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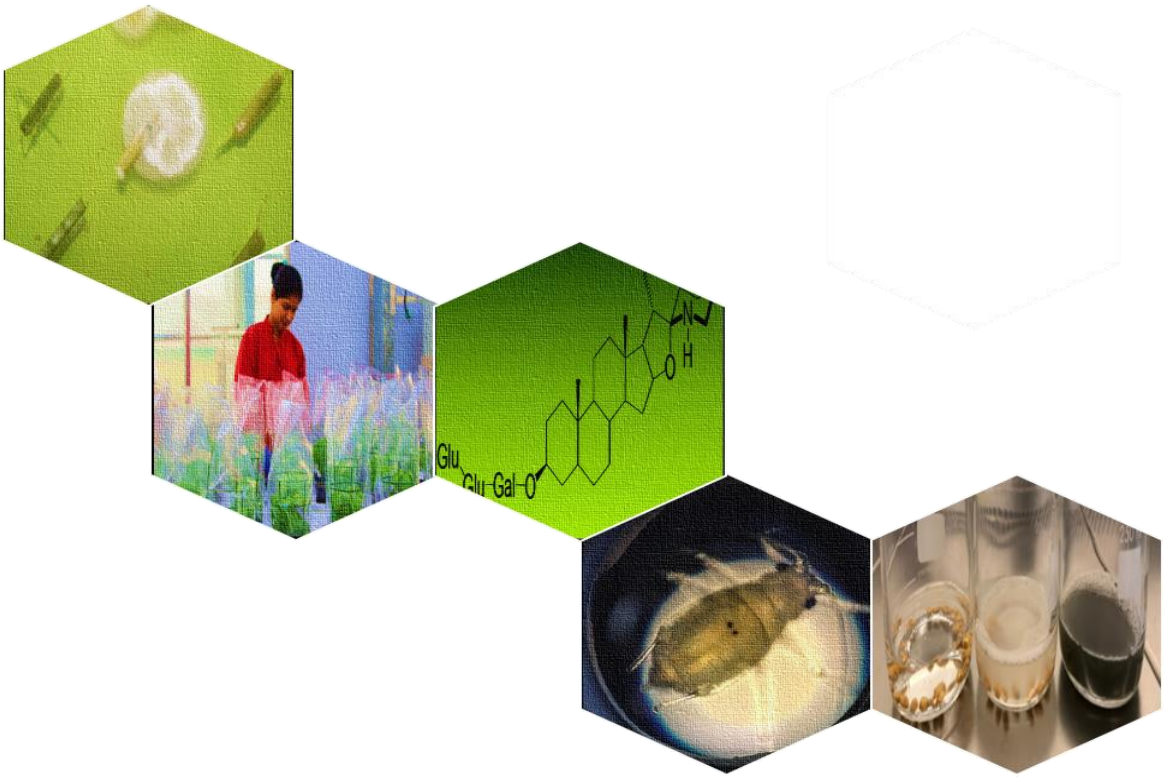
Entomopathogenic Fungal Endophytes in Plant-Fungus-Herbivore Interactions:

Exploring the importance of selected plant physiological responses in regulation of arthropod populations



Shumaila Rasool

PhD Thesis

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Title:	Entomopathogenic fungal endophytes in plant-fungus herbivore interactions: exploring the importance of selected physiological responses in regulation of arthropod populations
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PREFACE

The PhD work in the submitted dissertation was part of the Chief Minister Merit Scholarship program from Punjab Educational Endowment Fund, Pakistan. Most of the work described in the thesis was carried out at the Section for Organismal Biology, Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen from November 2016 to June 2020, under the supervision of Nicolai V. Meyling and Birgit Jensen.

During the PhD, two months period was spent in the lab of Inge S. Fomsgaard at the Department of Agroecology, Aarhus University, for the analysis of wheat and bean secondary metabolites (Manuscript II). The analysis of wheat enzymes (Manuscript I) was performed in collaboration with Thomas G. Roitsch at the Section for Crop Sciences, Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen. Whereas, the analysis of tomato secondary metabolites (Manuscript III) was performed in collaboration with Pablo D. Cárdenas and David I. Pattison at Section for Plant Biochemistry Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen. The research presented in this thesis generated three manuscripts as follows:

Manuscript I:

Rasool S., Jensen B., Roitsch T. G., Meyling N. V**.** Comparative activities of carbohydrate and antioxidant enzymes in wheat inoculated with entomopathogenic fungi in relation to effects against aphids. *In preparation.*

Manuscript II:

Rasool S., Vidkjær N. H., Hooshmand K., Jensen B., Fomsgaard I. S., Meyling N. V. Effects of entomopathogenic fungal endophytes against aphids correlate with alternations in bioactive plant secondary metabolite concentrations across plant families. *Prepared for submission to New Phytologist.*

Manuscript III:

Rasool S., Cárdenas P. D., Pattison D. I., Jensen B., Meyling N. V. entomopathogenic endophytic fungi affect population growth of two-spotted spider mite (*Tetranychus urticae* Koch) by changing profiles of steroidal glycoalkaloids in tomato. *Prepared for submission to Journal of Chemical Ecology.*

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Additionally, I would like to acknowledge all the colleagues and friends at Section of Organismal Biology for the good time and productive discussions that we had on Friday breakfasts and during lunch breaks. To my PhD coordinator Prof. Jørgen Eilenberg for always cheering me up and for helping me in different aspects and Prof. Jacob Weiner for reviewing my thesis introduction chapter and helping me always in scientific problems. I am happy to mention all the technicians and greenhouse assistants who helped me in my experiments. My sincere thanks to all my office mates and friends in Copenhagen. As Izaak Walton said "*good company in journey makes the way seem shorter*", you all made my journey beautiful.

Last but not least to my loving FAMILY. How I can find words to express my love for you MAMA and PAPA. You are the best parents, thanks for trusting, encouraging and praying for me. My adorable siblings Ubaid, Zain, Rani, Nomi and Dani thanks for the love and care that you show for me. Finally to my friends in Pakistan for a nice time during my visits back home.

Shumaila Rasool

LIST OF ABBREVIATIONS

EPF	Entomopathogenic fungi
EFE	Entomopathogenic endophytic fungi
PSMs	Plant secondary metabolites
FSMs	Fungal secondary metabolites
BXs	Benzoxazinoids
SGAs	Steroidal glycoalkaloids
ROS	Reactive oxygen species
cwInv	Cell wall invertases
vacIn	Vacuolar invertases
cytInv	Cytoplasmic invertases
Susy	Sucrose synthase
FK	Fructokinase
HXK	Hexokinase
Ald	Aldolase
PFK	Phosphofructokinase
G6PDH	Glucose-6-phosphate dehydrogenase
PGM	Phosphoglucomutase
PGI	Phosphoglucoisomerase
AGPase	ADP-glucose pyrophosphorylase
UGPase	UDP-glucose pyrophosphorylase
SOD	Superoxide dismutase
CAT	Catalase
POD	Peroxidase
GST	Glutathione-S-transferase
GR	Glutathione reductase
DHAR	Dehydroascorbate reductase
MDHAR	Monodehydroascorbate reductase
DIBOA	2,4-dihydroxy- 1,4-benzoxazin-3-one

DIBOA-Glc	2-(2,4-dihydroxy-1,4(2H)-benzoxazin-3(4H)-on)-β-D-glucopyranoside
DIBOA-Glc-Hex	Double-hexose derivative of DIBOA
DIMBOA	2,4-dihydroxy-7-methoxy-1,4- benzoxazin-3-one
DIMBOA-Glc	2-β-D-glucopyranosyloxy-4- hydroxy-7-methoxy-1,4-benzoxazin-3-one
HBOA	2-hydroxy- 1,4-benzoxazin-3-one
HBOA-Glc	2-β-D-glucopyranosyloxy-1,4- benzoxazin-3-one
HBOA-Glc-Hex	Double-hexose derivative of HBOA
HMBOA	2-hydroxy-7-methoxy-1,4-benzoxazin-3-one
HMBOA-Glc	2-β-D-glucopyranosyloxy-7-methoxy-1,4-benzoxazin-3-one
MBOA	6-methoxy-benzoxazolin-2-one

SUMMARY

Entomopathogenic fungi (EPF) are well-studied natural enemies of insect and mite pests and several isolates with relatively broad host ranges have been developed as biological control agents. Besides their direct interaction with insect and mite hosts as pathogens, these fungi are also able to associate symbiotically with plants as endophytes, hence living inside plant tissues asymptomatically. Several species of EPF can be experimentally established as endophytes in a range of plant species, including important crops, causing growth promotion and affecting plant-herbivore interactions. The effects of these endophytes on insect herbivores have been widely studied, but the mechanisms behind the reported effects are not documented. The general absence of fungal propagule production in colonized plant tissues and lack of infection in insects feeding on endophytically colonized plants support the notion of antibiosis and feeding deterrence over direct infection by EPF endophytes. Moreover, plants colonized by EPF below-ground can lead to effects on herbivores above-ground, suggesting complex interactions between the two organisms, potentially mediated by changes in the physiological response of the plant. This thesis addresses the question of how EPF seed inoculations can alter plant physiology with a focus on modifications of the activities of key carbohydrate and antioxidant enzymes and profiles of specific plant secondary metabolites (PSMs) and evaluate the potential role of these compounds in plant-fungal-herbivore tripartite interactions.

The capacities of three isolates of EPF, belonging to the species *Beauveria bassiana*, *Metarhizium brunneum* and *M. robertsii*, respectively, to establish as endophytes were evaluated through seed inoculation of important crop plants of three different plant families (Poaceae, Fabaceae and Solanaceae). The inoculations were evaluated for the effects on plant growth parameters, the population growth of arthropod herbivores and different physiological parameters in leaves of the host plants were measured with and without herbivore attack. Overall, *B. bassiana* was re-isolated from all tested plant parts (leaves, stem and roots), *M. brunneum* from stem and roots and *M. robertsii* only from roots of all tested plant hosts. Compared to control treatments, inoculations with *M. robertsii* significantly reduced herbivore population growth, while populations on *M. brunneum* inoculated plants unexpectedly increased. This pattern was observed across all tested crops. Inoculations with *B. bassiana* were related to negative or neutral effects on the population growth of arthropod herbivores as compared to control plants. Overall, the two isolates of *Metarhizium* spp. increased plant growth as compared to uninoculated control. Three scientific manuscripts were prepared based on current research.

In **Manuscript I**, the activities of key carbohydrate and antioxidant enzymes were measured in wheat leaves after fungal inoculations of seeds and infestation by the aphid, *Rhopalosiphum padi*. Inoculations with *M. robertsii* significantly reduced, *M. brunneum* increased while *B. bassiana* had no effects on population growth of *R. padi* as compared to control plants. The activities of most of the selected carbohydrate enzymes increased in wheat inoculated with both isolates of *Metarhizium* spp. while showing no clear correlation with a population growth of aphids. Whereas, activities of antioxidant enzymes showed minimal changes by EPF treatments and as a response to aphid feeding. However, *M. robertsii* increased the activities of superoxide dismutase while *M. brunneum* increased the activities of catalase and glutathione S transferase, which likely played a role in aphid population growth. This indicated that specific antioxidant enzymes played a role in aphid population growth by fungal inoculations while carbohydrate enzymes are likely linked with plant growth.

Manuscript II addresses the quantification of a range of PSMs in two separate host plants, belonging to benzoxazinoids in wheat and flavonoids in bean as response to EPF seed inoculation and aphid infestation to elucidate the role of these specific compounds in the plant-fungus-herbivore interactions. Wheat was infested with *R. padi*, while the aphid *Aphis fabae* was used to infest bean plants. Concentrations of more than half of the PSMs were affected by fungal and aphid treatments and the changes in aphid numbers were associated with this regulation rather than the endophytic colonization of above-ground plant tissues. Nor were PSMs related to growth promotion effects by EPF inoculations. The three fungal isolates produced comparable effects on aphid populations and PSMs across the two host plants. This study links for the first time the effects of EPF endophytes against aphids with plant physiological responses of wheat and bean.

In **Manuscript III,** the variability of two selected steroidal glycoalkaloids was determined in tomato leaves by LC-MS after fungal seed inoculations and infestations by two-spotted spider mite, *Tetranychus urticae*, a herbivore possessing a different feeding style than aphids. Population growth of spider mites was highest on plants inoculated with the isolate of *M. brunneum*, which also showed relatively low concentrations of steroidal glycoalkaloids in the leaves compared to the other treatments. In contrast, tomato plants inoculated with the isolates of *B. bassiana* and *M. robertsii* produced significantly higher amounts of the two secondary metabolites and harbored the lowest numbers of *T. urticae*. We conclude that EPF endophytes alter profiles of specific PSMs to influence the interactions between tomato and the herbivore, *T. urticae*. In addition, the effects on spider mites of the individual fungal isolates were comparable to observations on aphids on other host plants, indicating a general isolate specific effect on plant physiology.

Overall, the research reported in this thesis opens new insights into the effects of EPF endophytes on plant physiology to cause ecological effects against herbivores. The study provides a link between the ecological effects and the physiological responses of plants caused by EPF inoculations which may have implications in plant protection strategies. The understanding of plant physiological changes by beneficial fungi are important for future applications of EPF for herbivore management.

DANSK RESUME

Insektpatogene svampe er velkendte naturlige fjender for insekter og mider, og adskillige svampeisolater er udviklet som biologiske bekæmpelsesmidler. Ud over at have en direkte interaktion med insekter og mider som patogener er disse svampe også i stand til at etablere symbiotiske relationer med planter som endofytter, hvor svampen lever i plantevævet uden af forårsage symptomer. Flere arter af insektpatogene svampe er eksperimentelt blevet etableret endofyttisk i en række forskellige plantearter, inklusiv vigtige afgrøder, hvor de kan lede til forøget vækst og påvirke interaktionerne mellem planter og herbivorer. Effekterne af disse svampe som endofytter mod herbivorer er blevet grundigt studeret, men de bagvedliggende mekanismer som forårsager disse effekter er ikke blevet undersøgt. Den generelle mangel på produktion af infektive svampesporer fra det koloniserede plantevæv og manglende infektion af insekter, som lever på de koloniserede planter tyder på, at mekanismerne bag de rapporterede negative effekter på herbivorer skyldes indirekte interaktioner mellem svamp og insekt. Desuden kan etablering af insektpatogene svampe ved plantens rødder påvirke herbivorer på de overjordiske dele af planten, hvilket indikerer at effekter er medieret af ændringer i plantens fysiologiske respons. Denne ph.d. afhandling fokuserer på spørgsmålet om hvorvidt insektpatogene svampe, der inokuleres på plantefrø, kan påvirke plantens fysiologi, med fokus på central kulhydrat- og antioxidantenzymer og på specifikke sekundære plantemetabolitter, for at vurdere deres rolle i påvirkning af plante-svamp-herbivor interaktioner.

Evnen af tre isolater af insektpatogene svampe fra arterne *Beauveria bassiana, Metarhizium brunneum* og *M. robertsii* til at etablere sig endofyttisk gennem frøinokulering blev undersøgt i vigtige afgrøder fra tre plantefamilier (Græs-, Bælgplante- og Natskyggefamilien). Effekten af inokuleringerne blev undersøgt for udvalgte parametre for plantevækst, populationsvækst af bladlus eller spindemider samt for udvalgte fysiologiske parametre i bladene med og uden angreb af herbivorer. Generelt blev *B. bassiana* reisoleret fra blade, stængel og rødder fra alle tre værtsplanter, *M. brunneum* blev reisoleret fra stængel og rødder, mens *M. robertsii* kun blev reisoleret fra rødderne. Sammenlignet med kontrolbehandlinger førte inokulering med *M. robertsii* til signifikant reduktion af herbivorpopulationerne, mens planter inokuleret med *M. brunneum* overraskende havde de største populationstætheder. Dette mønster blev observeret for alle tre værtsplanter. Inokulering med *B. bassiana* førte enten til reduceret eller ingen ændring i herbivorernes populationsvækst i forhold til kontrolbehandlingen. De to isolater af *Metarhizium* spp. førte desuden til øget plantevækst sammenlignet med kontrolbehandlingerne. De opnåede resultater er præsenteret i tre manuskripter forberedt til videnskabelige tidsskrifter.

I studiet præsenteret i **Manuskript I** blev aktiviteten af centrale kulhydrat- og antioxidantenzymer undersøgt i hvedeblade efter inokulering af frø og angreb af havrebladlus *Rhopalosiphum padi*. Inokulering med *M. robertsii* førte til en reduktion af bladluspopulationen, inokulering med *M. brunneum* førte til en øgning, mens *B. bassiana* ikke havde nogen effekt på bladlusene, sammenlignet med kontrolbehandlingen. Aktiviteten af flere af de udvalgte kulhydratenzymer blev øget i planter inokuleret med *Metarhizium* spp., men der var ingen klar sammenhæng med vækst i bladluspopulationerne. Aktiviteten af antioxidantenzymerne vist begrænset ændring ved svampeinokulering og ved angreb af bladlus. Dog ledte inokulering med *M. robertsii* til øget aktivitet af superoxid dismutase, men *M. brunneum* førte til øget aktivitet af enzymerne catalase og glutathione-S-transferase, som sandsynligvis spiller en rolle i forbindelse med populationsvæksten hos havrebladlus. Resultaterne indikerer derfor at reguleringen af antioxidantenzymer har en betydning for effekter på bladlus, mens kulhydratenzymerne mere sandsynligt er koblet til plantevækst.

I **Manuskript II** præsenteres et studie hvor en række sekundære plantemetabolitter fra to værtsplanter blev kvantificeret, benzoxazinoider i hvede og flavonoider i bønne, som respons på frøinokulering med insektpatogene svampe og angreb af bladlus, for at belyse metabolitternes rolle i plante-svampe-insekt interaktionerne. Hvedeplanter blev angrebet af havrebladlus, *R. padi*, mens bønne blev angrebet af *Aphis fabae*. Koncentrationerne af mere end halvdelen af de sekundære plantemetabolitter blev ændret ved inokulering med svampe og ved bladlusangreb, og ændringerne i bladlusantal var i højere grad knyttet til disse ændringer end til hvorvidt svampen etablerede sig endofyttisk i planternes overjordiske dele. Ændringer i de målte koncentrationer af metabolitter var ikke relateret til ændringer i plantevækst. Inokulering med de tre svampeisolater resulterede i sammenlignelige effekter på bladluspopulationer og ændringer i metabolitter mellem de to plantearter. Dette studie er det første til at vise en sammenhæng mellem effekter af insektpatogene svampe på bladlus og ændringer i sekundære plantemetabolitter i hvede og bønne.

Manuskript III rapporterer om ændringer i to udvalgte steroide glykoalkaloider (SGA) i tomat, kvantificeret med LC-MS, efter frøinokulering med de tre svampeisolater og angreb af spindemider, *Tetranychus urticae*, som har en anden måde af optage føde på end bladlus. Populationsvæksten af spindemider var størst på tomatplanter som var inokuleret med *M. brunneum*, hvor der også blev fundet de laveste koncentrationer af SGA i bladene sammenlignet med de andre behandlinger. Tomatplanter inokuleret med *B. bassiana* og med *M. robertsii* havde signifikant højere koncentrationer af begge SGA i bladene og havde samtidig de laveste antal af spindemider. Vi

konkluderer at inokulering med insektpatogene svampe påvirker mængden af SGA i tomat hvilket påvirker interaktionen mellem tomatplanten og spindemider. Dog viser resultaterne at sammenhængen af afhængig af isolatet af svampen.

De opnåede resultater præsenteret i denne ph.d. afhandling giver ny indsigt i de økologiske effekter af insektpatogene svampes rolle som endofytter og sammenhængen mellem disse effekter og regulering af plantfysiologiske parametre. Resultaterne udgør dermed et forbindelsesled mellem effekter og mekanismer, som kan have betydning ved anvendelse af disse svampe i plantebeskyttelse. Forståelsen af ændringer i planternes fysiologi ved inokulering med insektpatogene svampe er vigtige for deres fremtidige udnyttelse i bekæmpelse af skadedyr.

STUDY RATIONALE

Agriculture plays a substantial role in the economy of a country. It not only provides food but also the raw material for the industry. Arthropod pests have always been a major threat to agriculture. They damage crops directly (by consumption) or indirectly (by transmitting diseases), causing extensive yield loss, reduction of plant quality and sometimes death of the crop plants (van der Goes van Naters and Carlso 2006). Many pest control strategies, such as the use of chemical and biological control methods, have been established to diminish losses caused by insect herbivores. The widely recognized environmental risks associated with chemical pesticides have encouraged the development of biological alternatives such as the use of living organisms (Lacey et al. 2001). Entomopathogenic fungi (EPF) have shown great bio-control potential against sucking, chewing, above-ground and below-ground arthropod pests (Faria and Wraight 2007; Meyling and Eilenberg 2007; Meyling et al. 2011). Although EPF showed promise in research, several biotic and abiotic factors reduce the persistence of the desired effects in the field, limiting efficacy (Meyling and Eilenberg 2007; Lacey et al. 2015). Innovation in application methods could address this limitation.

In recent years, research has shown that EPF are associated with plants as endophytes (reviewed by e.g. Vidal and Jaber 2015; Vega 2018). Endophytic fungi can live asymptomatically inside plant tissues during their life cycle, as obligate or facultative symbionts (Wilson 1995). Entomopathogenic fungal endophytes (EFE) can be naturally present or experimentally inoculated in many crop plants and can play a beneficial role in plant growth promotion, disease resistance, and they often have negative effects on herbivores (Vidal and Jaber 2015; Jaber and Enkerli 2017; Vega 2018; Barra-Bucarei et al. 2020). It has been hypothesized that EFE have a mutualistic relationship with plants by transferring insect-derived nitrogen to plants in return to photosynthates (Behie et al. 2012, 2017). Although EFE have been implicated in several beneficial effects in plants, the mechanisms behind their ecological effects are still largely unknown.

Most of the studies of EFE reporting negative effects on insect herbivores did not demonstrate that conidia were produced from inoculated plant tissue surfaces or that mycosis developed in insects feeding on the inoculated plants (Vega 2018). This supports the notion of an indirect, rather than a direct, mode of action of EFE against arthropod herbivores. One crucial facet is the ecological importance of physiological changes *in planta* after the endophytic colonization by EPF. The production of fungal secondary metabolites (FSMs) after the endophytic colonization by EPF has been reported in a few studies (Golo et al. 2014; Ríos-Moreno et al. 2016). Both plant and fungal secondary metabolites are considered to effect herbivore-plant interaction (Ludwig-Müller 2015;

Jaber and Ownley 2018). Nevertheless, the observations that most of the studies reported reduced population growth of arthropod herbivores rather than mortality and that negative effects on herbivores are observed even when colonization rates of EFE are low, suggests that the effects are mediated indirectly by plant secondary metabolites (PSMs). However, there is no study addressing changes in plant biochemical profiles associated with EFE colonization e.g. enzymes and bioactive PSMs, and their related impact on herbivores. The consideration of the mechanisms behind the EFE mode of action against insect herbivores is important to boost EFE utilization in biological control systems.

To better understand how EFE may alter the profiles of specific PSMs and what role these bioactive compounds play in plant-fungal-herbivore interactions, studies were designed using the same EPF isolates in different plants and against different insect herbivores. The results of the same EPF isolates across plant families and the changed activities of specific PSMs can provide important information on the role of biochemical changes affecting insect herbivory *in planta* after fungal inoculations. The research reported in this thesis work was designed to provide new insights into endophytic research of how EFE may change plant physiological processes leading to ecological effects and test the hypothesis that chemical changes in plants after fungal inoculations constitute the main mode of action against arthropod herbivores.

AIM OF THE STUDY

The main aim of this thesis is to advance the knowledge of the effects of plant inoculations with EPF and evaluating the potential mechanisms related to these effects on host plant growth and population growth of insect herbivores, with an emphasis on changes in plant physiological responses following EPF inoculation and to compare these responses to the effects on arthropod herbivores.

The following research questions were investigated:

- How will three isolates of different species of EPF (*Beauveria bassiana*, *Metarhizium brunneum* and *M. robertsii*) (a) colonize different host plants (wheat, bean and tomato) after seed treatments, (b) affect population growth of arthropod herbivores (aphids and spider mites) and (c) affect host plant growth across plant families?
- How will EPF colonization affect selected plant physiological responses, specifically the activities of key carbohydrate and antioxidant enzymes of wheat and specific plant secondary metabolites (benzoxazinoids in wheat, flavonoids in bean and steroidal glycoalkaloids in tomato)?
- What are the relationships between these plant physiological responses in the host plants and on the population growth of arthropod herbivores after seed inoculation with EPF?

HYPOTHESES

- The isolates of *B. bassiana* and *Metarhizium* spp. will display isolate specific colonization patterns in plant tissues in a similar manner among the three host plants.
- The EPF isolates will negatively affect the population growth of aphids and spider mites after seed inoculations.
- EPF inoculations will increase the growth of the inoculated plants in comparison to uninoculated plants in an isolate specific manner.
- The activities of antioxidant and carbohydrate enzymes in wheat will be modified after EPF inoculations and aphid feeding to reduce oxidative stress caused by herbivory and to improve plant growth, respectively.
- The levels of related plant secondary metabolites will be changed in plant tissues after EPF inoculations and the change will be related to negative effects in arthropod herbivores in wheat, bean and tomato.

MODEL SYSTEM AND THESIS OUTLINE

The thesis research shed light on different aspects of seed-inoculated entomopathogenic fungi, including endophytic colonization ability, plant growth effects, defense against herbivores and biochemical changes in several crop plants. Three fungal isolates were study namely, *B. bassiana* strain GHA (obtained from BotaniGard®), *M. robertsii* strain ESALQ 1622 (isolated from the soil of a cornfield, Mato Grosso, Brazil) and *M. brunneum* strain KVL 04-57 obtained from infected larvae of *Cydia pomonella* (same isolation origin as active ingredient of Met52, Novozymes, Salam, VA). The fungal isolates were inoculated through seed treatments in three model plants, wheat - *Triticum aestivum* (**Manuscript I and II**), bean - *Phaseolus vulgaris* (**Manuscript II**) and tomato - *Solanum lycopersicum* (**Manuscript III**). The endophytic colonization was evaluated via culture-dependent methods by plating sterilized plant tissues (leaf, stem and root) on selective media. The growth promotion was evaluated by measuring plant parameters including plant height, fresh and dry weights of root and shoot. Effects of seed inoculation on population growth of aphids – bird cherry-oat aphid (*Rhopalosiphum padi*) in wheat, black bean aphid (*Aphis fabae*) in bean and two-spotted spider mites (*Tetranychus urticae*) in tomato were evaluated. Finally, levels of bioactive plant secondary metabolites, benzoxazinoids in wheat and flavonoids in bean leaves (**Manuscript II**), steroidal glycoalkaloids in tomato leaves (**Manuscript III**) and levels of key carbohydrate and antioxidant enzymes in wheat leaves (**Manuscript I)** were analyzed after EPF colonization and herbivore feeding (Fig 1).

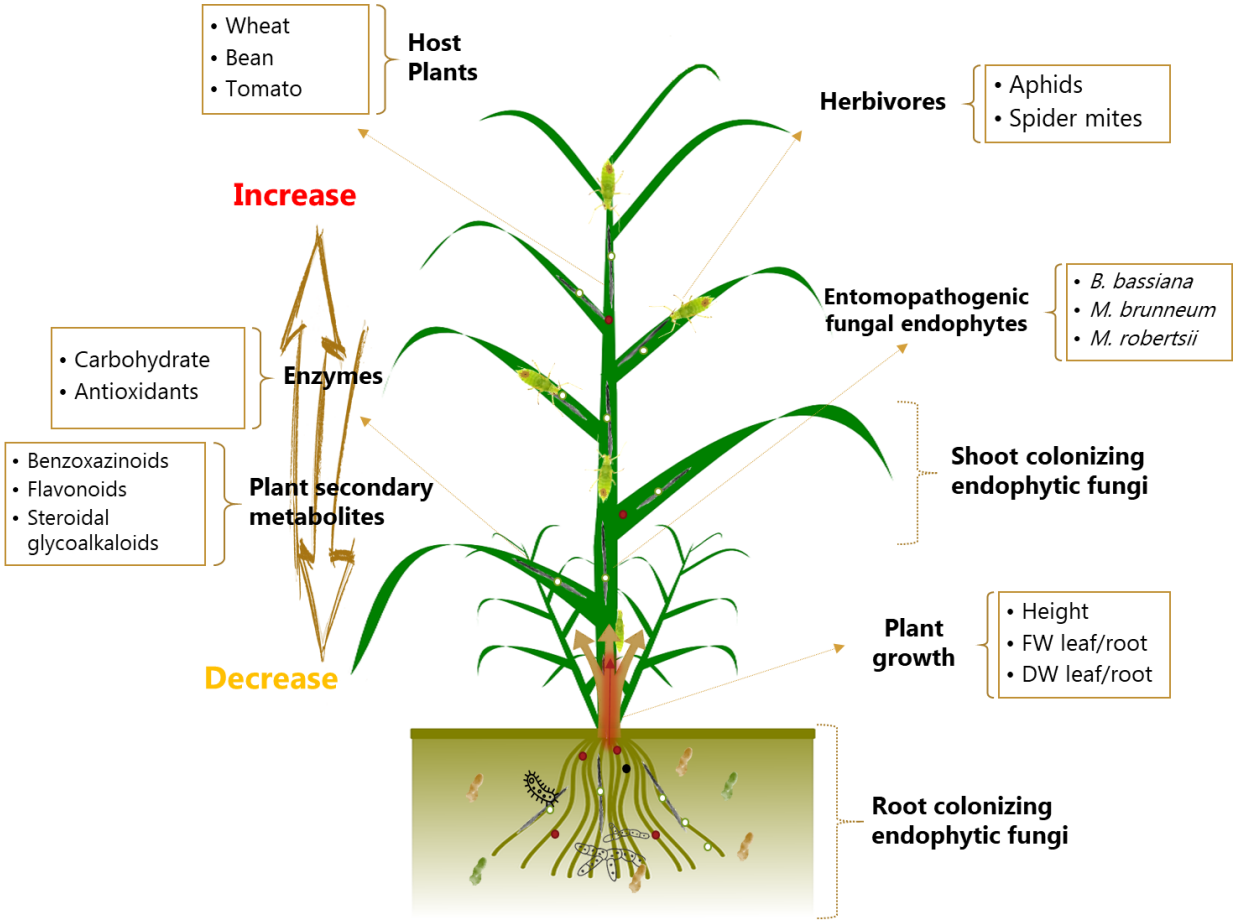

Figure 1. Model study system. Entomopathogenic endophytic fungi (*Beauveria bassiana*, *Metarhizium brunneum* and *M. robertsii*) are expected to colonize shoot (leaf and stem) and root of different plants hosts (wheat, bean and tomato), affect plant growth parameters (height and fresh/dry weight of root/shoot), affecting herbivore (aphid and spider mites) population growth and changes plant secondary metabolite (benzoxazinoids, flavonoids and steroidal glycoalkaloids) and enzyme (carbohydrate and antioxidants) profiles.

1. INTRODUCTION

Endophytic *Metarhizium brunneum* from tomato stem on selective media

1.1 Entomopathogenic Fungi

Entomopathogenic fungi (EPF) are ubiquitous natural enemies of insects in agroecosystems (Meyling and Eilenberg 2007; Lacey et al. 2015). Among the more than 5.1 million estimated species in the Kingdom Fungi (Brien et al. 2005), around 750-1000 fungal species are insect pathogenic (Vega et al. 2012), and 170 commercial products have been developed from 12 fungal species for pest control in agriculture (Faria and Wraight 2007). For infection, dispersed asexual infective spores (conidia) land on the cuticle of the host, attach and germinate initiating enzyme activation reactions from both fungi and the susceptible host (Zimmermann 2007). Fungi have evolved special mechanisms for entering insects by enzymatic degradation of the cuticle (Vega et al. 2009). After passing through the cuticle, the fungus invades the host body obtaining nutrients and causing the death of the host insect in 3-7 days due to starvation of the host and production of secondary metabolites (Shah and Pell 2003). Sporulation occurs outside the cadaver and new infective conidia are produced for transmission by dispersing in the environment (Shah and Pell 2003; Roy et al. 2006).

Many species of the order Hypocreales (phylum Ascomycota) are important components of agroecosystems and ubiquitous inhabitants of soils as they have been recovered from wide ranges of ecological and geographic zones (Meyling et al. 2009; Lacey et al. 2015). The distribution of *Metarhizium* spp. (Hypocreales: Clavicipitaceae) and *Beauveria* spp. (Hypocreales: Cordycipitaceae) have been extensively studied, with *Metarhizium* spp. preferentially affecting below ground arthropods while *Beauveria* spp. are mostly associated with above-ground insect pests in temperate climates (Meyling et al. 2011). Members of these genera generally exhibit broad host ranges, as *B. bassiana* has been found to naturally infecting > 700 host species (Inglis et al. 2001). It is thought to be associated with almost every major insect taxon in temperate regions (Meyling and Eilenberg 2007). Species of the genus *Metarhizium* have been investigated for years due to their promising potential as biocontrol agents against several pest species (Zimmermann 2007). Furthermore, *Metarhizium* spp. have also been investigated as root colonizers with the potential to improve plant growth by nutrient acquisition (Behie and Bidochka 2014a; Keyser et al. 2016). The recent upsurge of research has focused on the symbiotic endophytic associations of EPF with plant hosts and their potential beneficial effects such as against insect herbivores (Vega et al. 2008; Vega 2018).

Glossary of terms

Entomopathogenic fungus (EPF)	A fungus that infects insects and causes mortality.
Endophytic fungus	A fungus living inside plant tissues asymptomatically
Entomopathogenic endophytic fungus (EFE)	An entomopathogenic fungus which can also colonize plant tissues without any apparent symptoms of disease.
Obligate endophytic fungus	An endophyte which depends entirely on their host plants to fulfill its life cycle and has no free-living life stage.
Facultative endophytic fungus	An endophyte which does not depend entirely on a single host and has free-living life stage outside of plant host.
Vertical transmission of endophytes	The transmission of endophytes from one host (one generation) to another (second generation) via seeds or vegetative structures.
Horizontal transmission of endophytes	The transmission of endophytes from one infected host to another dispersed spores.
Systemic colonization of endophytes	Colonization of endophytic fungus throughout the plant. Distant colonization from the point of inoculation.
Localized colonization of endophytes	Non-systemic colonization localized in specific plant organs or tissues that were inoculated.

1.2 Endophytic fungi

Most land plants (> 90 % of known species) form symbiotic relationships with mycorrhizal (endo/ectomycorrhizal) or endophytic fungi (Behie et al. 2013). The term “endophyte” was coined by a German scientist Heinrich Anton De Barry (1884) and is used for microorganisms, mainly bacteria and fungi, which occur inside living plant tissues without any apparent symptoms of the disease for all or a part of their life cycles (Wilson 1995). Endophytes display a great diversity in host plant specificity, host tissue specificity (e.g. leaf, stem and root), transmission routes to new hosts

and phylogeny (Porras-Alfaro and Bayman 2011). The vertically-transmitted grass endophytes within the genus *Epichloë* (anamorph: *Neotyphodium*) have received most attention from researchers as they confer potential benefits for the host plants (Kuldau and Bacon 2008; Rodriguez et al. 2009; Jaber and Ownley 2018). Whereas horizontally-transmitted endophytes, which are dominated by Ascomycetes with several insect pathogenic genera (Ascomycota: Hypocreales), have received less attention (Arnold and Lutzoni 2007; Jaber and Ownley 2018).

1.3 Entomopathogenic endophytic fungi

Entomopathogenic fungi have been studied traditionally as insect pathogens, although the recent increase in research has uncovered their additional role as plant endophytes in nature. The phylogenetic history of the ascomycete entomopathogenic fungi links them to plant endophytes irrespective of their potential as biocontrol agents (Gao et al. 2011). The entomopathogenic fungal endophytes (EFE) have been isolated from various plant species as natural endophytes and have been experimentally inoculated in many host plants to evaluate their potential roles in plant protection (Vega 2008). There are increasing evidence that these EFE have effects as plant growth promoters, and against plant pathogens and arthropod pests (Vega et al. 2009; Jaber and Enkerli 2017; Jaber and Ownley 2018; Barra-Bucarei et al. 2020). A recent meta-analysis by Gange *et al.* (2019) revealed that EPF in general cause negative impacts on herbivores across insect taxa and feeding guilds after endophytic colonization in different plant families.

The fungus-plant interaction could be beneficial, neutral or antagonistic for both the partners depending upon the EPF isolates and plant species (Vidal and Jaber 2015). Among EPF, species of *Metarhizium* and *Beauveria* have received most attention and have successfully been established as endophytes experimentally using different inoculation techniques in different crop plants (Vega 2018; Jaber and Ownley 2018). *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) is well studied for its ability to colonize different plant species naturally or by artificial inoculations (Vega 2008) resulting in protection against insect herbivores (Vidal and Jaber 2015). In contrast, species of the genus *Metarhizium* are less characterized as plant inoculants (Jaber and Enkerli 2016) and isolates of *Metarhizium* spp. have shown both negative and positive effects against insect herbivores (Clifton et al. 2018; Canassa et al. 2019b).

1.3.1 Plant colonization by EFE

The ability of entomopathogenic fungi to colonize plant tissues symptomlessly is now well established (Vidal and Jaber 2015), both naturally and by artificial inoculation. These endophytic

associations indicate a complex lifestyle of EPF species, which can include invertebrate hosts, free living in the soil environment, or utilizing plant hosts as facultative endophytes (Allegrucci et al. 2017). *B. bassiana* has been isolated from several plant species naturally and inoculated artificially by using different inoculation methods in many host plants including; cotton (*Gossypium hirsutum*), banana (*Musa* spp.), corn (*Zea mays*), sorghum (*Sorghum bicolor*), coffee (*Coffea arabica*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*), bean (*Vicia faba*) and wheat (*Triticum aestivum*) (Reviewed by Vega 2018). Seed treatments and root inoculations with different *Metarhizium* spp. led to the successful colonization of different plant parts of soybean, wheat, switchgrass (*Panicum virgatum*), bean and maize (Sasan and Bidochka 2012; Clifton et al. 2018; Jaber 2018; Canassa et al. 2019b; Ahmad et al. 2020).

Fungal localization in plant tissues (leaf, stem and root), transmission to other hosts and fungal structures colonizing the host plants are important phenomenons to discuss in relation to the endophytic colonization ability of entomopathogenic fungi. In a study to evaluate the plant tissue localization of naturally occurring EPF, *Metarhizium* spp. were entirely found in roots while *B. bassiana* was found all over the host plant (Behie et al. 2015). However, few studies also showed the above-ground colonization of plants with *Metarhiuzm* spp. (Clifton et al. 2018; Jaber 2018). Likewise, seed inoculation with *B. bassiana* colonized all plant parts, *M. brunneum* colonized stem and roots while *M. robertsii* colonized mostly roots in wheat, bean and tomato plants (**Manuscript I, II and III**). The ability of *Metarhizium* spp. to colonize plant roots is dependent on plant species, environmental factors, evaluation techniques as well as fungal species and strains (Behie et al. 2015; Barelli et al. 2018). The above-ground colonization of plant tissues after seed inoculations with *B. bassiana* and in few reports with *Metarhizium* spp. confirms the systemic colonization ability of EFE (Vega 2018). The colonization specificity of EPF in host plant parts could be dependent upon the biochemical and physiological properties of host tissues (Tefera and Vidal 2009; Jaber and Enkerli 2016).

