29'01"W), Mato Grosso State, Brazil and the *B. bassiana* ESALQ 3375 isolate was obtained from soil of a strawberry field in Senador Amaral (22°33'12"S; 46°13'41"W), Minas Gerais State, Brazil.

4.2.2. Experimental set up

The experiments were conducted in four commercial strawberry fields (Figure 1). The roots of the strawberry seedlings were immersed in one of the following treatments before planting: A) *M. robertsii* ESALQ 1622 in water + 0.05% Tween 80; B) *B. bassiana* ESALQ 3375 in water + 0.05% Tween 80; C) Water + 0.05% Tween 80 (control). A randomized block design was used in all four field experiments.

Three experiments were conducted in Atibaia, São Paulo State, Brazil, from March to September 2018 in three separate open commercial strawberry fields with black plastic mulching and drip irrigation (Open field-locations 1, 2, 3 are shown in Figure 1). At these locations, each experimental strawberry block consisted of a bed 60 m long (20 m per treatment), 1.1 m wide and contained 600 plants (200 plants per treatment). Experiments at location 1 (23º04'14.32''S; 46º40'58.2''W) and location 2 (23º04'33.5''S; 46º40'30.1''W) had 6 blocks, where the three treatments were randomized inside each block, totalling 3.600 plants, while at location 3 (23º08'00.7''S; 46º37'04.5''W) there were 4 blocks, where the three treatments were also randomized inside each block, totalling 2.400 plants. Cultivars of locations 1, 2 and 3 were Camarosa (University of California, 1993), Camino real (University of California, 2001), and Oso grande (University of California, 1989), respectively. At these three locations, bare root strawberry plants (*Fragaria* x *ananassa*) were planted at the 4 leaves stage in three rows distant 0.27 cm between each other.

The experiment at location 4 was conducted in Senador Amaral (22º33'12.1''S; 46º13'41.8''W), Minas Gerais State, Brazil from July 2017 to January 2018, in low tunnels (with white plastic), with black plastic mulching and drip irrigation (Tunnel-location 4 in Figure 1). This field experiment was established in 18 low tunnels representing four blocks, each with three strawberry beds of each treatment, i.e. 12 strawberry beds per treatment. Each bed was 20 m long, 1.1 m wide and contained 250 plants, totalling 3,000 plants per treatment. At location 4, bare root strawberry plants, cultivar 'Albion' (University of California, 2006) were planted at the 4 leaves stage individually in three rows distant 0.27 cm from each other.

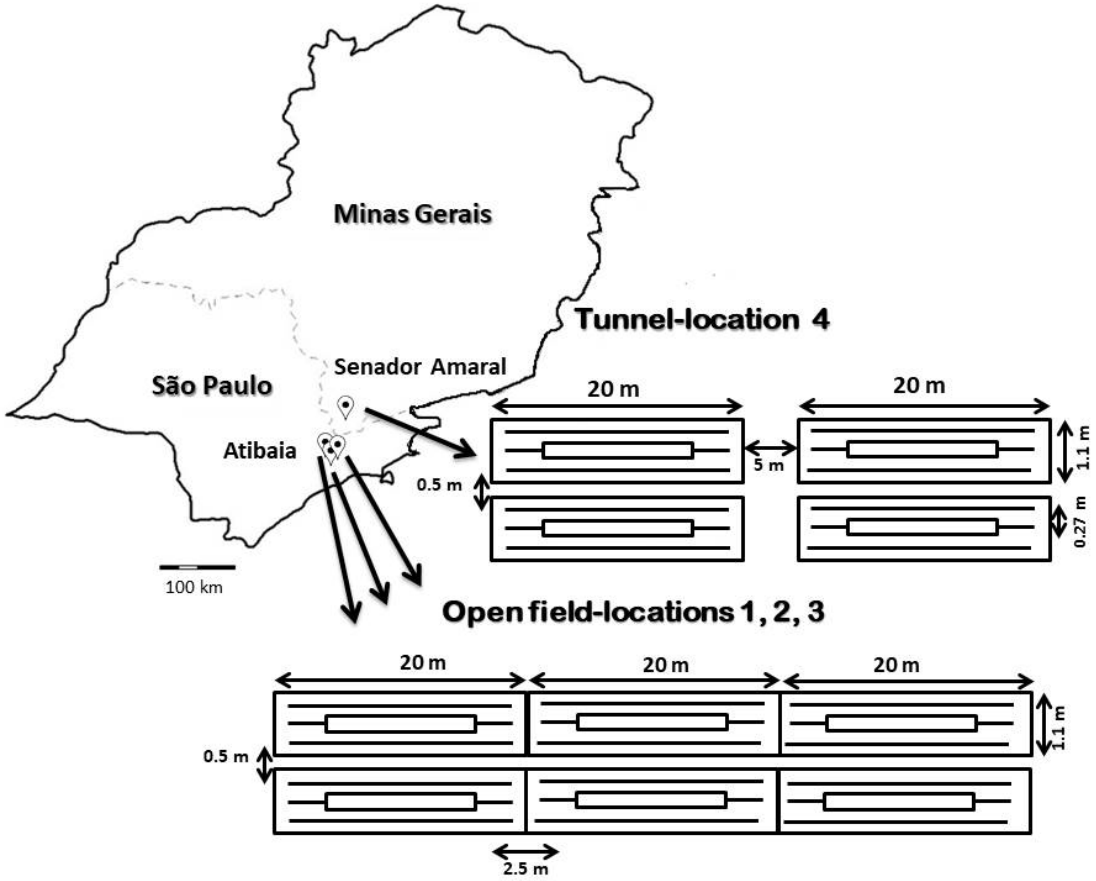

Figure 1. Experimental field set up in Open Field locations 1, 2 and 3 in Atibaia (1: 23°04'14.32"S 46°40'58.2"W, 2: 23°04'33.5"S 46°40'30.1"W, 3: 23°08'00.7 "S 46°37'04.5"W) and in Low Tunnel location 4 in Senador Amaral (22°33'12.1"S 46°13'41.8"W). Rows and area used for recording of data are indicated as a rectangle inside each bed.

