Effects of plant-beneficial fungi on plant growth and herbivore resistance under contrasting fertilizer conditions

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Abstract

Background and Aims

Plant-beneficial fungi play an important role in enhancing plant growth and protecting plants from biotic and abiotic stresses. However, context-dependency of such effects and differences among fungi often lead to inconsistent results that hamper their widespread use. Here, we investigated the effect of plant-beneficial fungi on plant growth and herbivore resistance, and how effects are mediated by fertilization.

Methods

Sweet pepper (*Capsicum annuum* L.) plants were root-inoculated with the plant-beneficial fungi *Beauveria bassiana* ARSEF 3097 and *Trichoderma harzianum* T22 and grown in a low-nutrient potting mix, with or without additional nutrients. Plant growth and herbivore resistance against the southern green stink bug (*Nezara viridula* L.) were compared between fungal treatments and fertilization levels by measuring several growth traits and quantifying feeding damage and plant defense-related gene expression.

Results

Fertilization significantly increased plant growth, but at the same time made plants more susceptible to herbivory. Irrespective of fertilization, *T. harzianum* stimulated plant growth and reduced feeding damage (number of leaf punctures), while *B. bassiana* only enhanced growth. For both strains, fungal inoculation generally increased the expression of marker genes involved in salicylic acid- and jasmonic acid-dependent defense responses upon herbivory, but this was less pronounced for salicylic acid-dependent defense signaling under fertilization.

Conclusions

We conclude that fungal inoculation improved plant growth and generally elicited a stronger defense response to stink bug feeding. Accordingly, plant damage was reduced by *T. harzianum*. Overall these results show that plant-beneficial fungi have the potential to promote plant growth and reduce feeding damage, irrespective of fertilization.

Keywords

Beauveria bassiana; herbivore resistance; *Nezara viridula*; plant growth promotion; *Trichoderma harzianum*

Introduction

The soil represents one of the most biodiverse ecosystems on Earth and is a vast source of various beneficial microorganisms, including bacteria and fungi, that are actively recruited by plants from the soil (Dini-Andreote 2020; Pang et al. 2021; Pieterse et al. 2014). These plant-beneficial microbes protect plants against biotic and abiotic stress and promote plant growth by a variety of mechanisms, including production of phytohormones, enhancement of nutrient uptake, activation of induced resistance, and production of bioactive secondary metabolites and antibiotics (Bamisile et al. 2018; Baron and Rigobelo 2022; Jaber and Ownley 2018; Woo et al. 2022). While the primary focus of research on plant-beneficial fungi has been on plant growth promotion and protection against plant pathogens, there is increasing evidence that plant-beneficial fungi also protect crops from insect herbivores (Pineda et al. 2010). For instance, it has been demonstrated that plant inoculation with arbuscular mycorrhizal fungi (AMF), root-colonizing fungi like *Trichoderma* or endophytes negatively influences the performance of aphids, spider mites, white flies, caterpillars, and stink bugs, correlating with enhanced defensive plant responses (Alınç et al. 2021; Contreras-Cornejo et al. 2018; Coppola et al. 2019; Getman-Pickering et al. 2021; Gupta et al. 2022; Jaber and Araj 2018; Rasool et al. 2021a, b; Wilberts et al. 2022). The use of plant-beneficial fungi has therefore become an emerging strategy to boost sustainable agriculture, but their persistence and functionality under field conditions are sometimes inconsistent and beneficial effects are often context-dependent (Baron and Rigobelo 2022; Lee Díaz et al. 2021).

Examples of major biotic factors determining the plant-beneficial effects of fungal inoculation include the plant species and developmental stage (Geisen et al. 2021), fungal species or strain (Raad et al., 2019; Rasool et al., 2021a), and interactions with other microorganisms (Alves et al. 2021). Furthermore, environmental factors such as temperature (Di Lelio et al. 2021), light availability (Konvalinková and Jansa 2016; Saha et al. 2022) and soil characteristics (Del Valle et al. 2020) can mediate the effects of plant-beneficial fungi. As a result, the positive effects of plant-beneficial fungi are often unpredictable, posing an important bottleneck for wide adoption in agriculture. A better understanding of the interplay between plants and beneficial fungi would provide opportunities to optimize the use of plant-beneficial fungi for sustainable agriculture and ecosystem management.

One of the most important factors affecting plant performance is nutrient availability in the soil (Miransari 2013). When nutrients are scarce, plants usually start developing a more extensive root system and release root exudates that favor microbial colonization in order to enhance nutrient uptake (Oldroyd and Leyser 2020). Also, soil microbes have been shown to mitigate the adverse effects of excessive nutrient concentrations on plant performance through enhanced nutrient extraction and sequestration (Miransari 2013; Zhuang et al. 2007). Furthermore, plant defense can also be directly linked to nutrient availability, since the production of chemical defensive compounds requires nutrients (Koricheva 2002; Neilson et al. 2013). Several studies have reported effects of soil nutrient availability on both direct and indirect plant defenses against herbivores (Lou and Baldwin 2004; Stout et al. 1998), ranging from positive to neutral or negative effects (Chen et al. 2010). However, despite the importance of soil nutrients for plant performance, remarkably little is known about how nutrient levels modulate the effectiveness of plant-beneficial microbes and consequently plant performance.

In this study, we evaluated the effects of root inoculation with beneficial fungi on plant growth and herbivore resistance, and how this is mediated by fertilization. Experiments were performed using sweet pepper (*Capsicum annuum* L.; Solanaceae) and the fungal strains *Beauveria bassiana* ARSEF 3097 (Hypocreales: Cordycipitaceae) and *Trichoderma harzianum* T22 (Hypocreales: Hypocreaceae). While *Trichoderma* constitutes a well-documented soilborne plant-beneficial organism (Kubheka and Ziena 2022; Woo et al. 2022), the beneficial effects of endophytic colonization by soil-dwelling entomopathogenic fungi like *B. bassiana* (Meyling and Eilenberg 2007; Quesada Moraga 2020) on plant performance have only been demonstrated more recently (Gange et al. 2019; Jaber and Ownley 2018; Vega 2018). Further, the southern green stink bug *Nezara viridula* L. (Heteroptera: Pentatomidae) was used as study species. This stink bug species is widely distributed across (sub)tropical and Mediterranean regions of the world, where it causes damage to a broad range of important crops such as soybean and cotton. More recently, due to global warming, *N. viridula* has expanded its distribution range to north- western Europe, where it attacks diverse vegetable crops, including tomato, sweet pepper and cucumber (Conti et al. 2021; Geerinck et al. 2022). With their piercing-sucking mouthparts, the stink bugs puncture plant tissues and cause major damage to fruits, seeds, growing shoots, flowers and leaves (Conti et al. 2021).

