# Early stages of germination, plant colonization and production of indole-3-acetic acid (IAA) by species of *Metarhizium*

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**Abstract**

Fungi in the genus *Metarhizium* are known for their ability to successfully colonize both insect and plant hosts. Some species, called generalists, can infect a broader range of insects, while infection by specialists is restricted to specific insects. Generalists and specialists require different environmental cues to initiate conidial germination, an essential step in the process of host colonization. In this study, the early morphological development of several strains of *Metarhizium* was evaluated under different nutritional conditions and substrates. Overall, generalist strains have less restricted nutritional requirements to initiate germination, while specialists germinated poorly in the tested conditions. Several *Metarhizium* germinated in corn (*Zea mays*) and bean (*Phaseolus vulgaris*) root exudate, indicating an ability to utilize plant derived compounds for their development. Change in surface hydrophobicity had a significant effect in rates of differentiation of germ tubes into appressoria, infective structures required for insect colonization. However, some strains can still produce some appressoria in hydrophilic surfaces, particularly *M. anisopliae*. Microscopic analysis showed that the generalist *M. robertsii* germinates in both insect and plant tissues, but with morphological variations. Appressorial formation was restricted to insect tissues while hyphal growth was observed in plant root tissue. In addition to early morphological development, generalists and specialists were evaluated for their ability to produce the plant hormone indole-3-acetic acid (IAA), which is used by plant-associated microorganisms to promote plant root growth and is also known to be involved in insect pathogenicity by *Metarhizium*. Regardless of lifestyle (differences in environmental condition preferences and life cycle), all strains were able to produce IAA *in vitro* in the presence of the exogenous precursor L-tryptophan. *M. anisopliae* strains showed high intraspecific variation in level of IAA production and were selected for plant inoculation assays. No correlation was observed between IAA production *in vitro* and effects on corn plant growth or rates of root colonization, as the most competent endophytic colonizer was not the

highest IAA producer. Our results indicate that other mechanisms might be involved in successful root colonization by *Metarhizium*, and IAA production could play other important roles in their ecology.

Keywords: *Metarhizium*, endophytic, appressoria, IAA

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# Chapter 1: Introduction and Literature Review

## 1.1 Introduction

*Metarhizium* (Ascomycota: Hypocreales) is a genus of cosmopolitan entomopathogenic fungi that live as saprophytes and endophytes. Their natural distribution range spans multiple biomes around the world, and they are routinely isolated from soils in both native and agricultural areas (Nishi et al., 2011; Schneider et al., 2021; Keyser et al., 2015; Botelho et al., 2019). Given their effective pathogenicity against arthropod hosts, some species of *Metarhizium* are used as biological control agents against pest insects in agricultural crops; at least 200 species of insects have been reported as potential hosts for these fungi (Zimmermann, 2007). *Metarhizium* species can be classified as specialists or generalists depending on their host range. While generalists can infect a broad range of insects, specialists have specialized to infect only a selection of hosts (Barelli et al., 2016; Stone & Bidochka, 2020; St. Leger, 2024). The process of insect infection by *Metarhizium* starts with dispersive spores called conidia and involves complex signalling pathways and a series of critical steps, including host surface attachment and recognition, production of insect cuticle degrading enzymes, differentiation of infective structures, and release of several metabolites including insect toxins (Wang & St. Leger, 2007; Fang et al., 2009; Wang et al., 2012).

Apart from being entomopathogenic fungi, *Metarhizium* also establish complex symbiotic associations with plant hosts. They have been reported as rhizosphere competent fungi for over two decades (Hu & St. Leger, 2002) and are known endophytes and plant growth promoters for several species of plants, including commercially relevant crops such as maize (*Zea mays*), beans (*Phaseolus vulgaris*), and others (García et al., 2011; Khan et al., 2011; Liao et al., 2014; CIifton et al., 2018; Krell et al., 2018; Ahmad et al., 2020b; Hao et al., 2021; González-Pérez et al., 2022). Compared to our understanding of *Metarhizium* as insect

pathogens, relatively less is known about the mechanisms involved in the establishment and maintenance of these plant associations are topics for ongoing research.

As the result of a complex evolutionary history, *Metarhizium* have large genomes with specialized plant- and insect-association genes and a conserved ability to produce a vast array of secondary metabolites, many of which are still uncharacterized and potentially essential in their remarkable ability to adapt to multiple ecological niches (Gao et al., 2011). Achieving a comprehensive understanding of mechanisms employed by *Metarhizium* to successfully colonize hosts will involve investigating metabolic processes as aspects of their morphological development during these processes. Given the richness and complexity of the diverse interactions established by *Metarhizium*, this genus provides an ideal subject for research about topics in fungal ecology, morphology, genetics, and evolution.

## 1.2 Generalists and specialists: phylogeny and evolution of entomopathogenicity and plant association traits in *Metarhizium*

*Metarhizium* are historically recognized as asexual, green-spored fungi and the causal agents of the green muscardine disease, named after the green layer of conidia produced on the surface of the insect cadaver after host infection (Boucias & Pendland, 1982). The *Metarhizium* genus has just over 30 recognized species and is composed of entomopathogenic fungi who also live as plant endophytes or across the rhizosphere, where they can act as plant growth promoters (Hu & St. Leger, 2002; Kepler et al., 2014; Liao et al., 2014). This ability to successfully colonize both plant and animal hosts can only be achieved through a rich arsenal of both plant- and insect-association genes, and the complexity of these relationships is a demonstration of the incredible genetic plasticity that this fungal lineage has managed to retain along its evolutionary trajectory.

The order Hypocreales likely derives from an ancestor whose primary source of nutrients was plant-based, but which now comprises fungi with multiple lifestyles including endophytes, insect pathogens, nematode pathogens, mycopathogens, and plant pathogens (Gao et al., 2011). It is likely that this diversity in lifestyle arose from multiple host-switching events during evolution within this lineage (St. Leger & Wang, 2020). It is estimated that *Metarhizium* diverged from the strictly endophytic lineage of *Epichloe festucae* about 307 million years ago, forming a monophyletic clade with *Pochonia chlamydosporia* from which it diverged about 180 million years ago (Spatafora et al., 2007; Gao et al. 2011). Like *Metarhizium*, *Pochonia* are also multitrophic fungi that thrive as pathogens of soil nematodes and are also very competent endophytes (Maciá-Vicente et al., 2009; Avelar et al., 2017).

One of the main hypotheses regarding the evolution of the *Metarhizium* is that insect pathogenicity is a derived trait that showed up later in this lineage following the divergence from their ancestral endophytic lineage. Analyses of *Metarhizium* genomes indicate that they are more closely related to endophytes and plant pathogens than to other animal pathogenic fungi (Gao et al., 2011). The ability to colonize and kill insects is likely a result of successful co-optation and adaptation of plant association genes towards insect pathogenicity, in a process that included genetic adaptations such as gene duplication and horizontal gene transfer (HGT) events (St. Leger, 2024). Insect pathogenicity also showed up in other fungi descending from endophytes such as *Beauveria bassiana* (Cordycipitaceae), indicating convergent evolution of this trait in different fungal lineages (Xiao et al. 2012). There is evidence that plant association remained a major force driving speciation and spatial distribution in *Metarhizium* (Bidochka et al., 2001b; Wyrebek et al., 2011; Wyrebek & Bidochka, 2013). *Metarhizium* species are not randomly distributed across the soil but are rather found in association with specific plant hosts that vary from one *Metarhizium* species to another (Wyrebek et al., 2011). This hypothesis is further supported by the fact that *Mad2*, a major protein involved in root surface attachment,

shows more divergence between *Metarhizium* species than the insect surface attachment protein *Mad1*, which is largely conserved within the genus (Wyrebek et al., 2013; Couceiro et al., 2022).

Entomopathogenicity most likely arose in plant-associated *Metarhizium* as an adaptation to concomitantly explore new sources of nutrition, especially insect-derived nitrogen (Gao et al., 2011; Stone & Bidochka, 2020). The ability to access nitrogen from insects, with which *Metarhizium* are in close contact in the soil environment, could then be used to further support their ongoing rhizospheric association by providing this nutrient to the plant host. This hypothesis is supported by reports of *Metarhizium*'s ability to transfer insect-derived nitrogen from insect hosts to plant hosts, although this specific nutrient transfer is not mandatory to establish and maintain successful rhizoplane colonization (Behie et al., 2012; Barelli et al., 2019).

Generally, *Metarhizium* species can be classified into two main categories regarding their host ranges and optimal lifestyles: generalists, who have a wide range of insect hosts and are also often found in association with plant hosts, and specialists, whose insect host range is much narrower (St. Leger, 2024). A proposed third category classifies as transitional those species with intermediate host ranges, such as *M. guizhouense* (St. Leger, 2024). Specialist strains tend to have smaller genomes, while a more generalist lifestyle is consistently associated with a higher number of protein-coding genes, including enzymes and toxins (Gao et al., 2011). Overall, all *Metarhizium* have more genes that encode secreted proteins than other closely related fungi, an indication of the complexity of their multitrophic interactions (Gao et al., 2011; St. Leger et al., 2011).

A major group of *Metarhizium* generalists is represented by the PARB clade (acronym standing for *M. pingshaense*, *M. anisopliae*, *M. robertsii*, and *M. brunneum,* respectively).

These fungi are closely related phylogenetically and have similar morphological characteristics, especially *M. anisopliae* and *M. robertsii*, which for a long time were classified as a single species (Bischoff et al., 2009). The PARB clade is often found in association with multiple plant species, besides having very broad insect host ranges (Wyrebek et al. 2011; Botelho et al., 2019). *M. robertsii*, for example, can infect some ticks (Ment et al., 2012; Marciano et al., 2021), lepidopterans (Wang et al., 2017), orthopterans (Rangel et al., 2021), and coleopterans (Oliveira et al., 2018; Oliveira & Rangel, 2018), and is also commonly found in association with several types of plants. Contrasting with generalists, specialist *Metarhizium* species can only infect a very narrow range of insect hosts (Stone & Bidochka, 2020). For example, *M. acridum* is a specialist in infecting orthopterans, while *M. rileyi* is highly virulent only against lepidopterans (Wang & St. Leger, 2005; Fronza et al., 2017).

In a detailed review about *Metarhizium*'s evolutionary history, St. Leger (2024) describes a major hypothesis for this diversification of lifestyles within the genus. Since they descend from a plant-associated ancestral, especially in the root region, *Metarhizium* fungi had multiple opportunities for host jumping due to close and constant contact with the rich biodiversity harboured in the rhizosphere surrounding plant roots. While this interaction eventually led to the development of entomopathogenicity, in those regions with a seasonality in the abundance of insect hosts (temperate regions with harsher winters, for example), generalist strains maintained their associated with plant roots, which would allow their survival in the absence of insect hosts. In the transition from strict plant symbionts to insect pathogens, the *Metarhizium* lineage readapted the use of pre-existing metabolic pathways towards insect-based nutrition.

Specialist *Metarhizium* lineages probably arose in warmer areas with an abundance of insects year-round. The constant supply of insect hosts, including above-ground species, would eventually lead some strains to drift away from the rhizosphere region as their main ecological

niche and shift towards more frequent events of insect pathogenicity (St. Leger, 2024). In establishing a close relationship with specific hosts, generalists would eventually end up with a metabolism more specialized for insect pathogenicity for their more frequent hosts, resulting in smaller genomes and less encoded proteins involved in the insect infection processes (Gao et al., 2011; St. Leger, 2024). In contrast, generalist have large genomes marked by extensive gene duplication, which grants these strains the ability to synthesize a substantially larger number of metabolites (St. Leger, 2024). A comparative analysis of the genomes of generalist *M. anisopliae* and specialist *M. acridum* showed that *M. anisopliae* possesses expanded gene families that are involved in insect pathogenicity and detoxification, including proteins, chitinases and toxins, which probably facilitate their infection of insects from different families (Gao et al., 2011).

Remarkably, despite their smaller genomes and specialization for specific insect hosts, specialist *Metarhizium* strains maintain their ability to colonize plant roots (Golo et al., 2014; Moonjely & Bidochka, 2019; Hu et al., 2023; Liao et al., 2023). Some strains show some level of conidial germination when grown in root exudate extracts, however, the germination rate observed is lower than that of generalist strains (Pava-Ripoll et al., 2011). Specialists also lack certain genetic features that enable successful plant growth promotion, although no effect on efficacy of plant colonization is observed (Liao et al., 2023). Overall, despite variations in genomic size and gene contents between specialists and generalists, *Metarhizium* are ubiquitously plant root colonizers, with higher levels of colonization at the surface (rhizoplane) level and a preference for monocot hosts instead of dicot plants (Moonjely & Bidochka, 2019).

### 1.3 The morphological development stages of *Metarhizium* during insect infection

*Metarhizium* have been described in literature for over a century as insect pathogens (Metschnikoff, 1879). As other entomopathogens in Clavicipitaceae, these fungi have a dimorphic development when infecting insects, with an apical growth phase during initial infection and a yeast-like phase when growing inside the insect body cavity (Pendland & Boucias, 1997). The conidium is the main dispersive structure of *Metarhizium* and are the starting point of host colonization. Conidia are dry, aerial spores that adhere to the insect cuticle mostly through hydrophobic forces, prior to initiating germination and penetration into the cuticle by using physical force and cuticle-degrading enzymes (St. Leger et al., 1987; Boucias et al., 1988). Conidia are economically important structures since most *Metarhizium*-based commercial products for insect biological control use them as the active ingredient in their formulations (Faria & Wraight, 2007).

Boucias et al. (1988) determined that adhesion by *Metarhizium* is nonspecific and highly driven by hydrophobicity of the conidia. As is the case with other fungal species, the conidial surface of entomopathogenic fungi contains hydrophobin-like proteins that, in this group, are involved in identifying the hydrophobic insect cuticle, adhering to the cuticle and inducing germination (Bidochka et al., 2001; Zhang et al., 2011). Adhesion to the cuticle does not determine virulence by itself, as the conidial surface contains specific antigen markers that vary between species and strains and are positively correlated with virulence against specific insect hosts; that is, conidia are able to adhere to the cuticle of a variety of insect species in a non-specific manner, but virulence will depend on successful completion of further steps such as germination, penetration of the cuticle and capacity to supress the insect's defences (Rath et al., 1996).

Even prior to initiating germination, conidia utilize enzymes including esterases, chitinases and proteases to access nutrients of the insect cuticle in proximity with the conidial cell surface. St. Leger et al. (1990) found that enzymatic activity by *Metarhizium* conidia was higher in insect cuticle than in artificial, nutrient-rich agar medium and showed that, already from this early stage, conidia detect environmental cues that will later influence their course of morphological development during host colonization. Conidia adapt rapidly to nutritional conditions as shown by the fast change in gene expression patterns to access different nutrients (Pava-Ripoll et al., 2011). Nitrogen contents of the substrate are of particular importance during fungal development, and it has been shown that the nitrogen response regulatory gene *Nrr1* is expressed consistently throughout the insect infection process (Fang & Bidochka, 2006). Overall, *Metarhizium* conidia tend to germinate faster and turn out to be more virulent when grown under nutritional stress, in part due to increased appressorial differentiation and production of virulence-related enzymes such as Pr1, which supports degradation of proteins in the insect cuticle (Ibrahim et al., 2002; Rangel et al., 2008).