4.2.3. Preparation of fungal inoculum

The two fungal isolates (*M. robertsii* ESALQ 1622 and *B. bassiana* ESALQ 3375) were retrieved from the -80°C culture collection and plated in Petri dishes (90 x 15 mm) containing 20 ml Potato Dextrose Agar (PDA; Merck, Darmstadt, Germany). The cultures were then kept in darkness at 25ºC for 10 days until harvesting of conidia. This was done by adding 10 ml sterile 0.05% Tween 80 (Oxiteno, São Paulo, Brazil) to the culture and scraping off the conidia with a sterile spatula. Conidial concentrations were estimated using a Neubauer hemocytometer (Merck, Darmstadt, Germany) and adjusted to 1 x 10^8 conidia ml^{-1}. Later, 10 ml of each suspension was inoculated with a pipette into individual polypropylene bags (35 cm length x 22 cm width) containing

300 g autoclaved (121ºC, 20 min) parboiled rice, inside an aseptic laminar flow chamber.

The fungal inoculated rice kernels were mixed in the plastic bags and incubated in darkness at 25ºC for 10 days. The bags were gently shaken every two days to ensure evenly distributed fungal growth on rice kernels. Prior to use in the experiment, the conidial viability was checked by preparing a conidial suspension by adding 1 g of rice with sporulating fungi from the plastic bag to 10 ml sterile 0.05% Tween 80. From the third dilution, 150 µl of the conidial suspension were transferred with a pipette onto PDA. The percentage of conidia germination was then evaluated according to Oliveira et al. (2015). Suspensions were only used if germination rates were higher than 95%.

4.2.4. Fungal inoculation of strawberry roots

Rice kernels colonized with each fungal isolate were added into water plus 0.05% Tween 80 as described below. For the Open Field experiments at locations 1, 2 and 3, the original conidia concentration per g of rice kernels for each isolate was estimated at 2.5 x 10^8 /g rice for *M. robertsii* and 1.3 x 10^9 /g rice for *B. bassiana*. The concentration was then adjusted to 1.5 x 10^{12} conidia of *M. robertsii* on 3.0 kg rice and *B. bassiana* on 0.56 kg rice. The rice was mixed with 100 L of well water plus 50 ml 0.05% Tween 80, resulting in 1.5 x 10^6 conidia/ml. Control consisted of 100 L of well water plus 50 ml 0.05% Tween 80. The final suspensions for the experiments contained 1.5 x 10^6 conidia/ml.

For the Low Tunnel experiment at location 4, the original conidia concentration per g of rice kernels for each isolate was estimated at 1.8 x 10^8 /g rice for *M. robertsii* and 7.5 x 10^8 /g rice for *B. bassiana*. The concentration was then adjusted to 1.5 x 10^{12} conidia of *M. robertsii* on 8.3 kg rice and *B. bassiana* on 2.0 kg rice. The rice was mixed with 750 L well water plus 375 ml 0.05% Tween 80, resulting in 2.0 x 10^6 conidia/ml. Control consisted of 750 L of well water plus 375 ml 0.05% Tween 80.

Strawberry roots were inoculated by immersing the root system of each plant completely into the respective treatment suspensions for 2 minutes. The inoculated plants were transported to the correct position in the rows inside plastic trays to avoid dripping suspension, and then were immediately planted. The suspensions were continuously mixed with a wooden stick during the strawberry root inoculation to ensure homogeneous concentrations.

4.2.5. Evaluations: arthropod pests, natural enemies and plant pathogens

All four field experiments were evaluated monthly for six months. However, the results obtained at location 4 (Low Tunnel experiment) are only reported up to 120 days after inoculation, because the producer applied a synthetic chemical pesticide at this time, which may have influenced observations at 150 and 180 days after inoculation.

In the Open Field experiments, we observed one leaflet and one flower from each of 15 plants of the central row of the strawberry bed as indicated in Figure 1. In the Low Tunnel experiment, we observed 15 leaflets (= one leaf from a triplet) and 15 flowers from each group of six plants (i.e. 2 or 3 leaflets per plant) in the central row of the strawberry bed as indicated in Figure 1. Each leaflet was destructively sampled by hand and visually observed in the field, and the arthropod pests and predatory mites were identified to species level and counted.

The predatory mites were transferred to plastic vials (500 ml, 8.5 cm high, 10 cm diameter) containing 70% ethanol and taken to the laboratory where they were mounted in Hoyer's medium for identification to species by comparing their morphology with information from original descriptions and redescriptions provided in the literature.

Leaflets with characteristic symptoms of the plant pathogenic fungi *Mycosphaerella fragariae* Tul. (Lindau), *Dendrophoma obscurans* (Ell & Ev.) and *Pestalotia longisetula* Guba were recorded and the percentage of leaflets with the diseases was calculated.

