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Multitrophic interaction of co-inoculated endophyte entomopathogenic fungi

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Multitrophic interaction of co-inoculated endophyte entomopathogenic fungi

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Summary

The application of entomopathogenic fungi (EPF) as biocontrol agents of insect herbivores is constrained by their limited efficacy due to high sensitivity to UV-light radiation, water leaching, and fungicide application. In order to overcome these constraints and to enhance the efficacy of entomopathogenic fungi as biocontrol agents, a possible way would be the introduction of these fungi into crop plants tissues as endophytes thus helping to overcome the environmental instability obstacle and building up a systemic protection against insect herbivores.

Entomopathogenic fungi are typically applied in single application strategies; however, a combination of multiple antagonists may improve the control efficacy of insect herbivores compared to single antagonist applications. Antagonist combinations might provide protection at different time intervals or under different conditions, occupying different niches and complementing each other. This study focusing on the establishment of EPF, such as *Beauveria bassiana* or *Metarhizium brunneum*, as combined applications is so far limited. By using a root immersion inoculation method of the spore suspensions, we assessed the effects of these treatments towards the establishment of an endophyte fungal community in tomato plants (cultivar: Moneymaker), and evaluated the effects on plant pathogens, insect herbivores and their parasitoid species.

Our research confirmed that both *B. bassiana* and *M. brunneum* strains were able to establish in the tomato plants as endophyte either as a single species or as species combination inoculation. The co-inoculation method for species combination either with sequence or simultaneous inoculation method were affecting the endogenous bacterial and fungal community of host plant within the root, stem, and leaf. The predominant bacterial genera found were Ralstonia, followed by Rhodanobacter, and Pseudomonas in both inoculation method. Fungal genera were dominated by the genus Pseudogymnoascus, Chalastospora, Cladosporium, and Mycosphaerella in the sequence inoculation. Fungal genera of the genus Chalastospora, Pseudogymnoascus, Olpidium, and Mycosphaerella were dominance in simultaneous inoculation method. Bacterial diversity and abundance from both inoculation method was significantly different between plant compartment, with higher value in the roots than in the stem or leaves and simultaneous inoculation method significantly increased bacterial abundance. The

higher bacterial diversity reported when inoculating simultaneously instead of sequentially. In contrast, there was no significant effect of the plant compartment on fungal diversity, but a simultaneous inoculation method induced a significant effect on fungal diversity and abundance. Our result finding that the simultaneous inoculation significantly increased both bacterial and fungal abundance and diversity. Similarly, the structures of the microbial communities were affected in all plant compartments.

The combination of *B. bassiana* and *M. brunneum* strains were able to inhibit fungal plant pathogen *Fusarium oxysporum* Schlechtendahl Race 3 and provided protecting effect towards plant growth parameters. Significant amounts of both EPF species were found in roots.

Successful EPF colonization in tomato plant tissues affected plant volatile compound profiles qualitatively and influenced insect's response. The most abundant plan volatile compound in all EPF inoculation treatments was β-Phellandrene. Tetradecanal was the plant volatile compound correlated with the responses of the greenhouse whiteflies (*Trialeurodes vaporariorum*) and their parasitoid (*Encarsia formosa*). VOCs emitted from plants inoculated with a combination of the two EPF strains tested were less preferred by greenhouse whiteflies and *E. formosa* parasitoid in the olfactometer tests. High parasitization rate of *E. formosa* on GHW larvae instar 3 of all EPF inoculation treatment indicating that EPF application as endophyte are compatible with the parasitoid *E. formosa* and could be an option for GHW control.

The development of a biological control with the mixture of EPF species introduced as endophyte within tomato in this study was a novel approach and it could be a viable option for the alternative management of pests and plant diseases in crop plants.

General Introduction

Entomopathogenic fungi (EPF) are known since the early 1800s century when it was discovered that the muscardine disease of silkworms in France was caused by a fungal species. This discovery is triggering the idea of using fungal insect pathogens for pest control (Audoin 1837 in Vega et al., 2009). The utilization of entomopathogenic fungi as a biocontrol agent for pests is an interesting alternative compared with synthetic pesticides, considering the safety aspect for plants, animals, and the environment. Over time evidence grew that several entomopathogenic fungi or their specific isolates may play additional roles in nature beside their primary role as insect pathogens, being also plant endophytes, antagonists of plant pathogens, being able to promote plant growth, improving resistance of plants to environment stress, and promoting beneficial associations in the rhizosphere (Vega et al., 2009).

The term of "endophyte" was first coined by the German botanist Anton de Bary in 1884 for all organisms colonizing plant tissues. Wilson (1995) defined the endophytic status as an asymptomatic colonization of fungi or bacteria within plant tissues. In natural ecosystems numerous plants are apparently associated with endophytic fungi (Rodriguez et al., 2009). Various genera of fungal entomopathogens have been recovered as endophytes from different plant species (Vega, 2008; Vega et al., 2008;2009) revealed their role as plant endophytes. Other studies reported the successful inoculation of fungal entomopathogens within various crop plant (Bing and Lewis, 1991; Batta et al., 2013; Tefera and Vidal, 2009; Posada and Vega, 2005; Brownbridge et al., 2012; Parsa et al., 2013; Greenfield et al., 2016). Since the ecological function of endophytes in plants has been well recognized, more studies have been conducted focusing on artificially introduced beneficial fungi to host plants. This opens up the opportunity to develop and understand the mechanism of the role of entomopathogenic fungi as endophytes aiming at establishing a systemic protection against insect herbivores.

The exploration of fungal entomopathogens as endophytes initiated with *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales), a well-known and widely used biocontrol agents targeting different insect species. Particular *B. bassiana* strains have been reported as successful colonizers of various plants species by certain artificially introducing methods. *B. bassiana* strain IC-5486 and CS16-1 became established as endophytes in in vitro-grown cocoa seedlings (Posada and Vega, 2005).

Foliar sprays of a conidial suspension of *B. bassiana* strain ARSEF 3113 resulted in the colonization of corn plant (Wagner and Lewis, 2000)**.** Seed applications of *B. bassiana* strain 11-98 resulted in endophytic colonization of tomato or cotton plants (Ownley et al., 2008)**.** *B. bassiana* strain EABb 04/01-Tip isolate was used to determine the ability to endophytically colonize opium poppy via foliar spray with fungal conidia (Quesada-Moraga et al., 2006). A fungal inoculum of *B. bassiana* strain CIAT 359 and CIAT 405 was introduced by the soil drench inoculation method on Cassava (Greenfield et al., 2016). *B. bassiana* strain ATCC 74040, registered as NATURALIS®, was successfully introduced via seed treatments into broad beans (Jaber and Enkerli, 2016).

Another EPF (*Metarhizium anisopliae*) has also been shown to be able to colonize plant tissues endophytically. Alkhayat et al., (2014) reported the ability of *M. anisopliae* to endophytically colonize tomato and cotton plants. High recovery rates of *M. anisopliae* have been reported by Batta (2013) from oilseed rape cultivars when applied onto leaves and stems. The former species *M. anisopliae* is a complex of different species, which have been taxonomically revised recently, resulting in 9 terminal taxa, including *Metarhizium brunneum* (Bischoff et al, 2009). Interestingly, only few reports have been published so far reporting *M. brunneum* Petch (Ascomycota: Hypocreales) being a successful colonizer of different plant species. For example, the *M. brunneum* strain BIPESCO5 was able to endophytically colonize broad bean plants via seed treatment (Jaber and Enkerli, 2016), and tomato plants using mycelium in an encapsulated form (Krell et al., 2018a). The *M. brunneum* strain ART 2825 endophytically colonized tomato plants (Rodríguez, 2016) when inoculated via root immersion. Krell et al. (2018b) reported that the application of encapsulated *M. brunneum* strain CB15 resulted in the colonization of potato plants.

The previous studies cited above demonstrated that the EPF *B. bassiana* and *M. brunneum* could be successfully inoculated as endophytes in various plants species. However, the question came up about their role as biocontrol agent of insect herbivores? Negative effects of endophyte entomopathogenic fungi (EEF) towards insect herbivore were initially reported by Bing and Lewis (1991). They endophytically colonized corn plant tissues with an isolate of *B. bassiana* via foliar and direct injection. This resulted in reduced tunneling in corn stalks and also suppressed the number of the European corn borer larvae (*Ostrinia nublialis* Hübner). Endophyte *B. bassiana* has been reported to reduce damage of the banana weevil, *Cosmopolites sordidus* Germar (Akello et al., 2008); other strains significantly reduced the numbers of cotton aphids

(*Aphis gossypii* Glover) and negatively affected the survival of cotton bollworm (*Helicoverpa zea*) (Lopez et al., 2014; Lopez and Sword, 2015) Transient endophytic colonization of *B. bassiana* and *M. brunneum*, via foliar application of fungal conidia caused mortality in the larvae of the beet armyworm (*Spodoptera littoralis* Boisduval) (Resquín-Romero et al., 2016) and nymphs of the sweet potato whitefly (*Bemisia tabaci* Gennadius) (Garrido-Jurado et al., 2017). Recent work of Hettlage (2018) reported that an inoculation of potato tubers by *M. brunneum* significantly reduced the number of eggs deposited by the Colorado potato beetle on potato plants (*Leptinotarsa decemlineata* Say).

Most studies aiming at endophytically colonizing host plants studies used sterile growth media for the host plants. For example, Jaber and Enkerli (2016) used inoculated broad been seeds treated by entomopathogenic fungi strains planted in disinfected pots containing sterile (autoclaved) planting substrate, regularly watered with sterile distilled water. Steam-sterilized loam soil in disinfected pots was used by Greenfield et al. (2016) in order to establish entomopathogenic fungal strains in cassava roots. Parsa et al. (2013) used sterile mixture soil and sand for endophytic colonization of common bean with *B. bassiana*, followed by watering the seedling plants with sterile distilled water. However, an earlier study of Tefera and Vidal (2009) revealed that there was no colonization of *B. bassiana* in stems and leaves of sorghum in non-sterile soil, while there was substantial endophytic colonization in vermiculite and sterile soil using the same plant species and fungal strain. Particularly, Parsa et al. (2018) studied the effect of soil sterilization on endophytic colonization, wherein colonization of *B. bassiana* and *M. anisopliae* in common bean seed treatment was least variable in sterile vermiculite and most variable in sterile soil:sand:peat mixture demonstrating that soil sterilization resulted in the largest impact on colonization explaining 70.8% of the total variance. As a matter of fact, the sterilization process kills not only harmful organisms, but also beneficials; therefore, the microbial diversity in the sterile soil will not be the same as in the non-sterile soil (Bennett et al., 2003). Using sterilized substrate might not be comparable to the same substrate when it is non-sterile, in terms of biological, chemical and physical properties. Hence, the result reported from studies using sterile plant growth substrates might not be practical for field application purposes. The specificity of fungal strains, the fungal inoculation method, the growth condition, and the host plant species are considered as successful factors of EEF inoculation (Vidal and Jaber, 2015). When all these factors are taken into account, the best option would be to use

non-sterile substrates, which does, however, not mimic natural conditions. It may also increase the chances of producing non-repeatable results due to the interactions between inoculated EEFs and their microbial community structure within host plant.

In attempts to improve the efficacy of biocontrol methods, several studies performed combinations between EPF with other biocontrol agents. For example, a combination of *B. bassiana* and the bacterium *Bacillus subtilis* effectively reduced the incidence of tomato fusarium wilt (*Fusarium oxysporum f. sp. lycopersici*) and results in a higher larval mortality of the tomato fruit borer (*Helicoverpa armigera*) compared with a single application of the biocontrol agent or the control (Prabhukarthikeyan et al., 2014). Mantzoukas et al. (2013) concluded that applying the entomopathogenic bacterium *Bacillus thuringiensis* ssp. kurstaki simultaneously with an isolate of the entomopathogenic fungi *B. bassiana* or *M. robertsii*, was able to control larvae of the stalk borer (*Sesamia nonagrioides*) and could be more effective than using each pathogen separately. Other studies showed the combination of two entomopathogenic fungi *B. bassiana* and *M. brunneum* effectively reduced tuber damage by the sweet potato weevil (*Cylas formicarius* F.) (Reddy et al., 2014). However, the combination of the two fungal species *B. bassiana* and *Nomuraea rileyi* did not have a synergistic effect on *Spodoptera litura* larval mortality due to the larval mycosis effect depends on the temperature. The larvae showed mycosis of *N. rileyi* at 25±1°C of all the isolate combination treatment. Whereas, at the temperature of 32±2°C all the dead larvae exhibited *B. bassiana* mycosis. This study revealed that only one of the fungal isolates sporulated on the larval cadaver but never both. Nevertheless, this results hint at the probable reason for the simultaneous occurrence of the two fungal isolates in the fields could be from different temperature range from usual mid-winter to early spring (Rao et al., 2006).

Mutual relationship between two biocontrol fungal species can add consistency of control by providing multiple mechanisms of action and effectiveness at the fluctuate temperature of certain time period (Inglis et al., 1997). The application of *B. bassiana* and *M. flavoviride* in combination may be a way to overcome some of the constraints of temperature on entomopathogenic fungi application, the application of both pathogens simultaneously resulted in a greater mortality of grasshopper nymph *Melanoplus sanguinipes* (Fabricius), rather than *M. flavoviride* in the high temperature environment, and equal mortality to the *B. bassiana* in the lower temperature environment (Inglis et al., 1997). Multiple antagonist combination may overcome inconsistencies in the

performance of individual antagonistic organisms (Larkin and Fravel, 1998). Despite their beneficial prospects, antagonistic responses may occur between biocontrol organisms, leading to unchanged control levels or even reduce control effects when compared to single applications of biocontrol agents.

To date there are no reports published regarding the combination of entomopathogenic fungal species as endophytes. In order to increase our understanding of potential benefits by co-inoculations, the development of an application containing a mixture of entomopathogenic fungi could be a viable option for an effective management of major pests and diseases in crop plants. This study thus aimed at assessing the effect of combined application of *B. bassiana* and *M. brunneum* strains in vitro and in planta.

Tomato was selected as a model plant for all experiments. Tomato, *Solanum lycopersicum* L, is a vegetable crop that is cultivated worldwide. Tomato can be grown directly in the field or in the greenhouse. Tomatoes are not only grown for the fresh markets but also for the processing industry. According to the latest short-term outlook report for European Union agricultural markets (European Commission, 2019), the total EU production of tomatoes was expected to increase to around 16.8 million tons in 2019, driven by a rise in the production of tomatoes for processing (+6%). However, the level of total EU tomato production in 2019 was 2% below the average of the past five year.

The tomato fusarium wilt disease, caused by *Fusarium oxysporum* f.sp. lycopersici, is one of the most destructive damaging diseases, caused significant yield loses in tomato production systems worldwide (Huang and Lindhout, 1997). Due to the persistence of the fungal pathogen spores in the soil and the endophytical growth within plant tissues, even the synthetic control options for this pathogen are limited (Amini and Sidovich, 2010). In attempts to improve the efficacy of biocontrol methods on plant pathogen, the EEFs might have a potential as antagonist of a plant pathogen (Jaber, 2015; Jaber, 2018; Jaber and Ownley, 2018). Tomato plants *Solanum lycopersicum* L, cultivar Moneymaker, were used in this study, since it is known to be susceptible to endophytic colonization by *B. bassiana* isolates (El-Deeb et al., 2012).

The polyphagous insect pests, the greenhouse whitefly *Trialeurodes vaporariorum* Westwood (Homoptera: Aleyrodidae) is one of the major pests of greenhouse vegetable production worldwide (Jauset et al., 1998). Whiteflies may deplete plant reserves, reduce primary production, and have direct phytotoxic effects or act as vectors of important plant viruses (Chen et al., 2004). Whiteflies also cause secondary damage

through honeydew excretion resulting in growth of sooty mold fungi reducing photosynthesis and ultimately reducing yield quantity and quality (Yee et al., 1998). The main damage resulting from this phloem sucking insect is reduced crop yields and damage of fruits (Byrne et al., 1990). Whitefly control is mainly managed by chemical insecticides in intensive greenhouse cultures (van Lenteren, 2000). Chemical control has resulted in the development of resistant whiteflies populations, and negative environmental impacts have encouraged the development of alternative pest management strategies, with microbial control playing an important role. Whiteflies feed by piercing the tissues of plants and sucking sap directly from the vascular bundles. Consequently, entomopathogenic fungi, which are the only insect pathogens infecting their hosts by direct penetration of the cuticle, show promise for their control (Faria and Wraight, 2001). Therefore, entomopathogenic fungal strains are regarded as an option for controlling whiteflies. Laboratory and field studies revealed that *B. bassiana* to be an effective pathogen for whiteflies when applied directly by concentrated conidial suspensions (Eyal et al., 1994; Fargues et al., 2003; Poprawski and Jones, 2000; Wraight et al., 1998; Wraight et al., 2000). Alternatively, a common biological control method used for greenhouse whitefly management is the application of their natural enemies, the parasitoid *Encarsia formosa* Gahan (Hymenoptera:Aphelinidae) (van Lentereen, 2000).

In this study we focused on the effects of EPF inoculations of tomato plants in single and combined treatments with specific fungal strains via root inoculation, on i) the interactions with the endophytic community, ii) on the interactions with the plant pathogen *Fusarium oxysporum* f.sp. lycopersici, iii) on the interactions with the greenhouse whitefly *T. vaporariorum*, and iv) on the interactions with the natural enemy *E formosa*.

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Chapter 1

Assessing the effect of entomopathogenic fungal co-inoculation on plant endophyte community structure

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Abstract

Despite the increasing number of studies reporting the successful inoculation of entomopathogenic fungi as endophytes within plants, only few studies have so far investigated the effect of an establishment of entomopathogenic fungi (EPF) as endophytes on the plant endophytic community structure. Here we analysed the effect of different inoculation methods of EPFs on the bacterial and fungal endophyte diversity and community composition using large-scale metabarcoding. Using strains of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium brunneum* we priorly test the compatibility of each EPF strain in in vitro assay and analysed the establishment of these fungal strains as endophytic colonizers in different plant tissue compartments of tomato plants. Moreover, we assessed the effects of colonization when inoculated simultaneously or in a sequence via root immersion of spore suspension applications. We observed the inhibition growth between the EPF strains when grown in in vitro assays, but no effect with regard to establishment when either inoculated in sequence or simultaneously. The sequence and simultaneous inoculation method affected the microbial community structure differently between root, stem, and leaf. The predominant bacterial genera found were Ralstonia, followed by Rhodanobacter, and Pseudomonas in both inoculation method. Fungal genera were dominated by the genus Pseudogymnoascus, Chalastospora, Cladosporium, and Mycosphaerella in the sequence inoculation. Fungal genera of the genus Chalastospora, Pseudogymnoascus, Olpidium, and Mycosphaerella were dominance in simultaneous inoculation method. Bacterial diversity and abundance from both inoculation method was significantly different between plant compartment, with higher value in the roots than in the stem or leaves

and simultaneous inoculation method significantly increased bacterial abundance. The higher bacterial diversity reported when inoculating simultaneously instead of sequentially. In contrast, there was no significant effect of the plant compartment on fungal diversity, but a simultaneous inoculation method induced a significant effect on fungal diversity and abundance. Our result finding that the simultaneous inoculation significantly increased both bacterial and fungal abundance and diversity. Similarly, the structures of the microbial communities were affected in all plant compartments. This study was conducted as a first step towards a possible advantage of combining EPF strains as endophytes within plants. Further studies are required to investigate how EPF combinations affect multitrophic interactions between plant microbiome, plant pathogen, plant herbivore and their natural enemies.

Keywords: bacterial, endophyte, entomopathogenic fungi, fungal, inoculation method, microbial community.

1. Introduction

Endophytic microorganisms including, bacteria and fungi are known from a wide range of plant species (Hardoim et al., 2015). These microorganisms have received specific attention in the last years due to their important ecological role mediating plant growth and health (Lodewyckx., 2002; Hardoim et al., 2015; Waqas et al., 2013). A specific group of these fungal endophytes, the entomopathogenic fungi (EPF) have recently gained specific attention due to their dual roles in nature; known for long as pathogens of insects (Resquin-Romero et al., 2016; Garrido-Jurado et al., 2016), they also play an important role as plant endophytes (Vega et al., 2009), interacting with plant pathogens (Ownley et al., 2008; 2010), or promoting plant growth (Sasan and Bidochka, 2012; Jaber and Erkenli, 2017; Krell et al., 2018).Various genera of fungal entomopathogen as endophytes have been recovered from different plant species (Vega et al., 2009). *Beauveria bassiana* and *Metarhizium* spp are the commonly EPF used as commercialized biopesticides worldwide (Faria and Wraight, 2007). Some studies reported the successful inoculation of these fungi within various crop plant (Bing and Lewis, 1991; Batta et al., 2013; Tefera and Vidal, 2009; Posada and Vega, 2005; Brownbridge et al., 2012; Parsa et al., 2013; Greenfield et al., 2016).

Several studies have been conducted to understand the effect of an EPF inoculation and growth on the host plant performance (Raya-Díaz et al., 2017; Quesada-Moraga, et al.,

2014; Gange et al., 2019). Recent studies also focused on the effect of an EPF inoculation on microbial communities associated with plants. However, since most of these studies focused on the soil microbiome interactions (Hirsch et al., 2013; McKinnon et al., 2018) data on the endophytic microbial communities following the colonization of plant tissues are still limited. Mayerhofer et al. (2017) reported that different formulations of the *Metarhizium brunneum* strain ART 2825, aiming at controlling wireworms in potato, caused only small shifts in the fungal communities in pot soil experiments. And in another experiment in the field, applications of the same fungal strain caused mainly spatial differences in the fungal and prokaryotic communities analysed. A study using *Metarhizium anisopliae* strain CQMa421 as foliar application to control the rice leaf roller *Cnaphalocrocis medinalis* Guenee showed the minimal impact of this EPF strain on endogenous microbial diversity with transient changes in bacterial abundance and diversity that may result in added benefits to plant growth promotion (Hong et al., 2017).

We hypothesis that EPF co-inoculation method as endophyte within tomato plants have an implication on plant host microbial community structure. A study was performed to assess the different methods of EPF inoculation, and how this inoculation method affects another endophyte (bacterial and fungal community) of the host plant.

2. Materials and Methods

2.1. Plant material

Tomato plants *Solanum lycopersicum* L cultivar Moneymaker (Rein Saat®, Austria) was used for EPF inoculation. Each seed was grown in a multi tray with a mixture of soil (Fruhstorfer Erde Typ T, Hawita Gruppe GmbH, Vechta, Germany) and non-sterile 0,3mm sand (3:1). Tomato seedling at the two-leaf stage were used in this study. Seedlings were removed from the substrate, and the roots were carefully washed with tap water prior to fungal inoculation. Seedlings inoculated with the spore suspension described at (2.4. Fungal inoculation), were individually transplanted into plastic pots (diameter 11cm) using the same soil mixture as described above. The plants were maintained under greenhouse conditions (21±2°C, 70-80% RH and 12h photoperiod) and irrigated regularly for a growing period of four weeks post inoculation.

2.2. Fungal material

Three strains of *B. bassiana* (1) EABb 04/01-Tip, (2) Bb 1022 and (3) BV 061 and a strain of *M. brunneum* (Cb15 III) were obtained from the culture collection of Agriculture Entomology working group, Department of Crop Sciences, Faculty of Agriculture, Georg-August-University Göttingen, Germany. For convenience, each strain will be abbreviated as flows: (1) Bb Que, (2) Bb Can, (3) Bb Col, and Mb Cb15.
The strains were grown in potato dextrose agar (PDA) at 24 ±2°C in dark conditions for two weeks to obtain enough spores for suspension preparation. Spores suspension production was carried out under a sterile bench (Thermo Fisher Scientific), starting by adding 5ml of Tween 20 (0.1 % v/v, DifcoTM) into two-week-old culture plates of each strain, followed by gently scraping off the culture surface with sterile microscope slide glass. Spores were then suspended in 10ml sterile distilled water. Spore concentration determine with a Thoma counting chamber (Marienfeld, Germany) and adjusted to 100 ml of $1x10^6$ spores/ml. To assess the viability of the spores of the different fungal strains used in this experiment, a germination test was carried out. 100µl from each spore suspension was spread out on PDA medium and incubated at 24 ±2°C for 3 days. Spore germination was checked under the microscope and average germination exceeded 90% for each strain.

2.3. Fungal co-culturing (in vitro assay)

Co-culture assays were conducted between different fungal species, aimed at comparing inhibition activities between strains, providing a basis for co-inoculations in planta. Two strains were placed on the opposite sides perpendicular of a Petri dish containing PDA. 10µl of $1x10^6$ spores/ml of each fungal strain suspension was dropped at 1cm from the margin of the plates. All pairings were carried out in six replicates. For the control, each fungal strain was inoculated on a separate Petri dish. The evaluation of mycelia growth measured by the colony diameter (cm) for each strain either in the control (R1) or in the co-inoculated Petri dishes (R2). All treatments were maintained at 24 ±2°C for 20 days, as the time of fungal mycelia growth ceased. Percent inhibition radial growth (PIRG) was calculated as follows (Skidmore and Dickinson, 1976):

$$\text{Percent inhibition radial growth (PIRG)} = \frac{R1-R2}{R2} x100$$

2.4. Fungal inoculation

To examine the effect of an EPF co-inoculation on the microbial endophyte community of tomato plants, we set up experiments using either a sequence inoculation (stepwise sequential inoculations of fungal strains) or a simultaneous inoculation (single mixture inoculations of fungal strains).

Sequence inoculation.

This inoculation method was set up by immersing tomato roots seedling into a 100ml of $1x10^6$ spores/ml suspension of the first strain of an EPF for 20 minutes. The same procedure was used for the control treatment, using distilled water instead of a spore suspension. Inoculated seedlings were individually transplanted into plastic pots (diameter 11cm) contained soil mixture (Fruhstorfer Erde Typ T, Hawita Gruppe GmbH, Vechta, Germany) and non-sterile 0,3mm sand (3:1). Seven days post initial inoculation, plant was removed from the pots, roots were gently shaken to remove most of the soil and thereafter roots were immersed in a 100ml of $1x10^6$ spores/ml suspension of the second fungal strain.