For endophytic detection of EPF, different culture-dependent (plating on culture media) and independent techniques (molecular detection methodologies e.g. PCR) have been used (McKinnon et al. 2017). Although PCR based detection is more sensitive, most of the reported studies used culture-dependent techniques for endophytic detection (Tefera and Vidal 2009; Gurulingappa et al. 2010; Mantzoukas et al. 2015; Mutune et al. 2016; Allegrucci et al. 2017; Canassa et al. 2019b; Ahmad et al. 2020). In a study to evaluate endophytic colonization, Quesada-Moraga et al. (2006) combined microbiological, molecular and microscopic methods to re-isolate and detected a strain of *B. bassiana*

(EABb 04/01-Tip) from the opium poppy, *Papaver somniferum* L., leaves inoculated by foliar spray. The hyphae of *B. bassiana* were detected in xylem vessels. Likewise, Landa et al. (2013) detected the same strain in intercellular spaces of aerial plant parts of *P. somniferum* by qPCR assay and confocal laser scanning microscopy. In an *in vitro* study, Sasan and Bidochka (2012) used light and confocal microscopy to show that *M. robertsii* conidia first adhere, then germinate and finally colonize the plant roots. These reports together with the present findings demonstrate that among EPF species *M. robertsii* showed more restricted colonization into roots while other tested *Metarhizium* species and *B. bassiana* also colonized above-ground plant parts. However, the methods of inoculation and detection techniques are worth considering when concluding on EPF colonization abilities (see also McKinnon et al. 2017). The combination of multiple endophytic detection techniques should be used where culture-dependent methods are the most straightforward, informing on viable fungal endophytes, while PCR and qPCR techniques are often more sensitive though not indicating whether the fungal material *in planta* constitutes living cells, and finally microscopic techniques are useful for the visual detection of specific colonizing plant parts and localization of fungal structures.

Although non-grass endophytic fungi are assumed to be exclusively transmitted horizontally (external penetration to host tissues after environmental spore dispersal; Carroll 1988), *B. bassiana* has been also reported to be vertically transmitted as an endophyte through infected seeds produced by the host plants including Monterey pine (*Pinus radiate*) and wheat (Lefort et al. 2016; Sánchez-Rodríguez et al. 2018). Various methods have been used for artificial inoculations of EPF in host plants, such as root dip, soil drench, foliage spraying and seed treatments (Tefera and Vidal 2009; Akello and Sikora 2012; Kasambala Donga et al. 2018; Canassa et al. 2019a). Seed inoculation by *B. bassiana* mostly result in endophytic colonization of above-ground plant parts effectively and cause effects against insect herbivores and effectively promote plant growth (Akello and Sikora 2012; Jaber and Ownley 2018; Canassa et al. 2019b).

In the present studies, seed inoculations with different EPF species resulted in the endophytic colonization of different plant families (**Manuscript I, II and III**). Seed treatment for endophytic colonization is considered the more reliable method as compared to spraying, which requires more fungal material and has more chances to degrade faster due to UV radiation etc. Interestingly, spraying of EPF species resulted in restricted colonization in the treated area for a short period in most of the studies (Gurulingappa et al. 2010; Biswas et al. 2012; Batta 2013) whereas, seed treatments caused systemic colonization of different plant parts (Jaber and Enkerli 2016; Jaber 2018) (**Manuscript I, II and III**). The duration of the seed treatment is also a very important factor to

consider, as it could affect the germination rate, growth and colonization percentages of the plants (Jaber and Enkerli 2016). In the present studies, depending upon the germination success, the seed treatment duration for wheat and tomato was 24 h while for bean was 2 h (**Manuscript I, II and III**). Pilot experiments showed that the germination rates of wheat and tomato seeds were increased after 24 h of seed treatments while the germination success of bean seeds immersed for 24 h was decreased but remained unaffected by 2 h of immersion. The most prominent reason behind the connection between seed treatment duration and germination rate is likely to be the size of the seeds, where larger seeds (e.g. bean) endured for a shorter time in suspensions, while smaller size seeds (e.g. wheat and tomato) showed a positive correlation. This hypothesis was not explored further, however.

1.3.2 Effects of EFE against arthropod herbivores

The endophytic associations of EFE with many plant species provide an indirect link between fungi and insect herbivores, often leading to negative effects on plant antagonists. The consequences of EFE on arthropod herbivores depends on growth conditions, interaction with other microorganisms and host attributes (Vidal and Jaber 2015). The insect pathogenicity and endophytic capacity of EPF are often considered mutually beneficial, as the fungus can transfer insect-derived nitrogen to the host plant (Behie and Bidochka 2014b; Behie et al. 2015), while the fungus can benefit from the carbon in the root exudates (Behie et al. 2017). The two most important hypocrealean fungal genera, *Beauveria* and *Metarhizium*, have great potential to infect insect herbivores, but more attention has been given to *B. bassiana* as plant endophytes to affect arthropod pests than has *Metarhizium* spp. (Vega 2018).

Treatments with *B. bassiana* reduced the growth and reproduction rate of vine mealybug, *Planococcus ficus* Signoret (Homoptera: Pseudococcidae) in grapevine (Rondot and Reineke 2018); tomato fruitworm, *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) (Powell et al. 2009) and two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae) in tomato (**Manuscript III**), cotton leaf-worm, *Spodoptera littoralis* larvae (Sánchez-Rodríguez et al. 2018) and bird cherry-oat aphid, *Rhopalosiphum padi* (Hemiptera: Aphididae) in wheat (**Manuscript I and II**); banana weevil, *Cosmopolites sordidus* Germar (Coleoptera: Curculionidae) in banana (Akello et al. 2008); cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) and Australian plague locust, *Chortoicetes terminifera* Walker (Orthoptera: Acrididae) in 6 different plant species (Gurulingappa et al. 2010). The seed treatments with *B. bassiana* and *Metarhizium robertsii* J.F. Bisch., Rehner & Humber decreased the population growth of *T. urticae* in bean under the greenhouse conditions (Canassa et al., 2019) and in strawberry in field conditions (Canassa et al. 2019a). While McKinnon et al. (2017)

and Gange et al. (2019) found negative effects of *B. bassiana* on aphids (phloem feeders), there are also reports of neutral or positive effects. The *B. bassiana* strain GHA showed no significant differences from control plants against soybean aphid (*Aphis glycine*) when inoculated on seeds of soybean (*Glycine max*; Clifton et al. 2018), while this strain increased the fecundity of second-generation black bean aphid (*Aphis fabae*) in fava beans (*V. faba*) after seed inoculation and leaf spraying (Jensen et al. 2019). Seed treatments with the same *B. bassiana* strain GHA showed negative effects on *A. fabae* in beans (**Manuscript II**) while it showed no differences against *R. padi* and *T. urticae* in wheat and tomato, respectively, compared to uninoculated plants (**Manuscript I, II and III**).

Metarhizium spp. also showed erratic performances against insect pests by either enhancing (Clifton et al. 2018) or reducing the population growth of insect herbivores (Canassa et al. 2019b). In a study conducted by Akello and Sikora (2012), *M. anisopliae* (Metchinikoff) Sorokin showed no effect against pea aphid (*Acyrthosiphon pisum* Harris) or bean aphid on fava bean, *Vicia faba* L. (Fabales: Fabaceae). An isolate of *M. brunneum* (Petch) KVL 04-57 increased the population growth of *R. padi* in wheat (**Manuscript I and II**), *A. fabae* in bean (**Manuscript II**) and *T. urticae* in tomato (**Manuscript III**). The hypothesized reasons for high reproduction rates of insect herbivores on fungal inoculated host plants have been 1) EPF reduced the general plant defense, 2) increased host plant quality or 3) insects invest more in reproduction due to stress (Clifton et al. 2018; Jensen et al. 2019). There is growing evidence regarding reduced damage of insect herbivores after the endophytic colonization of entomopathogenic fungi, but the unraveling of the mechanisms behind these reported effects is still the main challenge in EFE research.

1.3.3 Plant responses to EFE

Plant growth promotion mediated by isolates of different EPF genera applied via seed treatments, foliar spraying, root inoculations and soil drench methods has been demonstrated in different studies e.g. (Gurulingappa et al. 2010; Posadas et al. 2011; Jaber and Enkerli 2016, 2017). Seed inoculations using isolates of *B. bassiana*, *M. brunneum* and *M. robertsii* promoted different growth parameters in wheat (Jaber 2018) (**Manuscript I and II**), bean (Canassa et al. 2019b) (**Manuscript II**), tomato (**Manuscript III**) and maize (Ahmad et al. 2020). Plant height, biomass and number of leaf pairs were significantly enhanced by increasing the duration of seed treatment in broad beans (Jaber and Enkerli 2016). In a recent study, a positive correlation between the endophytic colonization rate and plant growth promotion was found in maize after seed inoculations with *M. robertsii* (Ahmad et al. 2020). The growth promotion effects of *Z. mays* seed treated with *B. bassiana* are associated with the

availability of abundant nutrients in the soil (Tall and Meyling 2018) suggesting that the effects are context dependent.

In a mutualistic relationship between the plant host and the fungus, the EPF receives photosynthates and returns nitrogen from insect cadavers to the host plant (Behie et al. 2017). Nitrogen is the most important nutrient for plant functionality but it is also a limiting nutrient for plants in temperate soils with a substantial loss due to insect herbivores (Brant and Chen 2015; Barelli et al. 2019). Interestingly, different EPF, including *Metarhizium* species with both broad and narrow host ranges and *B. bassiana*, were found to transfer insect-derived nitrogen to various host plants (Behie et al. 2012; Behie and Bidochka 2014a), indicating that this nutrient transfer between EPF and fungal associations could be the ubiquitous trait of these fungi that results in growth promotion in colonized plants. However, nutrient availability for host plant growth promotion is important to consider. A positive growth promotion effect has been found in experiments conducted under scare nutrient conditions (Jaber and Enkerli 2016, 2017; Jaber 2018), while also found dependent on high nutrient availability (Tall and Meyling 2018).

In the present studies, seed inoculation with both *Metarhizium* spp. resulted in higher plant growth in comparison to *B. bassiana* and uninoculated control plants (**Manuscript I, II and III**). Liao et al. (2014) suggested that irrespective of their role as entomopathogens, *Metarhizium* spp. are plant growth promoters and their beneficial effects are associated with their ability to colonize plant roots. An isolate of *M. robertsii* was found to produce indole-3- acetic acid (auxin), which plays a crucial role in growth promotion effects (Liao et al. 2017). Additionally, EPF isolates most prominently *M. brunneum* (EAMa 01/58–Su) were also found to increase Fe bioavailability in Fe containing calcareous and non-calcareous media (Raya-Díaz et al. 2017). In the same study, different inoculation techniques in sorghum plants enhanced Fe content of above-ground plant parts and improved root length, fine roots and leaf chlorophyll content (Raya-Díaz et al. 2017). The bioavailability of different essential nutrients could improve plant growth and development, however, it is still unknown if EPF can increase the uptake of other nutrients to improve plant growth.

1.4 Enzyme systems affected by fungal inoculations

Enzymes are biological macromolecules known as “biocatalysts” produced in living organisms to catalyze specific biochemical reactions (Khare and Yadav 2017). Some enzymes are “plant batteries”, protecting against adverse environmental conditions and other harmful organisms. A part of a plant’s defense against insect pests is through enzymes that harm digestive processes in the insect gut.

Carbohydrate and antioxidant enzymes are widely studied for their role in host plant growth and development, and as plant signaling molecules against numerous abiotic and biotic stresses to mediate defense responses in host plants (Gill and Tuteja 2010; Jammer et al. 2015). Endophytic fungi have been found to induce antioxidant enzyme production, leading to improved growth and maintenance of oxidative stress in plants (Hamilton et al. 2012). The role to plant enzymes (especially carbohydrate and antioxidant enzymes) after endophytic colonization by EPF and infestation by insect herbivores has not yet been studied. In **Manuscript I**, different carbohydrate and antioxidant enzymes after seed treatments with three EPF isolates, *B. bassiana*, *M. brunneum* and *M. robertsii* following *R. padi* infestations in wheat were analyzed. The activities of most carbohydrates and few antioxidant enzymes were significantly affected by EPF inoculations and aphid feeding (**Manuscript I**).

1.4.1 Carbohydrate enzymes

Carbohydrates including sucrose, glucose and fructose, not only regulate many developmental processes in the plant life cycle but also act as signaling molecules in plants (Gibson 2005; Halford et al. 2011). Enzymes are involved in carbohydrate production, regulation and dissemination from source (site of production, leaves) to sink (sites of consumption, new leaves, root and shoot) tissues, ultimately determining plant health and response to external stress factors (Jammer et al. 2015). Sucrose is a central molecule for plant metabolism, which undergoes hydrolytic irreversible cleavage by vacuolar (vacInv, in vacuole), cell wall (cwInv, in apoplast) and cytoplasmic invertases (cytInv, in the cytoplasm) into hexoses (glucose and fructose; Fig. 2; Roitsch and González 2004). Cell wall and vacInv are key metabolic enzymes producing nutritional and signaling molecules (help plants to respond to various external stimuli like stress and pathogen invasion), while the role of cytInv is not very well known (Roitsch and González 2004). Induced biosynthesis of cwInv in plant-pathogen interactions has been reported (Berger et al. 2007). Several biotic and abiotic stressors influence hexose/sucrose ratio, for example, pathogenic fungi utilize host carbohydrates through sophisticated structures leading to the downregulation of source metabolism (Wingler and Roitsch 2008). Likewise, in a symbiotic relationship with host plants, EFE are assumed to receive photosynthates from plants in return of nitrogen (Behie et al. 2017). This association of the fungus with the host plant could affect primary metabolism positively or negatively.

Enzymes involved in carbohydrate metabolism perform the following activities in plants: Invertases (cwInv, cytInv and vacInv) and sucrose synthase (Susy) are involved in sucrolytic activity (the breakdown of sucrose), fructokinase (FK) converts fructose to fructose-6-phosphate and is important for sucrose biosynthesis in reversible reactions; hexokinase (HXK), aldolase (Ald),

phosphoglucoisomerase (PGI) and phosphofructokinase (PFK) are essential for glycolysis; glucose-6-phosphate dehydrogenase (G6PDH) works in oxidative pentose phosphate pathway; phosphoglucomutase (PGM) and ADP-glucose pyrophosphorylase (AGPase) regulate starch biosynthesis and UDP-glucose pyrophosphorylase (UGPase) is vital for cell wall biosynthesis (Halford et al. 2011; Jammer et al. 2015; Fig. 2). All the above-mentioned enzymes are vital for plant growth and development, but the role of PFK is less clear (Jammer et al. 2015). Phytopathogens induce a series of changes, including a decrease in photosynthesis and an increase in respiration and invertases activity in plants (Berger et al. 2007). The induced changes by plant pathogens in primary and secondary metabolism ultimately affect the defense and developmental growth of the host plant (Berger et al. 2007).

In the recent increase in endophytic research regarding EPF, it is important to focus on plant physiological processes, such as identifying the activities of these carbohydrate enzymes in relation to fungus colonization, plant growth and insect herbivore performances. To test this, wheat seeds were treated with three EPF spp. isolates, *B. bassiana*, *M. brunneum* and *M. robertsii* and infested with or without *R. padi* to measure the activities of key carbohydrate enzymes (**Manuscript I**). Most carbohydrate enzymes showed higher activities in fungus inoculated plants as compared to uninoculated plants. However, the activities of cwInv and HXK were increased while of vacInv were decreased with *R. padi* feeding.

It has been suggested that enhanced primary metabolism support plants in defense against pathogens by fulfilling cellular energy requirements (Bolton 2009). However, in the present study, *M. brunneum*, which produced the highest number of *R. padi*, also increased the activities of most enzymes. This shows that high activities may play a role in fungus-plant symbiosis and plant growth but not in defense against biotic stress (**Manuscript I**). The slight changes due to *R. padi* infestations could be because of the feeding behavior of aphids causing minimal damage to plant tissues (Fig 5) (Züst and Agrawal 2016). As the analysis was only performed for source tissues further investigations are needed to evaluate the activities in sink tissues (e.g. roots and stem), where *Metarhizium* spp. showed higher colonization frequency. In addition, knowledge about the regulation of invertases in source and sink tissues is important, because it may determine the levels of carbon available or utilized for endophytic fungi.

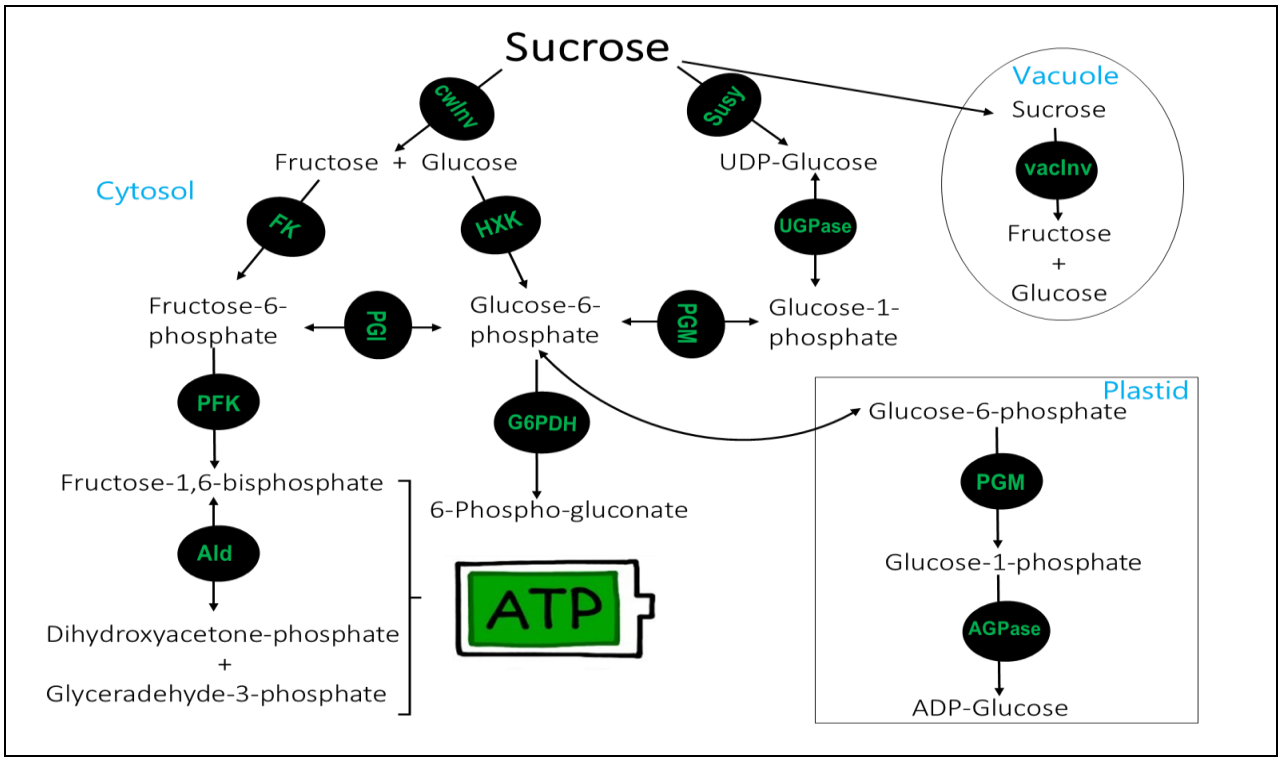

Figure 2. Simplified schematic representation of key enzymes involved in carbohydrate metabolism. cwInv - cell wall invertases, vacInv - vacuolar invertases, Susy - sucrose synthase, FK - fructokinase, HXK - hexokinase, PGM - phosphoglucomutase, PGI - phosphoglucoisomerase, UGPase - UDP-glucose pyrophosphorylase, AGPase - ADP-glucose pyrophosphorylase, PFK- phosphofructokinase, G6PDH - glucose-6-phosphate dehydrogenase and Ald - aldolase. Modified from (Jammer et al. 2015). All mentioned enzymes except Susy were analyzed in **Manuscript I**.

1.4.2 Antioxidant enzymes

Various plant metabolic pathways are known to produce reactive oxygen species (ROS) as by-products mainly localized in peroxisome, mitochondrion and chloroplast (Fig. 3 and 4; Gill and Tuteja 2010). ROS is mainly comprised of free radicals (superoxide - $O_2^{\bullet -}$ and hydroxyl radical - $OH^{\bullet}$) and non-radicals (hydrogen peroxide - H_2O_2 and singlet oxygen - $^1O_2$), which act as signaling molecules controlling processes such as plant growth and development, response to abiotic and biotic stresses and programmed cell death (Das and Roychoudhury 2014). However, overproduction of ROS in response to various stimuli (biotic/abiotic stress) leads to oxidative stress in plants. The resulting radicals are extremely toxic causing damage to carbohydrates, lipids, proteins and DNA leading to cell death (Das and Roychoudhury 2014). Therefore, a balance between ROS production and degradation is important to uphold a state of redox homeostasis (Fig 3), an equilibrium between ROS generation and elimination in plants (Concept et al. 2005).

Plants sustain redox homeostasis during stress via antioxidative enzyme systems consisting of enzymatic and non-enzymatic antioxidants (Fig 3). Enzymatic components include superoxide

dismutase (SOD), peroxidase (POD), glutathione-S-transferase (GST) glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and catalase (CAT), while non-enzymatic low molecular components are ascorbic acid, α-tocopherol, carotenoids, reduced glutathione, phenolics, proline and flavonoids (Miller et al. 2010; Das and Roychoudhury 2014). Metalloenzyme SOD (with metal co-factors) provides first-line defense against toxic ROS by scavenging $O_2^{\bullet-}$ into H_2O_2 and O_2 and improve plant tolerance to stress. Catalases are involved in the dismutation of H_2O_2 into H_2O and O_2 while POD reduces H_2O_2 with different reductants such as phenolic compounds (Fig 3; Gajewska et al. 2006). Other enzymes, including GR, DHAR and MDHAR, are involved in the ascorbate-glutathione cycle are also important to maintain redox homeostasis in plants (Gill and Tuteja 2010).

ROS and their scavenging enzymatic antioxidants are involved in different stress factors like salinity, drought, heavy metals, temperature and pathogens and pests attack (Dat et al. 2000; Mittler 2002; Das and Roychoudhury 2014). Caverzan et al. (2016) reviewed antioxidant responses of different wheat cultivars under several stressors, where SOD, CAT, POD and GR showed induction in most studies in response to external stimuli. Although ROS has shown an explicit role in plant responses to insect herbivory (Kerchev et al. 2012), plants need to maintain a state of redox homeostasis to tolerate stresses inflicted by pests such as aphids (Smith and Boyko 2007). The induction of antioxidants within the insect body and in infested plant hosts to detoxify excess ROS concentrations have been reported (Kerchev et al. 2012).

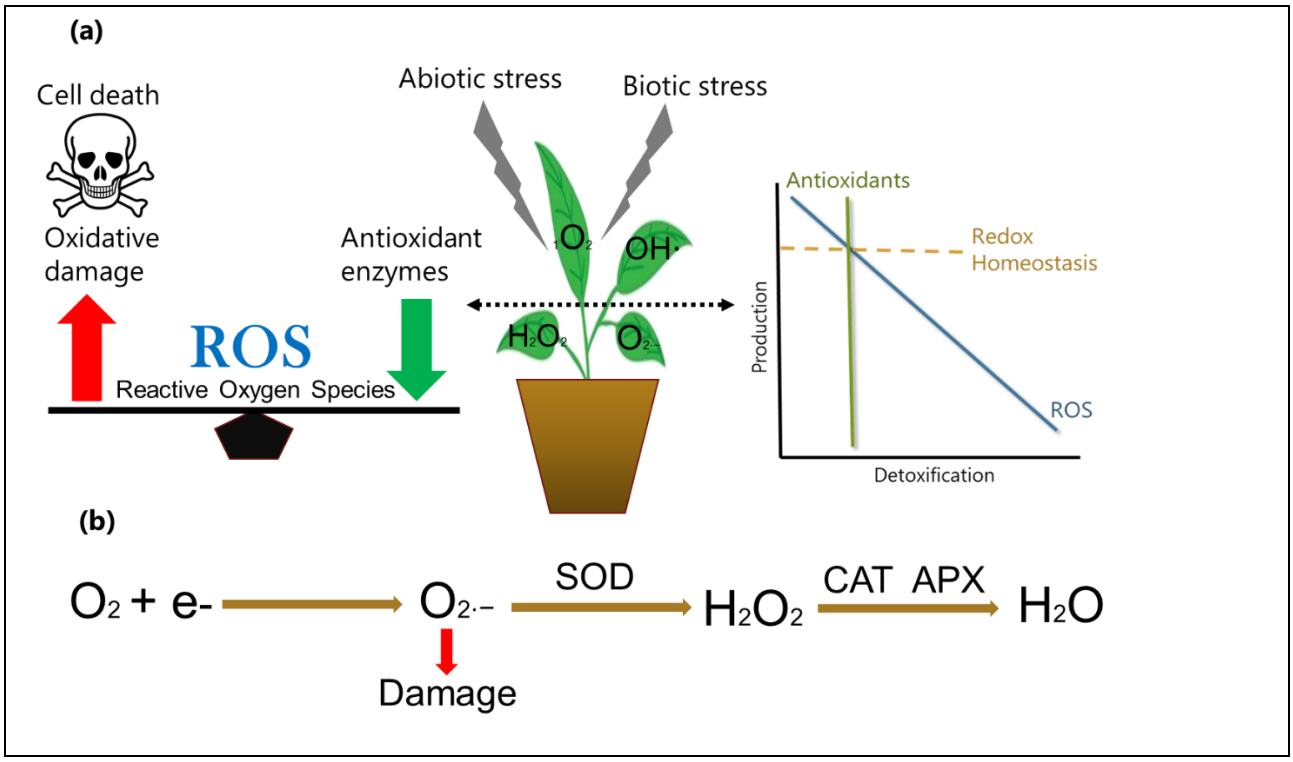

Figure 3. Reactive oxygen species (ROS) production and detoxification by antioxidant enzymes. (a) Plants produce ROS (superoxide - $O_2^{\bullet-}$, hydroxyl radical - $OH^{\bullet}$, hydrogen peroxide - H_2O_2 and singlet oxygen - $^1O_2$) when exposed to any biotic and abiotic stress. The overproduction of ROS can cause oxidative damage in plant tissues leading to cell death (showed by the red arrow). To avoid this oxidative damage in plant tissues antioxidant enzymes are produced to detoxify ROS (showed by the green arrow) to maintain a state of balance between production and detoxification of ROS (redox homeostasis). (b) For detoxification, the antioxidative enzyme, superoxide dismutase (SOD) converts $O_2^{\bullet-}$ into H_2O_2 while catalase (CAT) and ascorbate peroxidase (APX) converts H_2O_2 into H_2O and O_2 to remove oxidative stress in plants during stress.

Antioxidants transmit stress stimuli and help the plant to differentiate between a friend (benign or mutualistic endophytes) or foe (pathogens) through chemical communication between the invading fungus and the host plant (Fig 4; Hamilton et al. 2012). Regulation of the oxidative balance plays a vital role in plant-fungus symbiosis and endophytic fungi can produce antioxidants *in vitro* and *in planta*, particularly under stress (Hamilton et al. 2012). So, what about EPF endophytes? How will they affect the production of antioxidant enzymes? Will activities of antioxidant enzymes increase with EPF inoculations or herbivore feeding on fungus inoculated plants? To deal with these questions, wheat plants were inoculated with different EPF spp. isolates, infested them with *R. padi* and measured the levels of eight main antioxidant enzymes (**Manuscript I**).

The activity of SOD was increased in *M. robertsii* treated wheat plants in the presence of aphids. As mentioned earlier, SOD provides defense against oxidative stress in plants by scavenging $O_2^{\bullet-}$ into H_2O_2 and O_2 (Gajewska et al. 2006). The high production of H_2O_2 is thought to act as a signaling

molecule involved in resistance against aphids in plants (Maffei et al. 2007). The higher activities of H_2O_2 by aphid feeding have been found in oat, barley and wheat (Smith and Boyko 2007) which triggers other defense responses (e.g. increasing activity of peroxidase) to decrease aphid attack (Argandoña et al. 2001). Treatments with an endophytic bacteria (*Bacillus velezensis*) increased the induced systemic resistance against green peach aphid (*Myzus persicae*) in *Arabidopsis thaliana* by different factors such as callose deposition, cell death and accumulating H_2O_2 (Harun-Or-Rashid et al. 2017). This indicates that in the *M. robertsii* treatment, a higher activity of SOD was correlated with high levels of H_2O_2 leading to resistance against *R. padi* (**Manuscript I**).

In addition, CAT dismutates H_2O_2 into H_2O and O_2 (Gajewska et al. 2006) and higher activities of CAT in *M. brunneum* inoculated plants in presence of *R. padi*, strengthens the aforementioned role of H_2O_2 (**Manuscript I**). We assume that the increased CAT production in *M. brunneum* treated plants reduced oxidative stress caused by aphid feeding by scavenging H_2O_2 into non-toxic compounds (H_2O and O_2), leading to an increase in *R. padi* populations. A root-associated endophytic fungus *Serendipita indica* (formerly known as *Piriformospora indica*) and a root parasite, *Fusarium verticillioides*, increased CAT, GST, GR and SOD activities in maize roots compared to non-inoculated plants (Kumar et al. 2009), while a decrease in CAT activities was found when plants were first infected with *F. verticillioides* and after 10 days with *S. indica*. It was suggested that decreased activity helped plants to overcome disease load (Kumar et al. 2009). CAT induction is associated with high growth of infective fungi, overproduction is found to suppress resistance against pathogens while underproduction during biotic stress is important for programmed cell death (Mittler 2002; Kumar et al. 2009).

In addition, most of the antioxidant enzymes showed minimal changes, indicating that EPF treatments and aphid infestation did not stress plants (**Manuscript I**). Aphid infestation causes slight damage to plant tissues that reduces the chances of induced defense by plants (Pentzold et al. 2014). Although, aphid feeding has shown variable responses (enhanced, reduced or no changes) to antioxidative enzyme activities in different host plants (Khattab 2007; Ni et al. 2009; He et al. 2011).

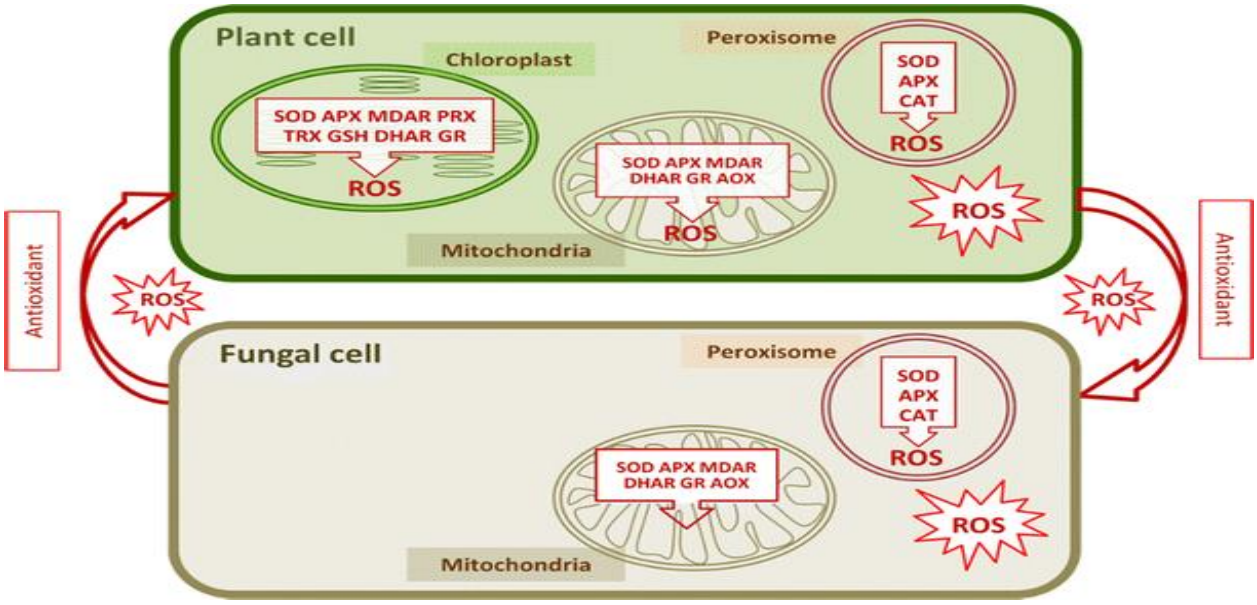

Figure 4. A plausible model for reactive oxygen species (ROS) and antioxidant production in plant and fungal organelles. Different antioxidant enzymes (SOD - superoxide dismutase, CAT - catalase AOX - alternative oxidase, PRX - peroxidredoxin, APX - ascorbate peroxidase, GR - glutathione reductase, DHAR - dehydroascorbate reductase, MDAR - monodehydroascorbate reductase, GSH - glutathione reduced, PRX - peroxiredoxin and TRX – thioredoxin) act in both organisms for ROS detoxification. The communication between fungal and plant cells is known to occur via ROS and antioxidants but details of the system are still unknown. Adapted from (Hamilton et al. 2012).

1.5 Plant secondary metabolites

Plant secondary metabolites (PSMs) are so-called bioactive compounds produced by plants in response to pest attacks to reduce damage (Howe and Jander 2007). The wide spectrum of defensive compounds may affect herbivore feeding, fecundity, population growth (Maag et al. 2015) and other reproductive as well as physiological processes by modulating the plant quality (Awmack and Leather 2002). The PSMs, therefore, play a crucial role in defense against insects and mites (Becerra 2015). The extent of production and quality of PSMs also limit the consumption rate and damage by herbivores (Huang et al. 2013) with lethal and sub-lethal implications (Chowański et al. 2016).

Endophytes have been called “chemical synthesizers” inside the plants (Owen and Hundley 2004) and fungal endophytes are well known for producing prolific amounts of bioactive secondary metabolites from various chemical classes (Suryanarayanan 2013; Stierle and Stierle 2015; Tidke et al. 2018). Entomopathogenic fungi are potential reservoirs of toxic secondary metabolites on its own and when associated with plants as endophytes as fungal secondary metabolites (FSMs; Fig. 5; Gurulingappa et al. 2011; Barelli et al. 2016; Ríos-Moreno et al. 2016). Barelli et al. (2016) reviewed the evidence that *Beauveria* and *Metarhizium* spp. are potential sources of toxic metabolites possessing insecticidal activities against insect herbivores, and Gurulingappa et al. (2011) reported

reduced reproductive period and fecundity of the cotton aphid, *A. gossypii* after exposure to fungal metabolites in culture filtrates by two EPF endophytes, *B. bassiana* and *Lecanicillium lecanii* (previously known as *Verticillium lecanii*).

In the case of EFE, it is strongly suspected that the negative impacts on arthropod herbivores reported in the literature are not linked to direct effects by the production of fungal chemicals, but rather by indirect effects mediated by the colonized plants through the production of PSMs (Fig. 5; McKinnon et al. 2017; Gange et al. 2019). It was expected that EPF would induce physiological changes *in planta*, for example, by increasing the levels of specific PSMs after endophytic colonization to carry out many processes such as defense against insect herbivores (Fig. 5). The present study has for the first time provided evidence for this hypothesis of chemical changes *in planta* after EPF inoculations affecting herbivore feeding (**Manuscript II and III**).

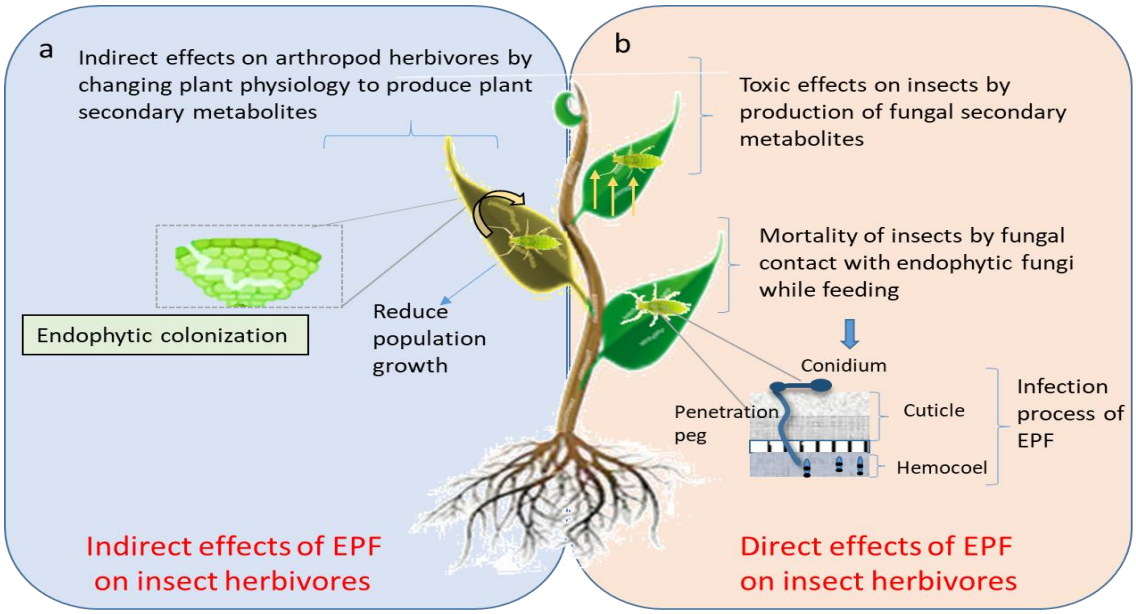

Figure 5. Schematic representation of the possible mode of action of entomopathogenic fungi (EPF) as endophytes. (a) After endophytic colonization, EPF affect arthropod herbivores indirectly by changing plant physiology to produce plant secondary metabolites or (b) EPF affect arthropod herbivores directly either by direct contact of herbivores with EPF while feeding or by producing fungal secondary metabolites.

1.5.1 Benzoxazinoids

The nitrogen-containing secondary metabolites, comprising 2-hydroxy-2H-1, 4-benzoxazin-3(4H)-one skeleton (benzoxazinones) with their derivatives (benzoxazolinones), are collectively called benzoxazinoids (BXs) (Wouters et al. 2016b). The BX compounds are mainly present in Poaceae family members including maize, rye and wheat but not in sorghum, barley, oat and rice (Niemeyer

2009). Hydroxamic acids (2, 4-dihydroxy-7-methoxy-1, 4-benzoxazin-3-one) are most active BXs mainly present as glucosides (i.e. DIMBOA-Glc, DIBOA-Glc, MBOA-Glc and HMBOA-Glc) in the vacuole and hydrolyze to their respective toxic aglucones (i.e. DIMBOA, DIBOA, HBOA and HMBOA) by the action of β-glucosidase upon any biotic or abiotic tissue damage (Niemeyer 1988, 2009). The bioactivity of BXs against insect herbivores is well documented. The pioneering work was conducted with maize, where a positive correlation between the concentrations of a BX aglucone (DIMBOA) and resistance to European corn borer (*Ostrinia nubilalis* Hübner) was found (Klun et al. 1967). The consequences of BXs vary from repellents to attractants, growth retardants to growth regulators and direct toxins depending upon the age of the plant and developmental stage of the insect herbivore (Niculaes et al. 2018). Biological effects of BX compounds on different genera of chewing and sucking insect pests in both *in vivo* and *in vitro* setups have been reported (Wouters et al. 2016a, b).

BXs have shown a wide range of antifeedant and antibiosis activities against aphids with no clear correlation between deterrence level and toxicity due to the feeding behavior of aphids (Wouters et al. 2016b). Aphids possess piercing-sucking mouthparts causing minimal damage to plant tissues compared to chewing insects (Guerrieri and Digilio 2008). Despite minimal tissue disruption while feeding, dynamic apportionment of BX compounds and activation of callose deposition (a sieve plate blockage protein) still make them effective defense compounds (Fig 6; Wouters et al. 2016b). The aphid stylet penetrates through apoplasts and the individual plant cells to reach the phloem and punctures mesophyll cell to release effector proteins. In response, the plant produces and transports a set of defensive compounds into the phloem that could be ingested during feeding, accumulated in haemocoel or excreted out of the body with other metabolites (Fig 6; Züst and Agrawal 2016). Therefore, BXs consequences in aphids can be more intricate than mere toxicity (Niculaes et al. 2018).