4.2.6. Evaluation of colonization of strawberry leaves and soil

Sampling of strawberry leaves and soil adjacent to plant roots was done 180 days after inoculation to evaluate the presence of entomopathogenic fungi. One strawberry leaf (= three leaflets) was randomly collected from one plant of the central row of each replicate plot treatment at each of the four locations. Collected leaves were placed in separate plastic bags and transferred to the laboratory for evaluation of endophytic colonization. The leaves were cut in sections of 4 x 1 cm, and then surface sterilized by following the method described by Greenfield et al. (2016). Three sections of leaves were plated in one Petri dish (90 x 15 mm) with the following selective media: 20 ml of PDA, 0.5 $g.L^{-1}$ of cycloheximide, 0.2 $g.L^{-1}$ of chloramphenicol, 0.5 $g.L^{-1}$ of

Dodine (65%) and 0.01 $g.L^{-1}$ of Crystal Violet (Behie et al., 2015). The sterilization efficiency was confirmed by plating 100 µl of the last rinsing water of the sterilization onto PDA (Parsa et al., 2013). Further, imprints of sterilized leaves were used as an additional method to confirm whether the sterilization was successful. This was done by gently pressing the leaf section with the cut edge onto the PDA medium (Greenfield et al., 2016) before placing sections in selective media plates. The Petri dishes were incubated at 25°C and after 15 days, the fungal colonization rate, i.e., the number of colonies forming units (CFUs) of *Metarhizium* or *Beauveria* was counted.

Soil samples adjacent to plant roots, from the plants where leaves were sampled without removing the plants by uprooting with a garden spade. Then soil with roots were placed into individual plastic bags and taken to the laboratory. Here, the soil was mixed, and subsequently 1 g was sampled and added to 10 ml of sterile 0.05% Tween 80, and vigorously vortexed for 30 s and serially diluted into distilled water + 0.05% Tween 80 to obtain the following concentrations: 1x10, $1x10^{-1}$, $1x10^{-2}$ and $1x10^{-3}$. Petri dishes (90 x 15 mm) containing selective agar medium as described above were divided into four equal quarter sections by marking the bottom part of the Petri dishes with a permanent marker. Then 100 µl from each soil dilution suspension was pipetted onto the selective media in each of the four sections. After the 100 µl was dried up inside a laminar flow chamber, the Petri dishes were incubated in darkness at 25°C for 15 days, before CFUs of *Metarhizium* or *Beauveria* were quantified.

4.2.7. Statistical analysis

We fitted Poisson generalized linear mixed models to the *T. urticae* counts obtained from locations 1, 2 and 3 (Open Field), including in the linear predictor the effects of block and different quadratic polynomials per each treatment and location combination over time (natural log-transformed) as fixed effects, and two random effects, namely, the effect of bed (since observations taken over time on the same bed are correlated) and an observation-level random effect to model overdispersion. Hence, the maximal model included 32 fixed effects and 2 variance components, totalling 34 parameters. We then performed backwards selection, using likelihood-ratio (LR) tests to assess the significance of the fixed effects. Treatments were compared by fitting nested models using grouped treatment levels and comparing them using LR tests; a significant test statistic means that the treatments cannot be grouped, as they

are statistically different (see e.g. Fatoretto et al., 2018). After model selection, the effects of proportion of occurrence of each plant pathogen species present (*M. fragariae*, *P. longisetula* and *D. obscurans*), damage by Coleoptera, and number of thrips (*F. occidentalis*) were added, separately, as covariates in the model and their significance assessed using LR tests.

For the other variables observed in locations 1, 2 and 3 (Open Field), we worked with the aggregated values across all time points. The proportion of leaflets infected by plant pathogens present (*M. fragariae*, *P. longisetula* or *D. obscurans*) and the proportion of leaflets damaged by Coleoptera were analysed by fitting quasi-binomial models with a logit link, including the effects of block, treatment, location, and the interaction between treatment and location in the linear predictor. The number of thrips was analysed by fitting quasi-Poisson models, also including the effects of block, treatment, location, and the interaction between treatment and location in the linear predictor. Significance of effects was assessed using F-tests, since the dispersion parameter was estimated (Demétrio et al., 2014). Multiple comparisons were performed by obtaining the 95% confidence intervals for the linear predictors.

For location 4 (Low Tunnel), Poisson generalized linear mixed models were fitted to the *T. urticae* counts, including in the linear predictor the effects of block and different intercepts and slopes per each treatment over time as fixed effects, and two random effects, namely, the effect of bed (since observations taken over time on the same bed are correlated) and an observation-level random effect to model overdispersion. Here, the maximal model included 9 fixed effects and 2 variance components, totalling 11 parameters. As for the models fitted for locations 1, 2, and 3 (Open Field), we then performed backwards selection, using likelihood-ratio (LR) tests to assess the significance of the fixed effects. Treatments were compared the same way, by fitting nested models using grouped treatment levels and comparing them using LR tests. Again, after model selection, the effects of proportion of occurrence of number of pests present and plant pathogens were added, individually, as covariates in the model and their significance assessed using LR tests.

For the other variables observed at location 4 (Low Tunnel), we worked with the aggregated values across all time points. The proportion of leaflets infected by plant pathogens was analysed by fitting quasi-binomial models with a logit link, including the effects of block and treatment in the linear predictor. The number of cucurbit beetles, white flies, thrips, and predatory mites were analysed by fitting quasi-Poisson models,

also including the effects of block and treatment in the linear predictor. Significance of effects was assessed using F-tests, and multiple comparisons were performed by obtaining the 95% confidence intervals for the linear predictors.

