Interactions between endophytic *Metarhizium brunneum* and oilseed rape plants: effects on plant health and possible mechanisms

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Submitted by

Catalina Posada-Vergara

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1st Referee: Prof. Dr. Michael Rostás

2nd Referee: Priv. - Doz. Rer. Nat. habil. Jürgen Gross

3rd Referee: Prof. Dr. Andreas von Tiedemann

4th Referee: Prof. Dr. rer. Nat. habil. Stefan Vidal

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Summary

Fungi of the genus Metarhizium, belonging to the order Hypocreales, are well-known entomopathogens that primarily infect insects. However, recent research has revealed their multifunctional lifestyles, including their ability to live as saprophytes in the soil and proliferate in the rhizosphere of various plant species. Metarhizium species have been found to be associated with plant roots, grow endophytically, and have shown beneficial effects on plant health, such as improved growth under stress conditions and protection against insect pests and plant diseases. Despite these findings, there is a lack of information on the mechanisms responsible for these interactions. Therefore, the objective of this thesis was to study the mechanisms behind the effects of fungal-plant associations on insects and plant pathogens. This thesis comprises five chapters, each focussing on different aspects of the interaction of *Metarhizium brunneum* with plants, insects, and plant pathogens.

Chapter 1 addresses the challenge of distinguishing among isolates of the same species and the importance of identifying specific fungal isolates to track their efficacy as biocontrol agents in the field. This study characterised a collection of *M. brunneum* isolates and developed a strain-specific PCR assay using random amplified polymorphic DNA (RAPD) markers and direct sequencing. The results demonstrate the usefulness of RAPD markers in developing sequence-characterised amplified regions (SCAR) to differentiate *M. brunneum* strains, providing a valuable tool to track specific isolates in field studies.

Chapter 2 focusses on the ability of entomopathogenic fungi to associate with two crop plants, winter oilseed rape and potato. This study evaluated the colonization of these plants by five *M. brunneum* isolates using quantitative polymerase chain reaction (qPCR) providing insights into the associations between the fungus and different crop species. These findings contribute to the selection of oilseed rape as a crop for further investigation of the fungus-plant association and its effects on insect pests, plant diseases, and potential mechanisms.

Chapter 3 examines the effects of *M. brunneum* on herbivores, specifically the chewing specialist *Psylliodes chrysocephala*, the generalist aphid *Myzus persicae* and the specialist aphid *Brevicoryne brassicae* using *Brassica napus* plants. This study revealed a feeding stimulant effect of *M. brunneum* on a specialist herbivore and a variable impact on aphid fecundity. The results are discussed in the context of plant-fungal interactions and their influence on nutritional quality, defence metabolites, and insect parameters.

Chapter 4 focusses on evaluating the direct and plant-mediated effects of *M. brunneum* on the cabbage root fly *Delia radicum* (CRF). This study demonstrates CRF infection by different fungal isolates and uses a split-root design to separate direct from systemic effects. It assesses their impact on herbivore damage, insect mortality, and the modulation of phytohormones, through measurements of plant hormone levels using HPLC-MS and examination of gene expression via qPCR. These findings show that root-associated *M.*

brunneum controls the CRF by direct insect infection, local and systemic priming of defence pathways, and modulation of phytohormones.

Chapter 5 explores the ability of *M. brunneum* to protect oilseed rape plants against the soil-borne pathogen *Verticillium longisporum*. The study investigates the mechanisms involved, employing in vitro and greenhouse experiments, gene expression analysis, and split-root setup to study direct competition, antibiosis, and plant-mediated effects. The results indicate that *M. brunneum* delays pathogen colonisation, exhibits competitive growth, and activates defence hormone pathways, resulting in decreased disease symptoms and improved plant growth.

In conclusion, this Ph.D. thesis advances our understanding of the interactions of *M. brunneum* with insects and plant pathogens, separating direct effects from plant-mediated effects as endophytes of plants. Characterisation of *M. brunneum* isolates, development of strain-specific assays, and evaluation of colonisation capabilities contribute to the tracking and utilisation of specific isolates effectively. The study highlights the potential of *M. brunneum* to reduce herbivore damage and protect plants against soil-borne pathogens. The findings contribute to advancing our understanding of the ecological roles and applications of *M. brunneum* in agricultural systems.

Zusammenfassung

Pilze der Gattung Metarhizium aus der Ordnung der Hypocreales sind bekannte Entomopathogene, die in erster Linie Insekten befallen. Neuere Forschungen haben jedoch gezeigt, dass sie multifunktionale Lebensweisen aufweisen, einschließlich ihrer Fähigkeit, als Saprophyten im Boden zu leben und in der Rhizosphäre verschiedener Pflanzenarten zu vermehren. Metarhizium-Arten werden mit Pflanzenwurzeln assoziiert, wachsen endophytisch und zeigen positive Auswirkungen auf die Pflanzengesundheit, wie verbessertes Wachstum unter Stressbedingungen sowie Schutz vor Insekten und Pflanzenkrankheiten. Trotz dieser Erkenntnisse gibt ein Mangel an Informationen über die Mechanismen, die für diese Interaktionen verantwortlich sind. Daher war das Ziel dieser Dissertation, die Mechanismen hinter den Auswirkungen von Pilz-Pflanzen-Assoziationen auf Insekten und Pflanzenpathogene zu untersuchen. Die Dissertation umfasst fünf Kapitel, die jeweils verschiedene Aspekte der Interaktion von *Metarhizium brunneum* mit Pflanzen, Insekten und Pflanzenpathogenen behandeln.

Kapitel 1 behandelt die Herausforderung, zwischen Isolaten derselben Art zu unterscheiden, und die Bedeutung der Identifizierung spezifischer Pilzisolate zur Verfolgung ihrer Wirksamkeit als biologische Schädlingsbekämpfungsmittel im Feld. Die Studie charakterisiert eine Sammlung von *M. brunneum*-Stämmen und entwickelt einen stammspezifischen quantitativer Polymerase-Kettenreaktion (PCR)-Test unter Verwendung von random amplifizierten polymorphen DNA (RAPD)-Markern und direkter Sequenzierung. Die Ergebnisse zeigen den Nutzen von RAPD-Markern bei der Entwicklung von sequenzcharakterisierten Amplifikationsregionen (SCARs) zur Unterscheidung von *M. brunneum*-Stämmen und bieten ein wertvolles Werkzeug zur Verfolgung spezifischer Isolate in Feldstudien.

Kapitel 2 konzentriert sich auf die Fähigkeit entomopathogener Pilze, mit zwei Kulturpflanzen – Winterraps (*Brassica napus*) und Kartoffel (*Solanum tuberosum*) - assoziiert zu sein. Die Studie bewertet die Besiedlung dieser Pflanzen durch fünf *M. brunneum*-Isolate mittels quantitativer PCR (qPCR) und liefert Einblicke in die Wechselwirkungen zwischen dem Pilz und verschiedenen Kulturarten. Die Ergebnisse tragen zur Auswahl von Winterraps als Kulturpflanze für weitere Untersuchungen zur Pilz-Pflanzen-Assoziation sowie deren Auswirkungen auf Schädlinge, Pflanzenkrankheiten und potenzielle Mechanismen bei.

Kapitel 3 untersucht die Auswirkungen von *M. brunneum* auf Herbivore, insbesondere den kauenden Spezialisten *Psylliodes chrysocephala*, den Generalisten-Aphid *Myzus persicae* und den spezialisierten Aphid *Brevicoryne brassicae* unter Verwendung von *B. napus*-Pflanzen. Die Studie zeigt eine futterstimulierende Wirkung von *M. brunneum* auf den spezialisierten Herbivoren und eine variable Auswirkung auf die Fortpflanzungsfähigkeit der Blattläuse. Die Ergebnisse werden im Zusammenhang mit Pflanzen-Pilz-Interaktionen und deren Einfluss auf die Nährstoffqualität, Abwehrmetaboliten und Insektenparameter diskutiert.

Kapitel 4 konzentriert sich auf die Bewertung der direkten und pflanzenvermittelten Auswirkungen von *M. brunneum* auf die Kohlfliege *Delia radicum* (CRF). Die Studie zeigt die Infektion der CRF durch die verschiedenen Pilzisolate und verwendet ein Split-Root-Design, um direkte von systemischen Auswirkungen zu trennen. Sie bewertet deren Auswirkungen auf Herbivorenschäden, Insektensterblichkeit und pflanzliche Abwehrmechanismen durch Messung von Pflanzenhormonen (HPLC-MS) und Genexpression (qPCR). Die Ergebnisse zeigen, dass mit den Wurzeln assoziierte *M. brunneum* die CRF durch direkte Insekteninfektion, lokale und systemische Priming von Abwehrwegen sowie Modulation von Phytohormonen kontrollieren.

Kapitel 5 untersucht die Fähigkeit von *M. brunneum*, Winterraps-Pflanzen vor dem bodenbürtigen Pathogen *Verticillium longisporum* zu schützen. Die Studie untersucht die beteiligten Mechanismen anhand von In-vitro- und Gewächshausversuchen, Genexpressionsanalysen und einem Split-Root-Setup, um direkte Konkurrenz, Antibiose und pflanzenvermittelte Effekte zu untersuchen. Die Ergebnisse deuten darauf hin, dass *M. brunneum* die Besiedlung durch den Pathogen verzögert, ein wettbewerbsfähiges Wachstum zeigt und Verteidigungshormonwege aktiviert, was zu verminderten Krankheitssymptomen und verbessertem Pflanzenwachstum führt.

Zusammenfassend trägt diese Doktorarbeit zu unserem Verständnis der Wechselwirkungen von *M. brunneum* mit Insekten und Pflanzenpathogenen bei und trennt dabei direkte Effekte von pflanzenvermittelten Effekten als Endophyt von Pflanzen. Die Charakterisierung von *M. brunneum*-Isolaten, die Entwicklung stammspezifischer Tests und die Bewertung der Besiedlungsfähigkeiten tragen dazu bei, spezifische Isolate effektiv zu verfolgen und zu nutzen. Die Studie hebt das Potenzial von *M. brunneum* zur Reduzierung von Herbivorenschäden und zum Schutz von Pflanzen vor bodenbürtigen Pathogenen hervor. Die Ergebnisse tragen dazu bei, unser Verständnis der ökologischen Rollen und Anwendungen von *M. brunneum* in landwirtschaftlichen Systemen voranzutreiben.

General Introduction

Among the millions of microorganisms living in the soil are fungi of the genus Metarhizium. They are members of the order Hypocreales, together with other well-known entomopathogens such as Beauveria and Lecanicillium spp. They are known as insect pathogens, but their multifunctional lifestyle has come to light in recent decades. They are able to live as saprophytes in the soil. They can also multiply in the rhizosphere of a wide range of plant species. Several publications have shown how they associate with plant roots and also grow endophytically. They have beneficial effects on plant health. These include improved growth under stress conditions and protection against insect pests and plant diseases. However, little is known about the mechanisms underlying these interactions, despite numerous reports of negative or interactive effects against herbivores and pathogens. Therefore, my research aimed to investigate the mechanisms underlying the effects of fungal-plant associations on insects and plant pathogens..

In this chapter, I will review the current state of knowledge on the phylogenetics of Metarhizium, its interactions with plant roots, and the effects of these interactions on insects and plant diseases. I will also give a general overview of possible mechanisms. Finally, I will conclude with an outline of the Ph.D. thesis, in which the main aims are presented.

Entomopathogenic fungi

Entomopathogenic fungi are natural enemies of arthropods and are mainly found in the orders Entomophthorales, which are obligate pathogens, and Hypocreales, which have both obligate and facultative entomopathogens (Roy et al., 2006; Moonjely et al., 2016). Within the Hypocreales, two well-known genera of facultative entomopathogens are Beauveria (Cordycipitacea) and Metarhizium (Clavicipitacea). *Metarhizium brunneum* is a species within the PARB clade, named after *M. **p**ingshaense*, *M. **a**nisopliae, M. **r**obertsii**,*** and *M. **b**runneum*, and proposed by Bischoff et al., (2009), and is found in natural and agricultural ecosystems worldwide (Rehner and Kepler, 2017; Sant et al., 2021). Species within the PARB clade cannot be easily distinguished by microscopic or colony morphology; therefore, molecular taxonomy and phylogenetic studies have explored different nuclear loci that better reflect the phylogenetic affinities among Metarhizium species (Driver et al., 2000; Pantou et al., 2003; Bischoff et al., 2009; Kepler and Rehner, 2013). To increase the resolution of phylogenetic studies in Metarhizium species, Kepler and Rehner (2013) selected new intergenic nuclear regions that showed intraspecific nucleotide variability. However, the identification of species using these genetic markers involves sequencing and phylogenetic analysis. Faster and more economical identification is possible using species-specific primers. Indeed, Mayerhofer et al. (2019) used the intergenic regions found by Kepler and Rehner (2013) to find species-specific

PCR primers for species in the PARB clade. However, rapid isolate-specific identification is not yet possible.

Phylogenetic studies have shown that entomopathogenic fungi such as Metarhizium species are more closely related to endophytes and plant pathogens than to animal pathogens (Wyrebek and Bidochka, 2013; Wang et al., 2016). They have retained the ability to interact with plants, as evidenced by proliferation in the rhizosphere, endophytic colonisation of plant roots, and persistence in the soil microenvironment after soil application (Hu and Leger, 2002; Bruck, 2005; St. Leger, 2008; Bruck, 2010; Sasan and Bidochka, 2012; Behie et al., 2015). Metarhizium species can colonize various crop plants and grow endophytically in root cortical cells (Bruck, 2010; Fisher et al., 2011; Sasan and Bidochka, 2012; Barelli et al., 2018). However, endophytic colonisation of the root system appears to be low level and transient (Barelli et al., 2018). Colonization of aboveground tissues has also been observed, especially after artificial inoculation with high concentrations of spores, both applied to the leaves or soil (García et al., 2011; Batta, 2013; Barelli et al., 2018).

Interaction of Metarhizium with the plant host

Roots that grow in the soil share the habitat with many microorganisms. Plant roots deposit up to 40% of the carbon in the rhizosphere, creating a microenvironment that promotes microbial communities different from those found in bulk soil. (Hartmann et al., 2009; Bakker et al., 2013). Roots interact with these microorganisms, some of which are pathogenic and some beneficial to the plant. Some interactions with beneficial microorganisms have been extensively studied, for example, plant growth-promoting and disease suppressing rhizobacteria or mycorrhizal fungi (Hampp et al., 2012; Poveda et al., 2020), and Trichoderma species (Alfiky and Weisskopf, 2021; Poveda, 2021).

Our understanding of the interaction of Metarhizium with plants is based on recent studies (St. Leger and Wang, 2020). It is now known that Metarhizium successfully associates with plant roots, and that this probably provides benefits to both organisms that compensate for the energetic cost of enabling this association (Morgan et al., 2005). The advantages of this association for the fungus are out of the scope of the present research, but we know that the fungus obtains photosynthates (Behie et al., 2017), and the rhizosphere can provide a refuge for the fungus to survive outside insect hosts (Meyling and Eilenberg, 2006). On the other hand, benefits to the plant include growth promotion (Jaber and Enkerli, 2017), and protection against abiotic and biotic stresses (St. Leger and Wang, 2020; Stone and Bidochka, 2020). Examples of growth promotion have been reported for soybean plants under salt stress (Khan et al., 2012), tomato, switchgrass (*Panicum virgatum*), common bean (*P. vulgaris*) and broad bean among other plants (García et al., 2011; Sasan and Bidochka, 2012; Jaber and Enkerli, 2016). Mechanisms that lead to growth induction can involve improved plant nutrition and the production of plant hormone-like molecules that stimulate plant growth (St. Leger and Wang, 2020). Metarhizium appears to improve nutrient acquisition; it transfers nitrogen from infected insects to plants (Behie et al., 2012), improves phosphorus uptake (Krell et al., 2018b) and releases iron from iron oxides *in vitro,* and decreases iron chlorosis in calcareous soils with

low Fe availability (Sanchez-Rodriguez et al., 2016). Furthermore, some species of Metarhizium produce auxin, a plant hormone involved in plant growth and development, and is particularly important for root formation and branching (Liao et al., 2017).

The ability of EEFs such as Metarhizium to have a negative effect on insect pests and plant diseases has been the topic of several reviews (Ownley et al., 2010; Vidal and Jaber, 2015; Jaber and Enkerli, 2017; Bamisile et al., 2018b; Bamisile et al., 2018a; Jaber and Ownley, 2018; Gange et al., 2019; St. Leger and Wang, 2020). Its multifunctionality as a direct controller of herbivore pests and as a plant-health booster makes it an attractive candidate for agricultural applications. However, there are also reports from Metarhizium and other fungal endophytes that increased insect attraction (Jallow et al., 2008; Aragón, 2016; Cotes et al., 2020), fecundity (Clifton et al., 2018; Rasool et al., 2021b) and feeding (Gange et al., 2012). Furthermore, few studies have explored the mechanisms behind the effects of Metarhizium on insects and plant pathogens. In fact, key publications have come out during the course of this thesis (Rivas-Franco et al., 2019; Cachapa et al., 2020; Gupta et al., 2022).

Possible mechanisms behind the effect of Metarhizium as plant endophyte on insects and plant pathogens

The effects of entomopathogenic endophytes on insect and plant pathogens can be the result of direct interactions with the insect or pathogen, or an indirect or plant-mediated effect, where beneficial fugus activate plant defence responses (Jaber and Ownley, 2018).

Direct effects

Metarhizium species produce a variety of secondary metabolites such as destruxins (Wang et al., 2012; Moonjely et al., 2016) that are toxic to insects and fungi (Lozano-Tovar et al., 2013; Sasan and Bidochka, 2013). One mechanism suggested in some reviews is that entomopathogenic endophytes produce mycotoxins inside plant tissues, causing detrimental effects on herbivores (Vidal and Jaber, 2015). However, research into the role of mycotoxins in the adverse effects of EEF on insects shows that the levels of fungal toxin found in plant tissues are too low to explain the effect on insects. (Resquín-Romero et al., 2016; Ríos-Moreno et al., 2016; Garrido-Jurado et al., 2017). Another proposed direct mechanism is the direct infection of the insect by the endophyte. However, the evidence is scarce; one study reported insect infection by endophytic *B. bassiana*, which is normally found in above-ground tissues, but mycosis could not explain the mortality found (Akello et al., 2008b; Akello et al., 2008a; Vidal and Jaber, 2015). In the case of Metarhizium, direct infection of above-ground herbivores is spatially improbable, given that Metarhizium is found primarily in the roots and rhizosphere. A study reported *Bemisia tabaci* mortality, but not mycosis, after foliar spraying with *M. brunneum* that led to a 'transient endophytic colonisation' of sweet potato leaves (Garrido-Jurado et al., 2017). Therefore, evidence of direct effects on insect performance is scarce, and it is plausible that the detrimental effects are not related to Metarhizium entomopathogenicity but are the result of the interaction of the fungus with the plant.

Furthermore, beneficial fungi can directly affect plant pathogens by hyperparasitism, competition for nutrients and space, and antibiosis (Ownley et al., 2010; Busby et al., 2016). Because Metarhizium lives in the soil and proliferates in the rhizosphere and plant roots, it shares the same habitat and hosts as soil-borne plant-pathogenic fungi. Consequently, this endophyte may have evolved direct mechanisms to compete against plant pathogens. A recent publication suggested direct interaction as the mechanism behind the reduction in *Verticillium dahliae* severity symptoms by *M. brunneum* (Miranda-Fuentes et al., 2020). As with insects, mycotoxins can affect fungal growth, and Metarhizium secondary metabolites have shown antagonistic activity against fungal pathogens, which correlate with reduced disease incidence (Lozano-Tovar et al., 2013; Sasan and Bidochka, 2013).

Indirect effects

Many beneficial associations of microorganisms with plants result in the induction of systemic resistance (ISR) (Pineda et al., 2010; Pieterse et al., 2014) which seems to be triggered both by specialised fungal symbionts like mycorrhiza (Hill et al., 2018; Dreischhoff et al., 2020) and generalised endophytes like *Trichoderma* spp. or nonpathogenic fungal strains (Shoresh et al., 2010; Pieterse et al., 2014). Induced systemic resistance is normally regulated by Jasmonic acid (JA) and ethylene signaling (van Wees et al., 2000; Verma et al., 2016) although several beneficial microorganisms can also induce salicylic acid (SA)-dependent systemic resistance (Pieterse et al., 2014). Some studies have shown how EEF can also induce changes on the plant defense system that could explain the negative effects on insect pests and plant pathogens. Inoculation with EEF can modify JA and SA hormone levels (Cotes et al., 2020; Rivas-Franco et al., 2020; Gupta et al., 2022), expression of genes involved in defense hormonal pathways (Hao et al., 2017; Ahmad et al., 2020; Qin et al., 2021; Gupta et al., 2022), transcriptional reprograming of plant defense system (Raad et al., 2019), plant volatiles (González-Mas et al., 2021) and plant secondary metabolites (Rasool et al., 2021b; Rasool et al., 2021a). These induced changes can influence the interaction of the plant with the insects and the pathogens (Pieterse et al., 2014; Fernandez-Conradi et al., 2018).

Induced systemic resistance by beneficial microorganisms generally involves priming (Pineda et al., 2010), which is characterised by increased sensitivity to JA and ethylene (Pineda et al., 2010; Shikano et al., 2017). In priming, the plant response to beneficial microorganisms is very mild, but upon perception of the stressor, there is a faster and/or stronger activation of the pland defence machinery, resulting in an enhanced level of resistance (Katz et al., 1998; Conrath et al., 2006; Pineda et al., 2010). Recent publications report that Metarhizium can also prime the plant against insects, by increasing SA hormone levels after herbivory (Rivas-Franco et al., 2020); or inducing higher myrosinase activity, a part of the defence response system of Brassicaceae (Cachapa et al., 2020). Plant priming by EEF against pathogens has also been suggested (Gómez-Vidal et al., 2009; Jaber and Ownley, 2018; Raad et al., 2019; Hu and Bidochka, 2021). However, none of these studies have included all the elements to recognise priming, which are naïve, primed, naïve-and-triggered, and primed-and-triggered plants (Martinez-Medina et al., 2016).

Thesis objective

Little is known about the mechanisms behind the beneficial effects of EEF on plant health. Furthermore, the results of the plant interaction with the entomopathogenic fungi are highly dependent on the plant species and the fungal isolates. This has been shown from rhizosphere competence to activation of plant defence responses (Pava-Ripoll et al., 2011; Angelone et al., 2018; Cachapa et al., 2020; Rivas-Franco et al., 2020; Rasool et al., 2021b).

Therefore, the main objective of my research was to identify and analyse in detail the effects of the association of the plant with *M. brunneum* on insect herbivores and a plant disease. During these investigations, I integrated different methods, using molecular biology tools, laboratory and greenhouse experiments.

Thesis outline

Chapter 1.

The main objective in this chapter was the molecular characterization of the *M. brunneum* collection of the Agricultural Entomology research group, in order to select the isolates for the next chapters. To address this objective, a phylogenetic analysis was done using intergenic nuclear regions that displayed high intraspecific nucleotide variability. A second objective was to develop isolate-specific primers using random amplified polymorphic DNA markers (RAPDs) and direct sequencing.

Chapter 2

The aim of this chapter was to select of a crop plant of economic importance that exhibited a good association in terms of root and plant colonization with *M. brunneum*, to use in the next chapters. For this, potato and oilseed rape plants were inoculated with five different fungal isolates, and root, shoot and leaf tissues were harvested three weeks later. After DNA extraction, the fungal detection in plant tissues by quantitative PCR (qPCR) was optimized. The results of this chapter lead to the selection of oilseed rape as the crop plant for the subsequent experiments.

Chapter 3

The aims of this chapter were to assess the effect of endophytic *M. brunneum* i) on the feeding of a specialist chewing herbivore, the cabbage-stem flea beetle (CSFB) *Physilliodes chrysocephala*, and ii) on the performance of two aphids: the specialist *Brevicoryne brassicae*, and a the generalist *Myzus persicae*. Plants were inoculated by root drenching with five different isolates. To evaluate the effect of plant fungal inoculation on CSFB feeding, a non-choice was used in which consumed leaf area was measured at different time points. Fungal colonization and endophytism was confirmed by qPCR, and plant growth parameters were measured. The effect of *M. brunneum* inoculation on the generalist and specialist aphids was analyzed by measuring aphid number, fecundity and pre-reproductive time of aphids feeding on leaves of two different ages. Fungal colonization and endophytism was also confirmed by qPCR.

Chapter 4

The aim of this chapter was to evaluate the direct and systemic effects of *M. brunneum* on the cabbage root fly (CRF). To assess direct effects, the pathogenicity of five isolates was measured in the presence and absence of oilseed rape plants. Parameters evaluated included CRF larval mortality, larval root damage and fungal colonisation of the rhizosphere and roots. In order to distinguish between local and systemic effects of the fungus-plant association, a split-root experiment was set up. In addition to the parameters already mentioned, the local and systemic response of plant roots to fungal inoculation and CRF herbivory was quantified. For this purpose, the phytohormones jasmonic acid, salicylic acid and abscisic acid were measured by high-performance liquid chromatography (HPLC) and the gene expression of genes involved in defence signalling and plant defence metabolites was measured (reverse transcription - qPCR).

Chapter 5

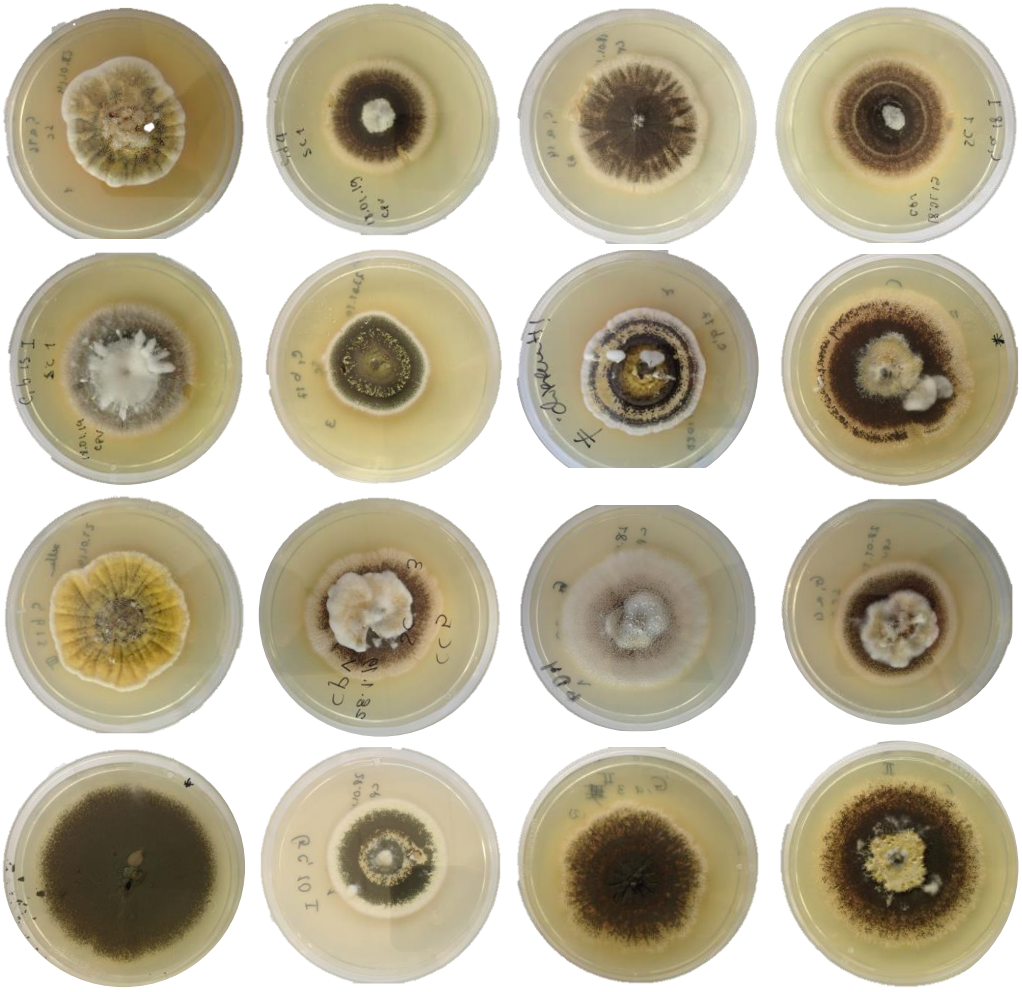
This last chapter was based on the results of my Master's thesis, in which I observed that the progression of symptoms of *Verticillium longisporum* disease was reduced when *M. brunneum* was co-inoculated with the pathogen. Therefore, the aim here was to assess whether the observed reduction in symptoms was a result of direct interaction between the fungi through competition or antibiosis, or whether there were plant-mediated effects involved. The direct effects of *M. brunneum* on the growth of *V. longisporum* were assessed using an in vitro confrontation assay and greenhouse experiments evaluating the colonisation of the rhizosphere, root and hypocotyl of both fungi. The systemic or plant-mediated effects were evaluated using a split-root experimental design and the parameters measured included Metarhizium rhizosphere, root and hypocotyl quantity, pathogen root and hypocotyl colonisation and disease development. In addition, the local and systemic root response to both fungi was analysed by measuring the expression of genes involved in defence signalling and plant defence metabolites..

Chapter 1.

Fast development of isolate-specific PCR primers for fungi based on SCARs: *Metarhizium brunneum* Gc2 as an example

Catalina Posada-Vergara[1], Stefan Vidal[1], Michael Rostás[1], Petr Karlovsky[1],

Georg-August-Universität Göttingen, Department of Crop Sciences



Abstract

Metarhizium brunneum, an entomopathogenic fungus, is used as a biological control agent against soil-borne insect pests. This fungus has been found in both natural and agricultural ecosystems, often associated with plant roots. Identification of specific fungal isolates is essential to monitor their efficacy as biocontrol agents in the field. However, it can be difficult to distinguish between isolates of the same species. In this study, we aimed to characterise a collection of *M. brunneum* isolates and develop a strain-specific PCR assay for selected *M. brunneum* isolates. We used an economical and rapid method using random amplified polymorphic DNA (RAPD) markers and direct sequencing to develop sequence-characterised amplified regions (SCARs) for the specific isolate. Although our phylogenetic analysis revealed low genetic diversity within our collection of *M. brunneum*, we were able to design two primer pairs suitable for intraspecific discrimination of one isolate. Our results demonstrate that RAPD markers are an economical and useful tool for the development of SCAR markers for the intraspecific differentiation of fungal strains. This study provides a valuable tool for tracking specific isolates in field studies, particularly in Metarhizium species of the PARB clade (*M. pingshaense, M. anisopliae, M. robertsii* and *M. brunneum),* which cannot be distinguished morphologically.

Introduction

Metarhizium brunneum is an entomopathogenic fungus that can be found in natural and agricultural ecosystems around the world. (Rehner and Kepler, 2017; Sant et al., 2021) .This fungus proliferates in the rhizosphere and associates with plant roots. (Hu and Leger, 2002; Bruck, 2005), and is a good candidate for the development of biological control products for soil-borne insect pests. In fact, the *M. brunneum* strain ARSEF 7711, also named F52 and Bipesco5, is used in commercial products (Met52®EC, Novozymes; GranMet-GRTM, Agrifutur s.r.l., Alfianello, Italy). In order to develop biological control products, it is important to monitor the fate of the fungus in soil, plants and insects under natural conditions. Due to the natural occurrence of Metarhizium species in soils, it is necessary to develop tools that allow intraspecific identification of a particular strain. However, it is not possible to distinguish isolates of the same Metarhizium species by microscopic or culture morphology. In addition, Metarhizium species belonging to the PARB clade (named after *M. **p**ingshaense M. **a**nisopliae, M. **robertsii,*** and *M. **b**runneum* are indistinguishable from each other (Bischoff et al., 2009). During the last two decades, molecular taxonomy and phylogenetic studies have explored different nuclear loci that reflect phylogenetic affinities among Metarhizium species (Driver et al., 2000; Pantou et al., 2003; Bischoff et al., 2009; Kepler and Rehner, 2013). Among these genetic markers, the 5'-intron-rich portion of the translation elongation factor one alpha (5TEF) is now used as a standard marker for species identification (Brunner-Mendoza et al., 2017; Rehner and Kepler, 2017; Moussa et al., 2021). To increase the resolution of phylogenetic studies in Metarhizium species, (Kepler and Rehner, 2013) selected new intergenic nuclear regions that displayed intraspecific nucleotide variability. However, species identification with

these genetic markers involves sequencing and phylogenetic analysis. A faster and more economical identification is possible using species-specific primers. In fact, Mayerhofer et al., (2019) used the intergenic regions found by Kepler and Rehner (2013) to find species-specific PCR primers for the PARB clade species. However, a fast isolate-specific identification of an isolate within the same Metarhizium species is not yet possible.

A low-cost and fast method to assess genetic diversity among organisms is the random amplified polymorphic DNA markers (RAPDs). RAPD markers can be used without prior DNA sequence information and are efficient in detecting intraspecific genetic differences (Bidochka and Small, 2005). For example, they have been used to assess the genetic diversity of Chilean M. anisopliae isolates (Becerra Velásquez et al., 2007). Nevertheless, they have fallen into disuse because they have low reproducibility. However, RAPDs can be used to develop high reproducible markers: isolate-specific amplicons derived from RAPDs bands can be used to develop 'Sequence-Characterised Amplified Regions' (SCAR) markers (Paran and Michelmore, 1993). These markers are quick and easy to use, since they involve a single PCR reaction, and they have a high reproducibility. If a specific band is found in one specific isolate, it can be used to develop isolate-specific primers. Developing SCAR markers normally involve a step of cloning the PCR product (Paran and Michelmore, 1993), which increases the time and cost of the method. Nevertheless, (Hernández et al., 1999) proposed a direct sequencing approach that is possible using combination of random primer pairs, to generate a band flanked by two different oligonucleotides.

We wanted to characterise a collection of *M. brunneum* strains isolated from crop and grassland soils, and specifically, we aimed to develop an isolate-specific PCR assay for *M. bruneum* isolate GC2.

Materials and Methods

Source of Metarhizium strains

Thirty-three *M. brunneum* isolates used in this study were sampled from soil samples in crop (51°33'58.3"N 10°04'10.1"E) and grassland (51°34'10.7"N 10°03'54.1"E) fields near Waake, Germany, all within a radius of 1 km (Table 3). Other *M. brunneum* isolates and Metarhizium species for the RAPD marker analysis and primer testing were obtained from the Westerdijk Fungal Biodiversity Institute, the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH DSMZ, and directly from different European research groups (Table 1).

Table 1. List of external fungal isolates used for the initial RAPD screening and primer testing

Strain	Species	Reference	Country origin
CBS[1] 257.90 [T]	M. pingshaense	Bischoff 2009	China
DSM[2] 1490/ ATCC 38630	M. anisopliae	Schoenian et al., 2010	USA, Oklahoma
DSM[3] 21704	M. anisopliae	Keyser et al., 2015a	Germany
KVL[4] 12-35	M. robertsii	Steinwender et al., 2014a	Denmark
KVL[4] 12-37	M. brunneum	Steinwender et al., 2014a	Denmark
BIPESCO5[5]/F52	M. brunneum		Innsbruck, Austria
ART 2824[6]	M. brunneum		

[1] Westerdijk Fungal Biodiversity Institute
[2] Plant Pathology and Crop Protection Collection, University Göttingen
[3] Deutsche Sammlung von Mikroorganismen und Zellkulturen
[4] Department of Plant and Environmental Sciences, University of Copenhagen
[5] Institut für Mikrobiologie, Leopold-Franzens Universität, Innsbruck
[6] Agroscope, Switzerland

Genomic DNA extraction

Fungal mycelium was grown in liquid culture from single spore colonies in potato dextrose broth (PDB) at 23 °C and 130 rpm for 10 days. DNA was extracted from 50 mg of freeze-dried and milled fungal mycelia (Zirbus VaCo freeze dryer; mixer mill Retsch MM 200, Retsch GmbH, Haan, Germany) with the CTAB extraction method previously described (Brandfass and Karlovsky, 2006). The quality and quantity of DNA was assessed by agarose electrophoresis.

PCR amplification and phylogenetic analysis

Phylogenetic analysis was performed using the 5' intron rich portion of the translation elongation factor one alpha (5TEF), and the MzIGS2 region, located between the pre-rRNA processing protein ipi1 and the DEAD / DAH box RNA helicase. Both Loci have been used for routine identification (5TEF) and phylogenetic analysis of Metarhizium species in the PARB clade, which includes *M. brunneum* (Bischoff et al., 2009; Mayerhofer et al., 2015; Rehner and Kepler, 2017). The sequences of the 33 isolates were submitted to GenBank, and their accession numbers are in Table 3. We also included in the analysis 51 sequences comprising 11 different Metarhizium species and included 30 *M. brunneum* isolates from previous phylogenetic studies (Table 2) (Kepler and Rehner, 2013; Rehner and Kepler, 2017; Mayerhofer et al., 2019). The primers used to amplify the regions of the local isolates are listed in Table 4. PCR amplification reaction mixtures for both regions consisted of 1x PCR ThermoPol® standard buffer (New England Biolabs GmbH, Frankfurt am Main, Germany (NEB)), 2.5 mM of $MgCl_2$, 200 µM dNTP, 0.2 µM of each primer, and 0.03 U/µl HotStart Taq polymerase (NEB). The PCR conditions started with an initial denaturation for 30 s at 95 °C, followed by 30 cycles of a 30 s at 95 °C, 30 s at 58 °C and 1 min at 68 °C, and a final incubation of 5 min at 68 °C. The PCR products were resolved on a 2% agarose gel stained with MIDORI Green Advance

(Nippon Genetics Europe). PCR products were purified by precipitation on isopropyl alcohol, and the amplicons were sequenced with primers used for amplification by Macrogen Inc. (Amsterdam, The Netherlands). Sequences were assembled and manually edited using BioEdit software (Hall, 1999). Sequences were aligned with the MUSCLE algorithm (Edgar, 2004) implemented in MEGAX (Version 10.0.5, (Kumar et al., 2018)) and then manually edited. Alignment lengths for each locus were 1058 for MzIGS2 and 761 for 5TEF. Alignments were concatenated with Mesquite 3.7 (Maddison and Maddison, 2021). The best partitioning scheme and evolutionary models for two predefined partitions (5TEF and MzIGS2) were selected using PartitionFinder2 (Lanfear et al., 2017), with greedy algorithm and corrected Akaike information criterion (AICc). Maximum likelihood phylogenetic analyses for single and concatenated dataset were performed using IQ-TREE (Nguyen et al., 2015) under Edge-linked partition model for 10000 ultrafast (Minh et al., 2013) bootstraps, as well as the Shimodaira–Hasegawa–like approximate likelihood-ratio test (Guindon et al., 2010).

Table 2. GenBank accession numbers of *Metarhizium* spp. strains used for the phylogenetic analysis.

		NCBI GenBank accession number	
Strain	**Species**	**TEF**	**MzIGS2**
ARSEF 324	M. acridum	EU248844.1	KX555241
ARSEF 7486*	M. acridum	EU248845.1	KC164543
ARSEF 7487*	M. anisopliae	DQ463996.2	KC164549.1
ARSEF 7450	M. anisopliae	EU248852.1	KX555249
ARSEF 4556	M. brunneum	CP058936.1	CP058936.1
ARSEF 4152	M. brunneum	EU248853.1	KX342425.1
ARSEF 4179	M. brunneum	EU248854.1	KX555250
ARSEF:2107	M. brunneum	EU248855.1	
ARSEF 3826	M. brunneum	EU248874.1	KX342437.1
ARSEF 5198	M. brunneum	EU248876.1	KX555251
ARSEF 6120	M. brunneum	EU248880.1	KX555252
ARSEF 6477	M. brunneum	EU248885.1	KX555253
ART 2825	M. brunneum	KR706488.1	MH605004.1
BIPESCO5	M. brunneum	KR706489.1	
ARSEF 472	M. brunneum	KX342754.1	KX342422.1
ARSEF 8515	M. brunneum	KX342755.1	KX342423.1
ARSEF 4164	M. brunneum	KX342756.1	KX342424.1
ARSEF 988	M. brunneum	KX342758.1	KX342426.1
ARSEF 3295	M. brunneum	KX342759.1	KX342427.1
ARSEF 6392	M. brunneum	KX342760.1	KX342428.1
ARSEF 4125	M. brunneum	KX342761.1	KX342429.1
ARSEF 1116	M. brunneum	KX342762.1	KX342430.1
ARSEF 1066	M. brunneum	KX342763.1	KX342431.1
ARSEF 8534	M. brunneum	KX342764.1	KX342432.1
ARSEF 5625	M. brunneum	KX342765.1	KX342433.1
ARSEF 1187	M. brunneum	KX342767.1	KX342435.1

		NCBI GenBank accession number	
Strain	**Species**	**TEF**	**MzIGS2**
ARSEF 4131	M. brunneum	KX342768.1	KX342436.1
ARSEF 4168	M. brunneum	KX342770.1	KX342438.1
ARSEF 7434	M. brunneum	KX342771.1	KX342439.1
ARSEF 1278	M. brunneum	KX342772.1	KX342440.1
ARSEF 2042	M. brunneum	KX342773.1	KX342441.1
ARSEF 2224	M. brunneum	KX342774.1	KX342442.1
ARSEF 2210	M. brunneum	KX342775.1	KX342443.1
ARSEF 2107*	M. brunneum	EU248855.1	KC164547.1
ARSEF 2133 / CBS218.56*	M. flavoviridae	KJ398787.1	NA
Mf98SSg	M. flavoviridae	KR706493.1	
ARSEF 4124*	M. frigidum	DQ463978.1	NA
ARSEF 2596*	M. globosum	na	KC164544
ARSEF 7507	M. guizhouense	EU248858.1	KU980454
CBS:258.90	M. guizhouense	EU248862.1	KX555259.1
ARSEF 7412	M. lepidiotae	EU248864.1	KC164551
ARSEF 7488*	M. lepidiotae	EU248865.1	KX342446.1
ARSEF 1946	M. majus	EU248867.1	KU980428
ARSEF 1914*	M. majus	KJ398801.1	KC164546.1
CBS 257.90 *	M. pinghaense	EU248850.1	KC164550.1
CBS:257.90	M. pingshaense	EU248850.1	
ARSEF 4342	M. pingshaense	EU248851.1	KX555256
ARSEF 727	M. robertsii	DQ463994.2	KC164548
ARSEF 2575*	M. robertsii	KR706486.1	MH605000.1
ARSEF 6472	M. robertsii	KX342740.1	KX342408

* ex-type culture

Table 3. Local *Metarhizium brunneum* isolates and GenBank accession numbers used in phylogenetic analysis

	NCBI GENBANK ACCESSION NUMBER	
ISOLATE	**TEF1**	**MZIGS2**
CA8 I	OQ282524	OQ283591
CA8 II	OQ282525	OQ283592
CA14	OQ282519	OQ283593
CA18 I	OQ282520	OQ283594
CA18 II	OQ282521	OQ283595
CA20-1	OQ282522	OQ283596
CA20-2	OQ282523	OQ283597
CB13 I	OQ282526	OQ283598
CB13 II	OQ282527	OQ283599
CB13 III	OQ282528	OQ283600
CB13 IV	OQ282529	OQ283601
CB15 II	OQ282530	OQ283602
CB15 IV	OQ282532	OQ283603
CB16 I	OQ282533	OQ283604
CB16 II	OQ282534	OQ283605
CB16 III	OQ282535	OQ283606
CB16 IV	OQ282536	OQ283607
CB16 V	OQ282537	OQ283608
CB17-B	OQ282538	OQ283609
CB19 I	OQ282539	OQ283610
CC5	OQ282540	OQ283611
CD4	OQ282541	OQ283612
GA3 I	OQ282542	OQ283613
GA3 II	OQ282543	OQ283614
GA4	OQ282544	OQ283615
GC1 I	OQ282546	OQ283617
GC2 I	OQ282548	OQ283618
GC2 II	OQ282549	OQ283619
GC10 I	OQ282545	OQ283616
GC20	OQ282547	OQ283620
GD2	OQ282552	OQ283621
GD12 I	OQ282550	OQ283622
GD12 II	OQ282551	OQ283623

Table 4. List of primers used for PCR amplification of selected genomic regions

Primer name	Direction	Region	Sequence
MZ_IGS2_2F	Forward	MzIGS2	CTGCAATGGATACACAAGATCTGCTCG
MZ_IGS2_3R	Reverse	MzIGS2	CCAGGCACCTGGGGCTGC
EF1T	Forward	5TEF	ATGGGTAAGGARGACAAGAC
EFjmetaR	Reverse	5TEF	TGCTCACGRGTCTGGCCATCCTT

RAPD analysis and PCR conditions

To test the feasibility of developing strain-specific PCR primers, four of the local isolates of *M. brunneum* were used: two isolates from grassland soils (Gc1I, Gc2II) and two from crop field soils (Ca8II, Cb16III). In addition, two external isolates of *M. brunneum* (KVL 12-37 and ART 2825, kindly provided by Prof. Dr Nicolai V. Meyling, Department of Plant and Environmental Sciences, University of Copenhagen and Agroscope) were included. DNA amplification was performed using pairwise combinations of 10-nucleotide random primers. These primers were selected as they produced polymorphic amplicons for Metarhizium species in previous studies (Fegan et al., 2009) (F06, F08, F10, H02, CK06, CK09, Ck12). Five additional random primers were included in the assessment (OPA3, OPA8, OPA11, OPB05, OPC18, Table 5). The PCR reaction mixture consisted of 10-20 ng of fungal DNA added to a 25 µl total reaction mixture containing 1x PCR ThermoPol® buffer (NEB), 4 mM of $MgCl_2$, 200 µM dNTP, 0.3 µM of each primer and 0.03 U/µl Taq polymerase (NEB). The PCR cycling conditions consisted of an initial denaturation for 30 s at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 37 °C and 30 s at 68 °C, with a final incubation of 5 min at 68 °C.