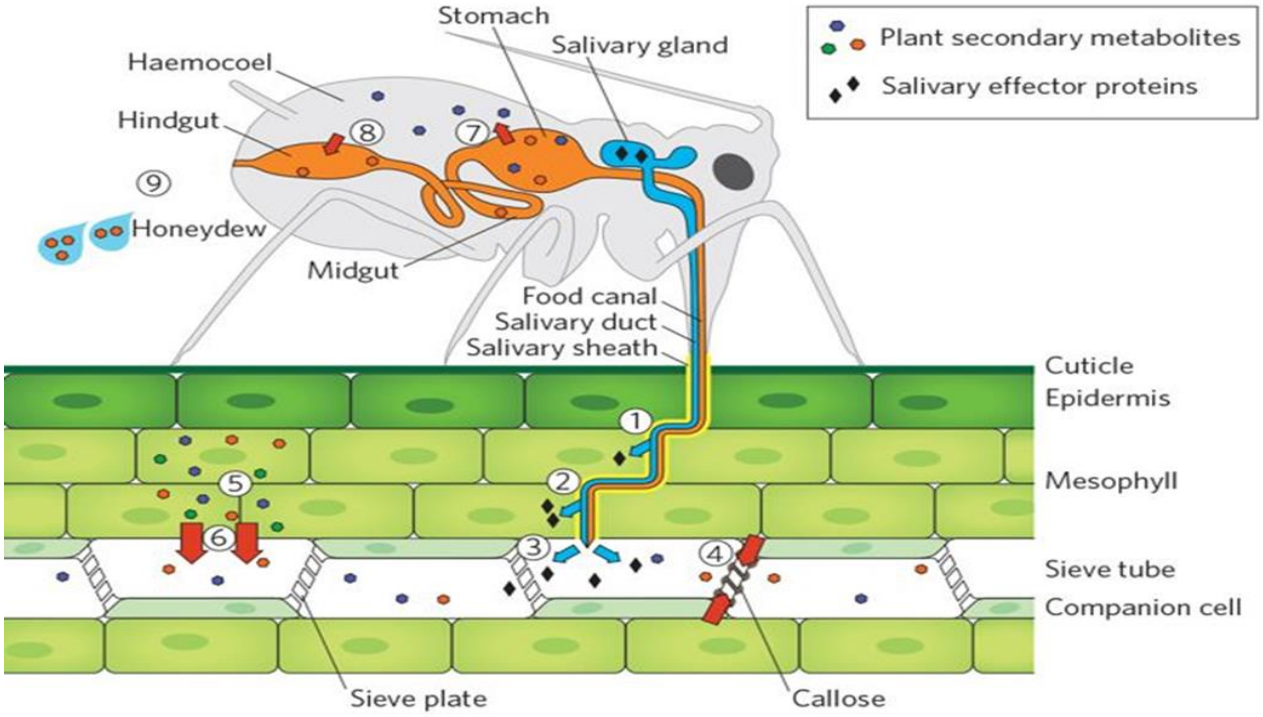

Figure 6. Schematic representation of aphid feeding and plant defense responses. (1) Aphid stylet penetrates the apoplast while projecting gelling saliva between cells to encase stylet and to block any leaks, (2) stylet puncture mesophyll cell and release effector protein-containing saliva, (3) suck plant phloem, (4) exude watery saliva to prevent callose deposition, (5) in response plant cells produce defensive plant secondary metabolites (PSMs), and (6) transport them into the phloem. (7) Aphid ingests secondary metabolites, (8) either accumulate in the haemocoel or excrete into hindgut or (9) exude out of the body with honeydew. Adapted from (Züst and Agrawal 2016). **Manuscript II** mostly focuses on the production of specific PSMs (benzoxazinoids in wheat and flavonoids in the bean) and their effect on aphid population growth (points 5-7).

The microbial associations with plants also depend upon the presence of defense compounds in colonized plant tissues (Suryanarayanan 2013). The production of BXs altered the endophytic community assembly in maize tissues (Saunders and Kohn 2008, 2009). Kudjordjie et al. (2019) found a correlation between BXs and microbial community assembly in rhizosphere, root and shoot of maize thus providing an insight into the bacterial and fungal taxa associated with maize. Seed inoculations using different strains of plant growth-promoting rhizobacteria (*Azospirillum* spp.), caused a strain-dependent modification in BXs contents in maize. However, the changing profiles of BXs after artificial inoculations with fungal endophytes especially EFE have not been documented previously. The present study for the first time showed that seed inoculations with different EPF isolates is related to changes of the levels of BXs compounds in wheat plants and showed a correlation between alterations in some BXs and aphid population growth (**Manuscript II**). The EPF isolates which showed negative effects against aphids, i.e. *B. bassiana* GHA and *M. robertsii* ESALQ 1622,

displayed high induction of most BX compounds as compared to the isolate *M. brunneum* KVL 04-57 which showed production of high number of aphids on inoculated plants (**Manuscript II**).

The *M. robertsii* ESALQ 1622 treatment (harboring the lowest number of aphids) coincided with the highest concentrations of DIMBOA in wheat both with and without *R. padi* feeding (**Manuscript II**). The metabolic analysis of durum wheat (*T. turgidum*) showed significant induction of DIMBOA and its glucoside (DIMBOA-Glc) after English grain aphid (*Sitobion avenae*) and bird cherry-oat aphid (*R. padi*) feeding (Shavit et al. 2018), which showed resistant and mild resistant responses to both aphid species, respectively. Hydroxamic acids (DIMBOA) can also cause indirect effects by triggering callose deposition (Maag et al. 2015) and are more active against insect herbivores and other organisms than benzoxazolinone (MBOA) and lactams (HBOA, HMBOA; Wouters et al. 2016a). Interestingly, *M. brunneum* KVL 04-57 treatment (harboring the highest number of aphids) produced the highest levels of MBOA and HBOA, which decreased significantly with *R. padi* feeding (**Manuscript II**). DIMBOA degrades to MBOA after tissue injury and a high reproduction of *S. avenae* was found at a specific concentration of MBOA (up to 0.1 mM) with negative effects as concentrations increased (> 0.1 mM; Hansen 2006). However, it is unclear whether MBOA and HBOA played a role in *R. padi* population growth or simply were degraded due to aphid abundance (**Manuscript II**).

The present findings suggest that most of the BXs compounds have potential against aphids and that the concentrations of different BX compounds increased with EPF seed inoculations, causing the negative effects on aphids (**Manuscript II**). It is still unclear which compounds played the actual role in reducing the population growth of aphids. Further molecular-based studies are needed to explore the biosynthetic pathways associated with the production of important BXs after fungus colonization and their effects on the ability of a fungus to colonize the plant and insect herbivores.

1.5.2 Flavonoids

Flavonoids (derivatives of 2-phenyl-benzyl-γ-pyrone) are a ubiquitous and chemically diverse group of PSMs possessing various biological activities, including UV protection, flower coloring, auxin transportation and defense against herbivores (Buer et al. 2010; Mierziak et al. 2014). Isoflavonoids, flavones, flavonols and anthocyanins are important flavonoid groups affecting plant-herbivore interactions (Simmonds and Stevenson 2001). Plants harbor a large variety of flavonoid compounds that confer resistance against arthropod herbivores or conversely increase the fitness of the herbivore by sequestering compounds in the cuticle of the body or wings for use in defense against natural enemies or for attracting mates (Simmonds 2003; Treutter 2005). The presence of flavonoids changes

the palatability and nutritional value of the host plant and affects their digestibility or even act as toxic substances against insect herbivores (Mierziak et al. 2014).

Comparative toxic and deterrent effects of flavonoids in numerous plant pests including aphids have been reported (Thoison et al. 2004; Frah et al. 2013; Goławska et al. 2014). When incorporated in the diet, Quercetin (flavonol) and naringenin (flavanone) showed detrimental effects on different population parameters (e.g. pre-reproductive period, development, fecundity and mortality) of pea aphids (*Acyrthosiphon pisum*; Goławska et al. 2014). Higher concentrations of quercetin and isorhamnetin chemotypes were found in cowpea (*Vigna unguiculata* L.) lines that showed resistance characteristic against *A. fabae* (Lattanzio et al. 2000), while *A. pisum* infestation induced flavonoid concentrations in leaves of pea (*Pisum sativum* L.). Simultaneously, some compounds, e.g. rutin (flavonols), behaved as phagostimulants or repellents towards many polyphagous insects depending upon concentrations and age of the insect (Simmonds 2003).

Flavonoids are often associated with plant-microbe interactions. Root exuded compounds act as signaling molecules for root-associated bacteria to induce gene transcription and improve root colonization, leading to improved plant growth and fitness (Treutter 2005). In addition, high accumulation of some flavonoid compounds (quercetin, acacetin and rhamnetin) was found in roots inoculated with arbuscular mycorrhizal fungus, *Glomus intraradices* (Ponce et al. 2004), although their role in plant-mycorrhizae symbiosis remains unclear (Treutter 2005). Flavonoids are involved in biological communication with other plant species, rhizobacteria, arbuscular mycorrhizal fungi, nematodes and pathogens (Sugiyama and Yazaki 2014). Some natural endophytic fungi and fungal entomopathogens have the potential to produce flavonoid compounds (Qiu et al. 2010; Zohri et al. 2016).

Based on the hypothesis that EFE cause physiological changes in plants, I intended to evaluate for the first time the correlation between EPF inoculations in plants and the production of specific flavonoids and their effects on insect herbivores. The EPF inoculated bean plants showed a correlation between resistance against aphids and the concentrations of some flavonoids (**Manuscript II**). However, modified levels of flavonoids depended on the EPF isolate: *A. fabae* feeding significantly increased the biosynthesis of quercetin, isoquercitrin, genistin and astragalin in the *M. robertsii* ESALQ 1622 treatment and of luteolin-di-Glc in the *B. bassiana* GHA treatment, resulting in a decreased of the number of *A. fabae* on bean plants (**Manuscript II**). These compounds have previously shown, adverse effects on various insect herbivores, including aphids (Lattanzio et al. 2000; Simmonds 2001; Goławska et al. 2008). Conversely, in the *M. brunneum* KVL 04-57 treatment,

the concentrations of isoquercitrin and rutin were decreased significantly in the presence of *A. fabae* (**Manuscript II**). It is suspected that rutin was utilized by *A. fabae* for increased reproduction with no direct relation to defense (**Manuscript II**). Simmonds (2003) concluded that rutin acts as a phagostimulant for many insect herbivores depending on the concentration.

How different fungal isolates interact with host plant physiology is an important factor to address in the future research. The fact that different EPF isolates changed the profiles of specific PSMs differently, strengthens the hypothesis that these EPF inoculations are involved in plant physiological changes. How these EPF, especially *M. robertsii*, which mostly colonized roots, interact with plants to induce specific compounds to affect above-ground insect herbivores need further study. Likewise, the treatment effects due to fungus in the rhizosphere and growing as epiphytes cannot be ruled out.

1.5.3 Steroidal glycoalkaloids

Steroidal glycoalkaloids (SGAs), the nitrogen-containing glycosylated forms of steroidal alkaloids, are the main bioactive metabolites of many members of the *Solanaceae*, e.g. potato (*Solanum tuberosum*), eggplant (*S. melongena*) and tomato (*S. lycopersicum*; Friedman 2002). Although SGAs are not associated with plant growth, they possess resistance properties against plant antagonists, including pathogens and herbivores (Milner et al. 2011). The toxicological effects caused by SGAs include the deformity of the reproductive system of herbivores, which is considered the most significant insecticidal response against plant pests (Chowański et al. 2016). The non-host glycoalkaloids similar to other glycoalkaloids could alter juvenile hormone activity by interacting with the insect endocrine system (Flanders et al. 1992). Also, high constituents of glycoalkaloids in insect tissues disturb molting and metabolic processes, leading to less consumption of plant tissues (Chowański et al. 2016). The synergistic increase in 20-hydroxyecdysone (hormone responsible for insect metamorphosis) activity has been found with high tomatine dose levels (Oberdörster et al. 2001).

Tomatine, a mixture of two SGAs α-tomatine and dehydro-tomatine, has been detected from all parts of the tomato plant, with high contents in unripe fruits and leaves (Friedman and Levin 1995; Friedman 2002). The compounds α-tomatine and dehydro-tomatine differ based on the absence and presence of a "double bond" in the ring structure, respectively (Fig. 7, Chowański et al. 2016). Several decades ago, Tingey (1984) appraised the bioactivity of glycoalkaloids on developmental as well as behavioral biology of potato insect pests and their potential as resistance factor after manipulation in breeding varieties. SGAs in edible African nightshade, *Solanum sarrachoides*, played an effective role against red spider mite, *Tetranychus evansi* (Acari: Tetranychidae; Jared et al. 2016). The

insecticidal effects of α-tomatine on pest physiology, induction of glycoalkaloids with pest damage and their field resistance against herbivores like Colorado potato beetle and potato leafhopper are reported in different studies reviewed by Milner (2011).

The bioactivity of SGAs and their potential to be incorporated in crop plants led to the idea to evaluate the activities of two important SGAs, α-tomatine and dehydro-tomatine after EPF inoculations in tomato plants and relate their activities to the effects of the EPF inoculation on population growth of two-spotted spider mites, *T. urticae* (**Manuscript III).** The fact that some endophytic fungi are capable to mimic the colonized plant's ability to produce the same metabolites after isolation (El-Hawary et al. 2016) favors the idea of toxic metabolites induction for defense *in planta* after fungal inoculation (Kusari and Spiteller 2011). Seed inoculations of tomato with *B. bassiana* GHA and *M. robertsii* ESALQ 1622 resulted in the lowest number of *T. urticae* and induced a significant amount of α-tomatine and dehydro-tomatine in tomato plants, while inoculation with *M. brunneum* KVL 04-57 that was related with higher *T. urticae* population growth and low SGAs activities (**Manuscript III**). It is possible that *B. bassiana* GHA and *M. robertsii* ESALQ 1622 imitated tomato plant's ability to produce SGAs to favor plant defense against *T. urticae* (**Manuscript III**).

The higher concentrations of both SGA compounds in specific EPF isolates and their induction by *T. urticae* feeding could be the induced defense reaction of the inoculated plants stimulated by the fungus (Moloinyane and Nchu 2019). Alternatively, it could be assumed that endophytic colonization by *M. brunneum* KVL 04-57 suppressed the biosynthesis of SGAs either directly or by modulation of other pathways involved in the production of these compounds. However, further studies are required to unravel the phenomena behind the contrasting outcomes of different fungal isolates belonging to the same genera. Different signaling pathways used in the production of specialized metabolites and integration of RNA sequencing and other transcriptomic, proteomic, metabolomics approaches could be used to obtain insight into the mechanisms.

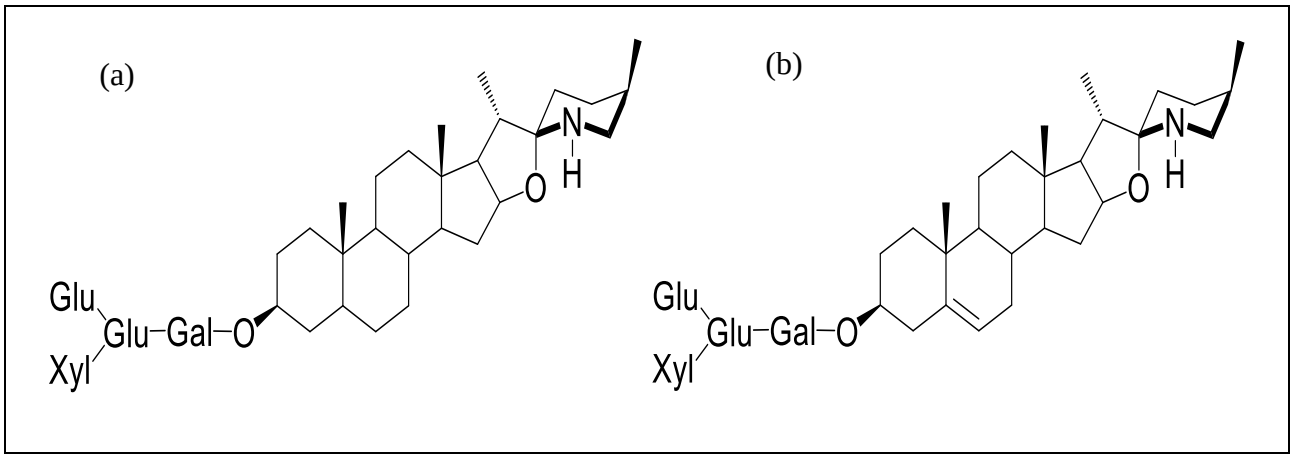

Figure 7. Structure of steroidal glycoalkaloids: (a) α-tomatine without a double in structure, (b) dehydro-tomatine, with double-bound in structure. Chemical structures were drawn using ChemDraw software.

2. CONCLUSION

Entomopathogenic fungi play a central ecological role when associated with plants in a symbiotic relationship as endophytes. The responses of EFE inoculated plants against arthropod herbivores are evident but the mechanisms behind these responses are still unclear. The research presented in this thesis will advance the current scientific knowledge of such modes of action with the following key conclusions:

- The three fungal isolates used for seed inoculations caused isolate specific effects towards insect and mite herbivores by effecting population growth rates positively (*M. brunneum*), negatively (*M. robertsii*) or negatively/neutrally (*B. bassiana*) compared to uninoculated control plants. The isolate specific effects were consistent among the three plant species tested.
- The activities of some carbohydrate enzymes increased with EPF seed inoculations and aphid infestation and decreased in uninoculated wheat plants with aphid feeding. Carbohydrate enzymes showed no direct relationship with the population growth of herbivores while the activities of antioxidant enzymes showed minimal changes in relation to fungal treatments and herbivore feeding.
- The concentrations of most of the plat secondary metabolites increased with *M. robertsii* and *B. bassiana* seed inoculated plants. Elevated concentrations of specific PSMs were related to the reduced population growth of herbivores from different arthropod classes in an isolate specific manner.
- After the inoculation of seeds, the three isolates of EPF species colonized wheat, bean and tomato plants in isolate specific manner, with *B. bassiana* colonizing all plant parts, *M. brunneum* in stem and roots, while *M. robertsii* was found mostly in roots and little in the stem.
- There was no straight forward relationship between colonization patterns and effects against herbivores. *B. bassiana* being able to colonize all tissues had a low negative impact, *M. brunneum* being a good stem and root colonizer increased herbivory while *M. robertsii* as a good root colonizer and least efficient stem colonizer had the highest negative impact on herbivory.
- The two isolates of *Metarhizium* spp. promoted plant growth parameters more consistently than the isolate of *B. bassiana.*

3. FUTURE PERSPECTIVES

There are still many aspects of EFE research that needs to be discovered and investigated to understand their real potential as biological control agents. This thesis work provides some fundamental conclusions, while it also originates new questions that should be addressed in the future to get deeper insights into the EFE based research.

- The fact that isolates of different EPF species from the same fungal genera showed incomparable results in various plant responses in the present study, emphasizes the importance of selection and screening of isolates. Further studies should consider carefully the potential variability among EPF isolates when investigating their potential for plant protection as endophytes.
- Regarding plant physiological changes caused by EPF endophytic colonization, more plant families, insect guilds, EPF species and isolates should be investigated to get a clear understanding of their interactions. While dealing with specialized plant secondary metabolites, the individual compound should be tested for its effects not only on insect herbivores but also on other plant characteristics (e.g. growth) and non-targeted organisms.
- The fact that different EPF species from the same fungal genera showed incomparable results in various plant responses in the present study, emphasizes the importance of selection and screening of potential isolates. Further studies should carefully investigate the changing pathogenic potential of EPF after they become endophytes.
- To better understand the mechanisms of the regulation of plant physiology by EPF, molecular-based studies in the combination of other transcriptomic, proteomic and metabolomic approaches are needed to get insights into the pathways involved in the production of specialized metabolites.
- The research presented here emphasizes the importance of cross-disciplinary research to evaluate different aspects of endophytic fungi. Experts from different areas, e.g. mycology, entomology, plant physiology and chemical ecology should work together to better understand the complexity of endophytic research. Collaborations among scientists from different research areas are necessary to understand the complex interactions among fungi, plants, herbivores, and their environment.

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Manuscript I

Comparative activities of carbohydrate and antioxidant enzymes in wheat inoculated with entomopathogenic fungi in relation to effects against aphids

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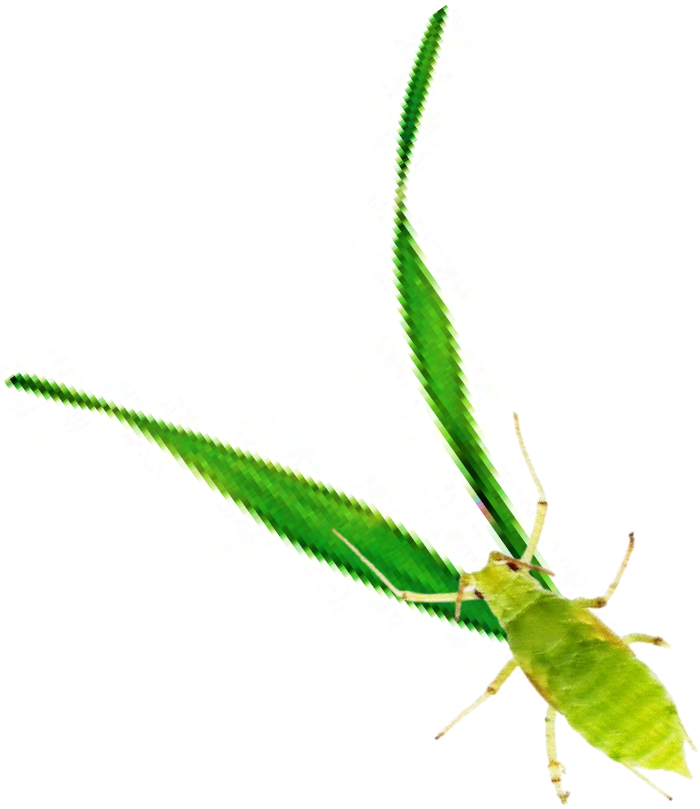
In preparation



Comparative activities of carbohydrate and antioxidant enzymes in wheat inoculated with entomopathogenic fungi in relation to effects against aphids

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Abstract

Entomopathogenic fungi (EPF) can colonize plants endophytically, promote plant growth and suppress the population growth of arthropod herbivores. However, the physiological changes in planta after EPF treatments and mechanisms behind these beneficial effects are still unknown. The present study aimed to evaluate the effects of seed inoculations with three isolates of EPF on the activities of key carbohydrate and antioxidant enzymes to plant growth and population growth of bird cherry-oat aphids, *Rhopalosiphum padi*. Seed inoculated *Beauveria bassiana* endophytically colonized leaves, stem and roots, *Metarhizium brunneum* colonized stem and roots and *M. robertsii* only colonized roots of wheat plants. Whereas inoculations with *M. robertsii* significantly reduced, *M. brunneum* unexpectedly increased while *B. bassiana* had no effects on population growth of *R. padi* as compared to control plants. Overall, *M. brunneum* showed significantly higher growth rates among other fungal treatments as compared to uninoculated control. The activities of most of the carbohydrate enzymes increased in wheat leaves inoculated with both *Metarhizium* spp., showing no clear correlation with the population growth of *R. padi*. Whereas, the antioxidant enzyme activities showed minimal changes by seed treatments and aphid feeding. However, *M. robertsii* treatment with the lowest number of aphids increased the activities of superoxide dismutase while *M. brunneum* with the highest number of *R. padi* increased the activities of catalase and glutathione S transferase. This study provides evidence of changed enzymatic profiles of wheat after EPF treatments in relation to plant growth and aphid population growth.

1. INTRODUCTION

Wheat, *Triticum aestivum* L. (Poaceae), is an important food crop cultivated worldwide and the most significant crop ensuring global food security (Tadesse et al. 2015). It contributes as a major resource for food, feed and raw material in different industries (Charmet 2011) and provides 20% of calories and protein to the world population (FAO 2014). However, insect herbivores substantially reduce wheat yield with an estimated threat of loss augmentation due to warming climate most likely in temperate regions (Deutsch et al. 2018). Aphids (Hemiptera: Aphididae) are phloem-feeding insects, which cause massive economic and nutritional losses by direct and indirect damage to crops (Guerrieri and Digilio 2008). While feeding, aphids insert toxic saliva at the feeding site affecting photosynthetic efficiency and nutritional value of crop plants causing slight visible damage (Zhou et al. 2015). Bird cherry-oat aphid (*Rhopalosiphum padi* L.) is one of the most damaging aphid specie of cereal crops (Blackman R 2000), predominantly found in wheat fields causing huge losses due to abundant feeding and through the transmission of viral diseases (Pereira et al. 2017). Aphid control is a challenge due to the high reproduction rate and resistance development against chemical insecticides that increase the demand for new control strategies (Guerrieri and Digilio 2008; Rebijith et al. 2017).

Entomopathogenic fungi (EPF) have been extensively explored as biological control agents of insect pests (Shah and Pell 2003; Meyling and Eilenberg 2007). Besides interacting with insect herbivores EPF have been found to be associated with plants forming a symbiotic relationship, colonizing living plant tissues during full or a period of their life cycle as endophytes (Wilson 1995; Vega 2008). Endophytes are often considered to have a mutualistic relationship with plants by enhancing competitive abilities and resistance against biotic and abiotic stressors of the host in exchange for nutrition and protection (Saikkonen et al. 1998). The entomopathogenic endophytic fungi are known as plant growth promoter conceivably by increasing the nutrient content of plants (Behie and Bidochka 2014; Tall and Meyling 2018) and insect pest protector possibly by producing fungal induced or plant-mediated secondary metabolites (Ríos-Moreno et al. 2016; Gange et al. 2019; Hu and Bidochka 2019).

The most studied entomopathogenic endophytic fungi are in the order Hypocreales (phylum: Ascomycota), where isolates of the genera *Beauveria* (Cordycipitaceae) and *Metarhizium* (Clavicipitaceae) have been successfully inoculated in different plant species to evaluate their ecological effects as plant protectants (Jaber and Ownley 2018). Generally, these genera showed

negative effects on the population growth from different insect feeding guilds, including sucking insect pests across plant families (Gange et al. 2019). *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin has been well explored as endophytic fungi and has shown potential to cause negative effects against many insect herbivores especially on the parthenogenetic reproduction of aphids (McKinnon et al. 2017; Vega 2018). For instance, seeds of fava beans inoculated with *B. bassiana* had detrimental effects on population growth and offspring fitness of *Acyrthosiphon pisum* and *Aphis fabae* (Akello and Sikora 2012). Seed inoculations with *B. bassiana* similarly reduced the number of *Aphis gossypii* in cotton compared to the control treatment (Castillo Lopez et al. 2014). Likewise, *Metarhizium robertsii* (J.F. Bisch., Rehner & Humber) have been found to reduce the reproduction rate of other arthropod pests after seed inoculations (Canassa et al. 2019). However, few recent studies have also shown neutral or positive effects of some *B. bassiana* and *Metarhizium* spp. isolates on population growth of aphids after seed inoculations in different plant families (McKinnon et al. 2017; Clifton et al. 2018; Jensen et al. 2019). These findings of dichotomous responses related to some fungal isolates have raised questions about the mechanisms behind the fungus mediated changes *in planta* after inoculations.

Enzymes are plant batteries, providing resilience against adverse biotic and abiotic stresses. Carbohydrate and antioxidative enzymes have been studied for their involvement in plant growth and as signaling molecules against plant external stresses to mediate defense responses, respectively (Gill and Tuteja 2010; Jammer et al. 2015). Carbohydrate enzymes are involved in the production, regulation and distribution of carbohydrates from source tissues (production site, leaves) to sink tissues (consumption site, root and shoot), determine plant health and respond to external factors (Jammer et al. 2015). Biotrophic symbionts particularly fungal pathogens utilize host synthesized carbohydrates through unique structures and decrease the levels of source metabolism (Wingler and Roitsch 2008). Likewise, endophytic EPF develop a symbiotic relationship with host plants and are assumed to obtain photosynthates in return of nitrogen to plants (Behie et al. 2017). This putative mutualistic association of a fungus with the plant could induce changes in primary metabolism.

Various plant metabolic processes produce reactive oxygen species (ROS) as by-products (Gill and Tuteja 2010). ROS assist plants in programmed cell death, in the detection of external stress stimulus and in maintaining plant growth and development (Das and Roychoudhury 2014). However, overproduction of these signaling molecules in response to different stress stimuli leads to oxidative stress in plants causing damage to essential plant processes and organelles ultimately leading to cell death (Das and Roychoudhury 2014). Therefore, an equilibrium between ROS generation and

degradation is crucial to maintain a state of redox hemostasis in plants which is achieved via antioxidative enzyme systems (Concept et al. 2005). The involvement of ROS and associated scavenging enzymatic antioxidants under different stress conditions such as high or low temperature, drought, salinity, heavy metals, pathogens and pests have been well documented (Dat et al. 2000; Mittler 2002; Das and Roychoudhury 2014). Plants require balanced cellular structures and redox hemostasis to endure herbivore pressure e.g. aphid infestation (Smith and Boyko 2007). Endophytic fungi have the potential to produce antioxidative enzymes both *in vitro* and *in planta*, particularly under stress (Hamilton et al. 2012). Antioxidants transfer stress stimulus and assist the host to distinguish between a friend (mutualist endophytes) or foe (plant pathogens) by chemical communication between an asymptomatic endophyte and the host plant (Hamilton et al. 2012). However, the role of EPF endophytes on the antioxidant system has not been reported.

We investigated here the effects of three EPF fungal isolates, *M. robertsii*, *M. brunneum* and *B. bassiana* on *R. padi* aphid populations in wheat after seed inoculations. Further, we examined the activities of key carbohydrate and antioxidant enzymes after EPF inoculations in the presence or absence of aphids. This was done with the objective to evaluate whether correlations between the activities of specific enzymes and the population growth of aphids after EPF inoculations could be identified.

2. MATERIAL AND METHODS

2.1 Host plant and insect

Untreated seeds of wheat (*Triticum aestivum* L., var. Sevin Sejet) were obtained from Sejet Plant Breeding and stored at 4 °C. Seeds were surface disinfected by immersing 30 sec in 70% ethanol (EtOH) and then 10 min in 2% sodium hypochlorite (NaClO, Sigma Chemical, St Louis, MO) followed by three repeated rinsings with double-distilled water (ddH_2O) and dried for 30 min under ventilation hood before inoculations. Sterilization efficacy was checked by spreading 100 µl of the last rinse on three Sabouraud Dextrose Agar (SDA; Sigma-Aldrich, Darmstadt, Germany) media plates for 10 days at 24 °C. No sign of contamination was found from any of the SDA plates.

Bird cherry-oat aphids (*Rhopalosiphum padi* L.) were obtained from already established colonies at the Department of Plant and Environmental Sciences, University of Copenhagen, Denmark. Aphid clonal population was maintained in BugDorm2 rearing tents (60 × 60 × 60 cm) in an insect room (20 ± 2 °C, 16:8 LD, 60-70% RH) on same wheat variety as used for the experiments. To obtain the same last instar nymphs, aphid adults were transferred to 30 ml medicinal cups containing 3-4 cm

wheat leaf pieces fixed into 3% water agar six days before releasing on experimental plants. In total 30 cups, containing 2 aphids each, were used to obtain the required number of nymphs. After 24 hours the adults were removed and leaves pieces with nymphs were transferred to a whole wheat plant for 5 days to develop into last instar nymphs.

2.2 Fungal isolates and suspensions

Three insect pathogenic fungal isolates including *B. bassiana* – Bb, strain GHA from the commercial product BotaniGard® (deposited as KVL 13-39), *M. brunneum* – Mb, strain KVL 04–57, isolated from infected larvae of *Cydia pomonella* collected in Austria (sharing active ingredient origin with Met52, Novozymes, Salam, VA) and *M. robertsii* – Mr, strain ESALQ 1622 isolated from corn soil in Mato Grosso – Brazil (deposited as KVL 16-38) were used for experimentation. The culture stocks for all isolates are stored at -80 °C at the University of Copenhagen.

The fungal cultures were propagated in Petri dishes (90 × 15 mm) containing 20 ml SDA media at 23 °C for 14 days in darkness. Conidial suspensions were prepared by scraping stock culture with sterile glass spatula by adding 10 ml of sterile 0.01% Triton X-100 solution. The suspensions were filtered through multiple layers of sterile cheesecloth to remove the hyphal fragment, conidial clumps and agar bits. The conidial concentrations were estimated by using a Fuchs-Rosenthal hemocytometer (Assistent, Sondheim von der Rhön, Germany, 0.0625 mm^2, depth 0.200 mm) and adjusted to a required final concentration of 1×10^8 conidia ml^{-1} using 0.01% Triton X-100. Conidial viability was assessed before using in experiments by spreading 100 µl of 1×10^5 conidia ml^{-1} dilution on three SDA plates and propagating at 23 °C. Germinated and non-germinated conidia were counted after 24 h of propagation. Conidia suspensions with > 90% germination rate were used for experiments.

2.3 Seed treatments and experimental setup

For seed inoculations, 30 ml of each fungal suspension (Bb, Mb and Mr) and control solution (Ct, 0.01% Triton X-100) was added in 250 ml screwed capped glass bottles. Sterilized seeds (approx. 18 seeds per treatment) were immersed in respective fungal and control solution for 24 h at 100 rpm under agitation. Treated seeds were dried on filter papers for 15 min and planted in 1 L plastic pots containing PG-mix peat soil (Krukväxtjord Lera & Kisel, Gröna linjen, Sweden) supplemented with limestone (pH: 5.5-6.5), 3% washed gravel, 4% silica and other micronutrients (NPK 182-91-194, Mg-247, S-99, Ca-2189, Zn-1.0, Fe-8.6, Cu-2.0, B-0.4, Mn-3.2, Mo-2.6 g/m^3). The pots were placed following a randomized complete block design comprising 6 blocks in the greenhouse at 25 ± 2 °C, 60-70% RH, 16:8 LD. Each block comprised two pots for each of the four treatments, eight pots per block, 12 pots per treatment and 48 pots per experiment (Supporting information, Fig. S1). A plastic

plate (17 × 3 cm) was placed under each pot for irrigation (every 3rd day) and to avoid cross-contamination by run-off. Ten days after sowing, plant heights (cm, from plant base to the tip of the fully emerged leaf) were measured with a ruler and five last instar aphid nymphs were transferred to the uppermost fully developed leaf of half of the plants from each treatment (six plants per treatment). All the plants were covered with a plastic cylinder (8 × 25 cm) having a mesh (0.09 mm) on the top to avoid aphid escape. The experiment was repeated on two occasions.

2.4 Data collection and sampling

The aphid population growth was recorded by counting the total number of aphids at 2, 4, 6, 8 and 10 days after release. After last counting aphids were removed with camel hairbrush and dipped into 70% ethanol to kill. Plant heights were measured again after 10 days of aphid release and 20 days after sowing. Blocks were randomly divided into two sets of three blocks each. One set of blocks was used for the evaluation of plant growth parameters (biomass) and second for enzyme analysis and detection of endophytic establishment of the fungal isolates (Supporting information, Fig. S1). For biomass measurement, shoots were cut from the base and roots were washed under running tap water to remove soil, dried on tissue papers for 5 min and fresh weight (g) was taken with an electronic balance (A&D model FA-2000, UK). Afterward, root and shoot samples were placed in paper bags and dried at 65 °C for 48 h in drying oven (A&D model FA-2000, UK) and weighed on the same balance. From the second set of blocks, leaf, stem and root samples were taken for endophytic detection of inoculated fungal isolates (see below). For enzyme detection, two leaves (one lowest old, 3-6 cm from the base and one new tiller) were harvested with scissors and pooled together as one biological replicate in a 15 ml falcon tube. Scissors were wiped with 70% EtOH between each treatment to avoid cross-contamination. Samples were flash-frozen immediately in liquid nitrogen and stored at -80 °C until further processing.

2.5 Detection of endophytic fungi

The leaf, stem and root samples were used for the detection of the fungal isolates as endophytes by plating surface-sterilized plant tissue pieces on selective media. From each selected plant three pieces of leaves (3 cm each), two pieces of stem (3cm each) and two pieces of roots (4 cm each) were excised and surface sterilized by dipping in 70% EtOH for 2 min, 2% NaClO for another 2 min followed by three repeated rinsing with ddH2O. Sterilization efficiency was checked by imprinting of all parts before and after sterilization (Tall and Meyling 2018) and by plating 100 µl of last rinsing water on SDA media (Parsa *et al.*, 2013). Subsequently, samples were trimmed from edges with a scalpel and divided into six pieces of leaves (0.5 – 1 cm^2), four pieces of the stem (0.5 – 1 cm) and four pieces of

the root (1 cm). The cut pieces from each tissue were randomly placed on selective media in different Petri dishes consisting of; Agar (6 g), Glucose (10 g), Peptone (5 g) and Dodine (0.2 ml) supplemented with antibiotics, Streptomycin (0.5 ml of 0.6 g ml^{-1}) Tetracycline (0.5 ml of 0.05 g ml^{-1}) and Cyclohexamide (1 ml of 0.05 g ml^{-1} (pH 6.3-6.5). The samples (mainly stem) were slightly pressed to ensure direct contact of any endophytic fungus with the selective media. Plates were sealed with parafilm and incubated for 21 days at 23 °C in darkness. The endophytic colonization was evaluated by checking any growing colonies from cutting edges visually and under stereomicroscope depending upon specific genera growth characteristics and resemblance with inoculated fungus.

2.6 Determination of carbohydrate and antioxidant enzyme activities

2.6.1 *Sample Extraction*

The extraction of leaf samples for the detection of carbohydrate and antioxidant enzyme activities was done following the protocol by Jammer et al. (2015) with few modifications. Briefly, the material of each frozen leaf was homogenized in liquid nitrogen using mortar and pestle and 500 mg was placed in a 2 ml Eppendorf tube. Afterward, 1 ml of extraction buffer (consisting of 40 mM TRIS-HCl (pH 7.6), 1 mM benzamidine, 1 mM EDTA, 0.1 mM PMSF, 24 μM NADP, 3 mM MgCl2, 14 mM β-mercaptoethanol and ddH_2O) was added to the ground plant material and mixed for 30 min on an electronic mixer and centrifuged at 4 °C and 20000 *g* for 10 min. The supernatant was collected and dialyzed overnight using dialysis tubes (~3-4 cm) hanging in 20 mM KPO4 buffer (pH 7.4), shifted to new tubes and stored as a crude dialyzed extract. The remaining pellet was washed three times with ddH_2O, re-suspended in 1 ml high salt buffer (40 mM TRIS-HCl (pH 7.6), 3 mM MgCl2, 1 M NaCl, 1 mM EDTA and ddH_2O) and processed by repeating the above procedure for cell-wall dialyzed extract (overview of extraction protocol is shown in Supporting Information, Fig. S2).