Penetration of the host cuticle involves both enzymatic and mechanical processes, as enzymes are deployed to breach the epicuticle and physical separation of lamellae is observed during procuticle penetration by the germ tube exerting physical pressure (Goettel et al., 1989). Proteases, lipases, aminopeptidases and chitinases are exuded to allow degradation of the cuticle and penetration of germ tubes towards the epidermis (St. Leger et al., 1986; St. Leger et al., 1987). Although protein is the main component of the insect epicuticle, a thin lipid outer layer, made up mostly of hydrocarbons, covers the insect body (Neville, 1975). Therefore, this is the first physical barrier entomopathogenic fungi must overcome in order to successfully infect their hosts. *Metarhizium* produce lipases and esterases that can break these molecules and seem to have an important role in pathogenicity, although their exact role in the infection process is not yet fully understood. One of such enzymes, the *M. anisopliae* spore surface lipase

(MASSL), has the ability to degrade a vast range of lipids (Silva et al., 2009). Lipase activity in *M. anisopliae* starts as early as 6 hours after contact with the cuticle and infers substantial degradation on the insect lipidic outer layer even before it initiates penetration with hyphae (Jarrold et al., 2007; Beys Da Silva et al., 2010a). *In vitro*, the fungus shows higher lipase production when the lipidic source is mixed with other cuticle components such as chitin and proteins, another indication that the whole composition of the insect surface plays a role in initiating infection (Beys Da Silva et al., 2010b).

Underneath the thin lipidic layer, the insect epicuticle is mostly composed of a protein matrix in combination with chitin microfibrils (Neville, 1975). Accordingly, proteases are probably the most critical enzymatic tools for cuticle penetration by entomopathogenic fungi. Pr1 and Pr2, a chymoelastase and a trypsin respectively, are the two major endoproteases produced by *Metarhizium* (St. Leger et al., 1986). Pr1 is a particularly effective enzyme and is four times more active than Pr2 in the conidial surface (Santi et al., 2010). It is also highly upregulated in the initial steps of infection during appressorial formation, aiding in degrading the cuticle for penetration, and at the end of the infection cycle as the fungal mycelia grow towards the outside of the insect cadaver and starts sporulation (Small & Bidochka, 2005). While *Metarhizium* produces chitinases, such as N-acetylglucosaminidase, these seem to be used in smaller quantities than proteases and are not produced at least during the first 40 hours after inoculation *in vitro* and *in vivo* (St. Leger et al., 1987a; Da Silva et al., 2005). Still, they have an important role in the course of infection as it has been found their disruption affects virulence against the insect host (Boldo et al., 2009). St Leger et al. (1996) hypothesize that initial degradation of the proteins in the cuticle is a necessary step before the chitin substrate is exposed to degradation by fungal chitinases, which would explain their production at a later timepoint of infection.

After initial germination, entomopathogenic fungi may form an appressorium, an expansion of the tip of the germ tube that adheres to the host and allows a penetrating hypha to grow and penetrate the body. Formation of appressoria is accompanied by the secretion of a mucoid sheath - which probably has a similar biochemical composition of beta-1,3-glucans that has been observed in other entomopathogenic fungi - that surrounds the apical swelling and adheres to the cuticle (St. Leger et al., 1989; Jarrold et al., 2007). In soft-bodied insects or structures, *Metarhizium* is able to penetrate the cuticle without the formation of an appressorium. However, it may still be produced in soft surfaces, indicating that other factors may induce its formation (Butt et al., 1995). Chemical and physical signals in the insect cuticle are recognized by germinating *Metarhizium* which may induce or repress appressorial formation. Differentiation is mainly influenced by surface hydrophobicity, relative nutritional stress and specific molecular markers in the cuticle, which Wang & St. Leger (2005) found to be mainly polar compounds through induction of appressorial formation by *Metarhizium* with dichloromethane extracts of insect cuticle. The signalling pathway cAMP-PKA is one of possibly multiple pathways involved in regulation of infection processes and induction of appressorial differentiation, as inhibition of protein kinase A (PKA) or disruption of the PKA subunit gene *MaPKA1* significantly affected appressorial morphology and impacted virulence against insects (Wang & St. Leger, 2005; Fang et al., 2009).

As the hyphae breach the cuticle and reach the haemolymph, the fungus switches its filamentous growth to unicellular, yeast-like forms called blastospores, and multicellular hyphal bodies, which multiply quickly until filling the entire body cavity. The haemolymph contains carbohydrate-binding proteins called lectins that are able to recognize and bind to certain markers in pathogen cell walls. These lectins act as opsonins, signalling to phagocytes the presence of pathogenic cells in the body (Pendland & Boucias, 1996). Cell wall surface is therefore an important carrier of molecular markers that may make entomopathogenic fungi

more susceptible to being attacked by the insect's immune cells. In a response to this threat, these fungi show differential cell wall carbohydrate composition at different life stages, with conidia, germ tubes, blastospores and hyphal bodies all exhibiting different epitopes (Wanchoo et al., 2009).

The specialist *M. rileyi*, highly virulent against Lepidopteran hosts, has blastospores that lack galactose residues, unlike other species which are easily recognizable by lectins in *Spodoptera* larvae haemocoel and are then exposed to granulocytes (Pendland et al., 1988). Wang and Leger (2006) discovered a specific protein produced by *M. anisopliae* hyphal bodies, *Mcl1*, that coats the fungal structures with a collagenous, hydrophilic layer to which insect haemocytes are unable to attach. The Mcl1 protein also serves as a mask to hide cell wall markers that are recognized by insect defences, such as β-glucans. Certain *Metarhizium* strains also produce cyclic peptide toxins called destruxins that lead to muscular paralysis in susceptible hosts (Roberts, 1966). However, not all insects are equally susceptible, and some strains have a reduced production or do not produce destruxins at all (Barelli et al., 2022). Kershaw et al. (1999) proposes that strains have different infection strategies, with some showing less production of blastospores *in vivo* and higher toxin production, while others go on the contrary direction and focus their energy into blastospore multiplication to kill the host, therefore having less need to synthesize toxins.

As infection continues, blastospores rapidly multiply and fill the insect haemolymph, and at a certain point, they synchronously germinate to form an invasive mycelium to penetrate the insect tissues (Pendland & Boucias, 1997). In *M. rileyi*, this transition involves a quorum-sensing system, probably involving multiple molecules, that triggers an apical growth switch after a threshold concentration of hyphal bodies is met (Boucias et al., 2016). Protease Pr1 is again up regulated during this stage, as it helps the mycelia breach the cuticle and make its way towards the outside of the insect body to initiate conidiation (Small & Bidochka, 2005). During

this process, the host dies as result of nutrient depletion and sustained damage to tissues; nutrient depletion might also be a trigger that initiates conidiophore differentiation and conidia formation on the surface of the insect (Boucias & Pendland, 1982).

### 1.4 Establishment of root colonization and growth promotion effects of plant-associated *Metarhizium*

The rhizosphere is the region surrounding the roots where plants release a complex mixture of compounds, including root exudates plentiful in organic carbon and soluble low-weight molecules, chemicals derived from the rhizodeposition of dead plant cells, and hydrophobic volatile compounds (VOCs) that diffuse through the soil (Massalha et al., 2017). These depositions confer the rhizosphere a unique chemical profile compared to non-rhizospheric soil and harbour an environment rich in microbial diversity, which can be beneficial or detrimental to plant health depending on microbiome profiling and the interspecific interactions established (Bais et al., 2006). A variety of factors influence the composition of the root microbiome, including the chemical communication between host plants and the microbes, as well as the microbe-microbe interactions between different species colonizing the rhizosphere (Lareen et al., 2016).

*Metarhizium* have been isolated from soil samples around the world for decades, but Hu and St. Leger (2002) were the first to demonstrate that, following field applications, concentration of *M. robertsii* propagules remained much higher in the rhizosphere region than in non-rhizospheric soil, providing solid evidence that these fungi benefit from the proximity with plant roots. Wyrebek et al. (2011) found that different species of *Metarhizium* have preferential plant hosts in natural environments, with *M. robertsii* being particularly competent in establishing rhizosphere associations with a broader range of plant types when compared to other species. The ability to establish associations with plants might have played a bigger role

in differentiation between populations of *Metarhizium* than insect parasitism, since habitat distribution of species and strains is highly associated to plant hosts and unrelated to the virulence of the strains (Bidochka et al., 2001; Wyrebek et al., 2011; Wyrebek et al., 2013).

Not only are *Metarhizium* spp. associated with rhizospheric soil, but they can also establish themselves as endophytes, as was first shown by García et al. (2011) who were able to isolate *M. anisopliae* from plant tissues after seed inoculation of tomatoes (*Solanum lycopersicum*). Since then, many studies have analysed the rhizosphere and endophytic competence of *Metarhizium* in a variety of plant species, particularly those with some commercial relevance, as well as the effects of these associations on the host plants. In corn (*Zea mays*) seed treatments, inoculation with *M. robertsii* resulted in increased height, increased above-ground biomass production and earlier establishment by young plants, showing that these fungi not only colonize plants but also act as plant growth promoters (Liao et al., 2014; Ahmad et al., 2020b). Inoculation with *Metarhizium* also led to increased biomass in other species, such as potato (*Solanum tuberosum*), wheat (*Triticum aestivum*), tomato (*S. lycopersicum*), soybean (*Glycine max*), and the model *Arabidopsis thaliana,* with higher growth benefits usually observed for plants under environmental stress conditions (García et al., 2011; Khan et al., 2011; CIifton et al., 2018; Krell et al., 2018; Hao et al., 2021; González-Pérez et al., 2022).

Plant growth promotion by *Metarhizium* is promoted by a few factors, which include suppressive effects against pest insects and plant pathogenic fungi, either by direct colonization of insect pests, competition and repression against phytopathogenic fungi, or indirectly by inducing the expression of defence genes by plant hosts (Sasan & Bidochka, 2013; Ahmad et al., 2020b; Hao et al., 2021; Gupta et al., 2022). Transportation of nutrients, particularly nitrogen, to the host plant also promotes plant growth. Behie et al. (2012) demonstrated that *Metarhizium* have the capacity to transfer insect-derived nitrogen from insect hosts to plant

hosts, establishing a complex tripartite relationship that involves modulation by nitrogen transporters such as ammonium permeases MepC and Mep2 (Moonjely et al., 2019).

As endophytes, *Metarhizium* usually associate more strongly to the plant root region, although they are eventually reisolated from stems and leaves, indicating some degree of mobility inside tissues during plant growth (García et al., 2011; Behie et al., 2015; Jaber & Enkeli, 2016; Ahmad et al., 2020b; Siqueira et al, 2020; Hu et al., 2023). Generally, *Metarhizium* inoculations result in lower rates of endophytic colonization than other filamentous endophytic fungi such as *P. chlamydosporia* (Moonjely & Bidochka, 2019; Hu et al., 2023). During advanced stages of colonization in switchgrass, observation of GFP-expressing *M. robertsii* shows abundant growth on the surface of roots, although endophytic colonization is also observed (Sasan & Bidochka, 2012). This provides further evidence that substantial fungal growth is concentrated in the outer surface of the roots. In industrial hemp (*Cannabis sativa* L.), both *Metarhizium* and *P. chlamydosporia* inoculations significantly increased plant growth, despite the rate of endophytic colonization of *P. chlamydosporia* in 30-day old plants being about 14-fold higher than endophytic *Metarhizium* (Hu et al., 2023). This indicates that colonization of internal structures might not be imperative for plant growth promotion effects of *Metarhizium* to be observed.

For reasons not yet elucidated, *Metarhizium* tends to colonize monocot plants at higher rates than dicot plants (Moonjely & Bidochka, 2019; Lahey et al., 2020). Moonjely & Bidochka (2019) found that both generalist and specialist strains of *Metarhizium* endophytically colonized corn and barley (*Hordeum vulgare*) roots at 10- and 20-days post inoculation, but no endophytes were reisolated from tomato, bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*) plants, with partial colonization reported for green bell pepper (*Capsicum annuum*). The same study showed that rhizoplane colonization was established in beans and peas, but at much lower rates than those recorded for corn and barley. When analysing the transfer of insect-derived

nitrogen to plant hosts, Behie et al. (2012) observed much higher translocation of insect nitrogen by *M. robertsii* to switchgrass (*Panicum virgatum*) than to haricot bean plants.

Mechanisms involved in *Metarhizium*-plant interactions are still a topic of investigation, as relatively little is known about this process compared to our knowledge about insect infections. Information regarding early-stage colonization and morphological development of root-associated *Metarhizium* is particularly lacking. It is known that plant root attachment involves expression of the cell wall adhesin gene *Mad2* and is an essential initial step for further colonization of the rhizoplane. *Mad2* disruption severely compromises the fungus' ability to colonize plant hosts (Wang & St. Leger, 2002). Liao et al. (2014) showed that *Mad2* expression is necessary for *Metarhizium* to promote growth in host plants, probably because improper adhesion impairs colonization of the rhizoplane. Sasan & Bidochka (2012) showed through electron scanning microscopy (SEM) that one hour after inoculation in switchgrass, *M. robertsii* conidia are already capable of effectively attaching themselves to root walls.

A few proteins have been proposed as important or imperative for proper establishment of root colonization by *Metarhizium*, including invertase MrInv, sugar transporters such as Mrt and Mst1, and fatty acid binding proteins Fabp1 and Fabp2 (Fang & St. Leger, 2010; Liao et al., 2013; Dai et al., 2021; Dai et al., 2024). These allow *Metarhizium* to utilize plant derived sugars and lipids for growth, which in turn stimulates further association with the plant host. Deletion of these genes significantly not only decreased rates of root colonization, but plant growth promotion effects were also impacted (Dai et al., 2021). Unlike obligatory plant-associated microorganisms, such as mycorrhizal fungi, plant-association is not strictly necessary for *Metarhizium*'s survival, and so, it is likely that nutrient transfer to the host plant will cease or at least decrease once plant-derived nutrients are no longer necessary to the fungus. Barelli et al. (2019) demonstrated that supplementing root-associated *Metarhizium*

with an external source of nutrients significantly decreased the rate of translocation of insect-derived nitrogen from the fungus to the plant, although root colonization symbiosis was maintained.