Table 5.Primers used for RAPD Analysis

Primer	Sequence	Reference
F06	GCCAATTCGG	(Fegan et al., 2009)
F08	GGGATATCGG	
F10	GGAAGCTTGG	
H02	TCGGACGTGA	
CK06	GCTTCGATACG	
CK09	TCACGATGCA	
CKl2	CGACGTTCAA	
OPA3	AGTCAGCCAC	(Becerra Velásquez et al., 2007)
Opa8	GTGACGTAGG	
Opa11	CAATCGCCGT	
OPB05	TGCGCCCTTC	
OpC18	TGAGTGGGTG	

A total of 66 PCR reactions were run with the different primer combinations. Five µl of each PCR product was resolved on a 2% agarose gel stained with MIDORI Green Advance (Nippon Genetics Europe). The gels were documented with a digital imaging system (Vilber Lourmat, Marne la Vallee, France), and images were visually inspected to detect bands

exclusive to one of the isolates. DNA from random amplified fragments exclusive to an isolate was recovered by running the remainder of the RAPD PCR reaction (±20 µl) in a 2% agarose gel stained with MIDORI Green Advance (Nippon Genetics EUROPE). The bands were briefly visualised under ultraviolet (UV) light and excised, and DNA was extracted from the band with the Prep-A-Gene kit (Bio-Rad Laboratories, Richmond, CA). To verify that the band amplified was flanked by both primers used, and not by only one of them, three different PCR reactions were run: one containing 0.3 µM of each oligonucleotide and two with 0.6 µM of each primer separately (see Hernández et al., (1999)). PCR conditions were as described for the RAPD markers, but the annealing temperature was increased. to 45 ° C and only 30 cycles. The amplification products were separated by electrophoresis on 2% agarose gels stained with MIDORI green. The amplicons exclusively amplified by the two primers (flanked by the two primers) were purified by isopropanol precipitation and sent to sequencing with each of the primers (Macrogen Inc. (Amsterdam, The Netherlands).

SCAR primer design and specific PCR amplification

The sequences from the different products were submitted for homology searches to candidate orthologues by the BLASTN 2.8.0 algorithm optimised for somewhat similar sequences (Altschul et al., 1990). Sequences without homologs from other *Metarhizium spp*. were identified as strain-specific to the respective isolate. The primers were designed with the program PRIMER3 (Koressaar et al., 2018) (http://primer3.ut.ee/). All primers were designed to have a similar melting temperature (~59 °C Tm). The annealing temperature was set 3 °C lower than the base stack Tm. Amplification reactions were carried out in 20 µl containing 10 to 20 ng of fungal DNA from six different isolates of *M. brunneum,* including BIPESCO5, and isolates from 4 other species of Metarhizium (see Table 1). The reaction mixture included 1x PCR ThermoPol® standard buffer (NEB), 2.5 mM of $MgCl_2$, 200 µM dNTP, 0.2 µM of each primer and 0.03 U/µl HotStart Taq polymerase (NEB). The PCR conditions started with an initial denaturation for 30 s at 95 °C, followed by 30 reaction cycles consisting of a 30 s denaturation step at 95 °C, an annealing step at 62 °C for 30 s, and 40 s at 68 °C. The final elongation was performed for 5 min at 68 °C. The amplicons were visualised in a 2% agarose gel electrophoresis as described above. Primer specificity was first tested in five isolates and, if they were specific to the targeted isolate, they were tested in all isolates of the collection.

Results

Phylogenetic analysis

Individual Maximum Likelihood (ML) analysis of the aligned 5TEF and MyIGS2 gave a tree topology similar to that of the concatenated alignment (Figures 1, 2 and 3). The isolates in the collection belong to *M. brunneum* and cluster into three distinct groups within the *M. brunneum* clade. All isolates collected from “crop” soils and eight from “grassland” soils clustered in one clade with 98% bootstrap support. Isolate GC10I clustered with isolate ART 2825 with 100% bootstrap support, and Gc2I and Gc2II grouped in another cluster with 99% bootstrap support.

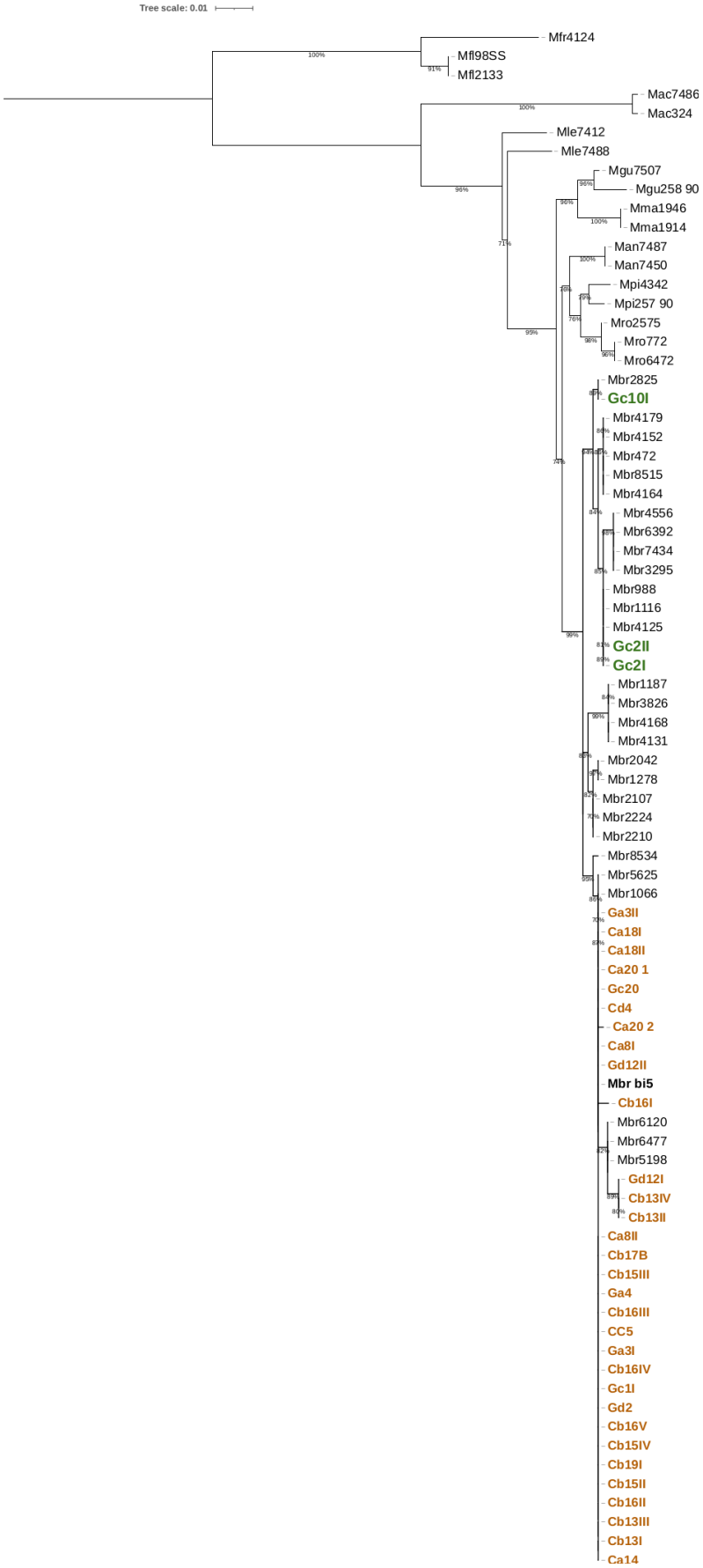


Figure 1. Maximum likelihood phylogeny of Metarhizium species and 30 isolates based on analysis of 5TEF region. Bootstrap values higher than 70% are shown below each node.

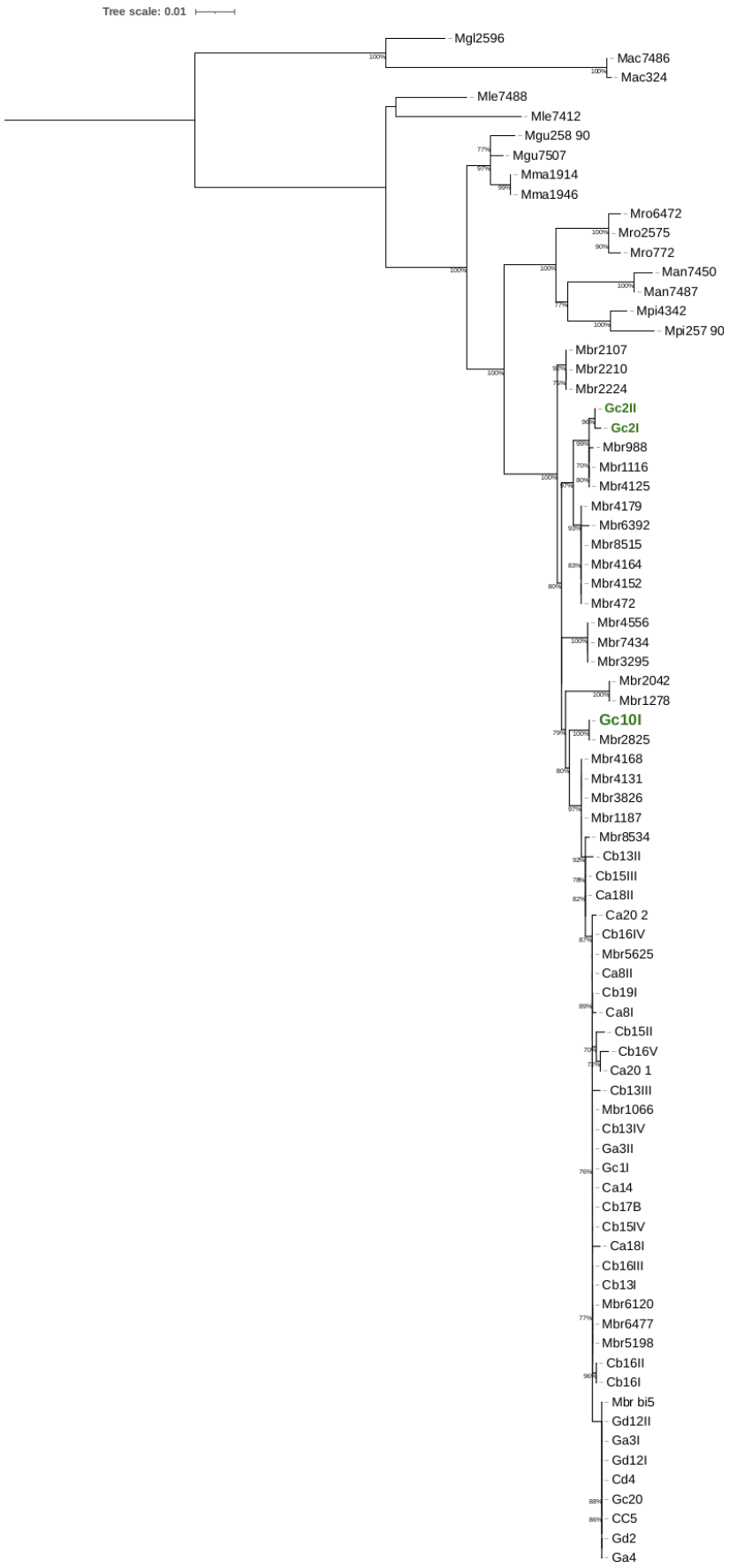


Figure 2. Maximum likelihood phylogeny of Metarhizium species and 30 isolates based on analysis of MzIGS2 region. Bootstrap values higher than 70% are shown below each node.

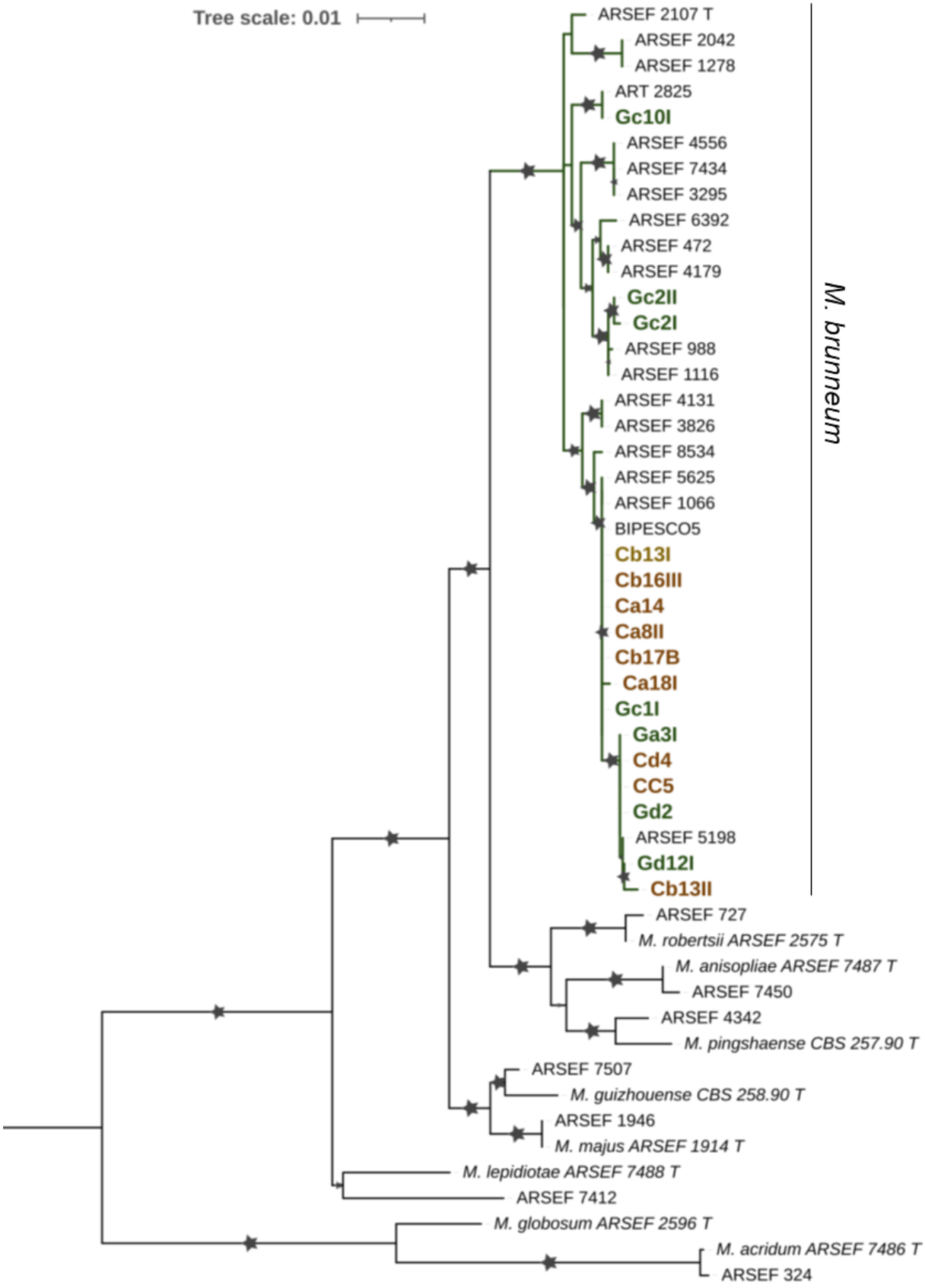


Figure 3. Maximum likelihood phylogeny of Metarhizium species and isolates based on analysis of a concatenated dataset of 5TEF-MzIGS2. Bootstrap values higher than 70% are shown below each node.

RAPD analysis

After visual inspection of the RAPID bands, 14 bands exclusive to only one, two or three isolates were selected (Figure 4a and 4c as an example). The band pattern of isolate Gc2II was different from all other isolates in several random primer combinations; therefore, six of the selected bands are exclusive to this isolate. Of the 14 bands selected, only four rendered specific products when using both primers. We obtained readable sequences from the amplified bands of the primers Ck09 x OPA8 (Figure 4b) and the primers F06 x H02 (Figure 4d). The band product of the amplification with the primers Ck09 x OPA8 was present in the field isolates (Ca8, Cb16, Figure 4a) with an approximate size of 170 bp; with a readable sequenced length of 140 bp. Blastn search resulted in only one hit with 100% identity with a hypothetical protein from *Pochonia chlamydosporia* 170, Genbank reference sequence XM_018289988. I designed four different pairs of primers (see Table 6) using as a template the complete XM_018289988 sequence. The 140-bp fragment only rendered one primer with the specified conditions (Primer3 default). The other primer sequences do not belong to the RAPD band sequence, but all primer pairs were designed to include the 140 bp fragment obtained in the band (Figure 5, sequence highlighted in yellow). Primers were first tested in a PCR with 6 isolates, where the 1L x 1R primer pair was the only one that amplified a band of ~ 370 bp in GC2II and a weak band in ART 2825. The pair of primers was then tested on all isolates DNA and resulted in the amplification of a band with the expected size (~370 bp) in the isolates ART 2025 (faint) GC2I, Gc2II and Gc10I (Figure 6a), plus multiple faint bands of higher size amplified in all isolates tested. It is important to note that the original RAPD band was exclusive to isolates Ca8, Cb16, but the designed primers resulted in specificity for a different set of isolates.

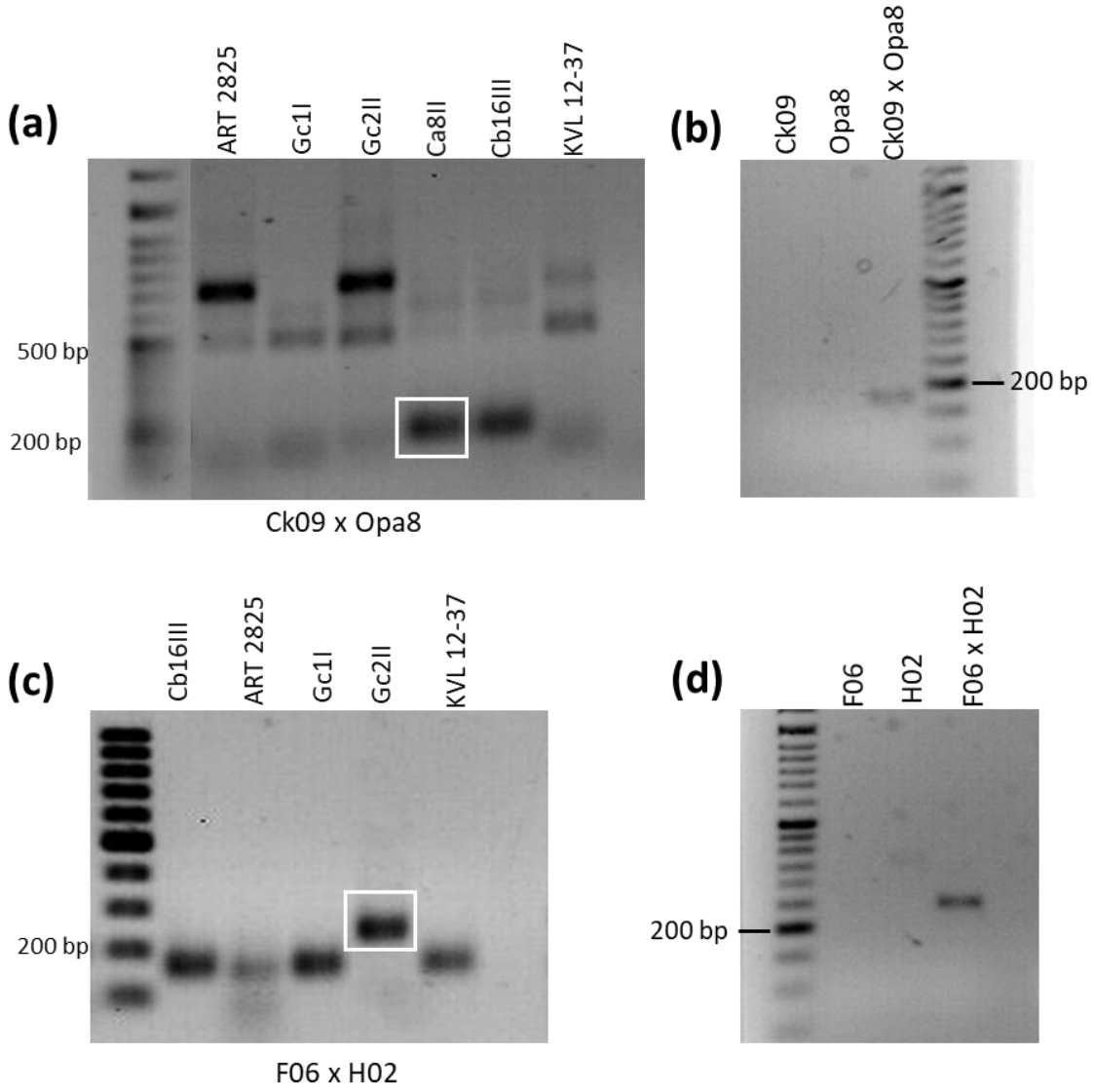


Figure 4. RAPD bands selected for sequencing, and confirmation of the flanking primers. (a) Primer combination Ck09 x OPA8 originated specific fragments for the isolates Ca8II and Cb16III, (b) the amplicon is flanked by the two different oligonucleotides. (c) Primer combination F06 x H02 generated amplicon exclusive to the isolate GC2II; and (d), the amplicon is flanked by the two different oligonucleotides. First lane in (a), (b) and (d): 50 bp marker, and (c): 100 bp marker.

Chapter 1. Fast development of isolate – specific PCR primers

Table 6. List of specific primers designed for the isolate Gc2II, based on exclusive RAPD bands of the isolate. The primers were designed based on the sequence obtained from the band (Location 'I', Gc2F-Gc2R and 2_F), or based on a homologous sequence, GenBank No. XM_018289988, (1-4).

Primer	**Sequence**		**Band size**		**isolates**
Primers Ck09 x Opa8		Location	Expected	Amplified	
1_F	CGGGGCTTCGAATTTCCAAT	556	245	~370	Gc2I, Gc2II, Gc10I, ART2825 (Faint)
1_R	CTTTTATGGTGACGGTGCCC	780			
2_F	TCAAGCTCCCGGACAATACT	I	240	-	-
2_R	CCACGGACACAGCTTCTTTC	O			
3_F	CGTCCCTTTCGCACTCTTG	O	278	-	-
3_R	GCCTCATGCCTCACTTCTTC	O			
1_F **4_R**	TAACTGATACGCGGGCTCAA	O	770	-	-
Primers F06 x H02		Location	Expected	Amplified	
Gc2F	TGGTGCCGACGATTTAATTGT	I	204	~200	Gc2I, Gc2II
GC2R	CAATTCTGGGAGGTGGAAGC	I			

The band product of the amplification with the F06 x H02 was present in GC2II (Figure 4c), with an approximate size of 250 bp, with a readable sequence of 217 bp (Figure 7). Blastn search resulted in a unique hit, 97% similarity to DNA fragment in the genome sequence of the *M. brunneum* strain ARSEF 4556 chromosome 3, bp 1904045 to 1903856 plus/minus, Genbank sequence ID CP058934. The fragment is located in a noncoding region between two hypothetical proteins; 'hypothetical protein G6M90_00g058400" and 'protein containing the CRAL-TRIO domain'. The Gc2F and GC2R primers were designed using the 217 bp sequence as a template. PCR reactions with this primer pair were then run with all isolates and resulted in the amplification of a band of approximately 200 bp, that was the expected size (207 bp) and was exclusive to the isolates Gc2I and Gc2II (see Figure 6b).

>XM_018289988.1 Pochonia chlamydosporia 170 hypothetical protein (VFPPC_11956), partial mRNA
ATGGCTGCGCGGTCAGGAGCGGTACCAAGTCGACGTCTACACCTTCGCATGGCGACAATGCGCGAGAC
AAATTTCAGAAATAAAGCGAATACACTCATGCAAAGAGACACATGTAACTGGTACGAATGGAGCGAGA
GAATCGCCTCACTAGGAGACCTACTAGGATACGTCCTACAGTGTTCCCCTCAATTTCCTGACAACCTC
AAACTGCTGGATGGCGTAACAGAAGCCGGCGAGATCATTAAGGCATACCAGAAGCACGCTGAAGAACA
TATTATGGTGGAGGAAGAGAAGGAGAGGAAAGAATGTCTCATCAATCTTATCAGGTATTATTTCCTTG
CGCACCTTCGCGTCTTAAAGGAATCTTATAAGCAGCTGTATGGTTCGAAAGCCTACAATAAAAAACTT
AAAGGCACAATCCTCAAGGGATTACAGGAATTCTGGAAATTAACCGGGGCACCGTCCGCGCTTAAATA
CTCTGAAGCGAACGATGTTATTGATTCTGTAGCGTACAGTGATCCGCTAATCTGTATCATTGTATGTC
AAATCGGGGCTCGGGGCTTCGAATTTCCAATTCACTACCGTCCCTTTCGCACTCTTGGCAGGGACTTC
AGACATGAGGACAACCAAGAGAAGACCCTCTTACAGCTCGGTCGGCGGTTCAAGCTCCCGGACAATAC
TAACATTCAACGCTGGGATTCTGACGCAGCCTCAGCAAGTAGGATTGTGGAGGCCACTCTCGGATACC
GCTTAAGTCGGAAAGACATCCTTTACTTCCTGGGGCACCGTCACCATAAAAGTCTTGAGCCCGCGTAT
CAGTTAATACATCTTGGTGAAGTCGAAGAAGTGAGGCATGAGGCTGTGGTATCGGCGCTTACCTTGAA
AGAAGCTGTGTCCGTGGGCTGTGGTACCCAAGGCGACTCTTCCGGCCAGACAGGTGGGAAACTCCACT
CATTCAGATTCACCAATGAATGCATGGCCTACTCAAACTTCTGCAATGACAACTTGGTTGTGTCACTG
AATCAAAGGTTGACACTCAAACGCCAAGAACCTTGGGGAAAATTTGTAGCCCGACTTGACGCGGACGC
ATGCCTATCGCCCACCGAGGGGTATATAAATGTTTTGCTCCCGCTGTTCGATGAGACTCAGATTATGA
AGCTCCAGGCGCCGAACTCGGGAGAGAAAGTAGTGTTCTCGTGGCCTCCAGACATGGCATTATGGTTT
CACAAATATGACCTTGAGTTACCGGAAGGTAAACTCCGTTATGTGCATCTACTTTACGAGTCTGATGC
AGTAGAAGATAAATCCGGCCAACGAACGGAACAGAGTCACAAAGATACCAGCGCATCAGGATACGGAC
CTTTTTGCATGCCCAAACACTCCGTGAGTCTACACCCAGATCTATTCATGTCTTCAGTATCCCCTGTC
TAA

Figure 5. GenBank sequence XM_018289988 used to design the intraspecific primers 1_F, 1_R. The sequence of the RAPD band Ck09 x Opa8 is coloured yellow. The primer search was limited to the sequence in blue. The locations of Primers 1_F and 1_F are highlighted in green.

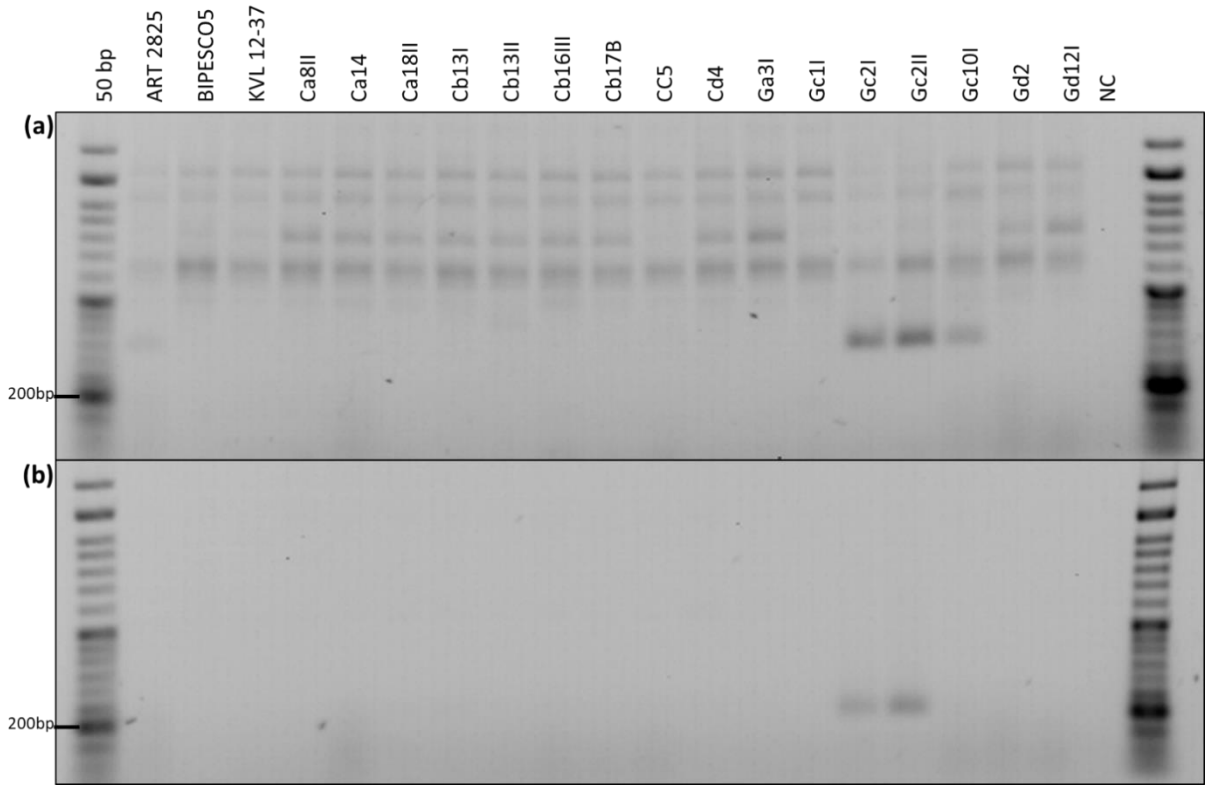


Figure 6. Isolate-specific PCR with primer pairs developed from the RAPD band (a) Ck09 x OPA8; and (b) F06 x H02 tested on different isolates of *M. brunneum.*

> F06 x H02
CGGCAATGCAATGGTGCCGACGATTTAATTGTTTGGTCCACCGAGATGCCGTCTGTCAGATGATAATG
CATAATTTCCAAGTGTGCATTTTCAAGATGCCCGATGCGCGTATCGGACCAACGCATCTCAAGAGAAC
TACGAAGTGGTGCGTATGTACGTCGCGAACTTGCCCCATGGTTATTGATAATAACTTAGGCTTCCACC
TCCCAGAATTGGC

Figure 7. Sequence from band exclusive to Gc2II in combination of primers F06 x HO2. In red are the designed locations of the Gc2 primers.

Discussion

Intraspecific and rapid identification of fungal isolates is a useful tool to follow the fate of specific isolates in field studies. This is even more necessary for Metarhizium species of the PARB clade, which are morphologically indistinguishable. (Bischoff et al., 2009). Here we phylogenetically characterised a local collection of Metarhizium isolates, all belonging to the species *M. brunneum*. The majority of isolates are closely related and belong to one intraspecific clade, with only three isolates belonging to a different clade. In a short time, we were able to develop intraspecific PCR primers in an economically feasible way. Although the primers cannot distinguish a single isolate, they are able to distinguish *M. brunneum* species within an intraspecific clade, thus increasing the resolution of identification.

Metarhizium brunneum has a strongly supported monophyletic origin (Bischoff et al., 2009; Kepler et al., 2014; Rehner and Kepler, 2017) but is phylogenetically diverse, so that

Rehner and Kepler (2017) have suggested that there could even represent seven distinct species. For the isolate analyses in this study, we selected the 5TEF region, which has become a standard for the identification of Metarhizium species, and the MzIGS2 region, which has the highest informative parsimony characters for *M. brunneum* (Rehner and Kepler, 2017). The maximum likelihood phylogenies obtained are consistent with previous phylogenetic studies of Metarhizium species (Bischoff et al., 2009; Kepler et al., 2014; Rehner and Kepler, 2017). *Metarhizium brunneum* forms a clade with 100% bootstrap support; and the isolates are distributed among several well supported terminal clades (> 70% bootstrap support). Of the 33 isolates analysed, 30 aggregate in one clade, some of them having identical sequences of these two regions analysed. Several of these isolates could be identical since they were collected in the same crop field (C). Only three grassland soil isolates cluster in a different clade; two of them are probably identical (Gc2I and Gc2II), and Gc10, which group together with ART 2825. The phylogenetic distance between these three isolates and the remaining ones analysed was reflected in the RAPD marker bands and explains why we obtained at least six unique bands for the isolate GC2II. We also detected unique and differential bands for GC1I, Ca8II and Cb16II, isolates that are closely related, but the two primers used for amplification did not flank the PCR products, and therefore we did not sequence them.

RADP markers are economical but are no longer used due to their low reproducibility. Nevertheless, they are inexpensive and useful tools for the development of SCARS markers and, as we show here, intraspecific primers. It is interesting to note that the RAPD bands used to develop the primers (1L-1R) were originally found in isolates Ca8II and Cb16III. However, we designed the primers based on the sequence we found in Genbank, *Pochonia chlamydosporia*, and the sequence of the primers was not part of the original sequenced region. These primers amplified a band in isolates of the Gc2II clade, suggesting that isolates CA8II and Cb16II do not have homologous sequences to the primes we designed. On the other hand, the Ck09 x OPA8 primers also amplified a band of ~700 bp in ART 2825 and Gc2II, as seen in Figure 4a. This band was not sequenced but indicates that these oligonucleotides may have amplified a segment in the same region, but longer, with sequence differences sufficient to be detected with the designed primers. *Pochonia chlamydosporia* is a species closely related to Metarhizium, and both belong to the 'Metacordyceps' clade (Kepler et al., 2014). The primers based on the unique sequence had a predicted length of 245 bp but rendered a fragment of ~ 370 bp. The primers developed are not exclusive to the isolate but may target isolates included in a big cluster that includes ART 2825, Gc10I and Gc2II (Figure 3).

The sequence of the RAPD band used to design the second pair of primers (Gc2F-Gc2R) gave a unique hit in the Blastn search and is from the genome sequence of *M. brunneum* isolate ARSEF 4556. Looking at the phylogenetic tree (Figure 3), this isolate forms a cluster with Gc2II and Gc2II with high bootstrap support (92%). The primers designed on the basis of this sequence are specific for GC2 (I and II) isolates from our collection.

We limited the initial screening with RAPD markers to five or six isolates only to make the process faster and more economic, but by doing this, we excluded the possibility of finding more isolate-specific primers. For example, we did not include Gc10I in the initial screening,

and the first primer pair did not differentiate this isolate. One key point of the process was the selection of the oligonucleotide primers to generate the RAPD bands. We selected primers based on published studies, where these primers have been proven to render informative bands in Metarhizium species. Since this technique has been widely used in the last decades, there are many studies that can be consulted to choose appropriate primers for different phylogenetic groups. Our approach was successful in rendering two different primers with different specificities in a short time, with equipment available to many laboratories.

Chapter 2.

Endophytic colonization of potato and oilseed rape plants by strains of *Metarhizium brunneum*: optimization of detection

Abstract

Several entomopathogenic fungi have been identified as endophytes in plants, with the ability to enhance host resistance against herbivorous insects and plant pathogens. Recent research has highlighted these beneficial effects in different plant and herbivore systems, stimulating interest in exploring the potential of endophytic entomopathogenic fungi (EEFs) for sustainable crop protection. Species within the genus Metarhizium, in particular, have shown promising properties; however, the efficacy of these fungi depends on their ability to associate with plant roots, as well as the specific fungal strain and plant species involved.

This first chapter of the Ph.D. thesis aims to achieve two main objectives. First, to optimize the quantification of fungal biomass in root tissues using the quantitative polymerase chain reaction (qPCR) to provide a reliable method for assessing fungal association with plant roots. Secondly, to identify a combination of crop-EEF with high potential for agricultural applications, allowing further investigation of their mode of action.

First, we evaluated the colonisation ability of five different *Metarhizium brunneum* isolates in oilseed rape (*Brassica napus* cv. Marathon) and potato (*Solanum tuberosum* cv Linda). The plants were inoculated by drenching the soil with a solution containing 10 ml of $1x10^7$ spores × ml^{-1}. After three weeks, we evaluated fungal colonisation by quantifying fungal DNA in roots, shoots, and leaf tissues using qPCR.

The results showed that the five fungal isolates successfully colonised both plant sppecies, although variations were observed depending on the specific plant and isolate. In oilseed rape plants, all isolates were detected in the roots and stems of most plants (70 to 100% of plants), and fungal DNA was also found in the leaves of 10 to 30% of the inoculated plants. In contrast, colonisation of potato plants was mainly restricted to the roots, except for one isolate, while certain isolates showed a lower colonisation frequency (40%-50% of plants).

In conclusion, qPCR proved to be an effective method for the quantitative detection of fungal DNA in plant tissues, although optimisation was necessary for each plant species. Furthermore, all *M. brunneum* isolates showed colonisation ability in both potato and oilseed rape plants, with higher fungal biomass and greater colonisation of the shoots and leaves in oilseed rape. Isolate-specific differences in plant colonisation were also observed.

Introduction

Entomopathogenic fungi such as *Metarhizium* sp. or *Beauveria* sp. (Ascomycota, Hypocreales: Claivicipitaceae) are well-known insect pathogens that can also associate with plants as endophytes. Research published in the last 30 years has shown that endophytic entomopathogenic fungi (EEF) can therefore increase host resistance to herbivorous insects (Fernandez-Conradi et al., 2018) and plant diseases (Ownley et al., 2010). Metarhizium species also proliferate in the plant rhizosphere and colonise root tissues as endophyte (Hu and Leger, 2002; Bruck, 2005; St. Leger, 2008; Sasan and Bidochka, 2012). For example, a survey of several plant species collected in southern Ontario found 730 Metarhizium isolates

in 11 different plant species. From these isolates, a total of 99.2% were found exclusively in roots (Behie et al., 2015). Studies in natural soils found that Metarhizium species form plant-rhizospheric specific associations (Wyrebek et al., 2011). For example, *M. guizhouense* was found almost exclusively in the rhizosphere of trees, while *M. robertsii* in grass roots and *M. brunneum* in shrubs and the rhizosphere of trees. However, recent studies with different species of Metarhizium show that they colonise the rhizosphere and rhizoplane of several dicot and monocot plants (Moonjely and Bidochka, 2019) and studies in agricultural soils do not support plant-rhizospheric specific associations of specific Metarhizium multilocus genotypes (MLG) (Steinwender et al., 2015). Within Metarhizium species*, M. brunneum* appears to be well adapted to the agricultural soil environment (Steinwender et al., 2014; Kepler et al., 2015; Steinwender et al., 2015). For example, it was the most common Metarhizium species (78.8%) in an ecosystem in Denmark, and in roots of oats, rye, and cabbage, the three most common cultivated plants in this country. Interestingly, its genetic diversity in agricultural soils appears to be low. For example, a single clone represented this species in soils from a long-term experimental farm in Maryland, USA (Kepler et al., 2015). Furthermore, in Denmark, a single MLG of *M. brunneum* represented more than 66% of total isolates obtained from roots of oats, rye, and cabbage (Steinwender et al., 2015).

The beneficial effects of Metarhizium on plants appear to depend on its ability to associate with plant roots. For example, *M. robertsii* mutants with a disruption in an adhesin MAD2 protein, that reduced rhizosphere competence (*Δmad2*) and in a rafinose transporter MRT that poorly utilised root exudates (*Δmrt*) had reduced or no beneficial effects on the plant, when compared to the wild type (Liao et al., 2014). In addition, differences in isolate performance within a species have often been reported, in terms of rhizosphere competence (Moonjely and Bidochka, 2019), rhizoplane colonisation (Razinger et al., 2014a; Razinger et al., 2018b) as well as endophytic colonisation (Moonjely and Bidochka, 2019; Rivas-Franco et al., 2020). Strain-specific variability of EEF has also been reported with respect to its virulence against insects (Vänninen et al., 1999a; Razinger et al., 2014b; Vidal and Jaber, 2015; Razinger et al., 2018b) as well as in terms of the physiological response of the plant (Hao et al., 2017; Raad et al., 2019; Rivas-Franco et al., 2019; Rivas-Franco et al., 2020; Qin et al., 2021).

In a previous project of our research group (Agricultural Entomology, University of Göttingen), several strains of *M. brunneum* were isolated from grassland and crop soils (Hettlage, 2018). Standard phylogenetic analysis of Metarhizium species is based on several intergenic nuclear regions, which showed intraspecific nucleotide variability (Kepler and Rehner, 2013). Phylogenetic analysis based on the 5'-intron-rich portion of the translation elongation factor one alpha (5TEF) and MzIGS2 region, located between the pre-rRNA processing protein ipi1 and the DEAD/DEAH box RNA helicase showed that most of these isolates are identical (this thesis, Chapter 2). However, they show morphological colony variability (per. observation) and differ in their aggressiveness towards the yellow mealworm (*Tenebrio molitor*) (Hettlage, 2018).

The prevalence of *M. brunneum* in agricultural soils makes it a good candidate for biological control in crops. Despite the apparent low phylogenetic diversity, I hypothesised that there would be isolate-specific differences in the fungal association with two different crop species. Since the beneficial effects on the plant seemed to depend on the association of Metarhizium with the roots, my first objective was to find a crop plant that showed good association with *M. brunneum*. I chose to assess fungal root association and endophytic colonisation using quantitative PCR, as this is known to be an accurate method for assessing fungal colonisation compared to culture-based methods (Tellenbach et al., 2010; Barelli et al., 2018). However, detection of fungal DNA in plant tissues can be challenging due to PCR inhibitors from the plant matrix remaining in DNA extracts (Nolan et al., 2013). Therefore, the second objective was to optimise the detection of fungal DNA in plant tissues by qPCR. The final goal was to have a plant-*M. brunneum* system and an optimised fungal detection in plant tissues for future studies of Metarhizium–plant-insect and Metarhizium–plant-pathogen interactions.

Materials and methods

Study system

Hybrid winter oilseed rape (OSR) (*Brassica napus* cv “Marathon”) (RAPOOL, Germany) and potato (*Solanum tuberosum*, cv “Linda”), (organic seeds harvest 2017, https://www.kartoffelvielfalt.de/) plants were used. The plants were grown in a greenhouse cabin in a soil consisting of a mixture of commercial soil (Fruhstorfer Erde Typ 25, Hawita Gruppe GmbH), sand and vermiculite (1:1:1, v:v) in a greenhouse with 18-28 °C (the experiments took place in June), supplemented with light to have a photoperiod of 16:8 h (L:D).

The *M. brunneum isolates* were obtained from the internal collection of the Division of Agricultural Entomology (see Table 1). To obtain the suspension of spores for inoculation, the isolates were grown on potato dextrose agar (PDA) (Carl Roth GmbH, Germany) at 23 °C for 14 days. The spores were removed from the hyphae by gently scraping the sporulating colony with a sterile glass slide and suspended in 20 ml of 0.1% Tween® 80 (Carl Roth GmbH, Germany). The conidia suspension was filtered through a plastic gauze and adjusted to a final concentration of 1×10^7 ml^{-1}. Spore viability was assessed prior to each experiment by plating 100 µl of 1 × 103 spores × ml-1 distributed in 10 µl drops in PDA plates and counting the germinating colonies 48 h later.

Table 4**. List of fungal strains used in the study (see Hettlage, (2018))**

Strain	Crop regime	Location
GC1I	Permanent semi-natural grassland for at least 7 years. Grassland harvested twice a year	51°34'10.7"N 10°03'54.1"E
Gc2II		
Ca8II	Winter wheat, barley or oilseed rape crop rotation, conventional farming	51 ° 33'58.3"N 10 ° 04'10.1"E
Cb15III		
Cb16III		

Plant inoculation

Oilseed rape seeds were surfaced sterilized with 70% ethanol for 1 min, 4% sodium hypochlorite (Carl Roth GmbH, Germany) for 1 min, rinsed three times, and then sown in sterile quartz sand. Two weeks after sowing, the seedlings were inoculated by drenching the roots and soil with 10 ml of spore suspension at the time of transplanting into square pots (11 x 11 cm, 1 L) filled with a non-sterile soil mix. The control seedlings were mock inoculated with a solution of 0.1 % Tween® 80. The experimental design consisted of six treatments (five *M. brunneum, one* control) with 10 replicate plants per treatment, randomly distributed in the greenhouse chamber.