2.6.2 *Carbohydrate enzyme activities*

The activities of invertases (cytoplasmic - cytInv, vacuolar - vacInv and cell-wall invertases – cwInv) were determined by endpoint assays by measuring absorbance rate at the wavelength of 405 nm (Sung et al. 1989; Jammer et al. 2015). The crude dialyzed extract was used for cytInv and vacInv activities while cell-wall dialyzed extract was used for cwInv activities. Glucose (0-5 nmol) standards were used for the calibration curve. The plant extracts with assay compounds (Supporting Information, Table S1) and glucose standards were incubated at 37 °C for 30 min, added GOD-POD reagent (GOD (10 U ml^{-1}), POD (0.8 U ml^{-1}) and ABTS (0.8 mg ml^{-1}) in potassium phosphate buffer (0.1 M, pH 7.0) and incubated again at room temperature for 20 min before analysis. The activities of other enzymes including fructokinase (FK), hexokinase (HXK), UDP-glucose pyrophorylase (UGPase),

ADP-Glucose pyrophosphorylase (AGPase), glucose-6-phosphate dehydrogenase (G6PDH), phosphoglucomutase (PGM), phosphoglucoisomerase (PGI), phosphofructokinase (PFK) and aldolase (Ald) were determined by kinetic assays by measuring absorbance rate at 340 nm (Jammer et al. 2015). The crude dialyzed extracts were used with assay compounds (mentioned in Supporting Information, Table S1) and ddH_2O was added to achieve the final reaction volume for the analysis. All the measurements were carried out in triplicate and the substrate was omitted from the control reactions (Supporting Information, Table S1). All the enzyme assays were carried out in 96-well microtiter plates using an Epoch Take3 spectrophotometer (Biotek, Bad Friedrichshall, Germany).

2.6.3 Antioxidant enzyme activities

The activities of antioxidative enzymes including, superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), glutathione S transferase (GST) were measured from crude dialyzed extract while of cell-wall peroxidase (CWPOX) was measured from cell-wall dialyzed extract photometrically in miniaturized 96-well plate format by kinetic assays (Jammer et al. 2015; Garcia-Lemos et al. 2019). All the measurements were carried out in triplicate and the substrate was omitted from the control reactions (Supporting Information, Table S2). The activities of carbohydrate and antioxidative enzymes were expressed in nkat g FW–1 calculated using Gen5 v3.04.17 (Biotek Instruments. Inc) software.

2.7 Statistical analysis

Data analysis and visualizations were performed in R (R Core Team, 2019), using the packages '*lme4'* (Bates et al. 2015), '*multcomp'* (Hothorn et al. 2008) and '*ggplot2'* (Wickham, 2016). Model assessments were carried out by residual and quantile-quantile (Q-Q plot) plots. The binomial logistic mixed-effect model was fitted to the fungus colonization data (presence/absence per plant pieces) using fungal isolates and plant parts (leaf, stem and root) as fixed effects with block and experimental repetitions as random effects. Fungal colonization percentages and confidence intervals were calculated using the same model assumptions. Poisson generalized linear mixed effect model (*log* link function) was fitted to aphid population data with seed treatments (fungal and control) as fixed effect while plant number, blocks and experiments were included as random factors. Linear mixed model was fitted to plant growth (height and biomass) and enzyme data with seed treatments, presence/absence of aphids and their interaction as fixed effects (date was also included in height data), while block and experimental replicates as random factors. Log transformations for Ald, FK and G6PDH enzymes were done to satisfy the model assumptions. Satterthwaite's approximations

using '*lmerTest'* package (Kuznetsova *et al*., 2017) were used for P-values and degrees of freedom (*df*). *post hoc* pairwise comparisons were done using Tukey's test for significant outcomes. Heat maps (fold change) for enzyme data were made by using MultiExperiment Viewer software (Ochs et al. 2010). Pairwise comparisons were done for enzymes with significant aphid (presence/absence) effects to check a significant increase in enzyme activities with aphid feeding shown in heat maps.

3. RESULTS

3.1 Population growth of *R. padi* on EPF inoculated wheat plants

Seed treatments (control or EPF inoculated) significantly affected the number of *R. padi* per wheat plant over 20 days after seed inoculation and 10 days of aphids infestation (df = 3, F = 11.33, P = 0.001). No significant differences were found between treatments at 2nd, 4th and 6th day after *R. padi* release, while the plants treated with *M. brunneum* supported a higher number of *R. padi* as compared to the other treatments at the 8th and 10th day of inoculations (Fig. 1). Plants of the *M. robertsii* treatment harbored significantly less *R. padi* than plants of the *M. brunneum* (P < 0.001) and control (P = 0.03) treatments at the 10th day of infestation. Furthermore, plants of the *B. bassiana* treatment supported significantly less aphids as compared to plants treated with *M. brunneum* (P < 0.001) 10 days after aphid application, but with no differences from aphid numbers of *M. robertsii* and control wheat plants (P > 0.05).

3.2 Activities of Carbohydrate Enzymes after EPF Inoculations and *R. padi* Infestation in Wheat

The activities of 12 key carbohydrate enzymes after seed treatments (control or fungal inoculated), with or without *R. padi* infestation are shown in Fig. 2. Concentrations of eight carbohydrate enzymes (cytInv, cwInv, FK, HXK, UGPase, AGPase, G6PDH and PFK) were significantly effected by seed treatments, three (vacInv, cwInv and HXK) were significantly affected by aphid feeding while a significant interaction between treatments and aphid challenge was only found for G6PDH (Fig. 2; Supporting Information Table S3). The overall activities of cwInv and HKX were increased with aphid feeding while vacInv was decreased (Fig. 2). The fold change activities with aphid feeding are shown in Fig. 3. Concentrations of enzymes decreased or increased with aphid feeding depending upon the seed treatment. The activities of cwInv, UGPase and PFK were significantly increased in *M. brunneum* treated plants, HXK and PFK increased in *M. robertsii* treated plants, HXK increased in *B. bassiana* treated plants while vacInv and G6PDH were decreased in control plants with aphid feeding (Fig. 3). In general, *M. brunneum* and *M. robertsii* presented higher activity profiles for most

carbohydrate enzymes as compared to *B. bassiana* and control treatments with aphid feeding (as indicated by red scale coloring in Fig. 3).

3.3 Activities of Antioxidant Enzymes after EPF Inoculations and *R. padi* Infestation in Wheat

The activities of eight antioxidant enzymes were measured after seed treatments, with or without *R. padi* infestation, shown in Fig. 4. Seed treatments significantly affected the concentrations of GR while the presence of *R. padi* significantly changed SOD, CAT and GST activities. The other four enzymes did not show any significant differences (Fig. 4; Supporting Information Table S4). The fold change activities within the same treatment with aphid feeding were more prominent in *M. brunneum* treated plants (red scale coloring in Fig 5). *M. brunneum* treated plants significantly increased activities of CAT and GST while the activity of SOD was significantly increased in *M. robertsii* treated plants with aphid feeding as compared to plants without aphid (Fig. 5).

3.4 Wheat growth promotion effects after EPF Inoculations and *R. padi* Infestation

Plant heights (cm) were not significantly affected by the inoculations after 10^{th} day of inoculations while significant seed treatments and aphid interaction was found at 20 days of inoculations ($df = 3$, $F = 8.58$, $P < 0.001$). At 20 day heights were significantly decreased in *B. bassiana* ($P < 0.01$) and control ($P < 0.001$) treatments, increased in *M. brunneum* treatment ($P < 0.01$) while showed no difference in *M. robertsii* ($P > 0.05$) treatment with aphid feeding as compared to plants without aphids. In fact, plants of the *M. brunneum* and *M. robertsii* treatments were higher than plants of the *B. bassiana* and control treatments in the presence of aphids (Fig. 6).

Shoot fresh weight was significantly effected by seed treatments ($df = 3$, $F = 3.08$, $P = 0.03$) and aphid challenge ($df = 1$, $F = 4.25$, $P = 0.04$), whereas dry weight was only effected by seed treatments ($df = 3$, $F = 5.55$, $P < 0.01$). Plants of the *M. brunneum* treatment showed significantly higher shoot biomass than *B. bassiana* and control treatments when plants were challenged with aphids (Fig. 6). A significant seed treatments effect was also found for root fresh ($df = 3$, $F = 5.40$, $P < 0.01$) and dry weights ($df = 3$, $F = 5.20$, $P < 0.01$). Both isolates of *Metarhizium* spp. showed higher root dry weight than control treatments in presence of aphids (Fig. 6).

3.5 Colonization of wheat tissues by entomopathogenic fungal isolates after seed inoculations

The three EPF isolates differently colonized wheat plants after 20 days of seed inoculations. Colonization percentages were calculated for colonized wheat plant tissue pieces of leaves, stem and roots (Fig. 7). A significant plant part effect was found for tissue pieces ($df = 2$, $F = 3.43$, $P = 0.03$).

Seed inoculations with *B. bassiana* colonized all plant tissues with a significantly high percentage in the stem than leaves (P = 0.02; Fig. 7). Whereas, *M. brunneum* was found in stem and roots and *M. robertsii* only in roots (Fig. 7). Overall, *B. bassiana* and *M. brunneum* were re-isolated from 58% of total inoculated wheat plants while *M. robertsii* was re-isolated from 41% of total wheat plants (n = 12). The plant was considered colonized when at least one of the tissue pieces showed signs of endophytic colonization. None of the targeted EPF isolates were detected in control plants although some infrequent outgrowth of unrelated endophytic fungus or bacteria were found in fungal or control wheat pieces.

4. DISCUSSION

Seed treatments with three isolates of entomopathogenic fungi successfully established as wheat endophytes with variable degrees of colonization in different plant parts. Although seed inoculation showed varied colonization rates, the degree of endophytic EPF in wheat was not related to the plant growth effects, the reproduction rate of *R. padi* and activities of some key carbohydrate and antioxidant enzymes in wheat. This study is first to demonstrate that seed inoculations with EPF changed the activities of some key carbohydrate and antioxidant enzymes in wheat supporting the hypothesis of physiological changes *in planta* after fungal seed treatments with EPF and aphid infestation.

Although a recent meta-analysis revealed that EPF endophytes had prominent negative effects against sucking insect herbivores (Gange et al. 2019), we demonstrate here diverse responses of the three EPF isolates against aphids. Seed treatments with *M. robertsii* ESALQ 1622, *B. bassiana* GHA and *M. brunneum* KVL04-57 showed negative, neutral and positive effects on *R. padi* population development in wheat, respectively, compared to the mock control treatment. The same isolate of *M. robertsii* (ESALQ 1622) has been found to reduce the population growth of two-spotted spider mites, *Tetranychus urticae*, in bean (*Phaseolus vulgaris*) following seed treatment (Canassa et al. 2019). A recent study demonstrated that *M. robertsii* seed treatments altered defense based gene expressions in maize (*Zea mays*) and decreased the relative growth rate of black cutworm (*Agrotis ipsilon*) feeding on the inoculated plants (Ahmad et al. 2020). The authors suggested that endophytic colonization with *M. robertsii* primed maize plant defenses against upcoming biotic stress by modulating gene expression. However, the feeding experiments with *A. ipsilon* were performed in assays with excised maize leaves and not whole plants as in the present study.

Conversely, *M. brunneum* treated plants enhanced the population growth of *R. padi* and aphid populations on *B. bassiana* inoculated plants showed no differences to control plants. These findings are in line with previous findings of Clifton et al. (2018), where *M. brunneum* (strain F52, same isolate origin as KVL 04-57) seed treatments increased the population sizes of soybean aphid (*Aphis glycines*) in soybean while treatment with *B. bassiana* (strain GHA) showed no effects. Likewise, two isolates of *M. anisopliae* (N1LT6 and S4ST7) showed no effects on different growth parameters of the aphids, *Acyrthosiphon pisum* and *Aphis fabae* after seed inoculations in fava beans (Akello and Sikora 2012). Several isolates of *B. bassiana* have been well explored for their ability to colonize plants as endophytes and have shown negative effects against several insect pests (Rondot and Reineke 2018; Sánchez-Rodríguez et al. 2018; Mahmood et al. 2019). However, Jensen et al. (2019) reported a high number of second-generation *A. fabae* nymphs in *B. bassiana* (GHA) seed treated and leaves sprayed fava beans (Jensen et al. 2019). It has been suggested that the particular EPF isolates may reduce the efficacy of general defense systems of plants or increase the nutritional composition of host plants to favor the reproduction rate of aphids (Clifton et al. 2018; Jensen et al. 2019).

Fungal endophytes interact with host plant physiology and enable plants to counter different stress factors e.g. herbivory (Rodriguez et al. 2009). To explore the mechanism behind distinct behaviors of EPF isolates after seed inoculations, we measured the activities of key carbohydrate and antioxidant enzymes 20 days after EPF inoculations and 10 days after *R. padi* infestation. In the presence of aphids, seed treatments with EPF isolates increased the activities of HXK, PFK, Ald (enzymes involved in glycolysis) and G6PDH (an enzyme involved in oxidative pentose phosphate pathway) as compared to control plants. Higher activities of these enzymes have been found in different plant species after abiotic stress and have been related to tolerance factors for stress (Klotz et al. 2006; Scharte et al. 2009; Mutuku and Nose 2012; Shu et al. 2014). The higher activities of these enzymes in fungal inoculated plants maintained plant health and tolerance level by producing energy under biotic stress but did not correlate with the aphid population growth in the present study. Plants in the *M. robertsii* treatment produced the highest levels of PFK, which have previously shown increased activity in resistant rice lines against a fungal plant pathogen, *Rhizoctonia solani* (Mutuku and Nose 2012). Nevertheless, the role of PFK in aphid resistance in the present study is unclear as plants treated with *M. brunneum* also showed higher activities of PFK than *B. bassiana* treated and control plants, but also supported the highest number of aphids.

The *R. padi* infestation caused no major changes in carbohydrate enzyme levels. However, activities of cwInv and HXK increased with *R. padi* feeding. cwInv is a central enzyme involved in

different processes including sugar and stress-related stimuli (reviewed by Roitsch and González 2004). Plant-pathogen interactions are found to induced biosynthesis of cwInv (Berger et al. 2007). Rehill and Schultz (2003) showed a positive correlation between cwInv activities and aphid fecundity but this correlation was not found in the present study. Conversely, the overall activities of vacInv were decreased with aphid feeding. Reduced vacInv activities have been found in drought-stressed young maize ovaries (Roitsch and González 2004). Although control plants showed overall lower activities than fungal inoculated plants, the levels of vacInv and G6PDH significantly decreased in control plants with aphid feeding. In host-pathogen interactions, increasing demand for sink assimilates decreases source metabolism (Roitsch and González 2004). During biotic stress, plants save energy by downregulation of primary metabolism and allocate it for defense (Rojas et al. 2014). The carbohydrate levels in the present study indicated that wheat plants in the EPF inoculations upregulated the activities of carbohydrate enzymes to produce energy as a stress response, but these changes in activity did not show an unambiguous role in defense against aphid infestations.

For antioxidant enzymes, most particularly the activity of SOD increased in *M. robertsii* inoculated plants infested with aphid compared to the other treatments. SOD scavenges $O_2^{\bullet-}$ into H_2O_2 and O_2 and provides first-line defense against oxidative stress in plants (Gajewska et al. 2006). The high production of H_2O_2 is expected to act as signaling molecules in plants as a resistance factor against aphids (Maffei et al. 2007). The induction of H_2O_2 in response to aphid feeding including *R. padi* has been found in barley, oat and wheat (Smith and Boyko 2007). Accumulation of H_2O_2 also triggers other compounds involved in plant defense responses (e.g. increase activity of peroxidases) to reduce aphid attack (Argandoña et al. 2001). The inoculations with an endophytic bacteria *Bacillus velezensis* YC7010 increased the levels of induced systemic resistance against green peach aphid, *Myzus persicae*, by increasing concentrations of H_2O_2, causing cell death and deposition of callose (a physical barrier in tissues) in *Arabidopsis thaliana* (Harun-Or-Rashid et al. 2017). This shows that the high levels of SOD in *M. robertsii* treatment were likely correlated to a high production of H_2O_2 leading to negative effects against *R. padi*.

Besides, CAT is involved in catalyzing the dismutation of H_2O_2 into H_2O and O_2 (Gajewska et al. 2006) and high production of CAT in *M. brunneum* treated plants with *R. padi* infestations strengthens the assumption of the aforementioned role of H_2O_2. We assume that *M. brunneum* treated plants increased CAT production to reduce oxidative stress caused by aphid feeding and scavenge H_2O_2 to non-toxic compounds, leading to an increase in population levels of *R. padi*. A root-colonizing fungus *Serendipita indica* (previously known as *Piriformospora indica*) and a root parasite, *Fusarium*

verticillioides induced the accumulations of SOD, CAT, GST and GR compared to non-inoculated plants in maize roots (Kumar et al. 2009). The overproduction of CAT is associated with suppression of pathogen resistance while underproduction is important for programmed cell death during biotic stress (Mittler 2002; Kumar et al. 2009). This association of CAT with pathogens could be correlated with *R. padi* feeding where overproduction increased aphid populations while low production caused a decrease of aphid populations. In addition, most of the antioxidative enzymes showed minimal changes indicating that fungal inoculations and aphid feeding did not stress wheat plants. The sophisticated feeding mechanism of aphids causes minor damage to plant tissues and reduces the possibilities of induced defense (Pentzold et al. 2014). Although, aphid herbivory showed inconstant responses (enhanced, reduced or elicit no changes) to antioxidant activities in different host plants (Khattab 2007; Ni et al. 2009; He et al. 2011).

The plant growth promotion effects were most prominently seen in wheat plants inoculated with *Metarhizium* spp. isolates. In the putatively mutualistic relationship, species of *Metarhizium* and *Beauveria* can translocate nitrogen from fungal killed insect cadavers to plant hosts (Behie et al. 2012; Behie and Bidochka 2014) in exchange for photosynthates from the plants (Behie et al. 2017). As nitrogen is considered a limiting factor for plants, the mobilization of nitrogen by EPF can improve plant growth (Jaber and Enkerli 2016; Hu and Bidochka 2019). In addition, EPF isolates are also found to improve the Fe bioavailability in calcareous and non-calcareous media with the most prominent effects seen with *M. brunneum* strain EAMa 01/58–Su (Raya-Díaz et al. 2017). The bioavailability of different nutrients could improve plant growth but it is still unknown whether EPF could also increase the uptake of other essential nutrients to improve plant growth. The high growth rates in plants inoculated with *Metarhizium* spp. also correlates with the higher activities of carbohydrate enzymes in both isolates in the present study.

However, *B. bassiana* treatment decreased plant growth specifically in the presence of aphids. The growth promotion effects from root colonization with EPF is also strain-specific as one strain of *B. bassiana* (BG11) increased plant growth of *A. thaliana* while another strain (FRh2) had no effects (Raad et al. 2019). Nutrient availability in the growing substrate is an essential factor for *B. bassiana* to improve plant growth (Tall and Meyling 2018). In the present study, we provided the same amount of nutrients to all plants so we could not evaluate the role of this factor for plant growth.

The tested EPF isolates showed various degrees of colonization success in leaf, stem and root of the wheat plant. *Metarhizium* spp. are mostly localized in roots while *B. bassiana* often is found

throughout the plant (Behie et al. 2015). Reports showed that seed inoculation of different host plants with *Metarhizium* and *Beauveria* species led to root colonization (Hu and Bidochka 2019). However, few studies also showed the above-ground colonization of plants with *Metarhiuzm* spp. (Batta 2013; Golo et al. 2014; Clifton et al. 2018; Jaber 2018). The pattern of fungus colonization did not correlate with effects on aphid population growth as the isolate with a prominent negative effect, *M. robertsii*, was only isolated from roots of wheat plants in the present study.

Our reported findings demonstrated that seed treated isolates of different EPF species had variable effects on the population growth of *R. padi* and on enzyme activity patterns. The activities of carbohydrate enzymes were not clearly related to levels of *R. padi* population growth but showed a correlation with plant growth. However, the activities of specific antioxidant enzymes increased with fungal inosculations and may, therefore, play a role in plant-herbivore interactions. These findings are important for further insights into the physiological roles of using EPF as plant inoculants and to understand their effects on insect herbivores.

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Figures

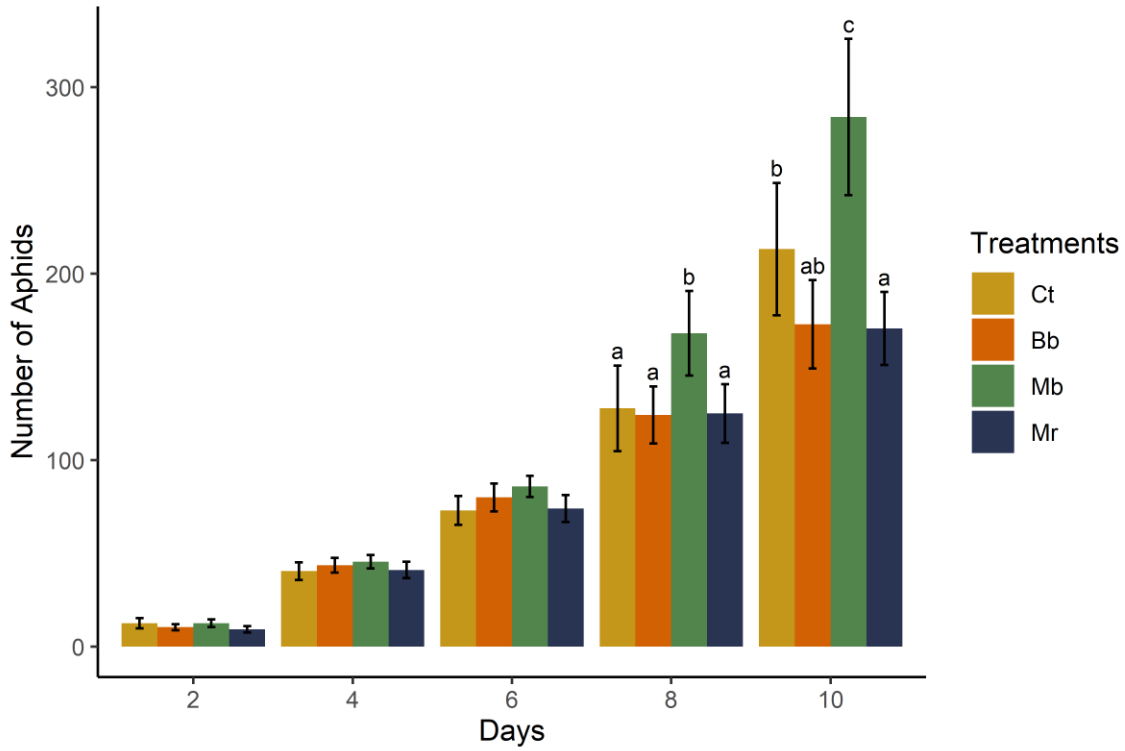

Figure 1: Effects of *Beauveria bassiana* (Bb), *Metarhizium brunneum* (Mb), *M. robertsii* (Mr) and control (Ct-Triton X-100 control) seed treatments on population growth of *Rhopalosiphum padi* in wheat over 10 days post infestation. Bars (mean number of *R. padi* ± SE) with the same letters are not significantly different by *post hoc* tests using *multcomp* function.

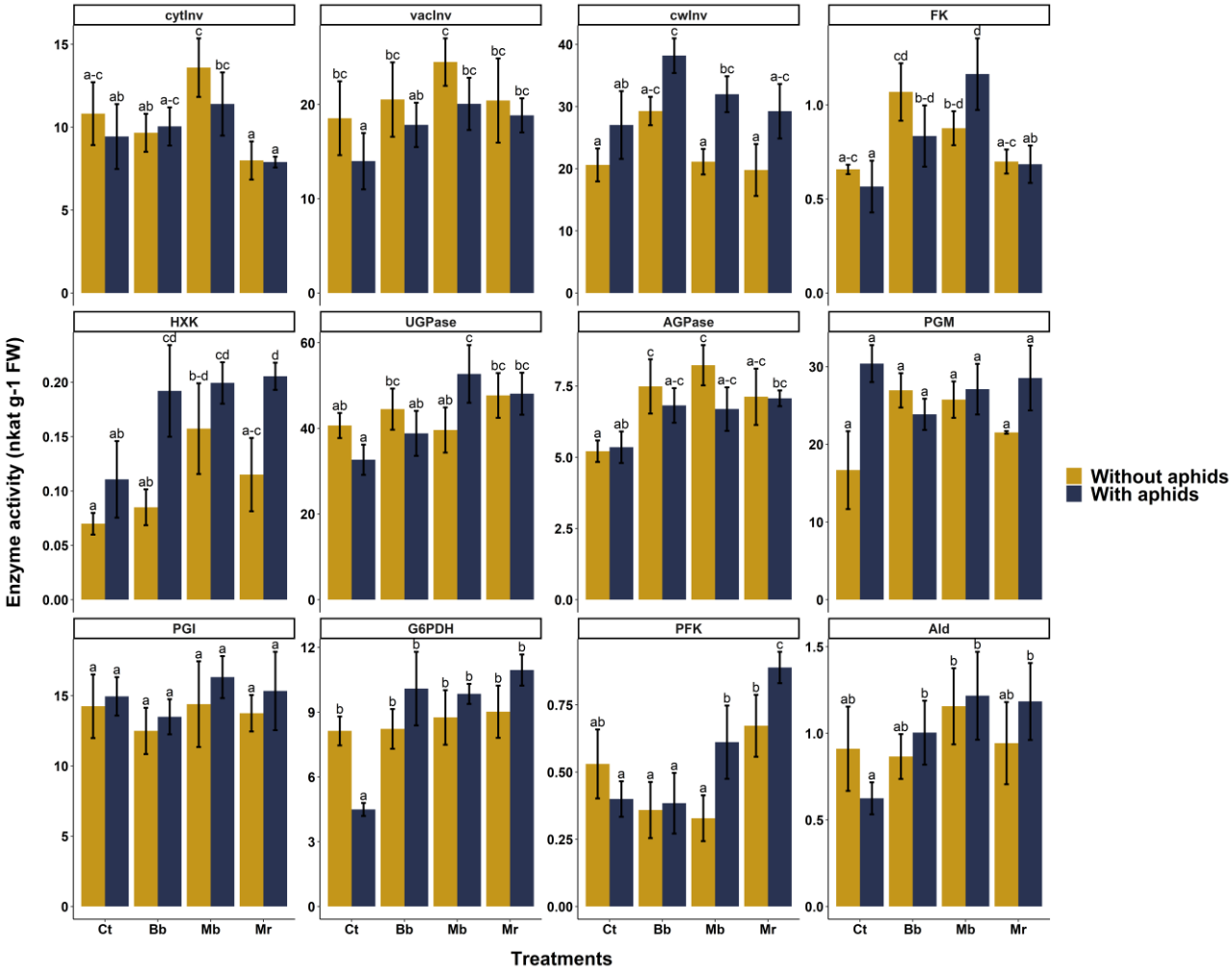

Figure 2: Effects of *Beauveria bassiana* (Bb), *Metarhizium brunneum* (Mb), *M. robertsii* (Mr) and control (Ct-Triton X-100 control) seed treatments and *Rhopalosiphum padi* infestation on key carbohydrate enzymes. Cytoplasmic invertases (cytInv), vacuolar invertases (vacInv), cell-wall invertases (cwInv) fructokinase (FK), hexokinase (HXK), UDP-glucose pyrophorylase (UGPase), ADP-Glucose pyrophosphorylase (AGPase), phosphoglucomutase (PGM), phosphoglucoisomerase (PGI), glucose-6-phosphate dehydrogenase (G6PDH), phosphofructokinase(PFK) and aldolase (Ald). The bars show enzyme activity nkat g FW^{-1} ± SE (n = 6) without *R. padi* (yellow bars) or with *R. padi* infestation (dark grey bars). Bars within a graph with same letters are not significantly different by *post hoc* tests using *multcomp* function; letters "a-c" = "a,b,c" and "b-d" = "b,c,d".

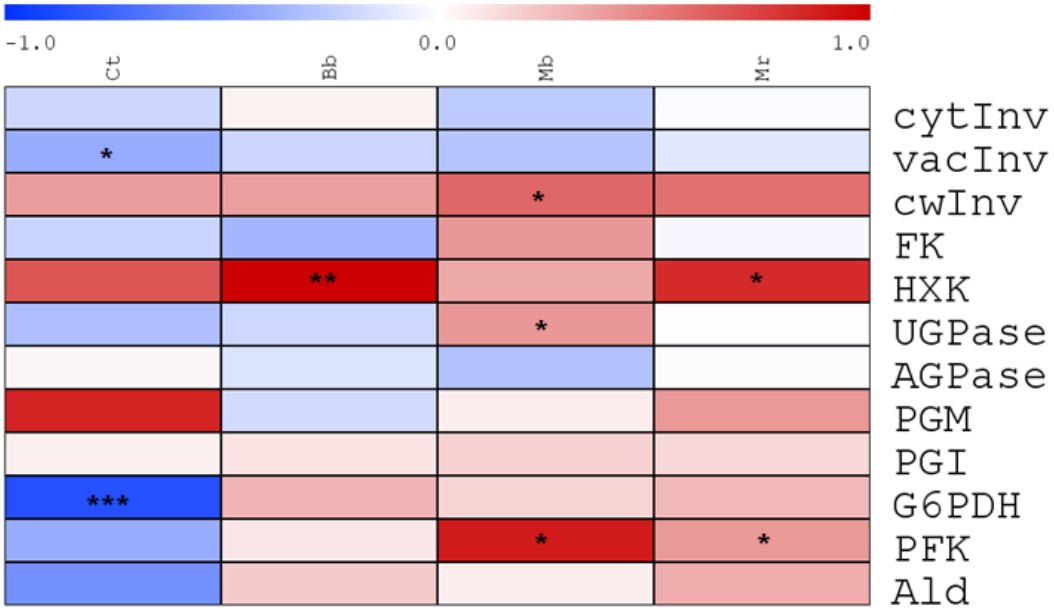

Figure 3: Fold change heat map analysis summarizing the activities of carbohydrate enzymes (see legend of Figure 2) in wheat with *Rhopalosiphum padi* infestation. The fold change results were calculated by Logarithm base 2 (Log_2) of the carbohydrate enzyme activities in wheat plants with *R. padi* divided by the activities without *R. padi.* Red cells indicate relatively higher activities of specific compounds in wheat plants after *R. padi* infestation, while blue cells show relatively lower activities of specific enzymes in wheat plants after *R. padi* infestation. White cells show no differences using false color scale visualization. The significant increase is indicated with asterisks (Significance codes: ***: P <0.001; **: 0.001<P<0.01; *: 0.01<P<0.05).

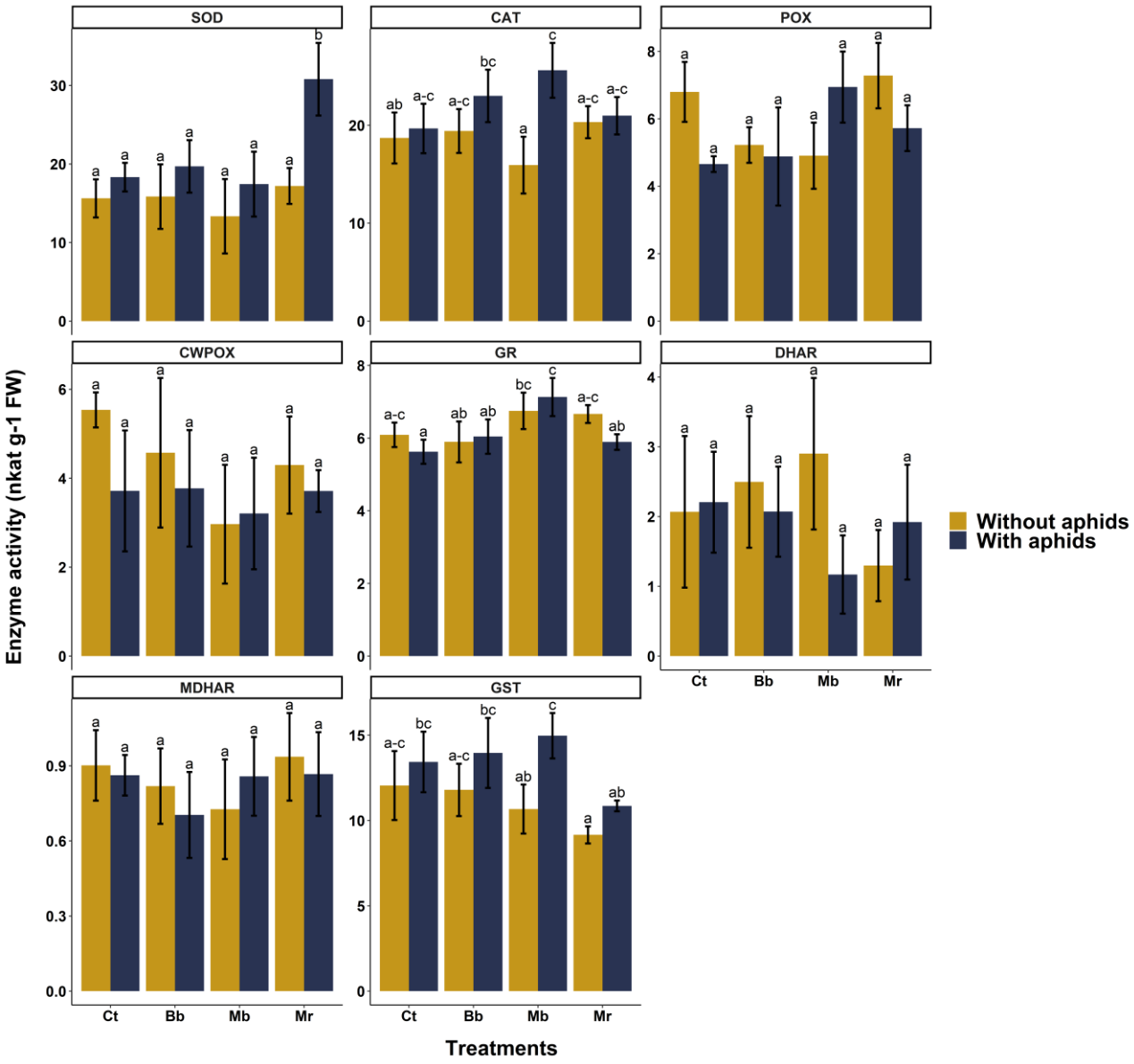

Figure 4: Effects of *Beauveria bassiana* (Bb), *Metarhizium brunneum* (Mb), *M. robertsii* (Mr) and control (Ct-Triton X-100 control) seed treatments and *Rhopalosiphum padi* infestation on key antioxidant enzymes. Superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), cell-wall peroxidase (CWPOX) glutathione reductase (GR), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and glutathione S transferase (GST). The bars show enzyme activity nkat g FW^{-1} ± SE (n = 6) without *R. padi* (yellow bars) or with *R. padi* infestation (dark grey bars). Bars within a graph with same letters are not significantly different by *post hoc* tests using *multcomp* function; letters "a-c" = "a,b,c" and "b-d" = "b,c,d".

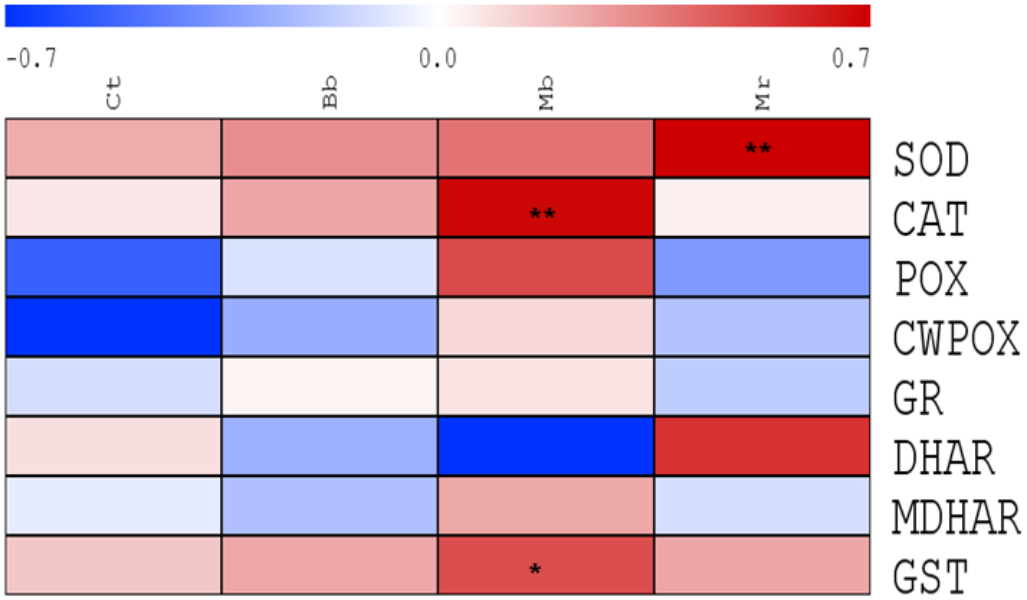

Figure 5: Fold change heat map analysis summarizing the activities of antioxidant enzymes (see legend of Figure 4) in wheat with *Rhopalosiphum padi* infestation. The fold change results were calculated as described for Figure 3 as are significance symbols.

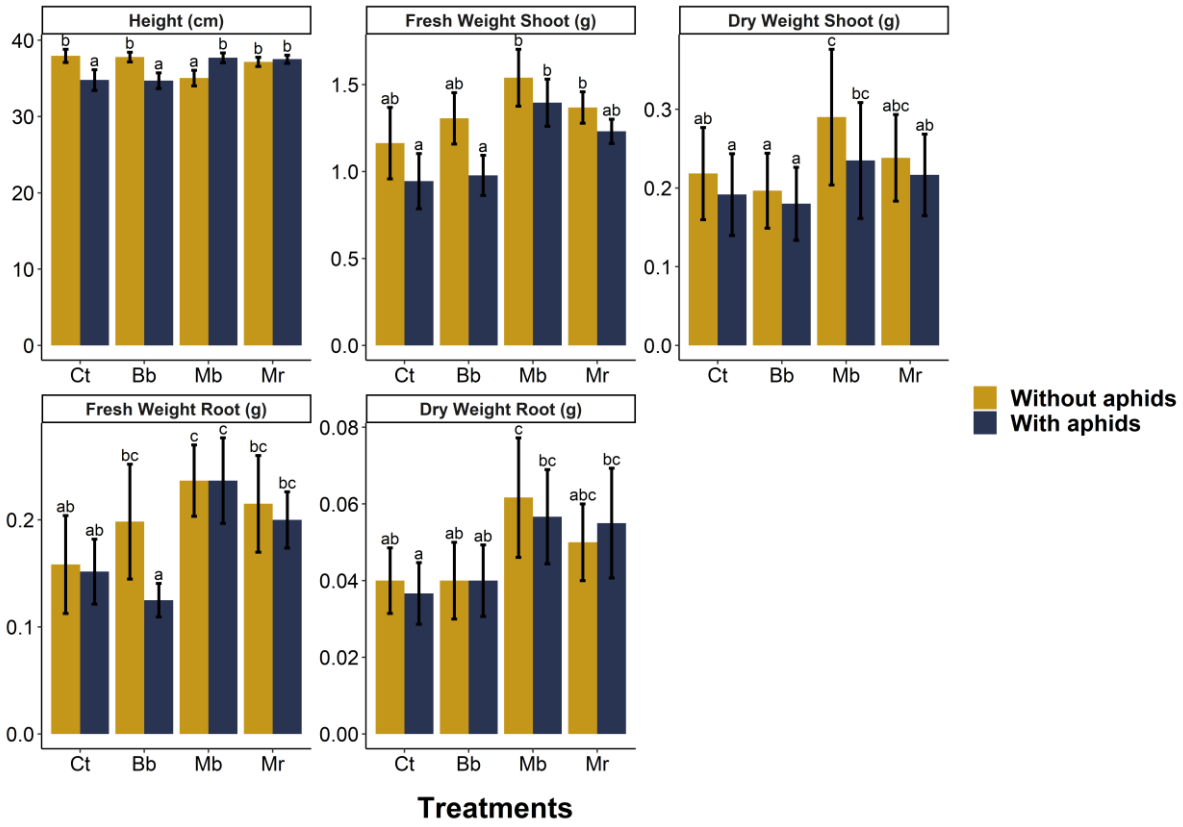

Figure 6: Effects of *Beauveria bassiana* (Bb), *Metarhizium brunneum* (Mb), *M. robertsii* (Mr) and control (Ct-Triton X-100 control) seed treatments on plant growth parameters of wheat. Mean ± SE values of height (cm), fresh weight of shoot (g), dry weight of shoot (g), fresh weight of root (g) and dry weight of root (g) 10 days after *Rhopalosiphum padi* infestation and 20 days after seed treatments are presented. Bars with the same letters are not significantly different by *post hoc* tests using *multcomp* function.

