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Root inoculation of strawberry with the entomopathogenic fungi *Metarhizium robertsii* and *Beauveria bassiana* reduce incidence of the twospotted spider mite and selected insect pests and plant diseases in the field

Fernanda Canassa[a,b], Fernanda C. N. Esteca[a], Rafael A. Moral[d], Nicolai V. Meyling[b,c], Ingeborg Klingen[c], Italo Delalibera Jr.[a*]

[a]Department of Entomology and Acarology, "Luiz de Queiroz" College of Agriculture/University of São Paulo (ESALQ/USP), 13418-900, Piracicaba, São Paulo, Brazil (fernanda.canassa@usp.br, ORCID: 0000-0001-9186-9278; fernanda.esteca@usp.br, ORCID: 0000-0001-8043-2433)

[b]Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark (nvm@plen.ku.dk, ORCID: 0000-0003-3025-4370)

[c]Norwegian Institute of Bioeconomy (NIBIO), Biotechnology and Plant Health Division, P.O. Box 115, NO-1431 Ås, Norway (ingeborg.klingen@nibio.no, ORCID: 0000-0002-8230-5857)

[d]Department of Mathematics and Statistics, Maynooth University, Maynooth, Co. Kildare, Ireland (rafael.deandrademoral@mu.ie, ORCID: 0000-0002-0875-3563)

*Corresponding author: Italo Delalibera Jr.

E-mail address: delalibera@usp.br

Telephone number: +55 (19) 3429-4199 (extension line 231)

ORCID: 0000-0001-9770-9216

Department of Entomology and Acarology, "Luiz de Queiroz" College of Agriculture/University of São Paulo (ESALQ/USP), 13418-900, Piracicaba, São Paulo, Brazil

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Key message

- Few studies have investigated the potential of plant inoculated entomopathogenic fungi as microbial control agents under natural field conditions.
- First report of reduced *Tetranychus urticae* numbers on strawberry plants receiving root inoculation with the entomopathogenic fungi *Metarhizium robertsii* and *Beauveria bassiana* under commercial cultivation regimes.
- Reduction in foliar plant pathogenic fungi and no harmful effects on naturally occurring predatory mites were also observed.
- This represent a new tool and an innovative biocontrol strategy that may be implemented in IPM and organic strawberry production.

Abstract

The effect of inoculation of strawberry roots by two 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 represented open field production while strawberries were grown in low tunnels at the fourth location.

Population responses of predatory mites to the fungal treatments were also assessed. Plants inoculated by the fungal isolates resulted in significantly fewer *Tetranychus urticae* adults compared to control plants at all four locations. The mean cumulative numbers ±SE of *T. urticae* per leaflet were: *M. robertsii* (225.6±59.32), *B. bassiana* (206.5±51.48) and control (534.1±115.55) at the three open field locations, while at the location with tunnels numbers were: *M. robertsii* (79.7±10.02), *B. bassiana* (107.7±26.85) and control (207.4±49.90). Plants treated with *B. bassiana* had 50% fewer leaves damaged by Coleoptera, while there were no effects on numbers of whiteflies and thrips. Further, lower proportions of leaflets with symptoms of the foliar plant pathogenic fungi *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%). No effect was seen on numbers of naturally occurring predatory mites. Our results suggest that both isolates tested may be used as root inoculants of strawberries to protect against foliar pests, particularly spider mites, and also against foliar plant pathogenic fungi without harming naturally occurring and beneficial predatory mites.

Keywords: Endophytic entomopathogenic fungi; Microbial control; Plant-microbe interactions; *Tetranychus urticae*; Integrated pest management (IPM).

1. Introduction

Strawberry is an important fruit throughout the world and in 2016 approximately 9.2 million tons of fruits were produced worldwide, with a yield of 22.690 kg/ha (FAOSTAT 2018). Cultivated strawberry, *Fragaria* x *ananassa* (Duch; Rosales: Rosacea), is attacked by a large complex of arthropod pests and plant diseases that may reduce the yield (Solomon et al. 2001). The twospotted 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* feed mainly on the underside of leaves and this feeding may lead to reduced photosynthesis and increased transpiration as well as injection of phytotoxic substances when feeding on mesophyll and parenchyma plant cells (Sances et al. 1979, 1982; Attia et al. 2013). The feeding damage therefore decreases foliar and floral development causing reductions in quality and quantity of fruits (Rhodes et al. 2006).