Large-scale transcriptome changes are observed in *Metarhizium* exposed to plant root exudate in comparison to insect cuticle or haemolymph (Wang et al., 2005). The protease Pr1 and the cell wall adhesin Mad2 are examples of up-regulated genes when *Metarhizium* are exposed to root exudates (Pava-Ripoll et al., 2011). Destruxins, cyclic peptides that are toxic to insect hosts and produced during insect infection, are also secreted by *Metarhizium* during interactions with plants, although this ability varies between strains and is not observed in all plants (Golo et al., 2014; Ríos-Moreno et al., 2016; Barelli et al., 2022). Generalists produce more types and bigger quantities of destruxins than specialists, and the effects of these compounds on plant hosts are not known. It is unlikely that destruxins are important for establishment of plant interactions, but since destruxin production is energetically costly, it is possible that they play a role yet unidentified in this ecological interaction (Barelli et al., 2022).

Understanding the rhizosphere competence of *Metarhizium* is especially important when considering the widespread use of these fungi in biological control programs across the world. They have already been studied in association to roots of important crop species. This shows that, when applied in the field with the purpose of controlling insect pests, *Metarhizium* could have other effects to the crops beyond their entomopathogenic ability. It is imperative to further investigate the mechanisms involved in the establishment of these endophytic relationships, as well as to study the effects of this relationship for both organisms involved. A deeper knowledge of such dynamics could have an impact on the practical applications of *Metarhizium* in agriculture and other fields, as well as provide valuable information for the understanding of its evolutionary history and the complex ecological relationships established by these fungi and their hosts.

## 1.5 Production of the plant hormone indole-3-acetic acid (IAA) by plant- and insect-associated *Metarhizium*

Several plant-associated fungi, both beneficial and pathogenic, are known for producing plant hormones to influence plant physiology and facilitate the establishment and maintenance of their relationship with their hosts (Chanclud & Morel, 2016). One of such hormones is the auxin indole-3-acetic acid (IAA), an indolic compound that influences many plant physiological processes such as formation of vascular tissue, development of flowers and fruits and, most importantly in the context of soil dwelling microbes, formation of lateral roots and root hairs (Luo et al., 2018).

In plants, IAA production is proposed to involve both tryptophan-dependent and tryptophan-independent pathways. The Trp-dependent pathways are far better understood, with involved genes, enzymes and middle products being extensively studied and described. Namely, four Trp-dependent IAA pathways are present in plants: tryptamine (TAM), indole-3-acetaldoxime (IAN), indole-3-acetamide (IAM), and indole-3-pyruvic acid (IPA); comparatively, tryptophan-independent pathways are barely understood (Di et al., 2015). IAA exerts its influence on plant morphological development through a transcription regulation pathway that starts with the binding to specific auxin receptors, which can possibly be found both intracellularly and extracellularly. Several proteins can bind to auxins, but a minority are actually able to initiate a cascade of downstream signalling (Yu et al., 2022). The signalling begins when auxin first binds to the transport inhibitor response 1 (TIR1)/auxin-binding protein (AFB) nuclear auxin receptors, which compose a subunit of F-box proteins in the ubiquitin ligase complex. This binding, in turn, promotes the degradation of the auxin/indole-3-acetic acid (Aux/IAA) transcriptional repressors through the ubiquitin-proteasome pathway (Grossmann, 2009). With the degradation of the Aux/IAA repressors, several auxin response factors (ARFs) transcriptional activator proteins are then capable of activating specific genes,

which will vary according to the plant tissue the process is taking place in. One of the effects of this pathway is the upregulation of expression of genes involved in the biosynthesis of other phytohormones, such as ethylene and abscisic acid (ABA), which will further affect the morphological development of the plant. In the presence of excessive amounts of IAA, long lasting effects in phytohormone production are observed and might result in deleterious effects for the plant (Grossmann, 2009; Yu et al., 2022).

*Metarhizium* fungi are reported producers of IAA, which has been found to be directly responsible for inducing plant root hair formation and is associated with increased host plant growth (Liao et al., 2017). The generalist *M. robertsii* produces IAA via a tryptophan (Trp)-dependent biosynthesis pathway that includes the conversion of Trp into tryptamine (TAM) by a Trp decarboxylase encoded by the auxin biosynthesis related gene *Mrtdc*, followed by TAM conversion into indole-3-acetaldehyde (IAAld) by an amine-oxidase, and subsequent IAAld conversion into IAA by an IAAld dehydrogenase (Liao et al., 2017). Other fungi have been reported to produce IAA by another biosynthetic pathway that achieves auxin production through the conversion of Trp into indole-3-acetamide (IAM) before the final product, and it is likely that *M. robertsii* also utilizes this pathway for IAA production (Liao et al., 2017). While microorganisms do carry tryptophan, the molecule's high energy concentration makes it costly to produce and, therefore, endogenous concentration of tryptophan tends to be low in microorganisms (Tang et al., 2023). Most tryptophan-dependent microbial IAA production is then tied to the supply of exogenous L-tryptophan in the environment to be used as a precursor for auxin biosynthesis.

Besides *M. robertsii*, *M. anisopliae*, *M. humberi, M. brunneum*, *M. marquandii,* and *M. pinghaense* are also auxin producers (Siqueira et al., 2020; Chaudhary et al., 2023). Siqueira et al. 2020 used a colorimetric assay to investigate the production of IAA by different species of *Metarhizium* and found that *M. anisopliae* produced indole compounds during liquid

fermentation in the absence of added Trp to the medium. However, since the colorimetric method used by the authors uses a non-specific reagent that might react with other indole compounds, it is not able to establish with certainty that *M. anisopliae* produces IAA via a Trp-independent pathway. Still, the results indicate an interesting research direction to elucidate whether all *Metarhizium* demand a Trp precursor to produce auxins or if it is possible that some employ other biosynthetic pathways for this end.

The ability of *Metarhizium* to produce auxins probably plays a role during the establishment of a symbiotic relationship in the rhizospheric region, since increased root hair formation would increase the root surface area for contact between fungi and plant host. Interestingly, Liao et al. (2017) showed that the generalist *M. robertsii* produces IAA not only during plant interactions, but also during insect infection, as immunolocalization showed that germinating hyphae released the auxin compound when growing on locust wings. Since insect cuticle is a potential source of L-tryptophan, in theory, entomopathogenic fungi could have access to this amino acid during infection and use it as a precursor for IAA biosynthesis; however, Trp content in the cuticle varies according to the developmental stage of the host and is associated, along with other aromatic amino acids, with the process of hardening of the cuticle (Dennell, 1958). Given these restraints to Trp access, further investigation is needed to establish if, and to which extent cuticle-derived Trp is a viable source of this precursor during *Metarhizium* infection. When grown in insect cuticle with the addition of exogenous L-tryptophan, *M. robertsii* showed earlier formation of appressoria (Liao et al., 2017). Additionally, the authors report that deletion of the *Mrtdc* gene, which allows conversion of L-tryptophan to tryptamine in the TAM pathway, not only impaired IAA production but also reduced formation of infective structures and virulence of *M. robertsii* against insects, indicating that the biosynthetic pathway used by these fungi to produce auxins might also be involved in regulating metabolic processes that impact entomopathogenicity.

Despite the apparent importance of IAA production in both plant and insect colonization processes by *Metarhizium*, the specific roles of IAA in insect pathogenicity are still not understood and only a small number of species have been tested for their ability to synthesize this compound. Notably, this information is only available for generalist strains. Investigating IAA production in a more varied selection of *Metarhizium* species, including specialist strains such as *M. acridum*, could provide an interesting insight into whether auxin production is reserved for strains strongly associated with plant roots, or if this ability is preserved across the genus independently of the lifestyle and ecological niches occupied by each species. Further research in this direction will increase our understanding of the possible uses of IAA by *Metarhizium* in establishing host colonization, especially in the greater context of potential adaptation of pre-existing plant-association biosynthetic pathways towards the development of entomopathogenicity in this fungal lineage.

### 1.6 Objectives and conclusion

The *Metarhizium* genus constitutes a group of highly adaptable fungi that exhibit remarkable plasticity in terms of niche adaptation that explain their successful distribution across multiple biomes (St. Leger & Wang, 2020). The rich evolutionary history of this group, with the emergence of entomopathogenicity from plant-associated ancestral lineages and ubiquitous retention of plant-association competence, makes *Metarhizium* a remarkably complex fungal lineage and provides opportunities to investigate aspects of fungal ecology and host niche adaptation, developmental traits associated with lifestyle transitions, and co-adaptation of complex metabolic pathways towards colonization of multi-kingdom hosts.

This study aims to investigate aspects related to the morphological development of *Metarhizium* strains with special focus on the initial stages of development, that is, the

germination of conidia and production of appressoria, which are known to be essential steps for the successful colonization of insect hosts. This typically occurs within the first 24 hours of contact between conidia and insect tissue, but whether the same morphological development within this timeframe is the same in plant roots has not been described in detail. By analysing early-stage development of a wide selection of generalists and specialists in substrates with different hydrophobicity and multiple nutritional sources, we describe strain-specific developmental characteristics of germinating *Metarhizium* conidia in different environmental conditions. Microscopy analysis of the generalist *M. robertsii* inoculated on plant and insect tissues allows us to observe different morphological characteristics in early-stage host colonization by the fungus. We also investigate the ability of generalists and specialists to produce the auxin plant hormone IAA, which is implicated in root associations and insect virulence by *Metarhizium*, and the inoculation of selected strains into plant hosts allow us to discuss whether IAA production *in vitro* is directly related to plant growth promotion and endophytic colonization. In summary, the main hypotheses investigated in this work are that (1) generalist strains of *Metarhizium* require less specific environmental cues to induce germination (i.e. rates of germination of generalists are higher than specialists in the tested conditions across different substrates and nutritional sources); (2) substrate hydrophobicity is a major factor in inducing appressorial differentiation in *Metarhizium* (i.e. rates of appressorial formation are lower in a non-hydrophobic surface); (3) *Metarhizium* employ different early-stage morphological developmental strategies during insect colonization in comparison to plant colonization (i.e. conidia do not produce penetration structures (appressoria) as frequently in plant tissues as in insect tissues); (4) generalist strains of *Metarhizium* are more efficient auxin producers than specialists given their closer association with plant hosts; and (5) higher auxin producers are more efficient in colonizing plant roots than lower auxin producers.

## Chapter 2: Assessment of early-stage morphological development by generalist and specialist strains of *Metarhizium* in different substrates and nutritional media

### 2.1 Introduction

Fungi in the genus *Metarhizium* are known for their ability to adapt to multiple lifestyles. They compose a lineage of insect pathogenic fungi who can also associated with with plant hosts, in the rhizosphere and within plant tissues as endophytes (Hu & St. Leger, 2002; Sasan & Bidochka, 2012). There is still a lack of understanding about certain mechanisms involved in the establishment of *Metarhizium* as rhizoplane colonizers or endophytes. In contrast, the developmental stages of *Metarhizium* in insect hosts is much better described, although specific mechanisms regarding germination and virulence are constantly under investigation. Regardless of the type of host, successful colonization starts with effective attachment of conidia to the host surface, a process mediated by adhesin proteins *Mad1* in insect cuticle and *Mad2* in plant tissues (Barelli et al., 2011). *Metarhizium* conidia are hydrophobic and attach in a non-specific manner to hydrophobic surfaces regardless of its nutritional contents (Zhang et al., 2011).

During insect colonization, entomopathogenic fungi often produce penetration structures called appressoria, apical swellings at the tip of germ tubes which attach to the insect and allow a penetration tube to breach the cuticle. Unlike conidial attachment, appressorial differentiation happens in a species-specific manner and requires specific physical and chemical cues from the substrate (Wang et al., 2005). Appressorial formation plays a major role in fungal virulence, with mutants lacking the ability to form this structure being severely impacted in their ability to infect insect hosts (Wei et al., 2017; Wang et al., 2021). Whether these structures are involved in the early process of plant colonization by *Metarhizium*,

however, is still unknown. However, long-term association between *Metarhizium* and plant hosts is well reported and has been confirmed through multiple techniques (Sasan & Bidochka, 2012; Hu et al., 2023).

In natural settings, plant-associated *Metarhizium* species display preferential associations with specific types of plant hosts and, overall, generalist strains are isolated much more often from plants than specialists (Wyrebek et al., 2011). However, the lack of a close association with plant hosts does not affect the ability of specialist *Metarhizium* strains to colonize plant hosts when inoculated into their roots (Moonjely & Bidochka, 2019). It is not known, however, how early-stage morphological development of specialist strains in the roots compare to that of generalist strains. In order to investigate mechanisms that are involved in the initial steps of host colonization by *Metarhizium*, such as germination and appressorial formation, this study aims to assess how surface hydrophobicity and nutritional cues affect stages of early development by generalists and specialist strains of *Metarhizium*. We also assessed the early developmental characteristics of a generalist *Metarhizium* strain (*M. robertsii* ARSEF 2575) in both insect and plant tissues to investigate the morphological differences in germinating conidia in these two types of hosts.

## 2.2 Material and methods

### 2.2.1 Phylogeny of selected fungal strains

A total of 21 strains from 6 species of *Metarhizium* and one strain of *P. chlamydosporia* were selected to construct a phylogenetic tree using partial CDS sequences of the 5' transcription elongation factor (TEF) gene (Table 1). Out of these strains, 16 *Metarhizium* were used in the experiments described in this work and are shown in bold (Table 1). Sequences for

the TEF genes are deposited in the National Center for Biotechnology Information (NCBI) and can be obtained through their accession numbers (Table 1).

Gene sequences were aligned with the Multiple Sequence Aligment Tool ClustalW inside the Mega X software version 11.0.13 (Tamura et al., 2021), using default parameters. Aligned sequences had overhanging gap regions manually removed and were evaluated for optimal statistical model. The phylogenetic tree was constructed using the neighbor-joining method with Kimura 2-parameter. Rates among sites were set to uniform and gap regions were partially deleted with a site coverage cutoff of 95%. The resulting tree is the result of 1000 bootstrap replications of the neighbor-joining tree for TEF sequences.

### 2.2.2 Preparation of biological material

A total of 16 strains from 5 *Metarhizium* species (*M. robertsii*, *M. anisopliae*, *M. brunneum M. pingshaense, M. guizhouense*, and *M. acridum*) were used in germination experiments (Table 1). To obtain conidia for the experiments, the strains were inoculated on PDA medium (Bioshop, Burlington, Canada) and incubated at 27°C for 14 days. Conidial suspensions were aseptically collected from PDA plates and suspensions were prepared using sterile deionized water with addition of 0.01% X-triton. The suspensions were filtered through glass wool to remove any mycelia and adjusted to a concentration of $1\times10^7$ conidia/ml using a counting chamber. To describe fungal colony morphology and assess rates of mycelial growth, all strains were inoculated on plates containing 10 ml of PDA by adding a 10 µl drop of conidial suspension to the center of each plate and incubating them at 27°C. Three biological replicates were prepared for each strain and colony diameters were measured at 7-, 14- and 21-days post inoculation.