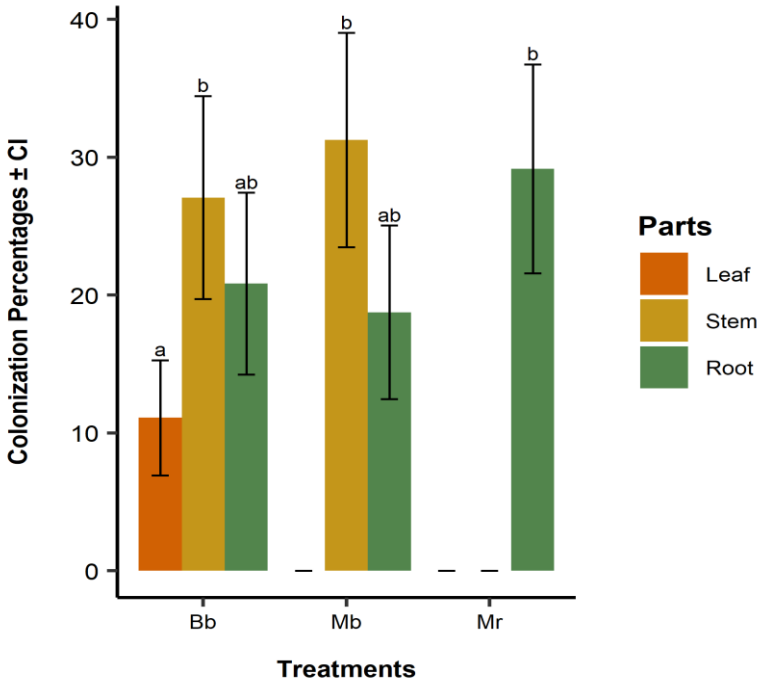

Figure 7: Percentages of endophytic colonization of wheat tissue pieces (leaf, stem and root), 20 days after seed treatments with *Beauveria bassiana* (Bb), *Metarhizium brunneum* (Mb) and *M. robertsii* (Mr). Bars (percentage ± 95% confidence intervals) with the same letters are not significantly different by *post hoc* tests using *multcomp* function.

Supporting Information

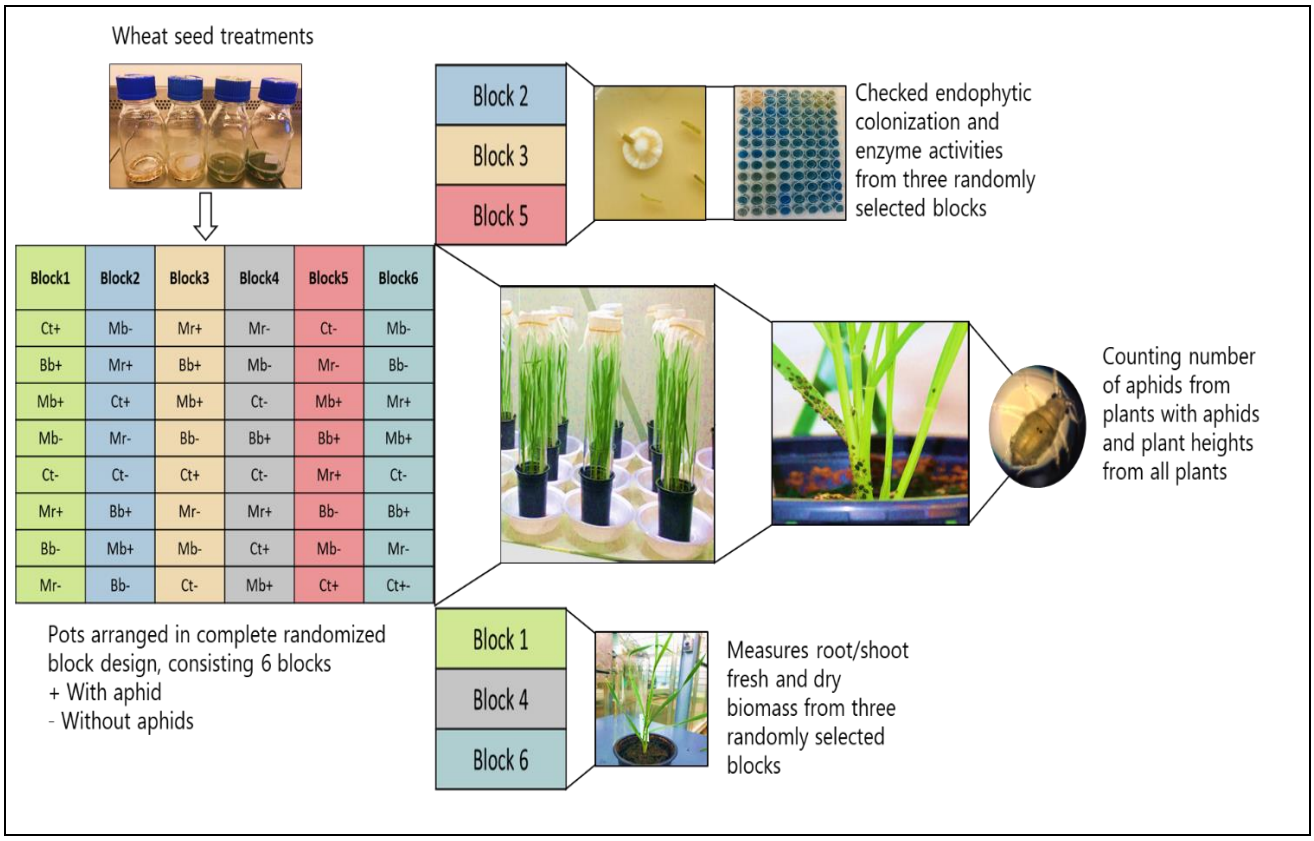

Figure S1. Schematic representation of experimental setup. Fungus (*Beauveria bassiana* - Bb, *Metarhizium brunneum* - Mb, *M. robertsii* - Mr) and control (Triton X-100 - Ct) treated wheat seeds were gown in randomized complete block design consisting six blocks. Number of aphids were counted at 2, 4, 6, 8 and 10 days after infestation from the plants with aphids (+) and plant heights of all plants were measures. Afterwrads, blocks were divided randomly into two sets (three blocks in each set). One set of blocks were used to check endophytic colonization and enzyme activities while the other set was used to measure plant growth parameters (root/shoot fresh and dry biomass).

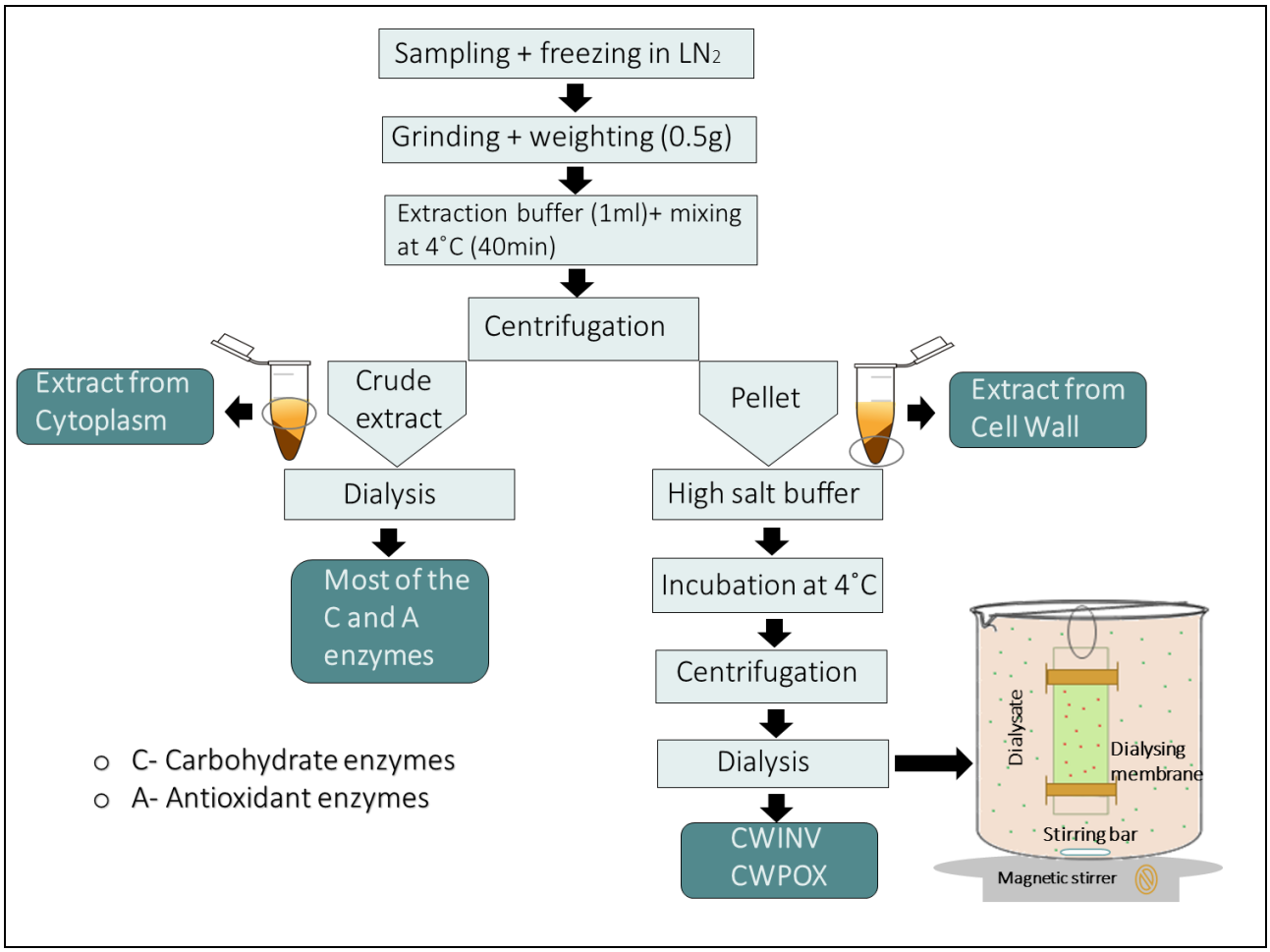

Figure S2. Enzyme extraction and dialysis protocol flowchart. The wheat leaf samples were ground with liquid nitrogen (LN_2), weighed (0.5 g), extracted using extraction buffer for 40 min at 4 °C, centrifuged and divided into crude extract (extract from cytoplasm) and pellet (extract from cell wall). Cell wall extract was washed with high salt buffer and both extracts were dialyzed using a dialyzing membrane. Cell wall extract as used for the analysis of cell-wall invertases and cell-wall peroxidase while cytoplasmic extract as used for all other carbohydrate enzymes (C enzymes) and antioxidant enzymes (A enzymes).

Table S1. Assay conditions for key carbohydrate enzymes analysis.

Enzymes	Assay compounds	Substrates	Wavelength	Extract volume	Reaction volume	References
cytInv	5 µl of sucrose (0.1 M) and 5 µl of reaction buffer (0.454 M Na_2HPO_4/0.273 M citric acid), pH 6.8	Sucrose	405 nm	5 µl	50 µl	(Jammer et al. 2015)
vacInv	Same as cytInv except reaction buffer pH 4.5	Sucrose	405 nm	5 µl	50 µl	(Jammer et al. 2015)
cwInv	Same as cytInv except reaction buffer pH 4.5	Sucrose	405 nm	5 µl	50 µl	(Jammer et al. 2015)
FK	Bis-TRIS (0.1 M, pH 8.0), $MgCl_2$ (0.5 M),ATP (0.1 M), G6PDH from *Leuconostoc mesenteroides* (1000 U ml^{-1}), NAD (0.05 M), PGI (3500 U ml^{-1}) and fructose (0.1 M)	Fructose	340 nm	5 µl	160 µl	(Appeldoorn et al. 1999; Petreikov et al. 2001)
HXK	Same as FK except PGI was excluded and fructose was replaced by glucose (0.1 M)	Glucose	340 nm	5 µl	160 µl	Same as FK
UGPase	TRIS-HCl (1 M, pH 8.0), EDTA (0.25 M), $MgCl_2$ (0.5 M), BSA (10%), Na-PPi (0.1 M), NADP (0.01 M), 3-PG (0.05 M), G6PDH from *Saccharomyces cerevisiae* (1.28 U ml^{-1}), PGM (1000 U ml− 1), UGP glucose (0.1M)	UDPGlc	340 nm	5 µl	160 µl	(Pelleschi et al. 1997; Appeldoorn et al. 1999)
AGPase	Same as UGPase except UGP glucose was replaced by ADP-Glucose (0.05 M)	ADPGlc	340 nm	5 µl	160 µl	Same as UGPase
PGM	TRIS-HCl (1 M, pH 8.0), $MgCl_2$ (0.5 M), DDT (0.5 M), NADP (0.01 M), G6PDH from *S. cerevisiae* (6000 U ml^{-1}), Glc-1,6-bisP (0.01 M), Glc-1-P (0.1 M)	Glc-1-P	340 nm	5 µl	160 µl	(Manjunath et al. 1998)
PGI	Same as PGM except Glc-1,6-bisP and Glc-1-P were replaced by fruct-6-P (0.1 M)	Fruct-6-P	340 nm	5 µl	160 µl	(Zhou and Cheng 2008)
G6PDH	TRIS-HCl (1 M, pH 7.6), $MgCl_2$ (0.5 M), NADP (0.01 M) and Glc-6-P (0.1 M)	Glc-6-P	340 nm	5 µl	160 µl	(Jammer et al. 2015)
PFK	TRIS-HCl (1 M, pH 8.0), EDTA (0.25 M), $MgCl_2$ (0.5 M), NADH(0.025 M), ATP (0.1 M), aldolase (372 U ml^{-1}), GDPH (2100 U ml^{-1}), TPI (600 U ml^{-1}) and fruct-6-P (0.1 M)	Fruct-6-P	340 nm	5 µl	160 µl	(Klotz et al. 2006)
Ald	Same as PFK except ATP and aldolase were excluded and fruct-6-P was replaced by F-1,6-bisP (0.025M)	F-1,6-bisP	340 nm	5 µl	160 µl	(Schwab et al. 2001)

Enzymes: Cytoplasmic invertases (cytInv), vacuolar invertases (vacInv), cell-wall invertases (cwInv) fructokinase (FK), hexokinase (HXK), UDP-glucose pyrophorylase (UGPase), ADP-Glucose pyrophosphorylase (AGPase), phosphoglucomutase (PGM), phosphoglucoisomerase (PGI), glucose-6-phosphate dehydrogenase (G6PDH), phosphofructokinase(PFK) and aldolase (Ald).

Table S2. Assay conditions for key antioxidant enzymes analysis.

Enzymes	Assay compounds	Substrates	Wavelength	Extract volume	Reaction volume	References
SOD	KPO4 buffer (0.05 M, pH 7.8), EDTA (0.1 mM), cytochrome C (0.05 mM), xanthine oxidase (0.0002 U mg^{-1}) and xanthine (10 mM)	Xanthine	550 nm for 40 min	5 µl	160 µl	(McCord, J. M., and Fridovich 1969)
CAT	KPO4 buffer (0.1 M, pH 7.0), antifoam (0.001%) and H_2O_2 (0.1 M)	H_2O_2	240 nm for 40 min	5 µl	160 µl	(Aebi 1984)
POX	KPO4 buffer (0.1 M, pH 7.0), guaiacol (2 mM) and H_2O_2 (0.15 mM)	H_2O_2	450 nm for 40 min	5 µl	160 µl	(Polle et al. 1994)
CWPOX	KPO4 buffer (0.1 M, pH 7.0), guaiacol (2 mM) and H_2O_2 (0.15 mM)	H_2O_2	450 nm for 40 min	5 µl	160 µl	(Polle et al. 1994)
GR	TRIS-HCl (0.1 M, pH 7.8), NADPH (25 mM) and glutathione oxidized (30 mM).	Glutathione oxidized	340 nm for 40 min	5 µl	160 µl	(Edwards et al. 1990)
DHAR	KPO4 buffer (0.1 M, pH 6.5), glutathione reduced (50 mM) and dehydroascorbic acid (50 mM).	Dehydroascorbic acid	290 nm for 40 min	5 µl	160 µl	(Dalton et al. 1986)
MDHAR	KPO4 buffer (50 mM, pH 7.2), NADH (25 mM), ascorbic acid oxidase (5U $µl^{-1}$) and ascorbate (50 mM).	Ascorbate	340 nm for 40 min	5 µl	160 µl	(Arrigoni O, Dipierro S 1981)
GST	KPO4 buffer (0.1 M, pH 7.4) and glutathione reduced and 2, 4-dinitrochlorobenzene (50 mM).	2,4-dinitrochlorobenzene	334 nm for 30 min	5 µl	160 µl	(Li et al. 1995)

Antioxidant enzymes: Superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), cell-wall peroxidase (CWPOX) glutathione reductase (GR), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and glutathione S transferase (GST).

Table S3. Linear mixed-effect model result table for carbohydrate enzyme activities in wheat showing the variation and significance due to seed treatments, aphids and their interaction.

Carbohydrate enzymes	Effects	F-ratio	Df	P-value
cytInv	Seed treatments	4.529	3; 32	0.009*
	Aphids	0.552	1; 33	0.462
	Seed treatments × Aphids	0.602	3; 32	0.618
vacInv	Seed treatments	2.735	3; 32	0.059
	Aphids	4.884	1; 32	0.034*
	Seed treatments × Aphids	0.552	3; 32	0.649
cwInv	Seed treatments	3.671	3; 32	0.022*
	Aphids	13.805	1; 33	<0.001***
	Seed treatments × Aphids	0.120	3; 32	0.947
FK	Seed treatments	4.704	3; 31	0.008**
	Aphids	0.941	1; 31	0.339
	Seed treatments × Aphids	0.887	3; 31	0.458
HXK	Seed treatments	4.297	3; 31	0.011*
	Aphids	11.858	1; 32	0.001**
	Seed treatments × Aphids	0.661	3; 31	0.582
UGPase	Seed treatments	4.280	3; 34	0.011*
	Aphids	0.022	1; 34	0.881
	Seed treatments × Aphids	2.846	3; 34	0.051.
AGPase	Seed treatments	4.413	3; 34	0.010*
	Aphids	1.210	1; 34	0.278
	Seed treatments × Aphids	0.602	3; 34	0.618
PGM	Seed treatments	0.258	3; 32	0.854
	Aphids	3.395	1; 33	0.074
	Seed treatments × Aphids	2.466	3; 32	0.079
PGI	Seed treatments	0.569	3; 34	0.638
	Aphids	0.981	1; 34	0.328
	Seed treatments × Aphids	0.066	3; 34	0.977
G6PDH	Seed treatments	7.718	3; 35	<0.001***
	Aphids	0.033	1; 35	0.855
	Seed treatments × Aphids	5.628	3; 35	0.002**
PFK	Seed treatments	10.736	3; 34	<0.001***
	Aphids	3.085	1; 34	0.088
	Seed treatments × Aphids	2.740	3; 34	0.058
Ald	Seed treatments	2.874	3; 33	0.050.
	Aphids	0.275	1; 33	0.603
	Seed treatments × Aphids	1.199	3; 33	0.325

All the individual or interaction effects with asterisks are significant (Significance codes: ***: P <0.001; **: 0.001<P<0.01; *: 0.01<P<0.05). The degrees of freedom (DF) column shows Numerator degree of freedom followed by Denominator degree of freedom (df.N; df.D).

Table S4 Linear mixed-effect model result table for antioxidant enzyme activities in wheat representing variation and significance due to seed treatments, aphids and their interaction.

Antioxidants	Effects	F-ratio	Df	P-value
SOD	Seed treatments	1.752	3; 34	0.174
	Aphids	4.700	1; 34	0.037*
	Seed treatments × Aphids	0.812	3; 34	0.495
CAT	Seed treatments	0.305	3; 34	0.821
	Aphids	4.402	1; 34	0.043*
	Seed treatments × Aphids	1.354	3; 34	0.273
POX	Seed treatments	1.031	3; 34	0.391
	Aphids	0.416	1; 34	0.523
	Seed treatments × Aphids	2.706	3; 34	0.060
CWPOX	Seed treatments	2.773	3; 30	0.058
	Aphids	1.420	1; 30	0.242
	Seed treatments × Aphids	1.030	3; 30	0.393
GR	Seed treatments	3.173	3; 32	0.037*
	Aphids	0.366	1; 33	0.549
	Seed treatments × Aphids	0.740	3; 32	0.535
DHAR	Seed treatments	0.538	3; 33	0.659
	Aphids	0.319	1; 33	0.575
	Seed treatments × Aphids	0.901	3; 33	0.450
MDHAR	Seed treatments	0.747	3; 34	0.531
	Aphids	0.023	1; 34	0.879
	Seed treatments × Aphids	0.409	3; 34	0.747
GST	Seed treatments	1.757	3; 34	0.173
	Aphids	5.851	1; 34	0.021*
	Seed treatments × Aphids	0.555	3; 34	0.648

All the individual or interaction effects with asterisks are significant (Significance codes: ***: P <0.001; **: 0.001<P<0.01; *: 0.01<P<0.05). The degrees of freedom (DF) column shows Numerator degree of freedom followed by Denominator degree of freedom (df.N; df.D).

Manuscript II

Effects of entomopathogenic fungal endophytes against aphids correlate with alternations in bioactive plant secondary metabolite concentrations across plant families

Shumaila Rasool, Nanna H. Vidkjær, Kourosh Hooshmand, Birgit Jensen, Inge S. Fomsgaard, Nicolai V. Meyling

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Effects of entomopathogenic fungal endophytes against aphids correlate with alternations in bioactive plant secondary metabolite concentrations across plant families

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Key words: Aphids, *Beauveria bassiana*, Benzoxazinoids, Flavonoids, *Metarhizium*, *Phaseolus vulgaris*, Wheat

Summary

- Entomopathogenic fungi (EPF) can colonize plants as endophytes. The effects on insect herbivores have been widely studied, but the mechanisms behind are not documented.
- Here we evaluated the colonization ability of three isolates of EPF, *Beauveria bassiana*, *Metarhizium brunneum* and *M. robertsii*, in wheat and bean following seed inoculation, and their effects on population growth of the aphids, *Rhopalosiphum padi* and *Aphis fabae*. In leaves, we quantified benzoxazinoids in wheat and flavonoids in bean after EPF inoculation and aphid infestation to elucidate the role of specific plant secondary metabolites (PSMs) in these plant-fungus-herbivore interactions.
- Inoculations of wheat and bean with *M. robertsii* and *B. bassiana* significantly reduced aphid populations compared to control treatments. Inoculations with *M. brunneum* unexpectedly increased the number of both aphid species. Concentrations of more than half tested PSMs were affected by seed inoculations and aphid feeding. The changes in number of aphids were associated with regulation in specific PSMs rather than endophytic colonization of above-ground and growth promotion effects by EPF.
- The study links the effects of EPF endophytes with plant physiological responses against aphids in wheat and bean. The understanding of PSMs regulations by beneficial fungi are important for future applications of EPF for herbivore management.

Introduction

Entomopathogenic fungi (EPF) within the order Hypocreales (Ascomycota) are well-studied natural enemies of insect pests (Meyling & Eilenberg, 2007) and several strains with relatively broad host ranges have been developed as biological control agents (Faria & Wraight, 2007). Besides interacting directly with insect hosts as pathogens, these fungi are also able to associate with plants as endophytes (Vidal & Jaber, 2015; Vega, 2018), colonizing plant tissues without causing symptoms (Wilson, 1995). In particular, fungal isolates from the genera *Metarhizium* (Clavicipitaceae) and *Beauveria* (Cordycipitaceae) have successfully been experimentally established as endophytes in many important crop plants such as tomato, cotton, potato, banana, maize, bean and wheat using different inoculation methods (Vega, 2018; Jaber & Ownley, 2018).

A recent meta-analysis revealed that EPF inoculations of plants often result in reductions of herbivore fitness across insect taxa, feeding guilds and plant families (Gange *et al.*, 2019). The most consistent effects were found in whole plant experiments after seed inoculations (Gange *et al.*, 2019). Furthermore, EPF has also been investigated for their effects as endophytes on plant growth (Barelli *et al.*, 2016; Jaber & Enkerli, 2017; Tall & Meyling, 2018) and plant disease control (Barra-Bucarei *et al.*, 2020). So far, most studies showed effects of *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin against a range of insect pests (Akello *et al.*, 2008; Sword *et al.*, 2017; Vega, 2018; Sánchez-Rodríguez *et al.*, 2018), while there are fewer reports on experimental inoculations by isolates of the genus *Metarhizium* (Jaber & Enkerli, 2016; Clifton *et al.*, 2018). *Metarhizium* spp. have been reported to exhibit diverse effects as plant inoculants against above-ground insect or mite herbivores by causing both positive and negative effects (Clifton *et al.*, 2018; Canassa *et al.*, 2019).

Wheat, *Triticum aestivum* Linnaeus (Poaceae: Poales), and common bean, *Phaseolus vulgaris* L. (Fabaceae: Fabales), are important nutritional crops cultivated worldwide (Guevara-González *et al.*, 2006; Charmet, 2011) but these crops experience massive losses due to insect pests, including aphids (Guerrieri & Digilio, 2008). Aphids (Aphididae: Hemiptera) are specialized piercing-sucking insects with modified mouthparts (stylet) adapted for feeding on phloem sap of vascular plants. The bird cherry-oat aphid, *Rhopalosiphum padi* L., and black bean aphid, *Aphis fabae* Scopoli, are important pests of wheat and bean, respectively. Aphids inject toxic saliva while feeding which disturbs photosynthetic efficiency and nutritional value of the host plant and the feeding can cause disease by transmitting viruses (Zhou *et al.*, 2015). The control strategies of aphids have traditionally been based on chemical insecticides, but biological methods such as the use of EPF as plant inoculants have

received increasing attention due to resistance development and prohibition of agro-chemicals (Gurulingappa *et al.*, 2010, 2011; Castillo Lopez *et al.*, 2014).

While feeding, the aphid stylet penetrates the apoplast and individual cells to reach the phloem where effector proteins are released and the plant transports a set of bioactive compounds into the phloem as defense response (Züst & Agrawal, 2016). Despite minimal tissue disruption by aphids while feeding, activation of callose deposition (physical barrier) make these substances effective defense compounds (Wouters *et al.*, 2016b). Plants generally produce specialized bioactive compounds, so-called plant secondary metabolites (PSMs), in response to herbivore attack to reduce damage and to retain fitness (Howe & Jander, 2007).

Wheat produces benzoxazinoids (BXs) which have effects against insect herbivores (Niemeyer, 2009). The compounds convert to their respective toxic aglucones (i.e. DIBOA and DIMBOA) by the action of β-glucosidase upon any biotic or abiotic tissue damage (Niemeyer, 2009). Likewise, flavonoids (derivatives of 2-phenyl-benzyl-γ-pyrone) play an important role in various biological activities (Mierziak *et al.*, 2014) and are the main phenolic compounds in bean (Guevara-González *et al.*, 2006). Flavonoids confer resistance against herbivores by acting as antifeedants or repellents and by increasing oxidative stress in exposed insect tissues (Simmonds, 2001; Łukasik *et al.*, 2011). Simultaneously, responses of some flavonoids vary in activity towards insects, e.g. quercetin and rutin can act as phagostimulants or repellents depending on concentrations (Simmonds, 2003).

The general absence of fungal conidia production in plants and lack of infection in insects feeding on endophytic colonized plants (Gange *et al.*, 2019) support the idea of antibiosis and feeding deterrence over direct infection by entomopathogenic fungal endophytes (Vega *et al.*, 2008). Few studies have focused on the production of fungal secondary metabolites *in planta* (Golo *et al.*, 2014; Ríos-Moreno *et al.*, 2016) but the evidence on the production of PSMs after endophytic colonization by EPF and the link between the amount of these compounds and effects against herbivores is still missing. Recently, it has been repeatedly suggested that the fungi are responsible for modulation of the chemical machinery *in planta* such as changes in the production of plant defensive compounds after endophytic colonization of EPF (Jaber & Ownley, 2018; Gange *et al.*, 2019; Hu & Bidochka, 2019). However, no study has so far addressed this hypothesis experimentally.

Here, we investigated aphid population growth on wheat and bean plants after seed inoculation using three entomopathogenic fungal isolates of the species *B. bassiana*, *M. brunneum* and *M. robertsii*. We further investigated, for the first time, the concentrations of selected specific PSMs

(benzoxazinoids in wheat and flavonoids in the bean) in the plant tissues to study their levels after seed inoculations of EPF with and without aphid attack in order to relate this to aphid reproduction rates. This study provides evidence that PSMs are integral components of fungal-plant-herbivore interactions and opens new insights into the research of EPF as endophytes.

Materials and Methods

Study organisms – Plant, Insect and Fungus

Untreated seeds of two plant species, wheat (*Triticum aestivum* L, cv. Sevin Sejet, Sejet Plant Breeding, Denmark) and common bean (*Phaseolus vulgaris* L, cv. Lasso, Olssons Frö AB, Helsingborg, Sweden) were used for the experiments. Seeds were surface sterilized by first rinsing with 70% ethanol and then soaking for 10 min in 2% sodium hypochlorite (NaClO, Sigma Chemicals, St Louis, MO) followed by 3 times rinsing with double-distilled water (ddH_2O) and air drying under sterile conditions for 6 hours.

Bird cherry-oat aphid (*Rhopalosiphum padi* L) and black bean aphid (*Aphis fabae* Scopoli) for experiments on wheat and beans respectively, were obtained from already established colonies at the Department of Plant and Environmental Sciences, University of Copenhagen, Denmark. Aphid rearings were maintained in laboratory cages (20 ± 2°C, 16:8 LD and 60-70% RH) in an insect room on the same plant species as used for experiments. Six days before experiments adult aphids were placed on a wheat or bean leaves set in 3% water agar in a 30 ml medicinal cup. The following day the adults were removed and leaves with nymphs were transferred to whole plants for development to last instar nymphs for 5 days. Twenty-five cups, each containing three aphids were prepared for each aphid species to obtain the required number of nymphs.

Three isolates of entomopathogenic fungi were used, *M. brunneum* (Mb) strain KVL 04–57, isolated from an infected larva of *Cydia pomonella* (Lepidoptera: Tortricidae) in Austria (same isolation origin as the active ingredient of the commercial product Met52, Novozymes, Salam, VA), *M. robertsii* (Mr) strain ESALQ 1622 (isolated from corn soil, Mato Grosso, Brazil) and *B. bassiana* (Bb) strain GHA (obtained from the commercial product BotaniGard® deposited as KVL13-39). All isolates are stored at -80°C at the University of Copenhagen.

Fungal suspensions

Fungal cultures were propagated for 14 days on Sabouraud Dextrose Agar (SDA; Sigma-Aldrich, Darmstadt, Germany) in darkness at 23 °C. Stock conidial solutions were prepared by adding 10ml of sterile 0.01% Triton X-100 solution followed by scrapping with glass spatula and filtering through

cheesecloth. The conidial concentrations for all suspensions were estimated by using a Fuchs-Rosenthal hemocytometer (Assistent, Sondheim von der Rhön, Germany, 0.0625 mm^2, depth 0.200 mm) and experimental suspensions of the required final concentration of 1×10^8 conidia ml^{-1} were prepared. Conidial viability was checked by spreading 100 µl from 1×10^5 conidia ml^{-1} serial dilution on three SDA plates and counting germinated and non-germinated conidia after 24 h at 23°C. The germination tests showed >90% viability rate for all experimental replicates. Thirty ml of fungal suspensions for each isolate containing 1×10^8 conidia ml^{-1} were added in 250 ml blue cap glass bottles for wheat and beans seed inoculations.

Treatments, seed inoculation and experimental setup

The study included three fungal (Mb, Mr and Bb) and two control treatments, Ct (0.1% Triton X-100-mock control) and Cu (untreated control without surface-sterilized seeds) either with (+) or without (-) aphids, resulting in 10 treatments for both wheat and beans. Seeds were immersed in respective fungal or mock control suspensions (except for the untreated control Cu treatment) for 24 h for wheat and 2 h for bean under agitation at 100 rpm. After inoculation, seeds were sown individually in 1 L pots containing PG-mix peat soil (Krukväxtjord Lera & Kisel, Gröna linjen, Sweden) comprising 4% silica, 3% washed gravel, limestone (pH: 5.5-6.5) and other micronutrients (NPK 182-91-194, Mg-247, S-99, Ca-2189, Fe-8.6, Mn-3.2, Cu-2.0, Zn-1.0, B-0.4, Mo-2.6 g/m^3) and incubated in a greenhouse at 25 ± 2°C, 16:8 LD, 60-70% RH. The plants received fertigation weekly containing: NPK 14-3-23 +Mg as macronutrients and B – 0.23%, Cu – 0.14%, Fe – 1.32%, Mn – 0.50%, Mo – 0.05% and Zn – 0.18% as micronutrients.

After 10 days, eight of the germinated wheat and bean plants from each treatment were arranged in a randomized complete block design. Wheat and bean treatments were setup on two separate tables (1.4 m x 3.8 m). Each pot (40 for wheat and 40 for beans) had a plastic plate (17× 3 cm) for the collection of irrigation water and to avoid cross-contamination. Five last instar aphid nymphs were released on each plant of the five treatments with aphids (+) for wheat (uppermost fully developed leaf) and bean (one of the fully developed leaf). Each plant was covered with micro-perforated polypropylene bags (28 cm × 50 cm, Sealed Air®) to avoid aphid escape. The experiment was repeated on three occasions.

Sampling and data collection

Recordings of aphid numbers, plant growth parameters and leaves samples for analyses of PSMs were collected 20 days after sowing corresponding to 10 days after aphid infestation. The total number of aphids on each plant of the five treatments with aphids (+) were counted, removed

individually with camel hairbrush and dipped into 70% ethanol to kill. Height (cm) of each plant was measured with a ruler. Leaves samples for PSMs analysis for wheat (two lowest old and two youngest top leaves) and beans (one lowest old and two youngest top leaves) were harvested with scissors, placed in a 50 ml falcon tube and immediately flash-frozen in liquid nitrogen and stored at -80°C until further processing. The four sampled leaves from an individual plant were pooled together to form one biological replicate. Samples for evaluation of endophytic colonization were collected from both roots and shoots (see below) and the remaining above-ground material was discarded. Roots were washed in running tap water, dried with tissue papers and weighed on an electronic balance (A&D model FA-2000, UK), then placed in paper bags and dried in an oven at 65°C for 48 h (A&D model FA-2000, UK) and weighed again on the same balance.

Detection of endophytic colonization by fungal isolates

Samples of roots, stem and leaves were collected to check for the presence of the entomopathogenic fungi as endophytes in experimental tissues. From each wheat and bean plant (with or without aphids) three pieces of leaves (3 cm from wheat and 3 × 3 cm for bean each), two pieces of the stem (3 cm each) and three pieces of roots (4 cm each) were cut with scissors. The plant parts were surface sterilized by immersing in 70% ethanol and 2% NaClO for 2 min in each solution, followed by three rinses in ddH2O (Parsa *et al.*, 2013). Sterilization efficacy was checked by plating 100 µl of last water rinsing (Parsa *et al.*, 2013) and imprinting before and after sterilization on SDA (Tall & Meyling, 2018). After sterilization, the edges of the samples were trimmed with a scalpel and cut into six pieces of leaves (0.5 – 1 cm^2), three pieces of the stem (0.5 – 1 cm) and four pieces of roots (1 cm). All the samples were placed on selective media consisting of; Agar 6 g, Glucose 10 g, Peptone 5 g, Dodine 0.2 ml of 0.1 g ml^{-1}, Streptomycin 0.5 ml of 0.6 g ml^{-1}, Tetracycline 0.5 ml of 0.05 g ml^{-1} and Cyclohexamide 1 ml of 0.05 g ml^{-1} (pH 6.3-6.5). The samples were carefully pressed into the agar to ensure contact with selective media and incubated for 14 days at 23°C in darkness.

Targeted plant secondary metabolite analysis using LC-MS/MS

Chemicals

HPLC grade methanol (MeOH) and acetonitrile (ACN) were obtained from Rathburn (Walkerburn, Scotland); MS grade MeOH and ACN from Fischer Scientific (Loughborough, UK); formic acid (FA) from Merck (Darmstadt, Germany) and acetic acid (AcOH) from Baker (Griesheim, Germany). The benzoxazinoid (BXs) standards, 6-methoxy-2-benzoxazolinone (MBOA), 2-hydroxy-1, 4-benzoxazin-3-one (HBOA), 2-hydroxy-7-methoxy-1, 4-benzoxazin-3-one (HMBOA), 2-β-D-glucopyranosyloxy-1,4-benzoxazin-3-one (HBOA-Glc), 2-β-D-glucopyranosyloxy-7-methoxy-1,4-

benzoxazin-3-one (HMBOA-Glc), double hexose derivative of 2-hydroxy-1, 4-benzoxazin-3-one (HBOA-Glc-Hex), 2, 4-dihydroxy-1, 4-benzoxazin-3-one (DIBOA), 2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-Glc), double hexose derivative of 2,4-dihydroxy-1, 4-benzoxazin-3-one (DIBOA-Glc-Hex), 2, 4-dihydroxy-7-methoxy-1, 4-benzoxazin-3-one (DIMBOA) were either obtained from an ongoing patenting process or synthesized with purity as mentioned in Adhikari *et al.* (2013) and 2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA-Glc) was isolated as described by Pedersen *et al.* (2017). Flavonoid standards were purchased as follows (purity in parenthesis): Quercitrin (98.5%), rutin (99%) and luteolin-di-Glc (95%) from Extrasynthese (Genay, France); quercetin dehydrate (98%) and astragalin (99%) from Sigma-Aldrich (Brøndby, Denmark); kaempferol (96%) and isoquercitrin (90%) from Fluka (Brøndby, Denmark); hyperoside (99%) from Roth (Karlsruhe, Germany); genistin (97%) from Lancaster (Brønshøj, Denmark). Kaempferol-Rha-Xyl-Gal, quercetin-Xyl-Gal, and quercetin-Rha-Xyl-Gal, originated from a previous experiment in which these compounds were extracted and purified from white clover (Carlsen *et al.*, 2008).

Extraction and sample preparation

Plant samples were freeze-dried, ground (Geno grinder, SPEX SamplePrep 2010, Ramcon, Denmark), weighed (0.025 g) and extracted using 80% MeOH + 0.1 % AcOH for BXs and 70% MeOH for flavonoids. One ml of the respective solvent was added to each sample, vortexed, ultra-sonicated (45 min) and centrifuged (Buch and Holm, Herlev, Denmark) at 15000 *g* for 10 min. The supernatant was collected and the extraction repeated once more. The supernatant was diluted 1:4 before the analysis of flavonoids and BXs, except DIMBOA and MBOA for which the extracts were diluted 1:40 (based on test sample calculations) followed by filtration (KX syringe filter, PTFE 13, 0.22 μm). For calibration curves (0.19-800 ng ml^{-1}), three standard mixtures for BXs and two standard mixtures (compounds analyzed in positive and negative mode respectively) for flavonoids were prepared.