I wanted to obtain potato plants that originated from a single bud and inoculate them when the roots had already emerged. Whole potato tubers were surface sterilized by inmersion in a solution of 2% NaOCl and 0.1% Tween 80 for 8 min, 70% ethanol for 10 min, and > three rinses with distilled water. The tubes were air dried and cut into wedges with one axillary bud per wedge (see fig1a). The wedges were placed in sterile moist vermiculite and kept in a growth chamber in the dark (21 °C, 65% RH) for two weeks, until the shoots and roots developed (see Figure 1b).

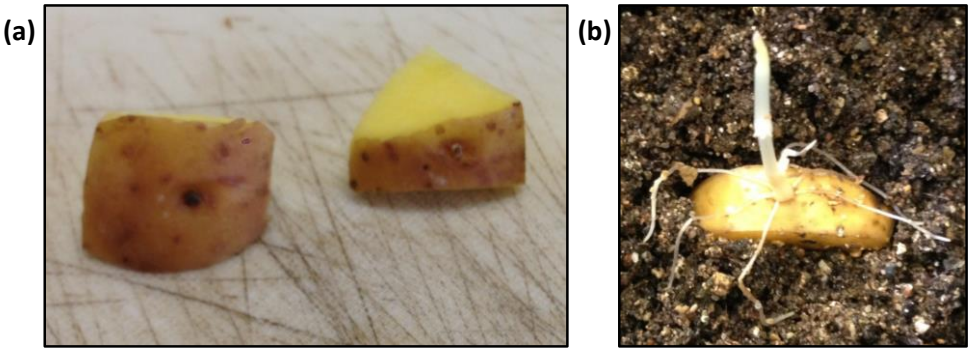


Figure 1. (a) Potato tuber wedges with one axillary bud before germination, and (b) after two weeks inside sterile moist vermiculite.

The germinated potato wedges were placed in a square pot (11 x 11 cm, 1 L) filled to 2/3 of its volume with non-sterile soil mix, inoculated by drenching 10 ml of spore suspension over the wedge, roots and soil surrounding the roots, and covered with more soil substrate, leaving only ~5 mm of the apical bud above the substrate. The experimental design consisted

of two plant species and six fungal treatments (five isolates, one control), and each treatment had 10 biological replicates, each consisting of a single pot containing one plant. Plants and treatments were randomly distributed in the greenhouse cabin.

For both species, the control seedlings were mock inoculated with 10 ml of 0.1% Tween 80. The height of the oilseed rape plants was measured weekly and the plants were harvested 3 weeks after inoculation. For fungal DNA detection, I collected the second and third leafs; a 4 cm section of the stem including 1 cm below the cotyledons (hypocotyl); and the entire root system washed with a high-pressure water jet. Roots were not sterilised since the aim was to measure root-associated fungi.

Height and weight of the potato plant were very variable because each tuber bud produced one or more stems and the stems varied greatly in size. Therefore, I only evaluated fungal colonisation of plant tissues. The plants were harvested three weeks after inoculation and the tissues collected were the third and fourth leaves, a 4 cm section of the stem cut after the first visible leaf, and the complete washed root system.

Samples of both species were frozen at -25 °C for 24 h, lyophilised for 72 h (VaCo 5, Zirbus Technology, Germany), and ground with a mixer mill (Retsch MM 400, Germany) in a stainless steel container with a 20 mm, 32 g steel ball (Retsch, Germany) for 30 s at maximum speed.

Quantification of root-associated and endophytic M. brunneum

Root-associated and endophytic colonisation was measured with real-time quantitative PCR (qPCR). DNA was extracted from 20 µg of powdered tissue described in section (2.2.2), and isolated with the peqGOLD plant DNA mini kit (VWR International, Belgium) according to the manufacturer's instructions, with a modification: 10 µl of proteinase K was added to the lysis buffer PL1, incubated for 10 min at 42 °C, followed by 10 min at 65 °C, after which 15 µl RNAse was added. The DNA quality was verified on agarose (0.8%) gels. The CFX384™ Real-Time System with a C1000™ Thermal Cycler (BioRad, Hercules, CA) was used for fungal DNA amplification and melting curve analysis. The primers used are specific for Metarhizium clade 1; Ma 1763 (CCAACTCCCAACCCCTGTGAAT) and Ma 2097 (AAAACCAGCCTCGCCGAT) (Schneider et al., 2011).

Optimization of M. brunneum detection in plant tissues:

The qPCR conditions were optimised for the detection of Metarhizium DNA in plant tissues using the 2x qPCRBIO SyGreen Low-ROX kit (PCRBIOSYSTEMS). A first test with the specifications of the recommended kit (primer concentration 0.4 µM), and annealing temperature of 62 °C resulted in the detection of a product with a melting peak of high temperature melting peak (~92 °C) that did not correspond to the one generated by the Metarhizium standard (86 °C). Gel electrophoresis showed that potato root samples amplified a smear of DNA of different sizes, indicating non-specific unwanted amplification. In order to increase the fidelity of amplification, a gradient qPCR with different temperatures (58 to 68 °C), and primer concentrations (0.2 to 0.4) was performed. I also evaluated the matrix effect of the

plant root DNA by amplifying a known fungal concentration (0.1 pg) in two different dilutions of DNA from a root control (mock inoculated). The final conditions amplified Metarhizium DNA from OSR root samples. There was still a small peak that formed at 92 °C, but gel electrophoresis confirmed amplification of the band with the expected size (334-339 bp). 5 µl of 2x qPCRBIO SyGreen Low-ROX (PCRBIOSYSTEMS), 0.2 µl of 10 µM of each primer, 1 µl of 1:50 DNA template solution for roots and 1:10 dilution for stem and leaves (showed no matrix effect), completed to a total of 10 µl of final reaction volume. The running conditions started with an initial denaturation for 2 min at 95 °C, followed by 40 reaction cycles consisting of a 5 s denaturation step at 95 °C, a 15 s annealing step at 66 °C, and a 10 s extension at 72 °C. The final elongation was carried out at 72 °C for 5 min. Melting curves were obtained by heating the samples to 95 °C for 60 s and cooling them to 55 °C for 60 s followed by a temperature increase from 55 °C to 95 °C by 0. 5 °C per cycle with continuous fluorescence measurement. Absolute fungal DNA g^{-1} of plant tissue was measured by comparing threshold cycle (Ct) values with DNA standards starting with a concentration of 100 pg $\times$ μl^{-1} and decreasing with a 1 : 3 dilution factor. Threshold cycle and standard curves were generated using the Bio-Rad CFX Maestro software. When a double peak was observed, the identity of the amplicon was verified by gel electrophoresis.

Data Analysis

Data exploration and statistical analyses were performed with R 4.0.3 (*R* Core Team, 2017). The biomass of oilseed rape leaves, shoots, and roots was analysed using a linear model (stats package), and the distributions were evaluated by checking Q-Q plots, histograms, and using the functions shapiro.test. The DNA values were logarithmically transformed to meet the assumptions of the linear model. When a model was significant, I used the Tukey honest significance *post hoc* test (Library multcomp (Hothorn et al., 2008)). Pearson's test and figure were created using ggscatter (ggpubr package).

Results

Optimization of fungal detection in root tissues by qPCR

The first qPCR evaluation of potato root samples using the parameters recommended by the manufacturer (PCR Biosystems), and the annealing temperature reported for the primers (61 °C (Schneider et al., 2011)) did not detect any fungal DNA. However, there was a product with a melting peak of high temperature-melting peak (~92 °C) that did not correspond to the melting peak of the Metarhizium standard (86 °C). Gel electrophoresis showed that potato root samples amplified a smear of DNA of different sizes, indicating nonspecific amplifications (Figure 2a). Fungal detection in oil seed rape root DNA extracts was also possible with the recommended settings of the kit, but the melting curve analysis revealed a double peak formation; one at the expected temperature (86 °C) and the second at ~92 °C.

The melting curve analyses of the three primer concentrations and the temperature gradient showed that the 0.2 µM primer per reaction and an annealing temperature of 66 °C resulted in positive detection of the Metarhizium specific band and reduced nonspecific

amplification as seen in Figure. 2b. However, there were still unwanted amplifications, evidenced in the gel as a grey smear above the Metarhizium band and in the melting curve as a second peak at higher temperature. (~91 °C)

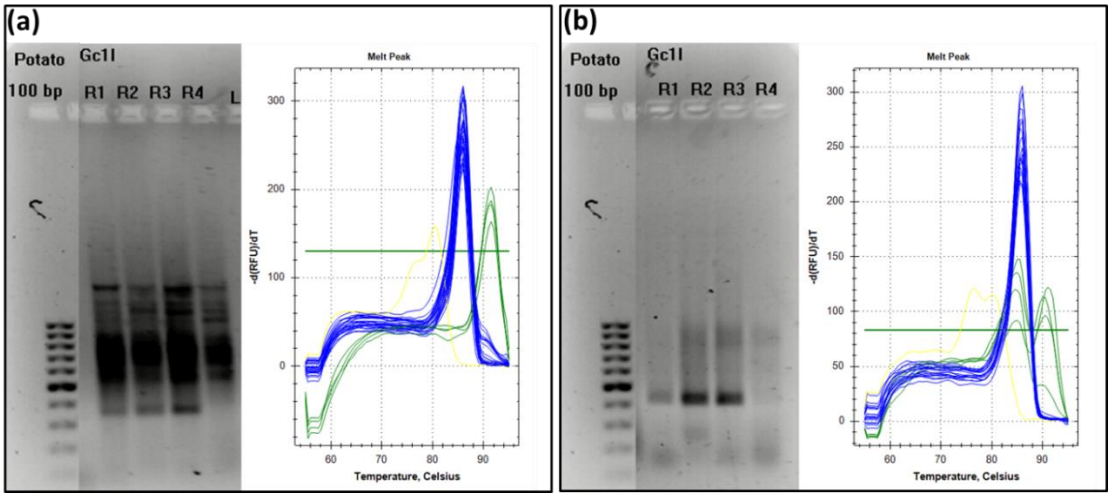


Figure 2. Gel electrophoresis and melting peak curves from qPCR products in potato root samples (a) with recommended kit settings (PCR biosystem) and (b) with optimised settings. Yellow: negative control (water); blue: *M. brunneum* standards; green: root samples.

To further optimise fungal DNA detection in potato root tissues, I evaluated the matrix effect of plant DNA. When 0.1 pg of fungal DNA was spiked with a 1:10 dilution of DNA from mock inoculated root tissue, a small double peak formed at ~90 °C (Figure. 3a). The 1:10 dilution without fungal DNA also produced the same high melting peak. When the fungal DNA was in a 1:50 root DNA dilution, the second peak was considerably reduced. Similarly, DNA from inoculated root samples diluted to 1:10 produced a double peak that was below the detection line, while the 1:50 dilution produced a detectable peak at the right temperature (86 °C), and the second peak was almost not existent (Figure.3b)

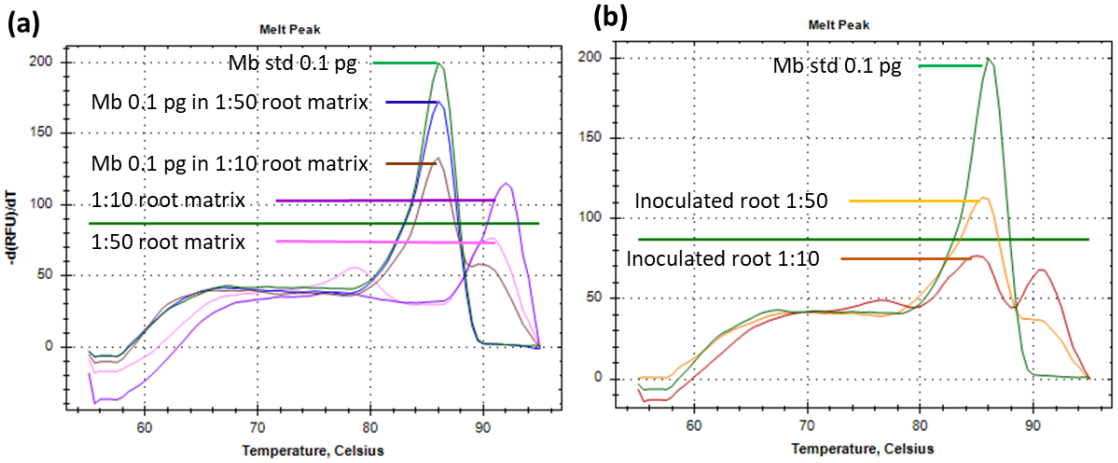


Figure 3. Melting curve analysis of the matrix effect of potato plant root matrix on the amplification of Metarhizium DNA. (a) *Metarhizium brunneum* standard (0.1 pg) alone (green), spiked with 1:10 root DNA (brown) or 1:50 (blue). Plant DNA without *M. brunneum* DNA 1:10 (purple) and 1:50 (pink). (b) DNA from roots inoculated with *M. brunneum*: standard (0.1 pg) (green), root sample 1:10 dilution (red); root sample 1:50 dilution (orange)

Colonisation of oilseed rape and potato plant tissues by Metarhizium brunneum

In oilseed rape plants, all isolates were detected in all plant roots from each fungal treatment and in at least 70% of the shoots (Figure 4a). However, leaf colonisation was scarce, with fungal DNA was detected in one or three of the 10 plants. The biomass of Metarhizium in the root tissues of the plants was significantly higher in Gc1I inoculated plants (see Figure. 4b) compared to plants inoculated with the other *M. brunneum* strains. Fungal biomass in the shoot was lower than in the roots and did not differ between isolates (Figure. 4b).

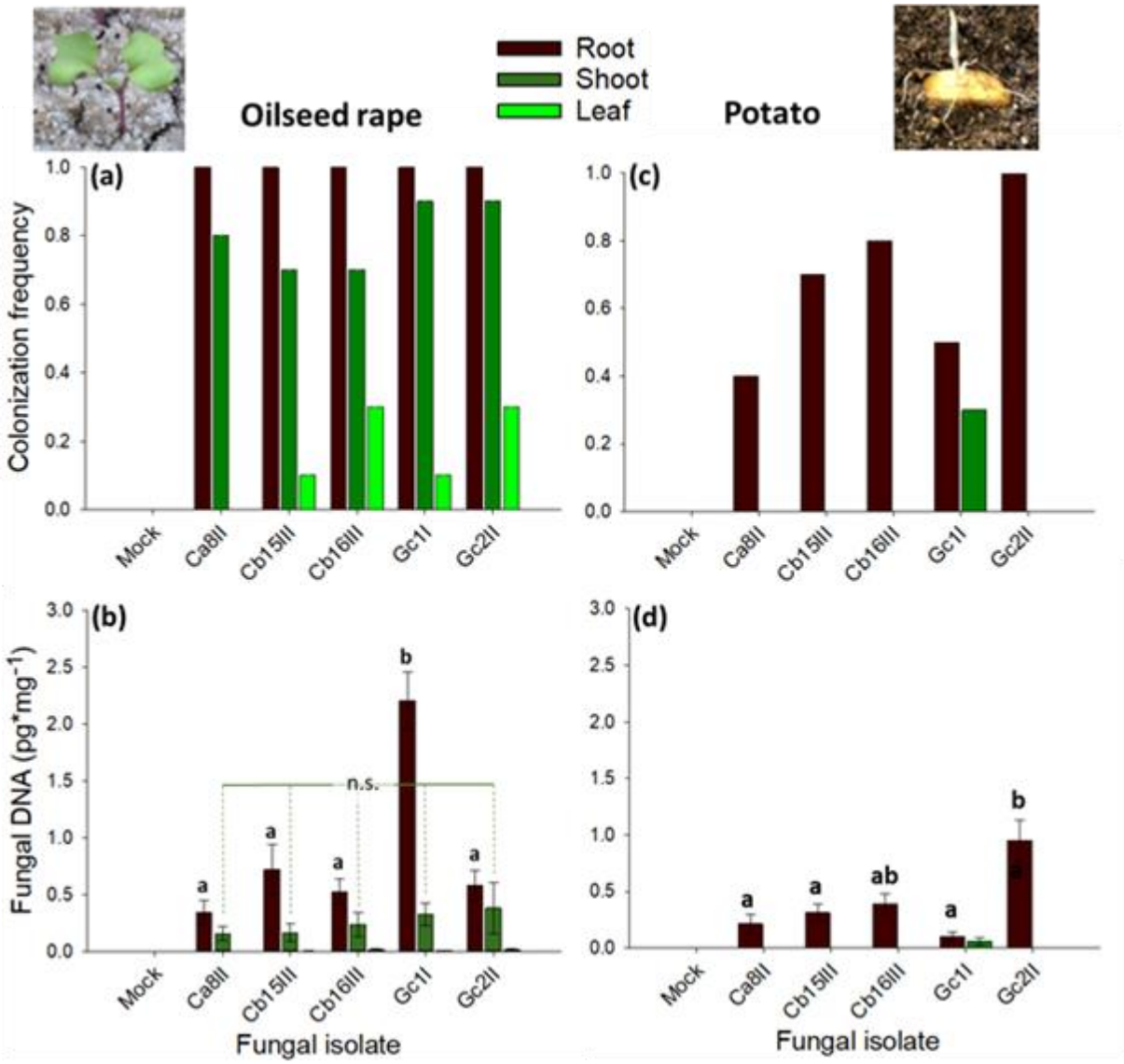


Figure 4. Plant colonization by five *M. brunneum* isolates. (a) and (b): *B. napus;* (c) and (d): *Solanum tuberosum*. (a) and (c): frequency of plants where DNA of the fungus was detected. (b) and (d) fungal DNA concentration (pg * g^{-1} DW) in plant tissues. Brown: roots; dark green: Shoot; light green: leaves. The different letters in (b) differ significantly from each other (Tukey HSD test, P< 0.05). Data represent means ± SE; n = 10

In potato plants, all isolates were detected in the roots, although not in all plants. Only Gc2II was found in all inoculated plants. In addition, only Gc1I was found in the leaves, in 3 out of 10 plants (Figure 4c). Fungal biomass in the root differed between isolates, with the highest amount found in Gc2II, followed by Cb16III (Figure 4d).

Metarhizium brunneum effect on oilseed rape biomass

Biomass was only measured in OSR plants, as potato plant growth was highly variable, as explained in the previous section. Shoot dry weight was not affected by the fungal treatment (Figure 5a). Root dry weight was lower in plants inoculated with Ca8II and Gc2II than in those inoculated with Gc1I, but none of the fungal treatments showed a significant difference from the control (Figure 5b).

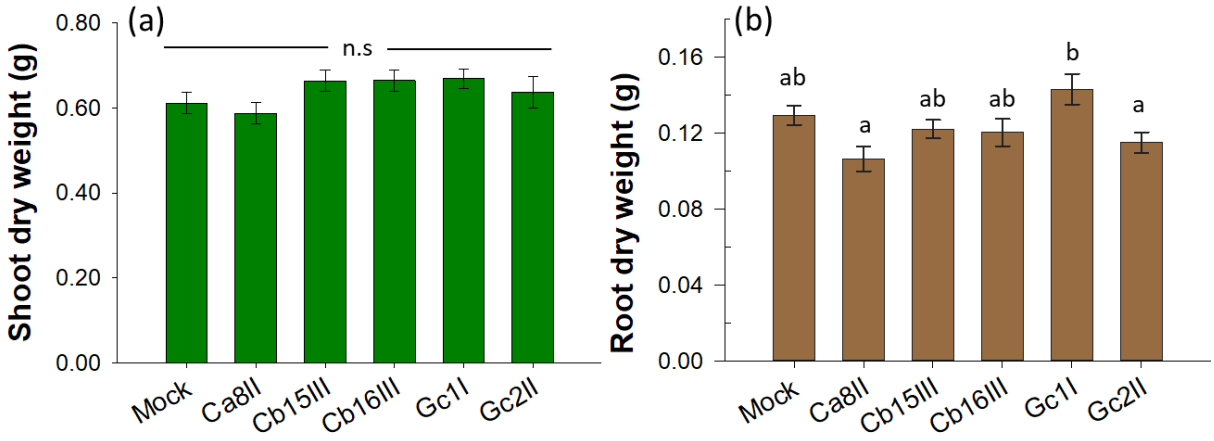


Figure 5. (a) Shoot dry weight and (b) Root dry weight of oilseed rape plants inoculated with different isolates of *M. brunneum.* Different letters in (b) indicate significant differences between treatments (Tukey HSD test, $p < 0.05$). Data represent means ± SE; n = 10.

Interestingly, fungal DNA in the roots had a strong positive correlation with root dry weight (Figure 6a, Pearson's test, r = 0.67, p < 0.0001), and even with dry weight (Figure 6b, Pearson's test, r = 0.39, p = 0.005). On the other hand, the fungal content in the shoots did not correlate with the plant biomass parameters.

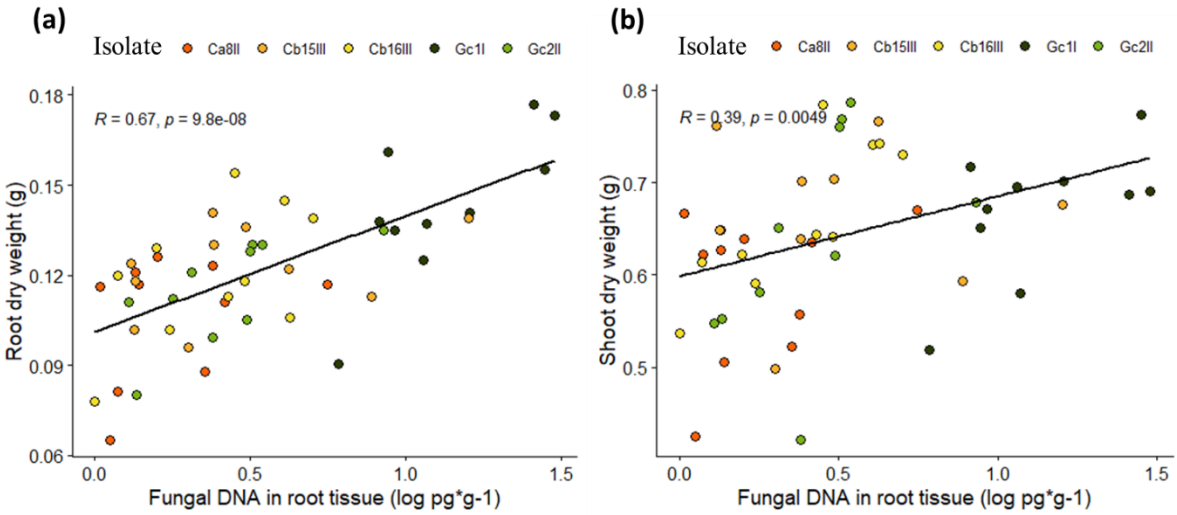


Figure 6. Pearson's correlation between (a) *M. brunneum* DNA in root tissue and root dry weight and (b) *M. brunneum* DNA in root tissue and shoot dry weight of oilseed rape plants inoculated with different isolates. Control plants were excluded from the analysis.

Discussion

The method for quantification of fungal DNA qPCR in plant tissues was optimised. I showed that *M. brunneum* colonised both the roots of potato and oilseed rape roots, but was more abundant in the latter.

Plant tissues are rich in metabolites such as polysaccharides and polyphenols which inhibit PCR amplification (Fang et al., 1992; Pandey et al., 1996; Porebski et al., 1997). We used a DNA kit designed for plant tissues, with a lysis step designed to remove polysaccharides, proteins, and cell debris by precipitation, and a further polysaccharide purification of column-bound DNA that promised to remove plant-derived PCR inhibitors (VWR-peqlab, 2018)(p. 13). In addition, low amounts of fungal DNA present in a plant DNA matrix can lead to unwanted non-specific amplifications; therefore, optimisation of the amplification protocol is a crucial step for the detection of fungal DNA in plant tissues.

The main problem encountered here was the lack of amplification of fungal DNA. The double peak in the melting curve analysis and the gel electrophoresis showed non-specific amplification, so the reaction mix was changed, to increase the stringency. Optimisation of the qPCR assay includes adjusting primers and magnesium concentrations and determining the ideal primer annealing temperature (Nolan et al., 2013). Measures to increase stringency include decreasing primer concentration to reduce the likelihood of non-specific primer binding; decreasing Mg^{+2} concentration to increase Taq polymerase specificity, and increasing primer annealing temperature to avoid non-specific primer annealing (Nolan et al., 2013). I could not manipulate the Mg^{+2} concentration because the kit already has a fixed and relatively high concentration (3 mM). However, I was able to optimise the assay and have a fungal detection method for the next steps of my research.

Metarhizium brunneum colonised both plant species, with better colonisation of oilseed rape, and isolate-specific differences in the amount of fungus. It is not surprising to find differences in colonisation of different plant species, and the results are consistent with previous studies (Steinwender et al., 2015; Moonjely and Bidochka, 2019). Differences in endophytic colonisation may be due to differences in inoculation; however, the roots of both species were exposed to the same concentration and number of fungal spores. Colonisation of potato plants by Metarhizium isolates has been reported previously (Ríos-Moreno et al., 2016; Krell et al., 2018a; Tomilova et al., 2021), although Ríos-Moreno et al., (2016) specify that this colonisation was 'transient', or temporary, after leaf spraying. Furthermore, Krell et al. (2018) reported only 20% endophytism after tuber inoculation with *M. brunneum.* However, it is not clear if this percentage refers to the total plant, since they collected roots, shoots and leaves, but only reported a total value. It would be interesting to know whether Solanaceae secondary metabolites affect Metarhizium growth, as has been reported for *B. bassiana* (Costa and Gaugler, 1989). On the other hand, Metarhizium species have been found to be naturally associated with the rhizosphere or to grow endophytically in Brassica species. For example, (Steinwender et al., 2015) found a strain of *M. brunneum* to be the most abundant in roots of cabbage plants (*Brassica oleracea*) from organically managed fields in Denmark. In another study (Keyser et al., 2015) *M. flavoviride* and *M. brunneum* were found in oilseed rape roots from agricultural fields. Metarhizium species have also been recovered from plant tissues after artificial inoculation; for example, (Hu and Leger, 2002) recovered a fourfold larger population of fungal propagules in the inner rhizosphere of cabbage plants than in the outer soil, after 4 months of an aerial application of *M. anisopliae*. Furthermore, a strain of the same species was recovered from leaves, petioles, and stems of oilseed rape plants after inoculation of plant leafs with a conidial suspension (Batta, 2013). Our results are consistent with previous findings and show that they also colonise stem and leaf tissues, but to a lesser extent. *Brassica* species including oilseed rape produce glucosinolates which are secondary metabolites involved in plant defense (Bruce, 2014). Isothiocyanates and other hydrolysis products of glucosinolates reduce the growth and virulence of Metarhizium species. For example, in one study, exposure to isothiocyanate vapour inhibited in vitro conidial germination and mycelial growth of *M. anisopliae* (Inyang et al., 1999), and in a second study, this fungal growth was completely inhibited by the addition of 2-phenylethyl isothiocyanate to the growth medium (Klingen et al., 2002). However, as observed by Klingen et al. (2002) and based on our results, it does not inhibit root colonisation.

Several studies have reported promotion of plant growth by Metarhizium and other EEFs (reviewed in (Bamisile et al., 2018a; Rigobelo and Baron, 2021)). I did not observe higher plant biomass with any of the fungal strains. However, root and shoot biomass was correlated with fungal biomass in the roots. Metarhizium is known to produce indolacetic acid (IAA, auxin), a plant hormone involved in root growth (Liao et al., 2017; St. Leger and Wang, 2020). However, disruption of fungal IAA production did not alter the ability to promote plant growth (Liao et al., 2017). The positive association between fungal colonisation and plant biomass may be the sum of all effects of the interaction, such as better nutrient acquisition (Behie et al., 2012; Raya-Díaz et al., 2017; Krell et al., 2018b), improved stress tolerance (Khan et al., 2012), etc.

Furthermore, the growth-promoting effect depends on the ability of Metarhizium species to associate with plant roots (Liao et al., 2014).

In summary, this chapter focuses on the optimisation of fungal DNA quantification using qPCR and the assessment of the colonisation ability of *M. brunneum* in oilseed rape plants. In addition, a positive correlation between fungal biomass in the roots of oilseed rape plants and plant biomass was observed. Based on these findings, the *B. napus* - *M. brunneum* system was selected for further investigation of the impact of the plant-fungus association on insect pests and plant diseases.

Chapter 3.

Effects of plant-*Metarhizium brunneum* association on feeding behavior of a specialist herbivore and performance of specialist and generalist phloem feeders

Abstract

Beneficial microbes, such as endophytic plant entomopathogenic fungi (EEF), can influence aboveground herbivores through plant-mediated effects. The entomopathogenic fungus *Metarhizium brunneum* is capable of associating with plant roots, and there is growing evidence suggesting both positive and negative effects on herbivores. The outcome of plant-microbe interactions on herbivores may vary depending on factors such as their feeding behavior (specialist or generalist) or feeding guild (chewers / sap feeders). In this study, using *Brassica napus* and three insect pests, I observed that *M. brunneum* had a feeding stimulant effect on the specialist *Psylliodes chrysocephala*. Additionally, a positive correlation was found between fungal colonization of plants and plant biomass. The effect of *M. brunneum* on aphid fecundity showed inconsistency across the three repetitions. Two of the repetitions indicated a positive effect on the fecundity of the generalist aphid *Myzus persicae* in older leaves, while a neutral or slightly negative effect was observed on the Brassica specialist aphid *Brevicoryne brassicae*. Although the mechanisms were not investigated in this chapter, the results are discussed in the context of existing research. The interaction between the fungus and the plant could potentially impact the nutritional quality of the plant and alter its response to insects, potentially modifying the concentration of defense metabolites. These changes may lead to increased feeding or improved insect parameters.

Introduction

The roots of the plants interact with many microorganisms in the soil. Fungal entomopathogens such as Metarhizium associate with plant roots and/ or grow as endophytes, resulting in beneficial effects for the plant (St. Leger and Wang, 2020). Several studies report that endophytic entomopathogenic fungi (EEF) and other fungal endophytes have negative effects on herbivores (Gange et al., 2019). The association with fungal endophytes can negatively affect herbivore preference and / or performance. Preference refers to the ability to discriminate between plants 'challenged' or inoculated with an endophyte from non-'challenged' plants. Effects on insect performance involve many aspects of the insect life cycle such as mortality, fertility, growth rate, development time, digestibility of consumed food, among others (Fernandez-Conradi et al., 2018).

However, other studies also show that fungal-plant interaction can lead to increased insect preference. For example, the moth *Helicoverpa armigera* laid more eggs on tomato plants inoculated with *Acremonium strictum* than on non-inoculated plants (Jallow et al., 2008), while *M. brunneum* increased the oviposition preference of *Delia radicum* flies on oilseed rape plants (Cotes et al., 2020). Endophytes can also lead to increased feeding; for example, two different leaf endophytes isolated from *Cirsium arvense*, *Chaetomium cochliodes,* and *Cladosporium cladosporioides,* increased the area consumed by the thistle tortoise beetle *Cassida rubiginosa* (Gange et al., 2012).

The association of plants with beneficial fungi can also improve aphid performance. For example, increased aphid fecundity has been observed in *Aphis glycines* feeding on soybean plants inoculated with *M. roberstii* (Clifton et al., 2018), in *Rhopalosiphum padi* feeding on wheat, and *A. fabae* feeding on bean leaves of plants inoculated with an isolate of the EEF *M. brunneum* (Rasool et al., 2021b).

The differential effects of endophytes on insects have been attributed to various factors, including the degree of feeding specialisation and insect gild. A meta-analysis conducted by Gange et al. (2019) (Gange et al., 2019) examined the effects of entomopathogenic and non-entomopathogenic endophytes on insect performance, which refers to insect growth, development, and reproduction. The study found that non-entomopathogenic endophytes had a detrimental effect on the performance of polyphagous insects, while the performance of both monophagous and polyphagous insects was negatively affected by entomopathogenic endophytes. This could be partly explained by the fact that specialist insects are less negatively affected by the allelochemicals of their host plants (Rothwell and Holeski, 2020), although this is not the case for all specialists (Ali and Agrawal, 2012). In terms of feeding gild, sap-sucking insects experience higher detrimental effects than chewing insects, especially when plants are inoculated with non-entomopathogenic endophytes (Gange et al., 2019).

In studies where fungal inoculation leads to increased consumption or improved performance, the effects have been attributed to a change in plant chemistry, either defensive or nutritional (Gange et al., 2012; Rasool et al., 2021b). On the other hand, increased feeding preference or oviposition has been linked to a change in the volatile profile of inoculated plants (Jallow et al., 2008; Aragón, 2016; Cotes et al., 2020).

Plants of the Brassicaceae family use glucosinolates (GSLs) as their main defence system against insects and pathogens (Liu et al., 2021). Although GSL and their hydrolysis products are normally repellent to herbivores, some specialist herbivores have evolved adaptations to detoxify them (Müller, 2009; Jeschke et al., 2016; Beran et al., 2018; Gikonyo et al., 2019) or to inactivate the myrosinases that hydrolyse GSLs leading to toxic compounds (Sporer et al., 2021)**,** and even to sequester them to use these compounds for their own defence against predators (Müller, 2009). In addition, some insect specialists use them as cues for finding and accepting host plants (Renwick. et al., 1991; Pivnick et al., 1994). GSLs may also act as feeding stimulants (Bartlet et al., 1994; Giamoustaris and Mithen, 1995).

The European cabbage stem flea beetle (*Psylliodes chrysocephala* L., Coleoptera: Chrysomelidae) is a specialist herbivore, highly adapted to GSL-containing plants of the Brassica family (Shukla and Beran, 2020). It is the most important pest of winter oilseed rape in Europe (Williams, 2010; Zheng et al., 2020). Adult beetles feed on the young seedlings during autumn, while larvae feed and bore into the petioles and stems, causing considerable damage to the plants (Alford, 1979; Williams, 2010). This species has developed various strategies to degrade, sequester, and inactivate GSLs (Beran et al., 2018), in addition to a symbiosis with an isothiocyanate detoxifying bacterium *Pantoea spp.* (Shukla and Beran, 2020). Glucosinolates are feeding stimulants for *P. chrysocephala* (Bartlet et al., 1994; Giamoustaris and Mithen, 1995), which feeds only on plants containing GSLs (Bartlet and

Williams, 1991). Another Brassica specialist is the cabbage aphid *Brevicoryne brassicae* (Hemiptera, Aphididae). It uses GSLs as host cues, and similar to *P. chrysocephala*, GSLs act as feeding stimulants (Nault and Styer, 1972). In addition, *B. brassicae* performs better on plant varieties with higher concentrations of some GSLs (but see (Mewis et al., 2005)), and can also sequester GSLs that are used against predators (Francis et al., 2001; Powell et al., 2006; Hodge et al., 2019).

On the other hand, the aphid *M. persicae* (Sulzer) is a generalist feeding on more than 400 plant species (Blackman and Eastop, 2006). It is one of the most important pests of several crops, not only causing direct damage through feeding and honeydew production, but also acting as a vector for several plant virus diseases (Ng and Perry, 2004). This aphid is also a pest of Brassica plants, although affected by GSLs. Several studies have shown reduced fecundity, growth, population size, and choice in plants with high GSL concentrations, especially indole GSLs and its breakdown products (Levy et al., 2005; Mewis et al., 2005; Kim and Jander, 2007; Kim et al., 2008; Pfalz et al., 2009). However, GSLs may also act as phagostimulants; *M. persicae* populations feed on non-host broadleaves treated with the glucosinolate sinigrin (Nault and Styer, 1972).

In this chapter, I evaluated the effects of *M. brunneum* inoculation on the specialist beetle *P. chrysocephala* and on the fertility and survival of two aphid species, the specialist *B. brassicae* and the generalist *M. persicae*. The hypothesis was that fungal inoculation would either have no significant effect or potentially enhance the performance and feeding of the specialist insects, assuming that the fungal-plant interaction triggers the up-regulation or induction of defense-related compounds. It was expected that these specialist insects, being adapted to the induced defense-related compounds, would not be significantly affected in terms of their performance and feeding. In contrast, the generalist M. persicae, which are not adapted to these changes, would experience negative effects on their performance and feeding due to the altered defense-related compounds induced by the fungal-plant interaction.

Material and methods

Plants, fungal isolates, and insects

Oilseed rape (*Brassica napus* var. Penn) plants were grown from seeds provided by Norddeutsche Pflanzenzucht, harvest 2018 (Hans-Georg Lembke KG, Holtsee, Germany). For all experiments, the seeds were surface sterilised in 70% ethanol for 1 min and 2% sodium hypochlorite (Carl Roth GmbH, Karlsruhe, Germany) for 5 min, rinsed three times with sterile water and sown in sterile quartz sand until the day of inoculation. In all experiments, the plants were grown on a non-sterile substrate consisting of washed river sand: commercial soil (Fruhstorfer Erde Typ 25, Hawita Gruppe GmbH) 1 : 3 (by volume).

Isolates of *M. brunneum* were obtained from the internal collection of the Division of Agricultural Entomology (see Table 1). To obtain spore suspension for all experiments, isolates were grown on potato dextrose agar (PDA) (Carl Roth GmbH, Germany) at 23 °C for 14 days. Conidia were removed from the hyphae by gently scraping the sporulating colony with a sterile

glass slide and the conidia were suspended in 20 ml of 0.1% Tween 80 (Carl Roth GmbH, Germany). The suspension was filtered through a plastic gauze and adjusted to a final concentration of 1 x 10^7 ml^{-1}. Spore viability was assessed before each experiment, by plating 100 µl of 1 × 10^3 spores ml^{-1} distributed in 10 µl drops on PDA plates and counting the germinating colonies 48 h later.

Table 5. *Metarhizium brunneum* isolates used in the experiments

Strain	Experiment	Crop regime	Location
GC1I	CSFB / aphids	Permanent semi-natural grasslands for at least 7 years. Grassland harvested twice a year	51°34'10.7"N 10°03'54.1"E
Gc2II	CSFB		
Gd12	Aphids		
Cb15III	CSFB / aphids	Winter wheat, barley or oilseed rape crop rotation, conventional farming	51°33'58.3"N 10°04'10.1"E
Cb17b	CSFB / aphids		
CC5	CSFB / aphids		

Myzus persicae came from the rearing of the Division of Agricultural Entomology and was maintained on oilseed rape (*B. napus* cv “Marathon” RAPOOL, Germany). An initial population of *Brevicoryne brassicae* was provided by Dr. Torsten Will of the Julius Kühn Institute.

The aphids were synchronised for two generations on oilseed rape (*B. napus* cv. Penn). For this, a mother aphid was placed in a leaf for 24 hours and the nymphs born in this time (~4) were left to mature (first generation). Once mature, each aphid was transferred to a new leaf for 24 h and the nymphs born during this time were left to mature for 8 d (*M. persicae*) or 11 d (*B. brassicae*) and used as mothers for the experiments.

Adult beetles of Psylliodes chrysocephala were provided from a laboratory culture by Dr. Beran of the Max Planck Institute for Chemical Ecology, Jena, The insects were reared on *B. rapa* cv. Yu-Tsai-Sum (Known-You Seed Co. Ltd) plants and used within 6 h of arrival. The insects had emerged within the previous 3 days and were therefore in the preestivation phase.

Effects of fungal endophytic colonization on P. chrysocephala

Ten-day-old seedlings were transplanted into 750 ml plastic cups filled with substrate to a depth of 5 cm. Plants were inoculated by drenching the roots with 5 ml of spore suspension for each isolate treatment, or 5 ml of 0.1% Tween for the control. Plants were watered daily. Twelve days after inoculation, two adult beetles were placed in each cup and closed with a 0.5 L inverted cup with a mesh at the bottom to allow ventilation. Insect herbivory was assesed non-destructively at 24, 48 and 72 h by counting the feeding units (fU) on each plant. The fU consisted of a circular hole of 1-2 mm left by the beetle after feeding. After 7 days of beetle feeding, the plants were harvested. The hypocotyls were surface sterilised by immersion in 70% ethanol for 1 min followed by immersion in 2% sodium hypochlorite for 5 min, rinsed three times in sterile water, rapidly dried on sterile paper towels, and stored at -25 ° C for DNA extraction. The plant leaves were photographed for image analysis: the eaten area on each

leaf was manually digitised and the leaf margin was reconstructed. Leaf area and herbivory were then analysed using Fiji software (Schindelin et al., 2012). Roots were washed, the roots and leaves were dried at 60 °C for 48 h, and the dry weight was measured. The experimental unit consisted of a cup cotaining the plant and two beetles. The six treatments consisted of five fungal isolates and one control, each with 15 replications.

Plant inoculation and insect infestation: aphids

The seven-day-old seedlings were inoculated by dipping their roots in a spore suspension for 20 min.The seedlings were then transplanted into 11-inch round pots. After inoculation, the plants were allowed to grow for three weeks before aphids were introduced. Prior to aphid infestation, plants were grown in a greenhouse under controlled conditions: temperature maintained at 24 ± 4 °C, relative humidity at 60-70%, and a 16-hour light/8-hour dark photoperiod, supplemented by high-pressure sodium lamps. One day before the aphid infestation, the plants were transferred top a growth room. For aphid infestation, two adult aphids were placed on the 3rd and 4th leaves of each plant within a clip cage and left undisturbed for 24 hours. After removal of the adult aphids, three newly born nymphs were left in each clip cage. The clip cages were then removed 24 hours after removal of the adults, and the leaves containing the nymphs were enclosed with perforated cellophane bags (bread bags) sealed with a wire at the base of the petiole.

Aphids were observed daily, and the time of maturity was recorded when the first nymph was born. Nymphs were counted daily for 7 d (*M. persicae*) or 9 d (*B. brassicae*) before the first-born nymph reached the time of reproduction based on preliminary observations. At the end of this period, the leaves in the bags were removed from the base of the petiole with a sterile scalpel, frozen at −25 °C to prevent further reproduction of aphids, the aphids were counted manually, collected in a 2 ml Eppendorf tube, and the leaves and aphids were stored at -25 ° C. The roots were washed and stored at −25 °C. Leaves, roots, and aphids were used for DNA extraction.

Each treatment consisted of 5 plants (replicates) and the experiment was repeated 3 times. Environmental conditions are known to affect the growth of plants and the outcome of microbe-plant-insect interactions. We wanted to test the effects of EEF-aphid-plant interactions under different conditions; therefore, each repetition of the experiment was placed in a different growth chamber. The first was in a growth chamber with neon white lights, 21 °C and 60-70% RH. The second replication; growth room, 19 °C and 60-70% RH with high pressure sodium lamps and the third was in a growth room, 21 °C and 60-70% RH with LED lights (GoLeaf E2 LED, 50 W, Bioledex, Germany).

DNA extraction and fungal quantification by qPCR

Fungal endophytic colonization of plants in both experiments and the possible fungal infection of aphids were measured by qPCR (Metarhizium DNA, pg $\times$ g^{-1} DW). Plant tissues were weighed, lyophilised for 72 h (VaCo 5 Freeze dryer, Zirbus technology GmbH, Germany), and pulverised with a mixer mill (Retsch MM 400, Germany) adding 4 stainless steel balls and

milling for 1 min at 30 × 1 s^{-1}. DNA was extracted using the CTAB extraction method described previously (Brandfass and Karlovsky, 2006). The DNA pellet was diluted in a final volume of 100 µl of TE buffer and DNA quality was verified by gel electrophoresis of agarose (0.8%) gel electrophoresis. The CFX384™ real-time system with a C1000™ Thermal cycler (BioRad, Hercules, USA) was used for fungal DNA amplification and melting curve analysis. Primers specific for Metarhizium clade 1 were used; Ma 1763 (CCAACTCCCAACCCCTGTGAAT) and Ma 2097 (AAAACCAGCCTCGCCGAT) (Schneider et al., 2011). Amplification was performed using 1:10 dilutions of DNA extracts. The qPCR reaction consisted of 5 µl of 2x qPCRBIO SyGreen Low-ROX (PCRBIOSYSTEMS), 0.2 µl of 10 µM of each primer, 3.6 µl of 1 µl of DNA template solution, completed to a total of 10 µl final reaction volume. qPCR running conditions started with an initial denaturation for 2 min at 95 °C, followed by 40 reaction cycles consisting of a 5 s denaturation step at 95˚ C, a 20 s annealing step at 63 °C, and a 10 s extension at 72 °C. The final extention was carried out at 72 °C for 5 min. Melting curves were obtained by heating the samples to 95 °C for 60 s and cooling them to 55 °C for 60 s followed by a temperature increase from 55 °C to 95 °C by 0. 5 °C per cycle with continuous fluorescence measurement. The absolute amount of fungal DNA per gram of plant tissue was measured against DNA standards starting at a concentration of 100 pg × μl^{-1}, and decreasing at a dilution factor of 1:3. The threshold cycle (Ct) and standard curves were generated using Bio-Rad CFX Maestro software. The identity of the amplicon was verified by comparing its size with gel electrophoresis in 10 random samples. The presence of DNA was assesed in all samples with two technical qPCR replicates.