Materials & Methods

Study organisms

Beauveria bassiana ARSEF 3097 is the active ingredient in the commercial bioinsecticide Naturalis®, and was obtained from the Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF; New York, USA). The strain has been shown to colonize diverse plant species endophytically upon artificial inoculation, including sweet pepper, in addition to its direct insect-killing capability (Jaber and Araj 2018; Vega et al. 2008; Wilberts et al. 2022). *Trichoderma harzianum* T22 (recently re-classified as *Trichoderma afroharzianum* (Chaverri et al. 2015); for consistency with previous research further referred to as *T. harzianum* in this manuscript) is a fungal strain produced by protoplast fusion (Harman et al. 2004). The strain is the active ingredient of a variety of biopesticides and biofertilizers (Vitti et al. 2015) including Trianum-P (Koppert Biological Systems, The Netherlands), from which it was isolated. Fungal strains were stored on potato dextrose agar plugs in 35 % glycerol at -80 °C until further use. All experiments were conducted using sweet pepper (*Capsicum annuum*) cv 'IDS RZ F1' (Rijk Zwaan, The Netherlands). Plants were sown in potting mix (DCM Zaaien & Stekken; DCM, Belgium) (see Table S1 (Supplementary Information) for the chemical composition of the potting mix) and put in a climate cabinet (MD1400, Snijders Labs, The Netherlands) at 23 ± 1˚C, 65 ± 2% RH and a 16L:8D photoperiod until fungal inoculation (see below). The cabinet was illuminated with LED lights to provide a photosynthetic flux density of 790 µmol photons m^{-2} s^{-1}. As focal insect species, *N. viridula* was used. A lab colony of *N. viridula* was originally established from a lab strain from the University of Palermo (Alınç et al. 2021). On a regular basis field-caught individuals were introduced in the colony to avoid inbreeding. Stink bugs were reared in insect cages (47.5 × 47.5 × 47.5 cm) (BugDorm, MegaView Science Co. Ltd., Taiwan) under controlled conditions (ECL02, Snijders Labs, The Netherlands) at 25 ± 1°C, 70 ± 2% RH and a 16L:8D photoperiod. Stink bugs were fed with seasonal organic vegetables (tomatoes, cabbage, beans and cauliflower) and organic seeds (sunflower, soybean and peanut). A wet

cotton roll was provided as an additional source of water. Furthermore, a sweet pepper plant was placed in the rearing cage, along with paper towels as oviposition substrates. Every two-three days the food and water were replaced and newly laid eggs were collected to maintain the colony. Nymphs obtained from the eggs were maintained under the same conditions as the adults, and newly emerged adults were used for continuing the rearing.

Experimental setup

In total, three experiments were performed in which the effect of fungal inoculation and the addition of fertilizer was evaluated on different aspects related to plant performance, including plant growth (Experiment 1), resistance to insect herbivory (Experiment 2) and activation of molecular plant defense responses (Experiment 3). Plants were either inoculated with one of the two fungi studied, or with physiological water (control). Fungal spore suspension preparation and plant inoculation were performed according to the procedures described in detail in Wilberts et al. (2022). Briefly, stored agar plugs were plated on PDA (Oxoid Holdings Ltd., United Kingdom) (*T. harzianum* T22) or Sabouraud dextrose agar medium supplemented with 0.25% yeast extract (SDAY) (Oxoid Holdings Ltd., United Kingdom) (*B. bassiana* ARSEF 3097), and plated once again onto the same agar medium before use. Fungal strains were then cultured on the corresponding agar media at 25°C for seven days. Subsequently, fungal spore suspensions were prepared by flooding plates with sterile physiological water (0.8% NaCl), and gently scraping spores from the dishes. Next, to remove mycelial fragments, the suspensions were filtered through a microcloth (Mira Cloth, Merck, USA) and washed two times with physiological water. The conidial concentration was determined using a Bürker hemocytometer. Finally, the suspensions were diluted to a final concentration of 1×10^7 conidia mL^{-1} for further use in experiments. Plants were inoculated when they reached the first-true leaf stage. After rinsing the seedling roots under a stream of tap water, the roots were submerged in 10 mL of conidial spore suspension or physiological water to obtain non-inoculated control plants. Afterwards, seedlings were planted in the same potting mixture as mentioned above in 17 cm diameter pots (2 L), and transferred to the greenhouse until further use in the experiments. In all experiments, plants received either no additional fertilizer (rainwater, not fertilized) or were fertilized with an additional nutrient solution (fertilized), recommended for the cultivation of sweet pepper (Table S2, Supplementary Information). Plants were put in the greenhouse according to a completely randomized design. Each plant was watered daily at regular time intervals by drip irrigation with individual irrigation tubes providing the water or nutrient solution for 'not fertilized' or 'fertilized' treatments, respectively. Greenhouse settings were a 14L:10D photoperiod and temperature and humidity settings at 20 ± 5°C and 65 ± 10%, and 18 ± 5°C and 70 ± 10%, during day and night, respectively. If incoming solar radiation was less than 450 W m^{-2} during the day, additional illumination was automatically supplied by high-pressure sodium lamps (Son-T 400 W).

Experiment 1: Assessing plant growth

In a first experiment, plant height, stem diameter, number of true leaves, number of flowers and leaf canopy area were recorded both four and eight weeks after inoculation. Measurements were performed on 14 biological replicates per treatment. Plant height was measured as the distance between the germ leaves and the apex, while stem diameter was measured with a digital caliper 0.5 cm above the position of the germ leaves. When determining the number of flowers, both the number of developing and completely developed flowers were counted. Canopy area was measured by

calculations from top view images as outlined in van Wesemael et al. (2019). Briefly, top view images were taken against a blue background, while the plant pot was covered with blue plastic as well. A red reference card of known size (10 cm × 5 cm) was put next to each plant. Next, raw images were transformed into an image containing only RGB-values by color segmentation using an in-house R program (van Wesemael et al. 2019), and canopy area (green plant pixels) was calculated. At the end of the experiment, eight weeks after inoculation, dry weight of the aboveground biomass was recorded, by putting the aboveground plant tissues (stem cut right above the germ leaves) individually in paper bags and drying in an oven until constant weight was reached. In addition, the mineral composition of the sampled and dried plant material was determined by the Soil Service of Belgium (Belgium). Contents of P, K, Mg, Ca, Fe, B, Cu, Na, Zn, Mn were analyzed by ICP-AES (inductively coupled plasma atomic emission spectroscopy) while total N and total C were determined using spectrometry, according to standard protocols. Analyses were performed on five pooled samples per treatment.