All analyses were carried out in R (R Core Team, 2018). Goodness-of-fit was assessed using half-normal plots with a simulated envelope, using package hnp (Moral et al., 2017). Generalized linear mixed models were fitted using package lme4 (Bates et al., 2015). All plots were generated using package ggplot2 (Wickham, 2009).

4.3. Results

4.3.1. Effects of *M. robertsii* and *B. bassiana* on *T. urticae*

Root inoculation of strawberry plants with the two fungal treatments (*M. robertsii* ESALQ 1622 and *B. bassiana* ESALQ 3375) significantly influenced the number of *T. urticae* adults over the six-month period (180 days) in Open Field locations 1, 2 and 3 (LR = 30.31, d.f. = 2, $p < 0.0001$) (Figure 2) and the Low Tunnel location 4 (LR = 10.39, d.f. = 2, $p = 0.0055$) (Figure 3). No difference between plants inoculated with the two entomopathogenic fungi were seen in locations 1, 2 and 3 (LR = 0.07, d.f. = 1, $p = 0.3092$) nor in location 4 (LR = 0.02, d.f. = 1, $p = 0.8793$).

There was no significant three-way interaction among Open Field locations (1, 2 and 3), treatment, and time (LR = 4.06, d.f. = 8, $p = 0.8516$), nor significant two-way interactions between Open Field locations (1, 2 and 3) and treatment (LR = 0.69, d.f. = 4, $p = 0.9524$) and between treatment and time (LR = 3.00, d.f. = 4, $p = 0.5574$). However, there was a significant interaction between location and time (LR = 49.91, d.f. = 4, $p < 0.0001$), which means that the population dynamics of spider mites changed differently between the inoculated and control plants over time at each location, with a significantly higher number of adults on the control plants in the three locations (LR = 30.31, d.f. = 2, $p < 0.0001$) (Figure 2). For the Low Tunnel location 4, there was no significant interaction between treatment and time (LR = 2.49, d.f. = 2, $p = 0.2879$), however, there were significant effects of time (LR = 43.02, d.f. = 1, $p < 0.0001$) and treatment (LR = 10.39, d.f. = 2, $p = 0.0055$), and hence there was a significantly higher number of *T. urticae* adults on the control plants at different times of evaluation, when compared to the two fungal treatments (Figure 3).