Other important pest of strawberries worldwide includes the western flower thrips, *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) which causes damage by the feeding of nymphs and adults resulting in flower abortion, fruit bronzing and malformation, and consequently yield loss (Solomon et al. 2001; Coll et al. 2007). Strawberries are also attacked by aphids of different species such as *Chaetosiphon fragaefolli* Cockerell, *Aphis forbesi* Weed, *A. gossypii* Glover and *Mizus persicae* Sulzer (Hemiptera: Aphididae) (Solomon et al. 2001; Bernardi et al. 2015; Dara 2016). The whitefly *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae) is also a significant pest of strawberry crop in many regions (Solomon et al. 2001; Bernardi et al. 2015; Dara 2016). Moreover, *Neopamera bilobata* Say (Hemiptera: Rhyparochromidae) and the spotted wing fruit fly, *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) have recently invaded and caused economic losses in the production of many strawberry fields in Brazil (Kuhn et al. 2014; Andreazza et al. 2016). High incidence of plant pathogens, especially fungal pathogens, is another challenge faced by strawberry farmers in all producing countries and 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 in strawberries is associated with undesirable effects on 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 continuous 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 the pests' natural enemies (Solomon et al. 2001; Sato et al. 2005). The use of invertebrate predators, parasitoids and microbial control agents in biological control is considered a sustainable alternative to synthetic chemical pesticides for control of arthropod pests (Garcia et al. 1988; Eilenberg et al. 2001). Except from the application of predatory phytoseiid mites to control *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; Attia et al. 2013).

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). The species *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Cordycipitaceae) and several species of *Metarhizium* (Clavicipitaceae) have been considered promising microbial control agents in strawberries (Sabbahi et al. 2008; Castro et al. 2018)

and 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 microbial 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 and quality, 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 on 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, only 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 common beans, *Phaseolus vulgaris* L. (Fabales: Fabaceae) with *B. bassiana* against *Liriomyza* leafminers (Diptera: Agromyzidae) (Gathage et al. 2016); of *Sorghum bicolor* L. (Moench) (Poales: Poaceae) with *B. bassiana, Metarhizium robertsii* Bisch., Rehner & Humber, and *Isaria fumosorosea* (Wize) Brown & Smith (Cordycipitaceae) (Mantzoukas et al. 2015); and of cotton *Gossypium* spp. (Malvales: Malvaceae) with *B. bassiana* against *Aphis gossypii* Glover (Homoptera: Aphididae) (Castillo-Lopez et al. 2014). These recent field studies report of significant effects against foliar arthropod pests under field conditions suggesting that

implementation of entomopathogenic fungi as plant inoculants into outdoor IPM programs has a major potential (Lacey et al. 2015; Jaber and Ownley 2018). Few field studies have been conducted on strawberry. One study was conducted on 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) and was 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.

The aim of the present study was therefore 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 species used were *M. robertsii* and *B. bassiana* and the origin of the isolates were from Brazil. They were selected based on the ability to reduce *T. urticae* numbers on strawberry (F. Canassa, unpubl.) and on common beans *P. vulgaris* (Canassa et al. 2019), in greenhouse experiments. 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.

2. Material and Methods

2.1. Fungal isolates

Based on earlier efficacy studies (F. Canassa, unpubl.), two entomopathogenic fungal isolates *M. robertsii* ESALQ 1622 and *B. bassiana* ESALQ 3375, identified to species level by molecular techniques according to Rezende et al. (2015) and Rehner and Buckley (2005), 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” at University of São Paulo (ESALQ/USP), Piracicaba, São Paulo, Brazil. The *M. robertsii* ESALQ 1622 isolate originated from soil of a corn field in Sinop City (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 City (22°33'12"S; 46°13'41"W), Minas Gerais State, Brazil.

2.2. Experimental set up

The experiments were conducted in four different commercial strawberry fields (Fig. 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 City, 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 Fig. 1). At all three locations, an experimental strawberry block was 60 m long (20 m for each treatment), 1.1 m wide and contained 600 plants (200 plants for each 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 (=strawberry beds), where the three treatments A), B), C) were randomized inside each block, totaling 3.600 plants, while at location 3 (23º08'00.7''S; 46º37'04.5''W) there were 4 blocks (=strawberry beds), where the three treatments A), B), C) were also randomized inside each block, totaling 2.400 plants. Strawberry 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 per bed with a distance of 0.27 cm between rows.