Experiment 2: Assessing plant damage

In a second experiment, the effects of fungal inoculation and fertilization on damage by *N. viridula* on sweet pepper leaves was evaluated by counting the number of salivary sheath flanges. Salivary sheath flanges are left behind when stink bugs have inserted their piercing-sucking mouthparts in plant tissues (Miles 1972). Since these stylet sheaths are formed more consistently than any other sign of plant tissue damage, such as external necrotic spots, they can be used as an indicator of crop damage by stink bug feeding (Bundy et al. 2000). The experiment was performed with plants eight weeks after fungal inoculation. One young adult female (2-4 days old), that had been previously starved for 24h, was confined for six days in a transparent plastic clip cage (3 cm diameter) covered with a fine mesh (0.27 mm × 0.77 mm) on the underside of the same leaf for all plants. To avoid plant damage from the clip cages, the rim of the clip cages was covered with soft compressible foam. Afterwards, stink bugs were removed and the leaf was cut off to count the number of sheath flanges. To visualize flanges, McBryde's staining solution (McBryde 1936) was prepared containing 0.2% acid fuchsin (Polysciences Europe, Germany) in 95% ethanol and acetic acid (1:1 vol:vol). Leaves were submerged in staining solution for 10 min after which they were rinsed with demineralized water. Subsequently, flanges were counted with the aid of a microscope. For each treatment, 15 replicates (with each replicate being one leaf coming from one plant) were included.

Experiment 3: Assessing molecular plant defense responses

In a third experiment, the effects of fungal inoculation and fertilization on modulation of plant defense against *N. viridula* were evaluated. To this end, the relative expression level of a set of different marker genes involved in major defense signaling pathways in sweet pepper was measured. Therefore, six weeks after fungal inoculation, plants of all fertilization-inoculation treatment combinations were randomly allocated to herbivory or no herbivory (five plants per treatment). For the herbivory treatment, two young (4-7 days old) adult females of *N. viridula*, that had been previously starved for 24 h, were enclosed in a transparent plastic clip cage (3 cm diameter) on the eighth fully expanded leaf of each plant, containing one female in the top compartment and one female in the bottom compartment of the clip cage. To determine gene expression levels without herbivory, plants received an empty clip cage. Stink bugs were allowed to feed for 8h, after which the clip cages and insects were removed to sample the exposed leaves. Leaf disks (25 mm

diameter) were punched out from the area where the clip cages were attached, and were immediately frozen in liquid nitrogen and stored at -80°C until RNA isolation. Extraction and purification of total RNA were carried out using the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Five biological replicates (each consisting of one leaf disk sampled from one plant) were included for each treatment. To eliminate potential contamination of DNA, on-column DNase digestion treatment was performed using the RNase-Free DNase set (Qiagen, Germany). After evaluation of the RNA concentration and purity, cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad, USA), following the kit's instructions. Expression levels of two genes involved in salicylic acid (SA)-dependent plant defense responses (*CaPR1* and *CaPR9*) and two genes involved in jasmonic acid (JA)-dependent plant defenses (*CaPINII* and *CaLOX2*) were evaluated by RT-qPCR (see Supplementary Information Table S3 for primer sequences). Quantitative PCR reactions were prepared in a 20 µL reaction volume using the iTaq Universal SYBR Green Supermix (Bio-Rad, USA). The thermal cycling program (StepOnePlus™, Thermo Scientific, USA) consisted of 95°C for 3 min followed by 40 cycles of 95°C for 15 s and 60°C for 45 s, followed by a melting curve analysis (steps of 15 s from 60°C to 90°C with a heating rate of 0.3°C s^{-1}) to confirm the specificity of the assay. Negative controls (nuclease-free water instead of cDNA template) were included in each qPCR run to confirm the purity of the reagents. All qPCR reactions were performed in triplicate. Expression levels were analyzed using the comparative Cq method (also known as $2^{-\Delta\Delta Cq}$ method) (Livak and Schmittgen 2001) and are presented as the average fold changes relative to the expression of the reference treatment (non-inoculated plants that were not fertilized and undamaged). The expression level of the reference gene *CaACTIN* was used to normalize qPCR data.

Statistical analysis

All data were analyzed using R version 3.6.3 (R Core Team 2019). For all analyses, a significance level of $\alpha = 0.05$ was used to assess significant differences. The effects of fungal strain and fertilization on plant growth traits (Experiment 1) were analyzed using two-way MANOVA (using Roy's Largest Root test) with fungal strain, fertilization and their interaction as independent factors. For the measurements four weeks after inoculation the MANOVA was performed on the variables plant height, stem diameter, number of leaves, number of flowers and canopy area, while for the measurements eight weeks after inoculation dry weight was included as well. Subsequently, effects of the treatments on the individual response variables were checked by two-way ANOVA, after checking assumptions of normality and homogeneity of variances. In case of unequal variances, White's heteroscedasticity-corrected standard errors were used. Tukey's HSD test was used for pairwise comparisons of fungal strains when a model was found to be significant. Statistical analyses were performed separately for both time points. In all the previous models the interaction factor proved to be non-significant; hence it was excluded from the models for further analysis. The mineral composition of the aboveground plant tissues was analyzed similarly as described above, using two-way MANOVA (using Roy's Largest Root test) followed by univariate ANOVAs. The interaction factor was again found to be non-significant, so it was removed from the model. To visualize differences between fungal strains and fertilizer treatments, the mineral composition of the plants was subjected to principal component analysis (PCA) on the correlation matrix (with centered and standardized data). The number of salivary sheath flanges counted after staining (Experiment 2) was analyzed using a generalized linear model (GLM) based on a negative binomial

distribution. A type III two-way ANOVA was performed on this model to evaluate the overall effect of fungal strain, fertilization and their interaction. Since the interaction term was found to be non-significant, it was further excluded from the model. To examine differences between fungal strain, a pairwise post hoc test was performed on the significant model. Differences in gene expression levels (Experiment 3) between different treatments were analyzed using three-way ANOVA, with herbivory, fertilization and fungal strain as fixed factors, and all possible interaction terms between the factors. Analysis was performed on the ΔCq-values. Post-hoc analysis was done using Tukey's HSD test after determining the best model. In all cases the best model was selected based on the AIC (Aikaike Information Criterion).