Data analysis

Data exploration and statistical analyses were performed using R 4.0.3 (*R* Core Team, 2016). Normality of all variables was assesed with the Shapiro-Wilk test, and the following transformations were performed to achieve normality: square root for root weight; and the Tukey ladder of powers was used for the *Mb* DNA with a lambda value of 0.075. All variables showed a homogeneity of variance, evaluated by Bartlett's K-squared test. To assess whether inoculation with the different *Mb* isolates had an effect on plant biomass (shoot and root dry weight and root length) and herbivory at the different time points, as well as on the amount of *Mb* DNA, the data were analysed using linear models (nlme library). Significance between treatments was evaluated by post hoc analysis with Tukey's test.

Results

Endophytic M. brunneum effect on P. chrysocephala feeding

The feeding behaviour of adult P. chrysocephala beetles was studied on oilseed rape plants in the presence of different isolates of *M. brunneum*. The results indicate that the amount of feeding varied with time and was significantly higher in plants inoculated with at least one of the isolates. Specifically, the feeding areas were found to be higher at 24 h, 72 h and 7 d in plants inoculated with the Cb17B isolate (Figures 1a, 1c and 1d), while at the final time point,

the beetles also fed more in plants inoculated with the CC5 and GC1 isolates, compared to non-inoculated plants (Figure 1d, $F_{(5, 83)} = 5.73$; $p = 0.019$, 0.017 and 0.001 respectively).

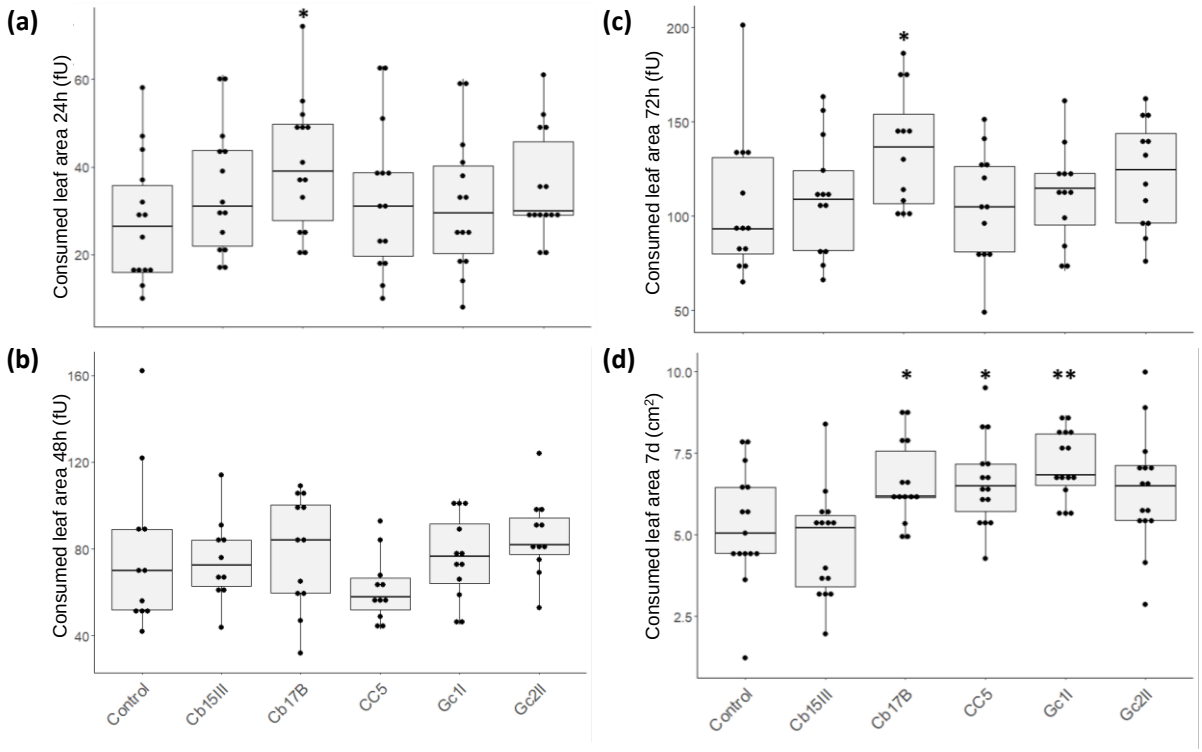


Figure 1 Leaf area consumed by oilseed rape plants inoculated with *Metarhizium brunneum* isolates by *Psylliodes chrysocephala* A) 24 h; B) 48 h, C) 72 h and D) 7 d after beetle release. The asterisks above the box plot indicate a significant difference between each treatment and the control (linear model, $P \leq 0.05$). The box plot shows all data points ($n = 15$), with the horizontal line representing the median, surrounded by the upper (25th) and lower (75th) percentiles. 'fU': 'feeding units', count circular area left after feeding (non-destructive). Data in (d) were measured by image analysis using the ImageJ software.

Standard screening tests are commonly used to evaluate seedlings resistance to flea beetle feeding. Typically, these tests measure the amount of feeding that occurs within 24 to 72 hours after beetles are introduced to fully emerged cotyledons. To better understand the relationship between early feeding and long-term feeding outcomes, a correlation analysis was performed between the amount of feeding observed at each time point (24, 48, and 72 hours) and the amount of feeding observed on day 7, as shown in Table 2. The analysis revealed no significant correlation between the feeding observed at 24 h and that observed on day 7. However, positive but weak correlations were observed between feeding observed at 48 hours, 72 hours, and on day 7.

Table 6 Correlation analysis of leaf area consumed by adult beetles at different time points after introduction into experimental units: Pearson's R values

Time after beetle introduction	**48 h**	**72 h**	**7 d**
24 h	0.61***	0.42***	0.19.
48 h		0.75***	0.24*
72 h			0.38**

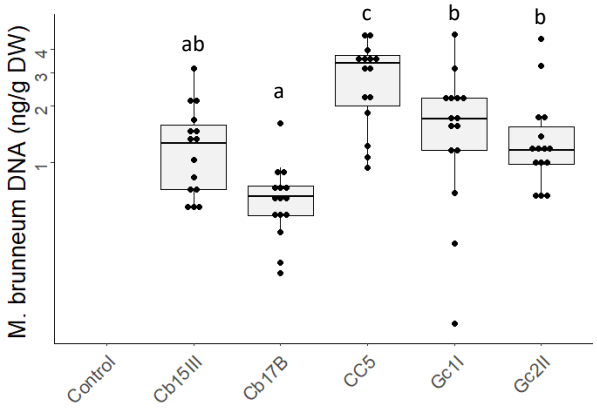


Figure 2. *Metarhizium brunneum* DNA (ng.g^{-1} DW) measured by quantitative PCR in hypocotyl tissue of *B. napus* plants 29 days after inoculation by root drench. The letters represent statistically significant differences in the amount of fungal DNA according to a linear model and Tukey's post hoc test. The box plot shows all data points (n = 15), with the horizontal line representing the median, surrounded by the upper (25th) and lower (75th) percentiles.

Metarhizium DNA was detected in hypocotyl tissues in all fungal treatments, with significant differences observed between isolates ($F_{4,70} = 11.11$, $p < 0.001$). Plants treated with the CC5 isolate showed the highest amount of fungal DNA, followed by Gci1, GC2II, Cb15III, and the lowest levels were detected in plants treated with Cb17B (Figure 2). Root and shoot biomass and total leaf area were not significantly affected by fungal inoculation, as shown in Figure 3a, b, and d. Only root length was reduced in plants inoculated with the Gc1I isolate (lm, $F_{5,84} = 1.187$, $p = 0.032$, Figure 3c). However, our analysis revealed a significant positive correlation between the amount of fungal DNA present in the hypocotyl and both leaf area (Figure 4a) and shoot dry weight (Figure 4b). In particular, when all plants were included in the correlation analysis, a negative correlation was observed between the amount of feeding and the weight of the shoots (Figure 4c).

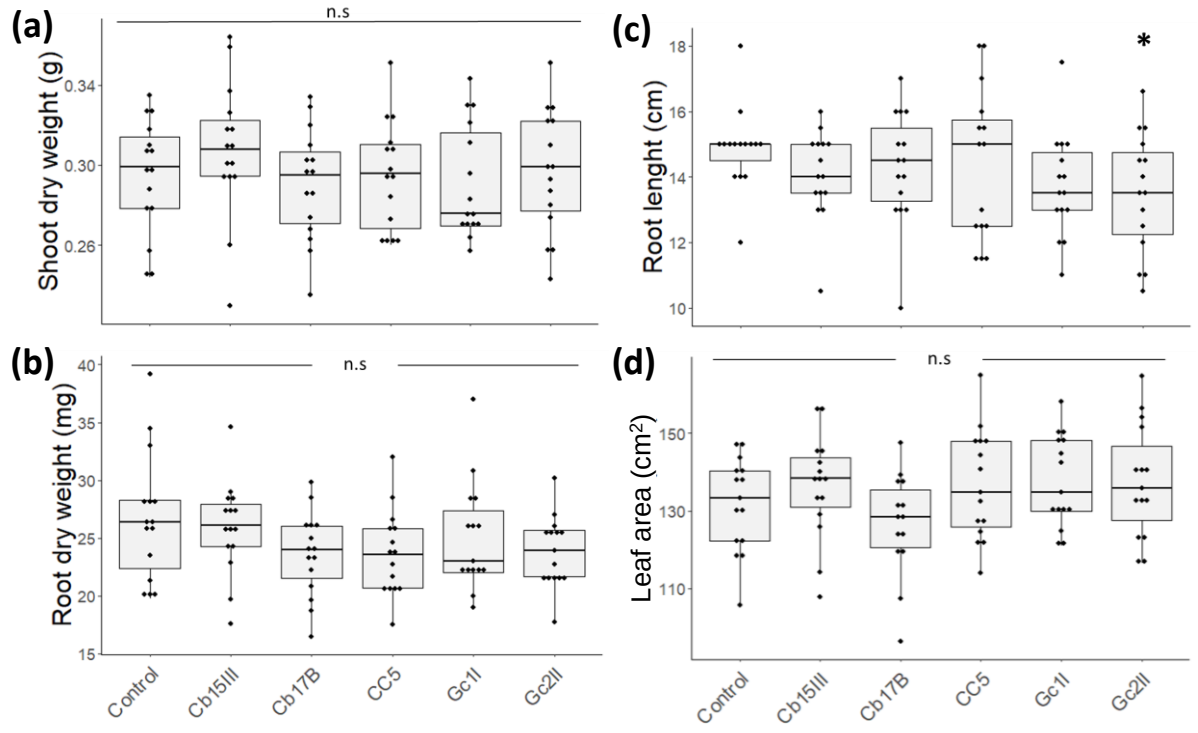


Figure 3 Plant biomass of *Brassica napus* plants inoculated with *M. brunneum* isolates 29 days after planting and 14 days after root drenching inoculation (5 ml, 1 * 10^7 spores/ml). Asterisks above the box plot in (D) indicate a significant difference between each treatment and the control (non-inoculated plants) (linear model, *$p < 0.05$). n = 15

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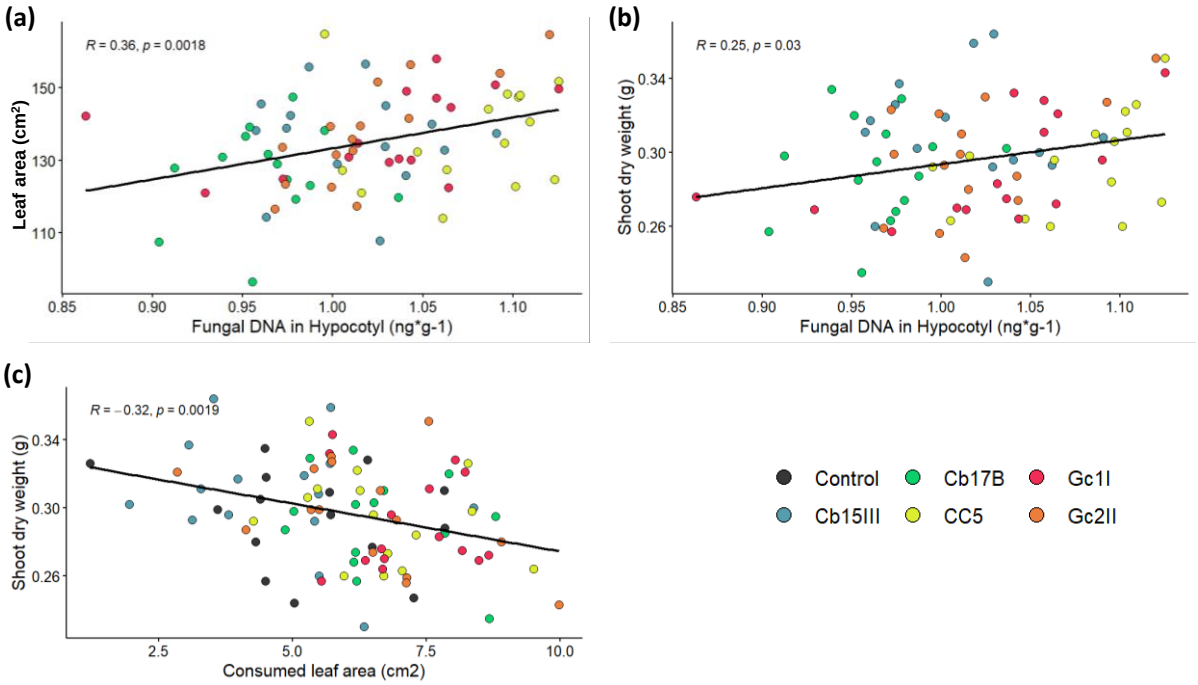

Figure 4 Pearson's correlation between *M. brunneum* DNA in the hypocotyl and (a) shoot and (b) root dry weight. (b) Pearson's correlation between the leaf area consumed and shoot dry weight. Each point represents a single data point, with different colours assigned to each isolate treatment, *n* = 15. (a) and (b) exclude control treatment with no fungal DNA.

Effects of endophytic M. brunneum under variable growth conditions on the performance of specialist (B. brassicae) and generalist (M. persicae) aphid species

The effect of different *M. brunneum* isolates on specialist *B. brassicae* and generalist *M. persicae* aphid populations varied according to growth conditions. Therefore, the results of each repetition are presented separately in individual figures and summarised in Figure 10. Leaf age was found to have a significant effect on generalist *M. persicae*, with more nymphs per adult observed on older leaves. The presence of fungal DNA on the roots was only confirmed in the second and third repetitions due to the light stress experienced by the plants during the first repetition when they were transferred from the greenhouse to the growth chamber. As a result, the leaf colour changed to reddish brown and older leaves (L3) began to senesce rapidly (Figure 5)

Figure 5. Oilseed rape plants showing stress symptoms after 10 days in the first growth chamber.

In the second and third repetitions, *M. brunneum* DNA was detected in the roots of all plants treated with different fungal isolates. However, in the second repetition, the amount of DNA detected in plants inoculated with CC5 and Gd12 was lower and was influenced by the species of aphid infesting the plant (Figure 6a). This was mainly due to the lack of DNA detection in two plants inoculated with CC5 and three plants inoculated with Gd12. In contrast, in the third repetition, there was no effect of the aphid species on the amount of DNA detected in the roots, and the plants inoculated with the Gd12 isolate had less or no DNA in the roots of plants with either aphid species (Figure 6b).

The first repetition of the experiment showed that the generalist and specialist aphid species responded differently to the treatments. Statistical analysis showed that leaf age significantly affected the total number of aphids per leaf and the number of aphids produced per nymph in the generalist *M. persicae* but not in the specialist *B. brassicae* when the fungal treatment was excluded from the model (Figure 7c, $F_{1,55} = 8.43$, $p = 0.005$; Figure 7d, $F_{1,55} = 13.7$, $p < 0.001$). In addition, the specialist showed a significant response to the fungal inoculation, resulting in a lower number of nymphs per adult in plants inoculated with CC5 and Gd12 isolates compared to control plants (Figure 7b).

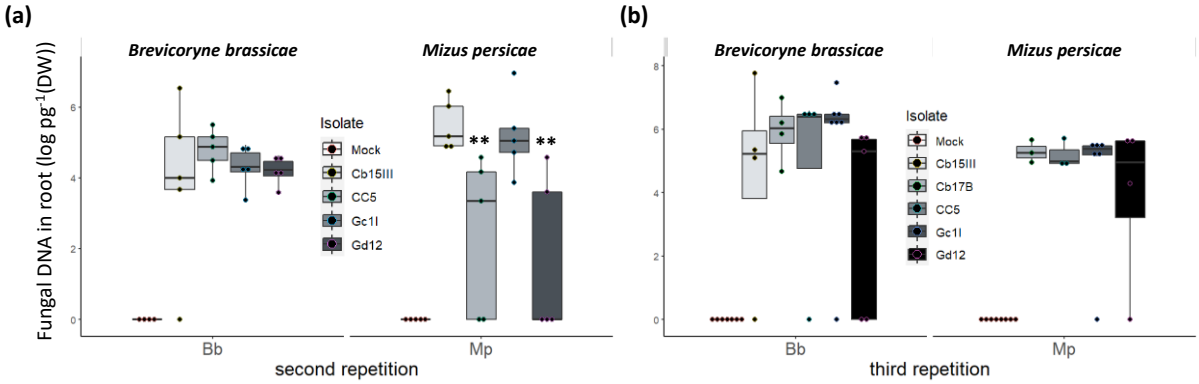

Figure 6 DNA content of *Metarhizium brunneum* in the roots of 5-week-old oilseed rape plants infested with *B. brassicae* or *M. persicae*. (a) second repetition; (b) third repetition. Seedlings were inoculated by root dip at 7 d and the aphids remained on the plant for 7 d (*M. persicae*) or 9 d (*B. brassicae*). Box plots show all data points ($n \leq 5$), with the horizontal line representing the median, surrounded by the upper (25th) and lower (75th) percentiles.

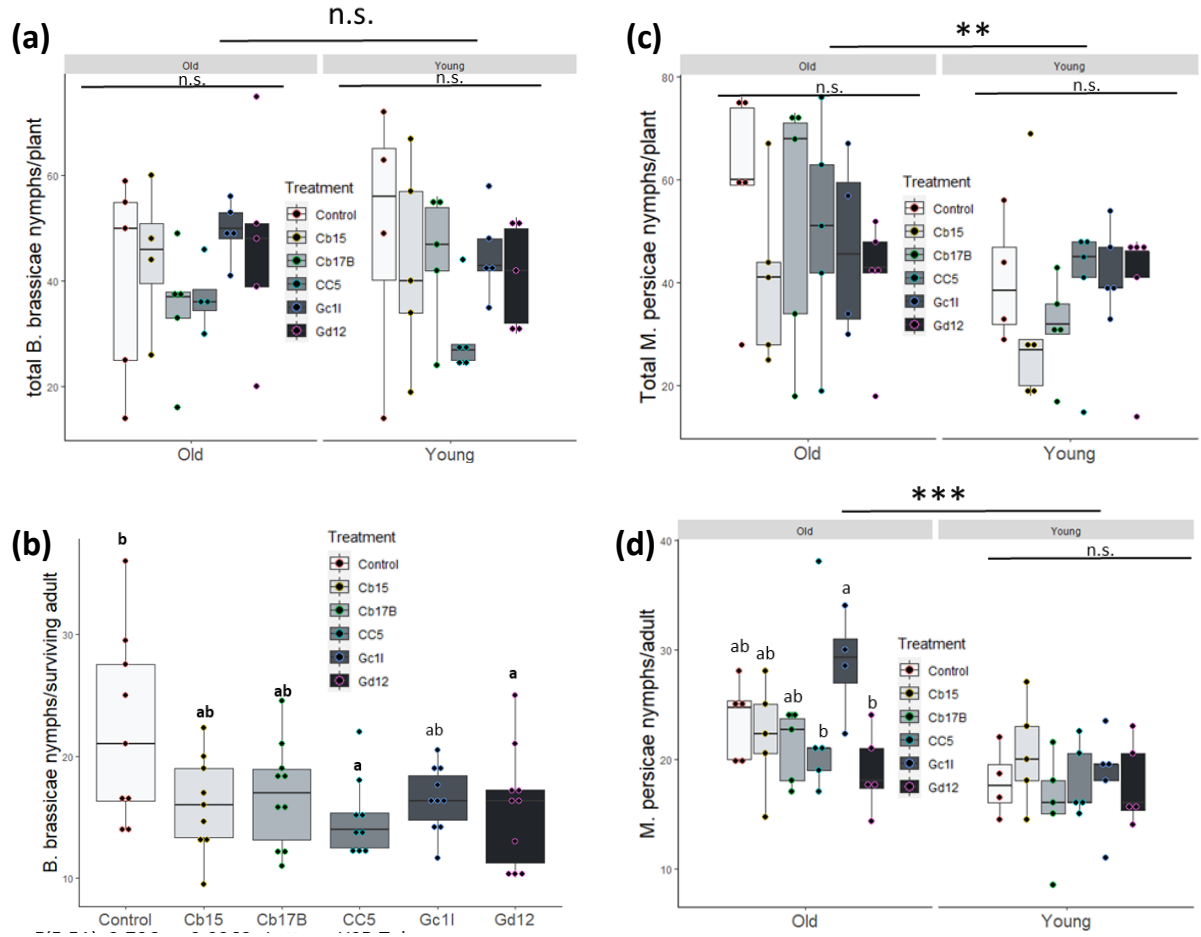

Figure 7 First repetition. Number of aphids per leaf in plants inoculated with different *M brunneum* isolates. (a) Total *B. brassicae* nymphs per leaf and (b) per placed adult; (c) total *M. persicae* nymphs per leaf and (d) per placed adult. Three newborn nymphs were left to develop from their birth on the third (old) and fourth (young) leaves of oilseed rape plants inoculated with 5 different fungal isolates. The aphids were left in the leaves for 9 d and 7 d after maturity for *B. brassicae* and *M. persicae,* respectively. Letters indicate significant differences according to a linear model and Tukey's post hoc test. Box plot shows all data points ($n \leq 5$), with the horizontal line represents the median, surrounded by the upper (25th) and lower (75th) percentiles.

In the second repetition, fungal inoculation and leaf age had no significant effect on the number of aphids per leaf for either species, as shown in Figures 8a and 8d, respectively. However, for *B. brassicae*, the final number of nymphs per adult was influenced by both leaf age and fungal inoculation. The control plants had a higher number of nymphs per surviving adult in the older leaves than in the younger ones (Welch Two Sample t-test = -3.81, $df = 5.99$, $p < 0.01$).

Fungal inoculation had an effect on the number of nymphs produced per surviving adult only in older leaves, where treatment with isolates CC5 and Gc1I reduced the number of nymphs per adult (GLM Poisson family, Tukey HDS, CC5: $p = 0.003$; Gc1I: $p = 0.02$). Similarly, the generalist *M. persicae* also showed a reduction in the number of nymphs per adult due to fungal inoculation, but with a different isolate, Cb15, in both young and old leaves (Figure 8e). Pre-reproductive time was not significantly affected by either treatment or leaf age (Figures 8c and 8f), but leaf age did affect the variability in pre-reproductive time of *M. persicae*. Almost all individual nymphs on old leaves had a pre-reproduction time of 7 days, with a coefficient of variation (CV) of 0.058. In contrast, nymphs feeding on younger leaves showed a wider range of pre-reproduction times between 7 and 9 days, except for the Gc1I treatment, which resulted in a higher CV (0.095).

The third repetition showed that leaf had no effect on *B. brassicae* performance. However, plants inoculated with the Gc1I isolate had a significant effect on the number of aphids per leaf (Figure 9a) and the number of surviving nymphs per adult (Figure 9b) of aphids feeding on younger leaves. On the other hand, there was an effect of leaf age on *M. persicae*, where aphids feeding on older leaves had a higher number of nymphs per surviving adult. Fungal inoculation also had an effect on aphids feeding on older leaves: plants that were inoculated with CC5, Cb17b, and Gc1I had a higher number of nymphs per surviving adult compared to control plants (Figure 9e).

The pre-reproductive time of none of the species was not affected by leaf age or fungal inoculation (Figures 9c, 9f). A significant negative correlation was found between the number of days taken by the nymphs to reach maturity, and the final number of nymphs per plant, as shown in Figure 10.

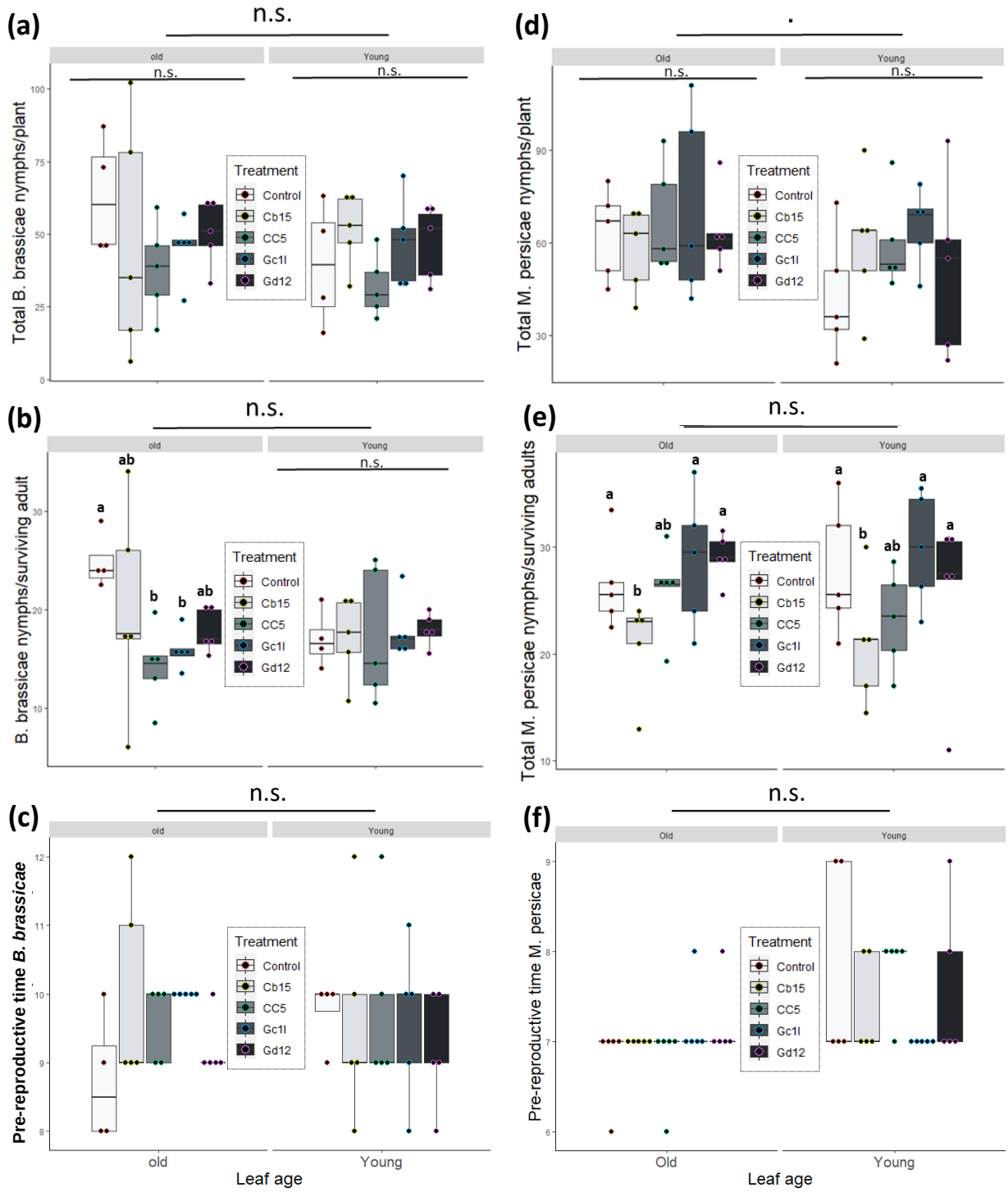

Figure 8. Second repetition. Number of aphids per leaf in plants inoculated with different *M brunneum* isolates. (a) Total *B. brassicae* nymphs per leaf, (b) per surviving adult, and (c) pre-reproductive time. (d) Total *M. persicae* nymphs per leaf, (e) per surviving adult, and (f) pre-reproductive time. Three newborn nymphs were left to develop from birth on the third (old) and fourth (young) leaves of oilseed rape plants inoculated with five different fungal isolates. The aphids were left on the leaves for 9 days after maturity for *B. brassicae* and 7 days after maturity for *M. persicae*. Letters indicate significant differences as determined by a general linear model (GLM) fitted with the Poisson family and Tukey's post hoc test. The box plot shows all data points ($n \leq 5$), whit the horizontal line representing the median, surrounded by the upper (25th) and lower (75th) percentiles.

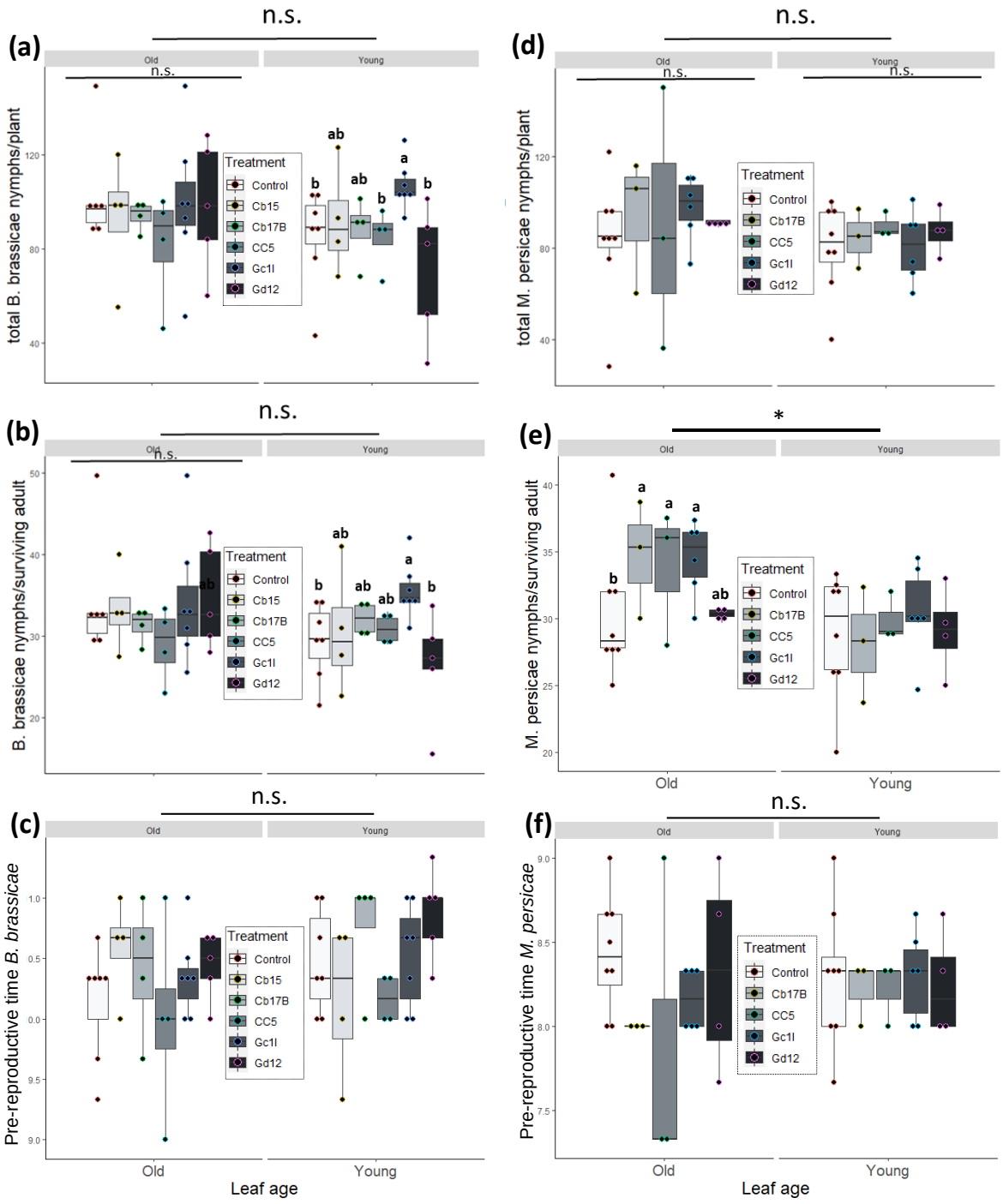

Figure 9 Third repetition. Number of aphids per leaf in plants inoculated with different *M brunneum* isolates. (a) Total number of *B. brassicae* nymphs per leaf and (b) per surviving adult; (c) days to nymph maturity; (d) total number of *M. persicae* nymphs per leaf and (e) per surviving adult; (f) days to nymph maturity. Three newborn nymphs were left to develop from birth on the third (old) and fourth (young) leaves of oilseed rape plants inoculated with five different fungal isolates. The aphids were left on the leaves for 9 days after maturity for *B. brassicae* and 7 days after maturity for *M. persicae*. Letters indicate significant differences as determined by a general linear model (GLM) fitted with the Poisson family and Tukey's post hoc test. The box plot shows all data points ($n \leq 5$), with the horizontal line representing the median, surrounded by the upper (25th) and lower (75th) percentiles.

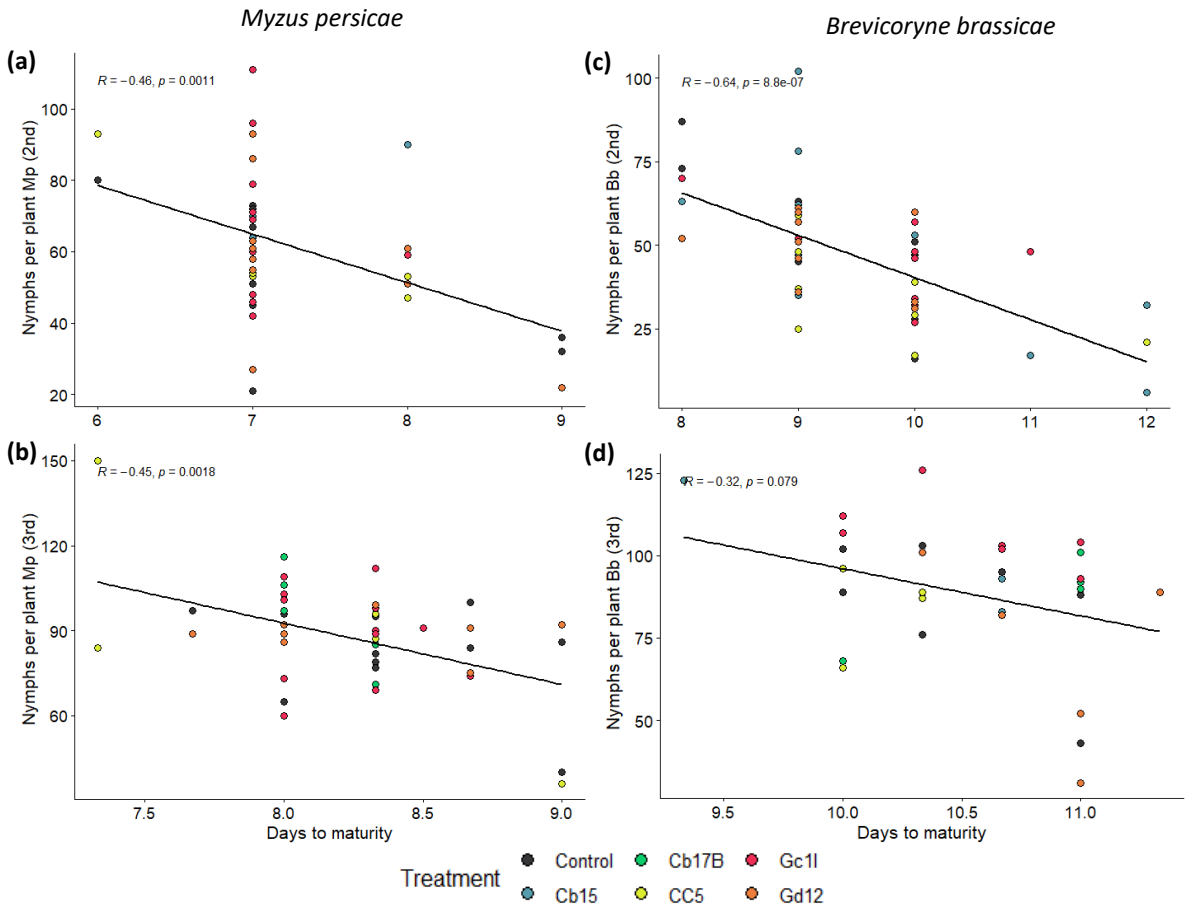

Figure 10 Correlation between the time that each nymph took to mature and the final number of nymphs per plant

A color-coded summary of the results of the three repetitions is presented in Figure 11.

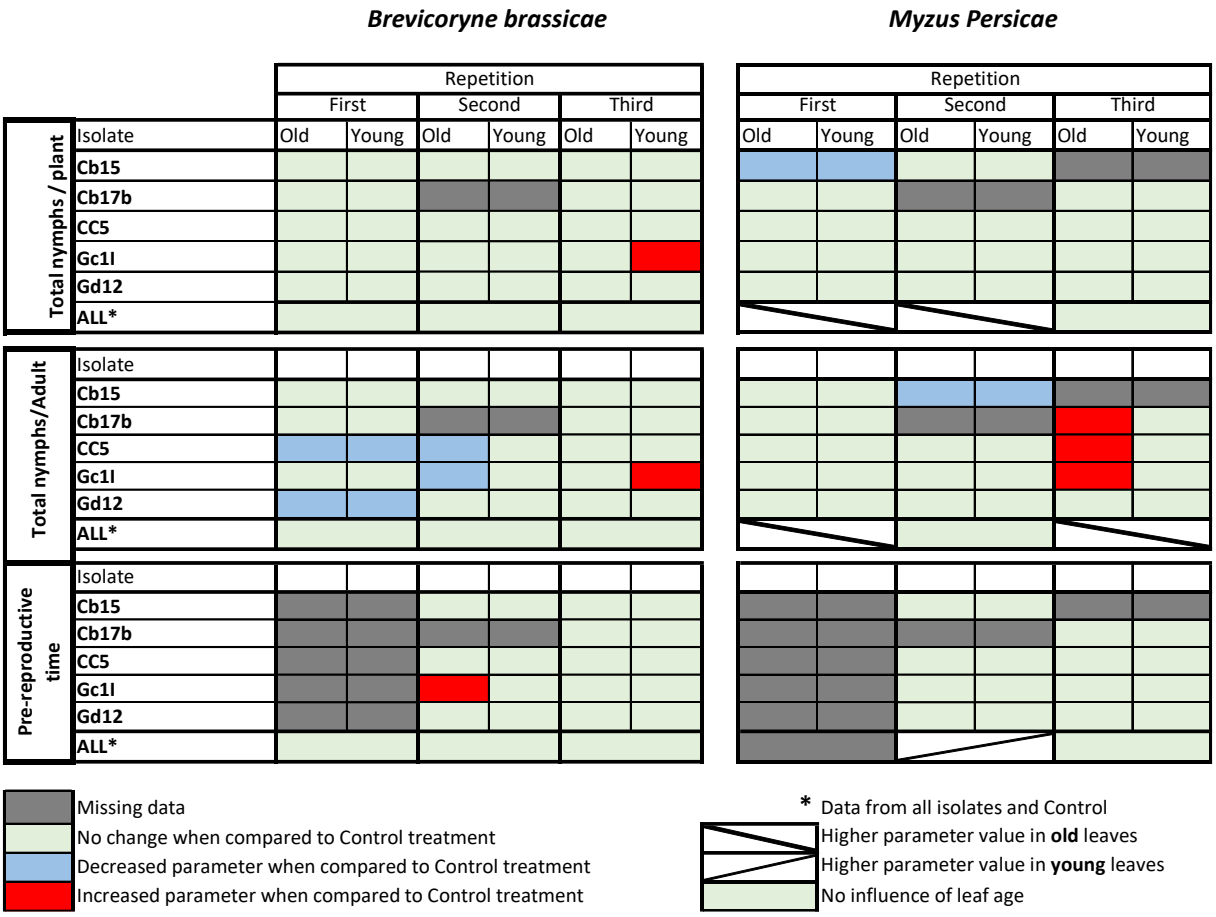

Figure 11 Color-coded summary of the performance of the aphids *B. brassicae* and *M. persicae* on oilseed rape plants inoculated with different *M. brunneum* isolates, with repetitions carried out under different conditions (see Methods)

Discussion

Plant interaction with *M. brunneum* increased leaf consumption by the Brassica specialist *P. chrysocephala* and increased the fecundity of the generalist aphid *M. persicae*, although not in a consistent manner. The present investigation and previously published studies show that the effects of entomopathogenic endophytes on insects are highly dependent on experimental conditions (Vidal and Jaber, 2015; Gange et al., 2019).

Endophytic M. brunneum effect on P. chrysocephala adult feeding

The cabbage flea beetle was found to consume more leaf area on plants that were inoculated with specific isolates of *M. brunneum*. Such increased consumption by herbivores may be due to changes in plant chemistry, which may involve changes in nutrition or defense mechanisms. Gange et al. (2012) observed a similar pattern with another specialist beetle, *C. rubiginosa*, where increased feeding occurred when *Cirsium arvense* plants were inoculated with either *C. cochliodes* or *C. cladosporioides* plant endophytes. According to the authors, these endophytes induced chemical changes in the host plant that benefited the specialist insect. Although plant metabolites were not measured in either the aforementioned study or the present study, the interaction between the plant and the fungus may have triggered

chemical changes that could be related to either nutritional aspects or plant defence responses.

Previous studies have reported that plant association with Metarhizium species results in growth promotion (see review by Jaber and Enkerli, 2017). Growth promotion has been attributed, in part, to improved plant nutrition due to nitrogen transfer from infected insects to plants and improved phosphorus and iron uptake (Behie et al., 2012; Sanchez-Rodriguez et al., 2016; Krell et al., 2018b; St. Leger and Wang, 2020). In addition, higher mineral and chlorophyll contents have been reported after EEF inoculation (Raya-Díaz et al., 2017; Krell et al., 2018c; Krell et al., 2018b; Alves et al., 2021).

No growth promoting effect was observed in this study. However, a positive correlation was found between fungal colonisation and aboveground plant biomass, specifically reflected in leaf area and shoot weight. This positive effect on plant biomass could possibly be attributed to improved nutrition. However, it is important to note that while improved plant nutrition may contribute to increased insect weight and performance, it does not necessarily result in increased consumption. In fact, lower nutritional quality in plants may induce compensatory feeding responses leading to increased herbivory. (Simpson and Simpson, 1990; Awmack and Leather, 2002; Wetzel et al., 2016).

Plants with improved nutrient availability could also produce higher levels of defence metabolites. For example, highly fertilized *B. napus* plants responded to the herbivory by *P. xylostella* by producing increased levels of sulphur, and this was associated with less foliage being consumed by the larvae (Sarfraz et al., 2009). Therefore, a second possibility is that the association with the fungus increased plant metabolites that stimulated the beetle to consume more. Indeed, entomopathogenic fungi (EPFs) directly affect plant defence responses. Studies indicate that *M. brunneum* and other EPFs can activate or inhibit plant defence responses by affecting gene expression and metabolite levels in an isolate-specific manner. For example, (Rasool et al., 2021b) found that while an isolate of *B. bassiana* and another of *M. robertsii* increased the production of benzoxazinoids in wheat and flavonoids in bean plants, whereas an isolate of *M. brunneum* reduced these metabolites, compared to the untreated control. Furthermore, inoculation with *M. anisopliae* increased isoflavonoids in soybean plants (Khan et al., 2012), and inoculation with a strain of *B. bassiana* increased terpene concentration in tomato plants (Shrivastava et al., 2015).

In this study, fungal inoculation may have increased plant defence metabolites, particularly glucosinolates, which are phagostimulants for *P. chrysocephala* (Bartlet et al., 1994; Giamoustaris and Mithen, 1995). In particular, the differences in leaf area consumption by the beetles were greater on the seventh day after the start of feeding compared to the first three measurement points. This suggests that the effect of the fungus-plant interaction was stronger after the onset of herbivory and became more evident after seven days. Plants treated with the fungus showed a greater response to herbivory, which may be due to priming, a phenomenon where the plant response to stress is stronger only after the plant has been challenged (Martinez-Medina et al., 2016a).

Influence of endophytic M. brunneum on the performance of specialist (B. brassicae) and generalist (M. persicae) aphids under different growth conditions

The experimental results highlight the significant influence of the microenvironment on the effect of fungal inoculation on aphid fecundity. This is demonstrated by the low consistency observed between repetitions, despite the use of the same fungal strains, plant variety, and soil mixture. Surprisingly, different and sometimes contradictory results were obtained in different repetitions of the experiments.