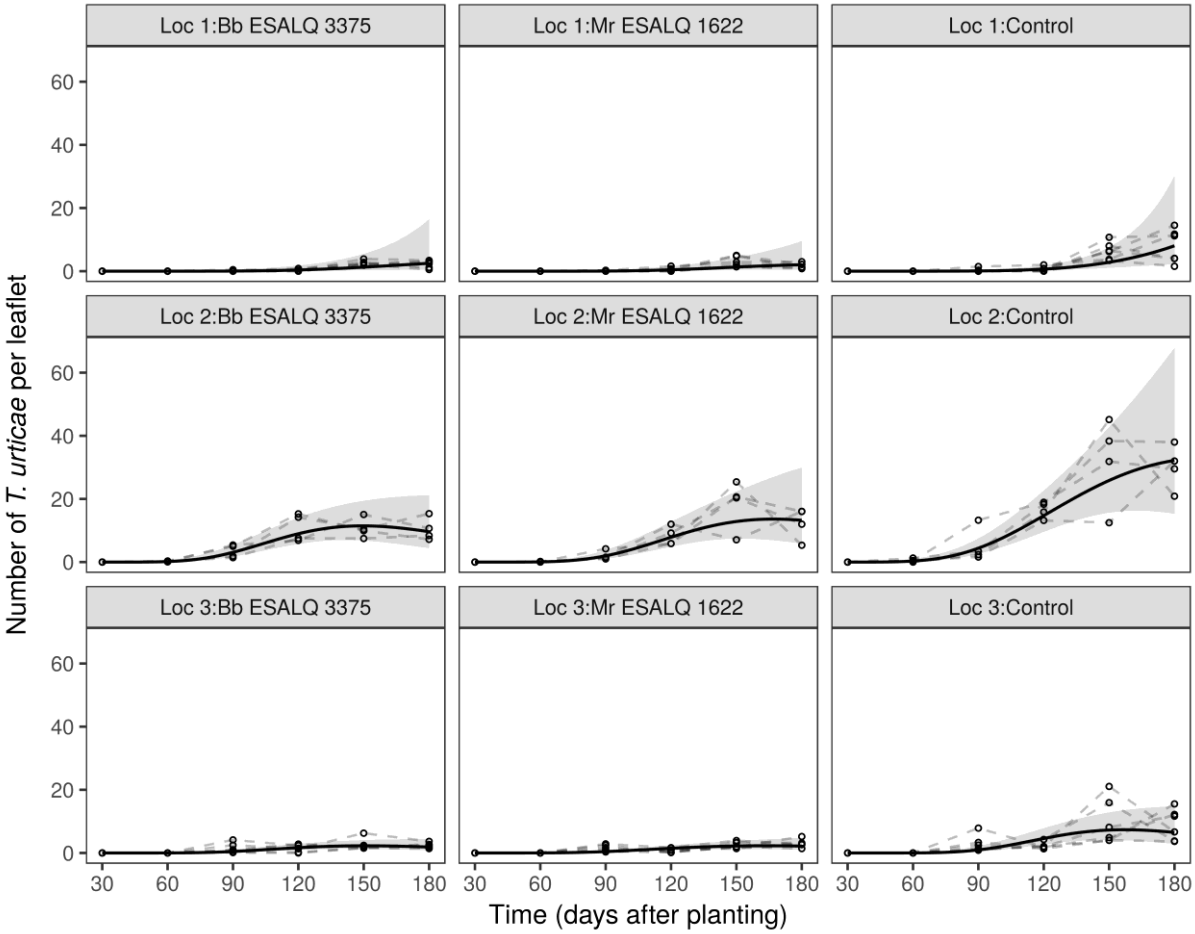

Figure 2. Effect of inoculation of strawberry root with *Beauveria bassiana* (Bb) isolate ESALQ 3375 or *Metarhizium robertsii* (Mr) ESALQ 1622 on numbers of adult *Tetranychus urticae* per leaflet 30, 60, 90, 120, 150 and 180 days after inoculation, at the Open Field locations 1, 2 and 3 in Atibaia, São Paulo State, Brazil (Loc 1: 23°04'14.32''S 46°40'58.2''W, Loc 2: 23°04'33.5''S 46°40'30.1''W, Loc 3: 23°08'00.7 ''S 46°37'04.5''W). The dots represent the observations; the solid lines are the fitted curves for the mean number of *T. urticae* per leaflet and the gray areas represent 95% confidence intervals of the curves.

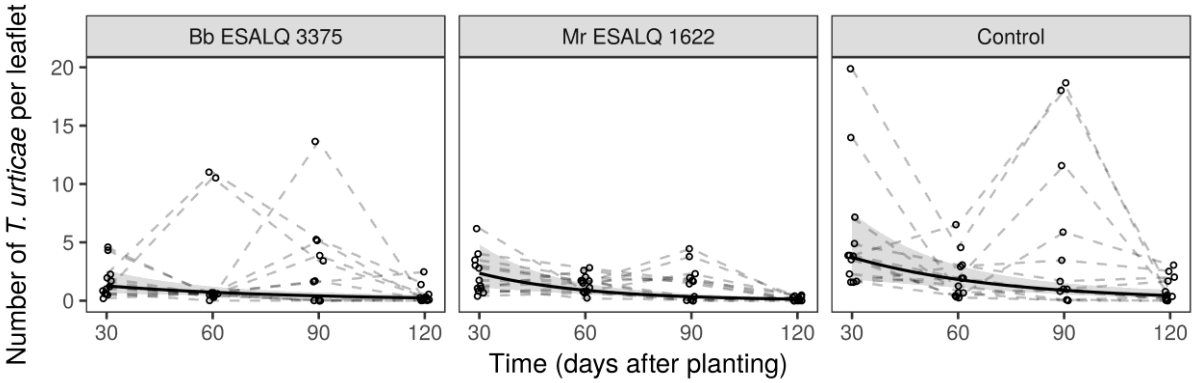

Figure 3. Effect of inoculation of strawberry root with *Beauveria bassiana* (Bb) isolate ESALQ 3375 or *Metarhizium robertsii* (Mr) ESALQ 1622 on numbers of adult *Tetranychus urticae* per leaflet from 30, 60, 90 and 120 days after inoculation at the Low Tunnel location 4 in Senador Amaral, Minas Gerais State, Brazil (22°33'12.1"S 46°13'41.8"W). The dots are the observations; the solid lines are the fitted curves for the mean number of *T. urticae* per leaflet and the gray areas represent 95% confidence intervals.

There was no significant effect of the proportion of leaflets infected by the plant pathogens *M. fragariae* (LR = 0.20, d.f. = 1, p = 0.6569), *P. longisetula* (LR = 1.89, d.f. = 1, p = 0.1693) and *D. obscurans* (LR = 1.90, d.f. = 1, p = 0.1686) on the number of *T. urticae* in Open Field locations 1, 2 and 3. However, there was a significant effect of the proportion of leaves damaged by Coleoptera (holes in the leaflets) on the number of *T. urticae* (LR = 5.13, d.f. = 1, p = 0.0235), suggesting that numbers of *T. urticae* were lower on leaflets damaged by Coleoptera (estimate of -1.60 in the logit scale, with an associated standard error of 0.72, indicating a negative relationship). Besides, in locations 1, 2, 3 there was no significant interaction between numbers of *T. urticae* and thrips in flowers (LR = 1.03, d.f. = 1, p = 0.3092). In Low Tunnel location 4, there was no significant interaction between numbers of *T. urticae* and thrips in flowers (LR = 0.73, d.f. = 1, p = 0.3929) or whiteflies (LR = 3.74 d.f. = 1, p = 0.0532).

4.3.2. Effects of *M. robertsii* and *B. bassiana* on other pests and diseases

Damage caused by Coleoptera (holes in the leaflets) was significantly reduced on strawberry plants inoculated with *B. bassiana* ESALQ 3375 compared to control plants in Open Field locations 1, 2 and 3 (Table 1). There was no significant interaction

between location and treatment ($F_{4,34}$ = 1.68, p = 0.1767), but there was a significant effect of location ($F_{2,40}$ = 12.61, p < 0.0001). The mean damage caused by Coleoptera (± SE%) in each location were: location 1 = 10.68 ± 1.57 a; location 2 = 3.89 ± 0.84 b; and location 3 = 4.54 ± 1.15 b.

There was no difference in the number of thrips in flowers between fungal inoculated strawberry plants and the control plants in Open Field locations 1, 2 and 3 (Table 1). There was no significant interaction between location and treatment ($F_{4,34}$ = 0.47, p = 0.7651), but there was a significant effect of location ($F_{2,40}$ = 11.98, p = 0.0001). The mean ± SE (%) in each location were: location 1 = 27.59 ± 4.28 b; location 2 = 14.26 ± 2.23 c; and location 3 = 40.09 ± 6.78 a.