Results

Experiment 1: Assessing plant growth

Both four and eight weeks after inoculation, fungal inoculation (four weeks after inoculation: Roy's Largest Root = 0.167, $F_{5,76}$ = 2.532, P = 0.036; eight weeks after inoculation: Roy's Largest Root = 0.267, $F_{6,75}$ = 3.198, P = 0.008) and fertilization (four weeks after inoculation: Roy's Largest Root = 0.305, $F_{5,75}$ = 4.580, P = 0.001; eight weeks after inoculation: Roy's Largest Root = 2.121, $F_{6,74}$ = 25.097, P < 0.001) significantly affected overall plant growth. None of the interaction terms were significant. When looking at the individual growth variables, both fungal inoculation and fertilization had a significant effect on all plant growth variables eight weeks after inoculation (Table 1; Fig. 1). On the contrary, four weeks after inoculation fungal strain only affected plant height and canopy area (Table 1; Fig. 1). Plant inoculation with *B. bassiana* resulted in larger plants than the control treatment (P = 0.003), while inoculation with *T. harzianum* increased canopy area compared to control plants (P = 0.017). The addition of fertilizer only caused a significant increase in stem diameter (P < 0.001) and canopy area (P = 0.044) four weeks after inoculation. No interaction effect was found between fungal strain and fertilization, for none of the measured response variables, at both time points. Most beneficial effects on plant growth were found for plants inoculated with *T. harzianum*. When compared with control plants, eight weeks after inoculation plants inoculated with *T. harzianum* had thicker stems (P = 0.026), more leaves (P = 0.017), more flowers (P = 0.009), a larger canopy area (P = 0.005) and a higher biomass (P = 0.031). Beneficial effects of inoculation with *B. bassiana* on plant growth were less pronounced, only resulting in slightly significantly more leaves (P = 0.046) and a larger canopy area (P = 0.049) eight weeks after inoculation compared to non-inoculated plants.

MANOVA performed on the nutrient concentrations of the aboveground plant tissues eight weeks after inoculation revealed significant effects of fungal strain (Roy's Largest Root = 3.147, $F_{12,16}$ = 4.196, P = 0.004) and fertilization (Roy's Largest Root = 110.943, $F_{12,15}$ = 138.678, P < 0.001), while no significant interaction effect was found between these factors. PCA showed a clear separation between the different fertilization and fungal inoculation treatments in the first two components explaining 63.71% and 15.48% of the variation (Fig. 2). While the first PC axis clearly separated samples according to fertilization treatment, the second PC axis separated samples according to fungal treatment (Fig. 2). Fertilization significantly affected the concentrations of P, K, Mg, Ca, Zn and Mn, and N and C content, while fungal inoculation significantly influenced the concentrations of B, Na and Mg (Table S4, Supplementary Information). More specifically, compared to control plants and plants inoculated with *B. bassiana*,

inoculation with *T. harzianum* resulted in significantly higher B contents ($P < 0.001$ and $P < 0.001$, respectively) and lower Na contents ($P = 0.002$ and $P = 0.042$, respectively), and significantly higher Mg contents compared to plants inoculated with *B. bassiana* ($P = 0.031$).

Experiment 2: Assessing plant damage

Fungal inoculation and fertilization had a significant effect on leaf damage (expressed as total number of salivary sheath flanges), while no significant interaction effect between both factors was found (Fig. 3). Plants that were fertilized had a higher number of stylet sheaths than plants that were not fertilized ($\chi^2 = 24.57$, $df = 1$, $P < 0.001$). On average, a 47% increase in feeding damage was found in fertilized plants. Moreover, fungal inoculation had a significant effect on the number of stylet sheaths ($\chi^2 = 20.44$, $df = 2$, $P < 0.001$). In particular, plants inoculated with *T. harzianum* had a lower number of stylet sheaths ($P = 0.008$) than non-inoculated plants and plants inoculated with *B. bassiana* ($P < 0.001$). By contrast, no significant difference was found between plants inoculated with *B. bassiana* and control plants ($P = 0.248$). The observed reduction in feeding damage on plants inoculated with *T. harzianum* compared to non-inoculated plants was 38% on average, 39% in fertilized conditions, and 37% for plants that did not receive additional fertilizer.

Experiment 3: Assessing molecular plant defense responses

Expression of *CaPR1* was mainly induced by the application of fertilizer and feeding by *N. viridula*, and there was an interaction effect between both factors (Fig. 4a; Table 2). Leaf transcript levels of *CaPR9* were affected by fungal inoculation, fertilization and herbivory, and the three-way interaction between these factors (Fig. 4b; Table 2). In the absence of herbivory, fungal inoculation did not affect the transcript levels of both marker genes for SA-signaling (*CaPR1* and *CaPR9*), irrespective of fertilization, while fertilization significantly enhanced constitutive SA-responses (Fig. 4a,b). By contrast, in plants exposed to stink bug feeding and grown without any additional fertilizer, inoculation with *T. harzianum* significantly increased transcript levels of *CaPR1*, while inoculation with *B. bassiana* increased transcript levels of *CaPR9*. However, in plants receiving additional fertilizer such increase in expression levels was not seen (Fig. 4a,b). The expression of the JA-marker gene *CaPINII* was affected by fungal inoculation, fertilization and herbivory, in a treatment-specific manner (Fig. 4c; Table 2). In the absence of herbivory, fungal inoculation or the addition of fertilizer did not result in an increase in *CaPINII* transcript levels. On the contrary, fertilization significantly enhanced expression of *CaPINII* in response to stink bug feeding, and inoculation with *B. bassiana* had the same effect (Fig. 4c). Transcript levels of *CaLOX2* were only affected by herbivory and fertilization, with both factors generally increasing *CaLOX2* expression, while fungal inoculation had no influence (Fig. 4d; Table 2).

Discussion

In this study, we assessed the ability of the plant-beneficial fungi *B. bassiana* ARSEF 3097 and *T. harzianum* T22 to promote plant growth and to induce resistance against *N. viridula*, and investigated to what extent these responses were mediated by fertilization. Overall, inoculation with *B. bassiana* and *T. harzianum* enhanced several plant growth traits, and stink bug feeding damage was reduced by 38% in response to inoculation with *T. harzianum*, irrespective of fertilizer application. We also found that fertilization, fungal inoculation and herbivory differentially influenced

plant defenses by inducing expression of defensive-related genes. Most considerable effects of plant-beneficial fungi on upregulation of SA-defensive responses were seen in response to stink bug feeding in unfertilized conditions, while the stimulating effect of plant-beneficial fungi on JA-defensive responses was not mediated by fertilization.