To obtain images of the early stages of fungal development in plant roots, a mCherry-expressing strain of *M. robertsii* ARSEF 2575 (kindly provided by Dr. St. Leger, University of Maryland, USA) was used in plant inoculation experiments. *M. robertsii* was selected due to its known ability to establish rhizosphere associations and promote growth and increased biomass production in different species of plant hosts (Wyrebek et al., 2011; Ahmad et al., 2020b; Gupta et al., 2022). To obtain conidia for inoculation, the mCherry-expressing *M. robertsii* was grown in the same conditions as described above.

Corn (*Zea mays*) and bean (*Phaseolus vulgaris*) seeds for inoculation assays and for root exudate preparation were obtained from OSC Seeds (Kitchener, Canada). Prior to use, seeds were first sterilized by immersion in 4% commercial bleach (7.55% w/w sodium hypochlorite) under agitation (120 rpm) for 20 minutes and subsequently rinsed three times with sterile deionized water to remove excess bleach. Bean seeds were immersed in the same solution three times for 5 minutes, washed with sterile distilled water once in between and rinsed three times after sterilization. All seeds were air dried under a flow hood and transferred to 4°C overnight to synchronize germination. To obtain the corn and bean root exudates, seeds were transferred to Petri dishes containing 1% water agar and incubated at 27°C for about 5 days. Then, 50 germinated seeds of each species were transferred to flasks with 100 ml of sterile deionized water and incubated at 27°C for 4 days under agitation (120 rpm). After the incubation period, the exudates were filtered through a 0.45µm filter into a centrifuge tube and stored in a freezer at -20°C until use. Seeds of switchgrass (*Panicum virgatum*) (OSC Seeds, Kitchener, Canada) were also used for assessment of root colonization by *M. robertsii*. Seeds were sterilized by immersion in 4% commercial sodium hypochlorite for 6 hours under agitation. Afterwards, they were rinsed three times remove excess bleach, air dried and stored at 4ºC prior to use to synchronize germination.

### 2.2.3 Germination assessments

Germination of conidia from the 16 selected strains was assessed under varying nutritional sources and in two different substrates. Polystyrene (Petri dish, VWR, USA) and cellophane (gel drying sheets, Sigma-Aldrich, USA) were used as substrates for germination due to their difference in hydrophobicity, with polystyrene being highly hydrophobic and cellophane being hydrophilic. To induce germination, small Petri dishes were covered in 2 ml of sterile deionized water with the addition of one of four nutritional sources: 0.01% yeast extract (YE, Sigma-Aldrich, USA), 0.005% L-alanine (Bioshop, Canada), 1% corn root exudate or 1 % bean root exudate. L-alanine was selected as a nutritional source since it is the most prevalent amino acid in the insect cuticle composition (St. Leger et al., 1986). In each Petri dish, 50 µl of conidial suspensions adjusted to a concentration of $1\times10^7$ conidia/ml were added to 2 ml of nutritional source. For the samples inoculated on cellophane, the material was cut into small pieces and placed at the bottom of the Petri dish, creating a hydrophilic surface for germination. After inoculation, the Petri dishes were sealed with Parafilm to avoid loss of moisture and incubated at 27°C for 24 hours. To observe the germinated conidia, samples were imaged under a light microscope (Leica Diaplan) coupled with a Leica DFC400 camera. Conidia were considered to be germinated when the emerging germ tube was at least as long as the conidium length. The apical portion of the germ tubes was observed and appressorial formation was considered when the germ tube tip had a visible and defined swelling, and its width was at least 1.5× the width of the germ tube. All strains were simultaneously inoculated in plates containing 10 ml of PDA to assess conidial viability.

**Table 1:** *Classification, host species/substrate, geographical origin, and accession numbers for TEF of selected sequences.* Bold indicates strains used in experiments in this work. "From lab" indicates TEF sequences obtained in lab and yet to be deposited in NCBI. ARSEF: Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, USA; UNAM: National Autonomous University of Mexico.

| Fungal strain | Species | Collection | Host/substrate | Geographical origin | Sequence accession number |
|---|---|---|---|---|---|
| AH-01 | *Pochonia chlamydosporia* | n/a | Soil | Ontario, Canada | [from lab] |
| **ARSEF 324** | ***Metarhizium acridum*** | **ARSEF** | **Orthoptera** | **Queensland, Australia** | **EU248844.1** |
| **ARSEF 549** | ***Metarhizium anisopliae*** | **ARSEF** | **Hemiptera** | **Brazil** | **AZNF01000002.1** |
| **ARSEF 2575** | ***Metarhizium robertsii*** | **ARSEF** | **Coleoptera** | **South Carolina, USA** | **KR706486.1** |
| **ARSEF 2974** | ***Metarhizium brunneum*** | **ARSEF** | **Diptera** | **Buenos Aires, Argentina** | **[from lab]** |
| ARSEF 5748 | *Metarhizium acridum* | ARSEF | Orthoptera | Colima, Mexico | EU248879.1 |
| ARSEF 6421 | *Metarhizium acridum* | ARSEF | Orthoptera | Senegal | EU248883.1 |
| **ARSEF 7486** | ***Metarhizium acridum*** | **ARSEF** | **Orthoptera** | **Niger** | **EU248845.1** |
| ARSEF 7487 | *Metarhizium anisopliae* | ARSEF | Orthoptera | Ethiopia | DQ463996.2 |
| **ARSEF 8680** | ***Metarhizium brunneum*** | **ARSEF** | **Lepidoptera** | **Ontario, Canada** | FJ229493.1 |
| **BRK 18-12** | ***Metarhizium robertsii*** | **n/a** | **Soil** | **Ontario, Canada** | **[from lab]** |
| **EH-473** | ***Metarhizium anisopliae*** | **UNAM** | **Hemiptera** | **San Luis Potosí, Mexico** | **KY616797** |
| **EH-798** | ***Metarhizium robertsii*** | **UNAM** | **Soil** | **Morelos, Mexico** | **KY616818** |
| **EH-802** | ***Metarhizium pinghaense*** | **UNAM** | **Coleoptera** | **Morelos, Mexico** | **KY616815** |
| **EH-809** | ***Metarhizium robertsii*** | **UNAM** | **Unknown** | **Morelos, Mexico** | **KY616820** |
| **EH-849** | ***Metarhizium anisopliae*** | **UNAM** | **Lepidoptera** | **Colima, Mexico** | **KY616804** |
| **EH-863** | ***Metarhizium anisopliae*** | **UNAM** | **Coleoptera** | **Jalisco, Mexico** | **KY616807** |
| **EH-865** | ***Metarhizium robertsii*** | **UNAM** | **Coleoptera** | **Jalisco, Mexico** | **KY616823** |
| **EH-869** | ***Metarhizium guizhouense*** | **UNAM** | **Coleoptera** | **Nayarit, Mexico** | **KY616814** |
| **HAB-5** | ***Metarhizium robertsii*** | **n/a** | **Soil** | **Mexico** | **[from lab]** |
| MLG4 | *Metarhizium brunneum* | n/a | Soil | Austria | OK423557.1 |
| MLG22 | *Metarhizium brunneum* | n/a | Soil | Austria | OK423548.1 |

To observe the morphological development of *M. robertsii in vivo*, conidial suspensions were added to different insect tissues (wings and larvae cuticle) as well as plant tissues (onion epidermis and plant roots). Wings of *Drosophila melanogaster* wild type were collected by being carefully removed from adult insects with tweezers and immersed in 0.5 µl drops of conidial suspension adjusted to $1\times10^6$ conidia/ml. Larvae of the greater wax moth (*Galleria mellonella*) were inoculated by placing 10 µl drops of conidial suspension onto the central dorsal area of the larvae cuticle. Onion epidermis from white onions was also used as substrate for *Metarhizium* germination. The epidermis was collected with tweezers and cut into square pieces (approximately 2 × 2 cm) to which several 1 µl droplets of conidial suspension were placed across the surface of the onion peel. To observe conidial germination in plant roots, corn, bean, and switchgrass seeds were first treated as mentioned above. After germinating on 1% water agar for about 5 days, seeds were transferred to individual Petri dishes lined with wet filter paper, and several 1 µl droplets of conidial suspension were inoculated across the length of the roots. All inoculated materials were placed in Petri dishes lined with wet filter paper and sealed with Parafilm to avoid loss of moisture and incubated at 27ºC for the defined length of each experiment. For samples set to be imaged under a confocal microscope, the mCherry-expressing strain was used in the inoculations to allow proper observation of the fungal structures.

### 2.2.4 Sample imaging

Imaging of the morphological development and initial germination steps of *M. robertsii* ARSEF 2575 in plant and insect tissues was performed using light microscopy, scanning electron microscopy (SEM) and spinning disk confocal microscopy. Red fluorescence channel images for the mCherry-expressing strain were obtained under a spinning disk confocal microscope (Zeiss

Axio Observer Z1/7 with Apochromat objectives) with an Alexa Fluor laser beam of 568 nm excitation wavelength and emission peak of 603 nm. Images were processed with the ZEN Blue software (version 3.6) to obtain measurements and display samples in varying depth by using serial Z-stack acquisition to generate three-dimensional imaging volumes.

In preparation for imaging under the SEM, inoculated samples were first fixed with Karnovsky's reagent, which was prepared by adding 2% v/v glutaraldehyde (Sigma-Aldrich, USA), 2% v/v paraformaldehyde (Sigma-Aldrich, USA) and 3% w/v glucose into a 0.1M sodium cacodylate buffer solution (Sigma-Aldrich, USA) at pH 7.2. Each sample was individually placed in 2 ml centrifuge tubes with approximately 1 ml of fixative and left at 4°C overnight. After fixation, the samples were rinsed with PBS buffer for about 10 minutes and dehydrated in a graded series of ethanol (30%, 50%, 70%, 80%, 90% and 100%) for 10 minutes each and rinsed three times with 100% ethanol at the end of the series. The samples were left to dry inside a fume hood, placed on conductive adhesive, SEM specific carbon tape mounted on aluminum stubs and subsequently coated with a layer of 15 nm of gold in a sputter coater machine. Imaging was performed in a Hitachi SU5000 FESEM scanning electron microscope under high vacuum, using secondary electron (SE) mode with a steady beam acceleration of 15 kV.

### 2.2.5 Statistical analysis of data

*Metarhizium* strains were inoculated into four different nutritional media (0.01% yeast extract, 0.005% L-alanine, 1% corn root exudate, and 1% bean root exudate) using two different substrates (cellophane and polystyrene). The experiment was performed twice with similar results. Results are displayed as the average of three counts of one hundred conidia each ± the standard

error for each sample (Fig. 4). For each medium, germination and appressorial formation of each strain was compared between polystyrene and cellophane using Student's t-test for significant difference between means. Asterisks on top of bars indicate significant difference between germination or appressorial formation in polystyrene compared to cellophane at the $p<0.05$ (*), $<0.01$ (**) or $<0.001$ (***) levels.

## 2.3 Results

### 2.3.1 Phylogeny of selected *Metarhizium* strains based on TEF sequences

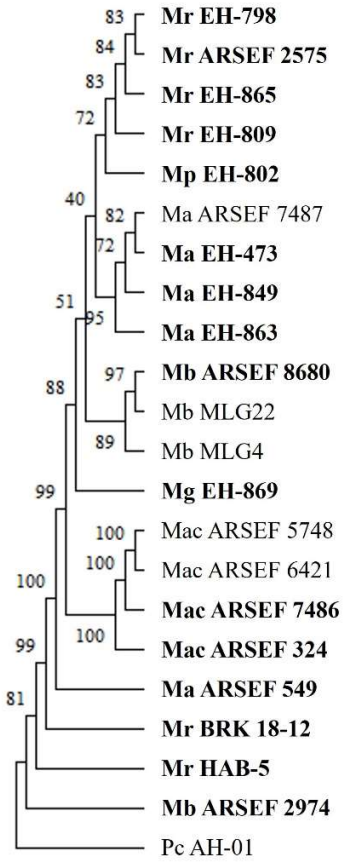


**Figure 1.** *Phylogenetic tree of translation elongation factor 1 alpha (TEF) for selected strains of Metarhizium. P. chlamydosporia* was set as the tree outgroup. CDS sequences of the TEF gene were aligned with ClustalW and the phylogenetic tree was constructed in MEGA X using the

neighbor-joining statistical method, with Kimura-2-parameter model and partial deletion of gap regions. The tree represents the results of 1000 bootstraps. Numbers next to nodes indicate the statistical probability of each node based on 1000 replicates for the phylogeny. Bold indicates strains used in the experimental tests described in this work.

### 2.3.2 Variations in *Metarhizium* colony morphology and mycelial growth rates

Significant morphological variations were observed between the *Metarhizium* strains inoculated in PDA plates (Fig. 2), including different colony growth rates in three weeks of incubation at 27ºC (Fig. 3). Some strains of *M. robertsi* had colonies composed mostly of mycelial growth, which visually distinguishes them from species which are marked by more abundant conidial formation. HAB-5, a *M. robertsii* strain isolated from soil in Mexico, showed smaller colony diameter after 21 days of incubation (5.98±0.15 cm) than all other *M. robertsii*. Both *M. brunneum* strains, ARSEF 2974 and ARSEF 8680, had the smallest colonies out of all species with 4.36±0.01 and 3.55±0.04 cm in diameter, respectively (Fig. 3).

The specialist species *M. acridum* is easily distinguishable from other species as it stands out for the big amounts of conidia with a vibrant green colour growing on top of the mycelia (Fig. 2). Strains ARSEF 324 and ARSEF 7486 had colony diameters of 6.80±0.12 and 6.62±0.14 cm after 21 days, respectively (Fig. 3). Other strains with visually abundant conidial formation in PDA include *M. robertsii* EH-809 and EH-798, *M. anisopliae* EH-473, and *M. brunneum* ARSEF 2974 (Fig. 2). In contrast, *M. robertsii* EH-865 and BRK 18-12, *M. anisopliae* EH-849, and M. *pingshaense* EH-802 had colonies composed mostly of mycelia, with comparatively poor conidial formation on the surface (Fig, 2).