In particular, in the first repetition, *M. persicae* showed higher fecundity in older leaves regardless of treatment, while in the third repetition, three of the five treatments with fungal isolates resulted in higher fecundity in older leaves. In contrast, no effect of leaf age was observed in the specialist *B. brassicae*. These results are consistent with previous observations on the behaviour of these two aphid species on *B. napus* plants. Weber et al., (1986) found that while *B. brassicae* reproduced equally well on both new and old oilseed rape leaves, *M. persicae* performed better on older leaves than on young leaves. The better performance of the generalist aphid on older leaves is probably due to a lower glucosinolate content as the leaves age. One of the factors contributing to lower nymph production in younger leaves could be a longer pre-reproductive period. Although there were no significant differences in this response variable, the correlation shown in Figure 10 indicates that a longer pre-reproductive period resulted in a lower number of nymphs per plant. Another study also shows that *M. persicae* prefers older parts of Sinapis alba plants wich have lower GSL levels, whereas *B. brassicae* feed mainly on young tissues, where GSL levels were the highest (Hopkins et al., 1998), suggesting that glucosinolate levels not only affect the reproductive performance of aphids, but also influence their preference. However, another study shows that *M. persicae* preferred and performed better on younger cabbage (*B. oleracea*) leaves, even though they had higher GSL levels (Cao et al., 2018).

The effect of fungal inoculation on aphid performance was found to be highly variable and inconsistent. For example, in the second repetition, both aphid species showed reduced fecundity due to fungal inoculation, but by different isolates. Conversely, in the third repetition, both aphid species showed increased fecundity when treated with at least one of the isolates, but only on young leaves for the specialist and only on older leaves for the generalist. In particular, in the first repetition, where the plants showed severe light stress symptoms, two isolates resulted in reduced fecundity of the specialist *B. brassicae*.

The inconsistent results between repeated experiments reflect discrepancies in the scientific literature. Previous studies have reported an increase in aphid progeny in plants inoculated with *M. brunneum* (Clifton et al., 2018; Rasool et al., 2021b). However, other investigations have shown that inoculation of faba bean plants with two isolates of *M. anisopliae* has no effect on the growth of the *Acyrsthosiphon pisum* aphid population (Akello and Sikora, 2012), or results in reduced *M. persicae* development and fecundity when feeding on sweet pepper plants inoculated with *M. brunneum* (Jaber and Araj, 2018). It is worth noting that the outcomes of plant-microbe interactions are highly context dependent (Hartley and Gange, 2009), and small experimental changes can lead to different outcomes. For example,

rhizobacterial colonisation of Arabidopsis roots has resulted in both induced systemic resistance and induced systemic susceptibility to *Mamestra brassicae*, with the only difference being the presence or absence of sand in the substrate mix (Pangesti et al., 2015).

The present chapter includes five different isolates because the impact of plant-fungal endophyte on herbivores varies depending on the specific isolate and species (Lu et al., 2021). Under the conditions of each repetition, there were significant differences between the effects of some isolates on aphid fecundity, but there was again no consistency in the results between the three repetitions.

In general, aphid feeding triggers the activation of the salicylic acid (SA)-dependent pathway, regulated by salicylic acid, although jasmonic acid (JA) may also be involved (Pineda et al., 2013; Züst and Agrawal, 2016). Numerous studies have compared plant responses to *M. persicae* and *B. brassicae* in terms of gene transcription, hormone activation, and biochemical responses (Cole, 1997; Mewis et al., 2005; Kuśnierczyk et al., 2007; De Vos et al., 2007; Züst et al., 2012; Hodge et al., 2019), with conflicting results. Previous research indicated that both species induced a similar response in *A. thaliana*, involving both the JA and SA signalling pathways, albeit with quantitative differences in the expression profiles, with *M. persicae* inducing a moderately stronger response compared to *B. brassicae* (Mewis et al., 2005). Another study showed that several genes related to the octadecanoid pathway, leading to JA production, were up-regulated upon infestation by both species (Kuśnierczyk et al., 2007). However, a recent publication found that foliar concentrations of JA and SA increased in response to *M. persicae* but not by *B. brassicae* (Hodge et al., 2019).

Metarhizium colonization has the potential to modify the plant response to aphids by inducing a stronger activation of defense hormone pathways. Chapter 4 of this thesis shows that *M. brunneum* increases JA levels and alters the JA response to a root-feeder (Posada-Vergara et al., 2022). In general, aphids are less affected by plant responses activated by SA (Züst and Agrawal, 2016) whereas they appear to be susceptible to JA-induced responses (Züst and Agrawal, 2016). Interestingly, the performance of the specialist aphid *B. brassicae* was positively correlated with the concentration of aliphatic GSLs such as progoitrin and sinigrin, but negative correlated with JA-induced indole GSLs (Cole, 1997). In addition, the generalist aphid *M. persicae* showed greater resistance to JA-induced responses than the specialist aphid. For example, *B. brassicae* showed lower fecundity when feeding on the *A. thaliana* ecotype "Cape Verde Islands" (Cvi), that showed a stronger activation of the JA-related defense response to aphid feeding compared to the "Wassilewskija" ecotype (Kuśnierczyk et al., 2007). Furthermore, in a mutant plant with impaired JA signalling (*coi-1*), the population growth of the specialist aphid was higher compared to wild type plants, while the population growth of *M. persicae* remained unchanged. This confirms that the generalist aphid is more resistant to JA-related plant defenses than *B. brassicae* (Mewis et al., 2005). Therefore, the initial hypothesis that Metarhizium colonization would negatively affect generalist aphids but not specialist aphids was not supported by the results.

Changes in the nutritional quality of plants can significantly affect the performance of aphids. Phloem feeding insects such as aphids are particularly influenced by the free amino

acids present in phloem sap and the ratio of amino acids to sugars (Douglas, 2003). Conversely, elevated levels of phosphorus and potassium in plants can reduce the nutritional suitability for aphids (Powell et al., 2006). As discussed above, Metarhizium inoculation can directly affect the mineral nutrient content of plants. In addition, the nature of the hormonal response induced by Metarhizium could also affect nutrient quality. For example, external application of the hormones SA and JA to the leaves of *Plantago lanceolata* resulted in a decrease in amino acids in the phloem exudate, with SA also causing a decrease in several carbohydrates and organic acids (Schweiger et al., 2014).

Furthermore, previous studies have shown both differential and similar effects on the performance of *B. brassicae* and *M. persicae* following the inoculation of *A. thaliana* with beneficial microorganisms. For example, *Pseudomonas fluorescens* improved the performance of *M. persicae* and primed the plant for a stronger JA pathway-mediated response, while no significant effect was observed on *B. brassicae* (Pineda et al., 2012). Conversely, *Kosakonia radicincitans* led to a reduction in the population of both the specialist *B. brassicae* and the generalist *M. persicae* (Brock et al., 2018). It is worth noting that studying plant-aphid interactions can be quite challenging, as even small variations in plant age, development, and environmental conditions can significantly affect bioassay outcomes (J.C. Schultz, unpublished data (Mewis et al., 2005).

In conclusion, the effect of fungal inoculation on aphid fecundity is significantly influenced by the microenvironment, as indicated by the low reproducibility observed between repetitions. Despite using the same fungal strains, plant variety, and soil mixture, different and sometimes contradictory results were obtained between repetitions. These results are consistent with previous observations on the behaviour of these two aphid species, where aphid reproductive performance is influenced by glucosinolate levels and the pre-reproductive period. The effect of plant-microbe interactions on aphids is highly dependent on the specific fungal isolate and aphid species, while the results are strongly influenced by contextual factors. The contrasting results both within repetitions and in the existing literature highlight the complexity of the effects of fungal inoculation on aphid performance, which remains incompletely understood. Further research is needed to elucidate into the underlying mechanisms that govern plant-fungal-aphid interactions, and to better understand the potential application of fungal inoculation in pest management strategies.

Chapter 4.

Root Colonization by Fungal Entomopathogen Systemically Primes Belowground Plant Defense against Cabbage Root Fly

Catalina Posada-Vergara [1], Katharina Lohaus [1], Mohammad Alhussein [2], Stefan Vidal [1] and Michael Rostás [1]

[1] Agricultural Entomology, Department of Crop Sciences, University of Göttingen, Grisebachstr 6,
37077 Göttingen, Germany

[2] Molecular Phytopathology and Mycotoxin Research, Department of Crop Sciences, University of Göttingen, Grisebachstr 6, 37077 Göttingen, Germany

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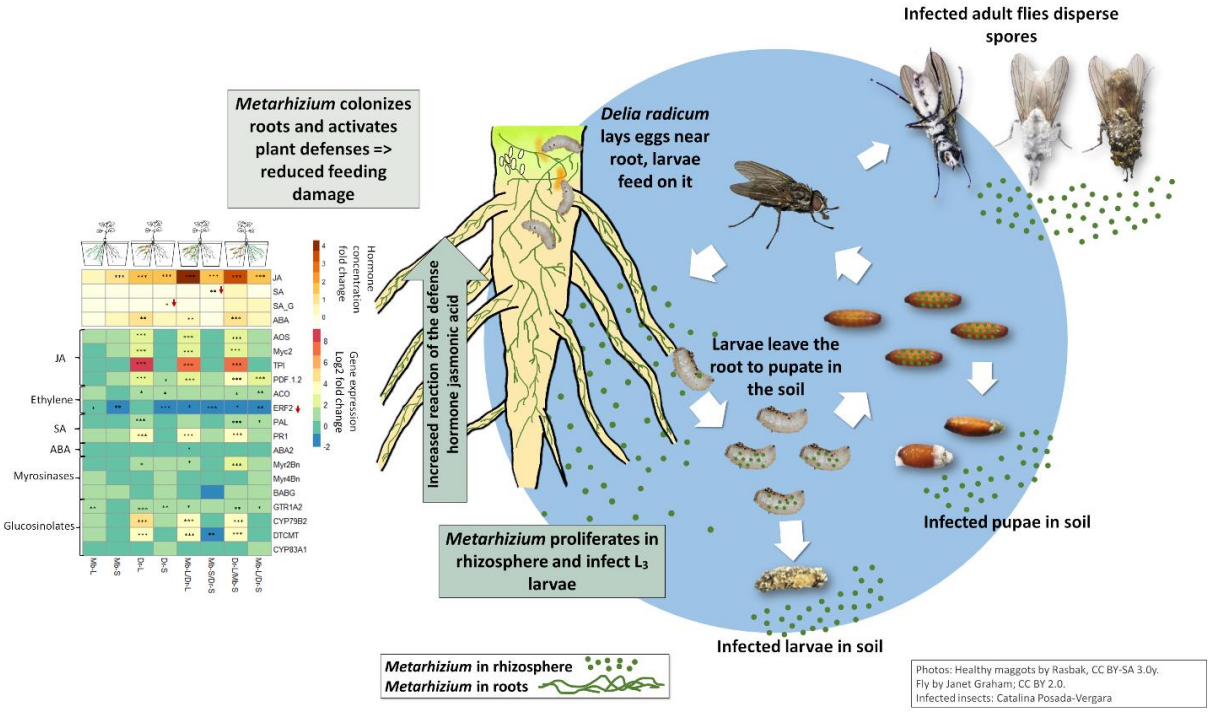

Abstract

Entomopathogenic fungi infect insects via spores but also live inside plant tissues as endophytes. Frequently, colonization by entomopathogens provides plants with increased resistance against insects, but the mechanisms are little understood. This study investigated direct, local, and systemic root-mediated interactions between isolates of the fungus *Metarhizium brunneum* and larvae of the cabbage root fly (CRF) *Delia radicum* attacking *Brassica napus* plants. All fungal isolates infected CRF when conidia were present in the soil, leading to 43–93% mortality. Locally, root-associated *M. brunneum* isolates reduced herbivore damage by 10–20% and in three out of five isolates caused significant insect mortality due to plant-mediated and/or direct effects. A split-root experiment with isolate Gd12 also demonstrated systemic plant resistance with significantly reduced root collar damage by CRF. LC-MS analyses showed that fungal root colonization did not induce changes in phytohormones, while herbivory increased jasmonic acid (JA) and glucosinolate concentrations. Proteinase inhibitor gene expression was also increased. Fungal colonization, however, primed herbivore-induced JA and the expression of the JA-responsive plant defensin 1.2 (PDF1.2) gene. We conclude that root-associated *M. brunneum* benefits plant health through multiple mechanisms, such as the direct infection of insects, as well as the local and systemic priming of the JA pathway.

Introduction

Species in the genus Metarhizium (Ascomycota, Clavicipitaceae) and Beauveria (Ascomycota, Cordycipitaceae) are widespread fungal pathogens of insects. These entomopathogens proliferate in the rhizosphere but can also live as endophytes inside plants, where they mainly colonize the root tissue (Hu and Leger, 2002; Bruck, 2005; St. Leger, 2008; Sasan and Bidochka, 2012). Due to these characteristics, entomopathogens are potential candidates for the control of soil-borne insect pests. Not only can the fungi infect insects that approach the root system in search for food, but as endophytes they may also induce changes in the plant that negatively affect insect performance (Vega, 2018; Gange et al., 2019). The mechanisms leading to reduced feeding or enhanced mortality in insects are not well understood, but recent studies have suggested that endophytic entomopathogenic fungi (EEF) may induce systemic resistance (ISR) in plants (Cachapa et al., 2020; Rivas-Franco et al., 2020). ISR is regulated by jasmonic acid (JA) and ethylene signaling and can be elicited by a variety of beneficial microbes, including plant growth promoting rhizobacteria and non-pathogenic fungi (van Wees et al., 2000; Pineda et al., 2010; Pieterse et al., 2014; Verma et al., 2016). However, apart from JA and ethylene, several beneficial microorganisms also induce salicylic acid (SA)-dependent resistance (Pieterse et al., 2014).

Plant responses to EEF vary considerably and happen to be isolate and species specific, ranging from suppression to the activation of hormonal pathways and defense-related metabolites. For instance, in *Arabidopsis thaliana* plants two endophytic strains of *Beauveria bassiana* showed significant differences in the upregulation of genes involved in JA and SA

signaling, phytoalexin synthesis, and other plant defense pathways (Raad et al., 2019). Likewise, a study with six different *B. bassiana* isolates demonstrated the differential activation of *Nicotiana benthamiana* genes involved in JA or SA signaling (Qin et al., 2021). Comparing two different EEF species, Rasool et al. (Rasool et al., 2021b) reported that *B. bassiana* and *M. robertsii* increased the production of defense-related benzoxazinoids in wheat and flavonoids in bean plants, while *M. brunneum* reduced the concentration of the same metabolites.

Apart from inducing plant responses, beneficial microorganisms can also mediate systemic resistance through priming (Pineda et al., 2010). Resistance conferred through priming is characterized by increased sensitivity to JA and ethylene due to microbe colonization, and in some cases involves the SA pathway (Martinez-Medina et al., 2013; Martínez-Medina et al., 2017). Primed plants show faster and/or stronger activation of cellular defenses when a biotic or abiotic stressor is perceived as a second trigger (Katz et al., 1998; Conrath et al., 2006; Pineda et al., 2010; Shikano et al., 2017). Priming has also been demonstrated in EEF recently. For example, *M. brunneum* primed cauliflower plants for increased myrosinase activity in response to herbivory by *Plutella xylostella* (Cachapa et al., 2020). Myrosinase is a crucial enzyme in the glucosinolate defense system of Brassicaceae.

So far, root-colonizing EEF have been shown to affect herbivorous insects systemically when feeding on aboveground plant parts (Cachapa et al., 2020; Rasool et al., 2021b). Whether similar indirect interactions also exist belowground, where EEF induce or prime defenses against herbivores feeding on uncolonized root tissues, has not been evaluated so far. However, split-root systems have been used to demonstrate the systemic effects of other root-associated fungi such as *Fusarium oxysporum*, *Pochonia chlamydosporia*, or *Trichoderma* spp. on phytopathogenic nematodes (Vu et al., 2006; Hao et al., 2012; Martinuz et al., 2015; Martínez-Medina et al., 2017; Ghahremani et al., 2019).

The cabbage root fly (CRF) *Delia radicum* (Diptera, Anthomyiidae) is an economically important pest of several Brassicaceae crops in the temperate zone (Ferry et al., 2009). The female fly lays its eggs into the soil, close to the base of the plant. After hatching, the first instar larvae feed on root hairs and then migrate to the tap root where they feed on periderm, phloem, and parenchyma tissue (McDonald and Sears, 1992). Wounded roots are entry points for soil-borne diseases (Keunecke, 2009) and when infestation rates are high, inflicted root damage can lead to decreased yields (Soroka et al., 2020).

Previous studies have shown that CRF larvae are susceptible to *Metarhizium* spp. (Vänninen et al., 1999b; Bruck, 2005; Razinger et al., 2014b; Myrand et al., 2015) and that plants inoculated with *M. anisopliae* were less damaged by the insect (Vänninen et al., 1999a). As with plant responses, isolate-specific differences can be found in pathogenicity towards insects (Bruck et al., 2005; Myrand et al., 2015; Razinger et al., 2018a) and in the ability of *Metarhizium* spp. to form associations with plants (Pava-Ripoll et al., 2011; Angelone et al., 2018). Hence, the first objective of this study was to compare the pathogenicity of different isolates of *M. brunneum* in the presence and absence of *Brassica napus* plants and, furthermore, to establish their rhizosphere competence and endophytic capabilities. The

second aim was to elucidate whether any reduction in root damage must be attributed entirely to *M. brunneum* infecting CRF larvae in the rhizosphere (direct effects) or, additionally, to fungus-mediated plant responses (indirect effects). To this end, a split-root setup was used that enabled differentiation between local and systemic responses (Vu et al., 2006; Cabanás et al., 2017; Martínez-Medina et al., 2017; Rubio et al., 2019; Kafle et al., 2022). The results suggest that endophytic *M. brunneum* can protect *B. napus* plants in both ways: by infecting CRF larvae in the rhizosphere and by priming for jasmonic acid-dependent plant responses locally and systemically.

Materials and Methods

Study System

Oilseed rape plants (*B. napus* var. Penn) were grown from seeds provided by Norddeutsche Pflanzenzucht (Hans-Georg Lembke KG, Holtsee, Germany) in a non-sterile soil mix, consisting of field loam, sand, and vermiculite in a ratio of 2:1:0.25 (by volume). Plants were grown in a rearing room at 19 °C, 60–70% RH and a 16:8 h (L:D) photoperiod with high pressure sodium lamps. All in vitro experiments were incubated in a growth cabinet (Mytron**,** Bio- und Solartechnik GmbH, Heilbad Heiligenstadt, Germany) at 19 °C and 65% RH in darkness if not specified otherwise.

Isolates of *M. brunneum* were obtained from the in-house collection of the Division of Agricultural Entomology (see Table S1). To obtain spore suspension for all experiments, isolates were grown on potato dextrose agar (PDA) (Carl Roth GmbH, Karlsruhe, Germany) at 23 °C for 14 days. The conidia were removed from the hyphae by gently scraping the sporulating colony with a sterile glass slide and the conidia were suspended in 20 mL of 0.1% Tween 80 (Carl Roth GmbH, Karlsruhe, Germany). The suspension was filtered through a plastic gauze and adjusted to a final concentration of 1×10^7 mL^{-1}. Spore viability was assessed before each experiment.

CRFs were reared under controlled conditions at 20 °C, 60–80% RH and a 16:8 h (L:D) photoperiod. Adults were kept in rearing cages (30 × 30 × 30 cm, BugDorm, Megaview Science Co., Ltd., Taichung, Taiwan) and fed with a diet of dry food, consisting of dextrose, skim milk powder, soy flour, and brewer's yeast in a ratio of 10:10:1:1 (by weight) and wet food consisting of honey, soy flour, and brewer's yeast in a ratio of 5:1:1 (by volume). To stimulate oviposition, a glass Petri plate filled with washed coarse sand and a piece of rutabaga (*B. napus* L. var. *napobrassica*) was placed inside the cage for 24 h. The eggs were extracted from the substrate by flotation. To obtain larvae, 100 eggs were placed on rutabaga slices (300 g) that were previously scratched to facilitate penetration by the neonate larvae. Eggs were then incubated in plastic boxes (1 L) filled with washed sand (2–5 mm). After 21 days, third instar larvae (L_3) were carefully extracted from the rutabaga. All eggs and larvae were used in the following 2 h after flotation and extraction, respectively.

Susceptibility of CRF to Different M. brunneum Isolates in Substrate without the Plant

The pathogenicity of *M. brunneum* was evaluated on L_3 larvae since this is the stage that leaves the root tissue to pupate in the soil. The experimental unit consisted of 35 mm black film canisters filled with either 20 cm^3 of sterile silica sand or with the non-sterile soil mix. The substrate was inoculated by adding 1 mL of spore suspension on top of the substrate. Controls received 1 mL of 0.1% Tween 80. Three L_3 larvae were then released into each container, making sure the larvae buried into the substrate. The film canisters were covered with Parafilm M and placed in the growth chamber. Daily emergence of adult flies was evaluated from day 16 to 21 until no more adults emerged. The remaining pupae were recovered by flotation, surface-sterilized with 70% ethanol for 30 s, and rinsed three times in sterile water. The mycosis of the adults and remaining pupae was further evaluated by placing them in Petri dishes lined with wet filter paper and incubated at 23 °C and 65% RH in darkness. Plates were observed daily for 10 days. The experimental setup consisted of six treatments (five fungal isolates, one control), two substrate types with 12 replicate plants per treatment. All treatments were kept completely randomized in the climatic chamber. Variables measured were the number of adult flies that emerged and the total mortality of CRF (%) defined as

$$Motality\ \% = \frac{(mycosed\ larvae) + (mycosed\ and\ dead\ pupae) + (mycosed\ flies)}{introduced\ larvae} \times 100$$

Total mortality was also used to calculate Abbott's corrected mortality (Abbott, 1925).

Rhizosphere Competence, Endophytism, and Plant Protection of M. brunneum Isolates

Rhizosphere competence and the endophytic ability of five *M. brunneum* isolates in oilseed rape plants were evaluated, and their pathogenicity towards CRF was assessed (for isolates, see Table S1). Seeds were surface sterilized in 70% ethanol for 1 min and 2% sodium hypochlorite (Carl Roth GmbH, Karlsruhe, Germany) for 5 min. Seeds were then rinsed three times and sown in sterile silica sand. One week after sowing, seedlings were inoculated by root dipping in spore suspension for 30 min and then transplanted to square pots (13 × 13 cm, 2 L) filled with non-sterile soil mix. Control seedlings were mock inoculated with a solution of 0.1% Tween 80. The experimental setup consisted of six treatments (five *M. brunneum* isolates, one control) with 12 replicate plants per treatment. A complete randomized design was used.

Four weeks after the inoculation of the spores, plants were artificially infested with eight CRF eggs per plant. The eggs were placed 1 cm below the surface on the root collar of each plant using a fine camel hairbrush, and eggs were covered by a thin layer of soil. The hatchling larvae were allowed to feed on the roots for 30 days. At this point, larval development was expected to be completed. The pupae were recovered as previously described. The mycosis of pupae was evaluated at 2-day intervals. Emerging adults were placed on a new plate to avoid cross-contamination. The difference between inserted eggs and recovered pupae was counted as *missing larvae*, assuming they died at the egg or larval stage. Total mortality (%) was calculated as

$$Total\ motality\ \% = \frac{(missing\ larvae) + (mycosed\ pupae) + (mycosed\ flies)}{introduced\ eggs} \times 100$$

Damage to the root collar of the plant was visually evaluated using a scale ranging from 0% (no damage) to 100% (whole collar root surface damaged) according to (Abbott, 1925). The amount of damage caused by each surviving pupae was estimated by the ratio damage (%) to the number of pupae recovered in each pot/root compartment.

Quantification of Rhizospheric and Endophytic M. brunneum

To evaluate fungal colony forming units (CFUs) in the rhizosphere, three lateral roots with the soil still attached were placed in 50 mL Falcon tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany) with 25 mL of 0.1% Tween 80. Tubes were vortexed for 10 s and inverted five times every 30 min for 3 h. After sedimentation for 20 s, 0.1 mL of a 1:10 dilution was plated on 9 cm Petri dishes with a semi-selective medium (Strasser et al., 1996). Petri dishes were then incubated at 23 °C and 65% RH for 21 days in darkness. The fungal colonies were counted every 3 days starting 10 days after plating until no new colonies appeared. Colonies of *M. brunneum* were identified by their morphology. This procedure was slightly modified in the split-root experiment (Sections 2.5–2.7): after adding Tween 80 to the samples, the tubes were vortexed for 10 s and placed on a shaker in horizontal position for 20 min at 250 rpm. Samples were sonicated for 30 s, briefly vortexed, left to sediment for 20 s, and then plated as described above. This shortened the processing time and the sonication step ensured better spore release from soil particles (Satomura, 2007).

Endophytic colonization was measured with real-time quantitative PCR (qPCR) in a 2 cm segment of root collar from each plant (Section 2.3) or from each half of the split-root (Sections 2.5–2.7). Root segments were surface sterilized with 70% ethanol for 1 min and 2% sodium hypochlorite for 5 min and rinsed three times with sterile water for 30 s. Roots were frozen at −25 °C for 24 h, lyophilized in a freeze dryer (Martin Christ Freeze Dryers, Osterode am Harz, Germany) for 72 h, and milled with a mixer mill (Retsch MM 200) in a stainless-steel container with a 20 mm/32 g steel sphere (Retsch GmbH, Haan, Germany) for 30 s at maximum speed. DNA was extracted from 30 mg of root tissue using the cetyltrimethylammonium bromide (CTAB) buffer extraction method described previously (Brandfass and Karlovsky, 2006). DNA quality was verified on agarose (0.8%) gels. The CFX384™ Real-Time System with a C1000™ Thermal Cycler (BioRad, Hercules, CA, USA) was used for fungal DNA amplification and melting curve analysis. The primers used were specific for Metarhizium clade 1: Ma 1763 (CCAACTCCCAACCCCTGTGAAT) and Ma 2097 (AAAACCAGCCTCGCCGAT) (Schneider et al., 2011). Amplification was performed with 1:10 dilutions of the DNA extracts. The reaction mix contained the following: 5 µL of 2x qPCRBIO SyGreen Low-ROX (PCRBIOSYSTEMS), 0.2 µL of 10 µM of each primer, 1 µL of DNA template solution, and 3.6 µ of water to complete a final volume of 10 µL. Running conditions were: denaturation for 2 min at 95 °C, 40 cycles of a 5 s at 95 °C, 20 s at 66 °C, and a 10 s at 72 °C, with a final step at 72 °C for 5 min. Melting curves were obtained by increasing the temperature to 95 °C for 60 s and decreasing it to 55 °C for 60 s with a subsequent temperature

increase from 55 °C to 95 °C by 0.5 °C per cycle with continuous fluorescence measurement. Absolute fungal DNA per g of plant tissue was measured by comparing threshold cycle (Ct) values against DNA standards starting with a concentration of 100 pg μL^{-1} and decreasing with a 1:3 dilution factor. The threshold cycle and standard curves were generated by the Bio-Rad CFX Maestro software. The identity of the amplicon was verified by comparing its size using gel electrophoresis. The presence of DNA in the root was evaluated for 12 replicate samples per isolate (Section 2.3) or 24 samples per treatment, 12 from each root compartment (Sections 2.5–2.7).

Direct and Systemic Effects of M. brunneum Isolate Gd12 on CRF Survival and Root Damage: Split-Root Setup and Bioassay

A split-root system was designed to differentiate between the direct effects caused by fungal infection and the indirect effects as a result of induced changes in plant metabolism. Gd12 was used, as this isolate had shown the strongest negative impact on CRF in the previous tests. To obtain seedlings with more than one tap root for the split setup, surface sterilized seeds were placed in Petri dishes with half strength Murashige and Skoog basal medium (Sigma-Aldrich, St. Louis, MO, USA), which contained sucrose and was adjusted to a pH of 6.8. The final concentration of agar was 10% (*w/v*). Petri dishes were then placed in a vertical position at 20 °C in darkness. Three days later, the seedling taproot was removed at the root base and the lower half of the Petri dish was covered with a light blocking fabric and left in a vertical position for five more days. Afterwards, seedlings were transplanted into a split-root system consisting of a pair of square pots (11 × 11 cm, 1.5 L) filled with non-sterile soil mix. The seedling was held in the center with a polypropylene cylinder (made from a 3 mL pipette tip; 13 mm Ø × 25 mm), and seedlings were inoculated by drenching the roots with 1 mL of either spore suspension or 0.1% Tween 80 as a control. The experimental setup consisted of five treatments with 12 replicate plants per treatment:

1. Control—one compartment mock-inoculated with 0.1% Tween 80;
2. Fungus—compartment A: *M. brunneum* (Mb-L), B: 0.1% Tween 80 (Mb-S);
3. Herbivore—compartment A: CRF (Dr-L), B: 0.1% Tween 80 (Dr-S);
4. Fungus/herbivore (local)—compartment A: *M. brunneum* + CRF (Mb-L/Dr- L), B: 0.1% Tween 80 (Mb-S/Dr-S);
5. Fungus/herbivore (systemic)—compartment A: CRF (Dr-L/Mb-S), B: *M. brunneum* (Mb-L/Dr-S).

In the treatment codes above “L” and “S” refer to the local and systemic roots, respectively. The position of the plants in the growth chamber was completely randomized. The plants were kept under the same conditions as described above. Infestation with CRF eggs, pupal recovery, and damage assessment were carried out as described in Section 2.3; however, only four eggs per root compartment were used in this experiment.

Direct and Systemic Plant Responses to Fungus and Herbivore: Gene Expression

Gene expression was analyzed in root tissues obtained from the split-root setup described in Section 2.5. Plants were harvested 7 days after egg inoculation when larvae had fed for about 24 h. Roots were washed and a 2 cm collar root segment from each root compartment was sliced, snap-frozen in liquid nitrogen, lyophilized, and milled as described in Section 2.4.

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) from 20 µg of lyophilized ground plant tissue following the manufacturer's instructions. The integrity of RNA was evaluated by denaturing gel electrophoresis. Concentration and purity were assessed by checking the absorbance ratios of OD260/OD230 and OD260/OD280 using a microplate spectrophotometer (Epoch, Bio-Tek (Agilent), Santa Clara, CA, USA). The first strand of cDNA was synthesized from 1 µg of total RNA using Fast Gene® Scriptase II (Nippon Genetics Europe, Düren, Germany) using a mix (1:0.5 by volume) of oligo dT and random hexamers following the manufacturer's instructions.

Most of the primers used were published in previous studies (Table S2). Primers for the *BABG, BnMyr4, Myr2.*Bn1, and *DTCMT*.a genes were designed with Primer3 (v.4.1.0) (Koressaar et al., 2018) using *B. napus* specific gene sequences from Genbank (https://www.ncbi.nlm.nih.gov/genbank/) and Plaza 4.0. (https://bioinformatics.psb.ugent.be/plaza/ versions/plaza_v4_dicots/, accessed on 8 August 2020) databases.

Gene transcripts were measured by qPCR as described in Section 2.4, with the following specific conditions. The reaction mixture contained the equivalent of 5 ng total RNA, 5 µL of 2x qPCRBIO SyGreen Low-ROX (PCR biosystems™, London, England), 0.2 µL of 10 µM of each primer, 1 µL of DNA template solution, and 3.6 µL of PCR-grade water to complete a total 10 µL final volume. The following temperature program was run: 95 °C for 2 min, 40 cycles of 95 °C for 10 s, and 60 °C for 30 s. Amplicon specificity was controlled by melting curve analysis as previously described. The relative expression of each gene was calculated using the $2^{-\Delta\Delta CT}$ method, with correction for primer efficiency (Pfaffl, 2001), normalized to the endogenous reference gene *ACTIN*, and subsequently normalized to those in the control plants. The selected genes are involved in defense responses (See Table S2 for gene description). As markers for phytohormonal pathways we used *ABA2* for abscisic acid (ABA); *ACO* and *ERF2* for ethylene (ET); *PAL* and *PR1* for SA; and *AOS, MYC2, PDF 1.2*, and *TPI for* JA. As markers for glucosinolate (GSL) metabolism, we used *CYP83A1* and *CYP79B2* for indol and aliphatic GSL, respectively, *GTR1A2* for GSL transport; BABG*, Myr2.Bn*, and *BnMyr4* for GSL degradation; and *BnDTCMT.a* for the phytoalexin brassinin.

Direct and Systemic Plant Responses to Fungus and Herbivore: Phytohormone Analysis

Phytohormones were analyzed in root tissues obtained from the split-root setup described in Section 2.5. The extraction was performed following a modified method of Müller

and Munne-Bosch (2011) (Müller and Munné-Bosch, 2011). Briefly, 40 mg of freeze dried and ground root tissue was suspended in 0.5 mL cold extraction solution consisting of methanol/isopropanol (20:80, *v/v*) with 0.1% formic acid (*v/v*), and placed in an ultrasonic bath at 4 °C for 10 min. Afterwards, samples were shaken at 280 rpm at 4 °C for 2 h and centrifuged at 13,000 rpm at room temperature for 10 min. The supernatant was further cleaned by centrifugation twice for 10 min at 13,000 rpm. Then, 200 µL of supernatant was transferred into a HPLC amber glass vial and analyzed instantly. The following chemicals were used as authentic standards: jasmonic acid (Cayman Chemical, Michigan, USA), salicylic acid (Sigma-Aldrich, Steinheim, Germany), salicylic acid glucoside (synthesized in the Division of Molecular Phytopathology and Mycotoxin Research, University of Göttingen, Gottingen, Germany), and abscisic acid (Sigma-Aldrich, Steinheim, Germany). HPLC-MS was used for the quantitative analysis of phytohormones. The system consisted of 1290 Infinity II UHPLC (Agilent Technologies, Waldbronn, Germany) equipped with a Zorbax Eclipse Plus C18 column (1.8 µm; 50 × 2.1 mm; Agilent Technologies; column temperature: 40 °C). The HPLC system was coupled to a 6460 triple quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany) with an electrospray ion source (capillary voltage, 4 kV; nebulizer pressure, 60 psi; nitrogen flow, 13 L min^{-1}; nitrogen temperature, 350 °C). The separation was achieved through a binary gradient elution program at a flow rate of 0.4 mL min^{-1}. The mobile phase A was water/formic acid (99.9:0.1, *v/v*), and B was methanol/formic acid (99.9:0.1, *v/v*). The gradient program was as follows: isocratic B at 5% for 0.2 min; 0.2 to 6 min, a linear increase from 5% to 75% B; 6 to 6.50 min, from 75% to 98% B; 6.50 to 9 min, isocratic B at 98%; 9 to 9.50 min, return to 5% B; 9.5 to 13 min, isocratic B at 5% for re-equilibration. The injection volume was 5 µL. Phytohormones were quantified in multiple reaction monitoring mode (MRM). The acquisition details are listed in Table S3. The calibration curve included 12 points from 0.48 to 1000 µg L^{-1}. Blanks and quality control standards were analyzed regularly. Limit of quantification *(LOQ*) and limit of detection (*LOD*) values were determined based on the standard deviation of the blank (Wenzl et al., 2016) with the following formulas:

$$LOD = 3.9\frac{\mathrm{Sy}}{\mathrm{b}}$$

$$LOQ = 3.3 \times LOD$$

where Sy = standard deviation of the blank and b = slope of the calibration curve.

Data Analysis

Data exploration and statistical analyses were performed with R 4.0.3 [49] . We used generalized linear models (GLM, library MASS (Venables and Ripley, 2002)) with a binomial family distribution to evaluate adult emergence, pupae recovered per plant, and CRF total mortality (including larvae and pupae with mycosis as well as adults that developed mycosis after emergence). The percentage of damage that larvae inflicted to the root collar, and the ratio of damage per pupa were analyzed with a beta regression model for percentages (library betareg (Cribari-Neto and Zeileis, 2010)). For DNA and CFU values we used linear models with a quasi-Poisson family distribution due to the over dispersion of the data. Pupal weight

was analyzed by one-way ANOVA after confirming normal distribution and homoscedasticity of residuals. Results from pupal survival were corrected using Abbott's formula (Abbott, 1925) which standardizes results based on control mortality. Gene expression data and phytohormone concentrations were analyzed by linear models, using compartment as a factor. All, except *ACO* transcript data, were logarithmically transformed to meet the assumptions of normality. When a model was significant, we used Tukey's honest significance test (Library multcomp (Hothorn et al., 2008)) for post-hoc analysis.

Results

Susceptibility of CRF to Different M. brunneum Isolates in Substrate

All of the *M. brunneum* isolates tested caused significant mortality in CRF larvae (L_3). The isolates Gc1I and Cb15III significantly reduced the percentage of emerged adults when larvae were exposed to conidia in non-sterile soil mix when compared with control treatment (Figure 1a). If the substrate was not considered in the model, Gc1I and Cb15III were significantly different from the control (GLM binomial model, p = 0.002 and p = 0.025 respectively). However, a significant effect was also found for the interaction fungal isolate × substrate (p = 0.049). In non-sterile soil substrate, we observed reduced adult eclosion (Chi square test, Holm p-Value adjustment method, $d\,f$ = 5, χ^2 = 19.12, p = 0.004).

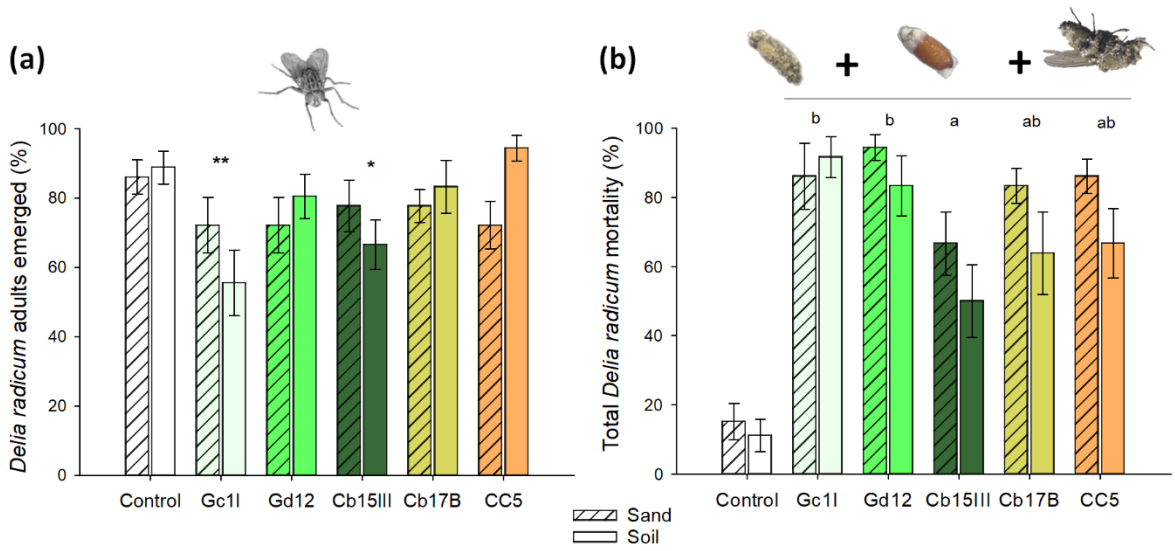

Figure 1. Susceptibility of Delia radicum (CRF) to isolates of M. brunneum in two different substrates (sterile sand = light grey bars; non-sterile soil substrate = dark grey bars). (a) Percentage of emerged CRF adults and (b) total mortality (larvae, pupae, and adult flies). The insects were exposed as larvae (L3) to substrate treated with fungal spores. Asterisks denote a significant difference from the control according to GLM, binomial distribution ($p < 0.05$) (Significance: *** 0.001; ** 0.01). Mortality caused by the isolates with different letters in (b) differ significantly from each other (Tukey HSD test, $p < 0.05$). Data represent means ± SE; n = 12.

When pupae and adults with symptoms of mycosis were included in the analysis, all isolates caused significantly higher mortality in CRF individuals compared to the control (Figure 1b). This was true for both soil and sand substrates (Table S4). Abbott's corrected mortality

was highest in Gd12 and lowest in Cb15III. With exception of the isolate Gc1I, significantly higher total mortality of CRF individuals was found in sterile sand compared to non-sterile soil mix (p = 0.005).

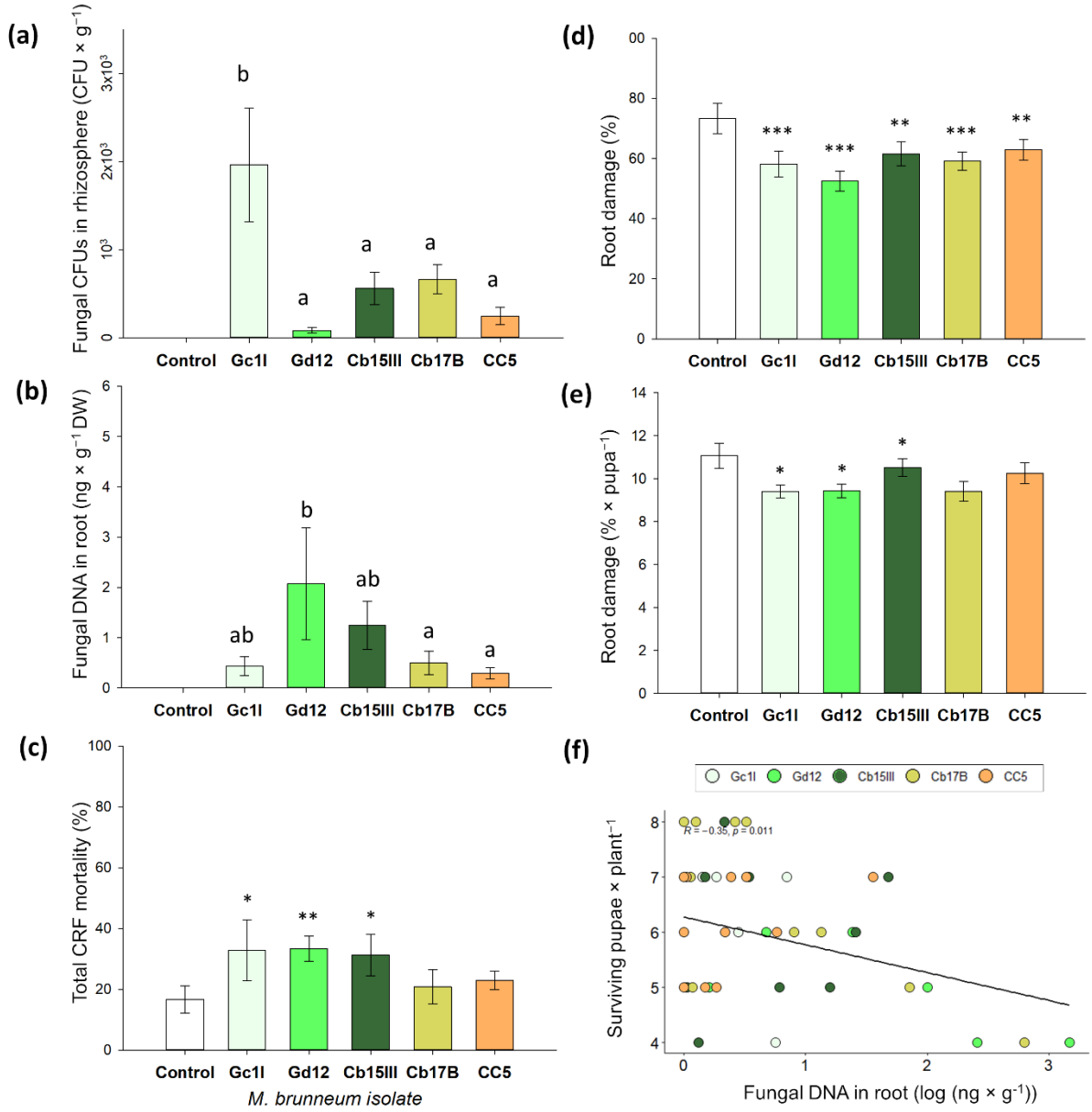

Figure 2 Colonization of rhizosphere and plant roots by isolates of *M. brunneum* and their effect on CRF survival and root damage. (**a**) Fungal colonization of rhizospheric soil (CFU g−^{1}); (**b**) fungal DNA in root collar tissue (ng × g^{-1} DW); (**c**) total CRF mortality; (**d**) root collar surface damage (%); (**e**) root damage caused per pupa recovered; (**f**) Pearson's correlation between *M. brunneum* DNA in root tissue and number of surviving pupae of CRF. Eight eggs of CRF were placed on the root collar of 5-week-old *Brassica napus* plants that had been inoculated with different *M. brunneum* isolates by root drenching at transplanting time (1 week). (**a**) Differences between isolates according to GLM with a quasi-Poisson family distribution (p < 0.05). Isolates with different letters differ significantly from each other (Tukey HSD test, p < 0.05). The asterisks above the columns indicate the significant difference from the control according to (**c**) GLM, binomial distribution (p < 0.05) and (**d**,**e**) percentage Beta-regression analysis (p < 0.05) (Significance: *** 0.001; ** 0.01; * 0.05). Data represent means ± SE; n = 12.

Rhizosphere Competence, Endophytism, and Plant Protection of M. brunneum Isolates

In this pot experiment, all five *M. brunneum* isolates were detected in the plant rhizosphere and in root tissue. Fungal CFUs varied from 84 spores g^{-1} of soil for Gd12 to 1963 spores g^{-1} for Gc1I, the latter being statistically different from the other isolates (Figure 2a; GLM, $p < 0.001$, Tukey HDS). Fungal DNA per gram of root collar tissue varied from 0.32 ng in CC5 to 2.07 ng in Gd12, the latter having significantly higher fungal DNA content than in isolates Cb17B and CC5 (Figure 2b; GLM, $p < 0.001$, Tukey HDS). In the control treatment, we found no CFUs in the rhizospheric soil and no fungal DNA in the root tissue.