Simultaneous inoculation

Mixture inoculations were set up by immersing root of tomato seedlings into a 100ml of $2x10^6$ spores/ml suspension, containing spores both strains of EPF (ratio 1:1) for 20 minutes. For the control treatments, the same procedure was set up with distilled water instead of a spore suspension. Inoculated seedlings were individually transplanted into plastic pots (diameter 11cm) containing the soil mixture mentioned above.

All plants were maintained in the greenhouse conditions (21±2°C, 70-80% RH and 12h photoperiod), and irrigated regularly. Four-week post inoculation, the plants were harvested by gently removed the plants from pots and washed the roots. The experiment performed as a randomized design with five replications.

2.5. Harvest and surface sterilization of plant material

Plant height of the sequence and simultaneous inoculation method were recorded prior harvesting, by measure the height (cm) from the growth point of cotyledon up to the new emerge leaves.

Different plant compartments were harvested: aboveground plants parts: first stage leaves and stem segments (2 cm sections measured from the cotyledon growth point);

and roots; these were cut using sterile scalpel and placed separately into Falcon tubes (Sarstedt AG&Co.KG) for further surface sterilization.

Aboveground plant samples (first stage leaves and stem) were surface-sterilized by serial washings in 70% ethanol for 1 minute, 2% sodium hypochlorite for 30 seconds and 70% ethanol for 1 minute, followed by two times immersion in sterile distilled water for 30 seconds and once in sterile diethylpyrocarbonate (DEPC)-treated water (modified from Andreote et al., 2010). Surface sterilization of roots was performed according to Li et al. (2010), with slight modifications. In this study, 2% sodium hypochlorite and sterile DEPC-treated water were used for the last step. To confirm successful disinfection procedures, aliquots of the DEPC water, used in the final washing step, were plated on PDA plates. These plates were incubated in the dark at 24 ±2°C for at least 1 week. No growth of microorganisms was observed.

In addition, water from the same aliquots was subjected to PCR targeting the bacterial 16S rRNA gene and ITS region of fungal rDNA as the negative control. No PCR products were detected. These results confirmed that the surface sterilization was successful in eliminating cultivable as well as non-cultivable epiphytic bacteria and fungi as well as potential DNA traces from the plant surfaces. Surface-sterilized plant material was ground to a fine powder in liquid nitrogen using an autoclaved mortar and pestle. Aliquots of the obtained powder were stored at−20°C until DNA extraction.

2.6. Extraction and amplification of total community DNA

DNA extraction

Total DNA of leaves, stem and roots was extracted employing the peqGOLD Plant DNA Mini kit (Peqlab, Erlangen, Germany) according to the manufacturer's instructions with two modifications as described previously (Wemheuer et al., 2016). DNA concentration of DNA extraction product was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies,Wilmington, DE, USA). In total, extracted DNA of 240 samples was subjected to PCR targeting the bacterial 16S rRNA gene and the fungal ITS region.

Amplification of the 16S rRNA gene

Bacterial endophyte communities of leaves, stem and roots were assessed by a nested PCR approach targeting the 16S rRNA gene. For details of the first PCR reaction mixture and the thermal cycling scheme see Wemheuer et al. (2016). Briefly, the

primers 799f (5′-AACMGGATTAGATACCCKG-3′) (Chelius and Triplett, 2001) and 1492R (5′-GCYTACCTTGTTACGACTT-3′) (Lane, 1991) were used in the first PCR to suppress co-amplification of chloroplast-derived 16S rRNA genes. Genomic DNA of *Bacillus licheniformis* DSM13 was used as a template in the positive control for the bacterial product. Negative controls performed using the reaction mixture without a template. PCR amplification resulted in two PCR products: a mitochondrial product with approximately 1.1 kbp and a bacterial product of approximately 735 bp. Bacteria-specific bands were purified and quantified as described in Wemheuer and Wemheuer (2017). PCR products subjected to nested PCR.

The V6-V8 region of the 16S rRNA gene was amplified with primers 968F and 1401R (Nübel et al., 1996) containing MiSeq adaptors (underlined) (MiSeq-968F 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAACGCGAAGAACCTTAC-3′; MiSeq-1401R 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCGGTGTGTACAAGACCC-3′) as described previously by Wemheuer and Wemheuer (2017) with one modification: 0.5 U of Phusion high fidelity DNA polymerase (Thermo Scientific, Waltham, MA, USA) was used. Three independent PCRs were performed per sample as technical replications. Obtained PCR products per sample were controlled for appropriate size, pooled in equal amounts, and purified using the magnetic plates with beads. Quantification of the PCR products performed using the Quant-iT dsDNA HS assay kit and a Qubit fluorometer (Thermo Scientific) as recommended by the manufacturer. Purified PCR products were barcoded using the Nextera XT-Index kit (Illumina, San Diego, USA) and the Kapa HIFI Hot Start polymerase (Kapa Biosystems, Wilmington, USA). The Göttingen Genomics Laboratory determined the sequences of the partial 16S rRNA genes employing the MiSeq Sequencing platform and the MiSeq Reagent Kit v3 (2 x 300 cycles) as recommended by the manufacturer (Illumina, San Diego, USA).

Amplification of the ITS region

Fungal endophyte communities of leaves, stem and roots were assessed by a nested PCR approach targeting the ITS region as described previously (Granzow et al., 2017). In the first PCR, the primers ITS1-F_KYO2 (5′-TAGAGGAAGTAAAAGTCGTAA-3′) (Toju et al., 2012) and ITS4 (5′- TCCTCCGCTTATTGATATGC-3′) (White et al., 1990) used to suppress co-amplification of plant-derived ITS regions. Genomic DNA of *Aspergillus nidulans* was used as a template in the positive control for the fungal

product. Negative controls performed using the reaction mixture without the template. Obtained PCR products were purified and quantified as described for the bacterial PCR products.

The ITS2 region subsequently amplified as described for the first PCR using approximately 50 ng product of the first PCR and the primers ITS3_KYO2 (Toju et al., 2012) and ITS4 (White et al., 1990) containing the MiSeq adaptors (underlined): MiSeq-ITS3_KYO2 (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGATGAAGAACGYAGYRAA-3′)

and MiSeq-ITS4 (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC -3′). Three independent PCRs performed per sample as technical replications. Obtained PCR products were pooled in equal amounts and quantified as described for the bacterial PCR products. Pooled PCR products were barcoded using the Nextera XT-Index kit (Illumina, San Diego, USA) and the Kapa HIFI Hot Start polymerase (Kapa Biosystems, Wilmington, USA). The Göttingen Genomics Laboratory determined the sequences of the ITS2 region employing the MiSeq Sequencing platform and the MiSeq Reagent Kit v3 (2 x 300 cycles) as recommended by the manufacturer (Illumina, San Diego, USA).

2.7. Processing of bacterial and fungal datasets

Obtained sequencing data were initially quality filtered with the Trimmomatic tool version 0.36 (Bolger et al., 2014). Low quality reads were truncated if the quality dropped below 10 in a sliding window of 4bp. Subsequently, all reads shorter than 100bp and orphan reads removed. Remaining sequences were merged, quality-filtered and further processed with USEARCH version 10.0.240 (Edgar, 2010). Filtering included the removal of reads shorter than 400 bp or longer than 450 bp (bacteria) or shorter than 290 bp and longer than 490 bp (fungi) as well as the removal of low quality reads (expected error > 1), it reads with more than one ambitious base.

Processed sequences of all samples were concatenated to one file and subsequently dereplicated into unique sequences. These sequences were denoised with the unoise3 algorithm implemented in USEARCH (Edgar, 2010). All OTUs consisting of one single sequence (singletons) were removed, in reference mode with the SILVA SSU Ref NR 99 132 databases (Quast et al., 2012) as reference data set for bacteria and the QIIME

release of the UNITE database version 7.2 (Kõljalg et al., 2013) for fungi. To assign the taxonomy of bacteria and fungi, unique and chimera-free sequences were classified by BLAST alignment against the SILVA database (Quast et al., 2012) and the UNITE database (Kõljalg et al., 2013), respectively, with an e-value threshold of 1e-20. Concatenated sequences of all sequences were mapped on the final set of unique sequences to calculate the evenness and abundance of each unique sequence in all samples. All non-bacterial or non-fungal OTUs were removed based on their taxonomic classification in the respective database. After the removal of plant-derived contaminations and zero OTUs of treatment replication, a total of 936.068 and 1.114.304 OTU reads obtained for bacteria and fungi, respectively. Filtered sequences mapped on remaining unique sequences to determine the occurrence and abundance of each unique sequence in every sample, resulted sequences grouped into 1.458 bacterial and 315 fungal OTUs. Total sequence number per OTUs of bacteria and fungi then used to calculate the relative abundance of microbial community composition (provided as supplementary tables S1 and S2).

2.8. Data analysis

All data sets were analysed using R version 3.4.0 (R Core Team, 2016). A t-test was conducted to compare the mean values of mycelia growth between EPF strains in the co-inoculation in vitro assays, as well as to compare plants height effect between sequence and simultaneous inoculation methods.

Organism taxonomy units (OTUs) data of bacterial and fungal communities obtained from sequence samples were analysed separately. As we were interested whether the inoculation method would influence the community structures within different plant compartments, leaves, stems, and roots microbial communities were analysed separately.

Potential differences in the community structure within the plant compartments of each inoculation method investigated by permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) with 1,000 random permutations using the *vegdist* and *adonis* function within the *vegan* package (Oksanen et al., 2016). To visualize the bacterial and fungal community composition within the plant compartments of sequence or simultaneous inoculation method, we generated a bar chart from the total sequence number of bacterial and fungal OTUs.

Alpha diversity indices (Richness and Shannon index of diversity) were calculated using the R-packages *vegan* 2.4.6 (Oksanen et al., 2016). OTU tables of bacteria were rarefied to 302 (root), 302 (stem) and 63 (leaves). Fungal data were rarefied to 45 (root), 55 (stem), and 3334 (leaves) sequences per sample prior to alpha diversity analyses using the *rrarefy* function in *vegan* package to reach at least more than 50% whole data coverage. Sample coverage was estimated using the Michaelis-Menten Fit function in *picante* package 1.6-2 (Kembel et al., 2014). The Michaelis-Menten Fit (MMF) was subsequently calculated from generated rarefaction curves using the *MM2* model within the *drc* package (Ritz and Streibig, 2005). Richness and diversity were calculated using the *specnumber* and *diversity* function, respectively. All alpha diversity indices were calculated ten times. The average from each iteration was used for further statistical analysis.

A Kruskal-Wallis test was performed to test the differences of bacterial and fungal richness and diversity on leaves, stems, and root from both inoculation method. To determine the effect of simultaneous and sequence inoculation method on bacterial and fungal diversity and richness Wilcoxon test used.

3. Results

3.1. Co-inoculation of entomopathogenic fungi strains (in vitro assay)

The growth of EPF strains in the dual culture assay reached an optimum 20 days after inoculation. Mycelium growth of strain Mb Cb15 was significantly inhibited when co-inoculated with the strain Bb Can (t-test [t (6) = 6.99, p<0.001]). There were no significance inhibition between Mb Cb15 and Bb Col (t-test [t (6) = 1.43, p<0.19]), and Bb Que (t-test [t (6) = 1.54, p<0.17]) (Figure 1).

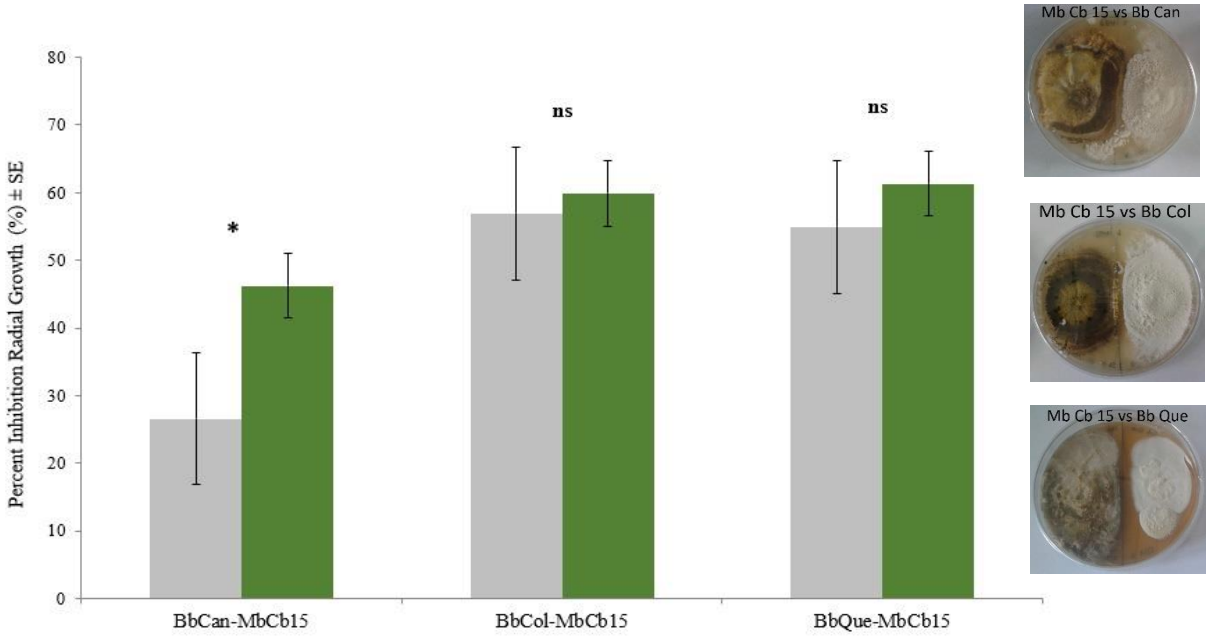

Figure 1. Percent inhibition of radial growth (PIRG) of co-inoculated EPF strains. Asterisks (*) above bar graph indicate significant differences, (ns) above bar graph indicate no significant differences within treatment.

3.2. Effect of sequence and simultaneous inoculation method on plant height

We found no significant effect of sequence or simultaneous inoculation of EPF treatments on plants height (t-test [t (39) = 0.71, p=0.47]) (Figure 2).

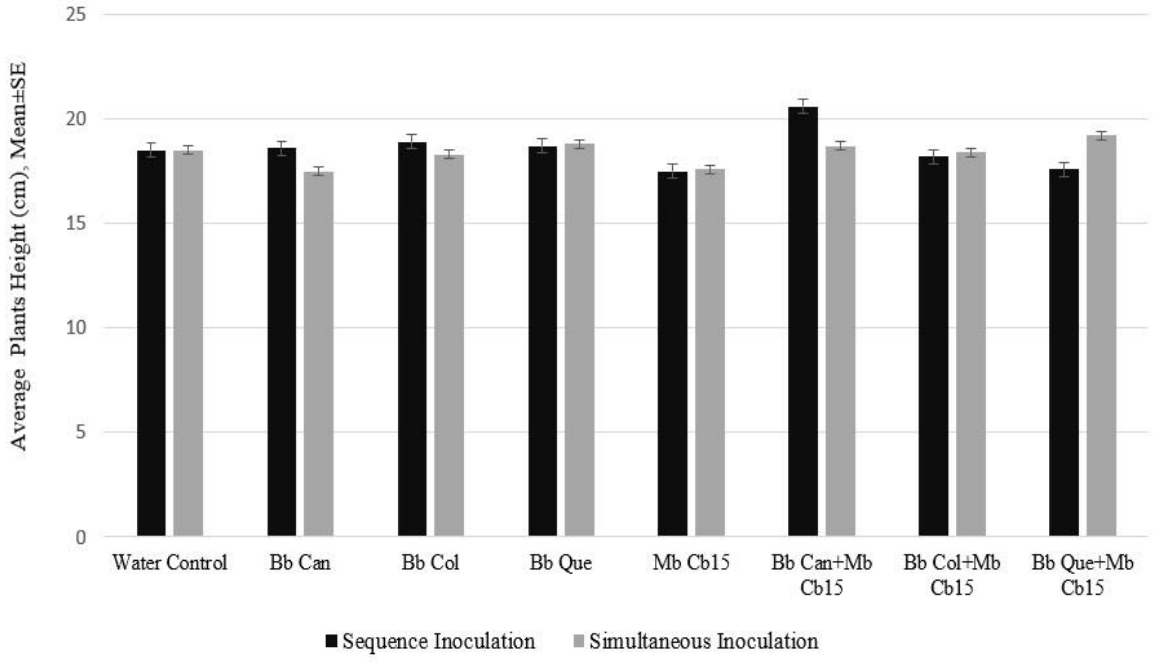

Figure 2. Average plant height (cm) of sequence and simultaneous inoculation method.

3.3. Effect of inoculation method on bacterial and fungal community composition between plant compartments

Bacterial and fungal community structure in plant compartments were influenced by the inoculation method (Table 1). The simultaneous inoculations method had a significant influence on the fungal community structure in all plant compartments tested, while the bacterial community structure was affected in root and stem compartments only. Sequence inoculations influenced the fungal community in root and stem compartments, but not in the leaves probably due to the high rarefied sequence cut in leaf data prior to the analysis. The bacterial community structures were influenced in stem and leaf compartments, but not in the roots.

Table 1. Effect of inoculation method on plant endophytes microbial community structures

Plant Compartment	Bacterial		Fungal	
	Sequence Inoculation	Simultaneous Inoculation	Sequence Inoculation	Simultaneous Inoculation
Root	R^2 = 22% p = 0.28	**R^2 = 20%** **p <0.05**	**R^2 = 23%** **p <0.05**	**R^2 = 27%** **p <0.05**
Stem	**R^2 = 23%** **p <0.05**	**R^2 = 21%** **p <0.05**	**R^2 = 25%** **p <0.05**	**R^2 = 27%** **p <0.05**
Leaf	**R^2 = 23%** **p <0.05**	R^2 = 15% p = 0.48	R^2 = 26% p = 0.08	**R^2 = 23%** **p <0.05**

Bacterial and fungal community structure was analysed with PERMANOVA (%). Significant difference (p<0.05, PERMANOVA) within plant compartment of each inoculation method are indicated in bold.

Bacterial and fungal genera were cluster to discover the endophyte microbial composition within the plant compartment and between the inoculation method. The dominant bacterial genus was Ralstonia (73%) both in the sequence inoculations and in the simultaneous inoculations (37%), followed by Rhodanobacter (21%) in the sequence inoculations and (26%) in the simultaneous inoculations and Pseudomonas ranked third with 19% in the sequence inoculations and 22% in the simultaneous inoculations (Figure 3).

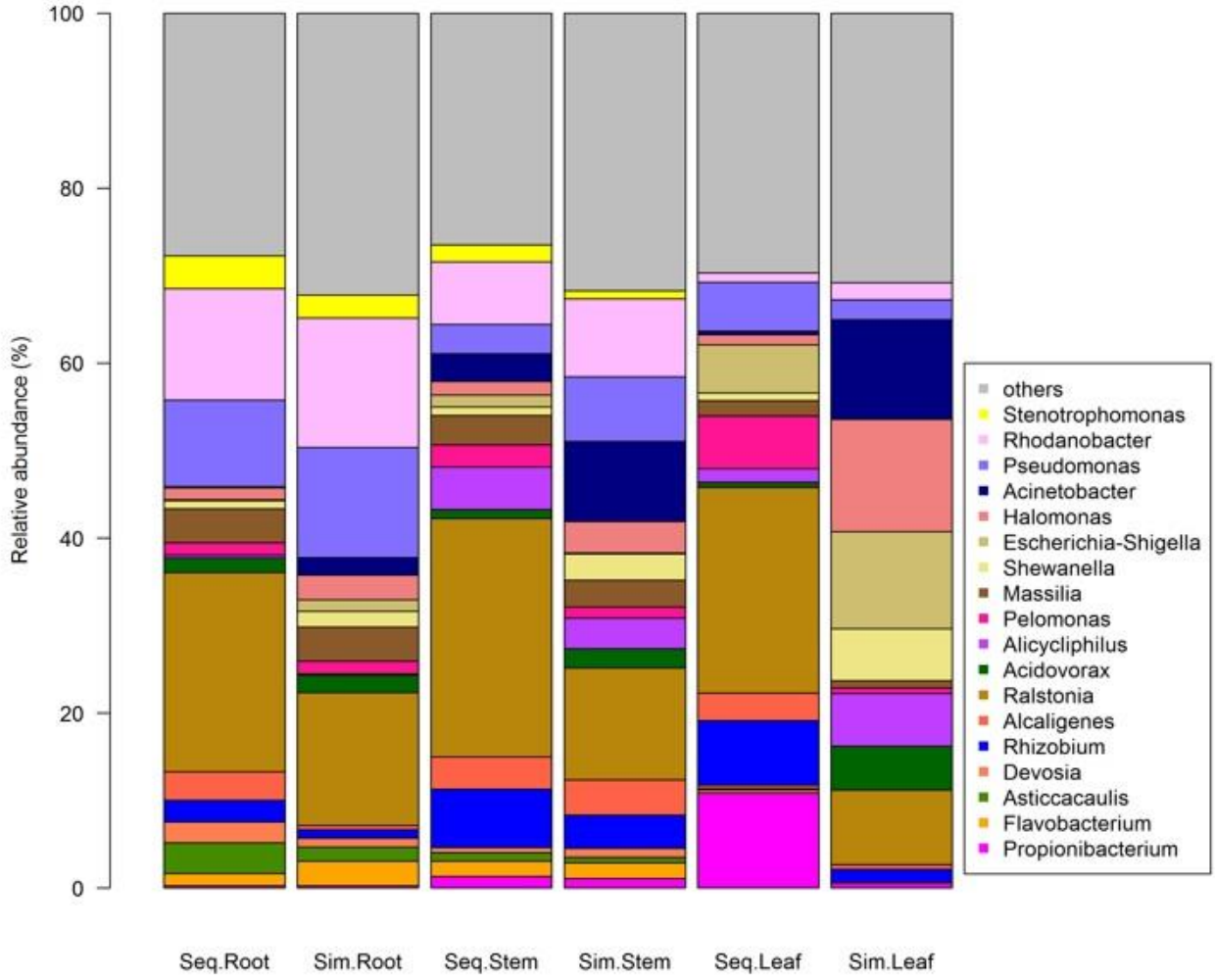

Figure 3. Dominant bacterial genera in the plant compartment according to the sequence (Seq) or simultaneous (Sim) inoculation method. Only groups with an average abundance of ≥1% showed.

Fungal genera proportions (Figure 4) were dominated by the genus Pseudogymnoascus (39%) followed by Chalastospora (20%), Cladosporium (15%), and Mycosphaerella (12%) in the sequence inoculations. On the other hand, fungal genera were dominated by Chalastospora was in simultaneous inoculations (37%), followed by Pseudogymnoascus (24%), Olpidium (24%), and Mycosphaerella (22%) (Figure 4).

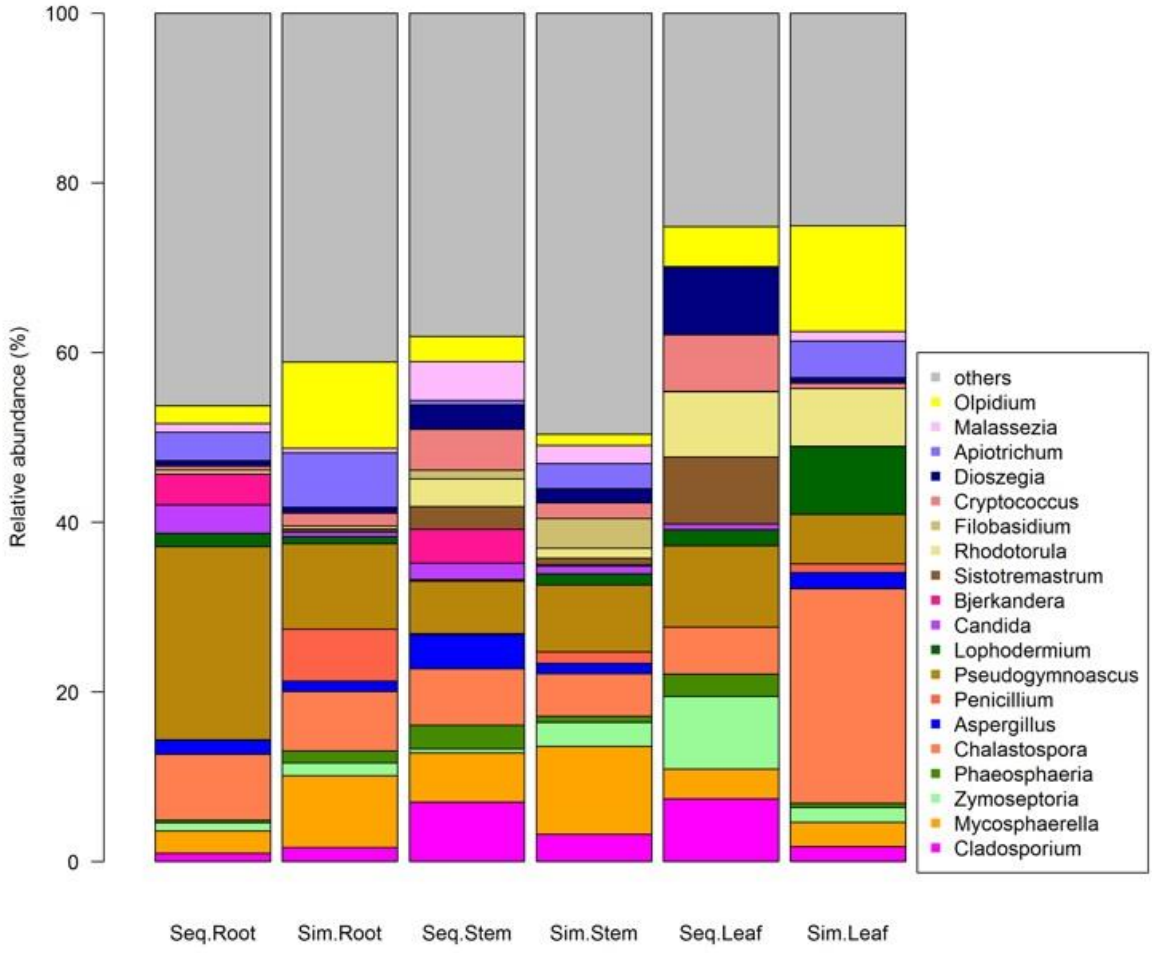

Figure 4. Dominant fungal genera in plant compartments according to the sequence (Seq) or simultaneous (Sim) inoculation methods. Only groups with an average abundance of ≥1% showed.