Quantification of PSMs

Analyses of BXs and flavonoids in wheat and beans respectively were performed by LC-MS/MS using an Agilent 1100 HPLC system connected with a 3200 QTRAP mass spectrometer (SCIEX, Foster City, CA) operated in multiple reaction monitoring mode (MRM). The tested BXs were benzoxazinones including hydroxamic acids and their glucosides (DIBOA, DIBOA-Glc, DIBOA-Glc-Hex, DIMBOA and DIMBOA-Glc), lactams and their glucosides (HBOA, HBOA-Glc, HBOA-Glc-Hex, HMBOA, and HMBOA-Glc) and benzoxazolinone (MBOA). Benzoxazinoids were

analyzed by electrospray ionization operated in negative mode following the method described for plant samples in Kudjordjie *et al.* (2019) with few modifications. The instrument parameters were as follows, Curtain gas, 40 psi; temperature, 500°C; ion source gas 1, 70 psi; ion source gas 2, 60 psi; collision gas, -2 psi; and ion spray voltage, 4300 V. Nitrogen was used as collision and source gas. The compound dependent parameters and MRM transitions monitored (quantifier and qualifier) are listed in Supporting Information Table SI. A Phenomenex (Macclesfield, UK) Synergi^{TM} Polar RP-80Å (250 × 2.0 mm id, 4 µm particle size) column was used to perform the chromatographic separation with a 300 µl min^{-1} flow rate and 25 µl injection volume at 30°C. The eluents were A: 7 % ACN+ 20 mM AcOH, B: 78 % ACN + 20 mM AcOH. The gradient was as follows: 8% B in 1 min, to 10% B in the next 2 min, followed by a 10 min increase to 70% B, another min to 90% B, maintained at 90% B for 2 min and finally returned to the initial conditions in 1 min and the column re-equilibrated for 8 min. All other LC-MS/MS conditions were as described before (Adhikari *et al.*, 2012).

Flavonoids including flavonols (quercetin, quercetin-xyl-gal, quercetin-rha-xyl-gal, quercitrin, isoquercitrin, rutin, kaempferol, kaempferol-rha-xyl-gal, astragalin and hyperoside), flavones (luteolin-di-glc) and isoflavones (genistin) were analyzed based on Czaban *et al.* (2018) with some modification. The mass spectrometer was operated in both positive and negative mode using electrospray ionization. The instrument parameters were as follows (neg. mode/pos. mode): Curtain gas, 30 psi/30 psi; temperature, 450 °C/ 450 °C; ion source gas 1, 60 psi/60 psi; ion source gas 2, 50 psi/50 psi; collision gas, 2 psi/2 psi; and ion spray voltage, -4200 V/4500 V. Nitrogen was used as collision and source gas. The compound dependent parameters and MRM transitions monitored (quantifier and qualifier) are listed in Supporting Information Table S2. A Phenomenex Synergi^{TM} Fusion column (250 x 2.0 mm, 4 µm particle size) with a flow rate of 200 µl min^{-1} (pos. mode) or 300 µl min^{-1} (neg. mode) and an injection volume of 25 µl was used to perform the chromatographic separation. Eluents A: 7% ACN + 0.2% FA, and B: 100% ACN + 0. 2% FA were used in the gradient system. For compounds analyzed in negative mode (Supporting Information Table S2), the gradient started with 0% B for 1 min, increasing to 64% B in 7 min, maintained at 64% B for 3 min, increasing to 100% B in 1 min, maintained at 100% B for 6 min, returned to the initial conditions in 1 min and the column re-equilibrated at 0% B for 9 min. For compounds analyzed in positive mode (Supporting Information Table S2), the gradient started with 0% B for 3 min, followed by an increase to 64% B in 5 min, maintained at 65% B for 3 min, increased to 100% B in 1 min, maintained at 100% B for 8 min, returned to 0% B in one min and the column re-equilibrated at 0% B for 10 min.

Quantifications of all compounds were done in Sciex Analyst 1.6.2 software based on standard curves prepared in the range 0.19-800 ng ml^{-1}, applying either a quadratic or linear function according to the best fit with a weighting of $1/x$ (correlation coefficient > 0.99). For confirmation of the presence of compounds, the ratios between peak areas of the quantifier and qualifier MRMs were inspected and compared with ratios for pure standards.

Statistical analysis

Data were predominantly analyzed in R (R Core Team, 2019) using '*lme4'* (Bates *et al.*, 2015) and '*multcomp'* packages for analysis (Hothorn *et al.*, 2008) and '*ggplot2'* package for plots (Wickham, 2016). Visual assessments of model fit were carried out by residual and quantile-quantile (Q-Q) plots. The binomial logistic mixed-effect model was fitted to the fungus colonization data (presence/absence per plant and plant pieces) using isolates and plant parts as a combined fixed effect with block and experimental repetitions as random factors. Colonization percentages and confidence intervals were calculated using the same model assumptions. Poisson generalized linear mixed effect models were fitted to aphid count data with seed treatments (three fungal and two controls) as fixed effect while blocks and experimental repetitions as random factors (log link function). Linear mixed models were fitted to plant growth data (plant height and root biomass) and to PSMs data with seed treatments, presence/absence of aphids and their interaction as fixed effects and block and experimental repetitions as random factors. Log transformations for root dry weight data and for few PSMs (DIMBOA-Glc, quercetin, isoquercitrin, kaempferol and genistin) were done to satisfy the model assumptions. The *P* values and degrees of freedom (numerator *df;* denumerator *df)* were calculated based on Satterthwaite's approximation using '*lmerTest'* package (Kuznetsova *et al*., 2017). In all analyses, *post hoc* pairwise comparisons were done using Tukey's test. Fold change heat maps for metabolite data were made by using MultiExperiment Viewer software (Howe *et al.*, 2010). To verify the hypothesis that aphid infestation induces changes in concentration of PSMs (represented in heat maps), the content of BXs and flavonoids were analyzed for each EPF treatment challenged or not challenged with aphids. PSM concentration data represent continuous variables. These data were analyzed by analysis of variance assuming a normal distribution. Variances were stabilized by appropriate transformation of data if necessary. These data were analyzed using PC-SAS (release 9.4; SAS Institute, Cary, NC).

Results

Effect of EPF inoculations on aphid reproduction

Seed treatment with EPF significantly affected population growth of *R. padi* (*df* = 4; 49, *F* = 79.8, *p* < 0.001) and *A. fabae* (*df* = 4; 49, *F* = 10.3, *p* < 0.001) in wheat and beans, respectively, after 10 days of infestation (Fig. 1). In wheat, plants treated with *M. robertsii* harbored significantly fewer aphids than all other treatments (Fig. 1a). In contrast, *M. brunneum* increased the aphid number per plant to the highest level of all the treatments. In the experiments with beans, plants grown from seeds inoculated with *B. bassiana* and *M. robertsii* harbored smaller *A. fabae* populations than both *M. brunneum* and the two control treatments; in fact, *M. brunneum* harbored more aphids than the mock control, Ct (Fig.1b). Furthermore, there were no differences in the size of the aphid populations between the two control treatments for neither wheat nor bean.

Effect of EPF inoculations and *R. padi* on benzoxazinoid levels in wheat

To explore BXs diversity in response to seed inoculations with EPF and *R. padi* infestation we quantified 11 BX compounds in wheat (Fig. 2). Concentrations of five BXs were unaffected by the treatments, while significant interactions between seed treatment and aphid challenge were seen for DIBOA-Glc, DIBOA-Glc-Hex, MBOA and HBOA (Supporting Information Table S3). However, concentrations of DIBOA-Glc and DIBOA-Glc-Hex were generally lower in the EPF treatments compared to the two controls, while wheat plants with *M. robertsii* treatment produced higher concentrations of DIMBOA than plants in *M. brunneum* with aphid challenge and the untreated control plants with and without aphids (Fig. 2). Concentrations of few BXs changed upon aphid challenge depending on seed treatment, i.e. MBOA and HBOA increased in the *B. bassiana* treatment when aphids were present whereas these two BXs decreased for *M. brunneum* upon aphid challenge (Fig. 3). Focusing on the relative change of BXs with and without aphids, the untreated control and *M. brunneum* treatments generally resulted in limited change (light colors in Fig. 3) while the mock control plants showed reduction of DIMBOA-Glc, but otherwise limited change. General relative accumulation in BX levels were seen in treatments with *B. bassiana* and *M. robertsii* of DIBOA, DIBOA-Glc, MBOA, HBOA, HBOA-Glc-Hex, HMBOA and HMBOA-Glc in response to aphid infestation (as indicated by red scale color in Fig. 3). These two seed treatments also resulted in lowest population growth of *R. padi* (Fig. 1a).

Effect of EPF inoculations and *A. fabae* on flavonoid levels in beans

In beans, 12 flavonoids were quantified. Of these, only the concentrations of four compounds were not significantly affected by the treatments while concentrations of six compounds were influenced by the interaction of seed treatment and aphid challenge (Fig. 4; Supporting Information Table S4).

Particularly, significant increases in concentrations upon aphid challenge were seen in the *M. robertsii* treatment for quercetin, quercetin-rha-xyl-gal, isoquercitrin, rutin, astragalin and genistin (Fig. 4) which was also the treatment affecting *A. fabae* populations most negatively (Fig. 1b). Plants of the *B. bassiana* treatment were the only to show increase of luteolin-di-glc when aphids were present, while aphid attack in general led to elevated levels of kaempferol in all treatments (Fig. 4). The relative levels of the 12 flavonoids upon aphid challenge are presented in Fig. 5, where plants in the *B. bassiana* and *M. robertsii* treatments generally resulted in flavonoid accumulations as reflected in red color while control treatments and *M. brunneum* plants exhibited reduced levels of several compounds as seen by blue colors (Fig. 5). These patterns of relative flavonoid levels correspond to the *A. fabae* populations sustained by the plants (Fig. 1b).

Effect of EPF inoculations on plant growth

Plant growth evaluated as height and root biomass after 20 days of seed inoculations is presented in Fig. 6. In wheat, significant interactions between seed treatment and aphid presence were found for both root fresh weight (df = 9; 108, F = 3.58, $p < 0.001$) and dry weight (df = 9; 108, F = 2.7, p = 0.001). This was most evidently reflected by larger root biomass in wheat plants with aphids that had received fungal treatments, irrespective of species, compared to the two control treatments (Fig. 6a). Likewise, a significant effect of treatments was observed for wheat plant heights (df = 9; 108, F = 1.77, P = 0.04).

In bean, significant interactions of seed treatment and aphid presence were found evaluating root biomass as fresh (df = 9: 108, F = 4.87, $p < 0.001$) and dry weight (df = 9; 108, F = 4.91, p = 0.001). As for wheat, the fungal treatments significantly increased fresh and dry root biomass in presence of aphids compared to the control treatments while root biomass was comparable among treatments in the absence of aphids (Fig. 6b). No effect of treatments was observed for bean plant heights (df = 9; 108, F = 1.44, $p > 0.05$) (Fig. 6b).

Endophytic colonization by fungal isolates in wheat and bean

All the fungal isolates displayed variable colonization levels in different plant parts (leaf, stem and root) 20 days after inoculations of wheat and bean seeds. The plants with and without aphids were pooled as one treatment, so individual treatment contained eight plants from each experiment. Colonization percentages were calculated for colonized plant tissues (leaf, stem and root) (Table 1a) and colonized plant tissue pieces from each treatment (Table 1b). For both wheat and bean,

colonization frequencies of plant tissues were significantly affected by fungal isolate and plant parts evaluated (wheat; $df = 7$, $F = 2.25$, $p = 0.04$; bean; $df = 7$, $F = 2.51$, $p = 0.01$) as were for colonization of plant tissue pieces (wheat; $df = 7$, $F = 16.6$, $p < 0.001$; bean; $df = 7$, $F = 8.33$, $p < 0.001$). The isolate of *B. bassiana* colonized all examined parts whereas *M. brunneum* and *M. robertsii* were only found in stem and root for both wheat and bean plants (Table 1). In wheat, the colonization rate of the stem was higher for *M. brunneum* compared to other isolates whereas *M. robertsii* colonized more roots than stem (Table 1a). The bean stem showed higher colonization rates for *B. bassiana* and *M. brunneum* than *M. robertsii*, while both *Metarhizium* isolates colonized more roots compared to *B. bassiana* (Table 1a). Bean plants showed comparatively low percentages of colonization than wheat except for more plant pieces of bean roots were colonized with *M. robertsii* (Table 1). None of the targeted isolates were detected in any mock (Ct) or untreated (Cu) control plants. The infrequent outgrowth of some other endophytic fungi or bacteria from the surface-sterilized plant parts (in fungal treated or controls) were observed with no apparent relation with the treatments. The surface disinfection method showed no signs of contamination from any microorganism from both plating of last rinse water and tissue imprints on SDA.

Discussion

Seed treatments of the three EPF isolates were successful in establishing the fungi as endophytes in different plant parts of wheat and bean although extent of colonization depended on the isolate. Though the colonization rates varied, the EPF isolates did affect plant growth, aphid reproduction and metabolite profiles supporting the hypothesis of physiological changes *in planta* activated by fungal colonization. More importantly, our analyses revealed that the EPF colonization modulated the concentrations of specific PSMs in both wheat and bean plants coinciding with significant changes in aphid reproduction rates. This study is the first to demonstrate a putative role of endophytic EPF in affecting levels of PSMs important for plant-fungi-herbivore tripartite interactions.

Plants inoculated with *B. bassiana* (GHA) and *M. robertsii* (ESALQ 1622) reduced the reproduction of aphids in both wheat and bean as compared to the plants inoculated with *M. brunneum* (KVL04-57) and the controls (Cu and Ct). In fact, *M. brunneum* treated plants resulted in higher densities of aphids compared to the other treatments. Correspondingly, Clifton *et al.* (2018) reported a higher abundance of *Aphis glycines* in soybean plants after seed inoculation with *M. brunneum* F52 (same isolate origin as KVL 04-57) as compared to un-inoculated plants whereas seed inoculation with *B. bassiana* GHA had no effect on aphid reproduction. In another study of fava bean, *M. anisopliae*

isolates (N1LT6 and S4ST7) displayed no effects against *Acyrthosiphon pisum* and *A. fabae* whereas *B. bassiana* isolates (G1LU3 and S4SU1) had detrimental effects on both aphid species (Akello & Sikora, 2012). Isolate ESALQ 1622 of *M. robertsii* being the most efficient aphid control fungus in the present study also negatively affected population growth of two-spotted spider mites, *Tetranychus urticae* following seed inoculation of the same bean cultivar (Canassa *et al.*, 2019). This indicates that the isolate has a promising potential for control of a range of herbivores. Furthermore, we also demonstrated that ESALQ 1622 reduced different aphid species on host plants from different families. The current study emphasizes the necessity of testing the outcome of each EPF isolate interacting with a given host plant and herbivore species especially as isolates being efficient entomopathogens (e.g. KVL04-57) may increase plant susceptibility when displaying an endophytic lifestyle.

The inclusion of the isolates of *M. robertsii* and *B. bassiana* which reduced and the isolate of *M. brunneum* that stimulated population growth of aphids both in wheat and bean enabled us to shed light on the importance of PSMs behind these dichotomous responses. Here, we focused on whether the fungal inoculations and aphid infestations modified the profiles of specialized benzoxazinoids (BXs) in wheat and flavonoids in the bean. Aphid feeding causes minimal tissue disruption and reduces the likelihood of the induction of β-glucosidase mediated defense, i.e. formation of toxic aglucones from glucosides with the *β*-glucosidase enzyme (Pentzold *et al.*, 2014). However, during aphid stylet penetration, the plant activates and transports a set of defensive compounds (e.g. BXs) (Züst & Agrawal, 2016). Furthermore, induced accumulation of plant responses such as hydrogen peroxide, cell death, and callose deposition could also be involved in the restriction of aphid growth. Recently, Harun-Or-Rashid *et al*. (2017) showed that the root endophyte *Bacillus velezensis* mediated the induction of such defense responses following aphid attack in *Arabidopsis thaliana.* In the present study, the differences in BXs and flavonoid accumulation strongly suggest that the *M. robertsii* and *B. bassiana* isolates played a role in inducing systemic resistance in plants triggered by aphid feeding. In contrast, some changes in PSMs for *M. brunneum* treatment were associated with induced susceptibility to aphids.

In wheat, the *M. robertsii* treatment which resulted in the lowest number of aphids coincided with the highest DIMBOA concentration both with and without aphids. The effect of DIMBOA on aphids is not fully understood. However, metabolic analysis of durum wheat showed induction of DIMBOA after *A. padi* feeding (Shavit *et al.*, 2018). The consequences of DIMBOA accumulation could also be indirect as DIMBOA can act as a signaling molecule triggering callose deposition to prevent

aphids from sucking phloem sap (Maag *et al.*, 2015). For *M. robertsii* none of the BXs were induced significantly upon insect attack suggesting that constitutive accumulation of BXs (e.g. DIMBOA) mediated by *M. robertsii* is highly important for the increased resistance to *R. padi*. While, for the *M. brunneum* treatment which stimulated aphid reproduction, the concentrations of most BXs were unaffected or even decreased after exposure to aphids.

Interestingly, *M. brunneum* induced a significant decrease in DIMBOA, MBOA and HBOA while *B. bassiana* significantly increased MBOA, HBOA and HBOA-Glc-Hex concentrations in plants challenged with aphids. Wouters *et al.* (2016a) suggested that hydroxamic acids (DIMBOA) are more active than lactams (HBOA, HMBOA) and benzoxazolinone (MBOA). DIMBOA degrades to MBOA after tissue injury (Hansen, 2006) and the relative high levels of MBOA in *M. brunneum* and untreated control indicate their rapid degradation from DIMBOA in the current experiment. Treatment with high levels of DIMBOA (*M. robertsii*) showed negative effects against aphids; while those with high levels of degraded MBOA (*M. brunneum* and untreated control) increased the aphid population. Hansen (2006) reported high reproduction of *Sitobion avenae* at specific concentrations (up to 0.1 mM) of MBOA that decreased with increasing concentrations, and Bravo *et al.* (2004) stated that HBOA and HMBOA had weaker effects than DIMBOA towards *R. padi*. However, differential antibiosis or antixenosis effects of BXs against aphids both *in planta* and *in vitro* conditions are frequently reported (Wouters *et al.*, 2016a). It is also possible that HBOA and MBOA were not directly linked with aphid population growth in wheat but instead, these compounds deplete due to high aphid abundance.

In wheat, all three fungal treatments reduced DIBOA-Glc and DIBOA-Glc-Hex concentrations as compared to controls with no differences for DIBOA which indicates that the high concentrations of DIBOA and its related forms did not affect the aphid population growth. In line with the hypothesis that the regulation of PSMs is affected by EPF and thereby plays an important role in the inhibition of aphid populations, the profiles of the *B. bassiana* and *M. robertsii* treatments were associated with accumulation of BXs upon aphid feeding. However, the relationship is not straight forward and do not unambiguously explain the importance of the specific metabolites. Hence, more different BXs accumulated in higher concentrations after aphid infestation in the *B. bassiana* treatment as compared to *M. robertsii* although not directly reflected in population levels of *R. padi*.

The varied distribution of flavonoids among different plant species and their ability to modulate insect feeding and oviposition behavior make them important compounds for insect-plant interactions

(Simmonds, 2001). Feeding of *A. fabae* in bean significantly increased biosynthesis of quercetin, isoquercitrin, genistin and astragalin in *M. robertsii* whereas only luteolin-di-Glc in the *B. bassiana* treatment was significantly elevated. Accumulation of flavonoid compounds was also found after *A. pisum* infestation in pea seedlings (Morkunas *et al.*, 2016). Goławska *et al.* (2014) demonstrated a reduction in fecundity of *A. pisum* after feeding on liquid diets containing quercetin, but Simmonds (2001) suggested that quercetin and derivatives thereof do not necessarily affect insect herbivores. The induced compounds observed in the present study in the *M. robertsii* and *B. bassiana* seed treatments have previously shown to have negative effects against different insect herbivores, including aphids (Lattanzio *et al.*, 2000; Simmonds, 2001; Goławska *et al.*, 2008). Interestingly, with aphid feeding the concentrations of isoquercitrin and rutin decreased significantly in the *M. brunneum* treatment. We suggest that rutin was utilized by aphid for increased reproduction with no direct relation to defense. Simmonds (2003) summarized that rutin act as a phagostimulant for many insects depending on the concentration.

EPF can establish a mutualistic relationship with plants by translocating nitrogen from insect cadavers (Behie *et al.*, 2012) and in return receive carbon (Behie *et al.*, 2017). Such transfers could play a role in how EPF affect aphid reproduction and plant growth. EPF isolates used in the current experiments improved root growth compared to controls when aphids were present. This indicates that fungus treated plants sustain the growth during biotic stress probably due to high nutrient acquisition (Behie *et al.*, 2012) to compensate lost biomass to better tolerate herbivory (McKinnon *et al.*, 2017). Similar trends of growth promotion were also found in other studies with *B. bassiana* (strain NATURALIS) and *M. brunneum* (strain BIPESCO5) used as seed treatments of broad beans, *Vicia faba* (Jaber & Enkerli, 2016) and wheat (Jaber, 2018). Besides, stimulation in root development was found by *M. robertsii* (strain Ma2575) in switchgrass, *Panicum virgatum* and *P. vulgaris* (Sasan & Bidochka, 2012). Seed treatment with *B. bassiana* (strain EABb 04/01-Tip) and soil surface application with *M. brunneum* (EAMb 01/58-Su) increased the bioavailability of different nutrients (e.g. Fe) leading to growth promotion of different dicot (tomato and sunflower) and monocot (wheat and sorghum) plants in calcareous soils (Sánchez-Rodríguez *et al.*, 2015, 2016). As all fungal isolates increased plant growth, no direct correlation between improved plant growth and high aphid reproduction rates was found. Although the differences in the nutritional composition of the plant by different isolates could be one of the reasons for high or low aphid abundance, additional experiments are required to address this hypothesis.

The *B. bassiana* isolate GHA established in the plant tissues of wheat and bean after seed inoculations with highest prevalence in the stem followed by leaves and roots. There are many supporting studies reviewed by Jaber & Ownley (2018) regarding *B. bassiana* establishment in plants using different inoculation methods showing variable degrees of colonization. Sánchez-Rodríguez *et al.* (2018) re-isolated *B. bassiana* from leaves, stem and roots of bread and durum wheat and Canassa *et al.* (2019) re-isolated *B. bassiana* from beans after seed treatments. *M. brunneum* and *M. robertsii* showed higher percentages of colonization in wheat than bean indicating different plant responses to the fungal isolates (Gurulingappa *et al.*, 2010). Here, both *Metarhizium* isolates were mainly re-isolated from roots followed by stem and no occurrence in leaves we observed. In previous studies, *M. brunneum* (strain BIPESCO5) has been found in all plant part of beans (Jaber & Enkerli, 2016) while only in stem and root of wheat (Jaber, 2018), while the *M. robertsii* isolate ESALQ1622 was only found in roots of beans after seed treatment (Canassa *et al.*, 2019). There was no straight forward correlation between endophytic colonization rate by the three isolates and their ability to affect aphid populations. These results further support the hypothesis that the effect of EPF endophytes against aphids, and potentially other insect and mite herbivores, is most probably associated with regulation of plants metabolism systemically.

Our findings reported here demonstrate that seed inoculations with the *M. brunneum* isolate presented a conducive host plant environment for *R. padi* and *A. fabae* while seed treatment with the *B. bassiana* and *M. robertsii* isolates mediated an environment limiting population growth for these aphids in two different host plants. The observed effects are likely linked to the regulation of plant defense systems, but this modulation is highly dependent on fungal isolate. Furthermore, there was no correlation between endophytic colonization capacity and effect against aphids feeding on those tissues which further indicates a systemic rather than local effect. These insights are important for future research on plant-fungus-insect interactions using EPF and in applications of these multifunctional microorganisms.

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Table and Figures

Table 1 Percentages of endophytic colonization of wheat and bean tissues (leaves, stems and root), 20 days after seed inoculations with *Beauveria bassiana*, *Metarhizium brunneum* and *Metarhizium robertsii*, respectively. (a) Percentage (95% confidence intervals) of colonized plant tissues and (b) percentage (95% confidence intervals) of the total number of tissue pieces colonized by the respective fungi.

a) Colonized plant tissues % (95% CI)

Treatments	Wheat			Bean		
	Leaf *n* = 24 (8 per exp.)	Stem *n* = 24 (8 per exp.)	Root *n* = 24 (8 per exp.)	Leaf *n* = 24 (8 per exp.)	Stem *n* = 24 (8 per exp.)	Root *n* = 24 (8 per exp.)
B. bassiana	41 (20.0-63.9)^{ab}	33 (13.5-54.4)^{a}	25 (8.2-44.2)^{a}	33 (16.8-54.7)^{abc}	37 (19.9-58.7)^{abc}	16 (6.0-37.2)^{a}
M. brunneum	0	62 (43.9-86.0)^{b}	41 (20.0-63.9)^{ab}	0	50 (29.9-70.0)^{c}	33 (16.8-54.7)^{abc}
M. robertsii	0	29 (10.7-49.4)^{a}	54 (27.6-72.5)^{ab}	0	20 (8.4-41.8)^{ab}	45 (26.4-66.3)^{bc}

b) Colonized plant tissue pieces % (95% CI)

Treatments	Wheat			Bean		
	Leaf *n* = 144 (6 per plant)	Stem *n* = 72 (3 per plant)	Root *n* = 96 (4 per plant)	Leaf *n* = 144 (6 per plant)	Stem *n* = 72 (3 per plant)	Root *n* = 96 (4 per plant)
B. bassiana	20 (14.5-28.6)^{abc}	27 (18.3-39.8)^{c}	11 (6.2-19.6)^{a}	14 (7.3-22.5)^{ab}	20 (10.2-33.1)^{bc}	8 (3.2-15.7)^{a}
M. brunneum	0	30 (20.6-42.7)^{c}	22 (15.1-32.7)^{bc}	0	23 (12.1-36.6)^{bc}	19 (10.1-30.5)^{bc}
M. robertsii	0	13 (7.4-24.2)^{ab}	27 (18.6-37.3)^{c}	0	12 (5.1-22.5)^{ab}	31 (18.5-44.4)^{c}

Same letters for colonized plant tissues and colonized plant tissue pieces of wheat and bean, respectively, indicate no significant differences at $\alpha = 0.05$ (by *post hoc* tests using *multcomp* function).

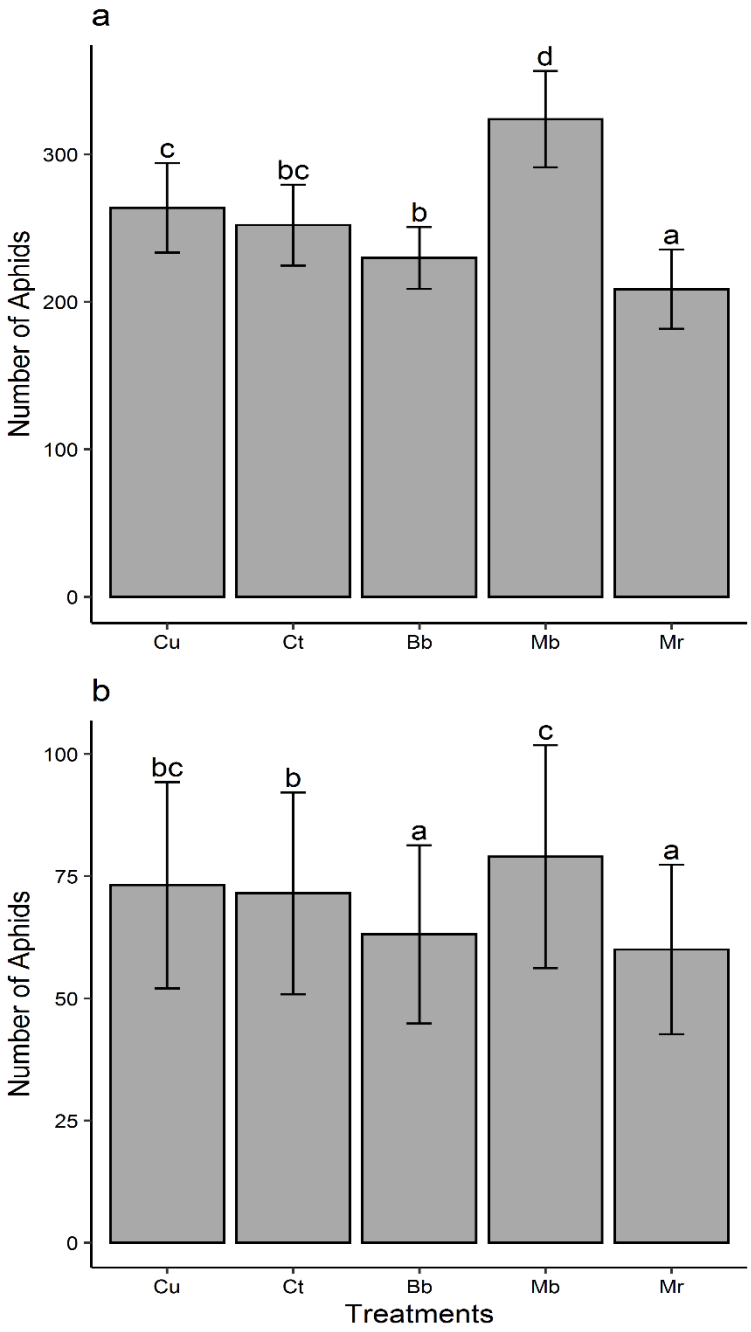

Figure 1 Effect of seed inoculations with entomopathogenic fungi (Bb- *Beauveria bassiana*, Mb- *Metarhizium brunneum*, Mr-*Metarhizium robertsii*) and controls (Cu-untreated control, Ct-Triton X-100, mock control) after 10 days post aphid infestation and 20 days post seed inoculations on aphid reproduction. (a) Mean number ± SE (Y-axis) of *Rhopalosiphum padi* on wheat per plant (b) and mean number ± SE (Y-axis) of *Aphis fabae* on bean per plant. Bars (mean ± SE) with same letters are not significantly different at $\alpha = 0.05$ (by *post hoc* tests using *multcomp* function).

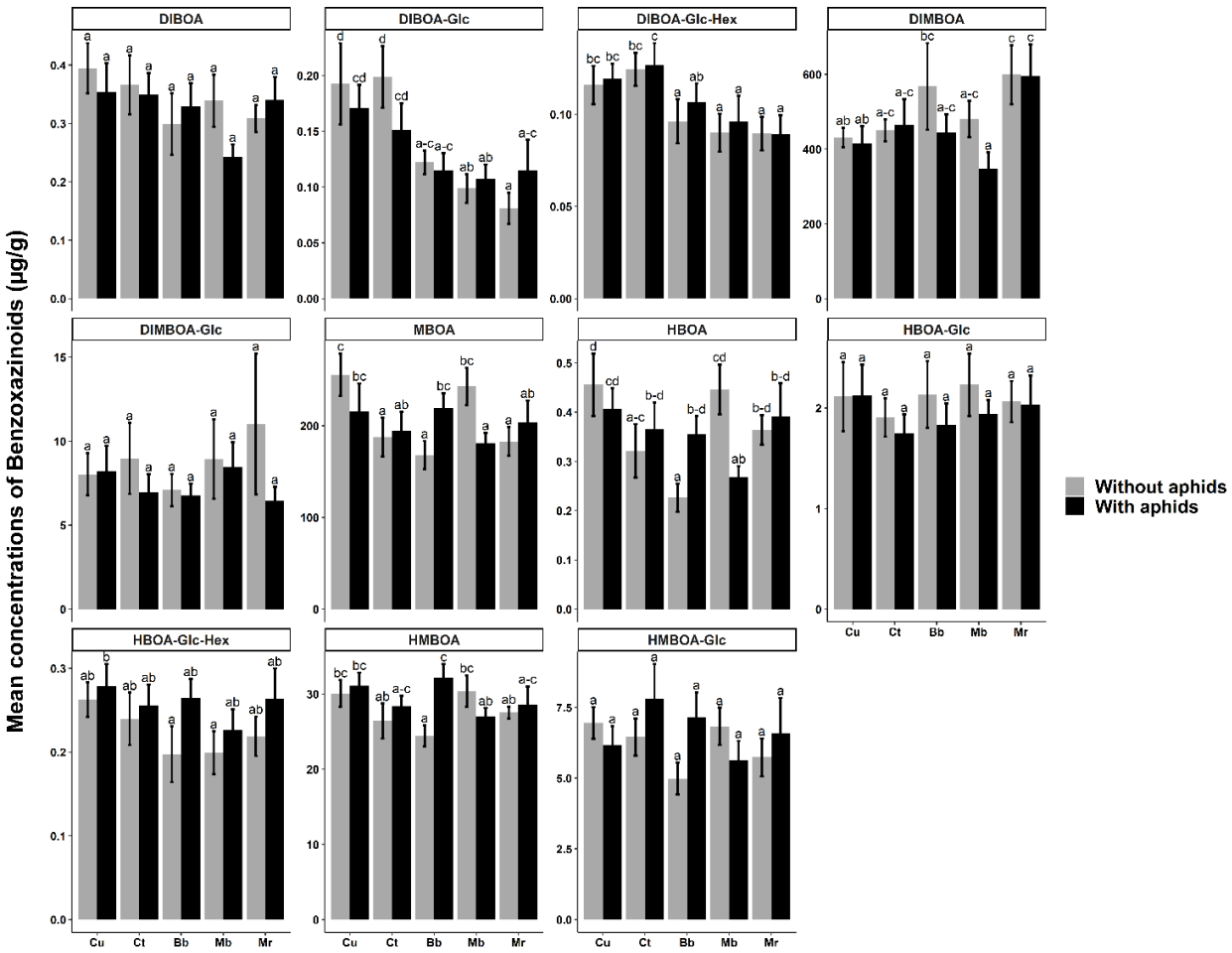

Figure 2: Effect of seed inoculations with entomopathogenic fungi (Bb- *Beauveria bassiana*, Mb- *Metarhizium brunneum* and Mr- *Metarhizium robertsii*), controls (Cu-untreated control, Ct-mock control) and *Rhopalosiphum padi* feeding on levels of 11 benzoxazinoids (BXs) in wheat. Bars show the mean concentrations ± SE (n = 12, total number of plants of the three pooled experiments) of BXs without aphids (grey bars) or with aphids (black bars). Bars within a graph with same letters are not significantly different (by *post hoc* tests using *multcomp* function); letters "a-c" = "a,b,c" and "b-d" = "b,c,d".

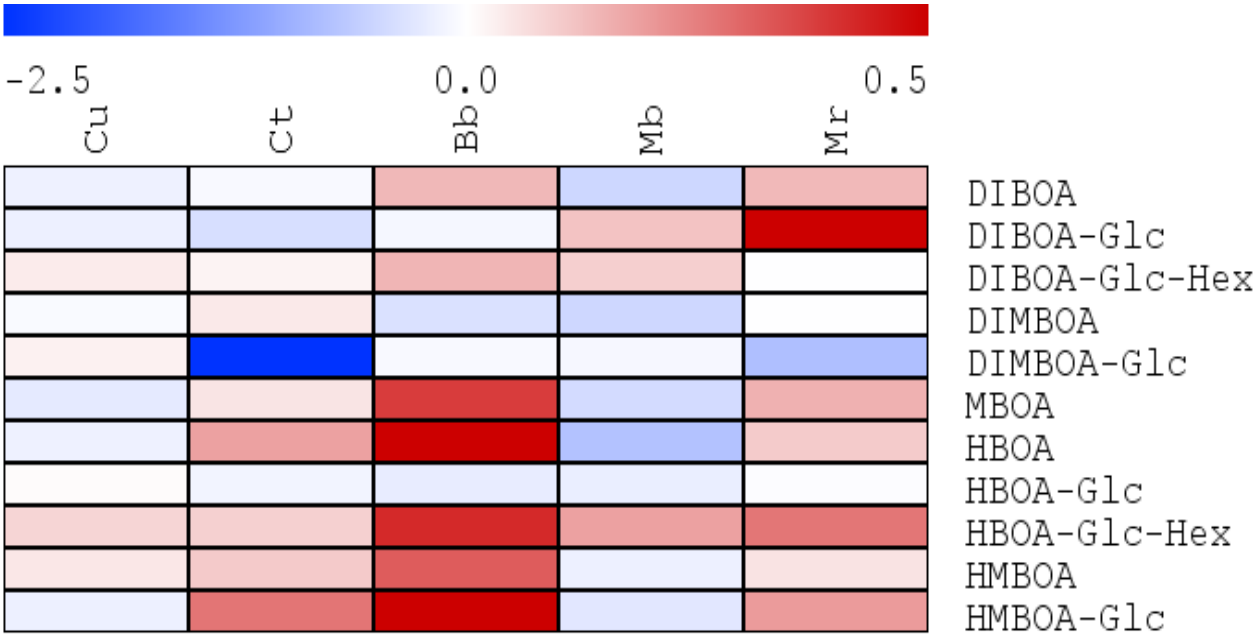

Figure 3: Heat map analysis summarizing the level of benzoxazinoids in wheat with *Rhopalosiphum padi* infestation. The fold change results were calculated by Logarithm base 2 (Log_2) of the metabolite concentrations in plant material with aphids divided by the concentrations in plant material without aphids. Red cells indicate relatively higher concentrations of specific compounds in plants after aphid infestation, while blue cells show relatively lower concentrations of specific compounds in plants after aphid infestation. White cells show no differences using false color scale visualization. The significant increase is indicated with asterisks (Significance codes: ***: P <0.001; **: 0.001<P<0.01; *: 0.01<P<0.05).

