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, *Tetranychus urticae*, plant growth, compatibility, *Phytoseiulus persimilis*.

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 Ownley, 2018; Jaber and Alananbeh, 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) 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; Donga et al., 2018; Bamisile 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. Seyedi 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. Material and Methods

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 2.3) 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. 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%.

2.3. 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.

2.4. 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; Cross 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.

2.5. 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 (2.4, plants with spider mites) and also on plants used in the experiments with predatory mites (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 (2.4; 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.

2.6. 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 2.3, 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).

2.7. 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 (2.4), 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.

2.8. 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.

2.9. 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.

3. Results

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, $p = 0.1146$).

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 *B. bassiana*, *M. robertsii*, 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

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.

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: *B. bassiana* = 34.9 ± 1.47; *M. robertsii* = 33.8 ± 1.79; *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).

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 fungi 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).

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%).

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.

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 *I. fumosorosea* (isolate 17) and *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 *M. persicae*. 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 un-inoculated 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 *A. colemani* 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 the 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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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.

Figure legends

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) *B. bassiana*, C) *M. robertsii* and D) is *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.

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) *B. bassiana*, C) *M. 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.

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) *B. bassiana*, C) *M. 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.

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 *P. 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.

Figure 1

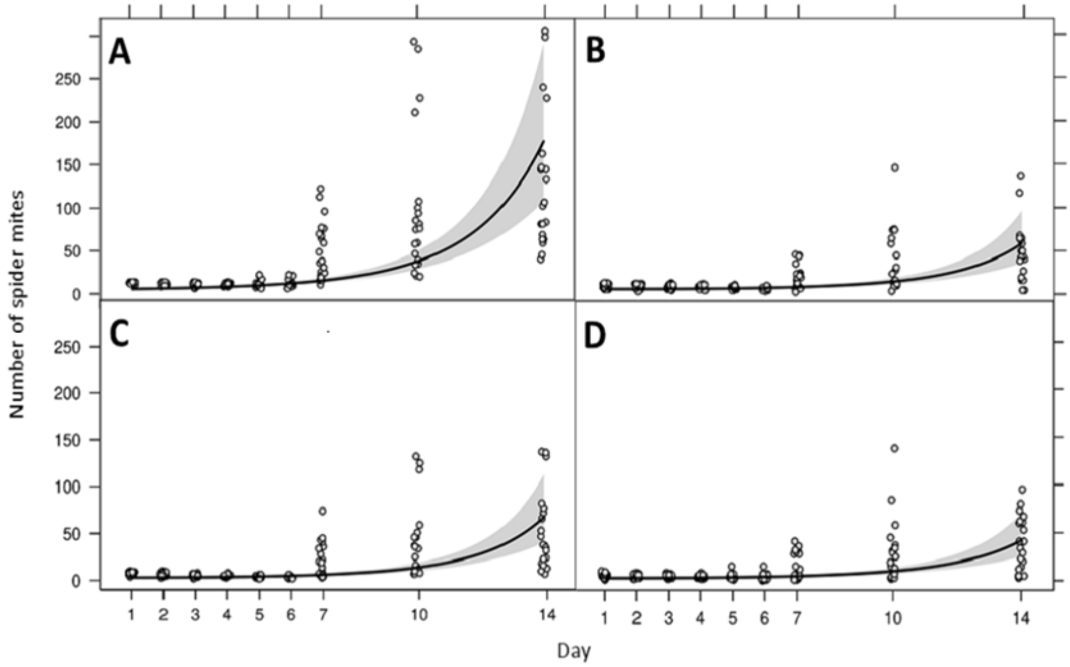

A
B
C
D
Number of spider mites
0
50
100
150
200
250
1
2
3
4
5
6
7
10
14
Day

Figure 2

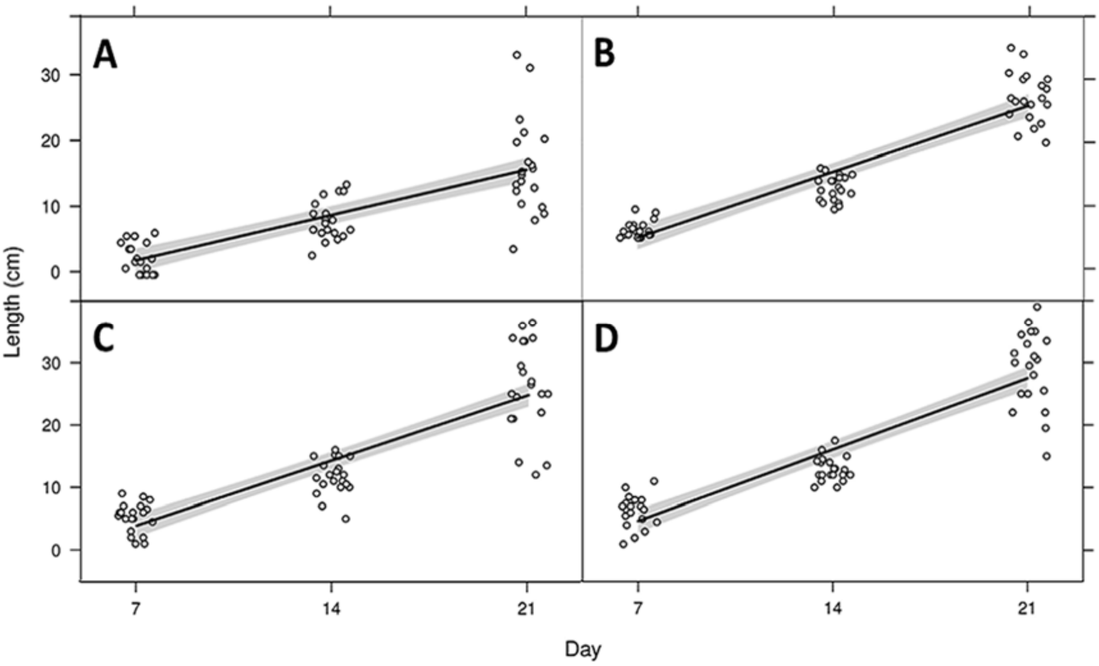


Figure 3

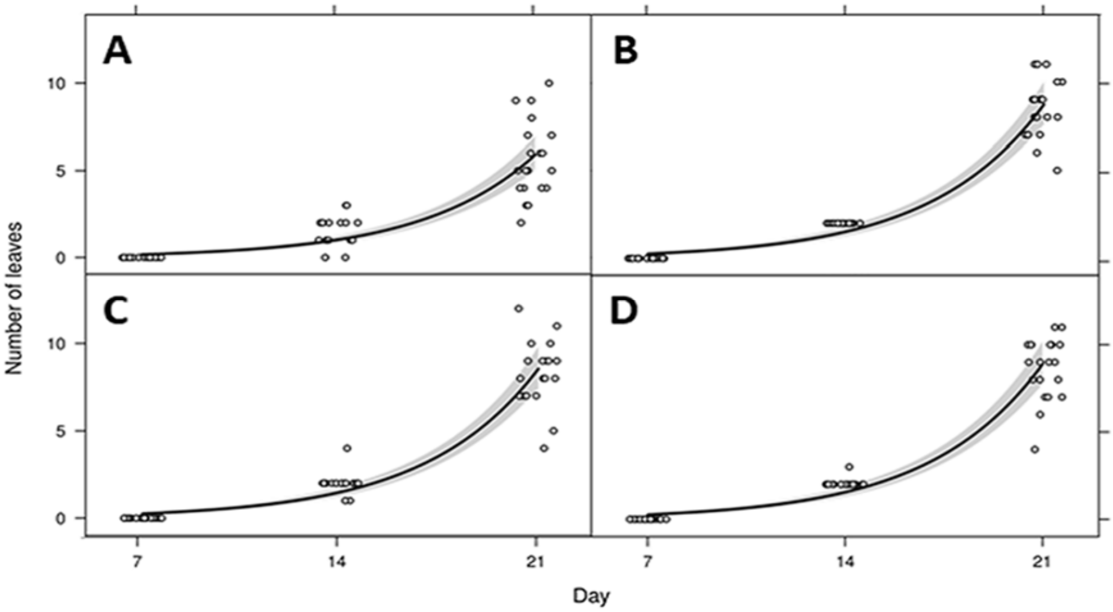
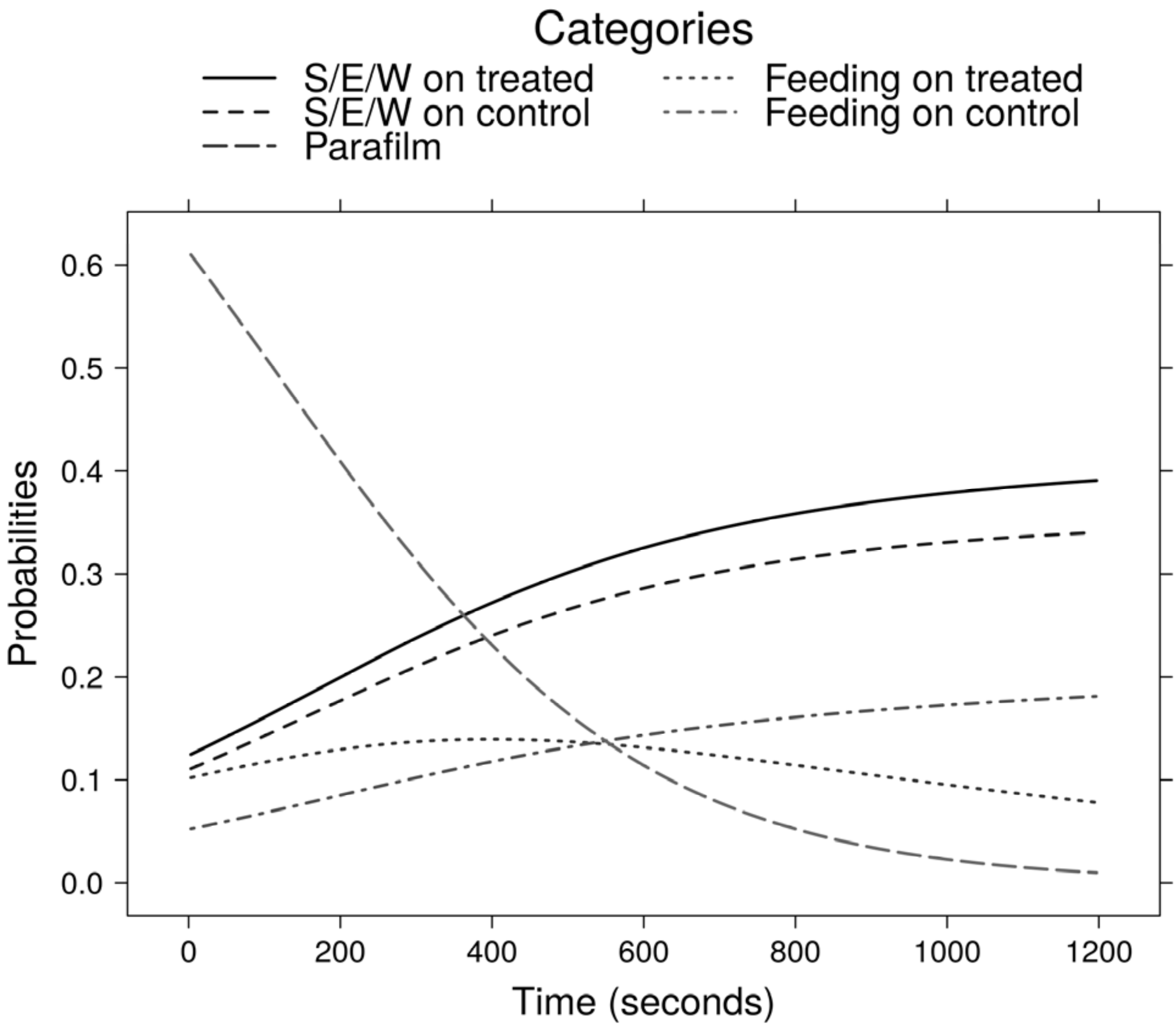


Figure 4


Categories
S/E/W on treated
S/E/W on control
Parafilm
Feeding on treated
Feeding on control
Probabilities
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
200
400
600
800
1000
1200
Time (seconds)