The experiment at location 4 was conducted in Senador Amaral City (22º33'12.1''S; 46º13'41.8''W), Minas Gerais State, Brazil from July 2017 to January 2018, in low tunnels (short hoop structures covered with white plastic), with black plastic mulching and drip irrigation (Tunnel -location 4 in Fig. 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, totaling 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 with a distance of 0.27 cm between rows.

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 onto 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%.

2.4. Fungal inoculation of strawberry roots

Rice kernels colonized with the two isolates (*M. robertsii* ESALQ 1622 and *B. bassiana* ESALQ 3375) were added into water plus 0.05% Tween 80 as described below. For the Open Field experiments at locations 1, 2, 3, the original conidia concentration per gram of rice kernels for each isolate was estimated to 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. The 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 gram of rice kernels for each isolate was estimated to 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. The 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 min. The inoculated plants were transported to the correct position in the rows inside plastic trays to avoid dripping suspension and then the plants were immediately planted into the row. The suspensions were continuously mixed with a wooden stick during the strawberry root inoculation to ensure homogenized concentrations.

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

All four field experiments were evaluated each 30 day 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 the following observations at 150 and 180 days after inoculation.

In the Open Field experiments at locations 1, 2, and 3, we observed 15 leaflets (= one leaf from a triplet) and 15 flowers representing 15 plants in each of the central rows of the strawberry beds as indicated in Fig. 1. In the Low Tunnel experiment at location 4, we observed 15 leaflets (= one leaf from a triplet) and 15 flowers from six plants (i.e. 2 or -3 leaflets per plant) in each of the central rows per strawberry bed as indicated in Fig. 1. Each leaflet was destructively sampled by hand and visually observed, and the arthropod pests were identified to species level and counted in the field.

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 for identification by observing each specimen under microscope. Each predatory mite was collected with a fine brush from the vial with 70% ethanol and mounted in Hoyer's medium for identification to species by comparing their morphology with information from original descriptions and redescriptions provided in Rowell et al. (1978), Chant and Yoshida-Shaul (1991), Moraes et al. (2004) and Tixier et al. (2008).

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.

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 and destructively collected from one plant per plot in the center 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 cm x 1 cm, and they were then surface sterilized by following the method described by Greenfield et al. (2016). Three sections of leaves were plated on 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 for 15 days before visually observed for fungal outgrowth of *Metarhizium* or *Beauveria* on each plant fragment. The frequency of occurrence was estimated as the number of plant fragments with entomopathogenic fungi present in relation to the total number of plant fragments.

Soil samples adjacent to plant roots were collected with a garden spade, from the same plants where leaves were sampled, without removing the plants. Then soil with roots were placed into individual plastic bags and brought back 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, and the presence of *Metarhizium* or *Beauveria* was detected according to fungal growth morphology in each plate. The frequency of occurrence was estimated as the number of soil samples with entomopathogenic fungi in relation to the total number of samples.

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, totaling 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 (holes in the leaflets most likely caused by *Colaspis* spp.), 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, totaling 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).

3. Results

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

347 (180 days) in Open Field locations 1, 2 and 3 (LR = 30.31, d.f. = 2, $p < 0.0001$) (Fig. 2) and the Low Tunnel
348 location 4 (LR = 10.39, d.f. = 2, p = 0.0055) (Fig. 3). No difference between plants inoculated with the two
349 entomopathogenic fungi were seen in locations 1, 2 and 3 (LR = 0.07, d.f. = 1, p = 0.3092) nor in location 4
350 (LR = 0.02, d.f. = 1, p = 0.8793).

351 There was no significant three-way interaction among Open Field locations (1, 2 and 3), treatment,
352 and time (LR = 4.06, d.f. = 8, p = 0.8516), nor significant two-way interactions between Open Field locations
353 (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. =
354 4, p = 0.5574). However, there was a significant interaction between location and time (LR = 49.91, d.f. = 4,
355 $p < 0.0001$), which means that the population dynamics of spider mites changed differently between the
356 inoculated and control plants over time at each location, with a significantly higher number of adults on the
357 control plants in the three locations (LR = 30.31, d.f. = 2, $p < 0.0001$) (Fig. 2). For the Low Tunnel location 4,
358 there was no significant interaction between treatment and time (LR = 2.49, d.f. = 2, p = 0.2879), however,
359 there were significant effects of time (LR = 43.02, d.f. = 1, $p < 0.0001$) and treatment (LR = 10.39, d.f. = 2, p
360 = 0.0055), and hence there was a significantly higher number of *T. urticae* adults on the control plants at
361 different times of evaluation, when compared to the two fungal treatments (Fig. 3).