Metarhizium brunneum isolates Cb15III, Gc1I and Gd12 significantly increased CRF total mortality when compared to the control (Figure 2c, Table S5). Abbott's corrected mortality was 20.0% for Gd12, 17.5% for Cb15III, and 15.0% for Gc1I. Additionally, all fungal isolates significantly reduced root damage compared to the control (Figure 2d, Table S6). Furthermore, we found a negative correlation between the amount of fungal DNA in the root collar tissue and the number of pupae recovered per plant at the end of the experiment (Figure 2f, Pearson's test, $r = -0.42$; $p = 0.0019$). We also detected significantly lower root damage per surviving pupae in isolates Cb17B, Gc1I, and Gd12 (Figure 2e).

Direct and Systemic Effects of M. brunneum Isolate Gd12 on CRF Survival and Root Damage: Bioassay

Plants successfully developed two similar root systems in our split-root setup. Root drenching of the seedling roots resulted in the colonization of the plant rhizosphere and root collar tissue. The fungus was only detected in the rhizosphere and roots of the compartments that were inoculated with *M. brunneum* (Figure 3a,b). No fungal CFUs in the rhizospheric soil or fungal DNA in the root tissue were detected in the untreated compartment of the split-root setup. The fungus was also absent in the controls.

Metarhizium brunneum (Gd12) caused a significant reduction in the number of CRF pupae when present in the same soil compartment as the larvae. The number of pupae recovered in roots of the systemic treatment was not significantly different from the control (Figure 3c, GLM binomial family, p (local) = 0.024; p (systemic) = 0.176). Abbot's corrected mortality was 32% in the local treatment and 16% in the systemic treatment. However, we observed a significant reduction in root damage (%) in both the local and systemic treatment when compared to the control (Figure 3d, beta regression model, p (local) < 0.001; p (systemic) = 0.03). Pupal weights did not differ significantly between treatments (control: 15.8 mg; local treatment: 16.2 mg, systemic treatment: 15.4 mg; F (2, 33) = 0.51, p = 0.603).

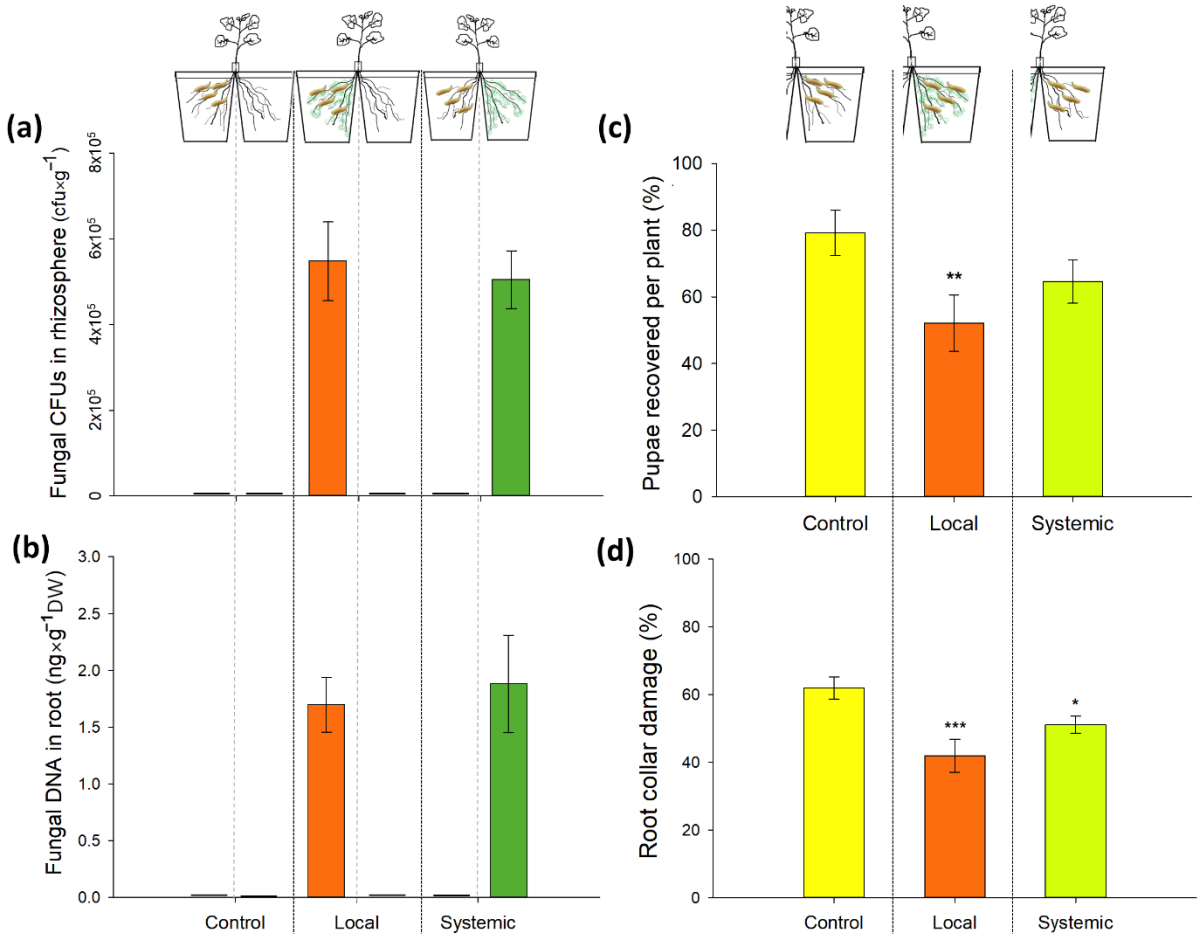

Figure 3. Local colonization of rhizosphere and plant roots by *M. brunneum* Gd12 and local and systemic effects on CRF survival and root damage. (**a**) Fungal colonization of rhizospheric soil (CFU g^{-1}); (**b**) fungal DNA in root collar tissue (ng × g^{-1} DW); (**c**) CRF pupae recovered; (**d**) root collar surface damage (%). Four eggs of CRF were placed on the root collar of 6-week-old plants in a split-root setup that had been inoculated with *M. brunneum* Gd12 either in the local or systemic compartment by root drenching at transplanting time (10 days after sowing). Asterisks above bars indicate significant differences from the control according to binomial GLM (**c**) or according to percentage Beta-regression analysis (**d**), ($p < 0.05$), (Significance: *** 0.001; ** 0.01; * 0.05). Data represent means ± SE; n = 12.

Direct and Systemic Plant Responses to CRF and M. brunneum: Gene Expression and Phytohormone Analysis

Local feeding by CRF larvae (Dr-L) led to an increased JA concentration ($\chi^2 = 6.07$, $p < 0.001$, Figure 4a) and an upregulation of the JA biosynthesis gene *AOS* ($\chi^2 = 5.92$, $p < 0.001$, Figure 5a) in all root compartments with CRF. Genes in JA downstream signaling were also upregulated as shown by higher expression of *MYC2* ($\chi^2 = 8.38$, $p < 0.001$; Figure 5b), *TPI* ($\chi^2 = 10.06$, $p < 0.001$; Figure 5c) and *PDF 1.2* ($\chi^2 = 4.16$, $p < 0.001$; Figure 5d). Larval feeding also induced the salicylic acid (SA) pathway but only at the gene expression level. While no increased concentrations of SA metabolites were observed (Figure 4c,d), there was an enhanced transcription of *PAL* ($\chi^2 = 4.44$, $p < 0.001$; Figure 5g). Enhanced expression was also measured for *PR1* ($\chi^2 = 5.42$, $p < 0.001$ Figure 5h). Abscisic acid (ABA) concentrations were higher in the compartment with larval feeding than in the control plants ($\chi^2 = 2.30$, $p =$

0.026 Figure 4b) but *ABA2* was not upregulated (Figure S1a). The ACO ethylene synthesis gene also showed higher levels of transcripts in response to insect feeding (χ^2 = 2.46, p = 0.018 Figure 5e) but not the downstream signaling gene *ERF.2* (Figure 5f).

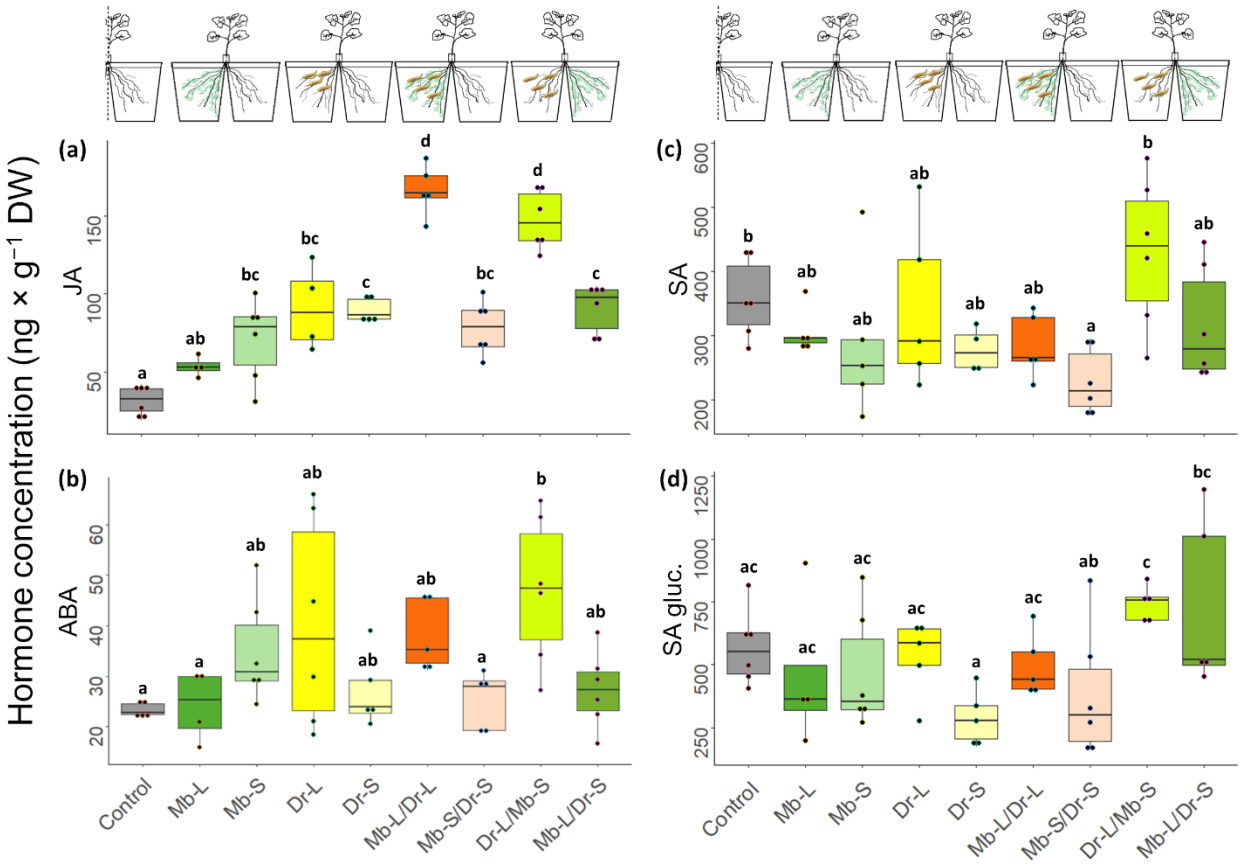

Figure 4. Phytohormone concentration of (**a**) jasmonic acid (JA), (**b**) abscisic acid (ABA), (**c**) salicylic acid (SA) and (**d**) salicylic acid glucoside (SA glu) in the tap roots of *B. napus* in a split-root setup. Local and systemic roots received the following treatments: mock inoculation with Tween 0.01% (control), *Metarhizium brunneum* in the local (Mb-L) or systemic (Mb-S) roots, CRF egg infestation in the local (Dr-L) or systemic (Dr-S) roots, both treatments in same roots (Mb-L/Dr-L), or different treatments on each split-root system (Dr-L/Mb-S; Mb-L/Dr-S). Plants were inoculated with *M. brunneum* at transplanting (10 d) and were harvested 7 days after egg infestation, 5 weeks after Mb inoculation. Different letters indicate statistically significant differences based on a linear model, and Tukey's post-hoc test. Boxplots show the distribution of the data, where the lower, middle, and upper lines represent the first quartile, the median, and third quartile, respectively. Data points represent independent biological replicates (n ≤ 6).

CRF feeding also activated the glucosinolate (GLS) defense system. The indole GLS synthesis gene *Cyp79B2* (χ^2 = 12.35, p < 0.001 Figure 5j) and the phytoalexin gene *DTCMT* (χ^2 = 10.69, p < 0.001 Figure 5k) showed enhanced expression when CRF larvae were present in the root compartment. *GTR1A2* was also upregulated (χ^2 = 4.11, p < 0.001 Figure 5i). Nevertheless, the expression of *CYP83A1*, involved in the synthesis of aliphatic GLS was similar to control plants (Figure S1d). Of the three myrosinase biosynthesis genes evaluated, only *Myr2Bn* (χ^2 = 2.56, p = 0.03 Figure 5l) was slightly upregulated by herbivory (other myrosinases, Figure S1b,c).

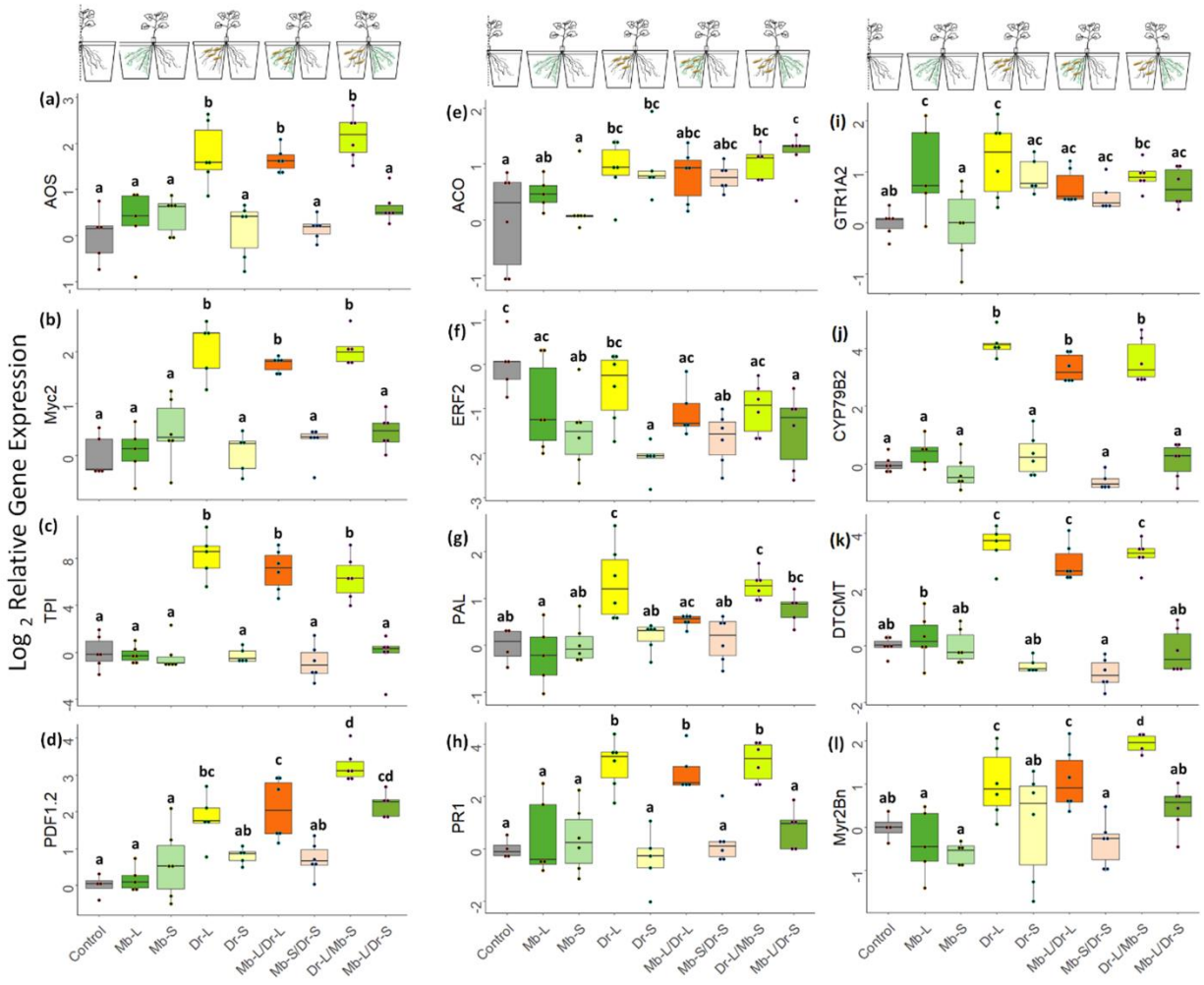

Figure 5. Expression of genes involved in defense signaling in taproots of *B. napus* in response to CRF herbivory or/and *M. brunneum* inoculation. Left panel shows genes related to jasmonic acid signaling (**a**–**d**). Central panel depicts genes involved in ethylene (**e**,**f**) and salicylic acid (**g**,**h**) signaling. The right panel shows genes involved in glucosinolate-related defense (**i**–**l**). The plants grew in a split-root setup in which each compartment had *M. brunneum* inoculation (Mb) in the local (L) or adjacent (S) compartment, CRF egg infestation (Dr) in the local (L) or adjacent (S) compartment, both treatments in the same compartment (Mb-L/Dr-L), or each in adjacent compartments of the same plant (Dr-L/Mb-S; Mb-L/Dr-S). Eggs were added 4 weeks after Mb inoculation. Plants were harvested 7 days after egg infestation. The letters represent statistically significant differences in the expression of the control treatment (Tukey HSD, $p < 0.05$). Boxplots show the distribution of the data, where the lower, middle, and upper lines represent the first quartile, the median, and third quartile, respectively. Data points represent independent biological replicates (n ≤ 6).

In general, biochemical responses of roots to CRF were limited to the root compartment with herbivory (Dr-L). We only detected a higher JA concentration in the systemic compartment (Dr-S) when compared with the control. (Dr-S; $\chi^2 = 6.47$, $p < 0.001$, Figure 4a). However, there was no change in the gene expression of any of the JA-associated genes (Figure 5a–d). Likewise, we did not detect a change in SA and ABA concentration (Figure 4b–d) or gene expression (Mb-S, Figures 5g,h and S1a) in the systemic compartment. The ethylene response factor *ERF.2* was downregulated (Dr-S, $\chi^2 = -4.53$, $p < 0.001$, Figure 5f). Glucosinolate

biosynthesis was not activated (Dr-S, Figure 5j), but there was upregulation of the GSL transporter gene *GTR1A2* (Dr-S, $\chi^2 = 2.70$, $p \leq 0.001$, Figure 5i). None of the genes involved in myrosinase synthesis were affected in the systemic compartment (Figures 5l and S1b,c).

Inoculation with *M. brunneum* alone (Mb-L) did not increase phytohormone concentrations (Figure 4) or the expression of genes involved in phytohormone biosynthesis or signal transduction (Mb-L, Figure 5), when compared to the control treatment. The expression of *GTR1A2* was upregulated ($\chi^2 = 3.23$, $p = 0.002$, Figure 5i) but the expression of myrosinase synthesis genes did not differ from the control. On the other hand, systemic roots (Mb-S) showed higher JA concentrations ($\chi^2 = 4.69$, $p < 0.001$, Figure 4a) and a lower gene expression of *ERF2* ($\chi^2 = -3.64$, $p < 0.001$, Figure 5f).

Metarhizium brunneum inoculation modified the jasmonate response induced by CRF both locally and systemically. Concentrations of JA increased 1.8-fold when the fungus colonized the same roots as the larvae and 1.6-fold when the fungus was in the systemic compartment (Mb-L/Dr-L and Dr-L/Mb-S, respectively, Figure 4a). At the gene expression level, the presence of the fungus in the adjacent compartment of the herbivore induced a higher expression of *PDF 1.2* (Dr-L/Mb-S, Figure 5d). *M. brunneum* did not modulate the expression of *GTR1A2*, *Cyp79B2*, *Cyp83A1,* or *DTCMT* in response to larval feeding (Mb-L/Dr-L and Dr-L/Mb-S). However, *Myr2Bn* was upregulated when *M. brunneum* was in the systemic compartment (Dr-L/Mb-S).

Discussion

Fungal endophytes and herbivores often associate with the root system of the same host plant. Therefore, the differentiation between the direct and plant-mediated effects of endophytic entomopathogens on herbivores is often not straightforward. To address this question, we first assessed the interactions between five *M. brunneum* isolates and CRF in the absence and presence of the host plant *B. napus*. The results showed that several fungal isolates were highly pathogenic in the soil substrate without plants and, after colonizing the roots, were capable of decreasing insect survival and plant damage. We then used a split-root setup to study the systemic plant-mediated effects of the isolate *M. brunneum* Gd12, and the results suggested that local and systemic plant defense responses against CRF play a role and can be primed.

Previous studies have shown that CRF is highly susceptible to different Metarhizium isolates in in vitro experiments. These bioassays were performed in the absence of soil (Vänninen et al., 1999a; Razinger et al., 2014b; Razinger et al., 2018a) or in sterile substrates (Bruck et al., 2005; Myrand et al., 2015). However, soil substrates may harbor a wide range of microorganisms that influence entomopathogenic fungi and their interactions with insects. As a result, the virulence of entomopathogens may be overestimated in sterile substrates (Lingg and Donaldson, 1981; Pereira et al., 1993; Parsa et al., 2018). Experiments with non-sterile soil were therefore carried out to confirm the pathogenicity of *M. brunneum* under more realistic conditions and to study isolate-specific effects. The bioassays showed significant reductions

in adult emergence when infected with the isolates Cb15III and GC1I and there was high mortality in adult flies for all isolates. The infected flies died shortly after eclosion and developed mycosis. So, overall, the tested isolates performed well in a non-sterile environment. It should also be noted that mycosis after adult fly eclosion has not previously been reported. From a biological control perspective, this aspect could be advantageous, since targeting juvenile CRF with soil application of *M. brunneum* may lead to increased infection rates in adult fly populations. The transmission of conidia between adult flies has been observed before and it is known that adult flies are susceptible to dry spores of *M. anisopliae* (Meadow et al., 2000).

In the experiment where *M. brunneum* was given the opportunity to colonize *B. napus* roots, three isolates were identified that significantly reduced pupal survival. All isolates though reduced the root collar area damaged by CRF larvae and were able to establish in the plant rhizosphere. Decreased herbivore damage was also observed under field conditions when cabbage plants were inoculated with *M. anisopliae* (Vänninen et al., 1999b). We expected lower pupal numbers in treatments where the fungal isolates showed higher colonization of the rhizosphere, as this may translate into more fungal spores being available to infect the insects. No negative correlation between CFUs and recovered pupae was apparent. However, this correlation was found for fungal DNA in the root tissue and the number of pupae recovered at the end of the experiment (see Figure 2f), confirming our expectation that endophytism may contribute to negatively affecting larvae. It is conceivable that more fungal biomass leads to stronger root defense priming. Nevertheless, this result must be taken with caution, as the negative correlation is driven mainly by two isolates.

Intraspecific variation in fungal performance is a common feature and fungal isolates may differ in their virulence to insects(Vänninen et al., 1999a; Razinger et al., 2014b; Razinger et al., 2018b), in rhizosphere competence (Moonjely and Bidochka, 2019), rhizoplane colonization (Razinger et al., 2014a; Moonjely and Bidochka, 2019), and endophytism (Moonjely and Bidochka, 2019; Rivas-Franco et al., 2020). Therefore, the selection of several isolates is necessary to understand the variability between isolates of a single fungal species. In this study, *M. brunneum* Gd12 was the most successful fungal isolate that caused high in vitro and in planta mortality in CRF. Interestingly, it was also the isolate with the lowest CFUs in the soil and the highest level of endophytic colonization. Hence, Gd12 was used in the split-root experiment to test whether the adverse effects of *M. brunneum* against CRF larvae were enforced by changes in plant metabolism.

Our observations confirmed that in the split-root setup, the fungus did not grow systemically in the roots of the adjacent compartment. Therefore, we assumed that the performance of CRF on systemic treatment was not the result of any direct interactions with the fungus. As in the previous experiment, *M. brunneum* Gd12 reduced both the number of pupae and the extent of plant damage by larvae in the local compartment. Similar effects were also found for the systemic compartment, although only the decrease in plant damage was statistically significant.

The negative effects on CRF that fed on roots in the systemic compartment (Dr-L/Mb-S) may be explained by defensive root responses as a result of fungal colonization and herbivore feeding. In general, the roots showed significantly increased JA concentrations in response to CRF, while the effects of *M. brunneum* on JA were considerably smaller. However, the highest concentrations of JA were measured in plants affected by both organisms, and this was irrespective of whether the fungus and herbivore were in the same (local) or different (systemic) compartments of the root system. The increased JA levels were accompanied by the upregulation of the myrosinase synthesis gene *Myr2Bn,* and the plant defensin gene *PDF1.2*, with higher expression in response to herbivory only when the fungus was in the systemic compartment (Dr-L/Mb-S). Interestingly, a priming response involving myrosinase enzymes was recently reported, where *M. brunneum* inoculation induced higher myrosinase activity in cauliflower plants attacked by *P. xylostella* (Cachapa et al., 2020). Moreover, the upregulation of *PDF1.2* was also observed in leaves of oilseed rape plants inoculated with another fungal endophyte, *Trichoderma harziarum,* in response to *Sclerotinia sclerotium* (Alkooranee et al., 2017). Therefore, our results suggest that the endophytism of *M. brunneum* primes the root for a stronger induction of JA upon herbivory.

Apart from JA, the phytohormones ABA and SA may be involved in plant responses to CRF (Karssemeijer et al., 2020). Abscisic acid signaling regulates responses to herbivory by co-activating the MYC branch of the JA pathway (Pieterse et al., 2012; Liu and Timko, 2021). However, in the present study, no clear pattern of ABA involvement was found (Figure 4b). Likewise, fungus and herbivore had no effects on the SA pathway as neither SA nor its glucosylated form (SA glu) were increased in the different treatments, when compared to control roots (Figure 4c,d). The SA pathway is important in the plant defense response to biotrophic pathogens (Pieterse et al., 2012) and also plays a role in modulating systemic responses to sap-sucking or cell content-feeding insects such as aphids and white flies (Van Poecke and Dicke, 2004; Lazebnik et al., 2014) and also chewing herbivores (Li et al., 2016; Karssemeijer et al., 2020). The observed upregulation of PAL and PR1 genes in our study may imply an involvement of SA signaling since PAL is at the base of the phenylpropanoid pathway that leads to SA biosynthesis. However, the phenylpropanoid pathway is also responsible for the biosynthesis of defense-related compounds such as lignin, coumarins, anthocyanins, and flavonoids (Lefevere et al., 2020). The pathogenesis-related protein PR1, a marker for SA downstream signaling, was strongly induced in all roots that were damaged by herbivory, irrespective of fungal presence in local or systemic roots. The results presented here confirm those of Karssemeijer et al., (2020) (Karssemeijer et al., 2020) who observed the increased expression of PAL in B. oleracea after 6 h and PR1 upregulation as a contributor in the Partial Least Squares Discriminant Analysis model of plant response to CRF herbivory at 24 h. However, PR-1 induction has also been reported as a response to *P. xylostella* herbivory in wild *B. oleracea* (Li et al., 2016). Pathogenesis-related genes are also induced by cys-nematodes (van Dam et al., 2018). Their activation in response to CRF larvae and cys-nematodes could have similar purposes; both organisms cause damage to the root tissues. Therefore, SA activation could also provide protection against pathogen infection. It would be interesting to explore the role of other components of the phenylpropanoid pathway in plant

defense against specialized Brassica herbivores, such as CRF. We observed a red–purple coloration of the root in the feeding zone in plants harvested for biochemical analysis (~24 h after initial feeding), suggesting the presence of anthocyanins. Furthermore, other Brassica specialists such as *Pieris brassicae* and *Phyllotreta nemorum* upregulated phenylalanine, flavonoids, and phenolic acids (Kovalikova et al., 2019). Furthermore, lack of induction of kaempferol-3,7-dirhamnoside levels after MYB75 overexpression, led to a loss of resistance of A. thaliana plants to *P. rapae* feeding (Onkokesung et al., 2014) and QTL resistance of canola to *Ceutorhynchus obstrictus* (cabbage seed pod weevil) correlated with a peak of the flavonoid kaempferol 3-O-sinapoylsophoroside 7-O-glucoside (Lee et al., 2014).

Glucosinolates are characteristic defense compounds in Brassicaceae that can be induced by herbivory via the JA pathway (Pierre et al., 2012; Van Geem et al., 2016; Hennies, 2016; Touw et al., 2020). The data presented here confirmed the upregulation of GSL marker genes in response to CRF (Sontowski et al., 2019; Touw et al., 2020). Larval damage to the roots of *B. napus* strongly increased the transcription of the indole-GSL gene *CYP79B2* and of *DCT-MT.* The latter gene encodes a dithiocarbamate S-methyltransferase, which catalyzes the final step in brassinin biosynthesis. DCT-MT induction in response to CRF has not been reported previously though. The phytoalexin brassinin contains a dithiocarbamate group that has insecticidal properties (Pedras and Yaya, 2010) and may thus play a role in defense against CRF larvae. Local induction of the myrosinase gene *Myr2Bn* was also found predominantly in roots with herbivore presence, although this was only statistically significant for the Dr-L/Mb-S treatment. Although induced by herbivory, GLS does not appear to greatly affect CRF development or feeding behavior. This is probably due to the microbiome in the larval gut that is capable of detoxifying GLS-derived isothiocyanates (Björkman et al., 2014; Van Geem et al., 2016). However, for the attacked plant, activation of the GSL defense system in the wounded roots could be a strategy to prevent secondary infection by microbial pathogens (Tsunoda et al., 2018; Touw et al., 2020).

Conclusions

This study showed that *M. brunneum* infects various life stages of the cabbage root fly and readily colonizes available plant roots. The capability of the different *M. brunneum* isolates to grow inside root tissue as an endophyte correlated positively with their impact on the herbivore, which suggests a supporting role for the plant in this interaction. By using a split-root setup, we demonstrated for the first time that endophytic colonization by an entomopathogenic fungus can prime plant defense responses against a root feeding herbivore, thus resembling similar interactions with other beneficial, root-associated microbes. The results further suggest an involvement of the JA pathway in the priming response against CRF, although the exact nature of the defense needs further exploration. Future studies using a split-root design and -omics approaches should help to find other genes and metabolites that take part in this plant–fungal–insect interaction. Additionally, it would be worthwhile to examine whether the priming response protects the plant against secondary soil-borne infections. In

summary, we conclude that EPF as a biological control agent of the rhizosphere and as an endophyte can benefit plant health by multiple mechanisms.

Supporting Information

Table S1. List of the fungal strains used in the study

Strain	Crop regime	Location
GC1I	Semi-natural permanent grassland for at least 7 years. Grassland harvested twice a year	51°34'10.7"N 10°03'54.1"E
Gd12		
Cb15III	Winter wheat, barley or oilseed rape crop rotation, conventional farming	51°33'58.3"N 10°04'10.1"E
Cb17b		
CC5		

Table S2. Primers used for qPCR amplification of genes of *Brassica napus* root tissues

ID	Gen/accession	Pathway	Gene description	Sequence 5' --> 3'	Reference
ABA2	LOC106300040	Abscisic acid biosynthesis	Xanthoxin dehydrogenase	GCATCGCTCGTCTGTTCCAC	Karssemeijer et al., 2021
	XM_013736089			CGGCGAAGTCAACAGCGTTA	
ERF2	At5g47220	Ethylene (ET) signaling	Ethylene Response Factor 2	ATGTACGGACAGAGCGAGGT	Yang et al., 2010
				AAGCTTCGAAACCAACAAGTAACTG	
ACO	EV102889	ET biosynthesis	ACC oxidase	TCCGTCTGGGCTATCACTCT	Maag 2014
				GTGAGTGGGTCGATGTTCCT	
PR1	XM_013877950	Salicylic acid (SA) signaling	Pathogenesis-related protein 1	AAAGCTACGCCGACCGACTACGAG	Alkooranee 2017
				CCAGAAAAGTCGGCGCTACTCCA	
PAL	LOC106342153	SA synthesis-Phenylpropanoid pathway	phenylalanine ammonia-lyase 1	TCGCTATGGCTTCTTACTGCTCTG	Karssemeijer et al., 2021
	XM_013781008			GAGGTCTTACGAGATGAGATGAGTCC	
AOS	LOC106327419	Jasmonic acid (JA) synthesis	Allene oxide synthase	ACCGCTTGCGACTAGGGATC	Karssemeijer et al., 2021
	XM_013765565			CAAAGTCCTTACCGGCGCAC	
MYC2	EV120351	JA signaling	Basic helix-loop-helix (bHLH) DNA-binding family protein	GCAAAGCCCAGACAGAGAAC	Maag et al., 2014
				AGCTCACGCAACACCTTCTT	
TPI	EV144353	JA signaling	Trypsin inhibitor B-like	GTGGTATCACCATGAACCTTG	Maag et al., 2014
				GTTGACCACCTTAACCGGAA	
PDF1.2	EV163328	JA signaling	Defensin-like protein 16	TCCATCACCCTTCTCTTTGC	Maag et al., 2014
				TTTTGGCACGCATAGTCGTA	
ACTIN	AF111812	Housekeeping gene	Housekeeping gene	ATCGTCCTCAGTGGTGGTTC	Maag et al., 2014
				TTGATCTTCATGCTGCTTGG	
GTR1A2	Bra018096	Glucosinolate (GSL) transport	Glucosinolate Transporter 1 A2	ATTCACCTTCGGGGAACTGG	MENDELEY CITATION PLACEHOLDER 1
				TCGCTTGCTTCTGCTTGGTC	

CYP79B2	At4G39950	Indole GSLs biosynthesis	CYTOCHROME P450, FAMILY 79, SUBFAMILY B, POLYPEPTIDE 2	AAGAGGTTGTGCTGCTCCG	Tytgat et al., 2013 Also in Marthur
				TCCAAGTGAAACCTTGAAGAAGTC	
CYP83A1	At4G13770	Aliphatic GSL biosynthesis	CYTOCHROME P450, FAMILY 83, SUBFAMILY A, POLYPEPTIDE 1	CTCCTTATCCCTCGTGCTTG	Mathur et al., 2013
				TGTCGTAACCAGCGATCTTG	
BABG	LOC106429220	Myrosinase biosynthesis	Beta-glucosidase 27-like	CCGAGCGAGCTATGGAGTTT	This study
	XM_022718558			CGGCTTGTCTGGATCCACTT	
BnMyr4	LOC106430598	Myrosinase biosynthesis	PREDICTED myrosinase 4-like	TCAACTGCGACAATCCCCTT	This study
	XM_013871387			ATCACAAGCAAGGTCTCCGG	
Myr2.Bn1	LOC106382545	Myrosinase biosynthesis	B.napus myrosinase, thioglucoside glucohydrolase	TTGAAGGAGGGAGAGGTCGT	This study
	NM_001316199			AGCATTGAGTTCGCCCATCA	
DTCMT.a	LOC106392535	Phytoalexin brassinin biosynthesis	Dithiocarbamate S-Methyltransferase	TGTTCCACTGGACCTAACACG	This study
	XM_013833342			GGCCAAAGAAAGATCCGGGA	

Table S3. Acquisition parameters for phytohormones analysis.

Compound	RT [min]	Polarity	Parent Ion [m/z]	Fragmentor V	Collision Energy V	Product Ion [m/z]
Trans-zeatin	1.93	+	220.1	100	15	136.1
					9	202.1
					22	148.1
Trans-zeatin-d5	1.92	+	225.1	105	16	137.1
					10	207.2
Abscisic acid (ABA)	4.68	-	263.1	85	4	153.1
					5	219.1
					12	204.1
Abscisic acid-d6	4.67	-	269.1	88	4	159.1
					8	225.1
Jasmonic acid (JA)	5.20	+	211.2	85	8	133.1
					8	151.1
					5	193
Jasmonic acid-d5	5.19	+	216.1	85	8	135.1
					9	153.2
					7	198.2
Salicylic acid (SA)	4.05	-	137	140	15	93.1
					35	65.1
Salicylic acid glucoside (SA-Glu)	2.41	-	137	140	15	93.1
					35	65.1

Table S4. Statistical summary of GLM analysis with binomial distribution of the total mortality on in vitro experiments. Mortality includes mycosed larvae, pupae, and adults that developed mycosis after emerging.

Isolate	**L3 Larvae**			
	Estimate	SEM	z value	Pr(>|z|)
Intercept	-1.6398	0.3706	-4.425	9.65E-06***
Cb15III	2.339	0.4349	5.378	7.52E-08***
Cb17B	3.0474	0.4525	6.735	1.64E-11***
CC5	3.1991	0.4588	6.972	3.11E-12***
Gc1I	4.1194	0.5248	7.85	4.15E-15***
Gd12	4.1194	0.5248	7.85	4.15E-15***
Sand	-0.7047	0.2525	-2.791	0.00525**

Table S5. Statistical summary of GLM analysis with binomial distribution of the total mortality in planta experiment. Mortality includes mycosed larvae, pupae, and adults that developed mycosis after emerging. RCD: root collar diameter, included as covariant.

Factor	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
(Intercept)	2.9015	0.9581	3.028	0.00246**
Cb15III	-0.8301	0.3658	-2.269	0.02324*
Cb17B	-0.2827	0.3721	-0.76	0.44746
CC5	-0.4439	0.368	-1.206	0.22774
Gc1I	-0.853	0.3887	-2.195	0.02818*
Gd12	-1.0107	0.3561	-2.839	0.00453**
RCD	-0.1567	0.111	-1.412	0.15794

Table S6.

Statistical summary of beta regression analysis with of percentage of damage of root collar in planta experiment.

Factor	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	1.1920	0.1789	6.662	2.71e-11 ***
TreatCb15III	-0.7290	0.2491	-2.926	0.003432 **
TreatCb17B	-0.8382	0.2379	-3.523	0.000427 ***
TreatCC5	-0.6718	0.2394	-2.807	0.005005 **
TreatGc1I	-0.8858	0.2623	-3.377	0.000732 ***
TreatGd12	-1.1614	0.2415	-4.808	1.52e-06 ***

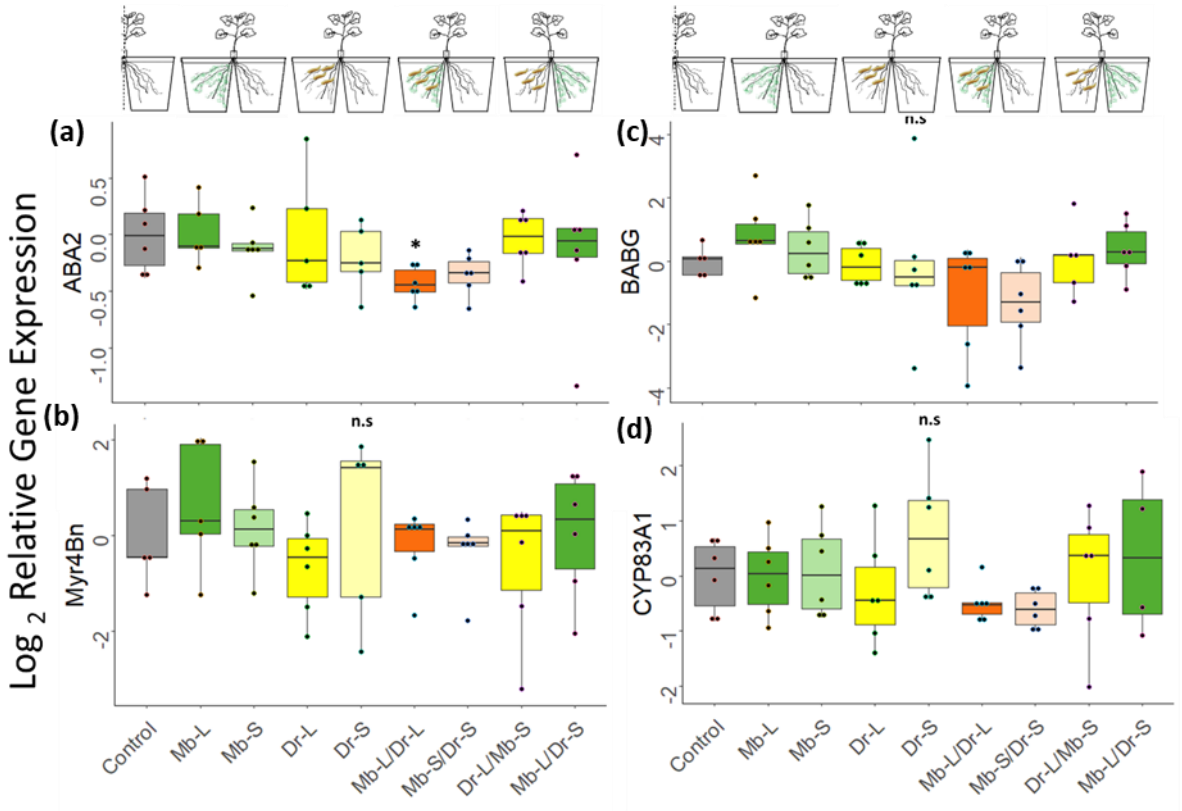

Figure S1. Normalized expression of representative genes of (a)abscisic acid biosynthesis *ABA2*, (b) myrosinase synthesis *Myr4Bn*, (c) beta-glucosidase biosynthesis *BABG* and (c) aliphatic GSL synthesis *CYP83A1*. Plants grew in a split root setup in which each compartment had either *M. brunneum* inoculation (Mb) in the local (L) or adjacent (S) compartment, *D. radicum* egg infestation (Dr) in the local (L) or adjacent (S) compartment, both treatments in same compartment (Mb-L/Dr-L), or each in adjacent compartments of the same plant (Dr-L/Mb-S; Mb-L/Dr-S). Eggs were placed 4 weeks after Mb inoculation. Plants were harvested 7 days after egg infestation. Gene expression was normalized to the housekeeping gene *AUXIN*. The boxplot shows all data points from at least 4 independent biological replicates (n ≤ 6) in which the horizontal line represents the median, surrounded by the upper (25th) and lower (75th) percentiles.

Chapter 5

Local competition and enhanced defense: how *Metarhizium brunneum* inhibits *Verticillium longisporum* in oilseed rape plants

Catalina Posada-Vergara [1], Stefan Vidal [1] and Michael Rostás [1],

[1]Agricultural Entomology, Department of Crop Sciences, University of Göttingen, Grisebachstr 6, 37077 Göttingen, Germany

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Abstract

Metarhizium brunneum is a soil-borne fungal entomopathogen that can associate with plant roots. Previous studies have demonstrated that root colonization by beneficial fungi can directly affect soil-borne pathogens through competition and antibiosis, and can activate a systemic response in plants, resulting in a primed state for a faster and/or stronger response to stressors. However, the mechanisms by which Metarhizium inoculation ameliorates symptoms caused by plant pathogens are not well known. This study evaluated the ability of *M. brunneum* to protect oilseed rape (*Brassica napus* L.) plants against the soil-borne pathogen *Verticillium longisporum*, and investigated whether the observed effects are a result of direct interaction and/or plant-mediated effects. In vitro and greenhouse experiments were conducted to measure fungal colonization of the rhizosphere and plant tissues, and targeted gene expression analysis was used to evaluate the plant response. The results show that *M. brunneum* delayed pathogen colonization of plant root tissues, resulting in decreased disease symptoms. Direct competition and antibiosis were found to be part of the mechanisms, as *M. brunneum* growth was stimulated by the pathogen and inhibited the in vitro growth of *V. longisporum*. Additionally, *M. brunneum* changed the plant response to the pathogen by locally activating key defence hormones in the salicylic acid (SA) and abscisic acid (ABA) pathways. Using a split-root setup, it was demonstrated that there is a plant-mediated effect, as improved plant growth and decreased disease symptoms were observed when *M. brunneum* was in the systemic compartment. Moreover, a stronger systemic induction of the gene *PR1* suggested a priming effect, involving the SA pathway. Overall, this study sheds light on the mechanisms underlying the protective effects of *M. brunneum* against soil-borne pathogens in oilseed rape plants, highlighting the potential of this fungal entomopathogen as a biocontrol agent in sustainable agriculture.

Introduction

Roots grow in the soil and share the same substrate with numerous microbes. It has been estimated that a gram of soil can contain up to 10 billion microorganisms (Torsvik and Øvreås, 2002). Moreover, plant roots deposit up to 40% of the carbon they fix during photosynthesis into the soil. This process, called rhizodeposition, creates a microenvironment that promotes a ten to 100 fold increase in microbial density around plant roots and a microbial community composition that is distinct from that found in bulk soil. (Hartmann et al., 2009; Bakker et al., 2013). This microenvironment, known as rhizosphere, is the site for multiple interactions between soil microbes and plant roots. Soil microorganisms can have beneficial effects on plants as they can facilitate nutrient uptake or protect against abiotic and biotic stresses (Berendsen et al., 2012; Pieterse et al., 2014).