Although there was no difference in the proportion of leaflets (n=15 leaflets per replicate) with symptoms of the plant pathogenic fungus *D. obscurans* in Open Field locations 1, 2 and 3 ($F_{2,38}$ = 1.02, p = 0.3710), the proportion of leaflets (n=15 leaflets per replicate) with symptoms of *M. fragariae* and *P. longisetula* were significantly smaller on plants inoculated with *B. bassiana* ESALQ 3375 and *M. robertsii* ESALQ 1622 in all fields (Table 1). Besides, for *D. obscurans*, there was no significant interaction between location and treatment ($F_{4,34}$ = 0.79, p = 0.5386), and among the three Open Field locations ($F_{2,40}$ = 1.54, p = 0.2300). For *P. longisetula*, there was also no significant interaction between location and treatment ($F_{4,34}$ = 0.58, p = 0.5676), and among the three Open Field locations ($F_{2,40}$ = 0.04, p = 0.8433). Regarding the disease caused by *M. fragariae*, there was no significant interaction between location and treatment ($F_{4,34}$ = 0.46, p = 0.7640), but there was a significant effect of location ($F_{2,40}$ = 39.84, p < 0.0001). The mean ± SE (%) in each location were: location 1 = 3.83 ± 1.06; location 2 = 14.20 ± 1.90; and location 3 = 0.56 ± 0.29.

Table 1. Mean ± SE of proportions of leaflets damaged by Coleoptera (%), cumulative number of thrips in flowers, and proportion of leaflets with symptoms of the pathogens *Dendrophoma obscurans*, *Pestalotia longisetula* and *Mycosphaerella fragariae* (%) representing the differences in the Open Field locations 1, 2 and 3, with summaries of generalized linear models below. Separate analyses were performed for each response variable.

Treatments[2]	Assessment[1]				
	Locations 1, 2, 3				
	Coleoptera damage	Nº of thrips	*D. obscurans*	*P. longisetula*	*M. fragariae*
B. bassiana	4.4 ± 0.88 b	24.5 ± 4.67 a	2.7 ± 1.23 a	1.3 ± 0.37 b	6.1 ± 1.66 b
M. robertsii	6.6 ± 1.15 ab	21.6 ± 3.34 a	2.5 ± 1.10 a	1.3 ± 0.48 b	4.6 ± 1.35 b
H_2O + Tween 80	8.7 ± 2.02 a	30.9 ± 6.27 a	4.5 ± 1.58 a	3.7 ± 1.24 a	9.8 ± 2.69 a
Test statistic	$F_{2,38} = 4.17$	$F_{2,38} = 1.97$	$F_{2,38} = 1.02$	$F_{2,38} = 4.92$	$F_{2,38} = 5.84$
p-value	p = 0.0240	p = 0.1549	p = 0.3710	p = 0.0158	p = 0.0066

[1]Data (mean ± SE) followed by different letters within a column are significantly different (GLM, followed by *post hoc* Tukey test, $P < 0.05$).

[2]Treatments included root inoculations of the entomopathogenic fungal isolates *Beauveria bassiana* ESALQ 3375 (*B. bassiana*), *Metarhizium robertsii* ESALQ 1622 (*M. robertsii*), and control treatment with H_2O + 0.05% Tween 80.

In Low Tunnel location 4, in addition to *T. urticae*, the other major pests were whiteflies and thrips in flowers, but there was no difference in the number of any of these among the three treatments (Table 2). In this location, the density of pest was always very low and very few leaves with symptoms of plant pathogens were observed. The cumulative proportion of leaflets with symptoms of all the diseases (*D. obscurans* + *P. longisetula* + *M. fragariae*) are presented in Table 2.

Table 2. Mean ± SE of cumulative numbers of whiteflies per leaflet and thrips per flower, and the mean ± SE proportion of leaflets with symptoms of foliar pathogens (combined % incidence of *Dendrophoma obscurans* + *Pestalotia longisetula* + *Mycosphaerella fragariae*) in the Low Tunnel location 4. Summaries of separate statistical analyses for each response variable using generalized linear models are presented below.

Treatments[2]	Assessment[1]		
	Whiteflies	Nº of thrips	Diseases
B. bassiana	6.6 ± 1.70 a	1.9 ± 5.33 a	0.5 ± 0.31 a
M. robertsii	6.0 ± 1.54 a	1.6 ± 3.70 a	0.5± 0.31 a
H_2O + Tween 80	5.9 ± 1.38 a	1.8 ± 2.91 a	1.2 ± 0.42 a
Test statistic	$F_{2,30} = 0.07$	$F_{2,30} = 0.18$	$F_{2;30} = 0.95$
p-value	p = 0.9359	p = 0.8358	p = 0.3988

[1]Data (mean ± SE) followed by different letters within a column are significantly different (GLM, followed by *post hoc* Tukey test, $P < 0.05$).

[2]Treatments included root inoculations of the entomopathogenic fungal isolates *Beauveria bassiana* ESALQ 3375 (*B. bassiana*), *Metarhizium robertsii* ESALQ 1622 (*M. robertsii*), and control treatment with H_2O + 0.05% Tween 80.