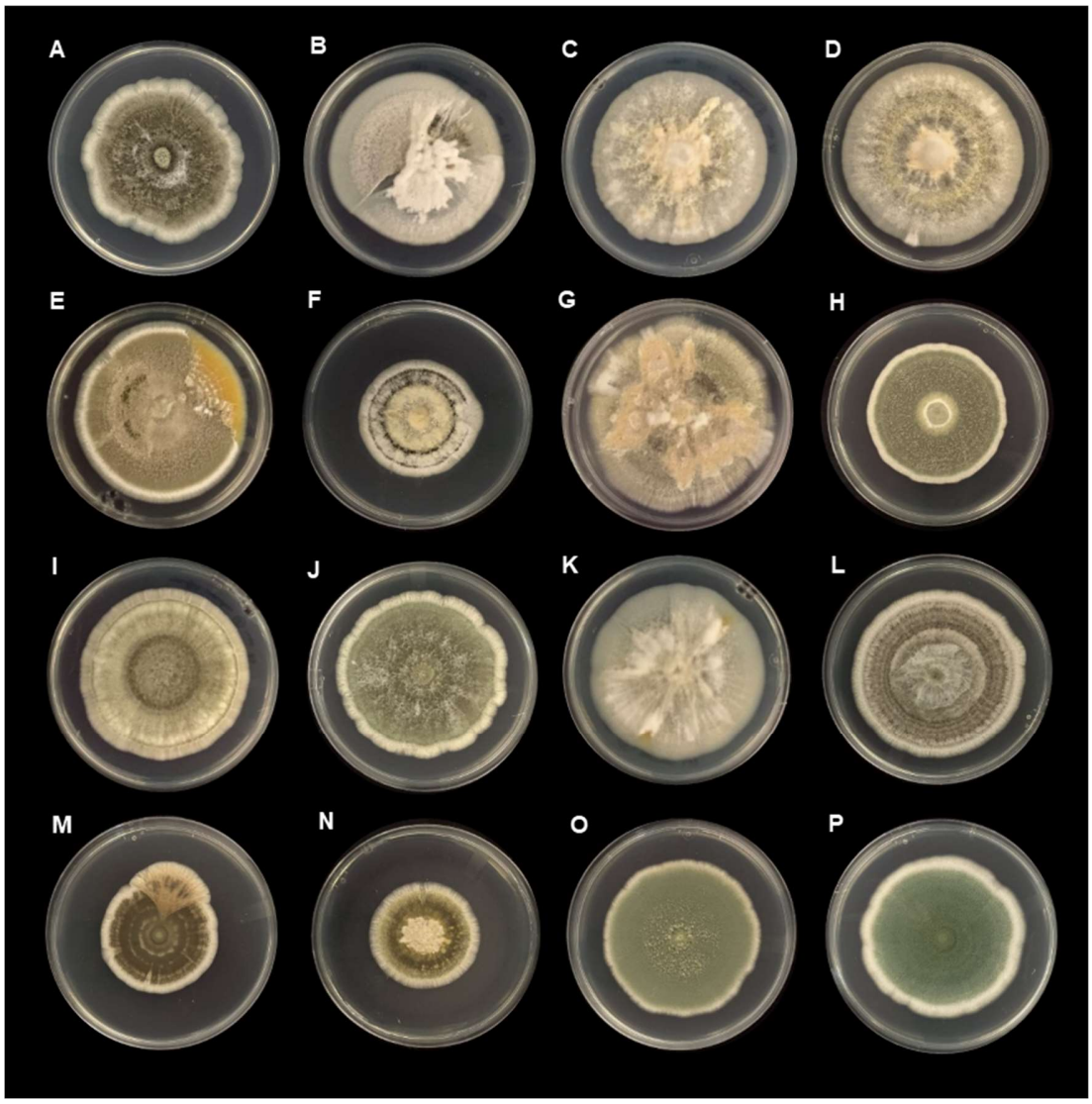


**Figure 2.** *Morphology of Metarhizium colonies growing in PDA plates 15 days after inoculation*. Strains from left to right and top to bottom: *M. robertsii* strains EH-809 (A), EH-865 (B), BRK 18-12 (C), ARSEF 2575 (D), EH-798 (E), and HAB-5 (F); *M. anisopliae* strains EH-849 (G), EH-863 (H), ARSEF-549 (I), and EH-473 (J); *M. pingshaense* strain EH-802 (K); *M. guizhouense* strain EH-869 (L); *M. brunneum* strains ARSEF 2974 (M) and ARSEF 8680 (N); and *M. acridum* strains ARSEF 7486 (O) and ARSEF 324 (P).

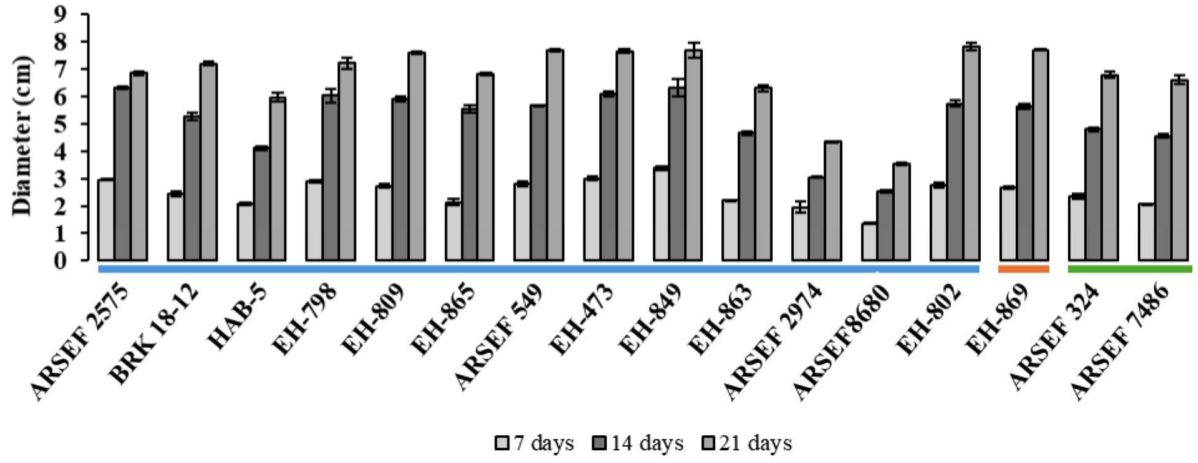


**Figure 3.** *Growth rates of Metarhizium colonies at 7-, 14- and 21-days post inoculation on PDA plates*. Plates were inoculated with 10 µl of conidial suspensions ($1\times10^7$ conidia/ml) and incubated in the dark at 27°C. Values are displayed in centimeters. Error bars indicate standard error based on the measurements of three biological replicates. Coloured lines under bars indicate generalist (blue), transitional (orange), and specialist (green) strains.

### 2.3.3 *Metarhizium* strains show different patterns of germination and appressorial formation in varying substrates and nutritional media

Conidial suspensions were incubated for 24 hours in four different nutritional media. Solutions of 0.01% yeast extract ("YE"), 0.005% L-alanine ("alanine"), 1% corn root exudate ("corn RE") and 1% bean root exudate ("bean RE") were used in combination with two artificial surfaces of different levels of hydrophobicity, with cellophane being hydrophilic and polystyrene being highly hydrophobic. All strains were simultaneously inoculated in PDA plates to confirm conidial viability in the experiment. Most strains showed high viability rates in PDA after 24 hours (>90%), with the exception of HAB-5, which had consistently lower viability rates in PDA than

the other strains (>50% only). An overview of rates of germination and appressorial differentiation in polystyrene and cellophane can be seen in Figures 4 and 5. Mean counts (%) and standard error values are presented in Table 9 (Appendix).

Not only did germination rates and morphological characteristics vary between different *Metarhizium* species, but significant variation was also observed between strains of the same species. Overall, *M. anisopliae* strains had the highest rates of germination when inoculated in polystyrene with YE, with 92.3±2.0%, 95.7±2.0%, 89.0±3.1%, and 84.3±2.9% of conidial germination observed for ARSEF 549, EH-473, EH-849, and EH-863, respectively (Fig. 4A). However, germination in the same nutritional medium lowered significantly in cellophane for all strains except EH-473 (t-test, $p < 0.05$). Generally, *M. anisopliae* germination remained relatively high in alanine, corn RE and bean RE when inoculated onto the hydrophobic polystyrene surface. In cellophane, germination rates decreased significantly for ARSEF 549 and EH-863 in alanine (Student's t-test, $p < 0.05$) (Fig. 4B), for ARSEF 549, EH-849 and EH-863 in corn RE (t-test, $p < 0.05$) (Fig. 4C), and for EH-473 in bean RE (t-test, $p < 0.01$) (Fig. 4D) when compared to polystyrene.

Two strains of the generalist species *M. brunneum* were tested in this experiment. ARSEF 8680 had a negligible germination rate in YE and polystyrene (2.7±0.3%), and did not germinate at all in any other media. Comparatively, ARSEF 2974 germinated very well in 0.01% YE in polystyrene (82.0±3.8%), although germination rates were lower in other media and in cellophane. Another generalist species, *M. pingshaense* germinated relatively well in all media when inoculated in polystyrene. While significant decrease in germination rates were recorded in cellophane with YE and alanine compared to polystyrene (t-test, $p < 0.05$), overall, mean germination rates in cellophane remained high for *M. pingshaense* in the hydrophilic surface.

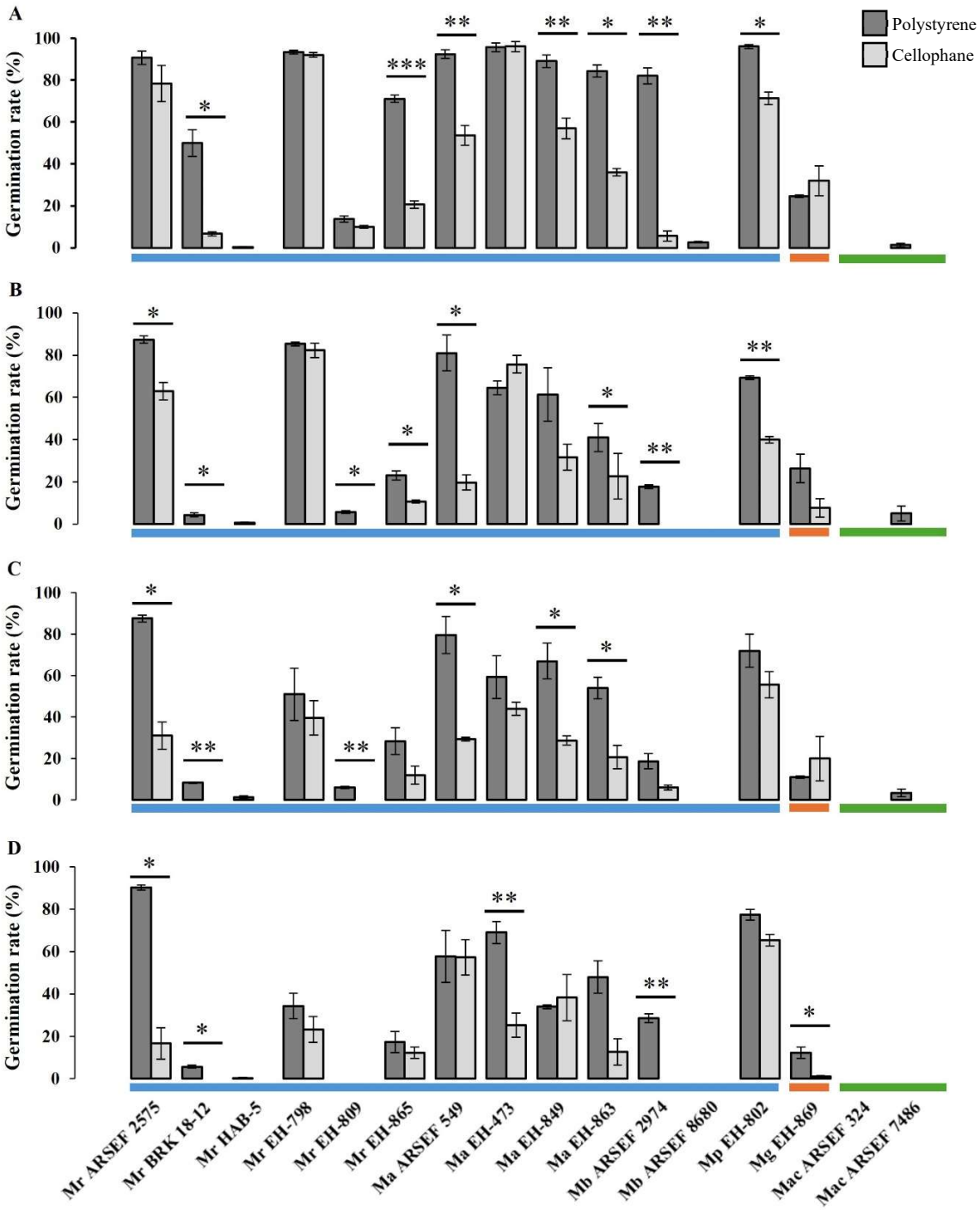


**Figure 4.** *Rates of germination of Metarhizium strains in artificial media and two substrates with different levels of hydrophobicity*. Graphs show mean percentage ± standard error of positive germination following three counts of one hundred conidia per treatment. Conidial suspensions

were inoculated into 0.01% yeast extract (A), 0.005% L-alanine (B), 1% corn root exudate (C), or 1% bean root exudate (D). Asterisks indicate that the rate of conidial germination in cellophane was significantly lower than that of polystyrene at the $p<0.05$ (*), $<0.01$ (**) or $<0.001$ (***) levels according to Student's t-test. Coloured lines under graphs indicate generalists (blue), transitional (orange), and specialists (green).

Intraspecific variation was remarkable in *M. robertsii*, with some strains showing high rates of germination and some germinating poorly or not at all. ARSEF 2575 had consistently high germination in all media when inoculated in the hydrophobic polystyrene surface (90.7±3.2% in YE, 87.3±1.8% in alanine, 87.7±1.7% in corn RE, and 90.3±1.2% in bean RE). However, germination lowered when conidia were inoculated in cellophane, with significant decreases observed in both corn and bean RE compared to polystyrene (t-test, $p < 0.05$). The Mexican strain of *M. robertsii* EH-798 also germinated well in YE (93.3±0.9% in polystyrene and 92.0±1.2% in cellophane) and alanine (85.3±0.9% in polystyrene and 82.3±3.4% in cellophane), with lower germination rates observed in corn and bean RE. Unlike ARSEF 2575, EH-798 germination did not vary significantly between polystyrene and cellophane in any nutritional media.

Three *M. robertsii* strains germinated very poorly in all conditions tested in this experiment. In polystyrene, the Canadian strain BRK 18-12 had some germination in YE (50.0±6.4%), but much lower rates in alanine and root exudates. Germination was very low (6.7±0.9% in YE) or inexistent when conidia were inoculated onto the cellophane surface. A similar pattern was observed in EH-809, with absolutely no germination recorded in bean root exudate for either surface. HAB-5, another Mexican strain, had virtually no germination in all tested conditions, and low germination was observed even in the control PDA plates (data not

shown). Interestingly, HAB-5 also has the lowest rate of mycelial growth compared to other *M. robertsii* strains (Fig. 2 and 3).

The specialist strains ARSEF 324 and ARSEF 7486 (*M. acridum*) did not have significant germination in any of the substrates or nutritional media used in this experiment, providing further confirmation of the strict environmental cues required by specialist *Metarhizium* strains to initiate conidial germination. The transitional strain EH-869 (*M. guizhouense*) had low but rather consistent germination rates across nutritional media in polystyrene (24.7±0.7% in YE, 26.3±6.7% in alanine, 11.0±0.6% in corn RE, and 12.3±2.6% in bean RE). Bean RE was the only medium in which germination decreased significantly in cellophane compared to polystyrene (t-test, $p < 0.05$).