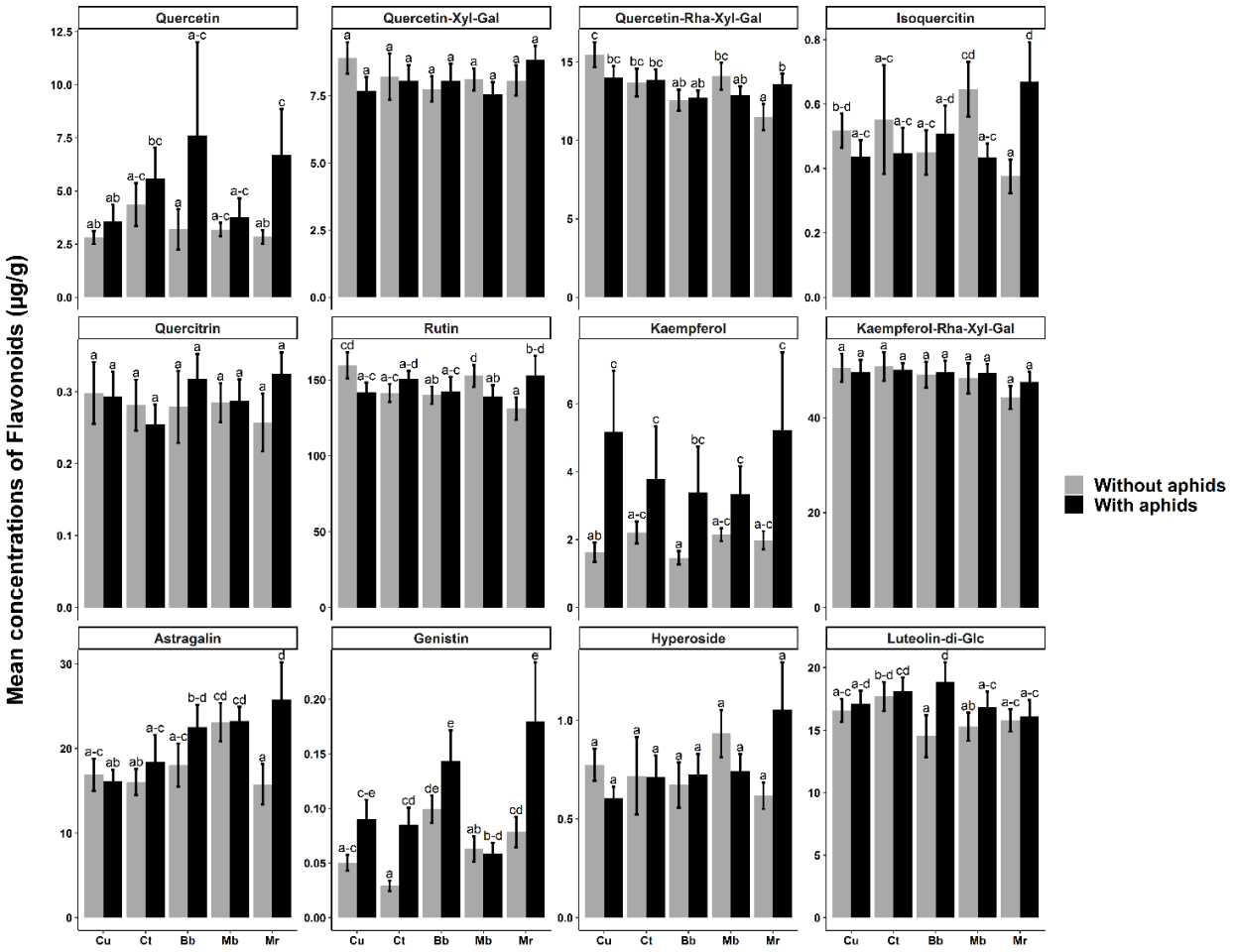

Figure 4: Effect of seed inoculations with entomopathogenic fungi (Bb- *Beauveria bassiana*, Mb- *Metarhizium brunneum* and Mr- *Metarhizium robertsii*), controls (Cu-untreated control, Ct-mock control) and the aphid *Aphis fabae* on flavonoid levels in bean. Bars show the mean concentrations ± SE (n = 12, total number of plants of the three pooled experiments) of flavonoids without aphids (grey bars) or with aphids (black bars). Bars within a graph with same letters are not significantly different (by *post hoc* tests using *multcomp* function); letters "a-c" = "a,b,c", "b-d" = "b,c,d", "c-e" = "c,d,e" and "a-d" = "a,b,c,d".

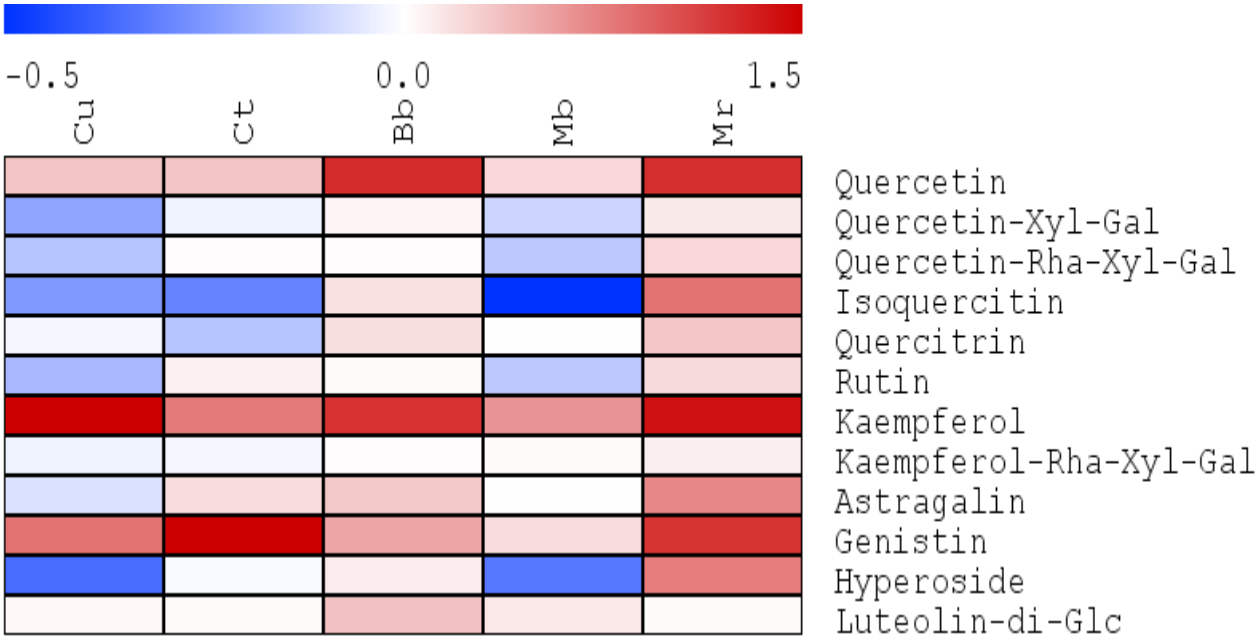

Figure 5: Heat map analysis summarizing the level of flavonoids in wheat with *Aphis fabae* infestation. The fold change results were calculated as described for Figure 3 as are significance symbols.

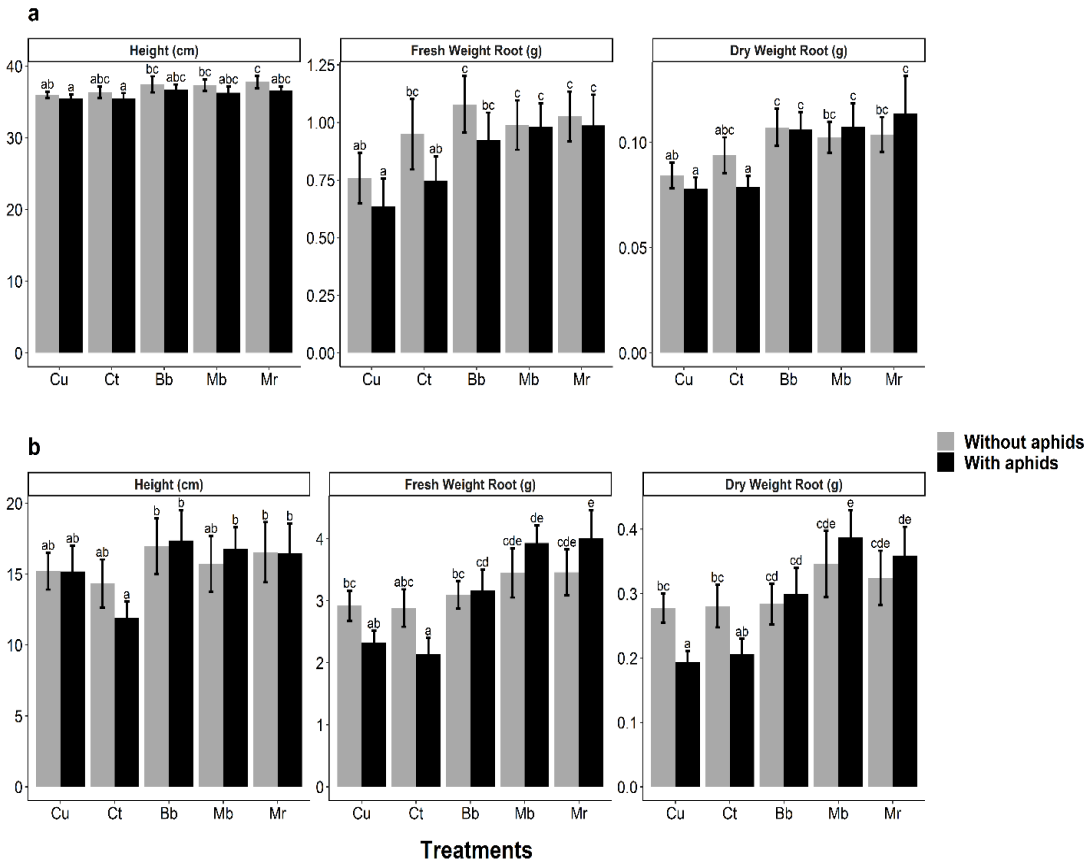

Figure 6: Effect of seed inoculations with entomopathogenic fungi (Bb- *Beauveria bassiana*, Mb- *Metarhizium brunneum*, Mr-*Metarhizium robertsii*) and controls (Cu-untreated control, Ct-mock control) on plant growth parameters of wheat (a) and bean (b). Mean ± SE values of height (cm), fresh weight of root (g) and dry weight of root (g) 10 days post aphid infestation and 20 days post-seed inoculations are presented. Bars with same letters are not significantly different (by *post hoc* tests using *multcomp* function).

Supporting Information

Table S1 Compound dependent parameters and MRM transitions monitored (quantifier and qualifier) for benzoxazinoids. Each parameter was optimized by direct infusion of the individual pure compounds into the mass spectrometer.

Compound	Ionization mode	Quantifier MRM[1]	Qualifier MRM[2]	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
DIBOA	ESI -	180/134		-15	-8.5	-14	-8	-2
DIBOA	ESI -		180/73	-15	-8.5	-14	-18	0
DIBOA-Glc	ESI -	342/134		-30	-11	-23.28	-22	-2
DIBOA-Glc	ESI -		342/162	-30	-11	-23.28	-16	-2
DIBOA-Glc-Hex	ESI -	504/134		-45	-9.5	-29.43	-42	0
DIBOA-Glc-Hex	ESI -		504/162	-45	-9.5	-29.43	-24	-2
DIMBOA	ESI -	210/149		-15	-8.5	-16	-12	-2
DIMBOA	ESI -		210/164	-15	-8.5	-16	-6	-4
DIMBOA-Glc	ESI -	372/149		-35	-10.5	-24.42	-28	-2
DIMBOA-Glc	ESI -		372/164	-35	-10.5	24.42	-20	-2
MBOA	ESI -	164/149		-30	-5	-14	-22	-2
MBOA	ESI -		164/121	-30	-5	-14	-22	-2
HBOA	ESI -	164/108		-30	-4.5	-12	-16	-2
HBOA	ESI -		164/118	-30	-4.5	-12	-16	-2
HBOA-Glc	ESI -	326/108		-40	-10	-22.67	-44	-2
HBOA-Glc	ESI -		326/164	-40	-10	-22.67	-20	-2
HBOA-Glc-Hex	ESI -	488/164		-80	-10.5	-28.83	-36	-2
HBOA-Glc-Hex	ESI -		488/108	-80	-10.5	-28.83	-38	-2
HMBOA	ESI -	194/123		-35	-10.5	-16	-28	-2
HMBOA	ESI -		194/138	-40	-8	-16	-16	-2
HMBOA-Glc	ESI -	356/494		-45	-9.5	-23.81	-40	-2
HMBOA-Glc	ESI -		356/138	-45	-9.5	-23.81	-40	-2

DP, declustering potential; EP, entrance potential; CEP, cell entrance potential; CE, collision energy; CXP, cell exit potential

Table S2 Compound dependent parameters and MRM transitions monitored (quantifier and qualifier) for flavonoids. Each parameter was optimized by direct infusion of the individual pure compounds into the mass spectrometer.

Compound	Ionization mode	Quantifier MRM[1]	Qualifier MRM[2]	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
Quercetin	ESI-	301/151		-55	-9.5	-23.32	-30	0
Quercetin	ESI-		301/179	-25	-10.5	-23.32	-24	-2
Quercetin-Xyl-Gal	ESI-	595/300		-70	-9.5	-34.2	-46	-10
Quercetin-Xyl-Gal	ESI-		595/271	-70	-9.8	-34.2	-68	0
Quercetin-Rha-Xyl-Gal	ESI-	741/300		-45	-12	-39.6	-60	-4
Quercetin-Rha-Xyl-Gal	ESI-		741/271	-45	-12	-39.6	-60	-4
Isoquercitin	ESI-	463/299		-55	-2	-29.33	-40	-4
Isoquercitin	ESI-		463/227	-55	-2	-29.33	-60	-2
Quercitrin	ESI-	447/300		-50	-9	-28.72	-28	-4
Quercitrin	ESI-		447/271	-50	-8	-28.72	-54	-4
Rutin	ESI+	611/303		26	5	27.25	25	4
Rutin	ESI+		611/465	26	5	27.25	25	4
Kaempferol	ESI-	285/185		-55	-5.5	-22.73	-36	-2
Kaempferol	ESI-		285/151	-55	-5.5	-22.73	-26	0
Kaempferol-Rha-Xyl-Gal	ESI-	725/284		-95	-9.5	-39.01	-58	-4
Kaempferol-Rha-Xyl-Gal	ESI-		725/255	-95	-9.5	-39.01	-88	-4
Astragalin	ESI-	447/284		-55	-6	-28.72	-38	-4
Astragalin	ESI-		447/227	-55	-6	-28.72	-66	-2
Genistin	ESI+	433/271		26	6	18	23	4
Genistin	ESI+		433/153	26	6	22.06	61	4
Hyperoside	ESI-	463/300		-60	-6	-29.31	-30	-3
Hyperoside	ESI-		463/271	-60	-6	-29.31	-58	-3
Luteolin-di-Glc	ESI+	611/287		40	8	27.25	50	4
Luteolin-di-Glc	ESI+		611/449	40	8	27.93	27	4

DP, declustering potential; EP, entrance potential; CEP, cell entrance potential; CE, collision energy; CXP, cell exit potential

Table S3 Linear mixed-effect model result table for benzoxazinoid compounds from wheat plants showing the variation and significance due to seed treatments, aphids and their combination

Benzoxazinoids	Effects	F-ratio	Df	P-value
DIBOA	Seed treatments	1.47	4; 109	0.215
	Aphids	0.55	1; 109	0.456
	Seed treatments × Aphids	1.13	9; 105	0.348
DIBOA-Glc	Seed treatments	7.05	4; 112	<0.001***
	Aphids	0.26	1; 112	0.016
	Seed treatments × Aphids	3.62	9; 108	<0.001***
DIBOA-Glc-Hex	Seed treatments	10.18	4; 109	<0.001***
	Aphids	0.97	1; 109	0.326
	Seed treatments × Aphids	4.56	9; 105	<0.001***
DIMBOA	Seed treatments	3.07	4; 109	0.026*
	Aphids	1.89	1; 109	0.236
	Seed treatments × Aphids	1.85	9; 105	0.116
DIMBOA-Glc	Seed treatments	0.12	4; 109	0.974
	Aphids	0.86	1; 109	0.355
	Seed treatments × Aphids	0.28	9; 105	0.977
MBOA	Seed treatments	1.94	4; 109	0.032*
	Aphids	0.14	1; 109	0.919
	Seed treatments × Aphids	2.312	9; 105	0.010*
HBOA	Seed treatments	2.22	4; 109	0.071
	Aphids	0.03	1; 109	0.855
	Seed treatments × Aphids	2.40	9; 105	0.015*
HBOA-Glc	Seed treatments	0.46	4; 109	0.764
	Aphids	1.00	1; 109	0.318
	Seed treatments × Aphids	0.38	9; 105	0.941
HBOA-Glc-Hex	Seed treatments	1.25	4; 112	0.292
	Aphids	4.06	1; 112	0.046*
	Seed treatments × Aphids	1.12	9; 108	0.349
HMBOA	Seed treatments	1.07	4; 112	0.370
	Aphids	1.46	1; 112	0.228
	Seed treatments × Aphids	1.49	9; 108	0.159
HMBOA-Glc	Seed treatments	0.57	4; 109	0.684
	Aphids	0.82	1; 109	0.365
	Seed treatments × Aphids	1.05	9; 105	0.402

All the individual or combined effect with asterisk (*) are significant (Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1) at $\alpha = 0.05$. The degrees of freedom (DF) column shows Numerator degree of freedom followed by Denominator degree of freedom (df.N; df.D).

Table S4 Linear mixed-effect model result table for flavonoid compounds from bean plants showing the variation and significance due to seed treatments, aphids and their combination

Flavonoids	Effects	F-ratio	Df	P-value
Quercetin	Seed treatments	1.12	4; 112	0.034*
	Aphids	5.51	1; 112	0.023*
	Seed treatments × Aphids	1.50	9; 108	0.027*
Quercetin-Xyl-Gal	Seed treatments	0.76	4; 109	0.551
	Aphids	0.43	1; 109	0.510
	Seed treatments × Aphids	1.19	9; 105	0.306
Quercetin-Rha-Xyl-Gal	Seed treatments	3.37	4; 109	0.012*
	Aphids	0.01	1; 109	0.894
	Seed treatments × Aphids	2.53	9; 105	0.011*
Isoquercitrin	Seed treatments	0.33	4; 112	0.852
	Aphids	0.00	1; 112	0.923
	Seed treatments × Aphids	2.03	9; 108	0.042*
Quercitrin	Seed treatments	0.25	4; 112	0.908
	Aphids	0.52	1; 112	0.470
	Seed treatments × Aphids	0.43	9; 108	0.911
Rutin	Seed treatments	0.80	4; 112	0.524
	Aphids	0.14	1; 112	0.706
	Seed treatments × Aphids	2.02	9; 108	0.043*
Kaempferol	Seed treatments	0.71	4; 112	0.322
	Aphids	11.80	1; 112	<0.001***
	Seed treatments × Aphids	1.85	9; 108	0.053
Kaempferol-Rha-Xyl-Gal	Seed treatments	1.25	4; 109	0.291
	Aphids	0.20	1; 109	0.651
	Seed treatments × Aphids	0.69	9; 105	0.715
Astragalin	Seed treatments	2.50	4; 109	0.046*
	Aphids	4.38	1; 109	0.038*
	Seed treatments × Aphids	2.33	9; 105	0.019*
Genistin	Seed treatments	5.71	4; 109	<0.001***
	Aphids	13.72	1; 109	<0.001***
	Seed treatments × Aphids	6.08	9; 105	<0.001***
Hyperoside	Seed treatments	0.74	4; 109	0.561
	Aphids	0.099	1; 109	0.753
	Seed treatments × Aphids	1.17	9; 105	0.319
luteolin-di-Glc	Seed treatments	1.25	4; 109	0.293
	Aphids	5.98	1; 109	0.015*
	Seed treatments × Aphids	2.17	9; 105	0.029*

All the individual or combined effect with asterisk (*) are significant (Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1) at $\alpha = 0.05$. The degrees of freedom (DF) column shows Numerator degree of freedom followed by Denominator degree of freedom (df.N; df.D).

Manuscript III

Entomopathogenic endophytic fungi affect population growth of two-spotted spider mite (*Tetranychus urticae* Koch) by changing profiles of steroidal glycoalkaloids in tomato

Shumaila Rasool, Pablo D. Cárdenas, David I. Pattison, Birgit Jensen, Nicolai V. Meyling

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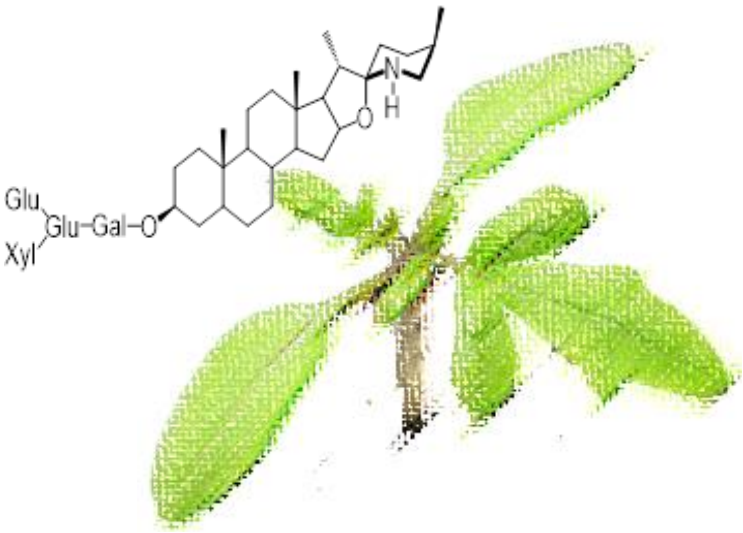

Entomopathogenic endophytic fungi affect population growth of two-spotted spider mite (*Tetranychus urticae* Koch) by changing profiles of steroidal glycoalkaloids in tomato

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Key Words Plant specialized metabolites, α-tomatine, dehydro-tomatine, *Beauveria bassiana*, *Metarhizium brunneum*, *Metarhizium robertsii*, Endophytes

ABSTRACT

Endophytic fungi live inside plant tissues asymptomatically forming a symbiotic relationship. Entomopathogenic fungi (EPF) can be experimentally established in several plant species as endophytes. The effects of EPF inoculations on growth promotion and plant-herbivore interactions have been demonstrated, likely by altering plant physiological responses. However, the role of plant physiological responses in plant-fungus-herbivore tripartite interactions has not been elucidated. Steroidal glycoalkaloids (SGA) are plant specialized metabolites harboring bioactive properties against arthropod herbivores. In the present study, the effects of seed treatments by three EPF isolates, representing *Beauveria bassiana*, *Metarhizium brunneum* and *M. robertsii*, on plant growth and population growth of the two-spotted spider mite (*Tetranychus urticae* Koch) were evaluated on tomato (*Solanum lycopersicum*). The variability in two SGA, α-tomatine and Dehydro-tomatine were determined in tomato leaves by LC-MS after fungal inoculations and *T. urticae* infestations. Population growth of *T. urticae* was highest on *M. brunneum* inoculated plants, which also showed low concentrations of SGA. In contrast, tomato plants inoculated with *B. bassiana* and *M. robertsii* produced significantly higher amounts of SGA and produced the lowest numbers of *T. urticae*. The results revealed that *B. bassiana* colonized all plant parts whereas isolates of *Metarhizium* spp. were only colonizing the stem and roots. We conclude that EPF endophytes alter concentrations of specific plant specialized metabolites to influence the interactions between tomato plants and the herbivore, *T. urticae*. However, the effects on SGA accumulation and population growth of *T. urticae* did not directly correlate with the EPF endophytic colonization patterns. The study sheds light on the physiological responses of tomato plants after EPF inoculations in comparison to the ecological effects which may have implications in plant protection strategies.

INTRODUCTION

It is estimated that insect pests cause about 35% of crop loss in agriculture directly or by transmitting phytopathogens (van der Goes van Naters and Carlso 2006) . The advances in pest control strategies mainly originate from the ecological and environmental impacts of specific control measures. Entomopathogenic fungi (EPF) in the order Hypocreales, particularly the genera *Beauveria* (Cordycipitaceae) and *Metarhizium* (Clavicipitaceae), are widely distributed taxa with potential as biological control agents for pest management (Meyling and Eilenberg 2007; Castro et al. 2018). However, despite showing pathogenic potential against several arthropod pests, these fungi have some caveats due to sensitivity to various biotic and abiotic factors (Meyling and Hajek 2010; Lacey et al. 2015) that enforces a need of innovation in methods to ensure their reliability and efficiency.

These EPF are also characterized as plant endophytes (Quesada-Moraga et al. 2014), being able to colonize plants both naturally and by artificial inoculation. Endophytic fungi live inside plant tissues asymptomatically during their life cycle (Wilson 1995) forming a symbiotic relationship (Behie et al. 2017). Among entomopathogens, *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin is well-studied for its potential to endophytically colonize different plant taxa with confirmed effects on feeding and reproduction of arthropod herbivores (Castillo Lopez et al. 2014; McKinnon et al. 2017; Sánchez-Rodríguez et al. 2018; Mahmood et al. 2019). In contrast, species of the genus *Metarhizium* are less characterized for their effects against herbivores as endophytes with most studies focusing on plant colonizing abilities (Jaber and Enkerli 2016; Vega 2018).

The two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) is a polyphagous pest that causes significant quality and yield losses of many important crops including tomato (Hoffland et al. 2000). During feeding, *T. urticae* individuals inject phytotoxic substances and produce webbing that disturbs the photosynthetic abilities of the host plant (Attia et al. 2013). Isolates of EPF have shown potential against *T. urticae* as an alternative to chemical pesticides when applied directly to produce infections (Chandler et al. 2005; Seiedy et al. 2010; Castro et al. 2018). In addition, EPF can cause negative effects against *T. urticae* through plant inoculations. For example, bean seed treatment with strains of *B. bassiana* and *Metarhizium robertsii* J.F. Bisch., Rehner & Humber resulted in endophytic colonization and decreased *T. urticae* populations under greenhouse conditions (Canassa et al. 2019b). Similarly, strawberry plants inoculated with the same fungal strains also showed reduced *T. urticae* populations in greenhouse and field trials (Canassa et al. 2019a, 2020). Interestingly, plants colonized endophytically with different isolates of *Metarhizium* spp. have shown

erratic effects against arthropod herbivores by either enhancing (Clifton et al. 2018) or suppressing population growth (Canassa et al. 2019b). To understand such dichotomous effects, further research is needed to unravel the underlying mode of action causing variable effects of endophytic EPF against arthropod herbivores.

One mechanism by which fungal endophytes can distress arthropod herbivores is by the regulation of bioactive responses of the plant's specialized metabolites (Hartley and Gange 2009; Tidke et al. 2018; Vega 2018). These metabolites play a crucial role as defense compounds against phytophagous insects and mites (Becerra 2015) by modulating host plant quality (such as nutrient constituents) which are key determinants for herbivore fecundity and fitness (Awmack and Leather 2002). There is currently no experimental evidence about the induction of such metabolites in plants after endophytic inoculations with EPF although it is strongly suggested that the negative impacts on arthropod herbivores are not linked to direct effects by fungal chemicals, but more likely to indirect effects mediated by the regulation of plant specialized metabolites (Canassa et al. 2019b; Gange et al. 2019).

Steroidal glycoalkaloids (SGA, glycosylated forms of steroidal alkaloids) are specialized metabolites mainly present in members of the *Solanaceae* family, e.g. potato and tomato (Friedman 2002), with properties to affect plant antagonists including arthropod herbivores (Milner et al. 2011). These compounds cause the malformation of the reproductive system of the herbivore, which is considered the most important insecticidal backlash against herbivores (Chowański et al. 2016). A mixture of α-tomatine and dehydro-tomatine is present in all parts of the tomato plant with high accumulations in leaves and unripe fruits (Friedman and Levin 1995; Friedman 2002). Many years back, Tingey (1984) reviewed the evidence of the adverse effects of these bioactive compounds on the behavioral and developmental biology of insects and their manipulation in breeding plant varieties as a resistance strategy. In addition, the adverse physiological disturbances caused by α-tomatine in insect herbivores like Colorado potato beetle and potato leafhopper were discussed by Milner (2011). The presence of SGA in African nightshades played a crucial role in defense against red spider mite *Tetranychus evansi* (Acari: Tetranychidae) (Jared et al. 2016). Despite this, there is no reported study to our knowledge focusing on the production of SGA after endophytic inoculations by EPF in tomato and comparing this production with the effects on arthropod herbivores feeding on the plants.

The present study was conducted to investigate whether three isolates of *B. bassiana*, *M. brunneum* and *M. robertsii*, could 1) colonize tomato plants by seed inoculations, 2) promote plant growth, 3)

affect population development of two-spotted spider mites feeding on the tomato plants and 4) change tomato plant physiology by altering the profiles of steroidal glycoalkaloids as a response to fungal inoculations and herbivore infestations.

METHODS AND MATERIALS

Study Organisms. Three entomopathogenic fungal isolates were used: *B. bassiana*, strain GHA, deposited as KVL13-39 (obtained from BotaniGard®), *M. brunneum*, strain KVL 04-57 obtained from infected larvae of *Cydia pomonella* (Lepidoptera: Tortricidae) collected in Austria (same isolation origin as active ingredient strain of the commercial product Met52, Novozymes, Salam, VA) and *M. robertsii*, strain ESALQ 1622 (isolated from the soil of a cornfield, Mato Grosso, Brazil). All isolates are stored at -80 °C at the University of Copenhagen, Denmark. The fungal cultures for experiments were propagated on Sabouraud Dextrose Agar (SDA; Sigma-Aldrich, Darmstadt, Germany) media in Petri dishes for 14 days in darkness at 23°C. Untreated seeds of tomato (*Solanum lycopersicum* L. var. Moneymaker) from Kings Seeds (www.kingsseeds.com) were used for the experiments. The tomato seeds were surface sterilized by dipping in 70% ethanol (EtOH) for 1 min and 1% sodium hypochlorite (NaClO, Sigma Chemical, St Louis, MO) for 10 min followed by five repeated washings with double-distilled water (ddH2O). 100 µl of the last rinse was plated on SDA media to check sterilization efficacy (Parsa et al. 2013). The seeds were then dried under sterile conditions for 30 min and used for inoculations. No signs of contamination were found from sterilization check plates. A colony of spider mites (*Tetranychus urticae*, obtained from EWH Bioproduction, Tappernøje, Denmark) was maintained in laboratory cages at ambient room temperature and light conditions on tomato plants.

Fungal Suspensions, Treatments and Seed Inoculations

Fungal suspensions were prepared under sterile conditions by harvesting conidia into 10 ml sterilized 0.01% v/v Triton X-100 (Sigma-Aldrich, Darmstadt, Germany) using a glass spatula. The resulting mycelial and conidial mixtures were filtered through multiple layers of sterile cheesecloth into 50 ml falcon tube and fungal concentrations were estimated using Fuchs-Rosenthal haemocytometer (0.0625 mm2, depth 0.200 mm, Assistent, Sondheim von der Rhön, Germany). For seed inoculations, 30 ml of 1×10^{8} conidia ml^{-1} for each of the three fungal isolates and 0.01% Triton X-100 for mock control (Ct) were prepared in 250 ml tightly capped sterile glass bottles. Tomato seeds (approx. 25 seeds per treatment) were immersed in respective control or fungal suspensions for 24 hours and agitated at 100 rpm. The conidial viability was estimated by spreading 100 µl of 1×10^{5} conidia ml^{-1}

serial dilution on SDA plates for 24 hours at 23 °C and counting germinated and non-germinated conidia for all three isolates. The suspensions were only used for experimentation when the germination test showed >90% viability.

Experimental Setup and T. urticae Bioassay

Seeds were sown individually in nursery trays and 20 days old seedlings were transferred to 3 L plastic pot in a controlled condition bioassay room (25 ± 2 °C, 16:8 LD and 60-70% RH) and kept for five days to establish in transplanted soil. The growing substrate for the plants contained PG-mix peat soil (Krukväxtjord Lera & Kisel, Gröna linjen, Sweden) with micronutrients (NPK 182-91-194, Mn-3.2, Cu-2.0, Ca-2189, Fe-8.6, Mg-247, S-99, Zn-1.0, B-0.4, Mo-2.6 g m^{-3}), silica (4%), washed gravel (3%) and limestone (pH: 5.5-6.5). In total, 32 pots representing eight biological replicates of each four treatments were arranged in a completely randomized design on a table. The experimental plants were irrigated every second day and fertilized with a balanced nutrient solution (Substral, NPK 6-1-5 with micronutrients) twice per week. The position of the pots was randomly changed at watering. To avoid cross-contamination and to collect excess irrigation water a plastic plate (17 × 3 cm) was placed under each pot. For the experiment, adult females of *T. urticae* were collected from the rearing colony and were placed on a new tomato plant for egg-laying. After 24 hours, the adults were removed and the plant with eggs was maintained for 15 days to obtain similar age adults of *T. urticae*. From this cohort, five female spider mites (recognized by the oval-shaped body and large size) were released on the 1st true leaf of each of four tomato plants per treatment, five days after transplanting corresponding to 25 days post fungal inoculations (DPI). Four other plants per treatment remained without *T. urticae.* All experimental plants (n = 8 per treatment) were individually covered with a micro-perforated polypropylene bag (28 cm × 50 cm, Sealed Air®) using plant support cages and sealed at the base with rubber bands to avoid spider mites escape (Supporting Information Fig. 1). The experiment was repeated on two occasions.

Data Collection and Sampling

The number of spider mites per plant was counted at 3, 5, 7, 10 and 15 days after infestation (DAI) using a handheld magnifier (illuminated loupe 20 × 21 mm). After the last count at day 15, all spider mites were individually removed with a camel hairbrush and killed in 70% EtOH. The heights (cm) of all plants were measured with a ruler. For SGA analysis, two leaves (terminal leaflet of 1st true leaf and 4th true leaf) were harvested from each plant with scissors, placed together in a 50 ml falcon tube representing one biological replicate, flash-frozen immediately in liquid nitrogen and stored at -80 °C

awaiting further processing. Afterwards, plants were cut at the base, roots were washed with running tap water and placed on tissue papers to absorb excess water and both root and shoot were transferred to individual paper bags. Plants were dried in an oven (Memmert 600, Germany) at 65 °C for three days and dry biomass was recorded on an electronic balance (A&D model FA-2000, UK).

Isolation of Endophytic Fungi

For the detection of endophytic establishment of the inoculated fungi, 12 extra tomato plants of each five treatments (without spider mites) were grown simultaneously in the same experimental setups placed randomly among the other experimental plants. The colonization was checked at the time of transplantation (20 DPI) and again at the end of the experiment (40 DPI). Six randomly selected plants from each treatment were sampled at each time point. The plants were carefully uprooted, divided into leaves, stems and roots and washed to remove adhered soil. For 20 days old plants, the whole set of leaves, stem and roots were cut, while for 40 days old plants three leaves (terminal leaflet from 1^{st}, 3^{rd} and 6^{th} true leaf of the plant), two 4 cm long pieces of the stem (bottom and top of the plant) and two roots (primary and lateral) were cut from the plant. The harvested plant parts were individually surface-sterilized by dipping for two minutes in 70% EtOH and 2% NaClO each followed by three rinses with ddH_20. The efficacy of surface-disinfection was checked by plating last rinse water on SDA (as mentioned above) and by gently imprinting the plant parts on SDA before and after sterilization (Tall and Meyling 2018). The substantial growth of different microorganisms was noticed from the imprints before sterilization while no contaminant or fungal outgrowth was observed after 21 days of incubation either from imprints or last rinses. The sterilized parts were then trimmed from the edges with a sterile scalpel and further cut into six pieces of each leaf (1-1.5 cm^2), four pieces of the stem (1.5-2 cm) and four pieces of the root (1.5-2 cm). The plant pieces were placed randomly on selective media (pH 6.3-6.5) consisting of; 6 g agar, 10 g glucose, 5 g peptone, 0.2 ml of 0.1 g ml^{-1} of dodine and antibiotics (streptomycin 0.5 ml of 0.6 g ml^{-1}, tetracycline 0.5 ml of 0.05 g ml^{-1} and cyclohexamide 1 ml of 0.05 gml^{-1}), by pressing carefully into the agar and incubated for 21 days in darkness at 23 °C. The endophytic identification of the fungal structures growing from the trimmed edges was done by checking colony morphology and conidial structures (Humber 1997) under the microscope. The unsystematic growth of some unidentified fungi was observed from both treated and control plants which did not resemble the inoculated fungi, and these were not further identified.

Analytical Standard and Chemicals

The authentic standard of α-tomatine (contains dehydro-tomatine as an impurity), purchased from Sigma-Aldrich was dissolved in methanol at the concentration of 0.1 mg ml^{-1}. Other solvents like acetonitrile and methanol (HPLS-grade, purity ≥ 99.9%) were purchased from Sigma-Aldrich (Schnelldorf, Germany), formic acid (Pierce™ LC-MS grade) was purchased from Thermo Fisher Scientific and sodium formate was purchased from Sigma-Aldrich (Steinheim, Germany).

Preparation of Plant Extracts for Targeted Analysis of SGA

To evaluate the effects of fungus colonization and its relationship with spider mite population growth on SGA metabolism, the relative amounts of α-tomatine and dehydro-tomatine were quantified. Metabolic profiling of α-tomatine and dehydro-tomatine was performed for the eight biological replicates representing each of the fungal inoculated and control treatments from each experiment. Among the eight biological replicates, four were with spider mites and four were without spider mites. Two leaves from each plant were ground in liquid nitrogen using mortar and pestle and extracted as described previously (Cárdenas et al. 2019). Briefly, 300 µl of 80% methanol was added to 100 mg frozen ground leaf tissue (1:3 w/v) and vortexed for 30 sec. Samples were then ultra-sonicated for 30 min at room temperature, vortexed again and centrifuged for 10 min at 14,000 rpm. The supernatants were collected and filtered by centrifuging for 10 min at 3700 rpm. Extracted samples were diluted 20-fold and 100 µl extract was transferred to an auto-sampler glass vial (2 ml) with a 200 µl conical glass insert. Two pooled samples (for each experimental set of samples) were prepared as quality controls by mixing all the samples from each experiment separately.

UHPLC-QqTOF-MS/MS Analysis of SGA

The relative amounts of α-tomatine and dehydro-tomatine were quantified using a Dionex Ultimate 3000RS UHPLC (Thermo Fisher Scientific) system equipped with a DAD detector, temperature-controlled auto-sampler (10 °C) and column oven (40 °C). Chromatographic separations were performed through a Phenomenex Kinetex XB-C18 (100 x 2.1 mm ID, 1.7 µm particle size, 100 Å pore size) column eluted with a flow rate of 0.3 ml min^{-1} by injecting 4 µl aliquots. The mobile phase eluents were, A: 0.05% formic acid in the water, B: 0.05% formic acid in acetonitrile. The extracts were eluted under gradient starting with the initial composition of 98% A and 2% B linearly increasing to 50% solvent B in 29 min and then increasing to 100% of B in 15 min before washing

for 5 min and finally decreasing to initial composition and re-equilibrating the column for 6 min. The UHPLC system was connected to a Compact™ (QqToF) mass spectrometer (Bruker Daltonics) with an electrospray ionization source operated in full scan positive mode with the following instrument parameters: nebulizer gas (nitrogen) at 2.0 bar; drying gas (nitrogen) at 8 L min^{-1} and 220 °C; capillary voltage, 4000 V; spectra acquisition rate, 6 Hz. MS/MS data were acquired in a data-dependent manner during the full scan acquisitions, using: collision energy, 18-45 eV (increasing with *m/z* of the precursor ion); precursor ion number, 3 and active exclusion, 3 spectra. Internal calibration of every chromatogram was performed by an automated infusion of 10 mM sodium formate at the beginning of each run using a syringe pump. The data acquisition was automated using a combination of Chromeleon Express (Thermo Fisher Scientific), Compass oTOF Control (Version 4.0.15.3248, Bruker Daltonics) and HyStar (Version 3.2 SR4, Bruker Daltonics) software. Metabolites were identified by comparison of their retention time and mass spectra to those of authentic standards analyzed in the same setup and extracted ion chromatograms (EIC) were used to locate compounds (Supporting information Fig. S1). Peak area quantification was performed using Compass DataAnalysis (Version 4.3, Bruker Daltonics) software.

Statistical Analysis

All the statistical analyses were performed in R (R Core Team, 2019). Plant colonization data, as presence/absence of fungus per sample, was analyzed using the binomial generalized linear mixed-effect model (*logit* link function) using fungal isolates, plant parts (leaf, stem and root), date (20 and 40 days post inoculations) and experiments as fixed effects while plant pieces and plant number as random factors. The respective fungus outgrowth from plant tissue piece was recorded as one, while no outgrowth was recorded as zero. The same model assumptions were used to calculate colonization percentages and confidence intervals. Poisson generalized linear mixed-effect model (*log* link function) was fitted to spider mite count data using seed treatments, counting days and experimental replicates as fixed effects and plant number as a random factor. Generalized linear mixed-effects models were fitted using the '*lme4'* package (Bates et al. 2015). Linear models using seed treatments and experiments as explanatory variables were fitted to plant growth (height, root and shoot dry weight and root: shoot ratio) and SGA peak area data. Residual and quantile-quantile (Q-Q plot) plots were used for visual assessments of model fit. The data for root dry weight and root: shoot ratio were log-transformed to satisfy the model assumptions. The P values and degrees of freedom were calculated based on Satterthwaite's approximation using the '*lmerTest'* package (Kuznetsova *et al*., 2017). Pairwise comparisons for all the significant effects were carried out using Tukey *post hoc* tests

by '*multcomp'* package. All the graphics were generated using the '*ggplot2'* package (Wickham, 2016).