4.3.3. Effects of *M. robertsii* and *B. bassiana* on predatory mites

At Open Field locations 1, 2 and 3, few arthropod natural enemies were observed, but at Low Tunnel location 4 there were many predatory mites, mainly of the species *Neoseiulus californicus* (McGregor) (Acari: Phytoseiidae). The numbers of these predatory mites at location 4 were not significantly different on plants inoculated with *B. bassiana* and *M. robertsii*, compared to the control ($F_{2,30} = 0.04$, p = 0.9642). The mean ± SE (%) for the three treatments at location 4 were: *B. bassiana* = 14.8 ± 3.06; *M. robertsii* = 14.3 ± 3.83; and control = 13.6 ± 2.57 predatory mites per leaflet accumulated for all sampling dates.

4.3.4. Colonization of *M. robertsii* and *B. bassiana* in strawberry leaves and soil

Low colonization levels of the plants by both *Metarhizium* spp. and *Beauveria* spp. were observed 180 days after inoculation of strawberry roots. At Open Field

location 1, neither *Beauveria* spp. nor *Metarhizium* spp. were recovered on selective media from leaf samples, but *Metarhizium* spp. was found in all soil samples while *Beauveria* spp. was not recovered from soil. From samples collected at Open Field location 2, 2/6 of leaf sections and 1/6 of soil samples were found to harbor *Beauveria* spp., while *Metarhizium* spp. was recovered from 1/6 of the soil samples but not from the leaves. At Open Field location 3, *Beauveria* spp. was recovered from 1/4 of leaves and soil samples while *Metarhizium* spp. was only found in 3/4 of the soil samples and not in leaves. At Low Tunnel location 4, *Beauveria* spp. was recovered from 5/12 of leaf samples and from 1/12 of soil samples. At this location *Metarhizium* spp. was not recovered from the leaves, but the recovery from soil samples was 9/12. None of the leaf or samples from the control plots were found to contain any of the target fungi at any of the four locations.

4.4. Discussion

Root inoculations of strawberry plants with *M. robertsii* ESALQ 1622 and *B. bassiana* ESALQ 3375 resulted in lower numbers of *T. urticae* adults compared to non-inoculated control plants. Few studies have investigated the potential of plant inoculated entomopathogenic fungi as microbial control agents under natural field conditions (reviewed by Jaber and Ownley, 2018, Vega, 2018) and the present study is the first report of the effect of strawberry root inoculation with *M. robertsii* and *B. bassiana* on *T. urticae* population under commercial cultivation regimes. The two fungal isolates were previously found to reduce *T. urticae* populations on bean *Phaseolus vulgaris* (F. Canassa accepted manuscript) and similar effects were also obtained under field conditions in strawberry indicating broad host plant indirect effects of these isolates against *T. urticae*. Further, predatory mite populations were not negatively affected by the fungal inoculations indicating that no adverse non-target effects should be expected using this pest management strategy.

The potential of *B. bassiana* as an endophyte for pest management has been reported in field studies with other crops. For example, Gathage et al. (2016) reported lower infestation levels of *Liriomyza* leafminers in bean leaves in a field experiment in Kenya where bean seeds previously inoculated with *B. bassiana* G1LU3 and *Hypocrea lixii* Patouillard (syn. *Trichoderma lixii*) F3ST1 were grown. Further, Castillo-Lopez et al. (2014) reported lower numbers of *A. gossypii* on cotton plants grown in the field in

Texas, USA, from seeds inoculated with the commercial product Botanigard® (BioWorks Inc, Victor, NY) based on the GHA strain of *B. bassiana.* The present results demonstrate that fungal inoculated strawberry plants also reduced the proportion of leaf damage caused by Coleopteran pests, while no effects on other pest damage, such as whiteflies and thrips in flowers, were observed in the experimental fields. Mantzoukas et al. (2015) reported from field studies of *Sorghum bicolor* that *B. bassiana* and *M. robertsii* suppressed tunneling *Sesamia nonagrioides* larvae by 60% and 87%, and increased larval mortality by 80% and 100%, respectively, compared to control plants after spray inoculations of plants.

We also recorded a significant reduction in the prevalence of the foliar plant pathogenic fungi *M. fragariae* and *P. longisetula* in strawberry plants inoculated with either of the isolates of *B. bassiana* and *M. robertsii*. According to Jaber and Alananbeh (2018), only few studies have been conducted on the effects of plant inoculated entomopathogenic fungi affecting plant pathogens and so far no field studies have been carried out. Jaber and Alananbeh (2018) reported that sweet pepper, *Capsicum annum* L. (Solanaceae), endophytically colonized with *B. bassiana* (strain NATURALIS) and *M. brunneum* (strain BIPESCO5) showed significantly reduced incidence and severity of three *Fusarium* species (*F. oxysporum*, *F. culmorum*, and *F. moniliforme*) using *in planta* bioassays in controlled greenhouse settings with sterile soil. So far, *B. bassiana* is the most studied entomopathogenic fungal species against plant pathogens and it has been reported to protect tomato and cotton seedlings against the plant pathogens *Rhizoctonia solani* and *Pythium myriotylum* (Ownley et al., 2008). Sasan and Bidochka (2013) reported 59.4% inhibition of *Fusarium solani* f. sp. *phaseoli* in bean, when co-cultured in pretreated sterile potting mixture with *M. robertsii*. In another study, the co-inoculation of wheat seeds with *Metarhizium brunneum* Petch and the mycoparasitic fungus *Clonostachys rosea* (Link) Schroers et al. (Hypocreales: Bionectriaceae) resulted in infections by *M. brunneum* in root-feeding coleopteran larvae and provided protection against the plant pathogen *Fusarium culmorum* (Keyser et al., 2016), but *M. brunneum* did not affect the plant pathogen individually. The present field study suggests that the tested isolates of *B. bassiana* and *M. robertsii* can provide long-term protection of strawberry against both arthropod pests and foliar pathogens using a single root application at the time of planting.