Inoculation with plant-beneficial fungi enhances plant growth, irrespective of fertilization

Both *B. bassiana* and *T. harzianum* improved plant growth, but most pronounced effects on plant growth were observed for *T. harzianum*. These results confirm previous studies showing the plant growth promoting capacities of *Beauveria* (e.g. Jaber & Enkerli, 2017; McKinnon et al., 2023; Raad et al., 2019) and *Trichoderma* species (e.g. Contreras-Cornejo et al., 2009; Nieto-Jacobo et al., 2017). The plant-growth promoting capacities of *T. harzianum* T22 (Harman et al. 2004; Vitti et al. 2015). However, plant growth promotion is not a universal trait of *Trichoderma*, and some *Trichoderma* strains can be neutral or even cause negative effects on plant growth (Nieto-Jacobo et al., 2017; Rasool et al., 2011). Similarly, in addition to positive effects, neutral or negative effects, such as reduced growth or biomass, have been reported for *B. bassiana* (Qayyum et al. 2015; Tall and Meyling 2018; Vega 2018). Results also showed that fertilization did not affect the ability of the fungi to increase plant growth. For AMF it is well established that they are most advantageous to plant growth in nutrient-limited conditions, while they can have neutral or even negative effects on plant performance at high fertilizer levels (Getman-Pickering et al. 2021; Johnson et al. 1997). For plant-beneficial fungi such as *B. bassiana* and *T. harzianum* only little is known in this regard, and contrasting results have been reported. For example, enhanced plant growth has been observed following seed inoculation with *B. bassiana*, but only at high nutrient conditions, while at low nutrient conditions plant growth was reduced or not affected (Tall and Meyling 2018). By contrast, *Trichoderma virens* was found to increase yield and biomass of lettuce irrespective of fertilizer conditions (Visconti et al. 2020), although most evident growth promotion was seen under conditions of low N availability (Fiorentino et al. 2018). Inoculation with *Trichoderma asperellum* caused improved growth of onions under different fertilization regimes, but not when no fertilizer was added (Ortega-García et al. 2015). On the contrary, growth promotion of wild turnip by *T. harzianum* was larger under unfertilized conditions and in less fertile soils (Caporale et al. 2019). In potato, inoculation with the endophytic entomopathogenic fungus *Metarhizium brunneum* enhanced plant productivity and vitality, irrespective of fertilizer addition, although strongest effects were found under fertilized conditions (Krell et al. 2018). In addition to effects on plant growth, our results also showed clear effects of fungal inoculation on shoot nutrient concentrations, irrespective of fertilizer level. These findings are in line with previous studies that have shown that inoculation with plant-beneficial fungi can increase the supply of macronutrients, but mostly micronutrients (Alves et al. 2021; Caporale et al. 2019; Rasool et al. 2011). Fungal inoculation significantly increased the concentration of B and Mg. While B is crucial for the development of flowers, fruits and seeds, as well as for the structural integrity of cell walls and membranes, Mg is indispensable for photosynthesis, as a key component of chlorophyll (Harris et al. 2018). Therefore, adequate contents of these minerals are required for plant growth and development. As such, plant-beneficial fungi may have important relevance in the context of reducing over-fertilization, since similar yields can be obtained by inoculation with these fungi while reducing the input of nutrients (Ortega-García et al. 2015; Visconti et al. 2020). Importantly, our results support the ability of *B. bassiana* to play a significant role as a plant-beneficial fungus by promoting plant growth, in

addition to its entomopathogenic nature (Jaber and Ownley 2018), comparable to the enhanced plant growth conferred by *Trichoderma* spp.

Fertilization results in increased feeding damage, while inoculation with *Trichoderma harzianum* T22 causes a reduction in damage

Addition of fertilizer resulted in a substantial increase in the number of salivary sheath flanges. This is in accordance with previous studies that have shown that soil fertilization can enhance host plant quality and attractiveness for phytophagous insects (Borowicz et al. 2005; Chen et al. 2010; Lu et al. 2007), for example by an increase in nitrogen content, a limiting nutrient for herbivores (White 1984). Further, our results showed a clear reduction in leaf damage in plants inoculated with *T. harzianum* T22 compared to non-inoculated plants. This is consistent with a study on tomato, reporting less foliar damage by *Tuta absoluta* and spider mites after inoculation with *T. harzianum* (Gupta et al. 2022). Similarly, a drastic reduction of foliar damage by *Spodoptera frugiperda* was found on maize inoculated with *Trichoderma atroviride* (Contreras-Cornejo et al. 2018) as well as of root damage by *Phyllophaga vetula* on maize inoculated with *T. harzianum* (Contreras-Cornejo et al. 2021). One possible mechanism for plant-beneficial fungi to affect insect damage is by altering the plant's nutrient composition, such as the C:N ratio, since plants with a low C:N ratio are more nutritional and attractive to herbivores (Getman-Pickering et al. 2021; White 1984). Yet, in this study, no evidence of altered C:N ratio in response to fungal inoculation was found. However, we did observe higher B contents in *T. harzianum*-inoculated plants (see Experiment 1), and previous research showed that B-deficient plants improved the performance of insect herbivores (Beanland et al. 2003). Rather than a result of the mineral requirements of herbivores, these findings were most likely linked to differences in primary and/or secondary metabolites, because B plays an important role in phenolic-based biosynthetic pathways with consequences for compounds involved plant defense. The reduced feeding damage by *N. viridula* we observed may be due to the involvement of *T. harzianum* T22 in affecting direct plant defenses (Coppola et al. 2019). By manipulating signaling pathways, plant-beneficial fungi induce drastic transcriptomic and metabolomics changes (Coppola et al. 2019) that result in the accumulation of specific defensive compounds with deleterious effects on herbivores (Pineda et al. 2010). The production of anti-feeding compounds can explain the observed reduction in feeding damage. Feeding deterrence as a result of fungal-induced metabolites is believed to be an important mechanism of increased herbivore resistance upon fungal inoculation (McGee 2002; Vega 2008), although evidence *in planta* is still scarce. There is ample evidence of *Trichoderma* spp. being able to produce anti-feeding compounds that directly harm pest insects *in vitro* (Contreras-Cornejo et al. 2021; Poveda 2021). However, there is little information available if this is also the case when *Trichoderma* spp. live in association with plants. In a study on tomato, inoculation with *T. harzianum* T22 caused no decrease in food consumption by *Spodoptera littoralis* larvae, while negative effects were observed on insect performance, suggesting anti-feeding compounds were not produced (Di Lelio et al. 2023). Further research is needed to find out how inoculation with *T. harzianum* T22 reduced feeding damage by *N. viridula*.