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

372

373 **3.2. Effects of *M. robertsii* and *B. bassiana* on other pests and diseases**

374

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 *M. robertsii* ESALQ 1622 and *B. bassiana* ESALQ 3375 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.

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*) can be viewed in Table 2.

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 *M. robertsii* and *B. bassiana*, compared to the control ($F_{2,30} = 0.04$, $p = 0.9642$). The mean ± SE (%) for the three treatments at location 4 were: *M. robertsii* = 14.3 ± 3.83; *B. bassiana* = 14.8 ± 3.06; and control = 13.6 ± 2.57 predatory mites per leaflet accumulated for all sampling dates.

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 *Metarhizium* spp. nor *Beauveria* 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, 33.3% (2 out of 6) of leaf sections and 16.7% (1 out of 6) of soil samples were found to harbor *Beauveria* spp., while *Metarhizium* spp. was recovered from 16.7% (1 out of 6) of the soil samples but not from the leaves. At Open Field location 3, *Beauveria* spp. was recovered from 25% (1 out of 4) of leaves and soil samples while *Metarhizium* spp. was found in 75% (3 out of 4) of the soil samples and not in leaves. At Low Tunnel location 4, *Beauveria* spp. was recovered from 41.7% (5 out of 12) of leaf samples and from 8.3% (1 out of 12) of soil samples. At this location *Metarhizium* spp. was not recovered from the leaves, but the recovery from soil samples was 75% (9 out of 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. Discussion

Our field experiment, replicated at four locations, show that 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

433 Ownley 2018; Vega 2018) and the present study is the first report of the effect on *T. urticae* numbers on
434 strawberry plants inoculated with *M. robertsii* and *B. bassiana* evaluated in the field under commercial
435 cultivation regimes. The two fungal isolates were previously found to reduce *T. urticae* populations on bean
436 *P. vulgaris* (Canassa et al. 2019) and since our strawberry field study show a similar effect this may suggest
437 that these isolates may be used as root inoculants of other crops to control *T. urticae*. Further, predatory mite
438 populations were not negatively affected by strawberry plants inoculated with *M. robertsii* ESALQ 1622 and
439 *B. bassiana* ESALQ 3375 indicating that adverse non-target effects on arthropod natural enemies may be
440 limited or non-existing.

441 The potential of *B. bassiana* as an endophyte for pest management has been reported in field studies
442 with other crops. For example, Gathage et al. (2016) reported lower infestation levels of *Liriomyza* leafminers
443 in bean leaves (*P. vulgaris*) in a bean field experiment in Kenya where bean seeds had been inoculated with *B.*
444 *bassiana* G1LU3 and *Hypocrea lixii* Patouillard (syn. *Trichoderma lixii*) F3ST1. Further, Castillo-Lopez et al.
445 (2014) reported lower numbers of *A. gossypii* on cotton plants grown in the field in Texas, USA, from seeds
446 inoculated with the commercial product Botanigard® (BioWorks Inc, Victor, NY) based on the GHA strain of
447 *B. bassiana.* Our field experiments also suggest that strawberry plants inoculated with *M. robertsii*
448 ESALQ 1622 and *B. bassiana* ESALQ 3375 reduced the proportion of leaf damage caused by Coleopteran
449 pests, while no effects on other pest damage, such as whiteflies or thrips in flowers, were observed. Mantzoukas
450 et al. (2015) reported from field studies of *Sorghum bicolor* that *B. bassiana* and *M. robertsii* suppressed
451 tunneling *Sesamia nonagrioides* Lefébvre (Lepidoptera: Noctuidae) larvae by 60% and 87%, and increased
452 larval mortality by 80% and 100%, respectively, compared to control plants after spray inoculations of plants.