It has been suggested that the mechanisms used by entomopathogenic fungi as plant associates and endophytes to antagonize plant pests or pathogens may result

through antibiosis, i.e., production of secondary metabolites by the associated fungus (Vidal and Jaber, 2015; McKinnon et al., 2017; Jaber and Alananbeh, 2018). Alternatively, another mechanism could be through induced systemic defense mechanisms of the inoculated plants, because the endophyte can be first recognized as a potential invader, which leads the plants to trigger its immune responses and consequently synthesize specific regulatory elements that may affect the arthropod pests and plant pathogen (Brotman et al., 2013; McKinnon et al., 2017). A third suggested mechanism could be that a plant pathogen and an endophytic fungus such as *B. bassiana* or *Metarhizium* sp. may compete with the pathogen for space and nutrients (Jaber and Alananbeh, 2018; Jaber and Ownley, 2018). The current field data demonstrate that the single inoculation events of strawberry roots with isolates of either *B. bassiana* or *M. robertsii* have negative effects against both *T. urticae* and selected plant pathogens in the foliage. The inconsistent re-isolation of fungi from leaf samples indicates that the effects are likely to be indirect, i.e. by systemic mechanisms, rather than direct such as the third hypothesis mentioned by Jaber and Ownley (2018). Given that effects were broadly observed against mites and plant pathogenic fungi it seems most likely that plant induced defenses were responsible for the reductions, but this will require further studies to elucidate.

Our data also suggest that natural populations of predatory mites, most of them identified as *N. californicus,* remained unaffected on strawberry plant inoculated with *M. robertsii* ESALQ 1622 or *B. bassiana* ESALQ 3375. The field experiments therefore indicate limited non-target effects in *T. urticae* control when the fungi are applied as root inoculants. Few studies have investigated the effects of plant associated entomopathogenic fungi on arthropod natural enemies and mostly focus have been on effects on parasitoids (Bixby-Brosi and Potter, 2012; Akutse et al., 2014; Jaber and Araj, 2018). The only study reporting on effects of plant-fungi interactions on predatory mites was by Schausberger et al. (2012), who showed that bean (*P. vulgaris*) colonized by the mycorrhizal fungus *Glomus mosseae* and infested with *T. urticae*, changed the composition of herbivore induced plant volatiles. This caused the fungal inoculated plants to become more attractive to the predatory mites, *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae), than non-mycorrhizal plants. It was suggested that the predatory mites associated the plant response with presence of prey (Patiño-Ruiz and Schausberger, 2014), and hence showed a higher oviposition rate on these plants resulting in more efficient *T. urticae* suppression (Hoffmann et al., 2011). The use of

B. bassiana (NATURALIS) and *M. brunneum* (BIPESCO5) as inoculants in sweet pepper combined with the aphid endoparasitoid *Aphidius colemani* Viereck (Hymenoptera: Braconidae) indicated compatibility in control of *Myzus persicae* Sulzer (Homoptera: Aphididae) as reported by Jaber and Araj (2018) under greenhouse conditions.

In the present study, *Metarhizium* and *Beauveria* were recovered at variable frequencies from samples of strawberry leaves and soil 180 days after inoculation of strawberry roots with fungal suspensions. In general, *Metarhizium* was mostly found in the soil samples, while *Beauveria* was only occasionally recovered from soil and seemingly more often from leaf samples. It has previously been reported that *B. bassiana* is a more extensive colonizer of foliar tissues than *Metarhizium* spp., when seed inoculations were used, while *Metarhizium* spp. have been reported as almost exclusively colonizing the rhizosphere of various plant species (Ownley et al., 2008; Quesada-Moraga et al., 2009; Akello and Sikora, 2012; Akutse et al., 2013; Behie et al., 2015). Behie et al. (2015) reported *M. robertsii* as being restricted to the roots while *B. bassiana* systemically colonized all parts of bean plants at field conditions. The present isolations were limited in effort and only performed at the end of the field trials, 180 days post inoculation, and we can therefore not exclude that transient endophytic colonization occurred during the field season. However, Klingen et al. (2015) found consistent establishment of two *M. brunneum* isolates and one isolate of *B. pseudobassiana* in rhizosphere soil of strawberries more than 1 year after inoculation of the substrate in Norway, indicating that related entomopathogenic fungi can persist long-term below-ground in the rhizosphere. However, abiotic conditions between Norway and Brazil are highly different and results may not be possible to compare directly. The sampling effort did not reveal any *Metarhizium* and *Beauveria* isolation in the samples from the control treatments although these fungi, particularly *Metarhizium* spp., are naturally occurring in soil of strawberry fields in this part of Brazil (Castro et al., 2016).

In conclusion, the present study demonstrates that entomopathogenic fungi can be applied as root inoculants in commercial strawberry fields to simultaneously control important arthropod pests, particularly *T. urticae*, and plant pathogenic fungi. There were no indications that the inoculations had negative effects on natural populations of predatory mites, particularly *N. californicus*. Hence, inoculation of strawberry plants with entomopathogenic fungi through root dipping may be used in combination with

predatory mites for control of *T. urticae.* This may represent a new tool and an innovative biological control strategy that may be implemented in IPM and organic strawberry production.

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