Differentiation of germ tubes into appressoria was largely influenced by surface hydrophobicity for most strains (Fig. 5). In polystyrene with YE medium, several strains had high rates of appressorial formation, including *M. robertsii* ARSEF 2575 (84.7±0.9%) and EH-798 (93.3±0.9%), *M. guizhouense* EH-869 (57.7±5.5%), and all four strains of *M. anisopliae*. In YE, mean counts of appressoria were lower in cellophane compared to polystyrene in all strains, with statistically significant decreases observed for ARSEF 2575, EH-473, EH-849, EH-863, ARSEF 2974, EH-802 and EH-869. However, some strains still had relatively high rates of appressorial differentiation in cellophane, even if lower than those recorded in polystyrene. That was the case for *M. anisopliae* strains EH-473 and ARSEF 549, for example, which had the two highest means of appressorial formation in cellophane at 74.7±5.0% and 63.0±6.8%, respectively. In contrast, EH-863, another *M. anisopliae* strain, formed lots of appressoria in polystyrene (92.3±0.9%) but had negligible differentiation (2.3±0.7%) in cellophane. A similar pattern was observed for *M. robertsii* ARSEF 2575 (84.7±0.9% in polystyrene and 1.7±0.3% in cellophane), *M. brunneum*

ARSEF 2974 (77.7±3.8% in polystyrene and 0% in cellophane), and the transitional species *M. guizhouense* EH-869 (57.7±5.5% in polystyrene and 0% in cellophane).

Compared to YE, the medium amended with 0.005% L-alanine resulted in considerably lower appressorial formation in almost all strains (Fig. 5B). EH-798 and EH-849 were exceptions, with 88.0±1.0% and 83.7±0.7% of appressorial differentiation in polystyrene, respectively. *M. brunneum* ARSEF 2974 had the most drastic decrease in appressorial formation in this medium, going from 77.7±3.8% in polystyrene with YE to near zero differentiation in alanine on the same surface. Once again, following the observations made in YE, most strains showed significantly lower appressorial formation in cellophane, with some having no differentiation at all. In corn and bean RE, appressoria remained relatively abundant for most strains when inoculated in polystyrene compared to alanine. However, most strains produced little to no appressoria in cellophane in both media; for example, *M. anisopliae* EH-473 had the highest rate of differentiation in cellophane in corn RE with only 24.3±9.2% of germ tubes resulting in appressoria, a significant decrease from the rate observed in polystyrene (t-test, $p<0.05$).

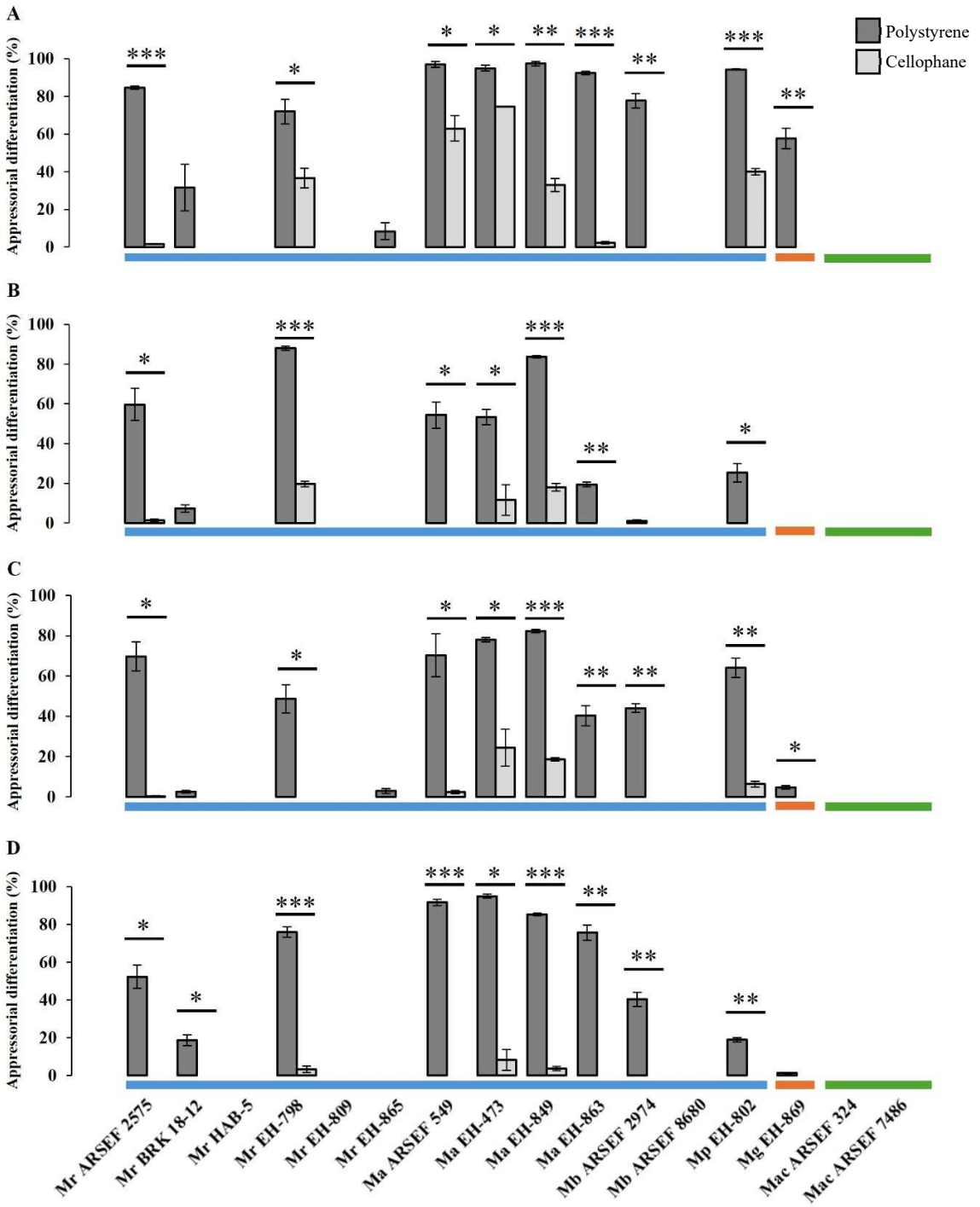


**Figure 5.** *Rates of appressorial differentiation of Metarhizium strains in artificial media and two substrates with different levels of hydrophobicity*. Graphs show mean percentage ± standard error of positive appressorial germination following three counts of one hundred germinated conidia per

treatment. Conidial suspensions were inoculated into 0.01% yeast extract (A), 0.005% L-alanine (B), 1% corn root exudate (C), or 1% bean root exudate (D). Asterisks indicate that the rate of appressorial differentiation in cellophane was significantly lower than that of polystyrene at the $p<0.05$ (*), $<0.01$ (**) or $<0.001$ (***) levels according to Student's t-test. Coloured lines under graphs indicate generalists (blue), transitional (orange), and specialists (green).

Figure 6 shows examples of morphological development of the generalist strain *M. anisopliae* ARSEF 549 growing in all four nutritional media and polystyrene and cellophane surfaces after 24 hours of germination. ARSEF 549 represents the general morphological features observed for other *M. anisopliae*, with germ tubes ranging from about 10 to 30 µm and clear apical swellings at the tip of the germ tubes indicating appressorial differentiation in different conditions (Fig. 6). Although appressorial differentiation happened in lower rates in cellophane compared to polystyrene (Fig. 4), these structures can still be seen at times even in the hydrophilic surface especially when conidia were grown in YE, as depicted by the black arrows in Fig. 6B. Appressoria were found in cellophane with corn RE but were very scarce; one of such structures is shown in Fig. F.

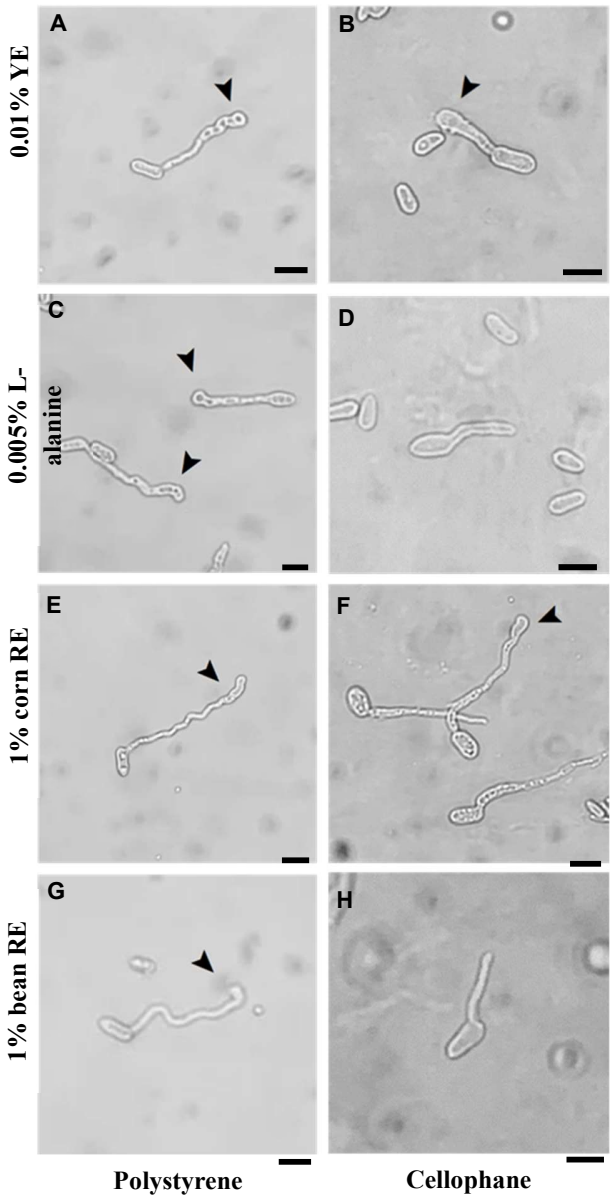


**Figure 6.** *Morphological features of generalist M. anisopliae strain ARSEF 549 germinating in artificial media. M. anisopliae* strain ARSEF 549 is shown germinating in polystyrene and cellophane (labelled rows) 24 hours after inoculation, using 0.01% yeast extract, 0.005% L-alanine, 1% corn root exudate and 1% bean root exudate as nutritional media. Black arrows indicate appressorial formation at the tip of germ tubes, which are produced in different rates in

polystyrene and cellophane. Morphological differences can be observed between treatments, including length and width of germ tubes, and presence of vacuoles in some germinating conidia. Bar: 5µm.

### 2.3.4 Generalist *M. robertsii* germinates in insect and plant tissues showing different morphological traits

Conidial suspensions of *M. robertsii* strain ARSEF 2575 were inoculated into fly wings, larvae cuticle, onion epidermis and plant roots to analyse initial fungal germination and development in each type of tissue. Rate of germination was compared between fly wing and onion epidermis and, overall, *M. robertsii* germinated extensively in both substrates, with an average of 94% germination in fly wings and 93% germination in onion epidermis (means of three counts of one hundred conidia). Differentiation of the germ tube into appressoria was observed frequently in conidia inoculated onto fly wing but were not observed in this experiment for conidia inoculated onto onion epidermis (76% in fly wing and 0% in onion epidermis, means of three counts of one hundred conidia) following 24 hours of inoculation at 27ºC.

To observe its developmental stages in different tissues and investigate the association between this species with plant hosts, germination of *M. robertsii* was analyzed under confocal microscopy and scanning electron microscopy. A comparison of the morphological characteristics of ARSEF 2575 germinating in onion and fly wings is depicted through SEM micrographs in Fig. 7. Appressorial formation is evident in the insect material (Fig. 7A), but germ tubes remained undifferentiated in onion peel. It was observed that, while conidia spread in an apparent random distribution across the surface of fly wings following inoculation with the conidial suspension,

conidia often seemed to accumulate in the division between onion epidermal cells when inoculated in the onion peel surface, possibly due to a difference in depth compared to the central region of the large onion cells.

Confocal micrographs of the mCherry-expressing strain of ARSEF 2575 show the germination of conidia after inoculation in onion epidermis, fly wing and larvae cuticle (Fig. 8). As was observed in SEM, different morphological development can be seen between the substrates, with conidia in onion epidermis forming long and uniformly thin germ tubes with no significant apical swelling that could indicate differentiation into appressoria. Inoculation in fly wing and larvae cuticle induces appressorial differentiation, and penetrating pegs can be seen breaching the insect cuticle as indicated in the pictures (Fig. 8). In order to observe *M. robertsii* germinating in plant tissues *in vivo*, corn, bean, and switchgrass roots were inoculated with conidial suspensions of mCherry-expressing ARSEF 2575. Results of these inoculations are depicted in Figures 9, 10 and 11. *M. robertsii* shows substantial germination and hyphal growth in both corn and switchgrass at 24 hours post inoculation (Fig. 9, 10). Interestingly, in corn roots, *M. robertsii* seems to germinate producing relatively long germ tubes which show a tendency to grow in a parallel direction in relation to the plant root cells (Fig. 9). In switchgrass, hyphal growth can be observed not only on the surface of the primary root structure but also associated to the lateral hairs as shown through the red fluorescence in Fig. 10. Comparatively to these two dicot species, *M. robertsii* seemed to germinate more poorly in bean roots after 24 hours of inoculation, although conidia seem properly attached to the root surface (Fig. 11). The use of Z-stacking (ZEN Blue, version 19.0) to image deeper regions of the root samples shows that no fungal structures are found internally in the plant root structure at this early stage of colonization, with only residual fluorescence from the upper layer being visible in the red fluorescence channels (Fig. 9, 10).