Inoculation with plant-beneficial fungi and fertilization alter plant defensive hormonal pathways in response to stink bug feeding

Fertilization enhanced basal transcript levels (expression levels in absence of herbivory) of SA-dependent defenses, while no effect was found on JA-dependent signaling. The latter is in agreement with previous research reporting no

effect of fertilization on constitutive proteinase inhibitor activity or total JA-production in the absence of herbivory (Chen et al. 2008; Mason et al. 2022; Stout et al. 1998). Furthermore, in inoculated plants stink bug feeding generally induced higher expression of SA- and JA-dependent defenses. This was mainly the case in unfertilized conditions, while the addition of fertilizer was found to overrule the effect of fungal inoculation on SA-dependent defenses. Previous studies have shown that inoculation of tomato plants with *T. harzianum* T22 increased transcript levels of several genes involved in direct defense induced by aphid infestation (Coppola et al. 2019) and *N. viridula* feeding (Alınç et al. 2021), which coincided with reduced herbivore performance. Likewise, inoculation with *B. bassiana* has been shown to drastically influence both SA- and JA-signaling pathways, even in the absence of herbivory (Gupta et al. 2022; Raad et al. 2019). It must be noted, however, that the regulation of plant defenses often shows a temporal pattern (Alınç et al. 2021). In our study, stink bugs were allowed to feed on leaves for 8 h. After this period of time transcript levels of *CaPR1* and *CaPR9* in non-inoculated plants were not found to be significantly increased compared to undamaged plants, while inoculation with *B. bassiana* and *T. harzianum* caused a significant increase in expression levels in response to stink bug feeding. This is in line with Alınç et al. (2021), who found a slower induction of SA-defense responses in tomato in response to *N. viridula* feeding, although they did not find evidence for enhanced SA-dependent responses in inoculated plants, which might be explained by the host-specificity of the regulation of plant defenses. Similarly, our findings regarding upregulation of JA-dependent defense responses (in fertilized conditions) are in agreement with Alınç et al. (2021), although we did not find a significant increase in expression levels of *CaPINII* after inoculation with *T. harzianum*, but only with *B. bassiana*, compared to non-inoculated plants.

Conclusion

Altogether, our results show that *T. harzianum* T22 and *B. bassiana* ARSEF 3097 have the ability to promote plant growth and reduce feeding damage by *N. viridula*, irrespective of fertilization. Largest beneficial effects were found for *T. harzianum* T22, leading to a 38% reduction in leaf feeding damage. Further research is needed to verify these effects in field-settings, and to determine the context to obtain the largest effects of plant-beneficial fungi. By optimizing the (a)biotic context (including fertilizer level) to support fungal plant partners, farmers and agricultural practitioners can potentially unlock the full benefits of plant-beneficial fungi, ultimately leading to more sustainable and resilient agricultural systems.

Statements & Declarations

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Author contributions

SVH, AC, HJ and BL contributed to the study conception and design. Material preparation and data collection were performed by SVH, IS and TA. Data analysis was performed by SVH. The first draft of the manuscript was written by SVH and all authors commented on previous versions of the manuscript and read and approved the final manuscript.

Data availability

All relevant data are presented in the manuscript and its Supplementary Information. The datasets generated during and/or analyzed during the study are available from the corresponding author on request.

Declaration of competing interests

The authors have no relevant financial or non-financial interests to disclose.

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Tables

Table 1 Effects[a] of fungal strain inoculation (three levels)[b] and addition of fertilizer (two levels)[c] on several plant growth traits of sweet pepper plants, four and eight weeks after fungal inoculation.

Response variable	Four weeks after inoculation				Eight weeks after inoculation			
	Fungal strain		Fertilizer		Fungal strain		Fertilizer	
	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value
Plant height	5.904	0.004 **	1.388	0.242	3.321	0.041 *	59.871	< 0.001 ***
Stem diameter	2.296	0.107	17.528	< 0.001 ***	3.937	0.024 *	28.040	< 0.001 ***
Number of leaves	1.794	0.173	0.845	0.361	4.395	0.015 *	79.726	< 0.001 ***
Number of flowers	1.567	0.215	0.023	0.879	4.88	0.010 *	100.953	< 0.001 ***
Canopy area	4.549	0.014 *	4.181	0.044 *	5.246	0.007 **	96.294	< 0.001 ***
Dry weight[d]	/		/		3.461	0.036 *	133.460	< 0.001 ***

[a] F-values and P-values from two-way ANOVA. Asterisks indicate significance of the factors ($0.05 > P > 0.01$: *; $0.01 > P > 0.001$: **; $P < 0.001$: ***).

[b] Plants were root-inoculated with *Trichoderma harzianum* T22 or *Beauveria bassiana* ARSEF 3097, or were mock-inoculated (control).

[c] Plants were grown in potting mix, with or without additional fertilizer.

[d] Dry weight was only determined eight weeks after fungal inoculation.

Table 2 Effects[a] of fungal strain inoculation (three levels)[b], addition of fertilizer (two levels)[c] and herbivory (two levels)[d] on the relative expression level of plant defense marker genes.

Gene	Fungal strain	Fertilizer	Herbivory	Fungal strain × Fertilizer	Fungal strain × Herbivory	Fertilizer × Herbivory	Fungal strain × Fertilizer × Herbivory
CaPR1	1.808 (0.178) *ns*	111.460 (< 0.001) ***	24.262 (< 0.001) ***	2.755 (0.076) *ns*	1.843 (0.172) *ns*	15.648 (< 0.001) ***	1.076 (0.351) *ns*
CaPR9	4.258 (0.022) *	54.826 (< 0.001) ***	127.439 (< 0.001) ***	2.281 (0.117) *ns*	2.084 (0.139) *ns*	3.491 (0.070) *ns*	4.288 (0.021) *
CaPINII	5.836 (0.006) **	29.936 (< 0.001) ***	130.700 (< 0.001) ***	/	4.093 (0.025) *	10.330 (0.003) **	/
CaLOX2	/	0.004 (0.949) *ns*	18.267 (< 0.001) ***	/	/	6.126 (0.017) *	/

[a] F-values from three (or two)-way ANOVA, with p-values given between brackets. Asterisks indicate significance of the factor ($0.05 > P > 0.01$: *; $0.01 > P > 0.001$: **; $P < 0.001$: ***; *ns*: not significant; /: not included in the final model).