3.4. Diversity and richness of bacterial and fungal between plant compartment and inoculation method

The bacterial and fungal endophyte relative abundance in plant compartments with regard to the inoculation method, was analysed by the diversity (Shannon index) and the richness (number of observed OTUs). Bacterial diversity (KW-test, H=66.24, df=5,

p<0.001) and richness (KW-test, H= 142.53, df=5, p<0.001) was significantly different between plant compartment of the inoculation method (Figure 5 a and b).

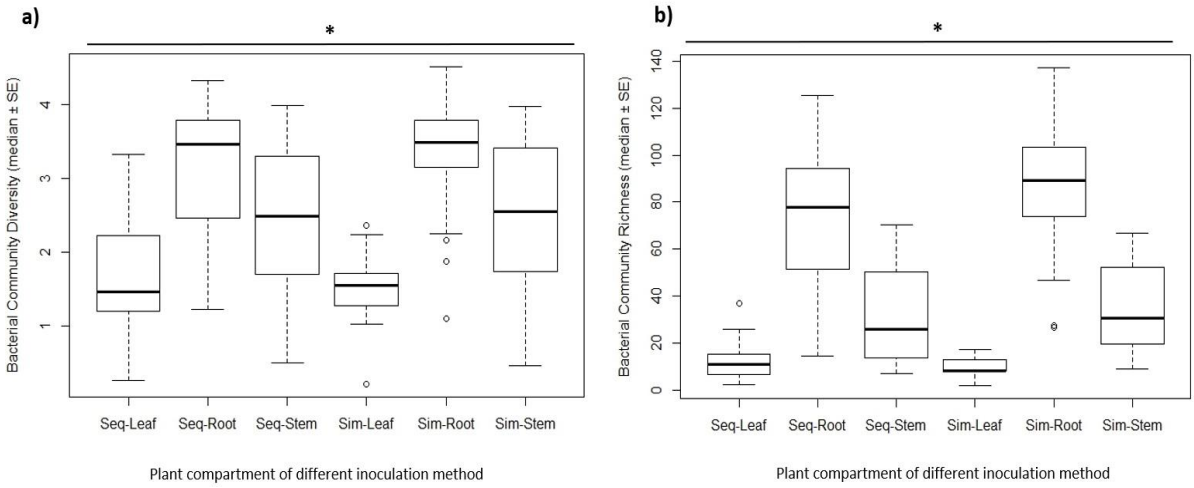

Figure 5. Bacterial community diverse (a) and richness (b) in different compartments of tomato plants with sequence (Seq) or simultaneous (Sim) inoculation method. Asterisks (*) above bar graph indicate significant differences between group (p<0.001, Kruskal-Wallis test).

The inoculation method influenced overall bacterial richness (Z = 4312, p<0.05) (Figure 6 b), but not the bacterial diversity (Z = 4704, p=0.12) of tomato plants (Figure 6 a).

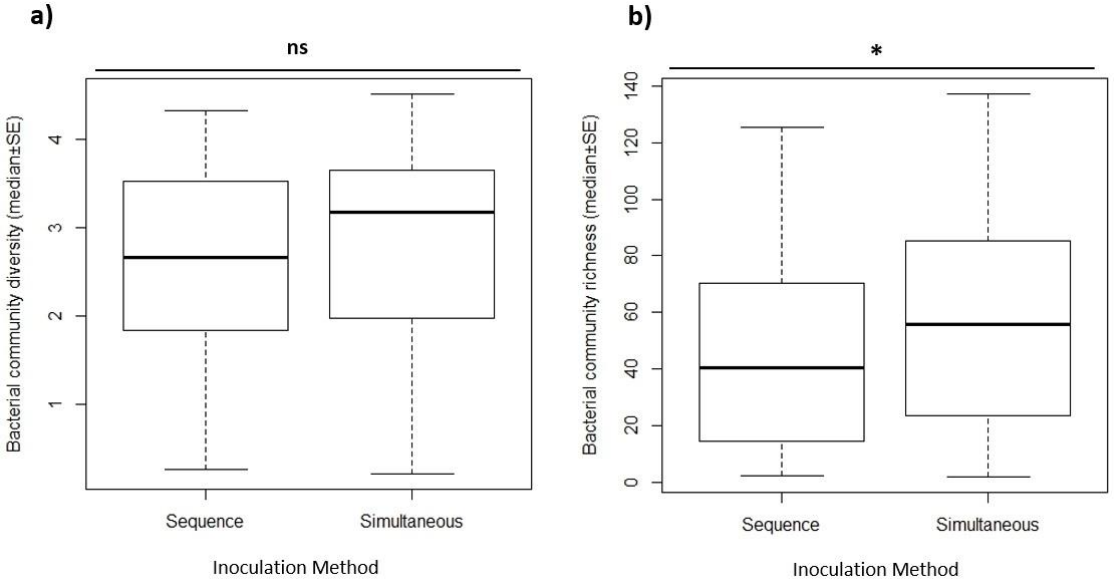

Figure 6. Bacterial community diverse (a) and richness (b) between different inoculation method. Asterisks (*) above bar graph indicate significant differences; (ns) above graph indicate no significant differences between sequence and simultaneous inoculation method (p<0.001, Wilcoxon test).

Fungal diversity did not differ between each plant compartment of different inoculation method (KW-test, H=3.89, df=5, p=0.14) (Figure 7 a), whereas there were significantly differences of the fungal richness (KW-test, H=14.36, df=5, p<0.001) (Figure 7 b).

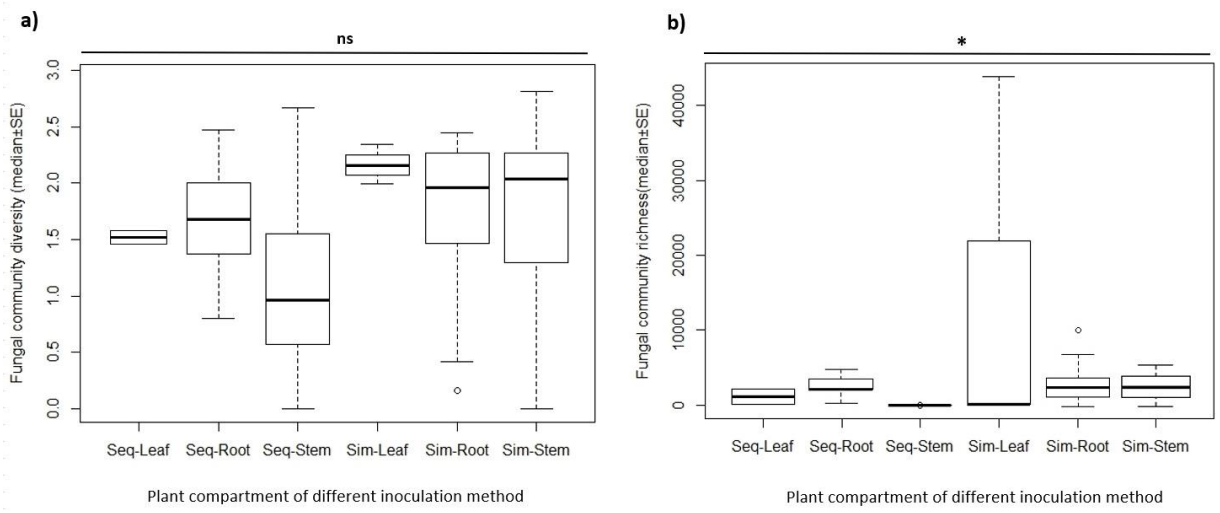

Figure 7. Fungal community diverse (a) and richness (b) in different compartments of tomato plants with sequence (Seq) or simultaneous (Sim) inoculation method. Asterisks (*) above bar graph indicate significant differences; (ns) above graph indicate no significant differences between group (p<0.001, Kruskal-Wallis test).

The inoculation method significantly influenced both fungal diversity (Z = 1585, p<0.001) and richness (Z = 1662, p<0.001) (Figure 8 a and b).

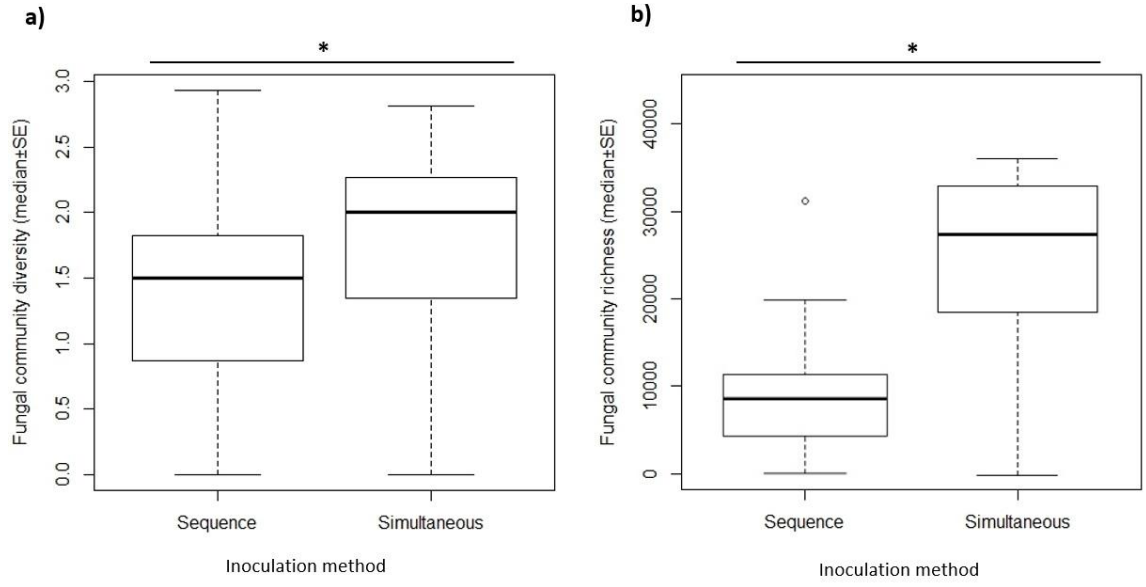

Figure 8. Fungal community diverse (a) and richness (b) between different inoculation method. Asterisks (*) above bar graph indicate significant differences between sequence and simultaneous inoculation method (p<0.001, Wilcoxon test).

4. Discussion

In this present study, inhibitory area was observed when *M. brunneum* strain Cb15 challenged with *B. bassiana* strain (Bb Can, Bb Col and Bb Que) were growing on the same plate. A dual culture method also used to examine the fungal endophyte interaction (Yan et al., 2015), resulted in antagonistic interaction between fungal isolates of the same species from the same leaf and from different plants with a clear inhibition zones in the culture plate. Dual culture assay of EPF *M. brunneum* and *B. bassiana* causing inhibition zones towards olive plant pathogens *Verticillium dahlia* and Phytophthora spp. supporting the mechanism of antibiosis (Lozano-Tovar et al., 2013). Inhibition zones in culture media of fungal antagonism interaction likelihood resulted from the production of inhibitory metabolites (antibiosis) produced by one or both fungal colonies because the recognition of other fungi lead to auto-inhibition (Jonkers, 2012). Antagonism between fungal species that occurred within the same plant are common (Gange et al., 2007), and lead to significant effects on foliar feeding insects due to chemical changes in the plant host caused by fungal endophyte (Gange et al., 2012). Metabolites secreted by EPF species display a wide array of biological activities, such as an antibiotic, antifungal, antiviral, and insecticidal (Lozano-Tovar et al., 2013). The EPF antagonism interaction in co-culture assay in our study utterly unreliable predictor to reveal the effect of inoculation EPF as endophyte impacting other endophyte communities within plants. Therefore, further studies were performed to assessing the effect of different inoculation method of EPF strains towards endophyte communities in tomato plants.

Simultaneous inoculation influenced bacterial community structures in roots and stem, but not in leaf. Moreover, this inoculation method affected fungal community in whole plant compartment. Sequence inoculation affected bacterial community in stem and leaf, yet fungal community only affected in root and stem. In accordance to this, plant compartment specific effects contribute to the endophyte community changed as Methylobacterium spp. inoculation lead to change on bacterial composition in potato shoots, whereas root endophytes were not influenced (Ardanov et al., 2012). Microbial communities varied between crop species and plant compartments resulting in different responses of these communities toward cropping regimes (Granzow et al., 2017).

All OTUs sequences were classified at genus level to capture the influence of the inoculation method on bacterial and fungal community composition. Dominant bacterial genera observed in both inoculation methods were Ralstonia, Rhodanobacter, and

Pseudomonas, respectively. Pseudomonas was reported as predominant bacterial genera in tomato stem (Miliute et al., 2015) and in tomato root (Lee et al., 2019). Another report of Granzow et al. (2017) revealed the predominant of genus Rhodanobacter across all samples of wheat and faba bean, followed by dominance of Ralstonia and Pseudomonas. Predominant Ralstonia and Pseudomonas were detected in Chardonnay and Merlot cultivar of grapevine; interestingly, higher abundance of Ralstonia genera were detected in both grapevine cultivar with integrated pest management (IPM) rather than organic production. The prevalence of genus Ralstonia likely linked to sampling of plants at the end of their vegetative cycle, which may enrich them in more saprophytic microbiota (Campisano et al., 2014).

Pseudogymnoascus, Chalastospora, Cladosporium, and Mycosphaerella were dominance fungal genera obtained from sequence inoculation. Simultaneous inoculation method was dominance by fungal genera of Chalastospora, Pseudogymnoascus, Olpidium, and Mycosphaerella, respectively. Manzotti et al. (2020) reported the relative abundance of genus Pseudogymnoascus was significantly different between tomato genotype UC82B and 8338, but there was no significant different of genus Olpidium. Genus Cladosporium was reported associated with tomato root (Poli et al., 2016), and leaf (Toju et al., 2019). The insecticidal properties of Cladosporium spp. has been reported against insect herbivores (Thakur et al., 2013; Bensaci et al., 2015). Genus Pseudogymnoascus and Mycosphaerella were occurred in leaf and root endosphere of wheat and faba bean plants associated with bulk soil and rhizosphere (Granzow et al., 2017). Fungal endophyte of wild barley from Chalastospora genus reported has salinity tolerance potential (Hammami et al., 2016).

Interaction of bacterial and fungal within the bulk soil, rhizosphere and endosphere likelihood that bacterial used fungal hyphae as a vectors to transferred to the host plant by the fungal route, since the fungal networks are considerably more mobile than bacterial cells (Le Cocq et al., 2017). The bacterial–fungal interaction occur due to the ability of fungal surfaces provide a niche for bacterial growth and movement within plants and the availability of nutrients and the secure access to the nutrient. The long lasting bacterial–fungal associations are based on a “give and take” nutrient policy, ensuring that mutual benefits are warranted for both parties survival (Haq et al., 2014).

In general, the relatively high abundance of bacterial and fungal genera varied according to the plant compartment and the inoculation method. Robinson et al. (2016) reported that the ecological niches of the surface and underground endosphere provide

different conditions for colonization and establishment of microbial endophytes. Therefore, microbial abundances and diversity in leaves and roots were different.

Bacterial diversity and abundance in the roots were significantly higher than in the stem or leaves and simultaneous inoculation significantly increased bacterial abundance. This was also true for the higher bacterial diversity when inoculating simultaneously instead of sequentially. In contrast, there was no significant effect of the plant compartment on fungal diversity, but a simultaneous inoculation method induced a significant effect on fungal diversity and abundance. We attribute the nonexistent effect of the plant compartment and inoculation method on the low OTUs numbers of fungal diversity.

It has been reported that microbial communities, either in the soil rhizosphere or in host plants as endophytes, are influenced by various factors, including soil type and the presence of certain fungal species used in the system (Aguilar-Trigueros and Rillig, 2016), plant species, root exudate production, root morphology, (Berg and Smalla, 2009), plant growth stage (Zhang et al., 2011) and pest control management (Campisano et al., 2014). Our results underline the importance of studying the effect of the fungal strain identity used in the system on plant-associated endophytic microbial communities.

Regarding the growth performance of the plants (plant height) there was no difference between sequence and simultaneous inoculation. In addition, the simultaneous inoculation significantly increased both bacterial and fungal abundance and diversity. Similarly, the structures of the microbial communities were affected in all plant compartments. We therefore recommend the method of simultaneous inoculation to establish different EPF as endophytes in plants. In order to simplify the application of combined EPF for further experiments, the method of simultaneous inoculation is also preferable.

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Supplementary table S1. Bacterial relative abundance (%) at genus level

Bacterial	Sequence inoculation			Simultaneous inoculation		
	Root	Stem	Leaf	Root	Stem	Leaf
Bacteria:Actinobacteria:Actinobacteria:Propionibacteriales:Propionibacteriaceae:Propionibacterium	0.24	1.32	10.85	0.25	1.07	0.58
Bacteria:Bacteroidetes:Flavobacteriia:Flavobacteriales:Flavobacteriaceae:Flavobacterium	1.43	1.74	0.37	2.85	1.80	0.00
Bacteria:Proteobacteria:Alphaproteobacteria:Caulobacterales:Caulobacteraceae:Asticcacaulis	3.50	1.00	0.16	1.56	0.64	0.10
Bacteria:Proteobacteria:Alphaproteobacteria:Rhizobiales:Hyphomicrobiaceae:Devosia	2.35	0.58	0.00	1.05	1.02	9.00
Bacteria:Proteobacteria:Alphaproteobacteria:Rhizobiales:Rhizobiaceae:Rhizobium	2.51	6.68	7.41	0.96	3.78	1.49
Bacteria:Proteobacteria:Betaproteobacteria:Burkholderiales:Alcaligenaceae:Alcaligenes	3.25	3.67	3.19	0.52	4.04	0.52
Bacteria:Proteobacteria:Betaproteobacteria:Burkholderiales:Burkholderiaceae:**Ralstonia**	22.78	27.26	23.46	15.14	12.83	8.53
Bacteria:Proteobacteria:Betaproteobacteria:Burkholderiales:Comamonadaceae:Acidovorax	1.64	1.00	0.57	2.03	2.17	5.01
Bacteria:Proteobacteria:Betaproteobacteria:Burkholderiales:Comamonadaceae:Alicycliphilus	0.33	4.86	1.56	0.14	3.50	6.03
Bacteria:Proteobacteria:Betaproteobacteria:Burkholderiales:Comamonadaceae:Pelomonas	1.42	2.55	6.07	1.43	1.26	0.62
Bacteria:Proteobacteria:Betaproteobacteria:Burkholderiales:Oxalobacteraceae:Massilia	3.88	3.33	1.75	3.92	3.09	0.80
Bacteria:Proteobacteria:Gammaproteobacteria:Alteromonadales:Shewanellaceae:Shewanella	0.95	1.00	0.87	1.77	3.01	5.94
Bacteria:Proteobacteria:Gammaproteobacteria:Enterobacteriales:Enterobacteriaceae:Escherichia-Shigella	0.10	1.34	5.47	1.35	0.08	11.09
Bacteria:Proteobacteria:Gammaproteobacteria:Oceanospirillales:Halomonadaceae:Halomonas	1.35	1.57	1.17	2.78	3.60	12.85
Bacteria:Proteobacteria:Gammaproteobacteria:Pseudomonadales:Moraxellaceae:Acinetobacter	0.17	3.18	0.39	2.03	9.19	11.42
Bacteria:Proteobacteria:Gammaproteobacteria:Pseudomonadales:Pseudomonadaceae:**Pseudomonas**	9.85	3.36	5.61	12.61	7.37	2.25
Bacteria:Proteobacteria:Gammaproteobacteria:Xanthomonadales:Xanthomonadaceae:**Rhodanobacter**	12.73	0.07	0.01	14.76	8.90	1.97
Bacteria:Proteobacteria:Gammaproteobacteria:Xanthomonadales:Xanthomonadaceae:Stenotrophomonas	0.04	0.02	0.00	2.63	0.95	0.00

Supplementary table S2. Fungal relative abundance (%) at genus level

Fungal	Sequence inoculation			Simultaneous inoculation		
	Root	Stem	Leaf	Root	Stem	Leaf
Fungi:Ascomycota:Dothideomycetes:Capnodiales:Davidiellaceae:**Cladosporium**	1.00	7.02	7.40	1.66	3.22	1.74
Fungi:Ascomycota:Dothideomycetes:Capnodiales:Mycosphaerellaceae:**Mycosphaerella**	2.60	5.81	3.52	8.45	10.37	2.90
Fungi:Ascomycota:Dothideomycetes:Dothideomycetes ord:Dothideomycetes fam:Zymoseptoria	0.99	0.49	8.55	1.50	2.80	1.74
Fungi:Ascomycota:Dothideomycetes:Pleosporales:Phaeosphaeriaceae:Phaeosphaeria	0.30	2.76	2.62	1.43	0.75	0.50
Fungi:Ascomycota:Dothideomycetes:Pleosporales:Pleosporaceae:**Chalastospora**	7.76	6.64	5.56	7.02	4.98	25.27
Fungi:Ascomycota:Eurotiomycetes:Eurotiales:Trichocomaceae:Aspergillus	1.67	4.06	0.00	1.19	1.22	1.90
Fungi:Ascomycota:Eurotiomycetes:Eurotiales:Trichocomaceae:Penicillium	0.02	0.09	0.01	6.15	1.40	1.03
Fungi:Ascomycota:Leotiomycetes:Leotiomycetes_ord_Incertae_sedis:Myxotrichaceae:**Pseudogymnoascus**	22.77	6.15	9.60	10.07	7.87	5.83
Fungi:Ascomycota:Leotiomycetes:Rhytismatales:Rhytismataceae:Lophodermium	1.60	0.20	1.89	0.82	1.32	8.00
Fungi:Ascomycota:Saccharomycetes:Saccharomycetales:Saccharomycetales_fam_Incertae_sedis:Candida	3.34	1.96	0.67	0.52	0.91	0.05
Fungi:Basidiomycota:Agaricomycetes:Polyporales:Meruliaceae:Bjerkandera	3.65	3.99	0.00	0.00	0.15	0.00
Fungi:Basidiomycota:Agaricomycetes:Trechisporales:Hydnodontaceae:Sistotremastrum	0.00	2.68	7.89	0.33	0.76	0.00
Fungi:Basidiomycota:Microbotryomycetes:Sporidiobolales:Sporidiobolales_fam_Incertae_sedis:Rhodotorula	0.43	3.24	7.69	0.00	1.19	6.79
Fungi:Basidiomycota:Tremellomycetes:Filobasidiales:Filobasidiaceae:Filobasidium	0.00	1.05	0.01	0.41	3.51	0.00
Fungi:Basidiomycota:Tremellomycetes:Tremellales:Tremellales_fam_Incertae_sedis:Cryptococcus	0.48	4.83	6.70	1.51	1.85	0.66
Fungi:Basidiomycota:Tremellomycetes:Tremellales:Tremellales_fam_Incertae_sedis:Dioszegia	0.65	2.87	7.94	0.66	1.64	0.61
Fungi:Basidiomycota:Tremellomycetes:Trichosporonales:Trichosporonaceae:Apiotrichum	3.35	0.54	0.00	6.42	2.96	4.35
Fungi:Basidiomycota:Ustilaginomycotina_cls_Incertae_sedis:Malasseziales:Malasseziaceae:Malassezia	1.03	4.58	0.09	0.57	2.14	1.11
Fungi:Chytridiomycota:Chytridiomycetes:Olpidiales:Olpidiaceae:**Olpidium**	2.10	2.96	4.70	10.14	1.32	12.44

Chapter 2

The effect of inoculation of entomopathogenic fungi as endophytes on plant pathogen inhibition

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Abstract

Entomopathogenic fungi (EPF) are known as biocontrol agent of insect herbivore. Over time evidence grew that several entomopathogenic fungi or their specific isolates may play additional roles in nature beside their primary role as insect pathogens, being also antagonists of plant pathogens. To improving biocontrol efficacy of EPF strain on plant pathogens, a study was conducted to examine whether the co-inoculation of EPF strain can colonize tomato plants as endophyte and subsequently provide protection against fungal pathogen (*Fusarium oxysporum* Schlechtendahl Race 3) causing fusarium wilt disease of tomato. Experiments were conducted by inoculating EPF strains *Beauveria bassiana* or *Metarhizium brunneum*, followed by co-inoculation of *F. oxysporum* strain 24 hour later. These experiments aimed at assessing the ability of the EPF to endophytically colonize tomato plant tissues and to inhibit the growth of the plant pathogen. Real Time PCR was conducted to quantify the presence of fungal strain inoculated in tomato plant tissues. Our result finding that co-inoculation strategies of the EPF strain were able to inhibit plant pathogen and enhanced plant growth parameter. Significant amounts of *B. bassiana* and *M. brunneum* DNA were found in roots as the EPF strains co-inoculation sites.

Keywords: *Beauveria bassiana*, Co-inoculation, Endophyte, *Fusarium oxysporum*, Inhibition, *Metarhizium brunneum*, Plant pathogen, RT-PCR.

1. Introduction

Fungal entomopathogens (EPFs), such as the entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) or *Metarhizium brunneum* Petch (Ascomycota: Hypocreales) have been recovered as endophytes from several plant species and have been shown to play additional roles in nature, such as plant pathogen antagonists, rhizosphere colonizers, and plant growth promoters (Vega et al., 2008; 2009).