453 We also recorded a reduction in the prevalence of the foliar plant pathogenic fungi *M. fragariae* and
454 *P. longisetula* in strawberry plants inoculated with *B. bassiana* ESALQ 3375 or *M. robertsii* ESALQ 1622.
455 According to Jaber and Alananbeh (2018), only few studies have been conducted on the effects of plant
456 inoculated entomopathogenic fungi affecting plant pathogens and so far no field studies have been carried out.
457 Jaber and Alananbeh (2018) reported, however, that sweet pepper *Capsicum annum* L. (Solanaceae)
458 endophytically colonized with *B. bassiana* (NATURALIS) and *M. brunneum* (BIPESCO5) showed
459 significantly reduced incidence and severity of three *Fusarium* species (*F. oxysporum*, *F. culmorum*, and *F.*
460 *moniliforme*) using *in planta* bioassays in controlled greenhouse settings with sterile soil. So far, *B. bassiana*
461 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). Furthermore, Sasan and Bidochka (2013) reported a 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 *F. culmorum* (Keyser et al. 2016), but *M. brunneum* did not affect the plant pathogen individually. The present strawberry field study suggests that the tested isolates of *B. bassiana* and *M. robertsii* can provide long-term protection of strawberries against both arthropod pests and foliar pathogens using a single root application at the time of planting.

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 a limited non-target effect on arthropod natural enemies 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). One of the few studies 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). Canassa et al. (2019) reported in short-term leaf disc experiments that *P. persimilis* showed no difference in the predation rate on spider mites from inoculated plants with *B. bassiana* (ESALQ 3375) and *M. robertsii* (ESALQ 1622) compared to control plants. 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) also indicated compatibility in control of *Myzus persicae* Sulzer (Homoptera: Aphididae) (in a greenhouse study (Jaber and Araj 2018). In another recent study, González-Mas et al. (2019) reported that the numbers of *A. gossypii*

parasitized by *A. colemani* was not influenced by whether the aphids had been feeding on seed inoculated melon plants with *B. bassiana* (isolate EABb 01/33-Su) or not. Further, application of *B. bassiana* on melon leaves did not influence the number of aphids consumed by larvae of the lacewing, *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae), and *C. carnea* showed preference to feed on aphids reared on inoculated rather than control plants in a choice bioassay (González-Mas et al. 2019). All these findings indicate that plant inoculated entomopathogenic fungi may be used in combination with parasitoids and predators to enhance the biocontrol efficacy of several plant pests in different crops.

In our study we were able to recover *Metarhizium* and *Beauveria* from strawberry leaves and soil adjacent to the roots at the end of the experiment and cropping cycle, meaning 180 days (for location 1,2,3) and 120 days (for location 4). The main aim of the present study was not to evaluate in-depth the dynamics of endophytism of the inoculated fungal isolates using a close-to-practice inoculation method in strawberry production systems and the use of commercial farm settings did not allow for repeated and complete destructive sampling of plant material. However, Castro et al. (2016) have previously reported the persistence in strawberry soil and rhizospheres in Brazil of the isolates *M. anisopliae* (ESALQ1037) and *M. robertsii* (ESALQ1426) for up to 12 months after soil drench application. Further, Klingen et al. (2015) report that two Norwegian isolates, one *B. pseudobassiana* and one *M. brunneum*, and an Austrian isolate of *M. brunneum* had long-term persistence (>1 year) in bulk soil and rhizosphere soil of strawberries in a semi-field experiment in Norway. 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), and similar results have been observed in our study. Although the observed effects of the inoculation on herbivore densities were consistent, endophytic colonization was not consistently detected in strawberry plants in our study. It has been previously reported that endophytic establishment may be influenced by several variables, such as host plant, fungal strain, environmental conditions, substrate and soil (Sánchez-Rodríguez et al. 2018). Moreover, previous research has showed that the establishment of entomopathogenic fungi within plant tissues may be transient (Garrido-Jurado et al. 2017) and the establishment success of fungal isolates is significantly reduced when inoculations are performed in natural soils (Parsa et al. 2018), as was the case in the present study. It should therefore be

519 expected that end-point measurements of endophytic colonization will be limited in field studies, particularly
520 over the 6-month time period.