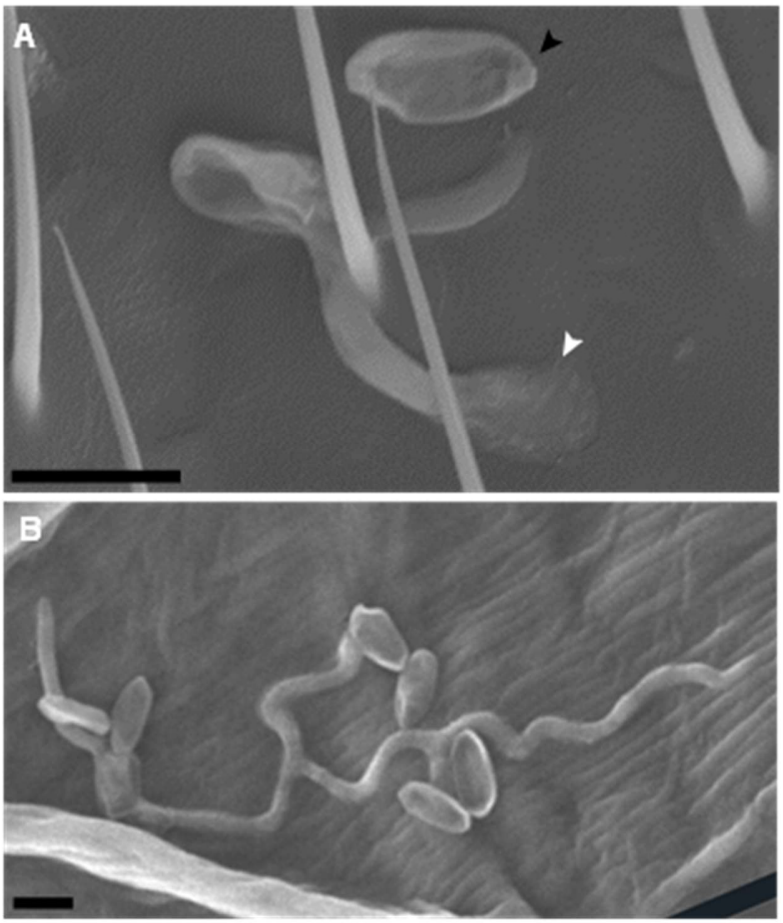


**Figure 7.** *M. robertsii germinating on plant and insect tissues as seen under scanning electron microscopy (SEM).* (A) *M. robertsii* growing on the surface of a *Drosophila* wing. Black arrow indicates ungerminated conidium, white arrow indicates appressoria at the tip of the germ tube. Wings hairs can be seen across the surface. (B) Germinated and ungerminated conidia of *M. robertsii* on the surface of onion peel. Most germ tubes are long and show no indication of appressorial differentiation on this tissue. Micrographs taken under high vacuum in secondary electron mode and beam acceleration of 15kV. Bar: 5μm.

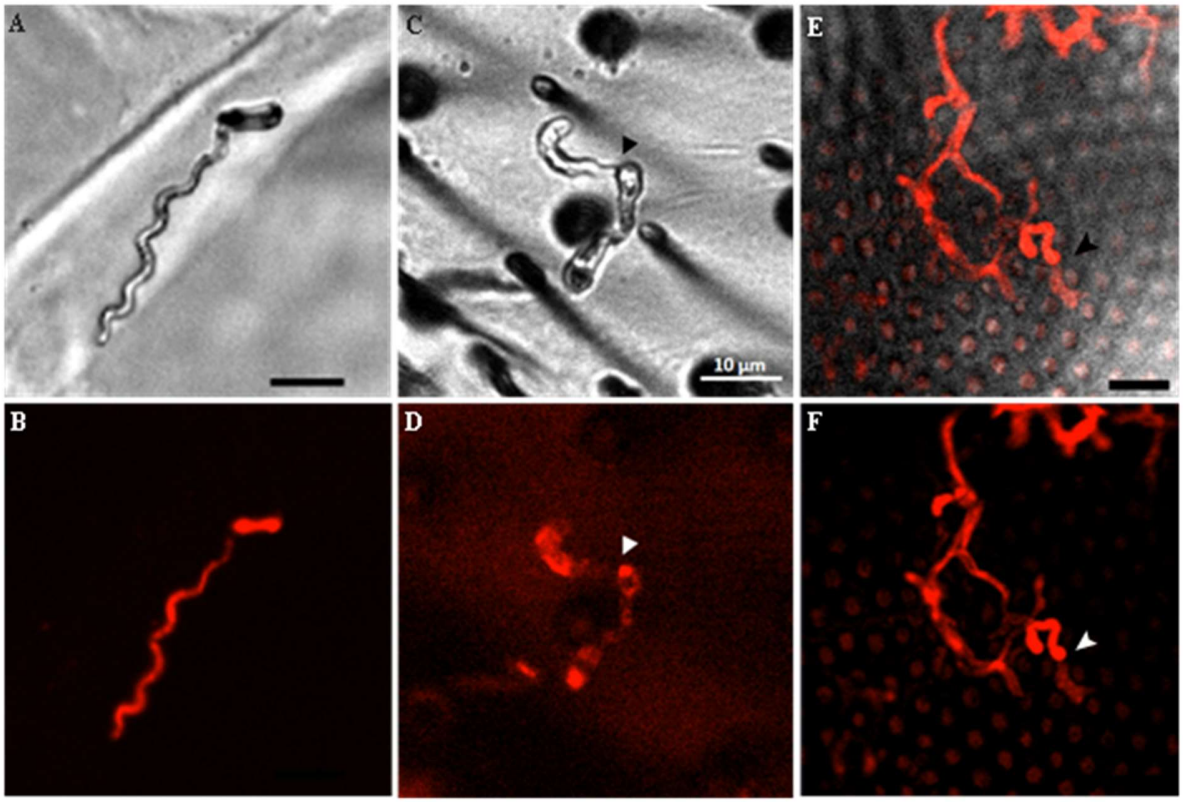


**Figure 8.** *mCherry-expressing M. robertsii strain ARSEF 2575 germinating on plant and insect tissues.* Brightfield images on the top row correspond to fluorescent channel images on the bottom row. (A-B) Onion epidermis, 24 hours after inoculation. Bar: 10µm. (C-D) Fly wing, 24 hours after inoculation. Arrows indicate region where germ tube differentiates into appressorium, and a penetrating tube can be observed. Bar: 10µm. (E-F) Larval cuticle, 48 hours after inoculation. Arrows indicate apical swelling with penetrating tube under the cuticle surface. (E) shows superposition of brightfield and red channels. Bar: 20 µm.

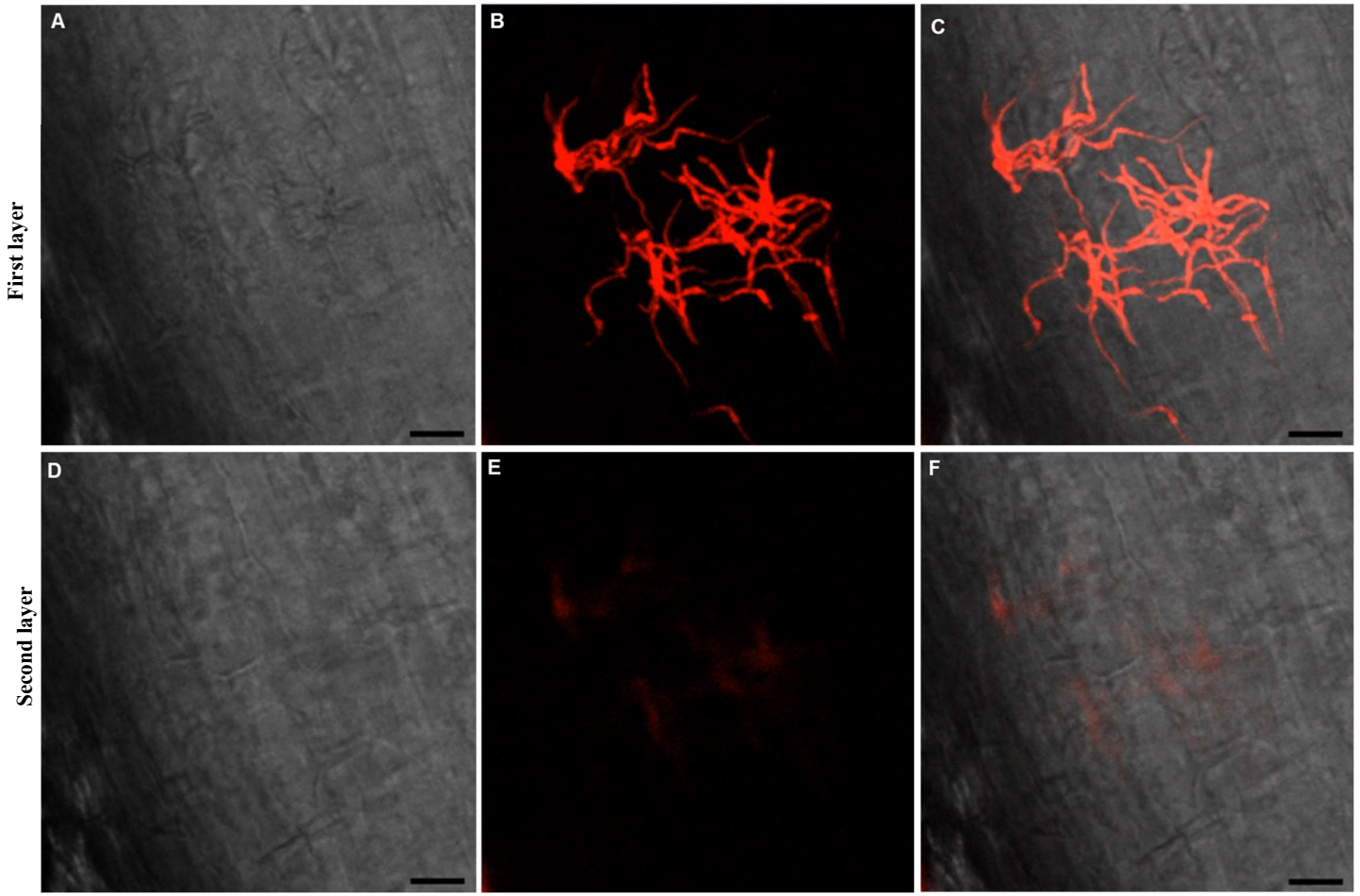


**Figure 9.** *M. robertsii conidia germinating on the surface of corn roots 24 hours post inoculation.* Columns show samples under brightfield (left), red fluorescence channel (center) and merged (right). Top row (A-C) shows the surface of the root, while bottom row (D-F) shows a deeper layer of the same region. Layers are approximately 20 µm apart. Bar: 20µm.

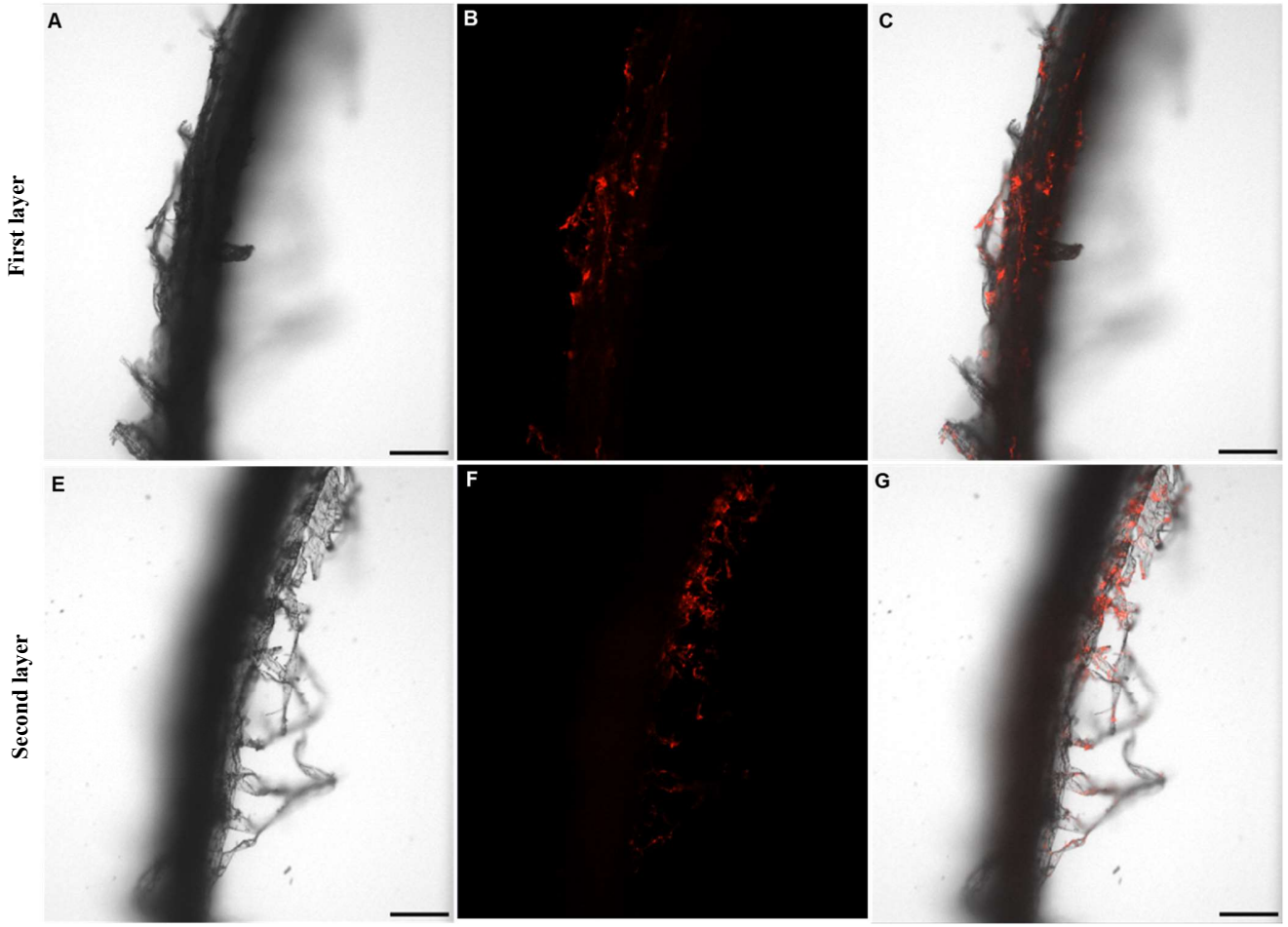


**Figure 10.** *M. robertsii germinating on the surface of switchgrass roots 24 hours post inoculation.* Images show sample under brightfield (left), red fluorescence channel (center) and merged (right). Top row (A-C) and bottom row (D-F) show the same region separated by about 110µm. Fluorescent signals indicate association with the surface (C) and lateral roots (F). Bar: 100µm.