Various studies, using *B. bassiana* isolates, have demonstrated that they can effectively reduce plant disease incidences when established in plant tissues as endophytes. The endophytic *B. bassiana* strain 11-98 suppresses damping-off of tomato caused by the soilborne plant pathogens *Rhizoctonia solani* (Ownley et al., 2008). it also reduced the severity of *R. solani* damping-off in cotton seedlings (Griffin, 2007) and severity of *Pythium myriotylum* damping-off in tomato seedling (Clark, 2006). Several *B. bassiana* strain; ATCC 74040 (registered as NATURALIS®), EABb04/01-Tip, ATP01, and ATP05 reported to significantly reduce disease incidence and severity of downy mildew caused by *Plasmopara viticola* on grapevine (Jaber, 2015). Seed inoculation treatment of *B. bassiana* strain ATCC 74040, registered as NATURALIS® or *Metarhizium brunneum* registered as BIPESCO5 has been reported of their endophytic establishment within wheat, can promote plant growth, and it is significantly reduce the disease incidence and severity of crown root rot (CRR) caused by *Fusarium culmorum* in wheat (Jaber, 2018).

The management of a soil-inhabiting fungal pathogen fusarium wilt disease (*Fusarium oxysporum f.sp. lycopersici*) is one of the main problems in tomato production systems, causing significant yield losses worldwide (Huang and Lindhout, 1997). Due to the persistence of the fungal pathogen spores in the soil and the endophytical growth within plant tissues, even the synthetic control options for this pathogen are limited (Amini and Sidovich, 2010). In attempts to improve the efficacy of biocontrol methods on plant pathogen, several studies performed with multiple antagonists. For example, a combination of certain fluorescent strains of Pseudomonas with the root associated fungus *Acremonium rutilum*, non-pathogenic *F. oxysporum* and *Verticillium lecanii* significantly suppressed the disease incidence of Fusarium wilt on radish (*F.oxysporum* f. sp. raphani) (Leeman et al., 1996). A combination of *B. bassiana* strain B2 and bacterium *Bacillus subtilis* effectively reduced the incidence of tomato fusarium wilt (*F. oxysporum* f.sp. lycopersici) compared with individual agent applications and control

treatments (Prabhukarthikeyan et al., 2014). A combination of *Trichoderma koningii* with a fluorescent Pseudomonas strain increased wheat yield and reduced crown root infection by *Gaeumannomyces graminis* var. tritici (Duffy et al., 1996).

Although the potential of biological control strategies using EPF species to control plant pathogens has been demonstrated repeatedly (Ownley et al., 2008;2010; Griffin, 2007; Clark, 2006; Jaber 2015; Jaber 2018); to date, there are no reports regarding the co-inoculation strategies of EPF as endophyte aim to control plant pathogens. Therefore, this study was performed to examine whether the co-inoculation of EPF strain can colonize tomato plants as endophyte and subsequently inhibit the growth of fungal pathogen (*Fusarium oxysporum f.sp. lycopersici*) causing fusarium wilt disease of tomato. We hypothesis that niche occupation by the inoculated EPF could inhibit the growth of the plant pathogen as a competition mechanism in inoculated site (root) of plant tissues.

2. Materials and Methods

2.1. Plant material

Tomato plants *Solanum lycopersicum* L cultivar Moneymaker (Rein Saat®, Austria) was used for EPF inoculation. Each seed was grown in a multi tray with a mixture of soil (Fruhstorfer Erde Typ T, Hawita Gruppe GmbH, Vechta, Germany) and non-sterile 0,3mm sand (3:1). Tomato seedling at the two-leaf stage were used in this study. Seedlings were removed from the substrate, and the roots were carefully washed with tap water prior to fungal inoculation. Seedlings inoculated with the spore suspension described at (2.5. Fungal strain co-inoculation), were individually transplanted into plastic pots (diameter 11cm) using the same soil mixture as described above. The plants were maintained under greenhouse conditions (21±2°C, 70-80% RH and 12h photoperiod) and irrigated regularly for a growing period of four weeks post inoculation.

2.2. Fungal material

Fusarium oxysporum

Four strains of *Fusarium oxysporum* used in the screening assays were kindly provide by the working group of Molecular Phytopathology and Mycotoxin Research, Department of Crop Science, Faculty of Agriculture, Georg-August-University Göttingen, Germany. These were (1) *F. oxysporum f. sp asparagi* BBA62286 (No.

0622), (2) *F. oxysporum f. sp conglutinans* Foc 5a (No. 1216), (3) *F. oxysporum f. sp ycopersici* CBS16730 (No. 0867), (4) *Fusarium oxysporum* Schlechtendahl: Fries (DSM No. 62338) Race 3. For convenience, each strain will be abbreviated as (1) F. oxy asparagi, (2) F. oxy conglutinans, (3) F. oxy lycopersici, (4) F. oxy Race 3.

The production of *F. oxysporum* spore suspensions used in the experiments were grown using mung bean medium. Mung bean broth (MBB) medium was prepared by boiling 40g of mung bean seeds in one liter of distilled water for 10 min. After cooling, the broth was filtered through cheesecloth and filled up with distilled water up to one liter, and divided per 100ml portions into Erlenmeyer flasks, follow by sterilization. Furthermore, sterile MBB inoculated with mycelium plaques of each strain were incubated in a shaker (28°C, 200 rpm) for 6 days. The spores of each *F. oxysporum* strain were collected by filtering through two layers of sterile cheesecloth into sterile Erlenmeyer flasks, followed by convert filtration into sterile 50ml Falcon tubes (Sarstedt AG&Co.KG), centrifugation (10 min, 4500rpm), discarding the supernatant and adding 1ml of sterile distilled water. Thus prepared, spore concentration determinations were performed with a Thoma counting chamber (Marienfeld, Germany) and adjusted to 100 ml of $1x10^6$ spores/ml.

Entomopathogenic fungi

Three strains of *B. bassiana* (1) EABb 04/01-Tip, (2) Bb 1022 and (3) BV 061) and a strain of *M. brunneum* (Cb15 III) were used for combined fungal growth assessments (in vitro assay) and co-inoculation treatments. Strains were obtained from the culture collection of the Agriculture Entomology working group, Department of Crop Sciences, Faculty of Agriculture, Georg-August-University Göttingen, Germany. For convenience, each strain will be abbreviated as (1) Bb Que, (2) Bb Can, (3) Bb Col, and Mb Cb15, respectively.

The strains were grown in potato dextrose agar (PDA) at 24 ±2°C in dark conditions for two weeks to obtain enough spores for the suspension. Spores suspension production was carried out under a sterile bench (Thermo Fisher Scientific), starting by adding 5ml of Tween 20 (0.1 % v/v, DifcoTM) into two-week-old culture plate of each strain, followed by gently scraping off the culture surface with sterile microscope slide glass. Spores were then suspended in 10ml sterile distilled water. Spore concentration determine with a Thoma counting chamber (Marienfeld, Germany), afterwards adjusted to 100 ml of $1x10^6$ spores/ml. To assess the viability of the spores of the different fungal

strains used in this experiment, a germination test was carried out. 100µl from each spore suspension was spread out on PDA medium and incubated at 24 ±2°C for 3 days. Spore germination was checked under the microscope and average germination exceeded 90% for each strain.

2.3. Screening of Fusarium strain inoculation

Screening of the successful inoculation of the *F. oxysporum* strain was performed by determining the endophytic colonization of each strain within tomato plant tissues via re-isolation from plant compartment and assessing the plant growth parameter. The inoculation was performed by immersing the tomato seedling roots into 100 ml of $1x10^6$ spores/ml suspensions of each strain of *F. oxysporum.* For the control treatments, the same procedures were conducted with distilled water instead of a spore suspension.

Colonization frequency for each *F. oxysporum* strain was evaluated four weeks post inoculation. From each plant treatment, three different plant compartments were harvested: lower leaves (the first stage of real leaves), stems (2 cm sections measured from the cotyledon growth point) and roots (primary roots). Surface sterilized samples (the procedure describes below in 2.6. Harvest and surface sterilization of plant material) of the respective plant compartment were placed inside a sterile bench (Thermo Fisher Scientific). Eight leaf disks of 6 mm diameter, 2 cm sections of stem segments were cut into eight pieces, and 1 cm of eight pieces of primary roots were placed into Petri dishes containing PDA. Fungal growth from each plant compartment was recorded after 14 days of incubation at 24 ±2°C in darkness. The colonization rate is calculated as percentage units:

$$\text{Colonization rate } (\%) = \frac{\text{Number of samples showed fungal growth}}{\text{Total number of plants samples}} x100$$

Ten plants per treatment were evaluated for plant growth parameter measurements at harvesting time.

2.4. Inhibition of fungal strain in co-inoculation in vitro assays

Co-inoculation assays were carried out between the *F. oxysporum* strain (F. oxy Race 3) and the EPFs, aiming to select the strain of the EPFs which would be able to best inhibit the growth of the fungal pathogen, for further use on co-inoculation experiments in planta.

The EPFs and F. oxy Race 3 were placed opposite side perpendicular in a Petri dish containing PDA. 10µl of $1x10^6$ spores/ml fungal strain suspension was dropped at 1cm apart from the edge of the plate. All pairings were carried out with five replications. As control, each fungal strain was inoculated on in a separate Petri dish. All treatments were maintained at 24 ±2°C for 20 days, the period of time mycelia reached their maximal growth, while no more growth was observed. Evaluation of mycelia growth for each strain in the control (R1) and the challenged Petri dishes (R2), was performed by measuring the final colony diameters in cm. Data were transformed into percent inhibition radial growth (PIRG) using the formula suggested by Skidmore and Dickinson (1976):

$$\text{Percent inhibition radial growth (PIRG)} = \frac{R1-R2}{R2}x100$$

2.5. Fungal strain co-inoculation

The fungal strain of *F. oxysporum* used in the screening experiment (F. oxy Race 3) and the EPF strain performing best in the co-inoculation in vitro assays (Mb Cb15 and Bb Can) were used for fungal strain co-inoculation experiments by establishing each strain as an endophyte. The first inoculation was performed by immersing the tomato seedling roots into 100 ml of $1x10^6$ spores/ml suspensions of a single EPF, or into 100ml of $2x10^6$ spores/ml suspension, containing spores both strains of EPF (ratio 1:1) as the combination treatment of EPFs for 20 minutes. Inoculated seedlings were individually transplanted into plastic pots (diameter 11cm) containing the soil mixture mentioned above. For the control treatment, the same procedure was conducted with distilled water instead of a spore suspension. 24 hours later, the inoculation was followed by co-inoculation of F. oxy Race 3 via root drenching with a 50 ml of $1x10^6$ spores/ml suspension. Treated plants were maintained under greenhouse conditions (21±2°C, 70-80% RH and 12h photoperiod), repeatedly checked for Fusarium wilt symptoms, and irrigated regularly for a growing period of four weeks post inoculation. The experiment was performed as a completely randomized block design with ten replications.

Table 1. Co-inoculation treatments of EPFs and *F. oxysporum* Race 3.

Prior inoculation	Co-inoculation	Treatment (mentioned as)
Bb Can	F. oxy Race 3	Bb Can + F. oxy Race 3
Mb Cb15	F. oxy Race 3	Mb Cb15 + F. oxy Race 3
Bb Can + Mb Cb15	-	Bb Can + Mb Cb15
Bb Can + Mb Cb15	F. oxy Race 3	Bb Can + Mb Cb15 + F. oxy Race 3
F. oxy Race	-	F. oxy Race 3
Water	-	Water Control

2.6. Harvest and surface sterilization of plant material

Above and below ground plant parts of each treatment was harvested four weeks post inoculation. Plant height of the plant was measured at the beginning of the experiment, by measuring the height (cm) from the growth point of cotyledon leaves up to the new emerge leaves. Aboveground part of the plants was then cut with a sterile scalpel and measured for aboveground fresh biomass weight (gram). Afterwards, the lower leaves (the first stage of real leaves), and the stem parts (2 cm sections measured from the cotyledon growth point) were cut with a sterile scalpel and placed separately into a Falcon tube for further surface sterilization. Furthermore, belowground part was gently removed from the pots and the roots were washed off under tap water to remove the rhizosphere soil, placed in sterile paper towels to remove the tap water residues, and primary root length (cm) was measured. Lastly, the roots were placed separately into a Falcon tube for further surface sterilization.

Aboveground plant material samples were surface-sterilized by serial washing in 70% ethanol for 1 minute, 2% sodium hypochlorite for 30 second and 70% ethanol for 1 minute, followed by two times immersion in sterile distilled water for 30 second and once in sterile diethylpyrocarbonate (DEPC)-treated water (modified from Andreote et al., 2010). Surface sterilization of roots was performed according to Li et al. (2010), with slight modifications. In this study, 2% sodium hypochlorite and sterile DEPC-treated water were used for the last step. To confirm the success of the disinfection procedure, aliquots of the DEPC water used in the final washing step were plated on PDA plates. The plates were incubated in the dark at 24 ±2°C for at least 1 week. No

growth of microorganisms was observed. Surface-sterilized plants samples from fungal strain co-inoculation experiment were freeze drying (Zirbus VaCo 5, Zirbus Technology GmbH Germany) for 72 hours. Freeze dried plant samples were finely ground in a mortar grinder (Mixer Mill MM 400, Retsch GmbH Germany) obtained powder samples, then stored at −20°C until DNA extraction.

2.7. Molecular detection of inoculated fungal DNA

DNA extraction of inoculated plant material was performed by using a total of 20mg dry weight sample of stem and roots, employing the peqGOLD Plant DNA Mini kit (Peqlab, Erlangen, Germany) according to the manufacturer's instructions with two modifications as described previously (Wemheuer et al., 2016). The DNA concentration of extraction product was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) for DNA dilution purpose. In total, extracted DNA of 120 samples was subjected for DNA quantification by real-time polymerase chain reaction (RT-PCR).

Endophytic growth confirmation of single and combination strains of EPFs followed by co-inoculation of F. oxy Race 3, was confirmed by real-time polymerase chain reaction (RT-PCR). To quantify each fungal DNA form extraction product, one standard curve was needed (Brandfass and Karlovsky, 2008). Standard curve dilution series of *B. bassiana*, *M. brunneum*, and *F. oxysporum* fungal DNA were prepared as one third from the highest 100 pg, 33.33 pg, 11.11 pg, 3.70 pg, 1.23 pg, 0.41 pg, 0.13 pg, 0.045 pg, 0.015 pg, and the lowest 0.0005 pg. Fungal DNA for the standard curve was obtained from a stock available at the Agriculture Entomology working group. Each RT-PCR plate was analysed separately with species-specific primers, contained 1:10 dilution of the DNA plant samples, and negative control from master mix with analytical triplicates of each RT-PCR running.

Species-specific primer for *B. bassiana* was Bsn1-2-forward "GCGTCAAGGTGCTCGAAGACAG" with Bsn1-2-reverse "TCTGGGCGGCATCCCTATTGT" (Zhang, 2014), and for *M. brunneum* Ma-1763-forward "CCAACTCCCAACCCCTGTGAAT" with Ma-2097-reverse "AAAACCAGCCTCGCCGAT" (Schneider et al., 2012) as performed previously to quantify *M. brunneum* strain ART 2825 (Rodríguez, 2016). Cycling conditions for both primers consisted of 2 min initial denaturation at 95°C, followed by 39 cycles of 10 sec denaturation at 95°C, 15 sec annealing at 65°C and 15 sec extension at 72°C and the

plate read at the end of each cycle. Followed by a final extension for 1 min at 72ºC, the amplification of melting curve analysis was performed ranging from 95°C to 55°C held for 30 sec each.

The species-specific primer for *F. oxysporum* was Clox1-forward "CAGCAAAGCATCAGACCACTATAACTC" with Clox2-reverse "CTTGTCAGTAACTGGACGTTGGTACT" (Mulè et al., 2004). Cyling conditions consisted of 2 min initial denaturation at 95°C, followed by 39 cycles of 10 sec denaturation at 95°C, 15 sec annealing at 61°C and 15 sec extension at 72°C and the plate read at the end of each cycle. Followed by a final extension for 1 min at 72ºC, the amplification of melting curve analysis was performed ranging from 95°C to 55°C held for 30 sec each.

The RT-PCR was performed with a reaction mixture containing 5µl 2× qPCRBIO SyGreen Master Mix Kit (PCR Biosystems Ltd., England), 0.2µl, 10µM of each primer, 3.6µl of sterile water and 1µl of sample DNA, and reached total reaction volumes of 10µl. RT-PCR was performed in an iCycler System CFX384 Real time system (Bio-Rad, Hercules, CA, USA).

2.8. Data analysis

Obtained data sets from the experiments were analysed by R version 3.6.3 for statistical computing (R Core Team, 2020). Prior to analyses a normality test of the residuals (Shapiro-Wilk) was used to test the assumption of normally distributed data, followed by assumptions for variances homogeneity by Levene's test. Furthermore, one-way ANOVA was performed followed by a post hoc test using Tukey's Honest Significant Difference (HSD) to identify significance differences between groups.

As an exception, the colonization rate of re-isolation *F. oxysporum* screening data and the fungal DNA quantification data were not normally distributed; therefore, a generalized linear model (glm) was performed with binomial distribution. Significance between groups was analysed as a post hoc test using Tukey's Honest Significant Difference (HSD).

3. Results

3.1. Screening strain of plant pathogen (*Fusarium oxysporum*)

Screening strain of fungal pathogen (*F. oxysporum*) for further co-inoculation experiment purpose was analysed in the following experiments.

3.1.1. Re-isolation of *F. oxysporum* strains colonized plant tissues

The endophytic colonization rates of each *F. oxysporum* strain were obtained from the re-isolation of different plant compartments (Figure 1). No significance different found of colonization rate between *F. oxysporum* strain in lower leaves (glm [$F_{(4, 45)} = 1.00$, $p = 0.41$]). Significance different of colonization rate between *F. oxysporum* strains were found in stem (glm [$F_{(4, 45)} = 87.98$, $p<0.001$]) and roots (glm [$F_{(4, 45)} = 188.20$, $p<0.001$]). Tomato plants treated with F. oxy Race 3 had the highest colonization rate among the strains tested both in stem (100%) and roots (90%). F. oxy lycopersici has 5% colonization rate of stem and 85% of roots. In contrast, colonization rate of F. oxy aspargi and F. oxy conglutinans were not recovered from any plant compartment. The highest colonization rate from re-isolation of stem and roots compartment of F. oxy Race 3 indicate successful establishment of this strain in tomato.

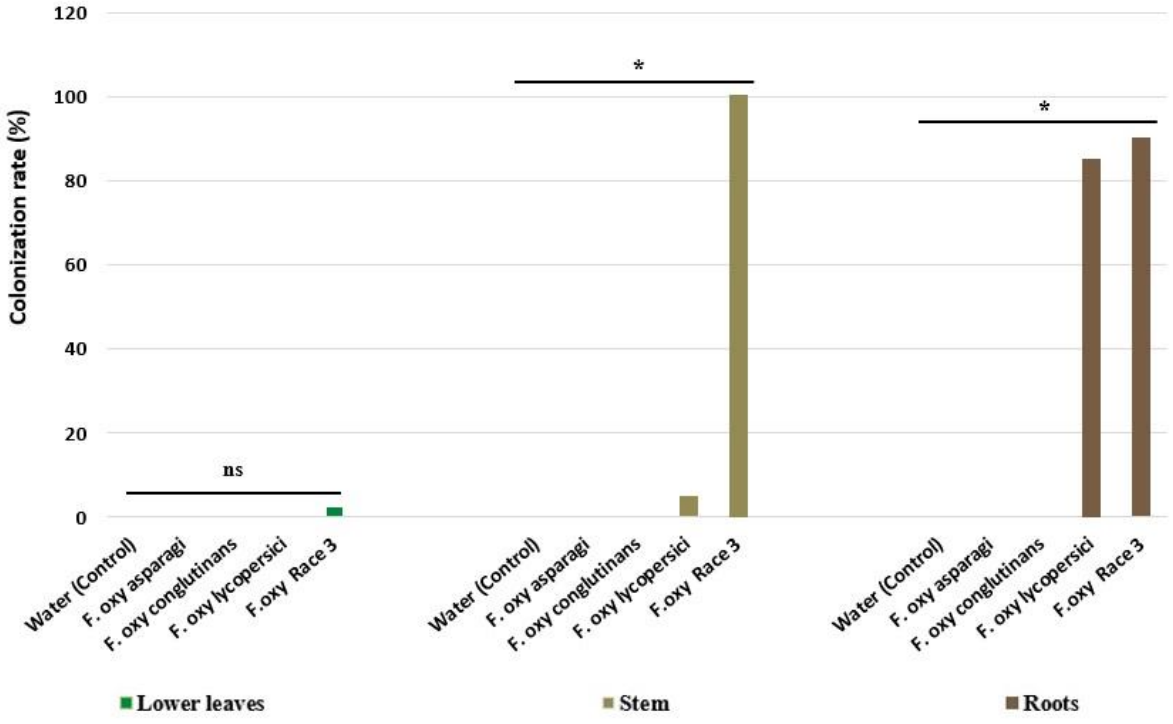

Figure 1. Colonization rate (%) of *F. oxysporum* strains from plant compartment re-isolation. Asterisks (*) above bar graph indicate significant differences, (ns) above bar graph indicate no significant differences between treatment ($p < 0.001$, generalized linear model).

3.1.2. Effects of inoculated *F. oxysporum* strains on plant growth parameters

Plant height

There was a significant effect of the treatment on growth of the tomato plants inoculated with the different fungal strains (ANOVA [$F_{(4, 45)} = 28.43$, $p<0.001$]). Post hoc comparison using the Tukey's HSD test indicated that the mean value of plant height for the F.oxy aspargi (17.14±1.26), F.oxy conglutinans (18.42±1.13), Water control (19.19±2.25), F.oxy lycopersicum (17.36±1.34) was significantly different than F. oxy Race 3 (12.07±1.98 Figure 2).

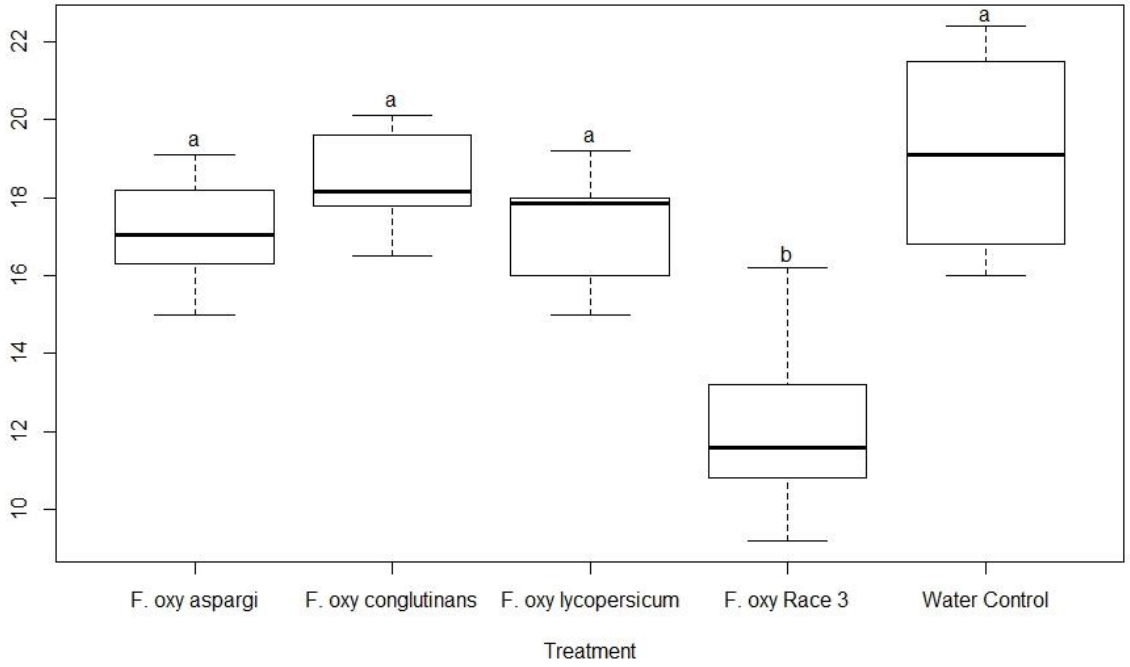

Figure 2. Effect of root inoculation treatments with *F. oxysporum* strains on tomato plant height (cm; means ± SE) compared to controls. Different letters refer to significant differences among treatments ($p < 0.001$, Tukey's HSD test, after one-way ANOVA).

Aboveground fresh biomass

There was a significant effect of the treatment on tomato plants aboveground fresh weight inoculated with the different fungal strains (ANOVA [$F_{(4, 45)} = 14.38$, $p< 0.001$]). The mean aboveground fresh weight value F. oxy Race 3 (13.50 ± 1.97) was significantly different compared to the other treatments, F. oxy aspargi (20.36±1.90), F. oxy conglutinans (18.69±2.67), Water control (19.76±2.56), F. oxy lycopersicum (18.19±2.06), respectively (Figure 3).