Figures

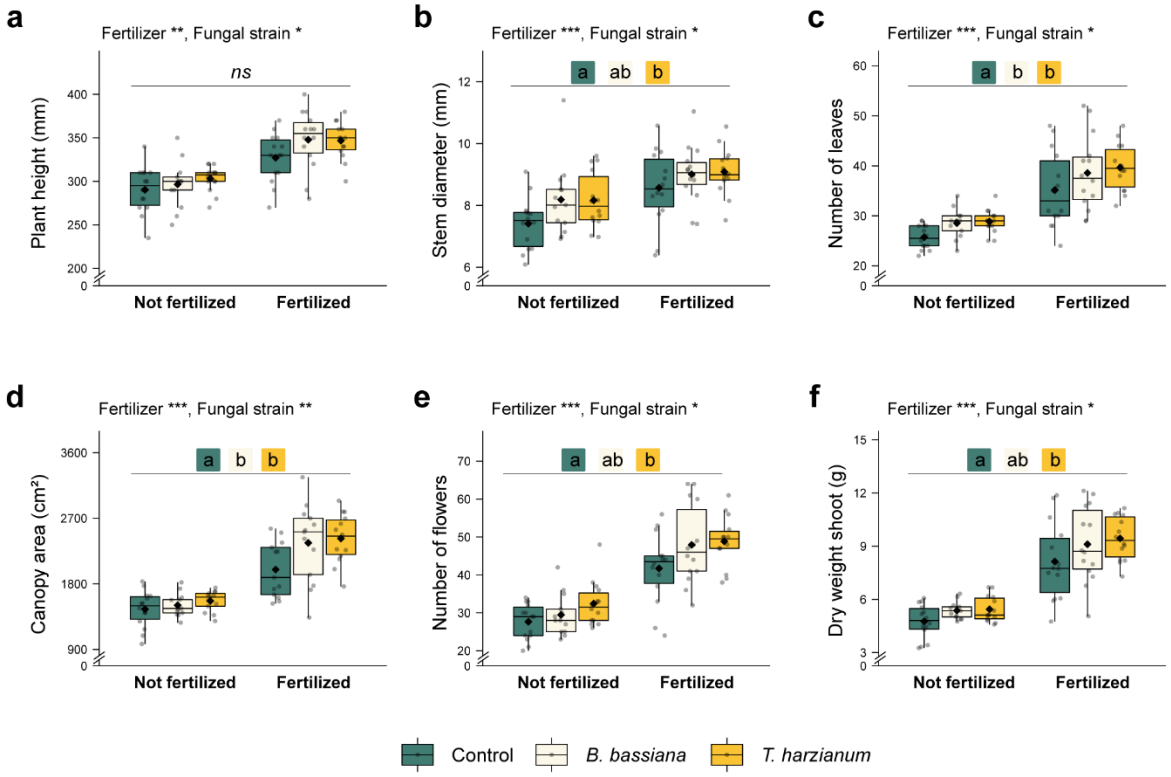


Fig. 1 Plant height **(a)**, stem diameter **(b)**, number of leaves **(c)**, leaf canopy area **(d)**, number of flowers **(e)** and shoot dry weight **(f)** of sweet pepper plants inoculated with *Beauveria bassiana* ARSEF 3097 (beige) or *Trichoderma harzianum* T22 (yellow) or non-inoculated control plants (green), under fertilized or non-fertilized conditions eight weeks after inoculation. The lower, middle and upper lines of the boxplots correspond to the first quartile, median and third quartile, respectively, while the whiskers represent the range from the minimum to the maximum and the diamond represents the average per treatment. Data points represent independent biological replicates (n = 14). Asterisks indicate significance of the factors (two-way ANOVA; 0.05 > P > 0.01: *; 0.01 > P > 0.001: **; P < 0.001: ***; *ns* = not significant). As there was no interaction between the factors fungal strain and fertilizer, differences between fungal treatments are indicated above the horizontal line, with different letters displaying overall significant differences between fungal treatments, from left to right for the control treatment, *B. bassiana* and *T. harzianum* (Tukey HSD, P < 0.05)

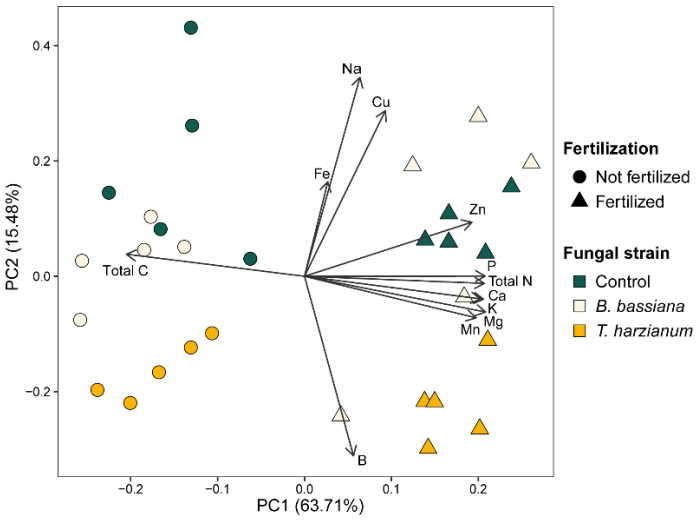


Fig. 2 Principal component analysis (PCA) visualizing the differences in mineral composition of aboveground plant tissues of sweet pepper plants inoculated with *Beauveria bassiana* ARSEF 3097 or *Trichoderma harzianum* T22, or non-inoculated control plants (represented by different colors) grown under fertilized or non-fertilized conditions (represented by different symbols) (n = 5)