RESULTS

Seed Inoculated EPF Isolates Differently affect Population Growth of T. urticae

Seed treatments with EPF isolates showed significant effects ($F = 10.63$, $df = 3$, $p < 0.001$) on number of *T. urticae* per plant. Likewise a significant combined effect of treatments and days was found ($F = 155.57$, $df = 19$, $p < 0.001$). Three to seven days after egg laying the population growth of *T. urticae* was very small and no differences between treatments were detected. However, at 15 days after the infestation significantly lower number of *T. urticae* was recorded on tomato plants seed treated with *B. bassiana* and *M. robertsii* as compared to *M. brunneum* treatment (Fig. 1). Furthermore, *M. robertsii* also resulted in a lower number of mites per plant than the untreated control at 15 days after infestation while there was no difference between the *B. bassiana* and the control treatment. Notably, tomato plants grown from *M. brunneum* treated seeds supported the highest number of spider mites both at 10 and 15 days after infestation (Fig. 1).

EPF Seed Inoculations and T. urticae Feeding Induce Accumulation of SGA

The relative amounts of α-tomatine and dehydro-tomatine in tomato leaves at 40 days post-seed inoculations and 15 days of *T. urticae* infestations were determined by LCMS. The concentrations of α-tomatine ($F = 7.21$, $df = 7$, $P < 0.001$) and dehydro-tomatine ($F = 4.40$, $df = 7$, $P < 0.001$) showed significant combined effects with seed treatments and *T. urticae* infestation (present/not present). Subsequently, for α-tomatine concentrations, a significant treatment effect was observed in presence ($F = 3.15$, $df = 3$, $P = 0.04$) or absence of *T. urticae* ($F = 2.99$, $df = 3$, $P = 0.04$). While dehydro-tomatine only showed a significant treatment effect when *T. urticae* were present ($F = 4.99$, $df = 4$, $P < 0.01$). A similar effect was also found in dehydro-tomatine in presence of *T. urticae*. In addition, *B. bassiana* treated plants displayed no significant differences than any other treatment (Fig. 2). The feeding of *T. urticae* was associated with significantly increased concentrations of α-tomatine ($F = 25.82$, $df = 1$, $P < 0.001$) and dehydro-tomatine ($F = 11.48$, $df = 1$, $P = 0.001$) in tomato leaves (Fig. 2).

Tomato Plant Growth Effects of EPF Seed Inoculations and T. urticae Feeding

Plant growth parameters including tomato plant height, root and shoot dry weight and root: shoot ratio were measured at 40 days post inoculations and 15 days after *T. urticae* infestation. The seed

treatment, *T. urticae* infestation (presence/absence) and their combined effects were measured for growth parameters. Plant heights were significantly reduced with *T. urticae* feeding (F = 4.34, df = 1, P = 0.041) in all treatments. No significant effects of fungal seed treatments (P = 0.3) or combined effects of seed treatment and *T. urticae* infestation (P = 0.09) was found. (Fig. 3). Dry weights of shoot showed significant insect effect (F = 1.21, df = 1, P = 0.04), while no differences in seed treatments (P = 0.3) or combined effects (P = 0.09) were found. Dry weight of root showed a significant seed treatment (F = 5.76, df = 3, P = 0.001) and combined effects (F = 4.36, df = 7, P < 0.001) with non-significant *T. urticae* feeding effects (P = 0.5). Both *Metarhizium* isolates significantly increased root dry biomass with *T. urticae* in comparison to *B. bassiana* and control treatment in the presence of *T. urticae* (Fig. 3). The root: shoot ratio also showed a significant seed treatment (F = 2.62, df = 3, P = 0.05) and combined effect (F = 2.80, df = 7, P = 0.01) with non-significant *T. urticae* effects (P = 0.1). All fungal treatments exhibited significantly higher relative allocation to root growth in the presence of *T. urticae* infestation while control treatment allocated oppositely (Fig. 3).

Endophytic Establishment EPF Isolates in Tomato Plants after Seed Inoculations

Seed treated with *B. bassiana, M. brunneum* and *M. robertsii* isolates colonized tomato plant tissue pieces (leaf, stem and root) differently at 20 and 40 days post inoculations. Colonization percentages were calculated for the total number of colonized pieces from each tissue (leaf, stem and root) (Table 1). A significant interaction between the plant parts (leaf, stem and root) and inoculated isolates was found for plant tissue pieces (F = 2.04, df = 1, P = 0.002). All three isolates showed different colonization patterns with *B. bassiana* colonizing all the plant parts while *M. brunneum* and *M. robertsii* were only recovered from the stem and root samples (Table 1). The isolate of *M. brunneum* colonized stems more frequently than *M. robertsii*, which was mostly recovered from roots (Table 1). Frequencies of endophytic occurrences for colonized tissue pieces significantly decreased from 20 to 40 days post inoculations (F =8.91, df = 1, P < 0.001) (Table 1). In general, *B. bassiana* was recovered from 66% and 25%, *M. brunneum* from 83% and 41% and *M. robertsii* 58% and 33% of tomato plants after 20 and 40 days post inoculations, respectively. The targeted fungal isolates were not detected in plant tissues of control treatment.

DISCUSSION

The majority of studies reporting negative effects of EPF endophytes on arthropod herbivores suggest that the mode of action is related to the bioactivity of fungal or plant specialized metabolites produced

in planta after fungal inoculations rather than direct pathogenicity of EPF, since mycoses of herbivores were absent in most of the studies (McKinnon et al. 2017; Vega 2018; Gange et al. 2019). The fact that most reports showed reduced population growth of arthropod herbivores rather mortality and that effects were observed even with low colonization rates, support the idea that EPF mediate the production of plant specialized metabolites over direct effects such as the production of fungal specific compounds *in planta*. Nevertheless, the accumulation of specialized plant metabolites in tri-trophic interaction with EPF having an endophytic lifestyle has not yet been studied. To investigate this hypothesis we quantified the levels of steroidal glycoalkaloids (SGA, α-tomatine and dehydro-tomatine) in EPF seed inoculated tomato plants challenged with two-spotted spider mites.

The seed inoculations with two *Metarhizium* spp. isolates caused opposite effects on the population growth of two-spotted spider mites under similar experimental conditions. Plants inoculated with *M. brunneum* KVL 04-57 and *M. robertsii* ESALQ 1622 harbored the highest and lowest number of spider mites, respectively. Moreover, *B. bassiana* KVL13-39 (strain GHA) supported relatively few numbers of spider mites at intermediate population levels. This is in line with the results of a greenhouse experiment with bean plants (*Phaseolus vulgaris*) where seed inoculation with the same *M. robertsii* isolate ESALQ 1622 and another *B. bassiana* isolate ESALQ 3375 also decreased spider mite populations as compared to mock control (Canassa et al. 2019b). The negative effects of these two fungal isolates were further confirmed by root inoculations of strawberry plants under commercial field conditions where the incidences of spider mites and other selected insect pests were observed over 180 days (Canassa et al. 2019a). However, in other studies, endophytic colonization by *B. bassiana* isolates have been reported to exhibit both negative or neutral effects against insect herbivores (McKinnon et al. 2017). The finding that *M. brunneum* KVL 04-57 can increase susceptibility to insects was recently demonstrated in both wheat and broad bean infested by aphids (Rasool et al. 2020). Furthermore, an isolate of *M. brunneum* (strain F52) was also reported to increase the population size of *Aphis glycines* in soybean plants after seed inoculations (Clifton et al. 2018). On the other hand, Jaber and Araj (2018) showed a negative effect on aphids in sweet pepper after root drench with *M. brunneum* strain BIPESCO5 (CBS123710). Collectively, our results emphasize the importance of evaluating the outcome of each unique EPF isolate which display an endophytic lifestyle after application to seed and root for insect pests control on shoots.

SGA from tomato plants have detrimental effects against several insect herbivore species (Friedman 2002). In relation to insect attack, the presence of SGA in African nightshades was found to be associated with plant defense responses against the red spider mite, *Tetranychus evansi* (Jared et al.

2016). Furthermore, the negative effects of α-tomatine on arthropod herbivore physiology, induction of glycoalkaloids with herbivore damage and their potential in field resistance against insect pests such as Colorado potato beetle and potato leafhopper are widely documented (Milner et al. 2011). In the present study, spider mite feeding and seed inoculations with EPF induced the biosynthesis of α-tomatine and dehydro-tomatine in tomato plants. Recently, it became evident that endophytes can induce or modify the biosynthesis of plant specialized metabolites (Ludwig-Müller 2015), which may affect insect herbivores (Hartley and Gange 2009). A current study showed that grapevine (*Vitis vinifera* L.) plants inoculated with *B. bassiana* produced more insect toxic volatile compounds as compared to control plants but had no effects on infestation levels of grapevine mealybug, *Planococcus ficus* (Moloinyane and Nchu 2019). We demonstrated that the concentrations of SGA were significantly increased with spider mite feeding in all treatments. The plant specialized metabolites are known to be induced by herbivores (Miresmailli and Isman 2014). The higher concentrations of both SGA compounds measured in tomato leaves from plants inoculated with EPF isolates and after spider mite feeding could reflect induced defense reactions of plants stimulated by the fungal inoculations, as suggested by Moloinyane and Nchu (2019). It is possible that *B. bassiana* and *M. robertsii* imitates tomato plant's ability to produce SGA to favor plant defense against *T. urticae.*

The tomato plants grown from *M. brunneum* treated seeds represented suitable hosts for spider mite populations and they produced low concentrations of α-tomatine and dehydro-tomatine. In line with these results, a high reproduction rate of *A. glycines* after seed inoculations of soybean plants with *M. brunneum* (strain F52) was suggested to be linked with reduced efficiency of salicylic acid (SA) and jasmonic acid (JA) defense pathways (Clifton et al. 2018). The molecular and chemical analysis by Shavit et al. (2013) suggested that increased developmental and survival rates of *Bemisia tabaci* was related to the reduced induced defense system (JA/ET and SA pathways) of tomato plants after pre-inoculation with plant growth-promoting rhizobacteria. Therefore, we expect that endophytic colonization by *M. brunneum* in the present study suppressed the biosynthesis of SGA either directly or by modifying related pathways involved in SGA production. However, more studies are required to unravel the phenomenon behind the contrasting effects of different EPF isolates belonging to the same genus.

High reproduction growth of insect herbivores on fungal inoculated plants has been suggested to be linked with the increased availability of nutrients resulting in improved plant growth (Clifton et al. 2018; Jensen et al. 2019). In the present study, tomato plants treated with the two isolates of

Metarhizium spp. showed overall improved growth with significant effects for root biomass when plants were challenged with biotic stress. This indicates that the high population growth of spider mites do not directly correlate with better growth in this specific setup. However, it is important to discern that *M. brunneum* maintained the growth with a significantly higher number of spider mites feeding than *M. robertsii*. The growth promotion concurs with other findings using different fungal isolates and plant species (Jaber and Enkerli 2016; Jaber 2018; Canassa et al. 2019b; Rasool et al. 2020). In the putative mutualistic relationship, EPF can translocate nitrogen from insect cadavers to plants and in return receive photosynthates (Behie et al. 2012, 2017; Behie and Bidochka 2014). The high nutrient acquisition by plants after EPF colonization can favor plant growth (Hu and Bidochka 2019) and they can sustain their fitness under biotic stress to compensate for the damage inflicted by herbivores and therefore better tolerate herbivory (McKinnon et al. 2017). The bioavailability of different nutrient content to plant also favor growth promotion (Tall and Meyling 2018).

The three isolate used in the present study varyingly colonized different tomato plant parts (leaf, stem and root) with *B. bassiana* systemically colonizing all tissues while the two isolates of *Metarhizium* spp. were mostly isolated from stem and root tissues showing a significant decrease from 20 to 40 days after inoculation. The pattern of colonization by *B. bassiana* in tomato plants concurs with other studies (Ownley et al. 2008; Barra-Bucarei et al. 2020). The highest colonization rate of *M. brunneum* was found in the stem which correlates with the findings of Jaber and Enkerli (2016), who reported the isolation of *M. brunneum* (strain BIPESCO5) from leaves after seed treatments of *Vicia faba*, unlike present study. In another study, *M. robertsii* was only found in *V. faba* roots after seed treatment (Canassa et al. 2019b) but not in the stem tissue. Notably, *M. robertsii* with high persistence as endophytes in roots resulted in reduced spider mite populations on inoculated plants comparable to observations in *B. bassiana* treated plants although the latter fungus was mostly recovered in aerial plant parts. This inverse localization with comparable effects supports the notion of an indirect effect via specialized plant metabolites over direct effects by the fungus itself. The low levels of fungal colonization at 40 days after inoculations may be due to several factors, such as increased competition with other microorganisms, the impermanence of fungi in plants (Resquín-Romero et al. 2016), appropriate tissue parts were not sampled to check colonization and the large size of the cut sections. However, the effects on SGA accumulation and population growth of *T. urticae* did not directly correlate with the EPF endophytic colonization patterns.

Our study suggests the occurrence of plant-mediated indirect effects on spider mite populations are variable, depending upon the entomopathogenic fungal isolate and the level of steroidal alkaloid

defense compounds in the leaf tissue. Seed inoculations caused plant colonization differently for all isolates and sustain plant growth under biotic stress, but these effects did not correlate with population growth responses of spider mites. Further studies should consider the plant-mediated and fungal induced species-specific defense compounds of plants in research on EFEs and elucidate the biosynthetic pathways leading to these chemical changes in plants after fungal inoculations. However, we cannot conclude whether the observed effects on herbivores and plant specialized metabolites are due to endophytic colonization, epiphytic growth of EPF or are due to other factors. These aspects should be investigated further in the future.

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Table and Figures

Table.1 Percentage (95% confidence intervals) of colonized tomato tissue pieces (root, stem and leaf) at 20 and 40 days post seed inoculations (DPI) with entomopathogenic fungal isolates: *Beauveria bassiana*, *Metarhizium brunneum* and *Metarhizium robertsii*, respectively.

Colonized plant tissue pieces % (95% CI)						
Treatments	20 DPI			40DPI		
	Root	Stem	Leaf	Root	Stem	Leaf
B. bassiana	18% (9.3 – 32.9)ab	25% (13.7 – 40)abc	19% (10.9 –31)ab	6% (1.8 – 17.6)a	12% (5.3-25.4)ab	0
M. brunneum	27% (15.3 – 42.3)bc	41% (27.1 – 57.4)c	0	16% (7.9 – 30.4)ab	22% (12.2-37.6)b	0
M. robertsii	35% (21.8 – 51.1)bc	10% (4 – 22.8)a	0	20% (10.7 – 35.3)b	0	0
	n = 48	*n* = 48	*n* = 72	*n* = 48	*n* = 48	*n* = 72

Same letters for 20 and 40 days, respectively, indicate no significant differences by *post hoc* tests using *multcomp* function.

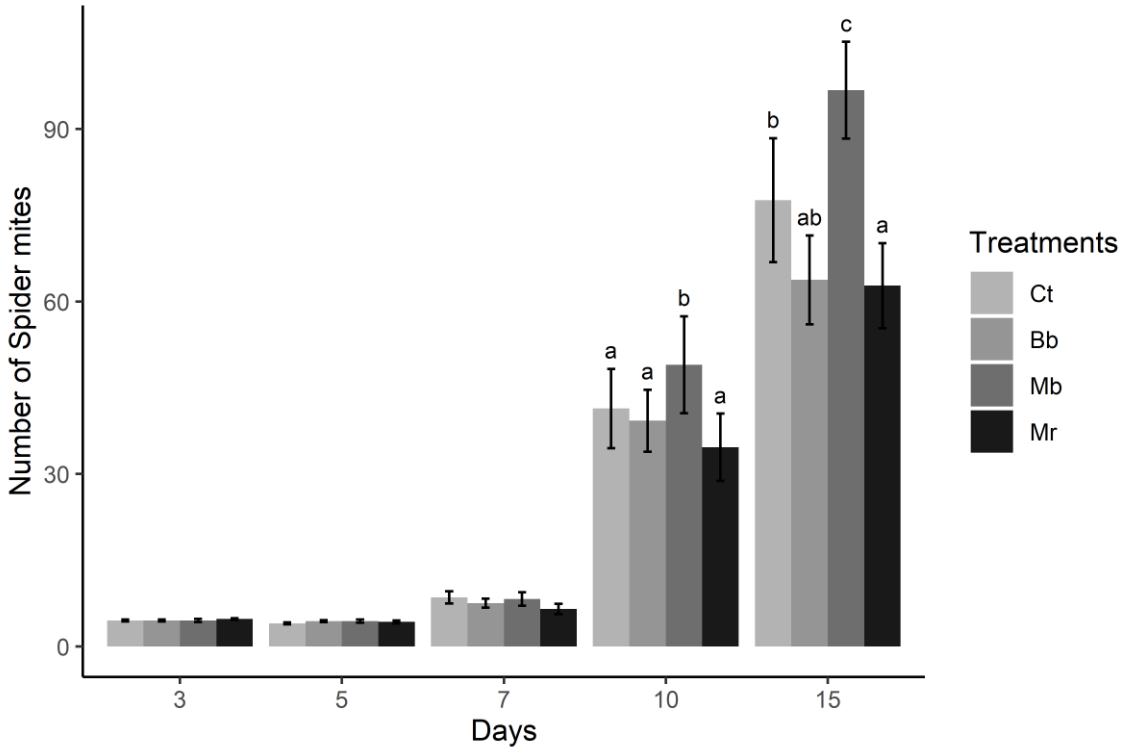


Figure 1: Population growth of spider mites (*Tetranychus urticae*) on seed inoculated tomato plants with entomopathogenic fungi (Bb: *Beauveria bassiana*, Mb: *Metarhizium brunneum* and Mr: *Metarhizium robertsii*) and control (Ct) over 15 days after spider mite infestation and 40 days after inoculations. Bars (means ± SE) with same letters are not significantly different by *post hoc* tests using *multcomp* function.

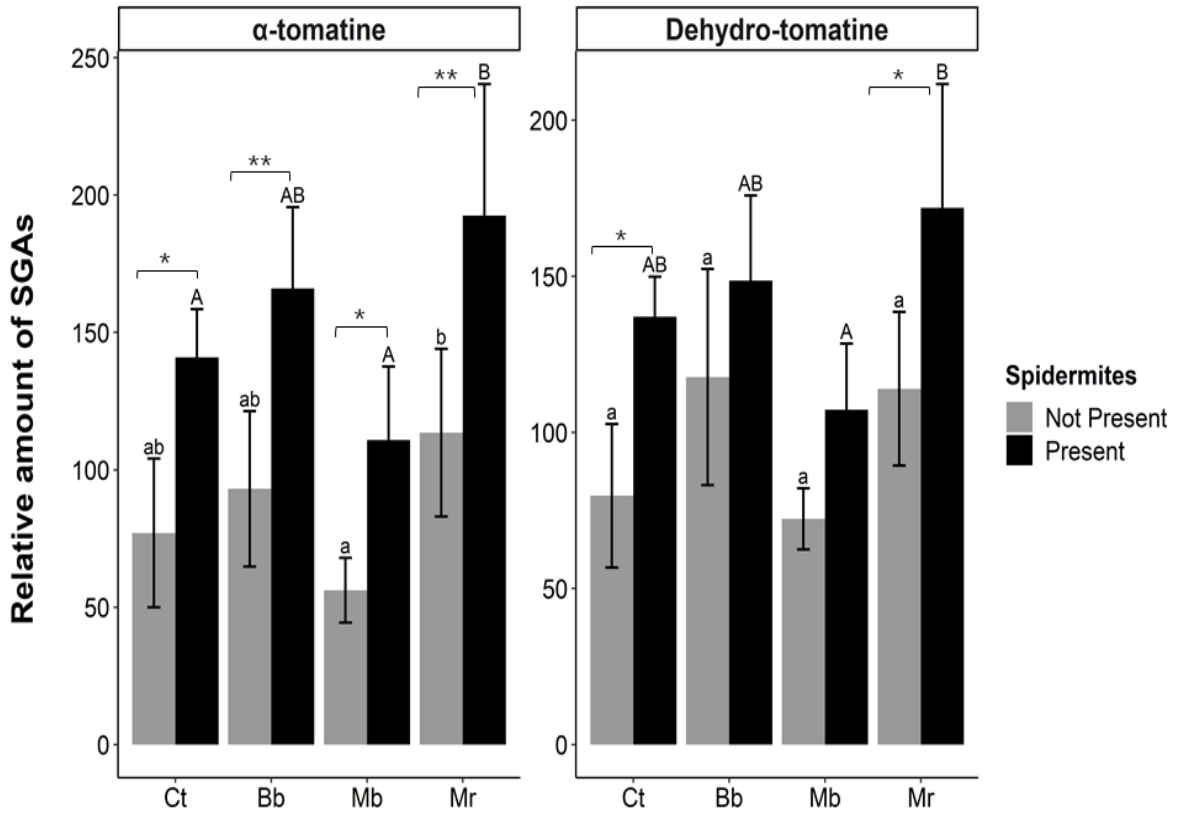


Figure 2: Steroidal alkaloids, SGA (α-tomatine and dehydro-tomatine) amounts after 40 days of seed inoculations with entomopathogenic fungi (Bb: *Beauveria bassiana*, Mb: *Metarhizium brunneum* and Mr: *Metarhizium robertsii*), control (Ct) and 15 days after spider mite infestation. Each bar shows the mean concentration ± SE (n = 8) of SGA with (black bars) or without spider mites (grey bars). The significance letters compare treatments without spider mites (grey bars; small alphabets), treatments with spider mites (black bars; capital alphabets) and with /without spider mites within the same treatment (lines with asterisks) for both SGA compounds. Same letters within a graph are not significantly different by *post hoc* tests using *multcomp* function.

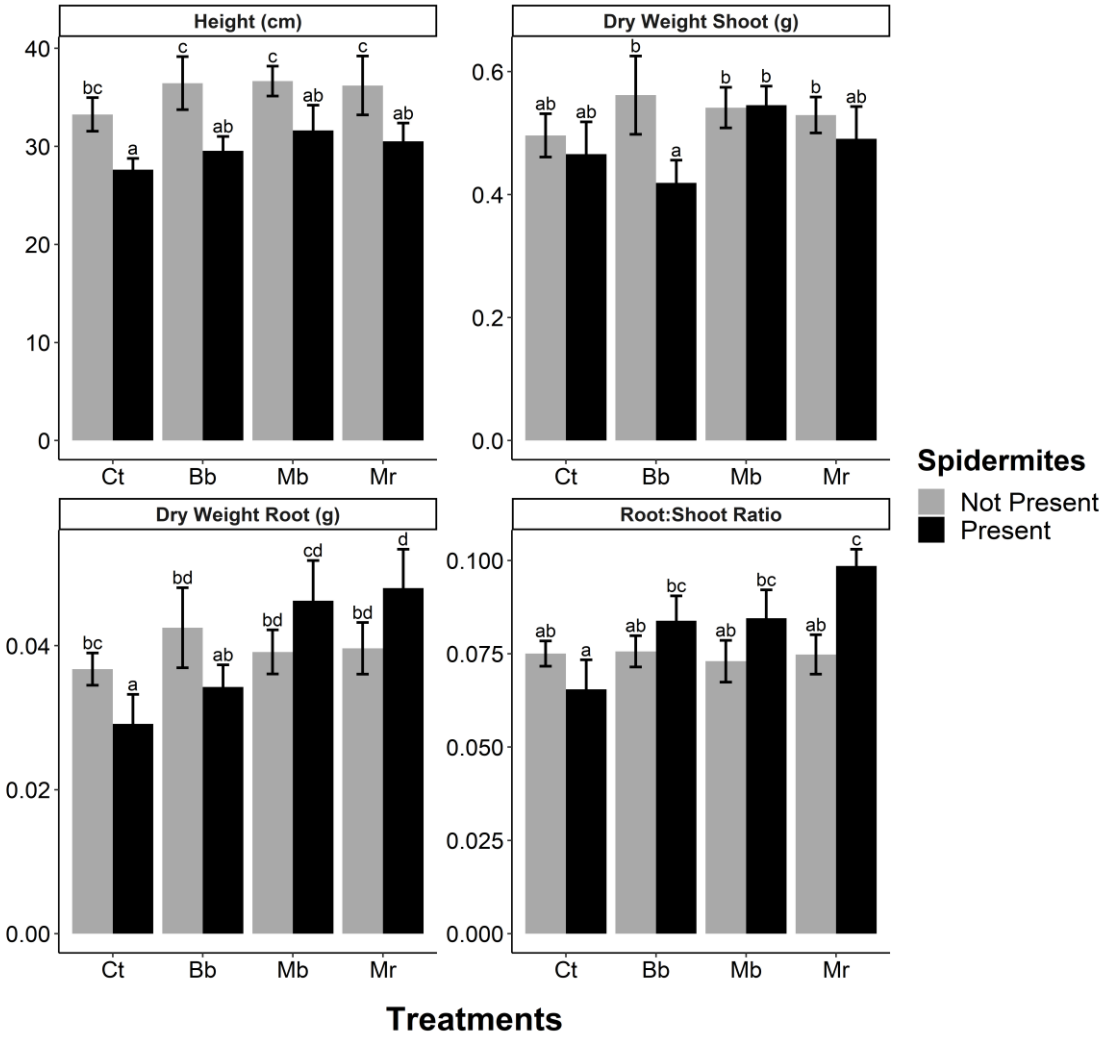


Fig. 3: Tomato plant growth (height (cm), dry weight of shoot and root (g) and root: shoot ratio) after 40 days of seed inoculations with entomopathogenic fungi (Bb: *Beauveria bassiana*, Mb: *Metarhizium brunneum* and Mr: *Metarhizium robertsii*), control (Ct) and 15 days after spider mite infestation. Each bar shows the mean ± SE (*n* = 8) of growth parameters with (black bars) or without spider mites (grey bars). Same letters within a graph are not significantly different by *post hoc* tests using *multcomp* function ; letters "a-c" = "a,b,c" ,"b-d" = "b,c,d" and "a-d" = "a,b,c,d".

Supporting Information

Table S1 Percentage (95% confidence intervals) of colonized tomato tissues (root, stem and leaf) at 20 and 40 days post seed inoculations (DPI) with entomopathogenic fungal isolates: *Beauveria bassiana*, *Metarhizium brunneum* and *Metarhizium robertsii*, respectively.

Colonized plant tissues % (95% CI)						
Treatments	20 DPI			40DPI		
	Root	Stem	Leaf	Root	Stem	Leaf
B. bassiana	25% (8.2 – 55.1)a	41% (18.4 – 69)abc	33% (13 – 62.4)ab	8% (1.1 – 41.3)a	16% (4.1 – 47.7)a	0
M. brunneum	41% (18.4 – 69)abc	66% (37.5 – 86.9)bc	0	16% (4.1 – 47.7)a	25% (8.2-55.1)a	0
M. robertsii	58% (30.7 – 81.5)bc	16% (4.1– 47.7)a	0	33% (13 – 62.4)a	0	0
	n =12			*n* =12		

Same letters for 20 and 40 days, respectively, indicate no significant differences by *post hoc* tests using *multcomp* function.

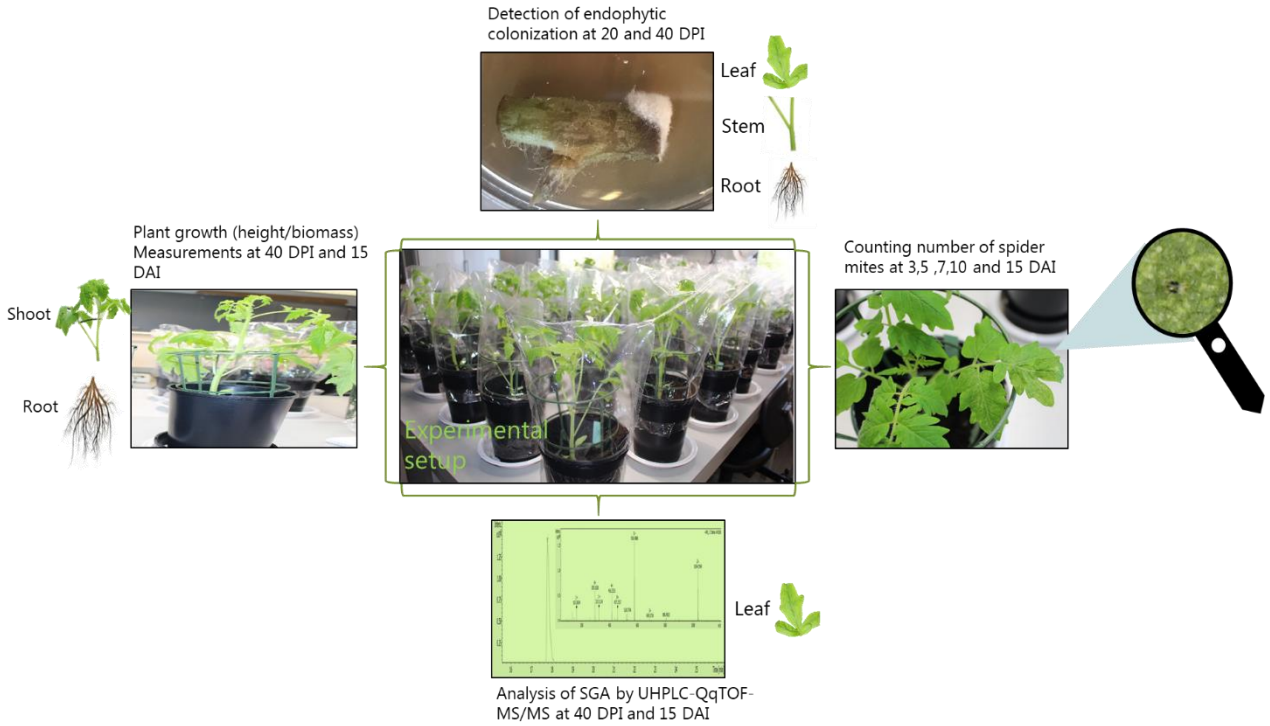
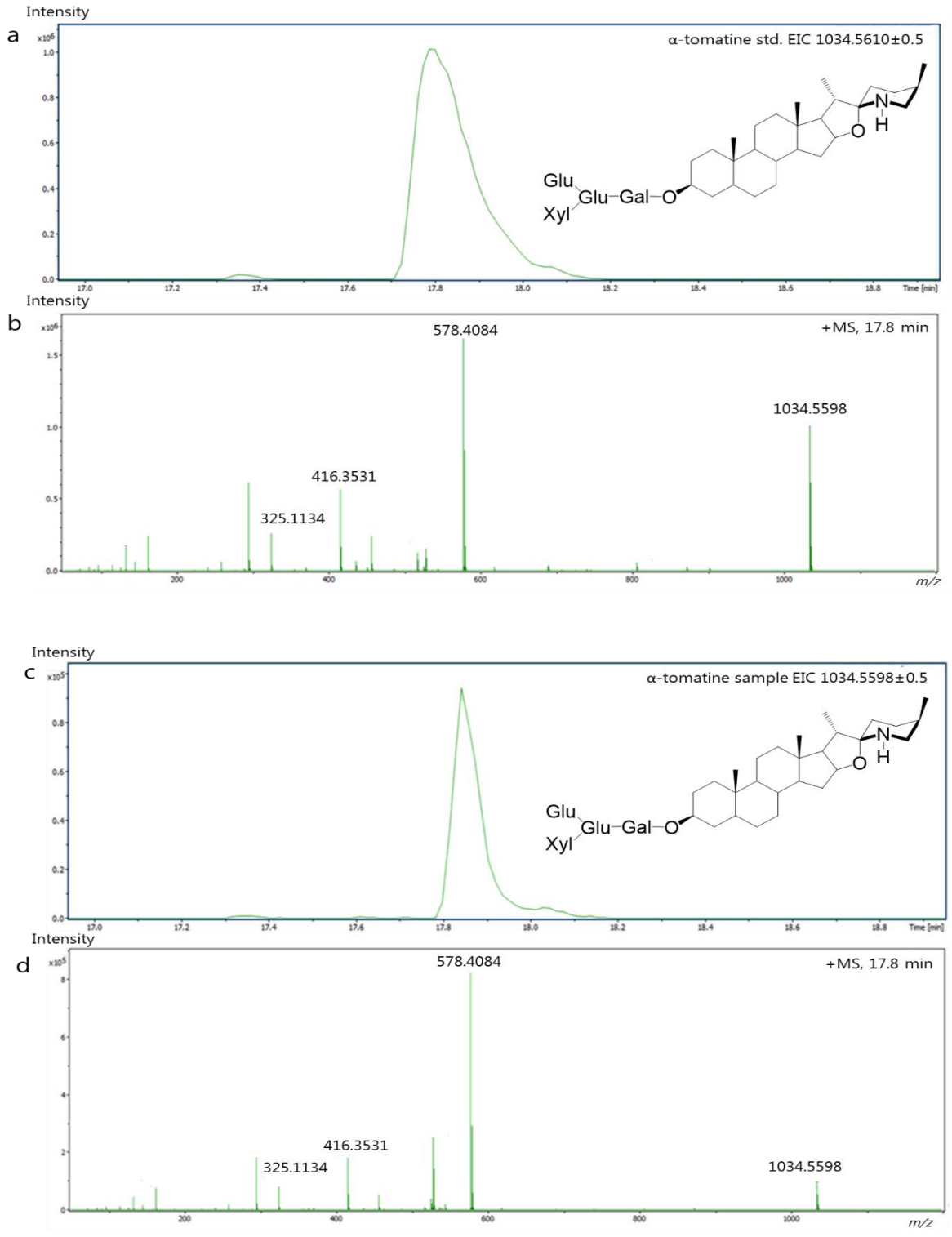


Figure S1. Experimental setup to check the endophytic colonization, population growth of two-spotted spider mites (*Tetranychus urticae*), plant growth and concentrations of steroidal glycoalkaloids (SGA) after seed inoculations with three entomopathogenic fungi (*Beauveria bassiana*, *Metarhizium brunneum* and *M. robertsii*) in tomato (*Solanum lycopersicum*). Endophytic colonization was checked from leaf, stem and root at 20 and 40 days post inoculations (DPI). The number of spider mites was counted at 3, 5, 7, 10 and 15 days after infestation (DAI). Tomato growth parameters (height and biomass) and the concentrations of SGA (α-tomatine and dehydro-tomatine) were measured at 40 DPI and 15 DAI.


a
Intensity
α-tomatine std. EIC 1034.5610±0.5
Glu
Glu–Gal–O
Xyl
N
H
O
Time [min]
b
Intensity
+MS, 17.8 min
578.4084
1034.5598
416.3531
325.1134
m/z
c
Intensity
α-tomatine sample EIC 1034.5598±0.5
Glu
Glu–Gal–O
Xyl
N
H
O
Time [min]
d
Intensity
+MS, 17.8 min
578.4084
416.3531
325.1134
1034.5598
m/z

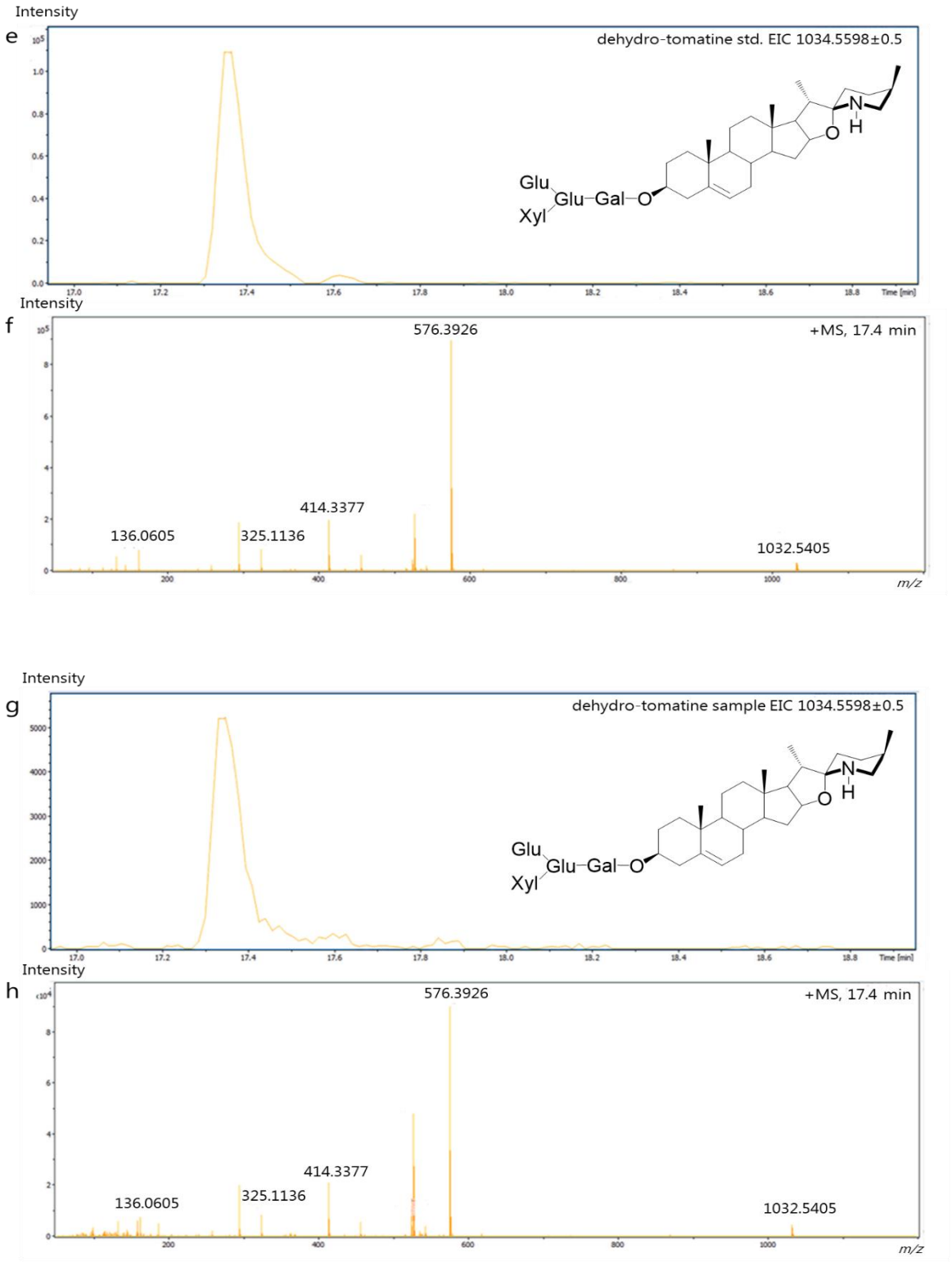


Figure S2: LC-MS analysis of steroidal glycoalkaloids in tomato after seed inoculations with entomopathogenic fungi. The extracted ion chromatograms (EIC) are based on *m/z* values ± 0.05 of 1034.5598(α-tomatine) and 1032.5405 (dehydro-tomatine). (a) α-tomatine standard EIC, (b) α-tomatine standard mass spectra, (c) α-tomatine sample EIC, (d) α-tomatine sample mass spectra, (e) dehydro-tomatine standard EIC (f) dehydro-tomatine standard mass spectra, (g) dehydro-tomatine sample EIC and (f) dehydro-tomatine sample mass spectra.

s