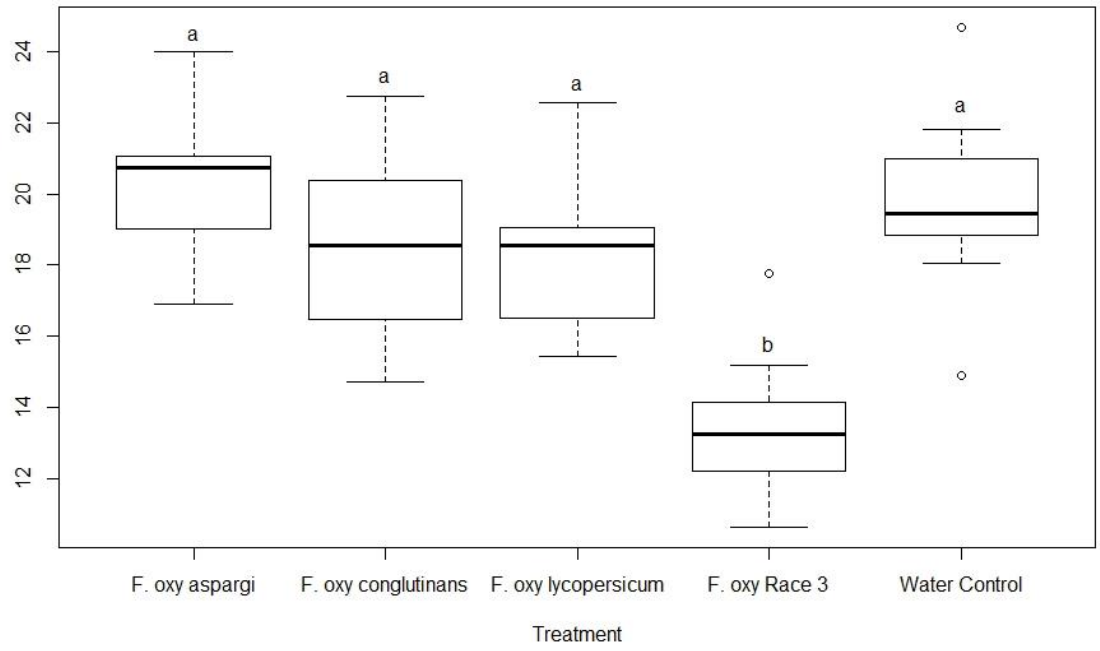

Figure 3. Effect of a root inoculation treatment with *F. oxysporum* strains on tomato aboveground fresh weight (gram; means ± SE) compared to controls. Different letters refer to significant differences among treatments ($p < 0.001$, Tukey's HSD test, after one-way ANOVA).

Root length

There was a significant effect of the treatment on root length of tomato plants inoculated with the different fungal strains (ANOVA [$F_{(4, 45)} = 2.07$, $p = 0.09$]). Post hoc test indicated that the mean value for the F. oxy conglutinans (29.08±4.03) was significantly different compared to F. oxy Race 3 (23.64±5.11). However, the treatment with F. oxy aspargi (26.35±3.62), F. oxy lycopersicum (26.99±3.68), and Water control (19.76±2.56) did not significantly differ from F. oxy conglutinans and F. oxy Race 3 (Figure 4).

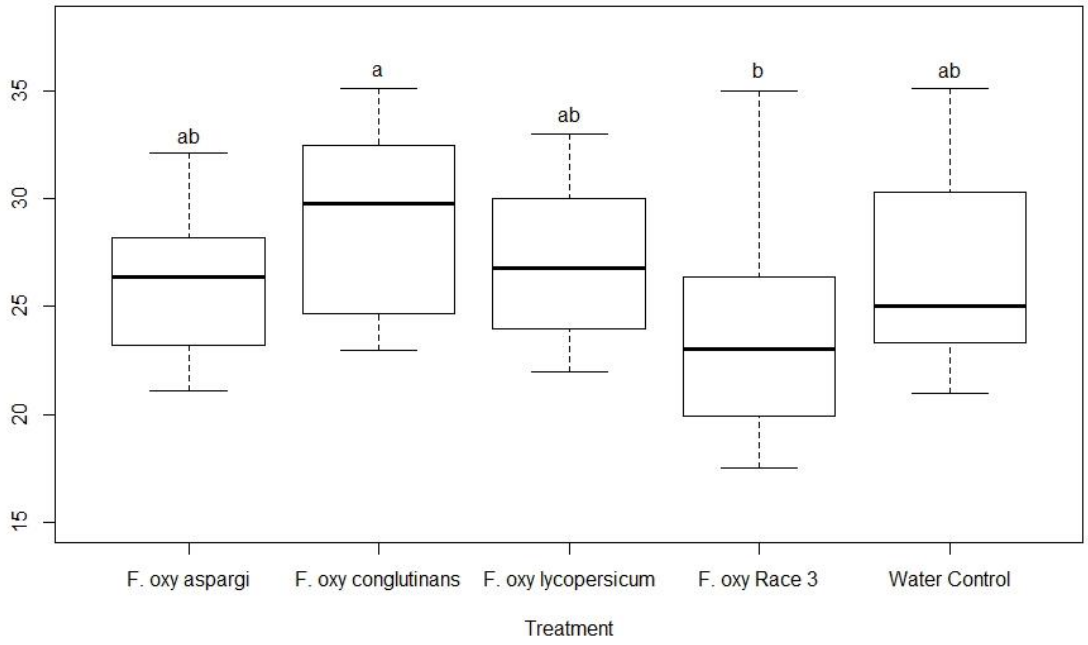

Figure 4. Effect of a root inoculation treatment with *F. oxysporum* strains on tomato root length (cm; means ± SE) compared to controls. Different letters refer to significant differences among treatments ($p < 0.001$, Tukey's HSD test, after one-way ANOVA).

The F. oxy Race 3 strain had the lowest plant growth parameter compared with other *F.oxysporum* strains. Hereinafter, F. oxy Race 3 was chosen for further co-inoculation experiment with EPF strains.

3.2. Co-inoculation of entomopathogenic fungi as endophyte towards plant pathogen

The effect of a co-inoculation of entomopathogenic fungal strains on suppressing the plant pathogen was analysed in the following experiments:

3.2.1. Co-inoculation assay of *F. oxysporum* Race 3 strain and entomopathogenic fungal strains in vitro

A significant inhibition of the F. oxy Race 3 growth when co-inoculated with the EPFs (ANOVA [$F_{(3, 16)} = 44.55$, $p < 0.001$]). Post hoc comparison indicated the mean value for Mb Cb15 (55.78 ± 7.76) and Bb Can (44.82 ± 5.97) was significantly different than Bb Col (11.00 ± 8.94) and Bb Que (11.57 ± 7.80; Fig. 5). Furthermore, Mb Cb15 and Bb Can was chosen for further co-inoculation experiment in planta.

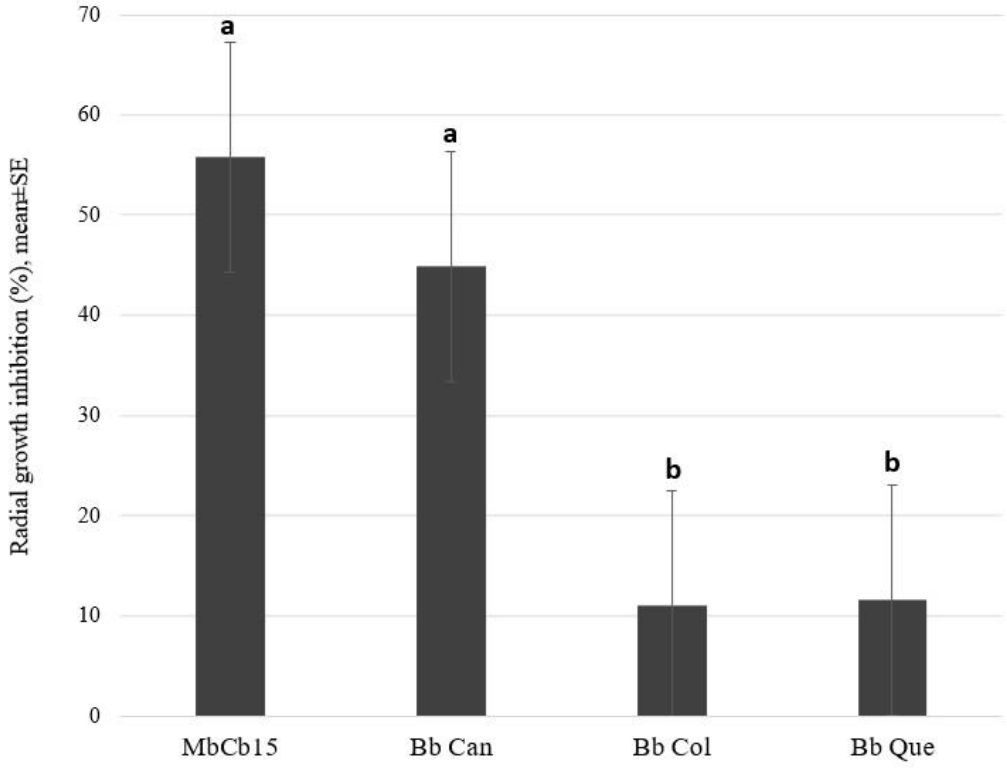

Figure 5. Radial growth inhibition (%; means ± SE) of *F. oxysporum* Race 3 when co-inoculated with entomopathogenic fungal strains. Different letters refer to significant differences among treatments ($p < 0.001$, Tukey's HSD test, after one-way ANOVA).

3.2.2. Molecular quantification of fungal DNA from co-inoculation experiments

Four weeks after fungal co-inoculation experiment, harvested stem and root were used to quantifying the fungal DNA of each treatment. Significant difference between treatment of *B. bassiana* inoculation (glm [$F_{(3, 76)} = 3.75$, $p < 0.01$]), *M. brunneum* inoculation (glm [$F_{(3, 76)} = 8.89$, $p < 0.001$]), and *F. oxysporum* inoculation (glm [$F_{(4, 95)} = 3.26$, $p < 0.01$]). In addition, there was no detection of any fungal DNA in the water control treatment (Table 2).

Table 2. Average fungal DNA isolated from co-inoculation treatments

No.	Treatment	*B. bassiana* (pg DNA)	*M. brunneum* (pg DNA)	*F. oxysporum* (pg DNA)
1	Bb Can+F. oxy Race3	2.90 ± 4.91 ab	-	0.00 ± 0.00 b
2	Mb Cb15+F. oxy Race3	-	0.31 ± 0.40 a	48.19 ± 19.06 a
3	Bb Can+Mb Cb15	1.81 ± 1.93 ab	0.02 ± 0.02 b	-
4	Bb Can+Mb Cb15+F. oxy Race3	7.61 ± 14.03 a	0.09 ± 0.15 b	0.00 ± 0.00 b
5	F. oxy Race3	-	-	0.32 ± 1.45 b
6	Water control	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b

Average pg DNA of analytical triplicates (pg DNA) of each fungal species. In a column, mean (± SE) followed by the same letter are not significantly different ($p < 0.001$, Tukey's HSD test, after generalized linear model)

The EPF combination of Bb Can+MbCb15 as well as Bb Can when co-inoculated with F.oxy Race 3 were able to inhibit the growth of the fungal pathogen. In contrast, Mb Cb15 co-inoculated with F. oxy Race 3 was not able to inhibit the fungal pathogen. The high amount of *F. oxysporum* fungal DNA when co-inoculated after Mb Cb15 was discovered only from 3 infected plants sites out of ten plants used, whereas other plants sites were occupied by Mb Cb15.

Fungal DNA quantification in different plant compartments

Significant pg DNA concentrations of *B. bassiana* (glm [$F_{(1, 78)} = 11.89$, $p<0.001$]) and *M. brunneum* (glm [$F_{(1, 78)} = 12.20$, $p < 0.001$]) were found in roots over all fungal co-inoculation treatment. The high concentration of. *F. oxysporum* DNA was found specify only in three localized infected site; yet, no significance between roots and stem (glm [$F_{(1, 98)} = 0.08$, $p = 0.77$]) (Figure 6).

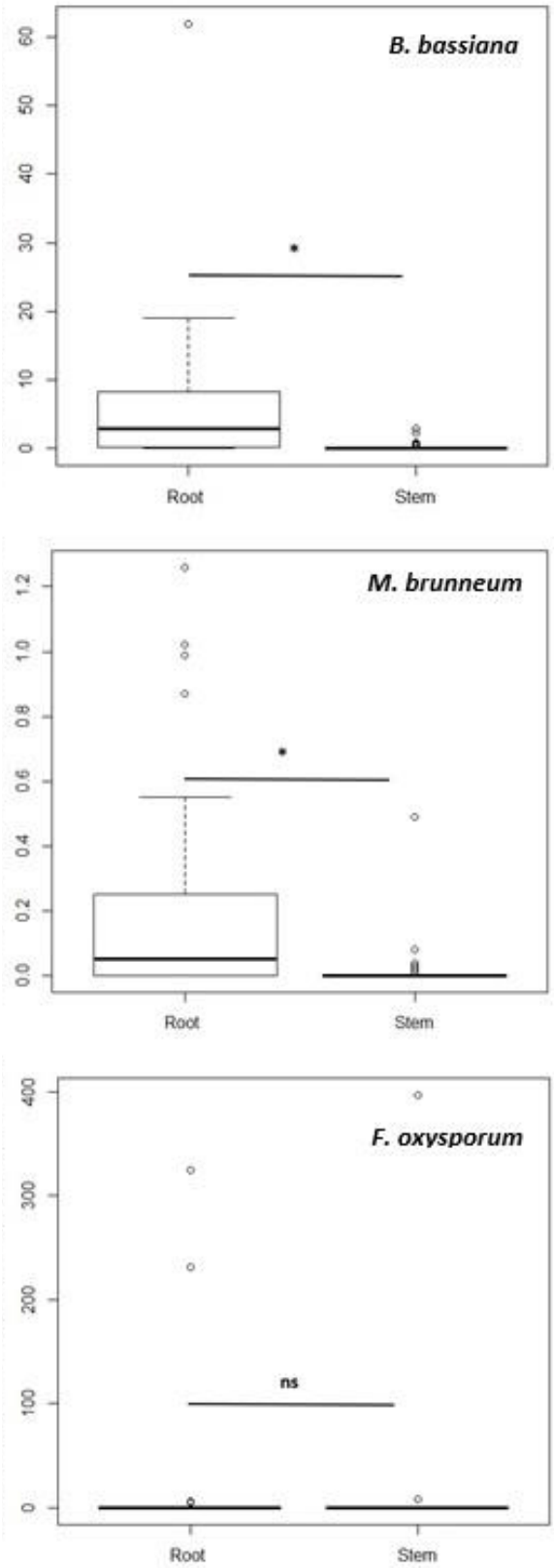

Figure 6. Average pg DNA of endophytic *B. bassiana*, *M. brunneum*, and *F. oxysporum* in plant compartments of all inoculation treatment. Asterisks (*) above bars indicate significant differences, (ns: not significant) (p<0.001, generalized linear model).

3.2.3. Effect of co-inoculated fungal DNA on plant growth parameter

There was a significant effect of the fungal co-inoculation treatment on plant growth parameter of plants height (ANOVA [$F_{(5, 54)} = 10.79$, $p<0.001$]), aboveground fresh biomass (ANOVA [$F_{(5, 54)} = 29.39$, $p<0.001$]) , and root length (ANOVA [$F_{(5, 54)} = 7.47$, $p<0.001$]) (Table 3).

Table 3. Plant growth parameters of tomato plants in co-inoculation treatments

No.	Treatment	Plant Height (cm)	Fresh Biomass (gram)	Root Length (cm)
1	Bb Can+F. oxy Race3	27.30 ± 3.38 a	28.29 ± 1.33 a	21.93 ± 3.55 a
2	Mb Cb15+F. oxy Race3	26.20 ± 4.20 a	27.77 ± 1.93 a	22.14 ± 3.73 a
3	Bb Can+Mb Cb15	25.40 ± 2.63 a	26.44 ± 3.14 a	21.66 ± 3.62 a
4	Bb Can+Mb Cb15+F. oxy Race3	25.25 ± 3.19 a	26.69 ± 3.40 a	20.21 ± 3.12 a
5	F. oxy Race3	17.65 ± 3.89 b	17.79 ± 1.41 b	14.52 ± 2.41 b
6	Water control	27.95 ± 4.00 a	27.04 ± 1.52 a	19.46 ± 3.33 a

Effect of fungal co-inoculation treatment on tomato plant growth parameter. In a column, mean (± SE) followed by the same letter are not significantly different ($p < 0.001$ of Tukey's HSD test, after one-way ANOVA.

4. Discussion

The high colonization rate of fungal plant pathogen strain F. oxy Race 3 occurred from stem and root confirmed their endophytic growth ability within tomato plant tissues (Amini and Sidovich, 2010), and suppress the plant growth parameter causing stunting on tomato plants (Cai et al., 2003). This present study confirmed the inhibition growth of F.oxy Race 3 when it challenged with between EPFs strain in same PDA plate. The ability of fungal endophyte to inhibition plant pathogen growth in in vitro assay worth consider as a potential antagonist biocontrol agent (Rocha et al., 2009; Garcia et al., 2012). In addition, the inhibition growth in culture media resulted from the production of inhibitory metabolites (antibiosis) of EPFs strain as antagonism interaction against plant pathogen (Ownley et al., 2010; Lozano-Tovar, 2013; 2017, Jaber, 2015;2018). Whether any of the EPF strains tested in the present study produces compounds with activity against F. oxy Race 3 is yet to be identified.

This study revealed the ability of EPF strain of Bb Can and combination of strain Bb Can+Mb Cb 15 to inhibit fungal pathogen of tomato plants. The ability of *B. bassiana* provide protection towards plant pathogens likely involve the competition for niche or

resources since this EPF strain was also plant colonizer (Ownley et al., 2010). The successful competition of EPF strain is likely due to earlier occupation of EPF strain within plant tissue confront the fungal plant pathogen as further intruder. Previous studies have compared different arrival inoculated time of microorganism effect to inhibit plants pathogen, revealed that prior inoculation of fungal endophytes strains strongly inhibit plant pathogen (Lee et al., 2009; Shittu et al., 2009; Adame-Ālvarez et al., 2014). A report from Silva et al. (2012) confirmed that inoculation of antagonist bacterial strains at 24 or 72 hours prior being challenged with the fungal pathogen, were significantly reduced coffee leaf rust severity (*Hemileia vastatrix*).

Our studies using root inoculation method have been tracing EPF DNA significantly in roots tissues. The colonization rate of *B. bassiana* was likely higher in roots and stem rather than leaves, when it inoculated via root drenching (Parsa et al., 2013). Establishment of *B. bassiana* was reported in banana root with root immersion inoculation method (Akello et al, 2007). Metarhizium species are more often reported as endophytes of roots and not the aboveground parts (Behie et al., 2015; Murphy et al., 2015). Accordance with those studies, fungal application were localized recovered from the plants part that was in direct contact with the fungal inoculum, and less likely or not at all in plant parts distant to the inoculation site (Akello et al., 2007; Tefera and Vidal, 2009). This found indicating that root as the entry point of inoculation play important role of EEF establishment, enhanced their ability to occupied niche of inoculation site, thus, be able to suppress fungal plant pathogen.

Beside the ability to inhibit plant pathogen, other additional ability of EPF to promote plant growth were confirmed in this study, our finding reported that tomato plants inoculated with EPF strains Bb Can, Mb Cb15 and combination of both fungal strains were able to enhanced plant height, aboveground fresh biomass, and root length compared with plants inoculated with the fungal plant pathogen. In line with a result of Jaber (2018), *B. bassiana* (NATURALIS) and *M brunneum* (BIPESCO5) can promote the growth of wheat following their endophytic establishment within plants through seed treatment.

Hereinafter, we highlighted the ability of EPFs strains to inhibit plant pathogen and the importance of fungal endophyte inoculated prior plant pathogen to facilitated niche establishment of EPF within host plant. Application of EPF as endophyte for biocontrol purposes need to be considered as precaution before plant pathogen invading crop plants.

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Chapter 3

Endophytic entomopathogenic fungi alter plant volatile profiles and influence the interaction of greenhouse whiteflies (*Trialeurodes vaporariorum)* and their parasitoid *Encarsia formosa*

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Abstract

Endophytic entomopathogenic fungi (EEFs) colonization of host plants can be regarded as bodyguards of through indirect interactions with insect herbivores. Volatile profiles emitted by plants upon feeding damage play an important role in mediating indirect multitrophic interaction among plant-insect herbivore and parasitoids. Experiments were conducted by establishing single and combination inoculations of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium brunneum* strains, followed by release of greenhouse whiteflies (*Trialeurodes vaporariorum*) or their parasitoid (*Encarsia formosa*). Behavioural responses of both organisms were tested using a six-arm olfactometer and whole inoculated plants. Real Time PCR was conducted to quantify the presence of each inoculated fungus within tomato plant tissues. GC-MS analyses were performed to analyse plants volatile profiles. Based on analytical, molecular and behavioral assay we demonstrate the successful EEF colonization of tomato plant tissues, and it affected plant volatile compound profiles qualitatively and influenced insect's response. The most abundant compound in all treatments was β-Phellandrene. Tetradecanal was the plant volatile compound that was correlated with the responses of the whiteflies and the parasitoid. VOCs emitted from plants inoculated with a combination of the two strains tested were less preferred by

greenhouse whiteflies and *E. formosa* parasitoid in the olfactometer tests. No effect of EPF inoculation treatment to parasitization rate of *E. formosa*. The results demonstrate for the first time that multitrophic interactions between herbivores and their natural enemy likelihood mediated by EPFs inoculated belowground, and an insect herbivore and its parasitoid interacting aboveground facilitated by host plants through indirect interaction of emitted VOCs.

Keywords: *Beauveria bassiana*, Endophyte, *Metarhizium brunneum*, Multitrophic interaction, Parasitoid, VOCs, Whiteflies.

1. Introduction

Plants harbor a complex microorganisms and insects, with interactions ranging from beneficial mutualism with natural enemies of herbivores and plant growth-promoting rhizobacteria or endophytes, to detrimental antagonism with insect herbivores and plant pathogens. To overcome antagonists and optimize mutualistic interactions, plants have developed specific mechanism to recognize interaction with another organisms. Plants release volatile secondary metabolites into the environment as a response to feeding damage or oviposition by herbivores (Dicke et al., 2003; Mumm et al., 2003), aiming to attract natural enemies which help to defend plants indirectly.

The theory of insect-plant interactions did not progress completely by not taking into account the third trophic level, which comprise parasitoids, and predators, but also microbes. Price et al. (1980) were the first proposing a framework for these multi-layer interactions. Natural enemies of insect herbivores (e.g. parasitoids or predators) utilize herbivore-induced plant volatile (HIPVs) emitted from plants to localize their herbivore hosts or prey. Turlings et al. (1990) were the first to demonstrate the important role these HIPVs play under field conditions. Since this benchmark paper for insects-plants odor cues, many studies on multitrophic interactions were carried out. An additional aspect of these interactions influencing plant volatile profiles are considered only recently. Endophytes, organisms living inside plant tissues without causing any visible symptoms, are now also important players in interactions between host plants and other organisms.

One important group of these endophytes comprise entomopathogenic fungi (EPFs). When growing inside plant tissues they have been shown to promote plant growth,

improve resistance to environment stress, and form mutualistic associations with the rhizosphere (Vega et al., 2009). Various studies revealed the effectiveness of EPFs as endophyte to reducing damage and suppressing growth of insect herbivores and plant pathogens (Bing and Lewis, 1991; Ownley et al., 2008, 2010; Akello et al., 2008; Vidal and Jaber, 2015), based on purported plant protection mechanisms such as induced systemic resistance, production of feeding deterrent or antibiotic compounds of fungal secondary metabolites.

Tritrophic interactions of microorganism-plant-insect herbivores were reported by the establishment of EPFs endophytes altering plant volatile profiles and host preferences of insect herbivores (Zhang, 2014; Rodríguez, 2016). Moreover, volatile profiles emitted from tomato plant inoculated by a root endophyte fungus (*Acremonium strictum*) were able to systemically influence the host selection of *Helicoverpa armigera* (Jallow et al., 2008). Menjivar et al. (2012) reported tritrophic interactions when inoculating the endophyte *Trichoderma atroviride* strain MT-20 and S-2 as well as endophyte *Fusarium oxysporum* strain 162, reducing the number of greenhouse whiteflies (GHW) attracted to inoculated tomato plants. In line with endophyte volatile profile changes, aboveground aerial endophyte establishment affected the root volatile emissions and influenced belowground insect responses (Rostás et al., 2014).

However, the specificity of indirect interaction of tomato plants, colonized by EPFs and their emitted volatile profiles towards the response of the greenhouse whiteflies (GHW), *Trialeurodes vaporariorum*, and their natural enemy parasitoid *Encarsia formosa* remains elusive. Therefore, this study aimed at assessing the ability of entomopathogenic fungi either applied as single and combination inoculations i) to endophytically colonize tomato plant tissues, ii) to identify changes in volatile bouquets emitted using headspace volatile profiles of inoculated tomato plants, and iii) to investigate behavioral responses of GHW and *E. formosa* exposed to these inoculated plants We hypothesis that EPF inoculations with *B. bassiana* and *M. brunneum* would alter headspace volatile profiles of tomato plants and would translate into different responses in higher trophic levels.

2. Materials and Methods

2.1. Plant material

Tomato plants *Solanum lycopersicum* L cultivar Moneymaker (Rein Saat®, Austria) was used for EPF inoculation. Each seed was grown in a multi tray with a mixture of soil (Fruhstorfer Erde Typ T, Hawita Gruppe GmbH, Vechta, Germany) and non-sterile 0,3mm sand (3:1). Tomato seedling at the two-leaf stage were used in this study. Seedlings were removed from the substrate, and the roots were carefully washed with tap water prior to fungal inoculation. Seedlings inoculated with the spore suspension described at (2.3. Fungal inoculation), were individually transplanted into plastic pots (diameter 11cm) using the same soil mixture as described above. The plants were maintained under greenhouse conditions (21±2°C, 70-80% RH and 12h photoperiod) and irrigated regularly for a growing period of three weeks post inoculation.

2.2. Fungal material

Two strains of entomopathogenic fungi, *Beauveria bassiana* Bb 1022 (referred to as Bb Can) and *Metarhizium brunneum* Cb15 III (referred to as Mb Cb15) were used for the inoculation treatments of endophytic establishment. Strains were obtained from the culture collection of Agriculture Entomology working group, Department of Crop Sciences, Faculty of Agriculture, Georg-August University Göttingen, Germany. Strains were grown in potato dextrose agar (PDA) plates at 24 ±2°C in dark conditions for two weeks to obtain enough spores for the suspension. Spores suspension production was carried out under sterile bench (Thermo Fisher Scientific), it started by adding 5ml of Tween 20 (0.1% v/v, DifcoTM) into two-week-old cultures of each strain, followed by gently scraping off the culture surface with sterile microscope slide glass. These spores were then suspended in 10ml sterile distilled water. Spore concentrations were adjusted to $1x10^6$ spores/ml with a Thoma Counting Chamber (Marienfeld, Germany). For all fungal strains used, a germination test was conducted. Therefore, 100µl of the spore suspension was spread on PDA agar and incubated at 24 ±2°C for 3 days. Furthermore, spore germination was checked under the microscope and average germination exceeded 90% for each strain.