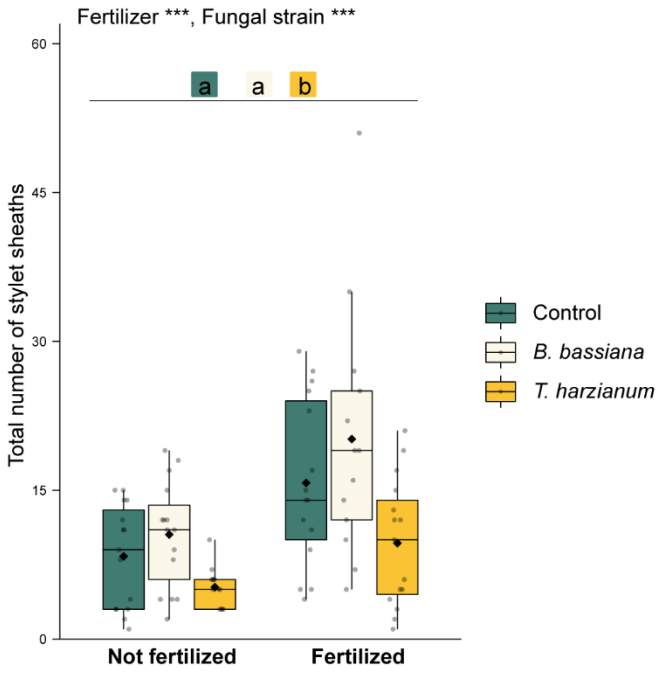
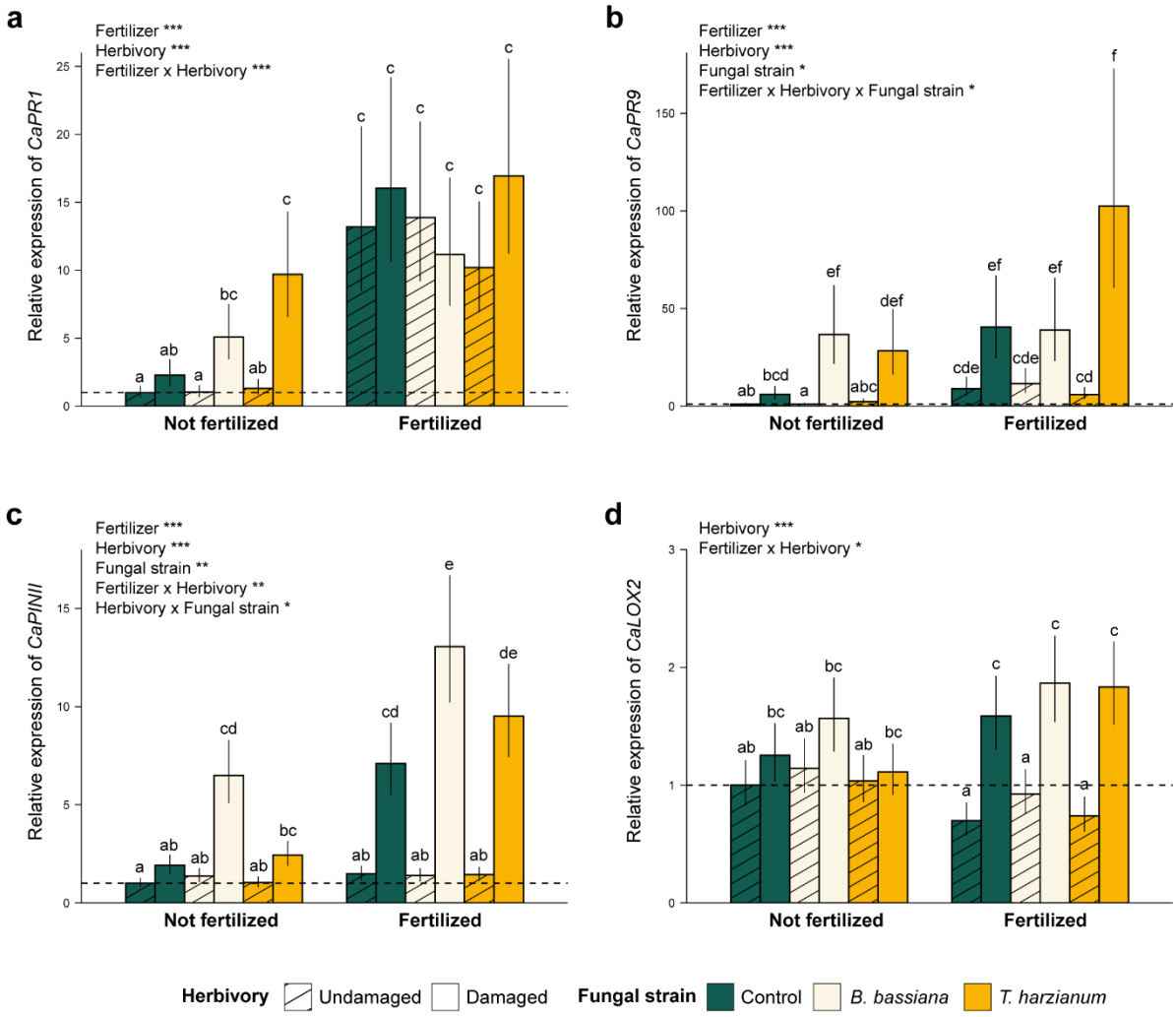


Fig. 3 Number of stylet sheaths counted after *Nezara viridula* feeding on leaves. Sweet pepper plants were inoculated with *Beauveria bassiana* ARSEF 3097 (beige) or *Trichoderma harzianum* T22 (yellow) or were non-inoculated (green), and grew under fertilized or non-fertilized conditions. The lower, middle and upper lines of the boxplots correspond to the first quartile, median and third quartile, respectively, while the whiskers represent the range from the minimum to the maximum and the diamond represents the average per treatment. Data points represent independent biological replicates (n = 15). Asterisks indicate significance of the factors (GLM; $P < 0.001$: ***). As there was no interaction between the factors fungal strain and fertilizer, differences between fungal treatments are indicated above the horizontal line, with different letters displaying overall significant differences between fungal treatments, from left to right for the control treatment, *B. bassiana* and *T. harzianum* (Tukey HSD, $P < 0.05$)



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676 **Fig. 4** Relative expression of *CaPR1* **(a)**, *CaPR9* **(b)**, *CaPINII* **(c)** and *CaLOX2* **(d)** in leaves of uninfested (striped
677 bars) or *Nezara viridula*-infested (empty bars) sweet pepper plants, inoculated with *Beauveria bassiana* ARSEF 3097
678 (beige) or *Trichoderma harzianum* T22 (yellow), or non-inoculated (green), and grown under fertilized or non-
679 fertilized conditions. Bars represent mean expression levels with standard error bars ($n \leq 5$). Expression levels are
680 normalized as $2^{-\Delta\Delta Cq}$, relative to undamaged, non-fertilized control plants (striped green bar; reference level indicated
681 with a dashed horizontal line). Asterisks indicate significance of the factors (three-way ANOVA; $0.05 > P > 0.01$: *;
682 $0.01 > P > 0.001$: **; $P < 0.001$: ***). Bars marked with different letters are significantly different from one another
683 (Tukey HSD, $P < 0.05$)

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