Several Metarhizium species, primarily known as fungal pathogens of insects, are also rhizosphere competent. They exhibit enhanced growth around roots (Bruck, 2005; Hu and Bidochka, 2021) and can colonize plant tissues (Sasan and Bidochka, 2012; Behie et al., 2015; Hu and Bidochka, 2021). Together with other genera of entomopathogens such as *Beauveria* and *Lecanicillium,* these fungi have been referred to as “endophytic entomopathogenic fungi” (EEF). Several studies have reported positive effects of EEF on plants, including ameliorating salt stress (Khan et al., 2012; Verma et al., 2014), promoting growth (Jaber and Enkerli, 2017; Jaber, 2018; Ahmad et al., 2020), improving nutrient acquisition (Behie et al., 2012; Sanchez-Rodriguez et al., 2016; Krell et al., 2018c) and reducing insect and pathogen damage (Vega, 2018; Gange et al., 2019).

In the past decade, an increasing number of studies have demonstrated that different Metarhizium species can inhibit several plant pathogens in vitro (Sasan and Bidochka, 2013; Jaber and Alananbeh, 2018; Miranda-Fuentes et al., 2020). Moreover, colonization by Metarhizium can reduce pathogen-induced symptoms in various plants (Sasan and Bidochka, 2013; Jaber and Alananbeh, 2018; Rivas-Franco et al., 2019; Miranda-Fuentes et al., 2020; Gupta et al., 2022). However, the mechanisms by which Metarhizium species antagonize plant pathogens are not well understood (Ownley et al., 2010; St. Leger and Wang, 2020). Plant protection can result from a combination of competition and antibiosis (Sasan and Bidochka, 2013; Jaber and Alananbeh, 2018; Miranda-Fuentes et al., 2020). Metarhizium has been shown to strongly inhibit the growth of pathogens in vitro through the production of secondary metabolites that are toxic to microorganisms and insects (Moonjely et al., 2016). Mycoparasitism has been suggested as another potential mechanism, although this has not been observed with Metarhizium (Jaber and Ownley, 2018). Another mechanism suggested, is the activation of plant defenses. Beneficial microorganisms can trigger induced systemic resistance (ISR) in plants (Pieterse et al., 2014), which activates plant defenses and helps protect against pathogens. This has been observed with obligate symbionts like mycorrhiza (Hill et al., 2018; Dreischhoff et al., 2020) and facultative endophytes like *Trichoderma* or other non-pathogenic fungal strains (Shoresh et al., 2010; Pieterse et al., 2014).

Some studies have shown that EEF may modify plant defense pathways. For instance, peanut plants inoculated with *M. anisopliae* strain M202-1 showed downregulation of genes involved in the hypersensitive response and synthesis of resistance proteins (Hao et al., 2017). Increased defense responses have also been observed. The jasmonic acid (JA) and salicylic acid (SA) pathways are essential in the defense response and were activated by *B. bassiana* BG11 in *Arabidopsis thaliana* (Raad et al., 2019) and by the strains Bb0062 and Bb02 in *Nicotiana benthamiana* (Qin et al., 2021). The inoculation of *M. brunneum* Mb7 induced the expression of pathogen resistance genes like *PR1* that play a role in the SA pathway (Gupta et al., 2022). Levels of SA and JA hormones were also higher in oilseed rape plants inoculated with *M. brunneum* F52 (Cotes et al., 2020) and in maize plants inoculated with *M. anisopliae* A1080 (Rivas-Franco et al., 2020) and *M. robertsii* strain ARSEF 14325 (Ahmad et al., 2020).

Recent publications suggest that Metarhizium primes plant defense responses against insects (Cachapa et al., 2020; Posada-Vergara et al., 2022; Qing et al., 2023). The phenomenon of priming is associated with ISR, whereby plants exhibit a faster and/or stronger defense response when encountering a stressor if they have been previously exposed to a beneficial agent. In addition, it has been demonstrated that pre-inoculation with *M. brunneum* Mb7 increased reactive oxygen species production in tomato plants elicited with the fungal protein ET-inducing xylanase, which is an inducer of plant defense responses (Gupta et al., 2022), indicating that Metarhizium could prime plants against pathogens. However, to our knowledge, there is currently little knowledge on how Metarhizium modifies plant responses towards a pathogenic fungus.

Verticillium longisporum is a soil-borne pathogen that causes Verticillium stem striping in oilseed rape (*Brassica napus* L. spp. *oleifera*) (Depotter et al., 2016). Melanized microsclerotia present in the soil germinate upon induction by plant root exudates, and the resulting hyphae grow towards the root, colonizing the surface of the root hairs and penetrating the roots through rhizodermal cells (Eynck et al., 2007). Subsequently, the hyphae grow towards the central cylinder and enter the xylem vessels, spreading into aboveground organs. Upon plant tissue death, the pathogen produces microsclerotia that can remain in plant debris in the soil for more than 10 years (Depotter et al., 2016).

In this study we investigated the interactions between *M. brunneum* and *V. longisporum* during their colonization of oilseed rape plants. Our hypothesis was that, as soil and root inhabitants, both fungi could directly affect each other's growth and plant colonization. We also hypothesized that *M. brunneum*, as a plant symbiont, could modify the plant's response to *V. longisporum.* To study the direct interaction, we carried out in vitro dual confrontation assays and co-inoculation of oilseed rape roots with both fungi, where we measured fungal colonization of the hypocotyl and plant disease progress. In order to distinguish direct effects from plant-mediated effects, we used a split-root experiment to further explore how each fungus affected the other's colonization of the rhizosphere, roots, and hypocotyl. We also measured the local and systemic plant responses to each fungus by analyzing the expression of marker genes involved in plant defenses and explored whether *M. brunneum* could modify the plant's response to *V. longisporum*.

Materials and Methods

Study system

Metarhizium brunneum Cb15III was obtained from the in-house collection of the Division of Agricultural Entomology. The pathogen *Verticillium longisporum* Vl43 was provided by the Division of Plant Pathology and Crop Protection, University of Göttingen. To obtain spore suspensions, fungi were grown on potato dextrose agar (PDA) (Carl Roth GmbH, Germany) at 23 °C for 14 days. Spores were removed from hyphae by scraping the surface of the colony with a sterile glass slide and were suspended in 20 ml of 0.1% Tween 80 (Carl Roth GmbH, Germany). The suspension was filtered through a plastic gauze and adjusted using 0.1% Tween 80 to a final concentration of 1×10^7 spores ml^{-1} for *M. brunneum,* and 1.5×10^6 spores ml^{-1} for *V. longisporum*. Spore viability was assessed before each experiment by plating 100 µl of a 1×10^3 spores ml^{-1} distributed in 10 µl drops on PDA plates and counting the germinating colonies 48 h later.

Winter oilseed rape (*Brassica napus* var. Falcon) (Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, NPZ, Hohenlieth, Germany) susceptible to *V. longisporum* (Eynck et al., 2009) was used in this study. For all experiments, plants were grown in a non-sterile soil mix consisting of a mixture of commercial soil (Fruhstorfer Erde Typ 25, Hawita Gruppe GmbH), heat-treated (steamed) compost and sand (2:1:1, v:v). Plants were kept in a greenhouse with 18-25 °C, supplemented with light to obtain a 16:8 (L:D) h photoperiod.

In vitro confrontation assay

Antibiosis between *M. brunneum* and *V. longisporum* was evaluated with a confrontation assay. Single colonies of each fungal species were produced through streaking a drop of spore suspension from a glycerol stock over PDA media on a 90 mm plate. The plates were then incubated at 23 °C in the dark for 48 h. Single colonies were then transferred to a 90 mm PDA Petri dish. Each fungus was grown either alone or in dual culture with a distance of 4 cm between colonies. Plates were placed completely randomized inside the incubator. The plates were scanned at 14, 17 and 21 days after starting the confrontation assay and the scanned images were used to measure fungal colony area with the ImageJ software (Ver. 1.53f51) (Schindelin et al., 2012). Growth inhibition (GI) was calculated using the area of the colonies growing alone (control) and in dual culture with the formula:

$$GI = \frac{(area\ control - area\ in\ dual\ culture)}{area\ control}$$

For each treatment (single culture - dual culture x 2 fungi) 5 replicates (plates) were carried out.

In planta co-inoculation assay

To evaluate the effect of *M. brunneum* on the disease progress of *V. longisporum* infecting oilseed rape plants, a greenhouse experiment was conducted. Oilseed rape seeds were surface-sterilized in 1% sodium hypochlorite for 2 min, followed by 75% ethanol for 2 min

and rinsed three times with sterile water. Seeds were then germinated in sterile silica sand and grown for 24 days. Afterwards, seedlings were uprooted, and the root tips were cut off. The roots were then immersed in fungal spore suspension for 30 min. The treatments included the following:

1. Control: roots mock-inoculated with 0.1% Tween 80
2. Mb: roots inoculated with *M. brunneum* Cb15III
3. Vl: roots inoculated with *V. longisporum* VL 43
4. Mb/Vl: roots inoculated with a mix of both fungi

After inoculation, the seedlings were transplanted into square pots (11 x 11cm, 0.5 L). Each treatment had 25 biological replicates, and each replicate consisted of an individual pot with a plant. The plants were arranged in a completely randomized design in a greenhouse cabin. Disease assessment, plant parameters and fungal quantification in plant hypocotyl were done as described below.

Split-root assay

A split-root system was used to differentiate effects caused by direct fungal interaction from indirect effects via induced changes in plant metabolism. Split-root ready seedlings were produced as in (Posada-Vergara et al., 2022). The inoculation of fungi was sequential to give *M. brunneum* time to associate with the plant roots before the inoculation with *V. longisporum.* Seedlings were transplanted into two bound square pots (11 cm) filled with non-sterile soil mix. One of the split-root pots was inoculated by drenching the roots with 3 ml of either *M. brunneum* spore suspension or 0.1% Tween 80 (mock inoculation). Seven days later, *V. longisporum* was inoculated by pipetting into the soil 3 ml of spore suspension. The experimental setup consisted of 5 treatments, with each plant having two root compartments (C1 and C2):

1. Control: C1 = mock inoculation, C2 = untreated soil
2. Mb: C1 = M. brunneum (Mb-L), C2 = mock inoculation (Mb-S)
3. VL: C1 = V. longisporum (Vl-L), C2 = mock inoculation (Vl-S)
4. Local: C1 = both fungi present (Mb-L/Vl-L) C2 = both fungi absent (Mb-S/Vl-S)
5. Systemic: C1 = M. brunneum present, V. longisporum absent (Mb-L/Vl-S), C2 = V. longisporum present, M. brunneum absent (VL-L/Mb-S).

Where “L” means local inoculation and “S” means systemic, or that the inoculation was performed in the adjacent split compartment. Each treatment had 35 plants, and plants from all treatments were randomly distributed in the greenhouse cabin.

To gather samples for gene expression analysis, a second split-root experiment was conducted. The pathogen was inoculated by root dipping to ensure better exposure to fungal spores; for this, seven days after *M. brunneum* inoculation, the roots of the compartment to be inoculated were carefully taken out and placed inside a 2 ml Eppendorf tube filled with *V. longisporum* spore suspension for 20 min, after which the roots were covered again with soil. Roots in one compartment of the control treatment had the same manipulation, dipped in 0.1% Tween 80.

Disease assessment

In all experiments, plant height and disease severity was evaluated weekly until 35 days after fungal inoculation (co-inoculation) or *V. longisporum* inoculation (split-root), using the scale described in (Eynck et al., 2007):

1 = no symptoms
2 = slight symptoms on oldest leaves (yellowing, black veins)
3 = slight symptoms on next younger leaves
4 = about 50% of leaves showing symptoms
5 = more than 50% of leaves showing symptoms
6 = up to 50% of leaves dead
7 = more than 50% of leaves dead
8 = only apical meristem still alive
9 = plant dead.

Disease severity values were used to calculate the AUDCP values (area under the disease progress curve) according to the following formula (Madden et al., 2007)

$$AUDPC = \Sigma ((y_i + y_{i+1}) / 2) \times (t_{i+1} - t_i)$$

Where y_i is the value of the disease severity for the "i" observation, and t_i is the time after inoculation when the observation "i" was taken.

The dry weight of roots (co-inoculation), shoots (all) and leaf area (co-inoculation) were determined at 21, 28 and 35 days post inoculation (dpi).

Quantification of rhizospheric and endophytic M. brunneum

In the split-root experiment, we evaluated whether *M. brunneum* colonized the rhizosphere, and if it was affected by *V. longisporum.* We measured fungal colony forming units (CFUs) in the soil closely attached to the roots at 7, 21 and 35 dpi of *V. longisporum*. The complete root system with soil attached was placed in 50 ml Falcon tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany) with 25 ml of 0.1% Tween® 80. In order to release the rhizospheric soil from the roots, tubes were vortexed for 10 s, after which the roots were taken out for DNA extraction (see below). Tubes with rhizospheric soil were placed in a shaker in horizontal position for 20 min at 250 rpm. Samples were sonicated for 30 s, briefly vortexed and left to sediment for 20 s. Then, 100 µl of the supernatant was diluted 1:10 (v:v) and 100 µl from the dilution were plated on 9 cm Petri dishes with semi-selective medium (Strasser et al., 1996). The Petri dishes were incubated at 23 °C and 65% RH for 21 d in darkness. Fungal colonies were counted in three-day intervals, starting 10 days after plating until no new ones appeared. Colonies were confirmed as *M. brunneum* according to their morphology. Tubes with the original soil dilution were dried in an oven (at 60 °C for 5 days, until they reached constant weight, and the dry weight was recorded.

Fungal DNA in root (split-root experiment) and in hypocotyl (first pot experiment and split-root experiment) was measured with real-time quantitative PCR (qPCR). Roots were washed,

dried and placed in −20 °C. For each plant, a 2 cm segment of hypocotyl was sliced, surface-sterilized in 70% ethanol for 1 min, 2% sodium hypochlorite for 5 min, rinsed three times with sterile water for 30 s, and placed in −20 °C. Roots and hypocotyls were lyophilized for 72 h (Martin Christ Freeze Dryers, Osterode am Harz, Germany), and milled with a mixer mill (Retsch MM 400, Haan, Germany) in a stainless steel container with a 20 mm, 32 g steel sphere for 30 s at maximum speed. DNA was extracted from 30 mg of root tissue with the cetyltrimethylammonium bromide (CTAB) buffer extraction method described previously (Brandfass and Karlovsky, 2006), and DNA quality was verified in agarose (0.8%) gels. The CFX384™ Real-Time System with a C1000™ Thermal Cycler (BioRad, Hercules, CA) was used for fungal DNA amplification and melting curve analysis. The primers used for *M. brunneum* detection are specific for the Metarhizium PARB clade: Ma 1763 (CCAACTCCCAACCCC TGTGAAT), Ma 2097 (AAAACCAGCCTCGCCGAT) (Schneider et al., 2011). Specific primers used for *V. longisporum* were: OLG 70 (CAGCGAAACGCGATATGTAG) and OLG 71 (GGCTTGTAGGGGGTTTAGA) (Eynck et al., 2007). Amplification was performed with 1:10 dilutions of the DNA extracts. The reaction mixture contained the following: 5 µl of 2x qPCRBIO SyGreen Low-ROX (PCR Biosystems, London, UK), 0.2 µl of 10 µM of each primer, 3.6 µl of 1 µl of DNA template solution, completed to a total of 10 µl of final volume reaction. qPCR running conditions started with an initial denaturation for 2 min at 95 °C, followed by 40 reaction cycles consisting of 5 s denaturation step at 95 °C, 20 s annealing step at 66 °C (for *M. brunneum*) or 60 °C (for *V. longisporum*), and 10 s extension at 72 °C. The final elongation was performed for 5 min at 72 °C. Melting curves were obtained by heating the samples to 95 °C for 60 s and cooling them to 55 °C for 60 s followed by a temperature increase from 55 °C to 95 °C by 0. 5 °C per cycle with continuous fluorescence measurement. Absolut fungal DNA amount per g of plant tissue was measured by comparing threshold cycle (Ct) values against DNA standards starting with a concentration of 100 pg $\times$ μl^{-1}, and decreasing with a 1 : 3 dilution factor. The threshold cycle and standard curves were generated by the Bio-Rad CFX Maestro software. The identity of the amplicon was verified by comparing its size with gel electrophoresis. The presence of DNA in the root was evaluated in 5 replicates (first pot experiment) or in each root compartments from 8 plant replicates (split-root experiment) for each sampling date.

Gene expression in plant roots

Gene expression was analyzed in root tissues obtained from the second split-root experiment. Plants were harvested 7 days after *V. longisporum* inoculation as previous studies have found clear plant responses to the pathogen (Behrens et al., 2019; Zheng et al., 2019). Whole roots were washed and snap-frozen in liquid nitrogen, lyophilized, placed in a 2 ml Eppendorf tube with 4 stainless steel 0.5 mm spheres, and milled with a mixer mill for 60 s at 30 Hz.

Total RNA was extracted with RNAzol®RT (Sigma-Aldrich, St. Louis, MO, USA) from 20-30 µg of lyophilized ground plant tissue, following the manufacturer's instructions. The integrity of RNA was evaluated by denaturing gel electrophoresis. Concentration and purity was assessed by measuring OD_{260}/OD_{230} and OD_{260}/OD_{280} absorbance ratios using a microplate

spectrophotometer (Epoch, Bio-Tek). First-strand cDNA was synthetized from 1 µg of total RNA using Fast Gene® Scriptase II (Nippon Genetics Europe) and oligo dT, following the manufacturer's instructions. Primers used were published in previous studies (Table S1) with the exception of ICS2, which was designed using Primer3 v.4.1.0) (Koressaar et al., 2018) with *B. napus* specific gene sequences from the Genbank (https://www.ncbi.nlm.nih.gov/genbank/) data base.

Gene expression was measured by qPCR with the equipment described in 2.6. The reaction mixture contained the equivalent of 5 ng total RNA, 5 µl of 2x qPCRBIO SyGreen Low-ROX (PCRBIOSYSTEMS), 0.2 µl of 10 µM of each primer, 3.6 µl of 1 µl of DNA template solution, completed to a total of 10 µl of final volume. The program consisted on 95 °C for 2 min, 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Amplicon specificity was controlled by melting curve analysis as previously described. The relative expression of each gene was calculated using the $2^{-\Delta\Delta CT}$ method, with correction for primer efficiency, tested using a five-dilution series of the template (Pfaffl, 2001), normalized to the endogenous reference gene *ACTIN* and subsequently normalized to those in the control plants. For this study, we selected the following genes in the hormonal and glucosinolate (GSL) pathway: abscisic acid (ABA) biosynthesis: *ABA2* (*xanthoxin dehydrogenase*), ethylene (ET) biosynthesis: *ACO* (*1-aminocyclopropane-1-carboxylic acid oxidase*), ET downstream signaling: *ERF2* (e*thylene-responsive transcription factor 2*), salicylic acid (SA) synthesis: *PAL* (p*henylalanine ammonia-lyase*), *ICS2* (i*sochorismate synthase 2*), SA downstream signaling: *PR1* (p*athogenesis-related protein 1*), jasmonic acid (JA) synthesis: *AOS* (a*llene oxide synthase*), JA signaling: *PDF 1.2* (*defensin-like protein 16*), aliphatic GSL biosynthesis: *CYP83A1* (*cytochrome P450 83A1*) and indole GSLs biosynthesis: *CYP79B2* (*cytochrome P450 79B2*).

Data analysis

Data exploration and statistical analyses were performed with the software R 4.0.3 (*R* Core Team, 2017). DNA, CFU and gene expression data were log transformed and plant weight was transformed to the power of two to meet the assumptions of normality and homogeneity of variance, and analyzed with one-way ANOVA. Significant differences between treatments were evaluated post-hoc with the Fisher LSD test (Library agricolae, (Mendiburu and Yaseen, 2020)). AUDPC values were analyzed with a generalized linear model (GLM) with quasipoisson family due to overdispersion.

Results

Direct and Systemic effects of M. brunneum on V. longisporum plant colonization and disease development

Metarhizium brunneum inhibited in vitro colony growth of *V. longisporum* when grown in dual culture. The inhibition was already evident after 14 d, when the fungal pathogen area was 13% smaller in dual culture than in single culture. By day 23, the growth inhibition was 42% (Table S1, Figure 1). Vice versa, *V. longisporum* did not affect *M. brunneum* growth when

grown in dual culture. The colony area of *M. brunneum* was marginally higher at 17 d in the dual culture (p = 0.091; $F_{1,8}$ = 3.68; Table S1). Increased mycelia production (17 d), and localized spore formation (23 d) were observed on the side confronting the *V. longisporum* colony (Figure 1b).

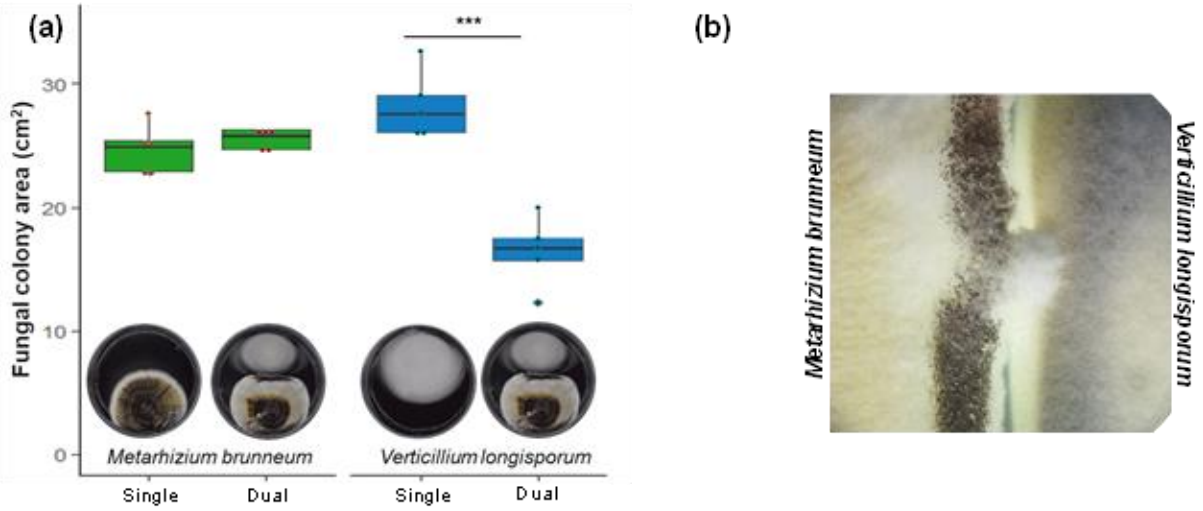

Figure 1. In vitro inhibition of *Verticillium longisporum* growth by *Metarhizium brunneum*. Fungal colonies grew in PDA media for 23 days either alone (single), or in confrontation with each other (dual). $F_{1,8}$= 44.8; p < 0.001, n=4. (**b**) Close-up of the confrontation zone.

Effect of co-inoculation M. brunneum on V. longisporum disease development

Co-inoculation with *M. brunneum* reduced the severity of symptoms of Verticillium stem striping disease. Although the shoot biomass of plants co-inoculated with both fungi was lower than that of control plants at 28 dpi, plants inoculated with both fungi had higher root dry weight (Figure 2b) and larger leaf area (Figure 2c) than plants inoculated with *V. longisporum* alone. The lower disease severity in plants co-inoculated with both fungi was reflected in the disease score which was lower at 21 and 28 dpi (Figure 2d). Furthermore, there was a delay in the colonization of the hypocotyl by the fungal pathogen in plants co-inoculated with *M. brunneum*. Specifically, the concentration of *V. longisporum* DNA increased between 21 and 28 dpi in plants inoculated with the fungal pathogen alone, while plants co-inoculated with both fungi had a lower amount of *V. longisporum* DNA at 28 dpi (Student's *t*-test, t = −2.35, df = 7.24, p = 0.025).

However, the protective effects of *M. brunneum* co-inoculation were no longer observed at 35 dpi, where there were no significant differences in plant growth parameters, disease score, or pathogen DNA in the hypocotyl between plants inoculated with *V. longisporum* alone and those co-inoculated with both fungi. We also measured *M. brunneum* in the hypocotyl tissues, which was detected only at 21 dpi (Figure 2f), with more plants showing endophytic growth in the dual inoculation treatment (3 out of 5 plants) than in the treatment with only *M. brunneum* (1 out of 5).

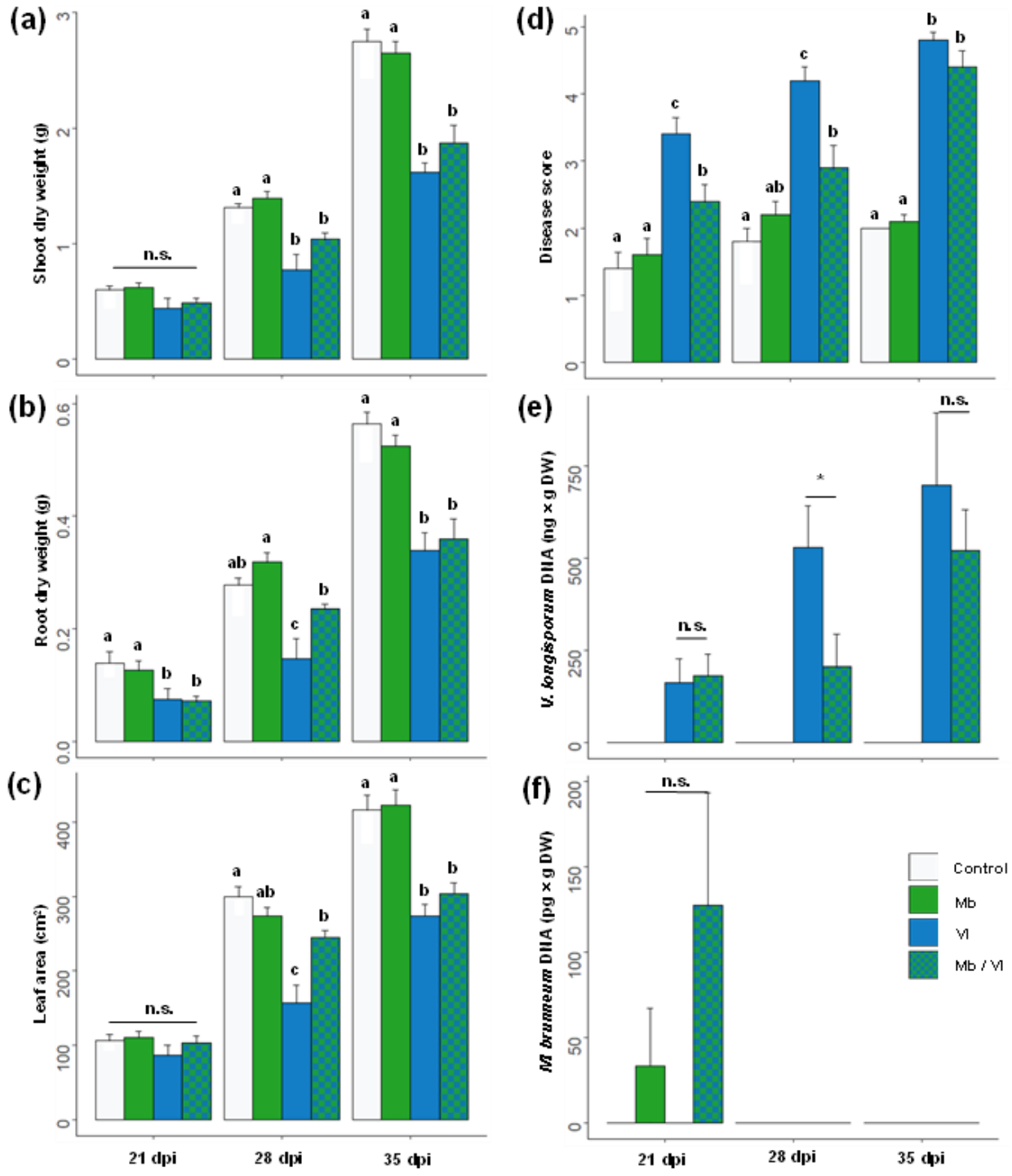

Figure 2. Effect of co-inoculation of oilseed rape roots with *M. brunneum* and *V. longisporum* on plant development and Verticillium disease progress. (a) plant shoot dry weight; (b) plant root dry weight; (c) leaf area; (d) disease score (see Eynck et al. 2007); (e) *V. longisporum* DNA in hypocotyl; (f) *M. brunneum* DNA in hypocotyl. Plants were inoculated by root dipping 21 days after germination. For the dual inoculation (Mb/Vl) a mix of spores was used. Plants were harvested at 21, 28 and 35 days post inoculation (dpi). Letters represent statistically significant differences within each date according to a linear model and Tukey's post-hoc test. *$p < 0.05$, Welch two sample *t*-test; n.s. = no significant differences. Bars represent means ± SE; n = 5.

The split-root experiment revealed both local and systemic effects of M. brunneum inoculation on *V. longisporum* DNA levels in the roots and hypocotyl, as well as on plant biomass and disease progression.

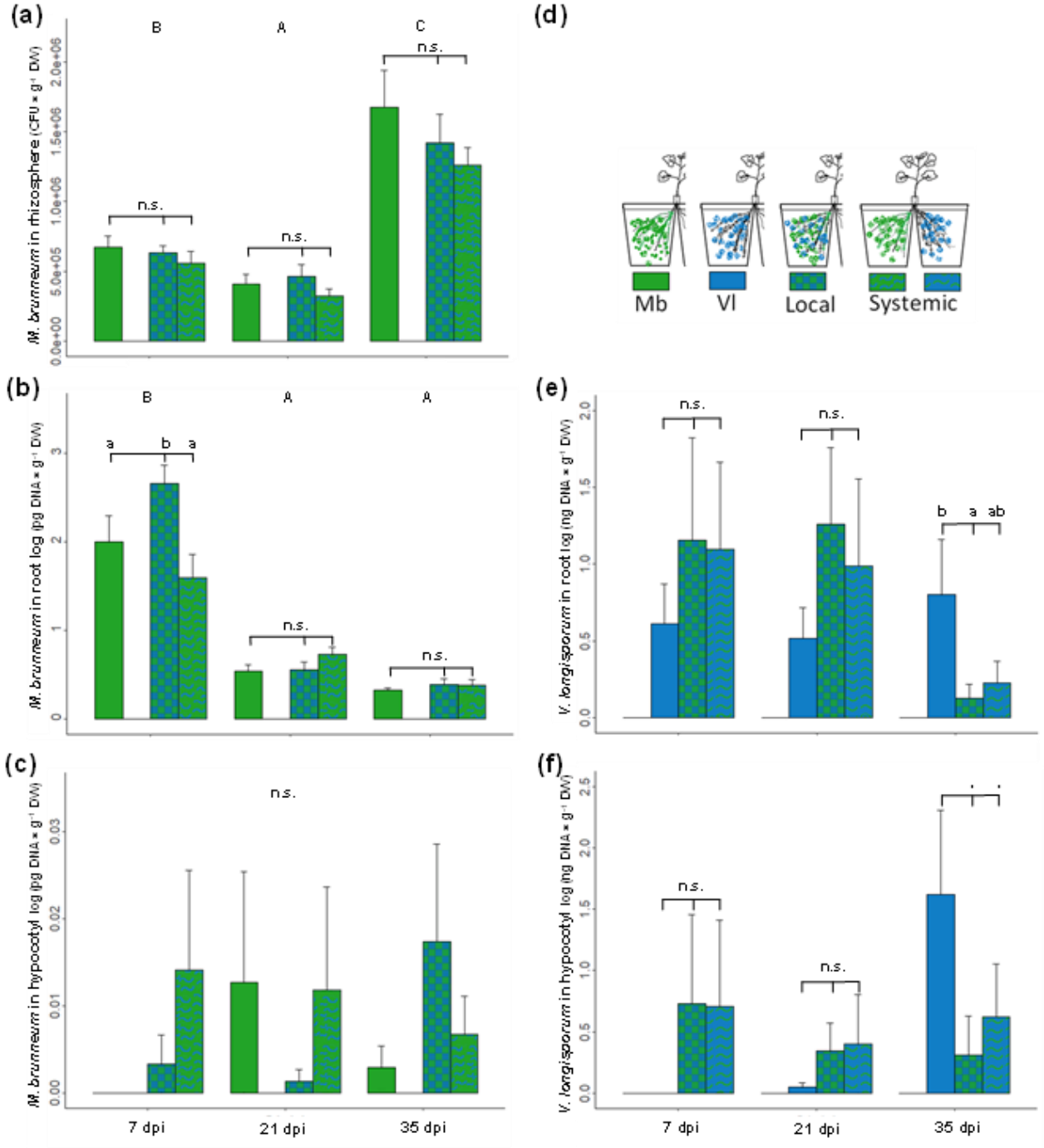

Figure 3. Colonization of rhizospheric soil (**a**), oilseed rape roots (**b, e**) and hypocotyls (**c, f**) by *M. brunneum* and/or *V. longisporum*. Plants grew in a split-root setup and were inoculated with fungal spores by root drenching. Treatment commenced with *M. brunneum* at transplanting (8 d), and was followed by *V. longisporum* after 7 d. Plants were harvested at 7, 21 and 35 dpi of *V. longisporum*. Panel (**d**) shows inoculation scheme and color patterns. Capital letters denote statistical differences between sampling dates, small letters denote significant differences among treatments within the same date. HDS Tukey; $p < 0.05$ according to a linear model; n.s. = no significant differences; bars represent means ± SE; n = 8.

Both fungi were detected only in the part of the root where they were inoculated. The biomass of *M. brunneum* in the rhizosphere, measured as the number of colony forming units (CFUs), was not affected by presence of *V. longisporum*. However *M. brunneum* CFUs changed over time, decreasing from 7 to 21 dpi and increasing at 35 dpi (Figure 3a). The amount of *M. brunneum* DNA in the roots was found to be significantly higher in the local compartment where *V. longisporum* was present, compared to roots where the entomopathogen was alone, or in the systemic compartment at 7 dpi (Figure 3b).

DNA of *M. brunneum* in the hypocotyl was low and detected in few plants; it was detected in one plant per treatment at seven and 21 dpi, and in about 30% of the plants at 35 dpi. There were no significant differences between treatments, but there was a tendency for a higher amount of DNA in the local treatment at 35 dpi (Figure 3c).

Verticillium longisporum concentration in plant roots was highly variable during the first three weeks (7 and 21 dpi) and was not affected by the presence of *M. brunneum.* However, pathogen DNA was lower in the local compartment at 35 dpi (Figure 3e, $p = 0.037$) and lower, though not significantly, in the systemic compartment ($p = 0.072$). Pathogen DNA in the hypocotyl (Figure 3f), was detected at 7 dpi, but only in one (Local) or two (Systemic) plants. At 35 dpi there was lower amount of *V. longisporum* DNA in the Local and Systemic treatments, but differences were only marginally statistically significant ($p = 0.061$ and $p = 0.092$, respectively).

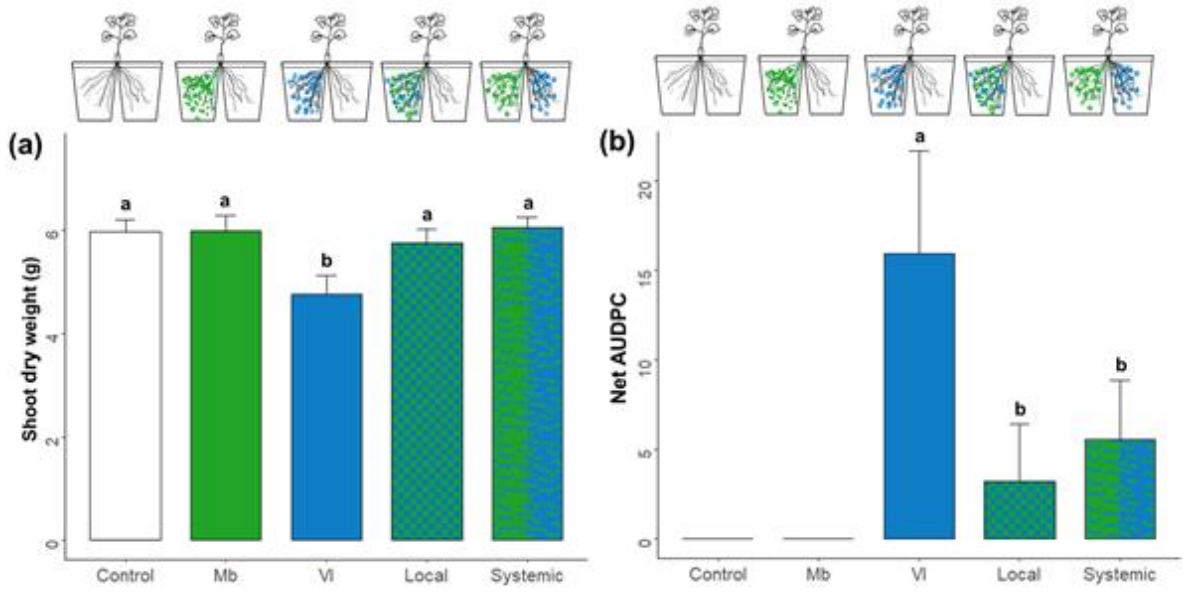

Figure 4. Shoot biomass (**a**) and area under the disease progress curve (AUDPC) (**b**) of oilseed rape plants inoculated with *V. longisporum* and/or *M. brunneum.* Plants grew in a split-root setup and were inoculated with fungal spores by root drenching. Treatment commenced with *M. brunneum* at transplanting (8 d) and was followed by *V. longisporum* after 7 d. Plants were harvested 35 d post inoculation (dpi) of *V. longisporum*. Inoculation scheme of a given treatment is represented above each bar. Letters represent statistically significant differences within treatments according to a linear model and Tukey's post-hoc test. *$p < 0.05$. Bars represent means ± SE; n = 8.

Inoculation with *M. brunneum* reduced plant stunting caused by *V. longisporum* infection as well as the AUDPC in both local and systemic treatments (Figure 4). We only observed

effects on plant biomass at 35 dpi, where the *V. longisporum* treatment had lower biomass than the other treatments (Figure 4a, lm, $F_{4,50} = 3.28$, $p = 0.018$; Vl vs Control $p = 0.006$). This was also observed for disease progression, where the *V. longisporum* treatment had a higher AUDPC (Figure 4b, lm, $F_{4,50} = 3.62$, $p = 0.002$).

Direct and systemic plant responses to V. longisporum and M. brunneum: Gene expression

Inoculation with *M. brunneum* alone affected the plant's SA pathway by upregulating the SA downstream signaling gene *PR1*, but not the SA biosynthesis genes *PAL* and *ICS*, compared to the control. Likewise, the JA/ET downstream signaling gene *PDF1.2* was upregulated in the local and systemic root compartments (Figure 5d). *Metarhizium brunneum* inoculation also induced changes in the ET pathway, upregulating ET synthesis (*ACO*) in the local compartment (Figure 5e) and decreasing the downstream signaling gene *ERF* both in the local and systemic compartment. The plant responded to *V. longisporum* infection by induction of the SA-responsive gene *PR1* (Figure 5c). The JA/ET downstream marker *PDF1.2* was also induced in the roots in both local (Vl-L) and systemic (Vl-S) compartments (Figure 5d). Interestingly, there was only a systemic but not a local plant response to the pathogen in the ET biosynthesis pathway with regard to *ACO* induction (Figure 5e), while *ERF* was slightly downregulated when compared with control roots (Figure 5f). There were no changes in the expression of genes involved in the biosynthesis of JA (*AOS,* Figure S1), abscisic acid (*ABA2,* Figure 5g) or glucosinolates (GSL) (*CYP83A1* and *CYP79B2*, Figure g h and j).

Interestingly, when *M. brunneum* was applied together in the same root compartment as *V. longisporum* (Mb-L/VI-L), roots had significantly higher *PAL* expression when compared with the control (Figure 5a). The induction of *PR1* in *V. longisporum* infected roots increased when *M. brunneum* was present in the systemic compartment (Vl-L/Mb-S), compared to the treatment with the pathogen alone (VL-L, Figure 5c). In the local treatment (Vl-L/Mb-L), induction of *PR1* was intermediate between the pathogen-only treatment and the systemic treatment and did not significantly differ from them. There was no local or systemic inhibition of the *ERF* gene (Figure 5f) when both fungi were colonizing the plant. The abscisic acid biosynthesis gene *ABA2* was induced in the compartments where *M. brunneum* was present (Mb-L/Vl-L and Mb-L/Vl-S, Figure 5 g). In the systemic treatment with *M. brunneum* and *V. longisporum* in separate root compartments, significant downregulation of the aliphatic GSL biosynthesis gene *CYP83A1* was observed in the Vl compartment (VI-L/Mb-S, Figure 5h), while for the indol GSL biosynthesis gene *CYP79B2* downregulation was found in the Mb compartment (VI-L/Mb-S, Figure 5i).

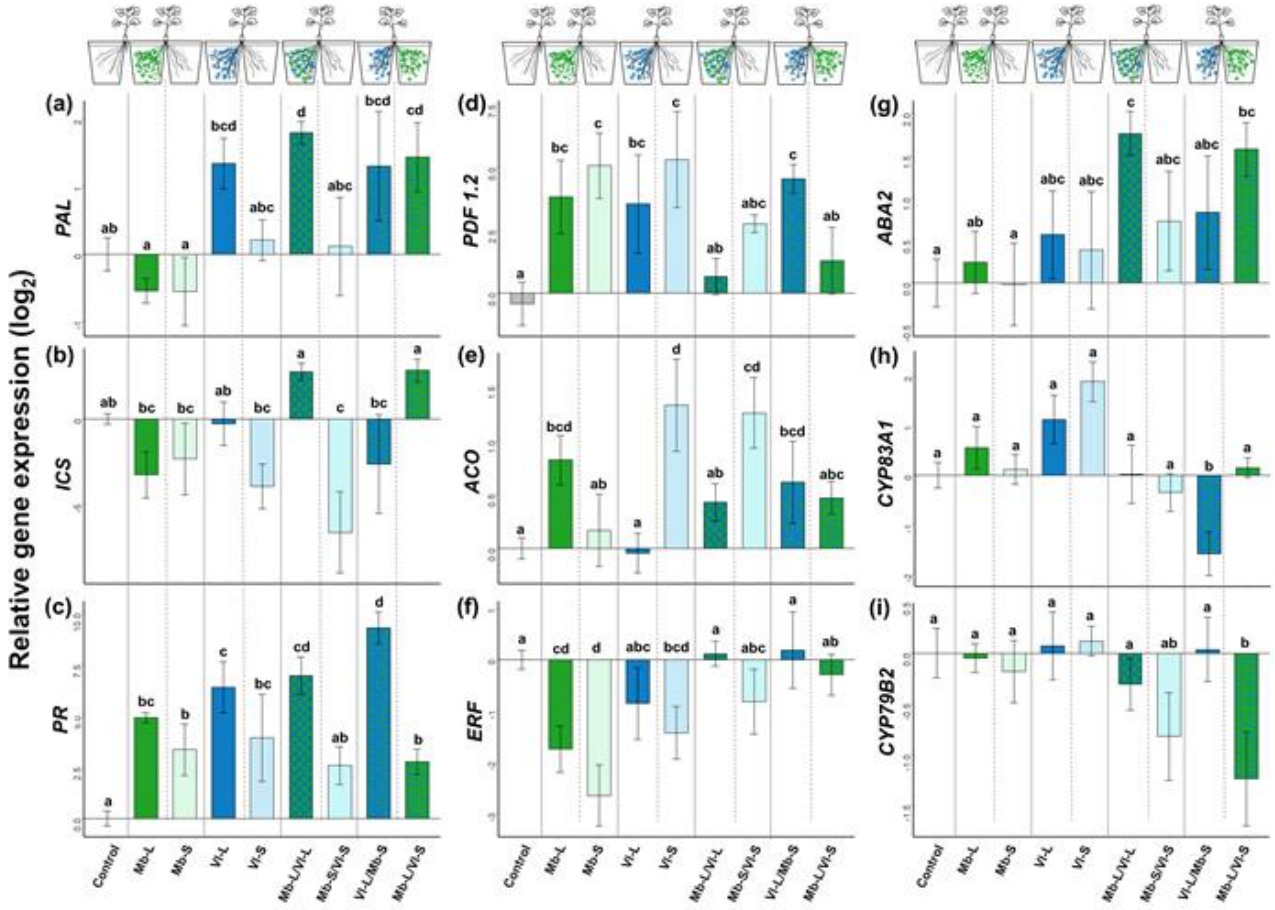

Figure 5. Expression of genes involved in defense signaling in roots of *B. napus* in response to *V. longisporum* and/or *M. brunneum* inoculation in a split-root setup. The left panel shows marker genes in the salicylic acid pathway: (**a**) *PAL,* phenylalanine ammonia lyase; (**b**) *ICS,* isochorismate synthase and (**c**) *PR,* pathogenesis-related protein 1. The middle panel shows (**d**) marker gene in the jasmonic acid pathway *PDF1.2* plant defensin 1.2, and for ethylene biosynthesis (**d**) *ACO,* 1-Aminocyclopropane-1-Carboxylic Acid Oxidase and downstream signaling (**f**) *ERF2,* ethylene response factor 2. The right panel displays genes involved in (**g**) abscisic acid biosynthesis *ABA2,* xanthoxin dehydrogenase; (**h**) aliphatic glucosinolate (GSL) *CYP83A1,* cytochrome P450 83A1 and (**i**) indol-GSL biosynthesis *CYP79B2,* cytochrome P450 79B2. The illustrations above the figures a, d and g show the fungal inoculation scheme, either with *M. brunneum* (Mb) or *V. longisporum* (Vl) in the local (-L) or adjacent (-S) compartment, and either treatments in same compartment (Mb-L/Vl-L), or each in adjacent compartments of the same plant (Vl-L/Mb-S; Mb-L/Vl-S). Fungi were inoculated by root dipping. Treatment commenced with *M. brunneum* at transplanting (8 d) and was followed by *V. longisporum* after 7 d. Plants were harvested 7 days after *V. longisporum* inoculation. Different letters indicate significant differences among treatments (Fisher LSD, $p < 0.05$). Bars represent means ± SE; n = 4 - 7.