2.3. Fungal inoculation

Fungal inoculation was performed by immersing the roots of the tomato seedlings into a 100 ml of $1x10^6$ spores/ml suspension of each strains of the entomopathogenic fungi for 20 minutes as a single strain inoculation. Mixture inoculations of Bb Can and Mb Cb15 were set up by immersing root of tomato seedlings into a 100ml of $2x10^6$ spores/ml suspension, containing spores of both strains (ratio 1:1) for 20 minutes. As a control treatment, the same procedure was conducted with distilled water instead of a spore suspension. Inoculated seedlings were individually transplanted into plastic pots (diameter 11cm) containing mixture of soil (Fruhstorfer Erde Typ T, Hawita Gruppe GmbH, Vechta, Germany) and non-sterile 0,3mm sand (3:1). The plants were maintained under the greenhouse conditions (21±2°C, 70-80% RH and 12h photoperiod), and irrigated regularly for a growing period of three weeks post inoculation.

2.4. Insect behavioral response analyses with a six-arm Olfactometer

These experiments were performed in a six-arm olfactometer system (see details in Turlings et al., 2004), with some modifications to optimize the insect movement. In this study we modified the bottom shelf by using the EPF inoculated plants enclosed within oven bags (Toppits ®. Minden, Germany). To accomodate the air pressure flow, the above part (air outlet) and below part (air inlet) of each oven bag was perforated to fit in with a 6mm rubber teflon air supply tube. Filtered (activated charcoal filter, 400cc, Alltech, Deerfield, Illinois, U.S.A.) and humidified air originating from a central in-house compressor was pushed into each vessel at a rate of 1.00 L/min (for GHW response) and at a rate of 0.5 L/min (for *E. formosa* response). With a vacuum pump (ME2, Vacuubrand,Wertheim, Germany) 0.5 L/min (for GHW response) and 0.25 L/min (for *E. formosa* response) of air was pulled through the trapping filter to collect the VOCs.

Figure 1. Six-arm Olfactometer system with modified at bottom shelf.

Greenhouse whiteflies (*Trialeurodes vaporariorum*) response assay

A GHW colony was maintained at the insect rearing collection of Agriculture Entomology working group. GHW were synchronized by establishing new colonies 1 month before being used by releasing 10 pairs of GHW adults into a rearing mesh cage with 2 weeks tomato plants cv. Moneymaker for 48 hours. Thereafter, the adults were removed from the rearing cage and the new colonies were maintained until use for the experiments. For the GHW response experiment, 20 adult GHW females were released in the middle chamber of the olfactometer system, replicated in four time series each 45 minutes. The response of GHW towards plant inoculated treatments were recorded using the number of GHW visiting each arm at each time series. The experimental design included two endophyte-free plants treated only with water as control (Water Control), three endophyte treatments Bb Can, Mb Cb15, Bb Can+Mb Cb15, and one enclosed oven bag without plants (empty). The experiment was performed as a randomized design with six replications.

Parasitoid (*Encarsia formosa*) response assay

Two-week post inoculation, each inoculated plant was exposed to two pairs of GHW within a mesh clip cage for 24 hours for egg laying. Hatching of larvae was observed on the underside leaf at 5 to 7 days post infestation. 20 GHW larval stages (L3) per plant were used for the further behavioral assay of *E. formosa*. Three weeks post fungal inoculation plants with larvae were enclosed in an oven bag (see above). The experimental design included two endophyte-free plants treated only with water as control (Water Control), three endophyte treatments, Bb Can, Mb Cb15, Bb Can+Mb Cb15, and one enclosed oven bag without plant (empty arm).

For the response assay, *E. formosa* was purchased from a commercial company (Katz Biotech AG, Germany). 5 strips of packaging were placed inside a 20 x 15cm plastic box with an aeration lid and stored at 24 ±2°C. Emerging parasitoids were supplied with honey water. Emerging females were separated daily from males before the experiment. 20 female *E. formosa* were released in the middle chamber of the olfactometer system, replicated four times with 45 minutes each. The response of *E. formosa* towards plant inoculated treatments were recorded using the number of *E. formosa* visiting each arm

at each time series. The experiment was performed as a randomized design with five replications. Immediately after the behavioral assay was performed, plants were removed from the oven bag and placed inside a cylindric mesh cage, and the 20 GHW larval were exposed to two pairs of *E. formosa* for 24 hours to assess the parasitization rate.

2.5. Plant volatile extraction and analysis

Trapping filters were attached into the above part of the oven bags, consisting of a 7 cm glass tube containing 30mg of 80–100mesh Super-Q Adsorbent (Altech, Deerfield, Illinois, USA). Before each experiment, devices were cleaned by rinsing with 1ml Dichloromethane. Furthermore, the aboveground volatile organic compounds (VOCs) of each plant treatment were collected in a total period of 4 hours for each experiment. Immediately at the end of the experiment, each volatile collected from each plant treatment trapped in the Super-Q filter were extracted with 150µl Dichloromethane. 10µl of an internal standard (Tetraline 20ng/µl) were added into each volatile sample. An aliquot of 2µl of each sample was analyzed by gas chromatography/mass spectrometry (Agilent GC7890B, MS5977B) with non-polar HP-5ms ultra inert column (30m length x 0.25mm internal diameter, 0.25µm thickness). Samples were injected in pulsed splitless mode, back inlet temperature was 220°C. The oven was held at initial 40°C (3 min), programmed at 8°C (1 min) to 320°C, final temperature was held for 8 min. Helium (2 ml/min) was used as carrier gas. The GC-MS chromatograms were analysed with Automated Mass Spectral Deconvolution and Identification System (AMDIS) given the integrated signal (IS) matrix. The peak area of each sample was calculated by integrated signal (IS), given the results of identified compound proportion within treatment:

$$\text{Identified compound proportion } (\%) = \frac{\text{Integrated signal of each compound}}{\text{Total integrated signal per treatment}} \text{x100}$$

Evaluation of the retention index (RI) was performed to determine compound identities from plant volatile as compared to standard n-alkanes. The RI of each compound was calculated based on the formula developed by van den Dool and Kratz (1963) :

$$Retention\ Index\ (RI) = 100\left[\frac{(tR - tRz)}{(tR(z+1) - tRz)} + z\right]$$

tR = retention time; tRz = retention time of previous standard n-alkanes; tR(z+1) = retention time of later standard n-alkanes; z = number of standard n-alkanes C-atoms.

Tentative identification of the compounds was based on RI matching of the mass spectra with NIST and Wiley mass spectrum library (version W11N17main.L), specified by van den Dool and Kratz-non polar-DB5 column. Regardless the quantity of each VOCs, our studies focused specifically on compound identities. The compounds identified in this study were based on several parameters: (1) retention time between 10 until 25 minutes, (2) quality of the retention index confirmed by probability of the NIST library match higher than 80%, and (3) presence in all the replicates of at least one treatment based on integrated signal (IS) of AMDIS.

2.6. Harvest and surface sterilization of plant material

Above and below ground plant material of treatments were harvested after the behavioral assays. Plant height of the experiment was recorded priorly, by measure the height (cm) from the growth point of cotyledon leaves up to the new emerge leaves. At this point, the measurement of fresh biomass weight (gram) of aboveground plants was carried out with a scale. Afterwards, stem segments (2 cm sections measured from the cotyledon growth point) were cut with a sterile scalpel and placed separately into a Falcon tube (Sarstedt AG&Co.KG) for further surface sterilization. Furthermore, belowground parts were gently removed from the pots and the roots were washed off under tap water to remove the rhizosphere soil, placed in sterile paper towels to remove tap water residues followed by placing the roots separately into Falcon tubes for further surface sterilization.

Stem samples were surface-sterilized by serial washing in 70% ethanol for 1 minute, 2% sodium hypochlorite for 30 second and 70% ethanol for 1 minute, followed by two times immersion in sterile distilled water for 30 second and once in sterile diethylpyrocarbonate (DEPC)-treated water (modified from Andreote et al., 2010). Surface sterilization of roots was performed according to Li et al. (2010), with slight modifications. In this study, 2% sodium hypochlorite and sterile DEPC-treated water were used for the last step. To confirm the success of the disinfection procedure, aliquots of the DEPC water used in the final wash step were plated on PDA plates. The plates were incubated in the dark at 24 ±2°C for at least 1 week. No growth of

microorganisms was observed. Surface-sterilized plants samples were freeze drying (Zirbus VaCo 5, Zirbus Technology GmbH Germany) for 72 hours. Freeze dried plant samples were finely ground in a mortar grinder (Mixer Mill MM 400, Retsch GmbH Germany) obtained powder samples, then stored at −20°C until DNA extraction.

2.7. Molecular detection of inoculated fungal DNA

DNA extraction of inoculated plant was performed as follows: a total of 20mg sample powder of stems or roots was extracted employing the peqGOLD Plant DNA Mini kit (Peqlab, Erlangen, Germany) according to the manufacturer's instructions with two modifications as described previously (Wemheuer et al., 2016). DNA concentrations of extraction products were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies,Wilmington, DE, USA) for DNA dilution purpose. In total, extracted DNA of 110 samples was subjected for endophytism confirmation by real-time polymerase chain reaction (RT-PCR). To quantify each fungal DNA extraction product, one standard curve was needed (Brandfass and Karlovsky, 2008). Standard curve dilution series of *B. bassiana* and *M. brunneum* fungal DNA were prepared as one third from the highest 100 pg, 33.33 pg, 11.11 pg, 3.70 pg, 1.23 pg, 0.41 pg, 0.13 pg, 0.045 pg, 0.015 pg, and the lowest 0.0005 pg. Fungal DNA for the standard curve was obtained from stock available at Agriculture Entomology working group. Each RT-PCR plate was analyzed separately with a species-specific primer, containing 1:10 dilutions of the DNA plant samples, and negative control from master mix with analytical triplicates of each RT-PCR running.

Species-specific primers of *B. bassiana* used was Bsn1-2-forward "GCGTCAAGGTGCTCGAAGACAG" and Bsn1-2-reverse "TCTGGGCGGCATCCCTATTGT" (Zhang, 2014). Species-specific primers of *M. brunneum* used was Ma-1763-forward "CCAACTCCCAACCCCTGTGAAT" and Ma-2097-reverse "AAAACCAGCCTCGCCGAT" (Schneider et al., 2012) as performed previously to quantifying *M. brunneum* strain ART 2825 (Rodríguez, 2016).

Cycling conditions for both species-specific primers consisted of 2 min initial denaturation at 95°C, followed by 39 cycles of 10 sec denaturation at 95°C, 15 sec annealing at 65°C and 15 sec extension at 72°C and the plate read at the end of each cycle. Followed by a final extension for 1 min at 72ºC, the amplification of melting curve analyses was performed ranging from 95°C to 55°C held for 30 sec each.

The RT-PCR was performed with reaction mixture containing 5µl 2× qPCRBIO SyGreen Master Mix Kit (PCR Biosystems Ltd., England), 0.2µl, 10µM of each primer, 3.6µl of sterile water and 1µl of sample DNA, resulting in total reaction volumes of 10µl. RT-PCR was performed in an iCycler System CFX384 Real time system (Bio-Rad, Hercules, CA, USA).

2.8. Data analysis

Data were analyzed with R version 3.6.3 for statistical computing (R Core Team, 2020). Fungal pg DNA quantification obtained from pooled data of stem and roots to determine successful endophytic colonization, as well as proportion of identified VOCs of each treatment were analyzed by a generalized linear model (glm) with binomial distribution, since priorly Shapiro-Wilk test assumed the data were not normally distributed. Significance between treatments were performed with post hoc tests using Tukey's Honest Significant Difference (HSD). Two-way ANOVA with treatment and identified VOCs proportion as main effects was performed for comparison of VOCs proportions between treatments.

Examination of GHW and parasitoid behavioral responses towards blends of VOCs offered in the six-arm olfactometer were analyzed by a generalized linear mixed model (glmm), treating the insect response to the EPF inoculation treatment as a fixed effect and day of experiment as well as time series as a random effect. The glmm with poisson distribution was used at it was likely that the number of insect responses occurring at a fixed amount of time was following this distribution. The estimation method was maximum likelihood with Laplace approximation. The model was evaluated with "glmer" function from "lme4" R-package (Bates et al., 2015).

Plant volatile data obtained from integrated signal (IS) given from AMDIS analyses was generated by using the MetaboAnalyst online platform (Chong et al., 2019) available at (https://www.metaboanalyst.ca/). It was used to visualize the effect of EPF inoculation treatment towards identified plant volatile compounds by generating a principal component analysis (PCA) to cluster the treatment factor. As well as to generate a heat maps to visualize the up-regulating or down-regulating of plant volatile compounds between treatments. Correlation of identified VOCs towards whiteflies responses were analysed by using the Pearson correlation coefficient.

3. Results

3.1. *T. vaporariorum* response assay to EPF inoculations

The effect of EPF strains inoculation alter plant volatile profiles and influence the interaction of greenhouse whiteflies (*T. vaporariorum)* was analyzed in the following experiments:

3.1.1. Endophyte colonization in planta

Fungal DNA quantification of inoculated plants was performed to confirm the endophytically colonization of EPF in single and combination treatments. We did not detect any fungal DNA on water control treated plants. However, a significant amount of fungal DNA was found in either single or combination treatments of *B. bassiana* glm $p<0.05$ [$F_{(2, 33)} = 3.09$, $p = 0.05$], and *M. brunneum* glm $p<0.05$ [$F_{(2, 33)} = 7.45$, $p = 0.02$], indicating successful colonization in planta (Table 1).

Table 1. Average fungal DNA isolated from inoculation treatment of greenhouse whiteflies experiment

No.	Treatment	*B. bassiana* (pg DNA)	*M. brunneum* (pg DNA)
1	Bb Can	1.03 ± 1.07 a	-
2	Mb Cb15	-	1.44 ± 1.59 a
3	Bb Can + Mb Cb15	1.35 ± 2.16 a	0.37 ± 0.37 b
4	Water control	0.00 ± 0.00 b	0.00 ± 0.00 b

Average pg DNA of each fungal species. In a column, mean (± SE) followed by the same letter are not significantly different at $p<0.05$, Tukey's HSD test, after generalized linear model.

3.1.2. Behavioral response of GHW towards EPF inoculation

The likelihood ratio test to evaluate the effect of EPF inoculation on GHW response response was significant different between EPF treatment (poisson glmm X^2 [4, N = 120] = 62.33, $p<0.001$). GHW significantly preferred to visit plants inoculated by Mb Cb15 (3.16±1.04) or Bb Can (2.75±0.84), compared to the combination of both strains (1.50±0.97), the water control (1.29±0.90), and the empty arm (0.58±0.71) (Figure 2).

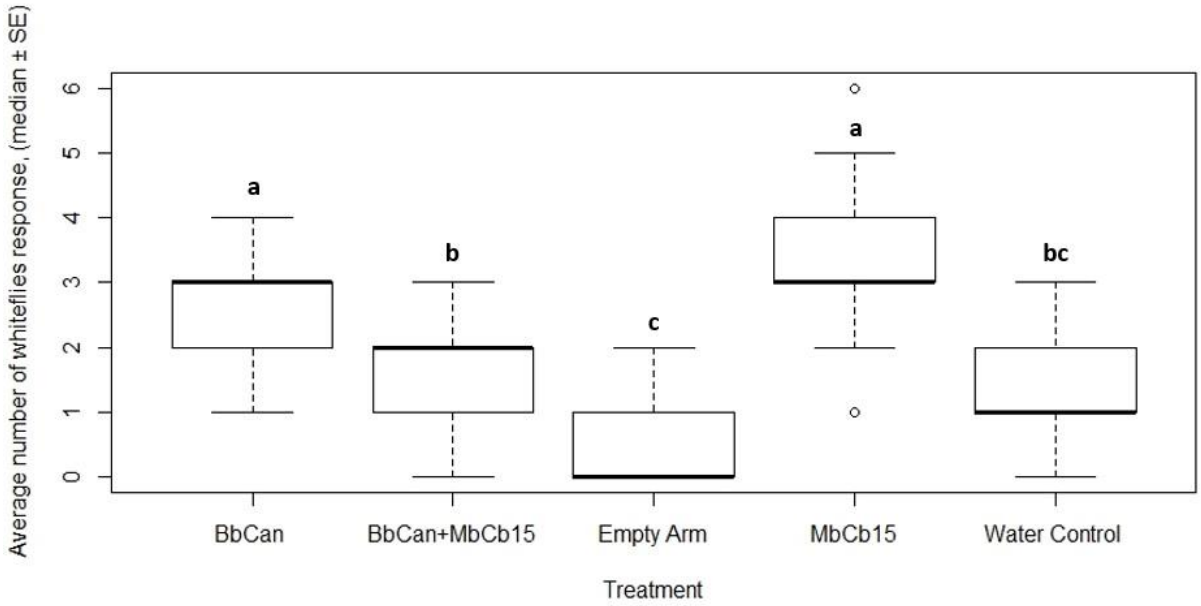

Figure 2. Average number of GHW responding to the treatments offered in six-arm olfactometer experiments. Different letters refer to median (± SE) significantly different among treatments (p<0.001, Tukey's HSD test, after generalized linear mixed model).

3.1.3. Influence of EPF inoculation on plant volatile profiles

The evaluation of the retention index (RI) identified 16 main plant volatile compounds collected from tomato plants in the GHW experiment (Table 2). Beta-Phellandrene was the most abundant volatile in all EPF inoculation treatment (glm p<0.01 [$F_{(63, 320)}$ = 45.42, p<0.001]). We found no significant effect of the EPF inoculation treatments on the proportion of each compound (Two-way ANOVA p<0.05 [$F_{(3, 15)}$ = 0.30, p = 0.82]).

Table 2. Proportions (%) of the 16 most abundant plant volatile compounds in tomato plants inoculated with different EPF compared to the water control of GHW behavioral experiment

No.	VOCs	Bb Can	Mb Cb 15	Bb Can+Mb Cb 15	Water Control
1	Beta-Phellandrene	28.11 ± 5.40 A, a	29.57 ± 5.80 A, a	25.45 ± 3.71 A, a	27.30 ± 8.94 A, a
2	p-Cymene	6.84 ± 5.01 A, b	5.65 ± 2.86 A, b	7.99 ± 3.93 A, b	7.29 ± 5.17 A, b
3	Caryophyllene	6.96 ± 4.63 A, b	5.72 ± 2.46 A, b	7.85 ± 3.29 A, b	5.91 ± 6.25 A, b
4	Dodecanal	4.25 ± 4.63 A, c	5.12 ± 2.64 A, c	4.62 ± 5.97 A, c	2.02 ± 3.11 A, c
5	Humulene	1.20 ± 1.08 A, d	0.80 ± 0.40 A, d	1.39 ± 0.75 A, d	1.26 ± 1.88 A, d
6	Tetradecanal	0.36 ± 0.37 A, d	0.61 ± 0.41 A, d	0.83 ± 1.40 A, d	0.13 ± 0.22 A, d
7	Dill ether	0.31 ± 0.29 A, d	0.34 ± 0.30 A, d	0.47 ± 0.22 A, d	0.38 ± 0.47 A, d
8	Galaxolide	0.37 ± 0.54 A, d	0.06 ± 0.05 A, d	0.72 ± 1.04 A, d	0.17 ± 0.15 A, d
9	1,3,5-Trimethyl-2-cyclopentylbenzene	0.25 ± 0.20 A, d	0.26 ± 0.12 A, d	0.30 ± 0.10 A, d	0.13 ± 0.09 A, d
10	Isoascaridol	0.08 ± 0.12 A, d	0.06 ± 0.07 A, d	0.10 ± 0.03 A, d	0.11 ± 0.11 A, d
11	Benzene, 1-ethyl-2,4,5-trimethyl-	0.07 ± 0.09 A, d	0.07 ± 0.09 A, d	0.11 ± 0.02 A, d	0.02 ± 0.04 A, d
12	Alpha-Copaene	0.04 ± 0.04 A, d	0.02 ± 0.03 A, d	0.05 ± 0.05 A, d	0.00 ± 0.00 A, d
13	Benzene, 1-(1-ethylpropyl)-4-methyl-	0.03 ± 0.07 A, d	0.03 ± 0.07 A, d	0.00 ± 0.00 A, d	0.02 ± 0.06 A, d
14	Benzene, 1-(1,1-dimethylethyl)-3-methyl-	0.04 ± 0.10 A, d	0.02 ± 0.06 A, d	0.00 ± 0.00 A, d	0.008 ± 0.02 A, d
15	Beta-Ocimene	0.003 ± 0.008 A, d	0.003 ± 0.008 A, d	0.02 ± 0.019 A, d	0.01 ± 0.02 A, d
16	3-Carene	0.005 ± 0.005 A, d	0.003 ±0.005 A, d	0.01 ± 0.00 A, d	0.006 ±0.005 A, d

Proportion of each plant volatile compounds (means ± SE) followed by the same uppercase letter within row are not significantly different at $p<0.05$, Two-way ANOVA. In a column, means (±SE) followed by different lowercase letter are significantly different at $p<0.01$, Tukey's HSD test, after generalized linear model.

The data variability of principal component analysis (component 1 and 2) coefficients (Table 3) and scores plot of plant volatile compounds from GHW experiment, visualized the cluster different of EPF and water inoculation to empty arm of the olfactometer (Figure 3).

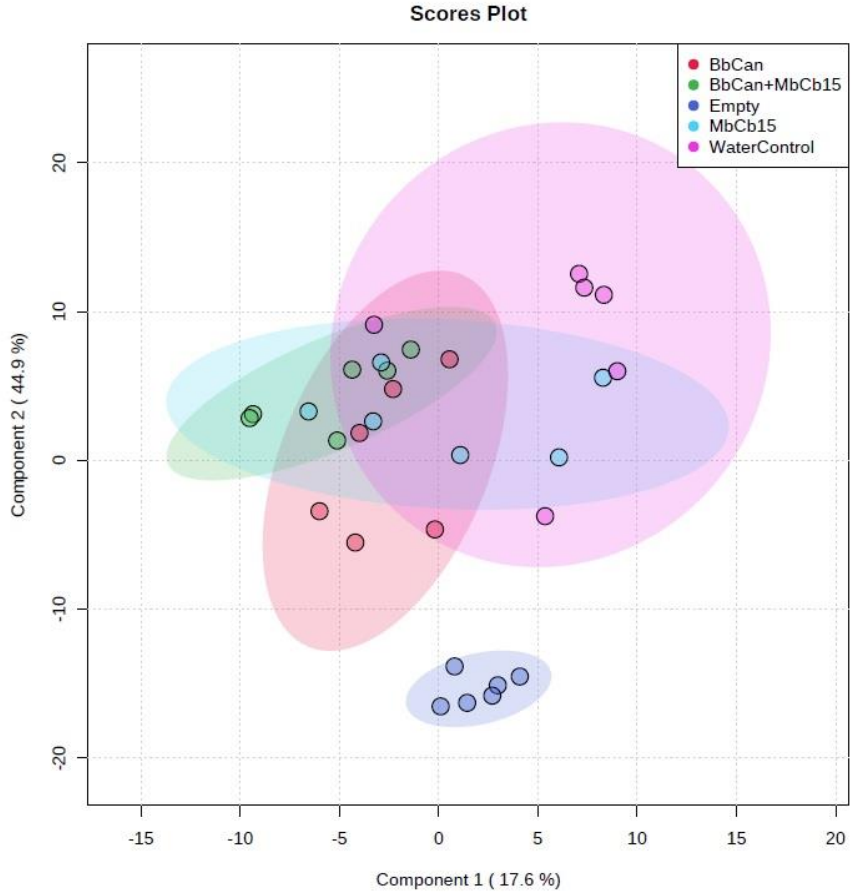

Figure 3. Scores Plot of principal component analysis (PCA) of plant volatile compounds emitted by EPF inoculation treatment from GHW experiment. Different colors cluster refer to inoculation treatment, and each dot represents treatment replication.

Table 3. Principal component analysis coefficients of plant volatile compounds emitted from EPF inoculation treatment of GHW experiments.

No.	VOCs	Compononet 1	Compononet 2
1	Beta-Phellandrene	0,09	0,10
2	p-Cymene	0,16	0,26
3	Caryophyllene	0,34	0,41
4	Dodecanal	0,45	0,42
5	Humulene	0,33	0,73

6	Tetradecanal	1,30	1,23
7	Dill-ether	0,94	0,95
8	Galaxolide	0,99	1,00
9	1,3,5-Trimethyl-2-cyclopentylbenzene	0,41	0,39
10	Isoascaridol	0,51	0,70
11	Benzene,1-ethyl-2,4,5-trimethyl	2,31	2,17
12	Alpha-Copaene	2,20	2,09
13	Benzene, 1-1-ethylpropyl-4-methyl-	0,86	1,10
14	Benzene, 1-1,1-dimethylethyl-3-methyl-	0,10	0,22
15	Beta-Ocimene	0,71	0,70
16	3-Carene	0,37	0,36

Principal component coefficient was performed based on correlation matrix between integrated signal (IS) of plant volatile compounds appears in each EPF treatment.