521 Given that negative effects were broadly observed against both *T. urticae* and selected plant pathogens
522 in the foliage after the single inoculation events of strawberry roots with isolates of either *B. bassiana* or *M.*
523 *robertsii*, and considering the inconsistent re-isolation of fungi from leaf samples; it seems most likely that
524 plant induced defenses were responsible for the reductions, but this will require further studies to elucidate and
525 conclude. It has been widely suggested that the mechanisms used by entomopathogenic fungi as plant
526 associates and endophytes to antagonize plant pests or pathogens may result through the production of
527 secondary metabolites by the associated fungus (Vidal and Jaber 2015; Yan et al. 2015; McKinnon et al. 2017;
528 Jaber and Alananbeh 2018). Alternatively, another mechanism could be through induced systemic defense
529 mechanisms of the inoculated plants, because the endophyte can be first recognized as a potential invader,
530 which leads the plants to trigger its immune responses and consequently synthesize specific regulatory
531 elements that may affect the arthropod pests and plant pathogen (Brotman et al. 2013; McKinnon et al. 2017).

532 In conclusion, the present study demonstrates that entomopathogenic fungi can be applied as root
533 inoculants in commercial strawberry fields to simultaneously control important arthropod pests, particularly
534 *T. urticae*, and plant pathogenic fungi. There were no indications that the inoculations of strawberry plant with
535 the entomopathogenic fungal isolates tested had negative non-target effects on naturally occurring predatory
536 mites, particularly *N. californicus*. Hence, inoculation of strawberry plants with entomopathogenic fungi
537 through root dipping may be used in combination with predatory mites for the control of *T. urticae.* This may
538 represent a new tool and an innovative biological control strategy that could be implemented in IPM and
539 organic strawberry production.

540

541 **Author contribution**

542

543 FC, IDJ, IK and NVM conceived and designed research. FC and FCNE conducted experiments. RAM
544 analysed data, prepared figures and wrote the statistical analysis section. FC, IK, IDJ and NV wrote the
545 manuscript. All authors reviewed and approved the manuscript.

546

547 **Compliance with Ethical Standards**

548

549 The authors declare that they have no conflict of interest.

550

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789 **Tables**

790

791 **Table 1.** Means ± SE of proportion of leaflets damaged by Coleoptera (%), cumulative number of
792 thrips in flowers, and proportion of leaflets with symptoms of the pathogens *D. obscurans*, *P.*
793 *longisetula* and *M. fragariae* (%) representing the differences in the Open Field locations 1, 2 and 3,
794 with summaries of generalized linear models below. Separate analyses were performed for each
795 response variable.

	Assessment[a]				
Treatments[b]	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

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

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

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

Treatments[b]	Assessment[a]		
	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

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

[b]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.