Discussion

This study examined how *M. brunneum*, a soil-borne fungal entomopathogen, protects oilseed rape plants from *V. longisporum*, a soil-borne pathogen. The results show that *M. brunneum* effectively delays the colonization of plant roots by *V. longisporum*, leading to a significant reduction in disease symptoms. The research highlights the importance of direct competition and antibiosis in this process, as *M. brunneum* thrives in the presence of the pathogen while inhibiting its growth. Additionally, using a split-root setup, we observed that *M.*

brunneum has a notable impact on the root's defense response both locally and systemically by priming the SA pathway.

The in vitro confrontation assays conducted in this study demonstrated that *M. brunneum* inhibited the growth of *V. longisporum* by forming an inhibition halo. This inhibition may be attributed to the production of secondary metabolites with antibiotic effects (Moonjely et al., 2016). Fungal inhibition in dual cultures with Metarhizium species was observed against a variety of other plant pathogens (Lozano-Tovar et al., 2013; Sasan and Bidochka, 2013; Jaber and Alananbeh, 2018; Miranda-Fuentes et al., 2020). Crude extracts and partially purified fractions of Metarhizium have also been reported to inhibit fungal pathogens in previous studies (Lozano-Tovar et al., 2013; Sasan and Bidochka, 2013; Lozano-Tovar et al., 2017; Guigón López et al., 2019). Moreover, *M. brunneum* volatile organic compounds (VOCs) also inhibit plant pathogenic fungi (Hummadi et al., 2022). Interestingly, our results indicate that the presence of the pathogen *V. longisporum* did not hinder the growth of *M. brunneum* in the confrontation assay. In fact, there was a slight inclination towards increased colony growth. Moreover, in pot experiments, the presence of *V. longisporum* did not affect the growth of *M. brunneum* in the soil. On the contrary, the entomopathogen was more abundant in the roots when *V. longisporum* was present. These findings suggest that the presence of other fungal mycelia may stimulate the growth of *M. brunneum*, leading to increased colonization of the root and potentially slowing down the spread of the pathogen's infection.

After penetrating the roots, *V. longisporum* colonizes the xylem vessels and grows into the shoot, where it stays restricted to single vessels until later, when the fungus starts its saprophytic phase by invading the stem parenchyma and producing microsclerotia in the shoot tissues (Eynck et al., 2007; Knüfer et al., 2017). In a previous study by Eynck et al. (2007), it was found that *V. longisporum* is initially detected at low levels in *B. napus* plants within the first four weeks after inoculation. However, its presence sharply increases in the hypocotyl at 35 dpi, as determined by quantifying the amount of its DNA. We aimed to investigate the impact of *M. brunneum* on the colonization of *V. longisporum* in the root and hypocotyl. Our observations revealed that at the root level (measured only in the split-root setup), the abundance of the pathogen was significantly lower at 35 dpi in root compartments where *M. brunneum* was present. In addition, during the co-inoculation experiment, we noticed that the abundance of *V. longisporum* in the hypocotyl was lower at 28 dpi. These findings suggest that while there were no apparent signs of *V. longisporum* inhibition in the roots at 7 and 21 dpi, a competitive interaction in the root zone resulted in reduced colonization of both the roots and hypocotyls by the pathogen. As a consequence, we observed diminished disease symptoms and improved plant growth in both the co-inoculation and split-root experiments. These results indicate that the presence of *M. brunneum* influenced the colonization dynamics of *V. longisporum*. In a previous study, it was found that pre-inoculation with the endophyte *V. isaacii* Vt305 also led to reduced plant colonization and symptom development caused by *V. longisporum* (Tyvaert et al., 2014). Although the specific mechanism was not investigated, the authors proposed that induced resistance could be responsible for this observed effect.

Our results from the split-root experiment further support the potential involvement of induced resistance in addition to the direct control mechanisms discussed earlier. Interestingly, even when *M. brunneum* was inoculated in a separate compartment from the pathogen, a protective effect was observed in the systemic treatment. In this case, the AUDPC values were lower, and there was no significant decrease in plant biomass compared to the treatment with the pathogen alone. Additionally, there was a marginal decrease in the amount of pathogen DNA detected in both the roots and hypocotyls.

Beneficial root-associated fungi can elicit induced systemic responses (ISR) in plants and prime them to respond more effectively against pathogens (Shoresh et al., 2010; Pieterse et al., 2014). The impact of Metarhizium species on plant defenses is not consistent, as studies have shown contradictory results. For example, *M. anisopliae* M202-1 was found to suppress plant defenses in peanut roots (Hao et al., 2017), while different species of Metarhizium induced the SA and JA pathways in other plants (Ahmad et al., 2020; Cotes et al., 2020; Rivas-Franco et al., 2020). In a study conducted previously, we observed no significant plant responses after five weeks of *M. brunneum* Gd12 inoculation (Posada-Vergara et al., 2022). However, our present study suggests that *M. brunneum* CB15III induces the up-regulation of two key pathways: the SA pathway, indicated by an increased transcription of the *PR1* gene, and the JA branch, resulting in up-regulation of *PDF1.2*. In contrast, we observed the down-regulation of *ERF2*, which is a protein involved in the transcriptional regulation of defense genes in response to ET and/or elicitors (Nakano et al., 2006). The inhibition of ET response has been observed both in *M. anisopliae* and the pathogen *F. oxysporum* (Hao et al., 2017). This down-regulation could potentially be a common response to root colonizers in plants.

ABA, SA, and JA/ET pathways play a crucial role in the response of oilseed rape plants to *V. longisporum*, as demonstrated by comparative transcriptomic analysis (Behrens et al., 2019). Infection by *V. longisporum* results in the suppression of genes involved in ABA biosynthesis, induction of the SA signaling pathway, and decreased response of the JA/ET pathway.

Another study investigated the phenylpropanoid and SA pathways in a resistant and susceptible variety of *B. napus*, demonstrating that the resistant line exhibited increased SA levels and elevated expression of SA marker genes PR1 and PR2 (Zheng et al., 2019)**.** SA signaling activation was further supported by a study showing a 2-fold increase in SA-activated PR1 expression in *A. thaliana*. Our findings align with these results, as we observed higher PR1 expression in both the local and systemic compartments of *V. longisporum*-inoculated plants. Furthermore, this study provides additional evidence supporting the role of the JA/ET signaling pathway in the plant response to *V. longisporum*. We found upregulation of the JA/ethylene downstream signaling gene PDF1.2 at both the local and systemic levels. A previous study reported the activation of PDF1.2 in oilseed rape (Hafiz et al., 2022) and *A. thaliana* but not in *B. napus* plants at 6 dpi (Behrens et al., 2019). Interestingly, we observed changes in ET markers exclusively in the systemic compartment. While the induction of ACO suggests activation of ET synthesis, the downregulation of ERF indicates inhibition of downstream signaling. Behrens et al. (Behrens et al., 2019) also reported the induction of

ethylene receptor 2 (ETR2) in *B. napus* at 3 dpi and *A. thaliana* at 6 dpi. The distinct systemic response of PDF1.2, ACO, and ERF to *V. longisporum* inoculation suggests that the pathogen may locally manipulate JA/ET signaling while the plant is capable of activating systemic signaling.

Root associated beneficial fungi can modify the plant response to *V. longisporum*. For example, *Trichoderma harzianum* and *Bacillus velezenis* induced a priming response on *B. napus* against this pathogen that involved the activation of the JA and ET hormonal pathways (Hafiz et al., 2022). Our observations suggest that *M. brunneum* altered the root response to *V. longisporum* both locally and systemically. Phenylalanine ammonia-lyase (PAL) is the first enzyme in the phenylpropanoid pathway, which is directly linked to increased resistance to *V. longisporum* (Zheng et al., 2019). We found that *M. brunneum* co-inoculation with the pathogen increased *PAL* gene expression. This is consistent with other studies showing that beneficial microorganisms can induce *PAL* gene expression or enzyme activity in response to a pathogen. For example, *P. fluorescens*, different strains of *B. bassiana*, and *T. asperellum* T-203 induced PAL expression or activity in olive, tomato, and cucumber plants infected with *V. dahliae*, *Rhizoctonia solani*, and *Pseudomona syringae*, respectively (Yedidia et al., 2003; Azadi et al., 2016; Cabanás et al., 2017).

Furthermore, we found that the gene expression of *PR1* was higher in *Verticillium*-infected roots when the entomopathogenic fungus was present in the adjacent compartment. This suggests that the plant mounts a stronger response to the pathogen through the SA pathway, which leads to a systemic induction of plant defenses. Although priming of plants against pathogens through the SA pathway has been reported for other beneficial endophytes, our study is the first to report priming in response to Metarhizium inoculation.

It is known that during the early stages of infection, *V. longisporum* requires the suppression of ABA biosynthesis to establish itself (Behrens et al., 2019). However, in our study we did not observe any downregulation of *ABA2* by *V. longisporum*. Instead, we found that *M. brunneum* induced an upregulation of this gene both locally and systemically when the pathogen was present. This finding suggests that the presence of *M. brunneum* may interfere with the pathogen's manipulation of ABA biosynthesis, which could lead to increased resistance in the plant.

Glucosinolates (GSL) are secondary metabolites found in *Brassica* plants that serve as a defense mechanism (Liu et al., 2021) by exhibiting antimicrobial properties (Bednarek et al., 2009; Vig et al., 2009). These compounds appear to be involved in the plant’s response to *V. longisporum*. For instance, CYP79B2, an enzyme involved in indolic GSL biosynthesis, is induced in *A. thaliana* plants when infected with the pathogen. Furthermore, the double mutant cyp79b2 cyp79b3 showed greater susceptibility to this pathogen (Iven et al., 2012; Fröschel, 2021). GSLs also play a role in endophyte-plant interactions, as the root endophyte *Piriformospora indica* DSM 11827 induces the CYP79B2 gene. In its absence, as found in mutant cyp79B2 cyp79B3, the endophyte grows uncontrollably (Nongbri et al., 2012). In our study, we found no evidence of CYP79B2 or CYP83A1 induction by *V. longisporum* in *B. napus* plants. Instead, we observed inhibition of CYP83A1 in the compartment inoculated with the

pathogen when *M. brunneum* was present in the adjacent compartment, and inhibition of CYP79B2 in the compartment inoculated with *M. brunneum*. These results suggest a possible synergistic inhibition of GSL biosynthesis by the two microorganisms. However, further investigations are needed to confirm this hypothesis.

Beneficial fungi can also induce plant resistance through the release of various metabolites in the zone of interaction (Harman et al., 2004; Shoresh et al., 2010). These metabolites can be recognized as microbe-associated molecular patterns (MAMPs) by the plant immune system and trigger defense responses (Shoresh et al., 2010; Newman et al., 2013). The mechanisms behind plant recognition of endophytic entomopathogenic fungi (EEF) and the induction of systemic resistance are not yet fully understood (Hu and Bidochka, 2021), but recent research suggests that the EEF *B. bassiana* (BG11 and FRh2) may elicit the upregulation of pattern recognition receptors in *A. thaliana* plants, indicating a potential for microbe-associated molecular pattern-triggered immunity (Raad et al., 2019; Hu and Bidochka, 2021). There is growing evidence that EEF induce systemic resistance in the plant, both against insects and plant pathogens (Raad et al., 2019; Cachapa et al., 2020; Rivas-Franco et al., 2020; Posada-Vergara et al., 2022). An important step in the future is to understand how these defense responses are elicited and to find MAMPs or other compounds responsible for the induction of plant defenses.

Conclusions

This study shows that *M. brunneum* delayed *V. longisporum* oilseed rape root colonization, resulting in decreased disease symptoms. The mechanisms involved include a faster colonization of *M. brunneum*, whose growth was stimulated by the pathogen's presence. This has likely resulted in preempting the space and a competition for resources, together with antibiosis. Moreover, *M. brunneum* changed the plant's response to the pathogen by locally activating *PAL and* ABA genes, suggesting an activation of the phenylpropanoid and abscisic acid pathways. Furthermore, with the split-root experiment we were able to prove that there is a plant-mediated effect, seen by improved plant growth and decreased disease symptoms when *M. brunneum* was in the systemic compartment. In addition, enhanced systemic induction of *PR1* suggested a priming effect. So far, several studies have demonstrated the induction of systemic resistance by EEF. To our knowledge, we provide the first evidence of ISR by *M. brunneum* against a soil-borne pathogen.

Supplementary material

Table S1. Primers used for qPCR amplification of genes of *Brassica napus* root tissues

ID	Gen/accession	Gene description	Sequence 5' --> 3'	Reference
ABA2	LOC106300040	Xanthoxin dehydrogenase	GCATCGCTCGTCTGTTCCAC	Karssemeijer et al., 2021
	XM_013736089.1		CGGCGAAGTCAACAGCGTTA	
ERF2	At5g47220	Ethylene Response Factor 2	ATGTACGGACAGAGCGAGGT	Yang et al., 2010
			AAGCTTCGAAACCAACAAGTAACTG	
ACO	EV102889	ACC oxidase	TCCGTCTGGGCTATCACTCT	Maag 2014
			GTGAGTGGGTCGATGTTCCT	
PR1	XM_013877950.1	Pathogenesis-related protein 1	AAAGCTACGCCGACCGACTACGAG	Alkooranee 2017
			CCAGAAAAGTCGGCGCTACTCCA	
PAL	LOC106342153 ?	phenylalanine ammonia-lyase 1	TCGCTATGGCTTCTTACTGCTCTG	Karssemeijer et al., 2021
	XM_013781008.1		GAGGTCTTACGAGATGAGATGAGTCC	
AOS	LOC106327419	Allene oxide synthase	ACCGCTTGCGACTAGGGATC	Karssemeijer et al., 2021
	XM_013765565.1		CAAAGTCCTTACCGGCGCAC	
PDF1.2	EV163328	Defensin-like protein 16	TCCATCACCCTTCTCTTTGC	Maag et al., 2014
			TTTTGGCACGCATAGTCGTA	

ACTIN	AF111812	Housekeeping gene	ATCGTCCTCAGTGGTGGTTC	Maag et al., 2014
			TTGATCTTCATGCTGCTTGG	
CYP79B2	At4G39950	CYTOCHROME P450, FAMILY 79, SUBFAMILY B, POLYPEPTIDE 2	AAGAGGTTGTGCTGCTCCG	Tytgat et al., 2013 Also in Marthur
			TCCAAGTGAAACCTTGAAGAAGTC	
CYP83A1	At4G13770	CYTOCHROME P450, FAMILY 83, SUBFAMILY A, POLYPEPTIDE 1	CTCCTTATCCCTCGTGCTTG	Mathur et al., 2013
			TGTCGTAACCAGCGATCTTG	
ICS2Bn	XM_022690315	Brassica napus isochorismate synthase 2, chloroplastic	GAATGATGCTCTTCCTCGCAGTT	This study
	LOC106431819		TCGGAGACAGAAACCTTCGGAT	

Table S2. Summary statistics of fungal colony area, and colony growth inhibition percentage of *M etarhizium brunneum* and *Verticillium longisporum* when growing in dual culture

	Df (1/8)				
Fungus	**dpi**	**F value**	**Pr (>F)**	Inhibition (% ± SE)	
***METARHIZIUM BRUNNEUM* CB15**	14	1.5843	0.2436		
	17	3.6803	0.0913·	-8.48%	±1.68
	23	0.7325	0.417		
***VERTICILLIUM LONGISPORUM* VL43**	14	21.203	0.0017**	12.97%	±1.90
	17	13.419	0.0064**	19.75%	±2.05
	23	44.798	0.0002***	41.84%	±4.49

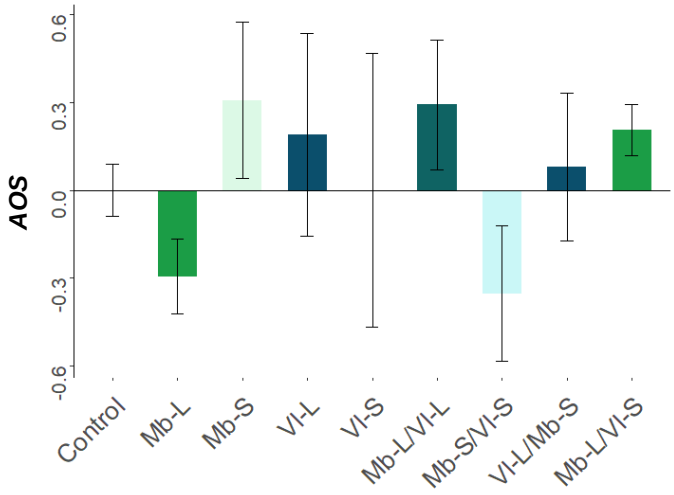

Figure S1. Expression of allene oxide synthase *AOS* gene

General discussion

Plant roots interact with many microorganisms in the soil and develop beneficial associations with some of them. Species of the genus Metarhizium are entomopathogenic fungi that can live as saprophytes in the soil, and associate with plant roots. Together with other fungal entomopathogens that associate and/or grow as endophytes, they are called endophytic entomopathogenic fungi (EEF). Over the last three decades, numerous publications have shown that EEFs protect the plants against pests and plant diseases, but few of them have investigated the mechanisms behind these phenomena. Furthermore, the beneficial effects seem to be highly dependent on the fungal isolate and plant species combination. The central aim of this thesis was to investigate the interaction between *Metarhizium brunneum*, the oilseed rape plant, one below-ground and two above-ground insect pests and a plant disease. The hypothesis of this thesis was that *M. brunneum* has a significant influence on root-feeding insects and plant pathogens, with both direct and indirect effects. Specifically, it is believed that the fungus has the potential to reduce the damage caused by root feeders and decrease their survival rates. In addition, the presence of EEF may alter symptom development and reduce pathogen colonisation of the plant. These effects could be achieved through direct interactions of the fungus with the pest and pathogen, as well as indirect effects through activation of plant defences

Association of *M. brunneum* with plants

Metarhizium species are commonly found in agricultural soil environments and have been shown to associate with the roots of various crop plants, as reviewed in Chapter 2 (Steinwender et al., 2014; Moonjely and Bidochka, 2019). Previous research by Liao et al., (2014) suggests that the ability of Metarhizium spp. to associate with plant roots may be a key factor in the beneficial effects of the fungi on plants. Specifically, a mutant of *M. robertsii* that was unable to adhere to plant roots lost its ability to enhance plant growth. Therefore, a first objective of the thesis was to optimize a reliable method for detecting the fungus-root association in plant tissues, and to evaluate the colonization of potato and oilseed rape plants by *Metarhizium brunneum*. When a plant is well colonized by *M. brunneum,* beneficial effects, such as improved plant growth and reduced pest and pathogen damage may be observed.

Previous publications have shown that fungal DNA detection by quantitative PCR is a reliable method to detect fungal DNA in plant tissues (Tellenbach et al., 2010; Barelli et al., 2018a). Therefore, this method was preferred over culture-based methods and its optimization for detection Metarhizium DNA in potato and oilseed rape tissues is presented in Chapter 2. The results confirmed that *M. brunneum* can colonize both crop plants, but colonization of oilseed rape was better, in terms of the number of plants colonized, the amount of DNA found in plant tissues, and the colonization of roots, shoots, and even leaves in a few plants.

Although previous publications have reported that endophytic colonization of the root system appears to be low level and transient (Barelli et al., 2018b), other publications have shown that Metarhizium can still be detected in the plant after several weeks after inoculation (reviewed in Greenfield et al., 2016; Hu and Bidochka, 2021)). In the present work, fungal colonization was analyzed along the different experiments, confirming the presence of *M. brunneum* in the roots and shoots at three weeks (Chapter 2), in the hypocotyl at four weeks (Chapter 3), in the root and hypocotyl at six weeks, and in the root base tissues at eight weeks after inoculation (chapter 4).

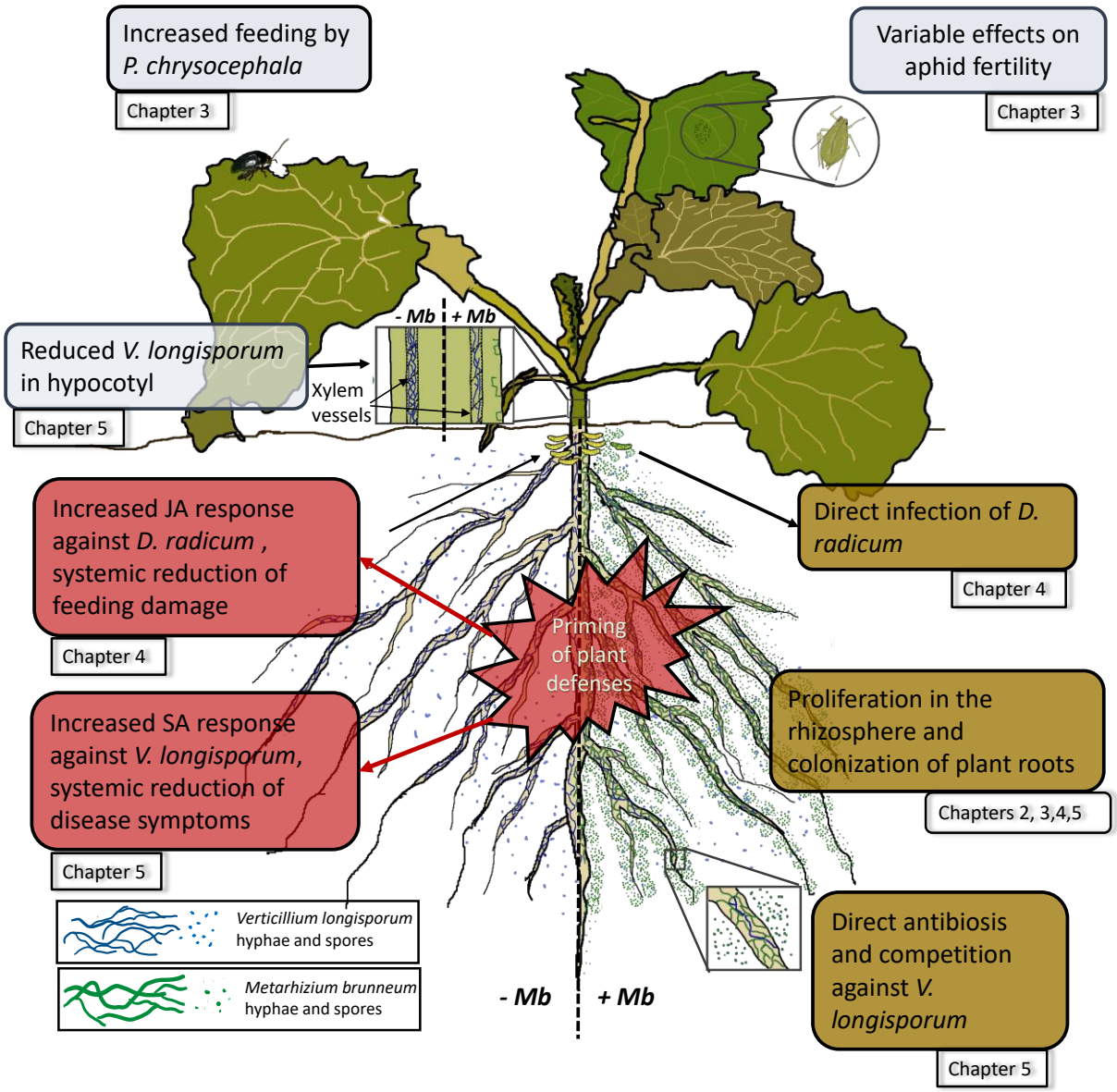

Figure 1. Overview of direct (dark yellow) and indirect (red and transparent background) effects of *Metarhizium brunneum* on plant health based on the results of this thesis. Representation of fungal mycelia in xylem vessels is based on Eynck et al., (2007) and on fungal detection by qPCR in the present study.

Comparisons among the different experiments must be taken with caution, due to different experimental setups. However, the present results show that the amount of fungal DNA in the shoot and hypocotyl was higher in younger plants (Chapter 3) than in older plants (Chapter 5), where the amount of DNA in the hypocotyl was very low and not present in all plants. A decrease in fungal DNA in roots was also observed along the time gradient (Chapter 5; Figure 3). Both observations suggest that endophytic colonisation may decrease with time, as has been reported previously (Barelli et al., 2019; Hu and Bidochka, 2021).

The persistence of entomopathogenic fungi in the soil is an important feature for long-term biological control of root pests, and Metarhizium spp. can multiply and persist in the rhizosphere of various crops for several months (Hu and Leger, 2002; Bruck, 2005; St. Leger, 2008; Bruck, 2010). In this thesis, I confirmed the presence of different isolates of *M. brunneum* in the rhizosphere up to 8 weeks after inoculation (Chapter 4) and even observed an increase in CFUs over time (Chapter 5), which was also observed by Bruck (2010). The establishment of a biological control agent such as Metarhizium in the soil after a single application can contribute to a more stable and sustainable control of root pests.

Intraspecific isolate differences

One of the questions that was transversal to some of the chapters in this thesis was whether isolates that were close, or even phylogenetically identical to each other (chapter 1) would have different effects in terms of rhizosphere and plant root colonization, pathogenicity against insects, and inducing plant-mediated effects on insect pests. Raad et al., (2019) suggested that the origin of the isolate could be one of the reasons for the different plant responses to two isolates of *Beauveria bassiana*, as one was isolated from plant tissues (endophyte) and the other from an insect. They proposed that depending on their origin, each fungal isolate may have produced different elicitor molecules (microbe-associated molecular pattern, MAMP) depending on its origin, which in turn induced different responses in the plant (Raad et al., 2019). Nevertheless, the isolates used here were all isolated from soil, and some of them are phylogenetically identical (Chapter 1). However, there were differences between isolates in plant colonization (Chapter 2), pathogenesis against the cabbage root fly (CRF, *Delia radicum*) (Chapter 4) and effect on aboveground herbivores (Chapter 3). The physiological plant response to the inoculation with different isolates was not compared in any of the experiments, so it is not possible to know whether the observed differences are due to activation of different plant pathways, as observed in Raad et al., (2019). However, the isolates used to study the plant-mediated effects against the CRF (Gd12) and *V. longisporum* (Cb15) were different, and while inoculation with Gd12 did not induce any of the plant defense marker genes (Chapter 4), Cb15 induced *PR1*, a marker of the SA pathway activation, and *PDF1.2*, a marker of the JA response (Chapter 5). However, the experimental conditions were different, in particular the time of sampling after fungal inoculation: four weeks for Gd12 and of two weeks for Cb15. As discussed in Chapter 5, the difference in the plant response to fungal colonization may be due to the plants being harvested at a different stage of fungal colonization, but an isolate-specific response cannot be ruled out.

Furthermore, there was variation in plant colonization for the same isolate between different experiments; for example, Gd12 DNA was higher in the roots of eight-week OSR plants infested with CRF compared to the other isolates (Chapter 4). However, this was not observed in OSR plants infested by the aphid species *M. persicae* or *B. brassicae*. It would be interesting to evaluate whether the growth of this isolate in plant tissues is stimulated by the presence of CRF larvae. Rivas-Franco et al., (2020) found that root colonization by two Metarhizium species decreased when maize plants were exposed to the root herbivore *Costelytra giveni*. However, the feeding strategy of the two insects is different: CRF larvae bore and feed inside the root, whereas *C. giveni* feeds from the outside. Therefore, a higher amount of fungal DNA in the root in Chapter 4 could also be due to the presence of Metarhizium-infected larvae inside the root.

Finally, the outcome of a plant-fungus interaction is highly context dependent. Small differences in temperature, humidity, light conditions, growth substrate, plant development stage and spore production, among others, can lead to different results. A reassuring observation is that even with the use of non-sterile substrates and different experimental conditions, there was good rhizosphere and root colonisation in all experiments, confirming that the *M. brunneum* isolates used are good colonisers of crops under natural conditions.

Direct effects of *M. brunneum* on CRF and *V. longisporum*

Metarhizium spp.and other beneficial microorganisms can have a direct effect on root pests and plant pathogens (Vega et al., 2009; De Silva et al., 2019; St. Leger and Wang, 2020; Sood et al., 2020). As mentioned above, *M. brunneum* colonized the rhizosphere, and the spores in the soil may have infected CRF larvae, similar to what was observed with the L3 larvae exposed to spores in the substrate. Furthermore, when CRF larvae were exposed to spores in the substrate, the fungus infected the larvae and remained latent during the pupation, allowing the adult to emerge, after which the fungus killed the fly and sporulated. These infected adults could be important for the dispersal of the fungal spores in the ecosystem.

Beneficial fungi can directly affect plant pathogens through mycoparasitism, competition, and antibiosis (Vinale et al., 2008; Ownley et al., 2010). In Chapter 5, the mechanisms of antibiosis and competition were investigated to understand how they reduce plant colonization and disease incidence by *V. longisporum*. Surprisingly, a specific isolate of Metarhizium increased in abundance in the roots when *V. longisporum* was present. This was unexpected, as it has not been previously reported for Metarhizium, Beauveria, or Trichoderma species when grown in the presence of a plant pathogen. On the contrary, one study has reported that *B. bassiana* did not grow in sunflower plants infected with *V. dahliae* (Miranda-Fuentes et al., 2020).

As discussed in Chapter 5, the higher amount of Metarhizium DNA in the roots in the presence of the pathogen may be evidence of a competitive process. The in vitro experiments also showed a tendency for a larger colony size of *M. brunneum* when grown in the presence

of the pathogen. A larger colony size could be the result of faster growth, which could lead to earlier occupation of the root epidermis, thus, slowing down the infection by *V. longisporum.*

Competition has been cited as one of the main strategies of beneficial fungi against pathogens (Ownley et al., 2008; Yan et al., 2015; Jaber and Alananbeh, 2018; Miranda-Fuentes et al., 2020) and antagonism between fungal species, and even within the same species (autoinhibition) is common (Yan et al., 2015). However, there is limited evidence of such competition between beneficial and plant pathogens outside of in-vitro studies. For example, case studies of fungal competition in the rhizosphere have been conducted, but have focused on beneficial fungi rather than plant pathogens. Specifically, fungal competition was studied for the beneficial fungi Trichoderma in the context of a possible effect of Trichoderma on other beneficial root symbionts such as mycorrhizal fungi (Martinez-Medina et al., 2016b; Thakur et al., 2019). These studies found that Trichoderma CFUs in the rhizosphere were reduced when plants were colonized by AM fungi (Thakur et al., 2019). Nevertheless, the role of competition in the control of plant pathogens by beneficial fungi should be investigated under more realistic (in planta) conditions.

Indirect effects of Metarhizium – plant interaction

Growth promotion is one of the most reported effects of beneficial fungi in general (Contreras-Cornejo et al., 2009; Rigobelo and Baron, 2021), and Metarhizium in particular (Jaber and Enkerli, 2017; St. Leger and Wang, 2020; Stone and Bidochka, 2020; González-Pérez et al., 2022). It can be the result of both improved nutrition and growth stimulation through hormonal manipulation. For example, through fungal auxin production, or by lowering ethylene levels through the fungal enzyme 1-aminocyclopropane-1-carboxylate deaminase (Liao et al., 2023). However, several studies also show that growth promotion is not always observed (Bamisile et al., 2018a; Rivas-Franco et al., 2019) or even negatively affected (Tomilova et al., 2021). Although I did not observe growth promoting effects in any of the experiments, there was a positive correlation between fungal DNA in root and shoot tissue and root and shoot dry weight (Chapter 2, Figure 6). The same was observed with regard to the relation between fungal DNA in hypocotyl and the leaf area, as well as shoot dry weight (Chapter 3, figure 4). Growth promotion by EEF may be dependent on specific isolate-plant or variety combination, as have been observed before (Rivas-Franco et al., 2019; Wilberts et al., 2023). Furthermore, EEF-mediated plant-growth-promotion effects are more evident under abiotic stress or nutrient limiting conditions (Krell et al., 2018b). For example, a meta-analysis of the literature on endophyte-plant interactions showed that endophytes had a greater beneficial effect on plants under stress than under control conditions (Rho et al., 2018), that was not the case in any of the chapters, therefore a possible growth stimulation effect cannot be ruled out.

Beneficial microorganisms can induce in the plant systemic resistance in plants against pest and diseases (Pineda et al., 2010; Pieterse et al., 2014). There is increasing evidence that specific isolates of Metarhizium can also induce plant systemic resistance (Ahmad et al., 2022; Gupta et al., 2022), involving a primed state (Rivas-Franco et al., 2019; Cachapa et al.,

2020). In priming, the plant defense response is absent or only slightly activated by the beneficial interaction (or priming trigger), but when an external stressor arrives, the plant's response to the pest or pathogen can be faster and stronger (Pineda et al., 2010).

In this thesis I studied the plant responses to a soil-bound pest and a soil-borne pathogen. It is difficult to separate direct from systemic effects when the beneficial microorganisms and the biotic stressors interact with the same part of the plant, the roots. Therefore, a split-root setup was used (Chapter 4), to separate the direct from the systemic effects of *M. brunneum* inoculation on the plant response to a root herbivore and to study how an isolate of *M. brunneum* affects the plant response to a fungal plant pathogen (Chapter 5).

The results of the experiments show that *M. brunneum* helped to reduce the damage caused by CRF, and to reduce the disease symptoms caused by *V. longisporum.* The split-root setup results suggest that both effects were not only caused by the direct effect of the fungus, as mentioned above, but there was a contribution of a systemic component. Furthermore, the hormone and gene expression results suggest that *M. brunneum* changed the plant response to both the pest and the pathogen in a tailored manner. Plant responses to herbivores are predominantly mediated by the JA pathway, and in Brassicaceae, activation of the glucosinolate-myrosinase system is part of the downstream response regulated by this hormone. Accordingly, *M. brunneum*-inoculated plants exhibited a greater JA production, and higher myrosinase gene expression when exposed to CRF herbivory than non-inoculated plants (Chapter 4). On the other hand, plant responses to plant fungal pathogens are often mediated by the SA pathway, and biotrophic and hemi-biotrophic pathogens such as *Verticillium* are sensitive to SA-dependent responses (Pieterse et al., 2012; Zhou and Zhang, 2020). Phenylalanine ammonia-lyase (PAL) is one of the two enzymes involved in SA biosynthesis (Huang et al., 2020), and *PR1* is a robust and commonly used marker for SA downstream expression (Pieterse et al., 2012). Accordingly, *PAL* expression was higher when *M. brunneum* was in the same root compartment as the pathogen, and *PR1* expression was higher in *Verticillium*-infected roots when the entomopathogenic fungus was in the adjacent compartment. Furthermore, suppression of ABA biosynthesis appears to be required for *V. longisporum* early infection (Behrens et al., 2019), and data presented in Chapter 5 suggest that *M. brunneum* induces up-regulation of the ABA biosynthesis (*ABA2*) in roots infected by the pathogen.

Metarhizium species are an important component of the soil microbiota with beneficial direct and systemic effects on plant health. However, results from Chapter 3 show that beneficial microbes such as Metarhizium can also have no effect or even increased feeding effects on insect pests. As discussed in the chapter, this could be due to an improvement in plant nutritional quality or a change in the concentration of plant secondary metabolites, as shown in previous publications (Khan et al., 2012; Krell et al., 2018b; Alves et al., 2021; Rasool et al., 2021b).This chapter also shows how the insect response depends on the isolate, as was also observed by Rasool et al., (2021a).

Conclusion

This thesis investigated the association between the entomopathogenic fungus *M. brunneum* and plants, focusing on the differentiation of direct and plant-mediated effects against herbivores and a plant pathogen. The study successfully developed a strain-specific PCR assay to distinguish *M. brunneum* isolates. It demonstrated the ability of *M. brunneum* to colonize two crops and highlighted the importance of quantitative PCR in assessing fungal association with plant roots. The research also revealed the effects of *M. brunneum* on herbivores, including feeding stimulation of a specialist herbivore and variable effects on the fecundity of generalist aphids. In addition, the mechanisms underlying plant-mediated resistance to herbivores were investigated, demonstrating both direct infection and systemic priming of defense pathways. The thesis also investigated the protective effects of *M. brunneum* against *Verticilium longisporum*, a soil-borne pathogen, revealing direct competition, antibiosis, and changes in plant defense responses. Importantly, in each case, the beneficial fungus appeared to prepare the plant in a tailored manner, activating the necessary defense mechanisms depending on the specific challenge. This adaptive response highlights the dynamic nature of plant-fungus interactions and the potential for *M. brunneum* to modulate plant responses depending on the presence of insects or plant pathogens. By unraveling the intricate mechanisms of these interactions, this research advances our understanding of plant-fungus associations and highlights the potential of *M. brunneum* as a biocontrol agent and its role in sustainable agriculture.

Outlook

In nature, plants form associations with multiple beneficial microorganisms and are challenged by multiple stressors simultaneously. Here, I have only examined how specific *M. brunneum* isolates alter the plant response to an insect and a plant pathogen. Nevertheless, plants face multiple abiotic and biotic stresses simultaneously, and the plant response in a complex scenario is likely to be very different from what is observed under controlled conditions.

Many questions remain unanswered regarding the interactions between plants and Metarhizium. For example, further investigation is needed to identify the components of early fungus-plant signaling, including signaling molecules and effector genes that promote plant colonization. Microbial-associated molecular patterns (MAMPs) serve as microbial-derived elicitors that are recognized by plant pattern recognition receptors (PRRs) to initiate plant defense responses (Pieterse et al., 2014; Zhou and Zhang, 2020). Effector genes, which encode small-secreted proteins, play a critical role in manipulating the host and establishing compatible responses while suppressing plant immune responses (Lucke et al., 2020; Constantin et al., 2021). Recent studies have suggested that Common Fungal Extracellular Membrane proteins (CFEMs), known for their effector roles in fungal pathogens, could potentially act as effectors in Metarhizium-plant interactions. In addition, evidence of effector-triggered immunity has been observed in plants inoculated with *M. brunneum*, suggesting the

need for further exploration of specific molecular patterns, fungal effectors, and plant receptors responsible for sensing these signals from Metarhizium (Cai et al., 2022; Gupta et al., 2022).

Metarhizium is only one of many beneficial microbes that inhabit the plant rhizosphere, where numerous microorganisms influence plant health. The application of -omics approaches, coupled with bioinformatics tools, has provided insights into the complex responses of plants and other players involved in multiple interactions (Chen et al., 2022). For example, tripartite interactions involving the beneficial fungus *Serendipita vermifera*, the cereal pathogen *Bipolaris sorokiniana*, and barley plants were investigated by analyzing the gene expression response of all three organisms using RNA-seq (Sarkar et al., 2019). In addition, modeling techniques provide a powerful tool to better understand the mechanisms and interconnections between different levels of rhizosphere interactions (Schnepf et al., 2022).

Finally, it is critical to view beneficial microorganisms such as Metarhizium as integral components of a healthy soil microbiome, rather than as mere replacements for insecticides or plant disease controllers. The benefits of rhizosphere microorganisms for plant health are well established, and significant progress has been made in developing commercial products for soil improvement and pest and disease management (Pallavi et al., 2017). Current research focuses on the development of synthetic microbial communities or consortia tailored for specific soil improvement purposes, highlighting the manipulation of soil microbiomes as a key aspect of sustainable agriculture (Kong et al., 2018; Liu et al., 2019; Tsolakidou et al., 2019; Du et al., 2021; Li et al., 2021; Pozo et al., 2021; Tabacchioni et al., 2021; Pradhan et al., 2022; Yin et al., 2022). However, studies of suppressive soils have shown that generalized suppression provided by microbial diversity can result in more stable disease and pest control, as well as improved abiotic stress tolerance, compared to a limited collection of antagonists. Therefore, soil management practices that prioritize soil health, including rich organic matter and diverse microbial composition, are critical and accessible to all farmers, including those with limited resources

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Curriculum vitae

Name	Catalina Posada-Vergara
Date of birth	29.03.1975
Address	Grisebachstr. 4, 37077, Göttingen
Contact information	+49 176 83324103 | cposadav@gmail.com

Education and qualifications

Year	Degree	University	Marks
2018 - 2023	Ph.D Program for Agricultural Sciences (PAG)	Göttingen University, Göttingen, Germany	
2014 - 2017	M.Sc. Sustainable International Agriculture	Georg-August Universität, Göttingen & Kassel University *Thesis title:* „Potential interactions among an endophytic entomopathogenic fungus, oilseed rape or tomato plants and their respective soil-borne pathogens"	Grade 1.5
2001 - 2005	Doctoral studies Integrative bioscience Major, Ecological and Molecular Plant Physiology Option	The Pennsylvania State University. Not finished, due to family reasons, returned to Colombia	Grade 3.9 / 4
1993 - 2000	B. Sc**.** Biology	Universidad Nacional de Colombia Bogotá, Colombia *Thesis title:* Germinable seed bank of a Paramo plant community under burning and grazing disturbances.(Parque Nacional Natural Chingaza)	Grade 4.3/5.0

Publications

Posada-Vergara, C.; Lohaus, K.; Alhussein, M.; Vidal, S.; Rostás, M. Root Colonization by Fungal Entomopathogen Systemically Primes Belowground Plant Defense against Cabbage Root Fly. J. Fungi 2022, 8, 969. https://doi.org/10.3390/jof8090969

Posada-Vergara, C.; Vidal, S.; Rostás, M. Local competition and enhanced defense: how *Metarhizium brunneum* inhibits *Verticillium longisporum* in oilseed rape plants. J. Fungi 2023, 9(8), 796 https://doi.org/10.3390/jof9080796

Participation in conferences

Posters

Posada-Vergara, C; Rostás, M; Vidal, S. Colonization of oilseed rape and potato plants by the insect pathogen Metarhizium brunneum varies with fungal isolate. DGaaE Entomology Congress 2019, Halle, Germany.

Posada-Vergara, C; Rostás, M; Vidal, S, Karlovsky, P. Fast development of isolate-specific PCR primers for fungi based on SCARs: Metarhizium brunneum Gc2 as an example. 63. Deutsche Pflanzenschutztagung, 26 - 29 September 2023, Georg-August-Universität Göttingen, Germany

Posada-Vergara, C., Vidal,S., Rostás, M. Local competition and enhanced defense: how Metarhizium brunneum inhibits Verticillium longisporum in oilseed rape plants. 63. Deutsche Pflanzenschutztagung, 26 - 29 September 2023, Georg-August-Universität Göttingen, Germany

Conference talks

Posada-Vergara, C., Lohaus, K., Vidal,S., Rostás, M. Susceptibility of Cabbage Root Fly (Delia radicum) to Metarhizium brunneum present in the soil or as endophyte of oilseed rape plants. 23. Jahrestagung des AK Biologischer Pflanzenschutz, 18-19.03. 2021

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Posada-Vergara, C.; Lohaus, K.; Alhussein, M.; Vidal, S.; Rostás, M Root colonization by fungal entomopathogen systemically primes belowground plant defense against cabbage root fly. Entomology Congress 2023, DGaaE. 20-23 February, Bozen-Bolzano, Italy

Posada-Vergara, C., Vidal,S., Rostás, M. Biocontrol at the root level: unraveling the mechanisms of Metarhizium brunneum in protecting oilseed rape from soil-borne pathogens. New IPM 2023. Swansea, 5 – 7th September, Swansea, UK

M.Sc. thesis direction

Maria Belen Ledesma “Evaluation of entomopathogenic fungi with endophytic characteristics against the planthopper *Pentastiridius leporinus* (L.) (Hemiptera, Cixiidae), an important pest in sugar beet”. Defended on 08.07.2021

Abimbola Lapeti. “Effects of endophytic *Metarhizium brunneum* in oilseed rape on the oviposition behaviour and direct effect on the larvae of *Athalia rosae”.* Defended 27.07.2021

Declarations

I hereby declare that this thesis has not already been submitted to other examination authorities in the same or a similar form.

Furthermore, I declare that I have not applied for a doctoral degree at any other university.

Göttingen, the 20th of July 2023

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(Signature)

I hereby declare in lieu of an oath that this dissertation was written independently and without unauthorised assistance.

Göttingen, the 20th of July, 2023

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(signature)