Graphical representation of up-regulate or down-regulate of each plant volatile compounds from each inoculation treatment was generated with a heat map (Figure 4). Isoascaridol, β-Ocimene, p-Cymene, 3-Carene, 1,3,5-Trimethyl-2-cyclopentylbenzene, Caryophyllene and Dill-ether were highly up-regulated in EPF combination (Bb Can+Mb Cb 15) inoculation treatment compared with single EPF inoculation treatment and water control. Plants inoculated with single EPF Mb Cb15 up-regulate Benzene, 1-1,1-dimethylethyl-3-methyl-, Benzene, 1-1-ethylpropyl-4-methyl-, Dodecanal, Tetradecanal, Humulene and 1,3,5-Trimethyl-2-cyclopentylbenzene. Plants inoculated with Bb Can up-regulate Benzene, 1-1,1-dimethylethyl-3-methyl-, Benzene, 1-1-ethylpropyl-4-methyl-,Galaxolide, Dodecanal, Alpha-Copaene, Beta-Phellandrene, and Benzene,1-ethyl-2,4,5-trimethyl. In contrast, none of plant volatile compounds were up-regulate by water control treatment.

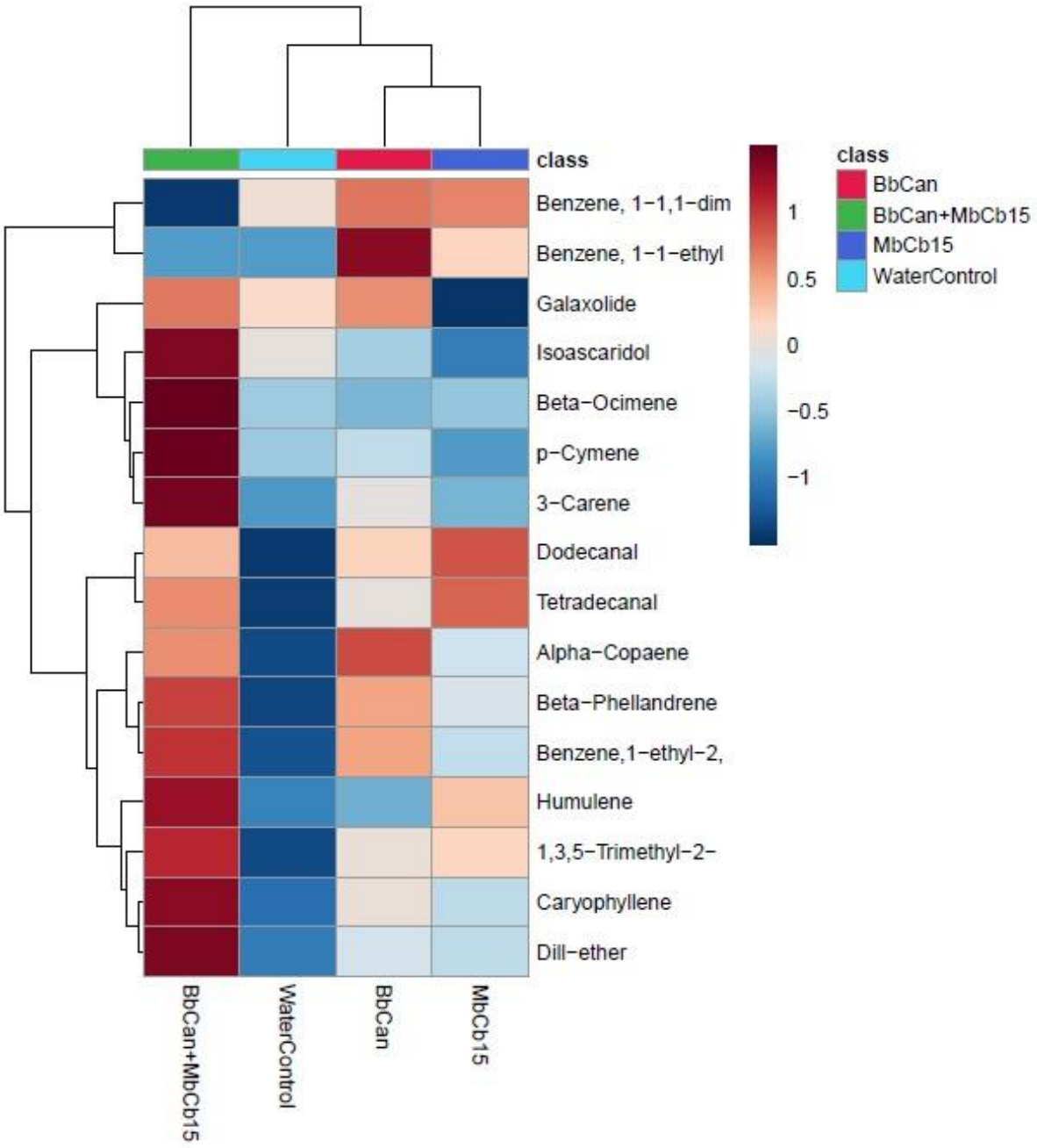

Figure 4. Up-regulating (red to reddish) or down-regulating (dark to light blue) heat map of identified plant volatile compounds affected by EPF inoculation treatment of GHW experiment.

3.1.4. Plant volatile correlated with GHW response

Pearson coefficient analysis was performed to examine the correlation between emitted plant volatile compounds to GHW response (Table 4). Tetradecanal, Dodecanal, α-Copaene and Benzene groups were correlated with GHW responses (Figure 6).

Table 4. Plant volatile compounds correlated with GHW response

No.	VOCs	r	*p*-value	FDR
1	Beta-Phellandrene	-0.030152	0.88877	0.94432
2	p-Cymene	-0.14125	0.51032	0.86755
3	Caryophyllene	-0.05456	0.80011	0.90679
4	**Dodecanal**	0.22127	0.29877	0.86755
5	Humulene	-0.07298	0.73469	0.90679
6	**Tetradecanal**	0.29872	0.15622	0.8074
7	Dill ether	-0.1633	0.4458	0.86755
8	Galaxolide	-0.088592	0.68059	0.90679
9	**1,3,5-Trimethyl-2-cyclopentylbenzene**	0.059182	0.78355	0.90679
10	Isoascaridol	-0.27705	0.18998	0.8074
11	**Benzene, 1-ethyl-2,4,5-trimethyl-**	0.1923	0.36801	0.86755
12	**Alpha-Copaene**	0.28218	0.18157	0.8074
13	**Benzene, 1-(1-ethylpropyl)-4-methyl-**	0.14297	0.50511	0.86755
14	**Benzene, 1-(1,1-dimethylethyl)-3-methyl-**	0.12097	0.57339	0.88615
15	Beta-Ocimene	-0.18749	0.38033	0.86755
16	**3-Carene**	0.012079	0.95533	0.95533

Pearson correlation analysis (r) of plant volatile compounds with GHW response. Compound in bold font was positively correlated to GHW response. FDR represent false discovery rate.

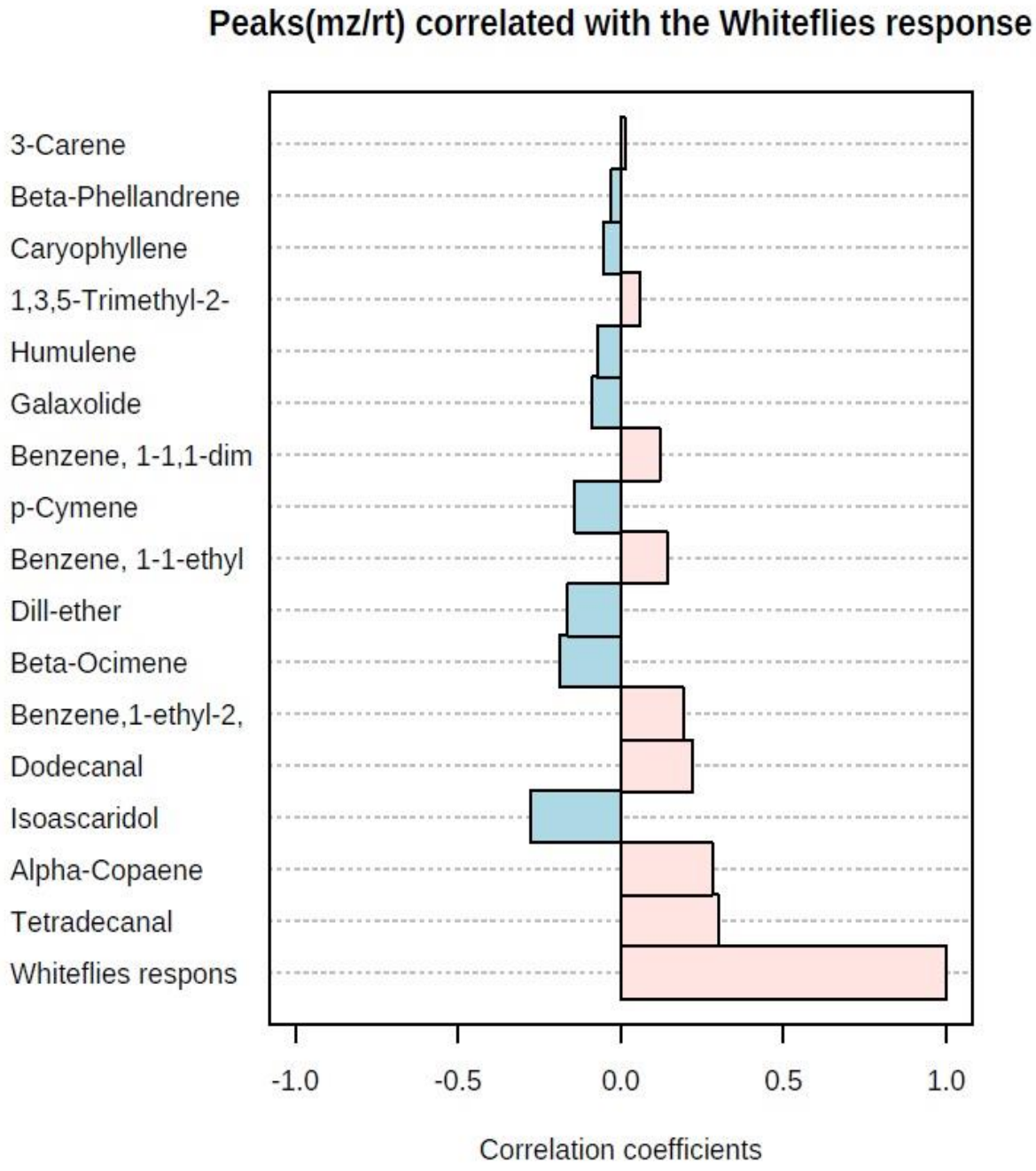

Figure 5. Plant volatile compounds correlated to GHW response.

3.2. Parasitoid (*E. formosa*) response assay to EPF inoculation

The effect of EPF strains inoculation alter plant volatile profiles and influence the interaction of parasitoid (*E. formosa*) was analyzed in the following experiments:

3.2.1 Endophyte colonization in planta

Fungal DNA quantification of inoculated plants was performed to confirm the endophytically colonization of EPF in single and combination treatments. We did not detect any fungal DNA on water control treated plants. However, fungal DNA quantification of *B. bassiana* (glm $p<0.05$ [$F_{(2, 27)} = 2.18$, $p = 0.12$], and *M. brunneum* (glm $p<0.05$; [$F_{(2, 27)} = 1.51$, $p = 0.23$] were not significant different among treatment (Table 5).

Table 5. Average fungal DNA isolated from inoculation treatment of parasitoid *E. formosa* experiment

No.	Treatment	*B. bassiana* (pg DNA)	*M. brunneum* (pg DNA)
1	Bb Can	0.70 ± 1.37 a	-
2	Mb Cb15	-	0.03 ± 0.07 a
3	Bb Can + Mb Cb15	0.17 ± 0.59 a	0.06 ± 0.13 a
4	Water control	0.00 ± 0.00 a	0.00 ± 0.00 a

Average pg DNA of each fungal species. In a column, mean (± SE) followed by the same letter are not significantly different at $p<0.05$, Tukey's HSD test, after generalized linear model.

3.2.2 Behavioral response of *E. formosa* towards EPF inoculation

The likelihood ratio test to evaluate the effect of EPF inoculation on *E. formosa* response was significant different between EPF treatment (poisson glmm X^2 (4, N = 120) = 160.27, $p<0.001$). *E. formosa* significantly preferred to visit Bb Can (3.04±0.90) over the other EPF inoculated plants; Mb Cb15 (2.25±0.67), combine Bb Can + Mb Cb15 (2.08±0.88), and water control (0.16±0.38). We observed no visit of *E. formosa* in the empty arm (Figure 7).

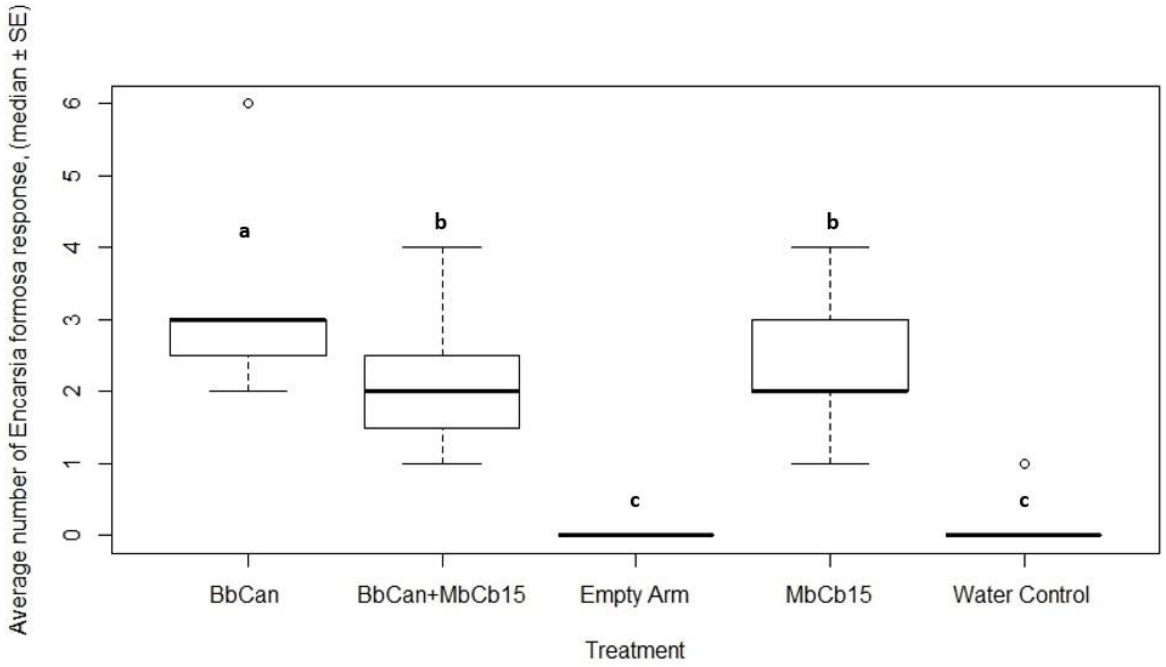

Figure 6. Average number of *E. formosa* responding to the treatments offered in six-arm olfactometer experiments. Different letters refer to median (± SE) significantly different among treatments (p<0.001, Tukey's HSD test, after generalized linear mixed model).

3.2.3. Influence of EPF inoculation on plant volatile profiles

The evaluation of the retention index (RI) identified 19 main plant volatile compounds collected from tomato plants in the *E. formosa* experiment (Table 6). In line with the identified compound result of GHW experiment, β-Phellandrene also the most abundant volatile overall EPF inoculation treatment (glm p<0.05 [$F_{(75, 304)}$ = 40.76, p<0.001]). We found no significant effect of the EPF inoculation treatments on the proportion of each compound (Two-way ANOVA p<0.05 [$F_{(3, 18)}$ = 0.59, p = 0.61]).

Table 6. Proportions (%) of the 19 most abundant plant volatile compounds in tomato plants inoculated with different EPF compared to the water control of *E. formosa* behavioral experiment

No.	VOCs	Bb Can	Mb Cb 15	Bb Can+Mb Cb 15	Water Control
1	Beta-Phellandrene	47.70±17.81 A, a	52.77 ± 13.38 A, a	52.88 ± 15.75 A, a	51.03 ± 17.58 A, a
2	p-Cymene	2.87 ± 2.16 A, b	3.23 ± 1.01 A, b	4.93± 3.61 A, b	3.90 ± 1.95 A, b
3	Heptane, 2,2,4,6,6-pentamethyl-	0.96 ± 0.91 A, b	0.89 ± 0.78 A, b	5.41 ± 6.50 A, b	6.24 ± 6.46 A, b
4	Dodecanal	3.32 ± 1.79 A, b	4.08 ± 1.94 A, b	2.34 ± 2.82 A, b	1.87 ± 1.36 A, b
5	Caryophyllene	0.87 ± 0.72 A, b	0.95 ± 0.19 A, b	3.86 ± 4.53 A, b	1.07 ± 0.63 A, b
6	Nonanal	2.42 ± 1.29 A, b	0.60 ± 0.20 A, b	0.70 ± 0.83 A, b	0.22 ± 0.33 A, b
7	Benzene, 1,4-dimethyl-2-(2-methylpropyl)-	0.28 ± 0.31 A, b	0.75 ± 0.55 A, b	1.00 ± 0.75 A, b	1.34 ± 0.66 A, b
8	2,4-Di-tert-butylphenol	0.88 ± 0.47 A, b	0.52 ± 0.08 A, b	0.38 ± 0.19 A, b	0.36 ± 0.12 A, b
9	Durene	0.13 ± 0.16 A, b	0.38 ± 0.19 A, b	0.57 ± 0.33 A, b	0.98 ± 0.29 A, b
10	Benzene, 1-(1,1-dimethylethyl)-3-methyl-	0.06 ± 0.09 A, b	0.30 ± 0.23 A, b	0.62 ± 0.35 A, b	0.91 ± 0.37 A, b
11	Indene	0.04 ± 0.06 A, b	0.00 ± 0.00 A, b	0.46 ± 0.08 A, b	0.65 ± 0.47 A, b
12	Tetradecanal	0.42 ± 0.29 A, b	0.49 ± 0.21 A, b	0.14 ± 0.25 A, b	0.00 ± 0.00 A, b
13	1,1,2-Trimethylcyclohexane	0.06 ± 0.06 A, b	0.22 ± 0.17 A, b	0.27 ± 0.24 A, b	0.46 ± 0.40 A, b
14	Beta-Guaiene	0.00 ± 0.00 A, b	0.10 ± 0.14 A, b	0.13 ± 0.19 A, b	0.19 ± 0.07 A, b
15	Decamethylcyclopentasiloxane	0.00 ± 0.00 A, b	0.00 ± 0.00 A, b	0.12 ± 0.14 A, b	0.27 ± 0.17 A, b
16	Alpha-Copaene	0.00 ± 0.00 A, b	0.09 ± 0.21 A, b	0.11 ± 0.25 A, b	0.09 ± 0.10 A, b
17	Carveol	0.00 ± 0.00 A, b	0.02 ± 0.06 A, b	0.00 ± 0.00 A, b	0.17 ± 0.12 A, b
18	Alpha-Pinene	0.003 ± 0.007 A, b	0.00 ± 0.00 A, b	0.055 ± 0.03 A, b	0.08 ± 0.05 A, b
19	cis-Valerenyl acetate	0.017 ± 0.015 A, b	0.05 ± 0.04 A, b	0.008 ± 0.01 A, b	0.01 ± 0.011 A, b

Proportion of each plant volatile compounds (means ± SE) followed by the same uppercase letter within row are not significantly different at $p<0.05$, Two-way ANOVA. In a column, means (±SE) followed by different lowercase letter are significantly different at $p<0.01$, Tukey's HSD test, after generalized linear model.

The data variability of principal component analysis (component 1 and 2) coefficients (Table 7) and scores plot of plant volatile compounds from *E. formosa* experiment, visualized the cluster different of EPF and water inoculation to empty arm of the olfactometer (Figure 7).

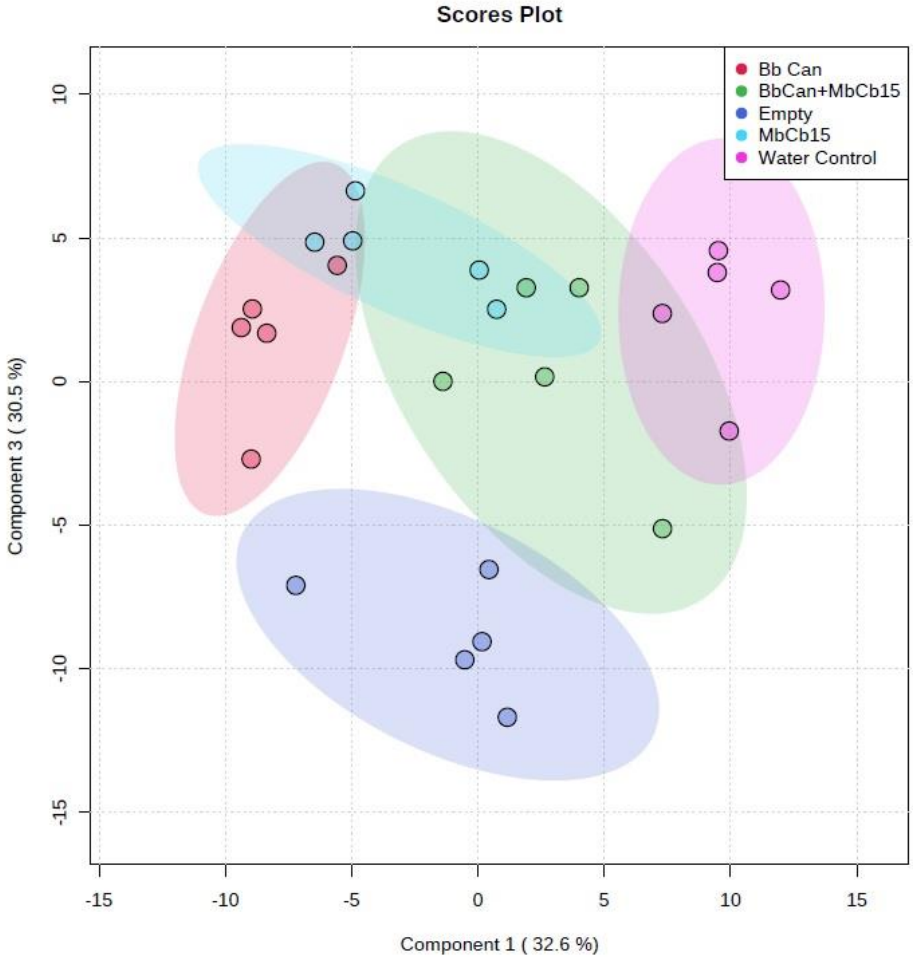

Figure 7. Scores Plot of principal component analysis (PCA) of plant volatile compounds emitted by EPF inoculation treatment from *E. formosa* experiment. Different colors cluster refer to inoculation treatment, and each dot represents treatment replication.

Table 7. Principal component analysis coefficients of plant volatile compounds emitted from EPF inoculation treatment of *E. formosa* experiments

No.	VOCs	Component 1	Component 3
1	Beta-Phellandrene	0,24	0,34
2	p-Cymene	0,33	0,39
3	Heptane, 2,2,4,6,6-pentamethyl-	0,64	0,97
4	Dodecanal	0,94	0,73
5	Caryophyllene	0,14	0,12
6	Nonanal	0,26	0,36
7	Benzene, 1,4-dimethyl-2-(2-methylpropyl)-	1,08	0,87
8	2,4-Di-tert-butylphenol	0,32	0,43
9	Durene	0,92	0,69
10	Benzene, 1-(1,1-dimethylethyl)-3-methyl-	1,43	1,13
11	Indene	0,63	1,96
12	Tetradecanal	1,42	1,25
13	1,1,2-Trimethylcyclohexane	1,60	1,31
14	Beta-Guaiene	1,49	1,15
15	Decamethylcyclopentasiloxane	1,06	1,01
16	Alpha-Copaene	1,27	0,97
17	Carveol	1,73	1,78
18	Alpha-Pinene	0,09	0,45
19	cis-Valerenyl acetate	0,61	0,79

Principal component coefficient was performed based on correlation matrix between integrated signal (IS) of plant volatile compounds appears in each EPF treatment.

Graphical representation of up-regulate or down-regulate of each plant volatile compounds from each inoculation treatment was generated with a heat map (Figure 8). Nonanal, Caryophyllene, cis-Valerenyl acetate, and Durene were highly up-regulated by Mb Cb15 inoculation. Alpha-pinene and Tetradecanal were highly up-regulated by Bb Can inoculation. In contrast with single EPF inoculation, EPF combination of Bb Can + MbCb15 were hihgly up-regulate 2,4-Di-tert-butylphenol, p-Cymene, Beta-Phellandrene, Heptane, 2,2,4,6,6-pentamethyl-, and Indene. Inoculation with water as

control treatment were up-regulate Decamethylcyclopentasiloxane, Carveol, Dodecanal, and β-Guaiene higher than EPF inoculation treatment.