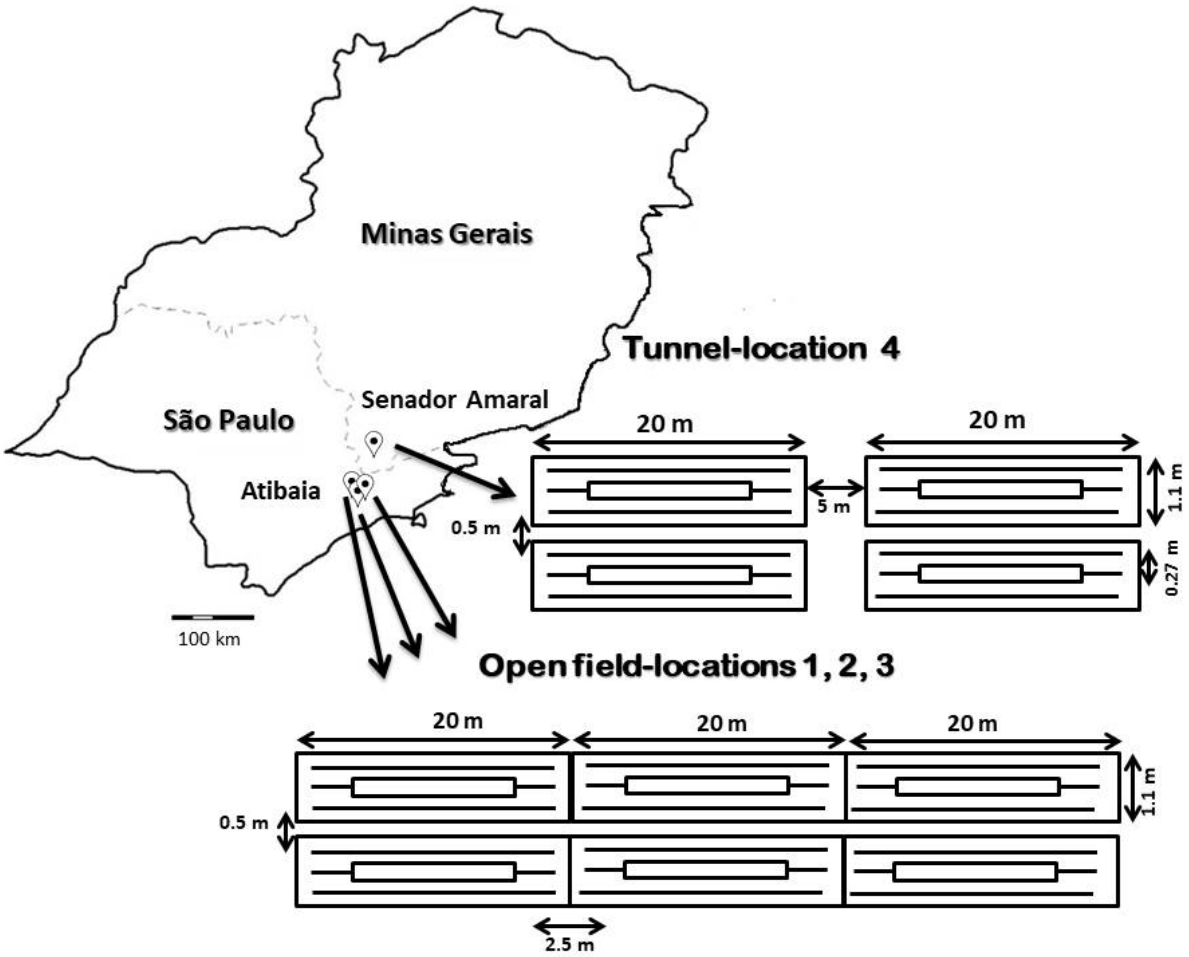


Fig. 1 Experimental field set up in Open Field locations 1, 2 and 3 in Atibaia (1: 23°04'14.32"S 46°40'58.2"W, 2: 23°04'33.5"S 46°40'30.1"W, 3: 23°08'00.7 "S 46°37'04.5"W) and in Low Tunnel location 4 in Senador Amaral (22°33'12.1"S 46°13'41.8"W). Rows and area used for recording of data are indicated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

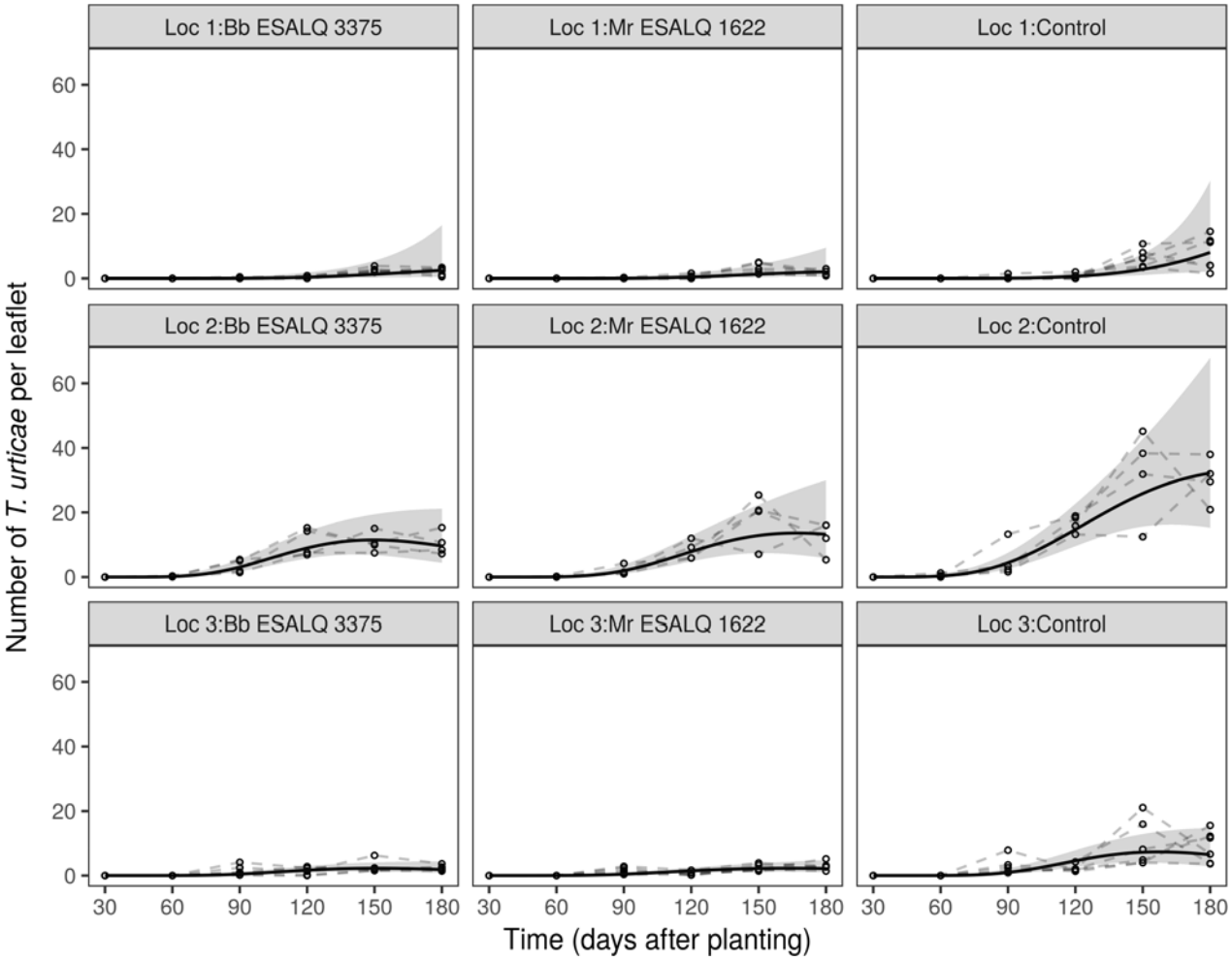
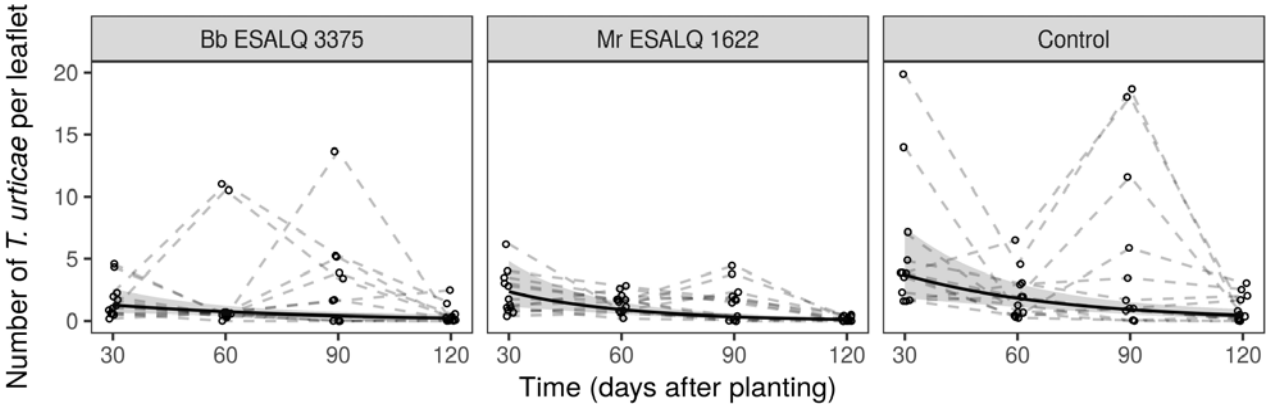


Fig. 2 Effect of inoculation of strawberry root with *Beauveria bassiana* (Bb) isolate ESALQ 3375 or *Metarhizium robertsii* (Mr) ESALQ 1622 on numbers of adult *Tetranychus urticae* per leaflet 30, 60, 90, 120, 150 and 180 days after inoculation, at the Open Field locations 1, 2 and 3 in Atibaia, São 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



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851 **Fig. 3** Effect of inoculation of strawberry root with *Beauveria bassiana* (Bb) isolate ESALQ 3375 or
852 *Metarhizium robertsii* (Mr) ESALQ 1622 on numbers of adult *Tetranychus urticae* per leaflet from 30, 60, 90
853 and 120 days after inoculation at the Low Tunnel location 4 in Senador Amaral, Minas Gerais State, Brazil
854 (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
855 number of *T. urticae* per leaflet and the gray areas represent 95% confidence intervals