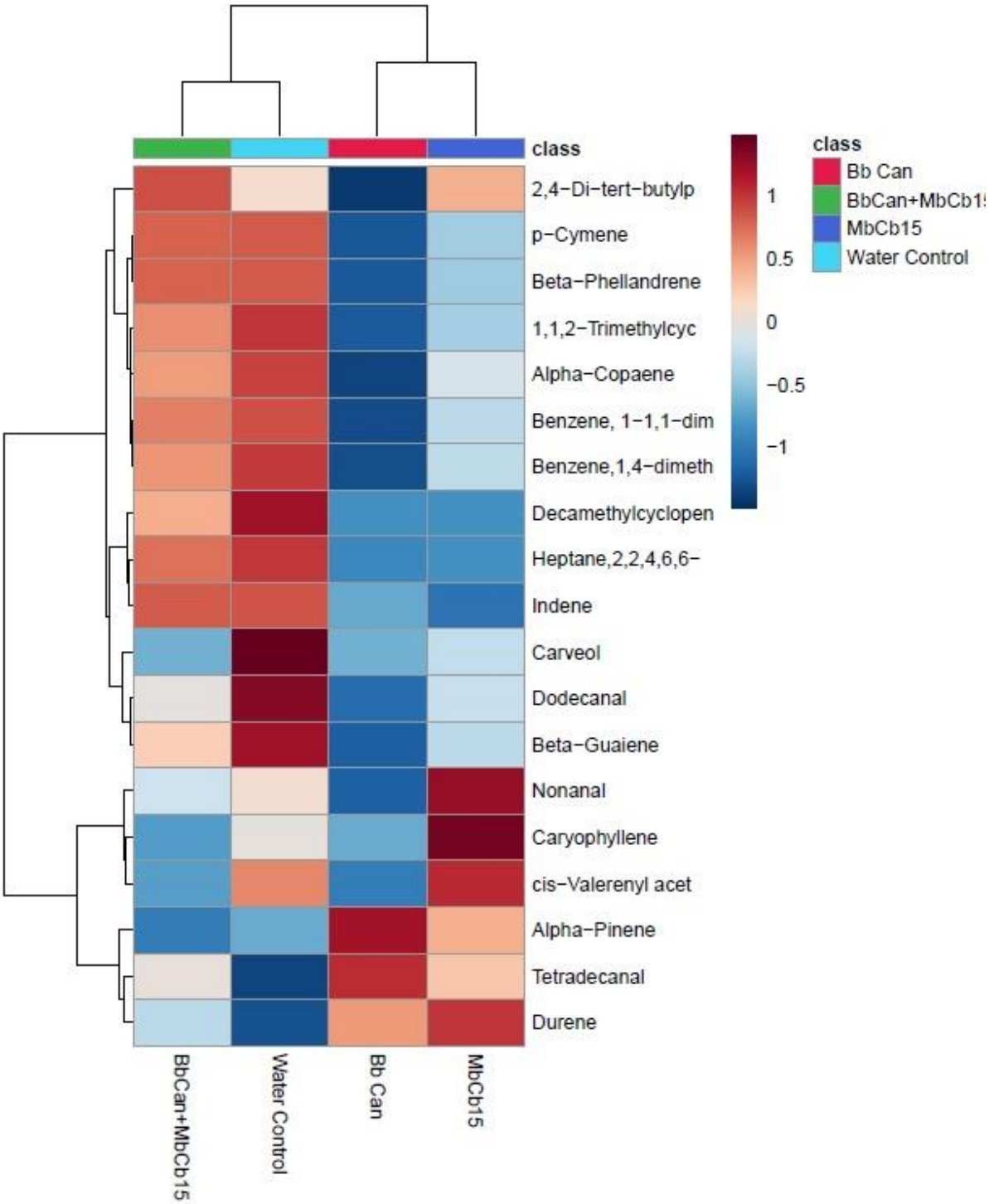

Figure 8. Up-regulating (red to reddish) or down-regulating (dark to light blue) heat map of identified plant volatile compounds affected by EPF inoculation treatment of *E. formosa* experiment.

3.2.4. Plant volatile correlated with *E. formosa* response

Pearson coefficient analysis was performed to examine the correlation between plant volatile compounds to *E. formosa* response (Table 8). Only Tetradecanal, Dodecanal, and Nonanal were correlated with *E. formosa* response (Figure 9).

Table 8. Plant volatile compounds correlated with *E. formosa* response

No.	VOCs	r	p-value	FDR
1	Beta-Phellandrene	-0.33358	0.15063	0.20084
2	p-Cymene	-0.31388	0.17776	0.22221
3	Heptane, 2,2,4,6,6-pentamethyl-	-0.56409	0.00957	0.03354
4	**Dodecanal**	0.060078	0.80135	0.83903
5	Caryophyllene	-0.048521	0.83903	0.83903
6	**Nonanal**	0.70843	4,7254	0.00315
7	Benzene, 1,4-dimethyl-2-(2-methylpropyl)-	-0.34865	0.13193	0.18847
8	2,4-Di-tert-butylphenol	-0.12532	0.59859	0.6651
9	Durene	-0.38558	0.09315	0.16937
10	Benzene, 1-(1,1-dimethylethyl)-3-methyl-	-0.42739	0.06016	0.13369
11	Indene	-0.52095	0.01851	0.04627
12	**Tetradecanal**	0.67279	0.00115	0.00575
13	1,1,2-Trimethylcyclohexane	-0.37238	0.10591	0.17046
14	Beta-Guaiene	-0.56104	0.01006	0.03354
15	Decamethylcyclopentasiloxane	-0.54771	0.01242	0.03549
16	Alpha-Copaene	-0.36762	0.11080	0.17046
17	Carveol	-0.84125	0.03367	0.33673
18	Alpha-Pinene	-0.40363	0.07759	0.15518
19	cis-Valerenyl acetate	-0.26441	0.25993	0.30580

Pearson correlation analysis (r) of plant volatile compounds with *E. formosa* response. Compound in bold font was positively correlated to *E. formosa*. FDR represent false discovery rate.

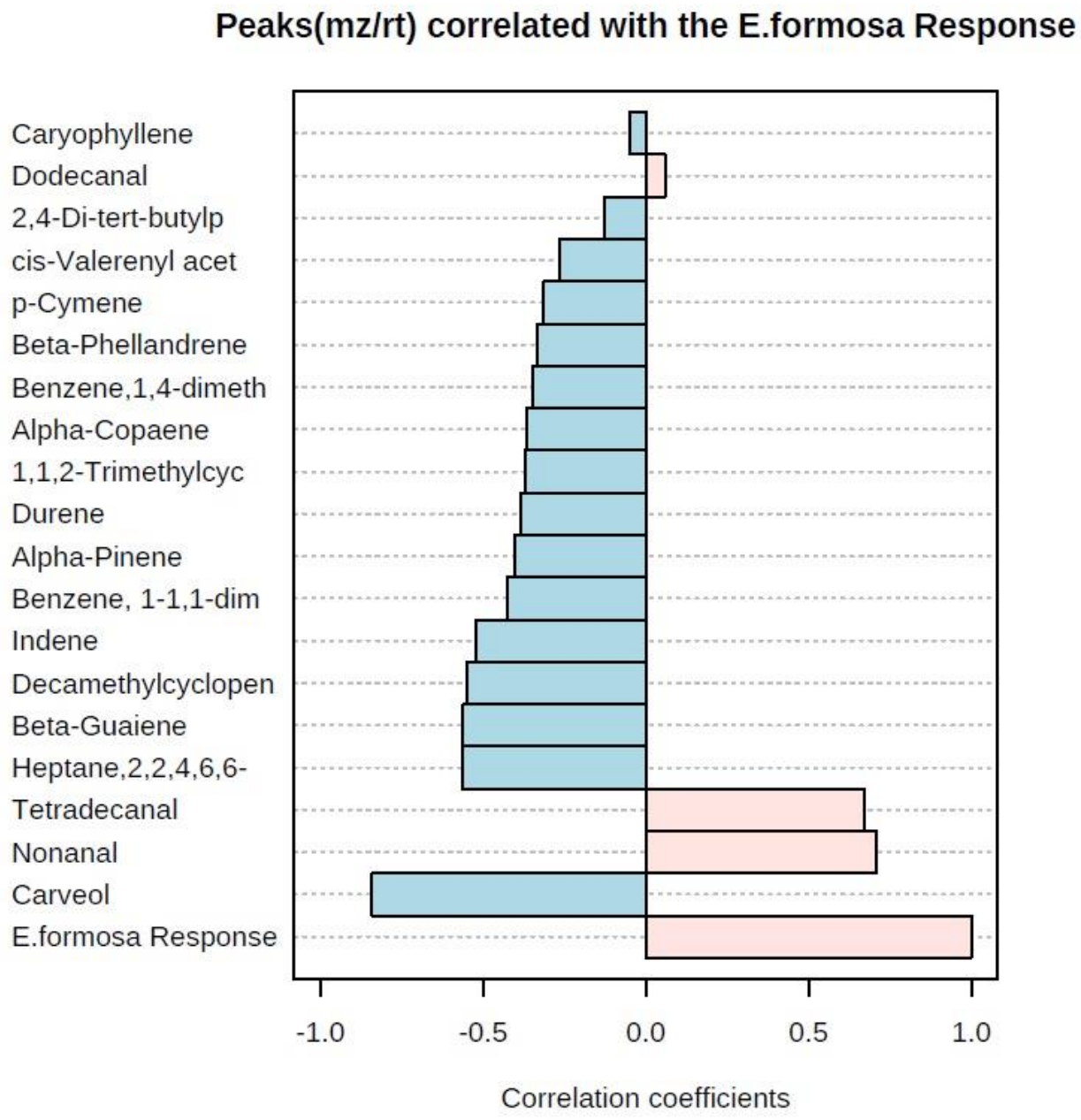

Figure 9. Plant volatile compounds correlated to *E. formosa* response.

3.2.5. *E. formosa* parasitization rate on GHW L3 in fungal inoculated plants

Parasitoid *E. formosa* did not discriminate the EPF inoculated plants (ANOVA $p<0.1$ [$F_{(3, 18)} = 0.76$, $p = 0.53$]), due to high parasitization rate among all treatment (Figure 10)

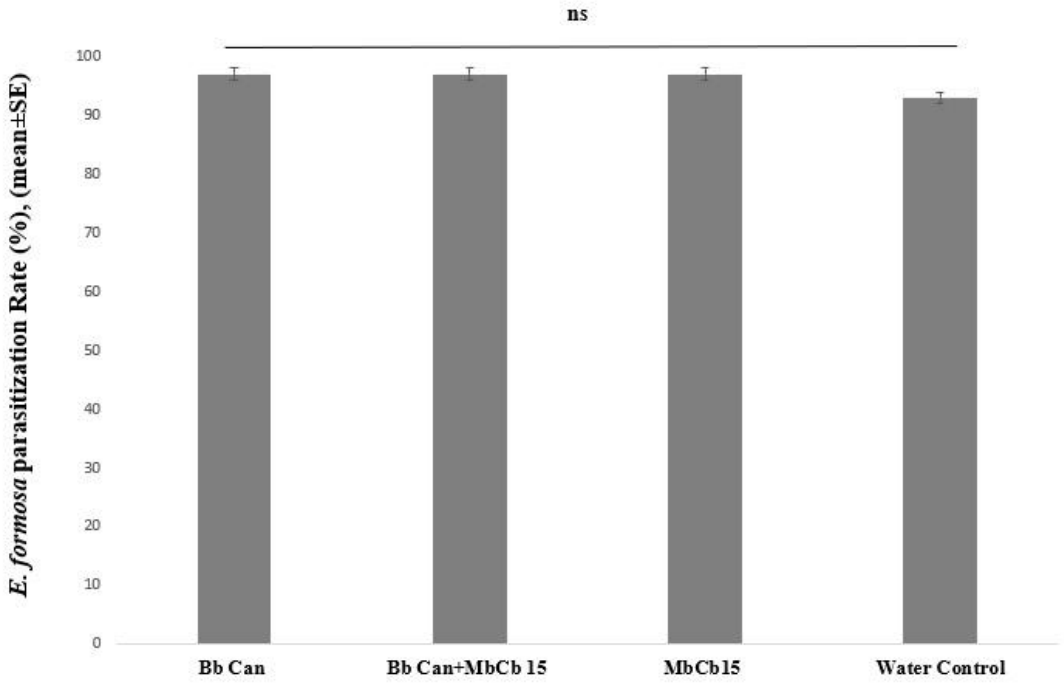

Figure 10. *E. formosa* parasitization rate on GHW L3 of EPF inoculation treatment; (ns) above bar graph indicate no significance between treatment, $p<0.1$, one-way ANOVA.

4. Discussion

Our study confirmed successful establishment of single EPF inoculation *B. bassiana* (Bb Can), *M. brunneum* (Mb Cb15), and combination of both EPF strain as endophyte within plant via root inoculation both in GHW experiment and *E. formosa* experiment. Successful establishment of EPF strain (Bb Can) as endophte used in this study were previosuly reported in tomato (Rodríguez, 2016), oil seed rape and broad bean (Vidal and Jaber, 2015). As well as EPF strain Mb Cb15 in potato (Hettlage, 2018) and tomato (Krell et al., 2018). In this study, we confirmed the colonization of both EPF strain in tomato.

The behavioral response of GHW were significantly visit olfactometer arm emitted plant volatile compounds from single EPF inoculation plants (Mb Cb15 or Bb Can) over EPF combination inoculation (Bb Can+Mb Cb15) and water control. Evaluation of

retention index (RI) identified 16 plant volatile compounds with significant proportion of β-Phellandrene, followed by p-Cymene, Caryophyllene and Dodecanal among inoculation treatment. However, EPF inoculation does not affect the proportion of plant volatile compounds. Tetradecanal, Dodecanal, α-Copaene and Benzene groups were correlated with GHW responses. We speculate that the significantly visit of GHW on Mb Cb15 inoculated plants due to up-regulate of Tetradecanal, Dodecanal, and Benzene group (Benzene, 1-1-ethylpropyl-4-methyl-, Benzene, 1-1,1-dimethylethyl-3-methyl-, and 1,3,5-Trimethyl-2-cyclopentylbenzene). And the significantly visit of GHW on Bb Can inoculated plants due to up-regulate of α-Copaene, Dodecanal, and Benzene group (Benzene, 1-1-ethylpropyl-4-methyl- and Benzene, 1-1,1-dimethylethyl-3-methyl-). EPF combinations (Bb Can+Mb Cb15) were up-regulate Isocardol, β -Ocimene, p-Cymene, 3-Carene, Humulene, Caryophyllene, and Dill-ether, these compounds were not correlated with the GHW response. Contrarily, Benzene, 1-1,1-dimethylethyl-3-methyl- and Benzene, 1-1-ethylpropyl-4-methyl-, which were correlated with GHW response being down-regulate. Water inoculation treatment were down-regulating the plant volatile compounds correlated with GHW response (e.g Tetradecanal, Dodecanal, α-Copaene and the Benzene groups).

Parasitoid *E. formosa* were significantly visit Olfactometer arm emitted volatile compounds from Bb Can inoculation over Mb Cb 15 inoculation, EPF combination inoculation (Bb Can+Mb Cb15), and water control. There were no *E. formosa* visit have been observed on empty arm. Evaluation of retention index (RI) identified 19 plant volatile compounds with significant proportion of β -Phellandrene over all inoculation treatments. Tetradecanal, Dodecanal, and Nonanal were correlated with *E. formosa* responses. We speculate that the significantly visit of GHW on Bb Can inoculated plants due to up-regulate of Tetradecanal. However, *E. formosa* responses on Mb Cb15 and water control were likelihood to the up-regulate of Nonanal and Dodecanal, respectively. In contrast, EPF combination inoculation (Bb Can+Mb Cb15) were down-regulate Tetradecanal, Dodecanal, and Nonanal.

In this present study we confirmed that inoculation of EPF strain affected the up-regulate or down-regulate of plant volatile compounds and influence the GHW or *E. formosa* response in olfactometer. Some studies reported root fungal endophyte (*Acremonium strictum, Trichoderma harzianum,* and *B. bassiana*) associated with plants were able to alter the VOCs emitted by plants and negatively affect insect pest

performance (Jallow et al., 2008; Zhang, 2014; Rodríguez, 2016). Among various secondary metabolites produces by plants, volatile organic compounds (VOCs) play a central role in the relationship between plants and herbivorous insects, as well as between insects and their natural enemies. In order to discriminate the diverse insects interaction (i.e: herbivore, parasites, pollinator, or natural enemies), emitted plant volatile function as reliable and precise signals by plants, as well as these volatile can be utilized as cues by insects to choose host plants (Kessler and Baldwin, 2001). Plant volatile organic compounds function as indirect plant defence against insect (Mumm et al., 2003) and phytopathogen (Rostás et al. 2003;2006), to overcome environmental stress and mechanical damage (Ameye et al., 2008), to attract pollinators (Dudareva and Pichersky, 2010), and as plant-to-plant communication (Baldwin et al., 2006). In addition, the olfactory receptor neurons of insect's antennae could detect the ratio of compounds for recognition of a host (Webster et al., 2010). VOCs emitted by host plants as response of environment stimulus such as fungal colonization or insect herbivore attacks were formed as blends volatile in particular ratio (Hammerbacher, 2019). Specificity of insect herbivore attacking certain plant species contributed to the composition of the HIPVs blend (Hare, 2011). The ratios of blend compounds likely determine as attractive for specialist parasitoids, that were mainly respond to specific volatile blends induced by their host (van Oudenhove et al., 2017). Accordingly, plant volatile compounds are crucial airborne signals in ecological systems.

Tetradecanal was the most correlated compound on insect response in GHW and *E. formosa* behavioral response experiment, followed by Dodecanal and Nonanal. Tetradecanal, Dodecanal, Nonanal classified as Aldehyde class of VOCs. Aldehydes are important components of plant volatile, it is belong to an important class of volatile that are indispensable to plants in response to environmental conditions (Hu et al., 2008), and mainly response to the damaged on tomato leaves (Buttery et al., 1987). Most of the aldehydes known as C6-C10 aldehydes green leafy volatile (GLVs) , such as (E)-2-Hexenal, (Z)-3-Hexenal, n-Hexanal, Acetaldehyde, Butanal, Pentanal, Heptanal, Octanal, Decanal, Undecanal, Nonanal, Dodecanal, and Tetradecanal (Buttery et al, 1987). These compounds as components of plant fragrance, can be emitted after plant damage and are considered as direct or indirect defence signals. However, the changes patterns in aldehyde levels stimulated by damage, are largely unknown in plants (Kishimoto et al., 2005).

Green leaf volatile (GLVs) such as β-phellandrene, Limonene, Caryophyllene, Humulene, 2-Carene, (E)-Z-Hexenal, n-3-Hexenal, (E)-2-Hexenal, Eugenol, 1,8-Cineole, and Linalool were emitted from tomato plants (Buttery et al., 1987). Collection of the plant volatile from tomato cv. UC82 revealed compounds of a-pinene, (Z)-3-hexen-1-ol, a-phellandrene, limonene, (E)-b-ocimene, p-cymene, methyl salicylate, (E)- and β-caryophyllene, were emitted at higher levels from aphid-infested plants than from undamaged control plants (Sasso et al., 2007). Large amount of β-Phellandrene collected from three tomato cultivar that was correlated to tomato leaf miner (*Tuta absoluta*) host-searching behaviour (Proffit et al, 2011). p-Cymene used as deterrent to control northern blowfly (*Protophormia terraenovae*) (Ibrahim et al., 2001). Solanaceae plants with lower amounts of p-Cymene were more attacked by whiteflies, thus it considered as repellent agent (Bleeker et al., 2009), whilst Janmaat et al. (2002) reported p-Cymene as a toxic agent to the western flower thrips (*Frankliniella occidentalis*). Beta-caryophyllene was able to exert the highest repellences rates against storage pest *Sitophilus zeamais* (Bougherra et al., 2015). Beta-Phellandrene, p-Cymene and Caryophyllene as the most abundant compound found in our study were reported in previous study as insect herbivore repellent, deterrent, and affected the searching behaviour. Our finding confirmed these compounds were un-correlated to GHW and *E. formosa* response. In line with our result, inoculated tomato plants with endophyte *B. bassiana* (Rodríguez, 2006) and with *A. strictum* (Jallow et al., 2008) also reported β-Phellandrene, p-Cymene, and trans-β-caryophyllene emitted from inoculated tomato plants. Our study attributes the mechanism of EPF inoculation alter plant VOCs mediated the indirect-complex interaction among host plant-insect herbivore-parasitoid.

In this present study, we found no effect of EPF inoculation treatment to parasitization rate of *E. formosa*. More than 95% (N=400) parasitization of *E. formosa*, indicated by black immature GHW on underside leaves of tomato plants were observed among all inoculation treatment. High parasitization rate of all treatment are plausible since *E. formosa* preferably GHW L3, and since hosts are available under exceptional circumstances (inside cylindric mesh cage). In natural condition *E. formosa* is attracted by honeydew produced as excreted of adult whiteflies (van Lenteren et al., 1996). However, in our study systems naïve *E. formosa* were used to encounter GHW L3, therefore the searching pattern of parasitoids were relied on olfactory cues. As reported

by Birkett et al. (2003), *E. formosa* uses volatile emitted by plants infested with GHW as host-locating response. Volatile emitted during insect herbivore feeding are often used by parasitoids and predators of herbivores to locate their prey (Turlings and Erb, 2018). Enhanced volatile emission by insect herbivore infestation might thus be used by parasitoid to locating their parasitic hosts.

The efficiency of the host finding process ultimately determine *E. formosa* capability to control GHW (van Lenteren et al., 1996). Foraging behavior of host location by *E. formosa* in our study was efficient to parasites GHW L3, since the study was performed inside mesh cage; hence, *E. formosa* do not have to search for hosts at broad environment. According to van Roermund et al. (1997 a,b), *E. formosa* will stayed at least minimum five minute on each leaflet for host encounter, it will increases the arrestment effect and ability to parasitize by lay their daily egg load at extremely high host densities, thereby reducing the chance that clustered hosts escape from parasitism. In line with our result, colonization of EPF strain *B. bassiana* (NATURALIS®) or *M. brunneum* (BIPESCO5) as endophyte did not affect the parasitization rate of endoparasitod *Aphidius colemani* on green peach aphid *Myzus persicae* (Jaber and Araj, 2018). Likewise, colonization of fungal endophyte *B. bassiana* strain ICIPE 279, G1LU3, S4SU1 or *Hypocrea lixii* (strain F3ST1) did not affect the parasitization rate of *Phaedrotoma scabriventris* and *Diglyphus isaea* on pea leafminer *Liriomyza huidobrensis* (Akutse et al., 2014). These finding indicate that EPF application as endophyte are compatible with the parasitoid *E. formosa* and could be an option for GHW control.

Regarding the multitrophic interactions investigation, the EPF strain used, the specificity of insect herbivore as host for the natural enemies, and the experiment set up for interaction mechanism are important elements to be considered. For a study development purpose, critical awareness needs to be raised up in order to performed studies that unequivocally demonstrate the effect of insect response towards endophyte inoculation is due to the direct effect of metabolites originating from the endophyte, and not by fungus-mediated changes in host plant metabolism. We strongly believe that unravelling as many aspects of direct and indirect mechanism is a paramount importance and will provide a deeper understanding of multitrophic interaction throughout microbes-plant-insect herbivore-natural enemies.

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Ph.D.| Georg August Universität-Göttingen, Germany.

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PhD degree in the field of Agriculture Entomology, under International PhD program of Graduate School Forestry and Agriculture (GFA) Georg August Universität Göttingen, Germany.

Dissertation tittle "Multitrophic interaction of co-inoculated endophyte entomopathogenic fungi"

M.Agr. | Udayana University, Bali-Indonesia.

2008 – 2010

Master degree in the field of Agriculture Biotechnology, under Postgraduate Programed of Udayana University in Bali-Indonesia.

Master thesis tittle "The Predator Fauna as the Potential Biological Control Agent against Cocoa Pod Borer *Conopomorpha cramerella* (Snellen) (Lepidoptera: Gracillaridae) in The Cocoa Ecosystem"

B. Agr. | Udayana University, Bali-Indonesia.

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Bachelor degree in the field of Crop Protection (Program study: Pests and Plant diseases), under Agriculture faculty of Udayana University in Bali-Indonesia.

Bachelor thesis tittle "Biological Aspects of Parasitoids *Hemiptarsenus varicornis* (Girault) (Hymenoptera: Eulophidae) on Leaf miner pest *Liriomyza sativa* (Blanchard) (Diptera: Agromyzidae) Arising from Different Host Plants".

PROFESSIONAL EXPERIENCES

Indonesian Agency for Agricultural Research and Development (IAARD), Ministry of Agriculture Republic of Indonesia | deployed at:

1. Junior Researcher | Bali Assessment Institute of Agricultural Technology (Bali AIAT), Denpasar, Bali-Indonesia.

2014-present.

Main task was to support national programs to carrying out the assessment, assembly and development of appropriate agricultural technology specific location in Bali province. Specifically, on plant protection field, to disseminate the recent plant protection technology to the farmers, implementing Good Agricultural Practice, Integrated Pest Management, pests and plant diseases observation in field and how to overcome the problem in easy practical approach for farmers.

2. Junior Researcher | Indonesia Vegetable Research Institute (IVEGRI), Lembang, West Java-Indonesia.

2011-2014

Main task was to support national programs to held research on component of vegetables technology system, including the vegetables crop health management. The aim of the research program was to create effective technology to support the development of the national vegetables agribusiness system.

ADDITIONAL EXPERIENCES

Coordinator | Research Collaboration and Development of IVEGRI

2012-2013

As a coordinator of division Research Collaboration and Development at Indonesia Vegetable Research Institute (IVEGRI). Main tasks were to facilitate other parties from Indonesia and overseas to conduct an experiment related with the vegetable's commodities aspect. The development, initiating and bridging the collaboration by preparing a meeting for the stakeholders, prepare the point of MoA (Memorandum of Agreement), and supervise the implementation of the agreement.

Field Technician | Democratica Republic of Timor Leste

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Field technicians at collaborating project "Survey of Pest and Disease Animal and Plant for Quarantine List of Democratica Republic of Timor Leste in all

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Exchange Student | Ibaraki University-Japan

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PUBLICATION

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Copyright| Software copyright

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Officially register by Copyright certificate of computer software.

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Abstract in Scientific Meeting

- **Hadis Jayanti** and Stefan Vidal. "Combining entomopathogenic fungi as endophytes for biocontrol". German Congress of Entomology- Deutsche Gesellschaft für Allgemeine und angewandte Entomologie e.V. Freising-Münich. Germany. 13-16 March 2017.

- **Hadis Jayanti**, Catalina Posada-Vergara, Stefan Vidal. "Assessing the effect of endophyte entomopathogenic fungal combinations on pathogen inhibition". International Symposium- Microbe-Assisted Crop Production Opportunities, Challenges and Needs. Microbe 2019. Vienna-Austria. 2-5 December 2019.

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Declarations

1. I, hereby, declare that this Ph.D. dissertation has not been presented to any other examining body either in its present or a similar form.

   Furthermore, I also affirm that I have not applied for a Ph.D. at any other higher school of education.
2. I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorized aid.

Gottingen, 27 June 2020

Hadis Jayanti