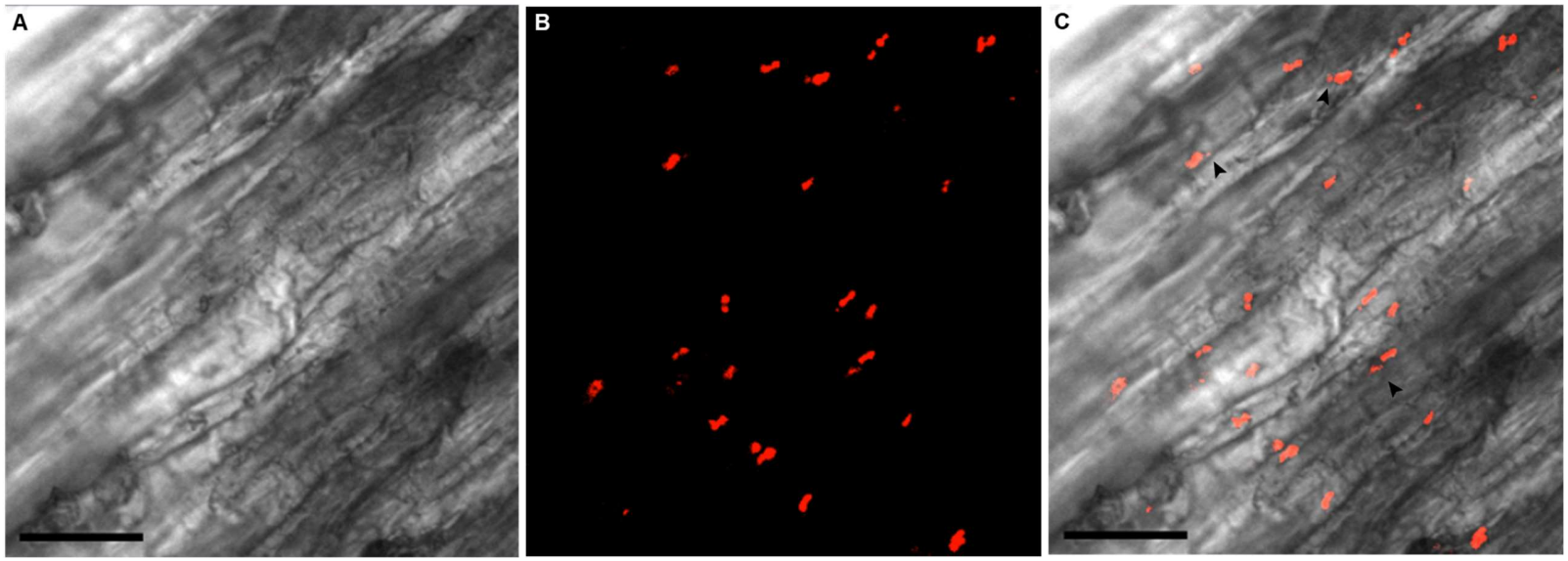


**Figure 11.** *M. robertsii conidia germinating on the surface of bean roots 24 hours post inoculation*. Images show sample under brightfield (A), red fluorescence channel (B) and merged (C). Most conidia are not yet germinated or have short germ tubes (black arrows). Bar: 50µm.

## 2.4 Discussion

Microscopical observations of *M. robertsii* ARSEF 2575 growing on plant roots and insect tissues indicate that varying morphological development is present in this strain in the first stages of host colonization, depending on the host it is starting to colonize. The use of an mCherry-expressing strain of ARSEF 2575 allowed us to confirm the establishment of close associations between *Metarhizium* and plant roots through confocal microscopy, mostly with the two dicot species used in this work (Fig. 9, 10). Conidia germinated abundantly in the surface of corn and switchgrass roots and showed well developed hyphae 24 hours post inoculation. In our observations, apical growth with no formation of appressoria and no visually recognizable colonization of internal root structures were identified in corn and switchgrass at this stage of germination.

In corn, despite the random deposit of conidia onto the surface of the roots, hyphal growth of germinated conidia seemed to lean towards the direction of growth of the host plant root (Fig. 10). From inoculations in switchgrass, not only was a strong root association confirmed, but the fungi also grew on the plant's lateral roots. In their assessment of plant growth promotion by IAA-producing *M. robertsii* ARSEF 2575, Liao et al. (2017) also reported observing an association of fungal hyphae with lateral roots of *Arabidopsis* grown in association with this strain. These results indicate that lateral roots represent an opportunity of rhizoplane colonization by *Metarhizium*, which coupled with the information that ARSEF 2575 possesses the ability to produce IAA and also stimulates lateral root formation in *Arabidopsis*, show us that IAA secretion by *Metarhizium* could be one of the mechanisms that explain the rhizosphere and rhizoplane competence of these fungi. In our observation of mCherry-expressing ARSEF 2575 through confocal microscopy, conidia were seen attached to the root surface of beans, but at regions mostly remained ungerminated (Fig. 11). It is possible that different morphological development would be observed in different regions of the root.

However, *Metarhizium* is known for its poorer association with monocots than with dicots. In an assessment of root colonization by multiple strains of *Metarhizium*, Moonjely & Bidochka (2019) found that, while they are still able to colonize the rhizosphere and rhizoplane of bean roots, rates of colonization of all strains were much lower in beans than in other species of monocot plants. Notably, no endophytic colonization was observed within bean root tissues in this study. The mechanisms that explain the greater rate of colonization in monocots than in dicots by *Metarhizium* are still not understood. In our experiments, we recorded a high rate of germination by *M. robertsii* in both corn and bean exudates (Fig. 4). The fact that *Metarhizium* germinates in both exudates and is able to colonize the rhizosphere and rhizoplane of both corn and bean indicates that these fungi can utilize nutrients derived from both types of plants to some extent. It is likely that other factors such as composition of the root surface or specific plant-fungus signalling could be at play to explain the reduced germination of *M. robertsii* ARSEF 2575 in bean roots, and the overall weaker association of Metarhizium with dicots compared to monocots.

*M. robertsii* is known as a competent rhizoplane colonizer, and its conidia attach effectively to the surface of plant roots prior to germination. Sasan & Bidochka (2012) have reported the successful attachment of *M. robertsii* to the surface of switchgrass roots, a process that involves the expression of specific plant-adhesion genes such as *Mad2* (Wang et al., 2007). Barelli et al. (2011) found that plant stems and root exudate are environmental cues that stimulate the production of this adhesin by *Metarhizium*, indicating the capability of these fungi to properly initiate physical attachment with the host prior to further morphological development and subsequent rhizoplane colonization. Sasan & Bidochka (2012) also confirmed the ability of *M. robertsii* to establish long-term associations with plant hosts - up to 60 days post inoculation - through multiple microscopy techniques such as confocal and scanning electron microscopy. It was also reported that, at this late stage, *M. robertsii* hyphae

could be seen colonizing the interior structures of the plant root. This observation agrees with some studies that have shown an ability of endophytic colonization by *Metarhizium*, although these fungi tend to be isolated in lower quantities from internal structures when compared to other endophytic fungi such as *P. chlamydosporia.* Our observation of *M. robertsii* in switchgrass showed that, after 72 hours of inoculation, the fungus displays extensive hyphal growth in the rhizoplane and entangled with lateral roots, but at this stage, no visible fungal structures were detected in internal plant structures. Along with the lack of observation of appressorial formation by ARSEF 2575 in plant tissues, it is possible that other mechanisms are utilized to allow internal colonization of the root, and this process probably takes place at a later stage following inoculation. These could also be part of the reason why *Metarhizium* are relatively poor endophytic colonizers when compared to other plant-associated fungi such as *P. chlamydosporia* (Moonjely & Bidochka, 2019). The strong root surface association observed in our microscopical observations of strain ARSEF 2575 supports the higher tendency of this fungus to firstly colonize the rhizosphere and rhizoplane of plant hosts, rather than producing penetrating structures that would allow it to grow internally in the roots from an early stage. Strong rhizoplane association by *Metarhizium* has been reported in other studies (Sasan & Bidochka, 2012; Moonjely & Bidochka, 2019). Hu et al. (2023) reported the ability of three species of *Metarhizium* species to successfully colonize the rhizoplane of *Cannabis sativa* L. plants, while the level of endophytic colonization was significantly lower when compared to the competent endophyte *P. chlamydosporia*.

In contrast to plant tissues, appressorial formation was observed when the fungus was inoculated onto insect tissues such as fly wings and larvae cuticle (Fig. 7). Besides the apical differentiation into appressoria, germ tubes were also observed in insect host tissues. These results support the hypothesis that physical cues in the surface of host insects, including the hydrophobicity of such cuticles, are important factors in stimulating appressorial differentiation

by *Metarhizium*. When inoculated in a hydrophilic surface, most *Metarhizium* strains showed a significant decrease in appressorial formation compared to a hydrophobic surface, including ARSEF 2575, which had virtually no appressorial differentiation in cellophane (Fig. 6). Interestingly, *M. anisopliae* strains had higher rates of appressorial germination in cellophane than *M. robertsii* and other *Metarhizium* species. The reason why this phenomenon is observed in *M. anisopliae* and not in other generalists is unknown, and whether this has any effect on host colonization by *M. anisopliae in vivo* remains to be investigated. When comparing root colonization, Moonjely & Bidochka (2019) report no significant difference in rates of rhizoplane or endophytic colonization by these two species. Both also showed equal rates of mortality against insect hosts such as mealworm (*Tenebrio monitor*) and grasshopper (*Melanopus sanguinipes*). Microscopical observations of *M. anisopliae in vivo,* such as those performed for ARSEF 2575 in this study by using confocal and scanning electron microscopy, could be interesting to determine whether any major morphological differences are present in the early-stage development of *M. robertsii* and *M. anisopliae* strains in insect and plant hosts.

Our experiments show that most generalist strains of *Metarhizium* germinate, at varying rates, in multiple nutritional media including alanine and corn and bean exudates (Fig. 5). L-alanine is a major component of insect cuticle and the most abundant amino acid in locust cuticle; *Metarhizium* proteases and hydrolases are efficient in releasing alanine from insect cuticle for use as a nutritional source, and inoculation with alanine supports *M. anisopliae* conidial germination and biomass growth (St. Leger et al., 1986; St. Leger et al., 1994). Our observations show that high rates of germination in 0.005% L-alanine were maintained for most of those strains who also germinated well in YE. Exceptions include *M. robertsii* BRK 18-12 and EH-865, as well as *M. brunneum* ARSEF 2974, for which germination decreased in alanine. At the chosen concentration (0.001%), alanine did not cause significant decrease in appressorial formation by most strains. St. Leger et al. (1989) showed that appressorial

differentiation *in vitro* by *M. anisopliae* decreased as concentration of alanine increased, reaching zero by 0.5% alanine solution. Appressorial formation is driven in some level by nitrogen deprivation, and L-alanine, being a readily usable amino acid, could repress germ tube differentiation at higher concentrations. Even though alanine is the most prevalent amino acid in locust cuticle, it alone was not able to induce germination in the acridid specialist strains *M. acridum* ARSEF 324 and ARSEF 7486. Jarrold et al. (2007) also found that *M. acridum* germinated poorly on alanine, although addition of glucose along with the amino acid was, for some reason, restore promotion of some level of germination.

Gene expression analyses have showed that competent root colonizers are able to utilize plant-derived nutrients, such as carbohydrates, to support fungal growth (Pava-Ripoll et al., 2011). However, this ability varies a lot between strains, even within the same species. In *M. robertsii*, ARSEF 2575 had particularly high germination rates, but BRK 18-12, HAB-5 and EH-809 did not show the same behaviour. Notably, these strains, along with *M. brunneum* ARSEF 8680, germinated poorly across all nutritional media tested indicating that, for some reason, their nutritional requirements to initiate germination are more specific. Similar disparities in germination rates among strains have been reported previously; Pava-Ripoll et al. (2011) tested different *M anisopliae* strains for their ability to germinate in bean root exudate at varying concentrations and found significant variation in germination rates between them. Interestingly, our experiment showed that substrate hydrophobicity also has an effect on conidial germination apart from influencing appressorial formation. Certainly, multiple environmental cues are involved in initiating germination and differentiation of appressoria in these fungi, and the degree to which these factors exert this influence changes significantly between strains.

Specialist strains *M. acridum* ARSEF 324 and ARSEF 7486 did not display any conidial germination *in vitro* in any tested conditions of our study. These results are in line with previous

reports of poor germination of specialists in root exudate and the requirement of specific nutritional cues for their germination and appressorial formation (Wang & St. Leger, 2005; Pava-Ripoll et al., 2011; Zhang et al., 2019; Zhang et al., 2020). Interestingly, despite this specificity and their close association with insect hosts, specialists are known to retain their ability to colonize plant roots, which contrasts with the implied loss of plant-association competence expected of specialists due to their smaller genomes and loss of plant-association genes during diversion of generalist strains (Moonjely & Bidochka, 2019; St. Leger et al., 2024). The lack of germination of *M. acridum* in root exudates indicate that these plant-derived compounds might not be involved in inducing fungal growth during their interaction with plant roots. However, root exudates used in our experiments were obtained under sterile conditions; since plants change the composition of their exudates based on several factors including root microbial composition, it is possible that *M. acridum* colonizing plant roots *in vivo* would encounter a different nutritional profiling. It remains a question whether root exudate has any effect on allowing specialists to retain their competence as plant colonizers, but it is likely that there are many other mechanisms at play in this plant-fungus interaction.

## 2.5 Conclusions

Overall, our microscopical observations of ARSEF 2575 confirm that this generalist strain of *Metarhizium* is able to germinate in both plant and insect tissues, although morphological differences are observed between these two substrates. Appressorial formation and penetration of host tissue were only seen in insect tissues in the tested conditions at an early stage of colonization. The low levels of internal root colonization by *Metarhizium* observed in studies that analysed long term association between fungi and plant host show that, while this ability exists in this lineage, other endophytes such as *P. chlamydosporia* tend to be

more competent in accessing internal plant structures (Moonjely & Bidochka, 2019; Hu et al., 2023).

It is unlikely that significant endophytic colonization by *Metarhizium* takes place in the period evaluated in this work, but it is possible that longer-term associations may lead to opportunistic establishment of the fungus as an endophyte, although the mechanisms through which this colonization happens are still not described. Besides root surface association, we also reported the association of *M. robertsii* ARSEF 2575 with lateral roots when inoculated with switchgrass plants (Fig. 11). *M. robertsii* is a known IAA producer as reported by some studies, with significant effects on lateral root formation and growth in host plants (Liao et al., 2017, Siqueira et al., 2020). The production of IAA by *Metarhizium* could be a strategy used by the fungus to increase the surface area of the hosts with which it is capable to form strong physical associations though the formation of hyphae adjacent to the lateral roots, further solidifying this symbiotic relationship in the rhizosphere.

# Chapter 3: *In vitro* IAA production by *Metarhizium* and assessment of plant colonization competence by selected strains

## 3.1 Introduction

The auxin indole-3-acetic acid (IAA) is a major phytohormone involved in regulation of multiple plant physiological traits, including lateral root formation, root growth, leaf and fruit formation, amongst others (Di et al., 2015). Several plant-associated microorganisms, including fungi and bacteria, also possess the ability to produce and release phytohormones such as IAA, which can then be used to induce morphological changes that will support the establishment of symbiotic relationships or, in the case of phytopathogens, to facilitate infection of plant structures (Chanclud & Morel, 2016).

The main biosynthetic pathways for IAA production both in plants and in plant-associated microorganisms are dependent on L-tryptophan as a precursor (Di et al., 2015). Two of these pathways have been suggested to be present in *M. robertsii*: the TAM pathway, which involves the decarboxylation of tryptophan into tryptamine (TAM), followed by formation of indole-3-acetaldehyde (IAAld) and subsequent dehydrogenation into IAA; and the IAM pathway, which starts with the formation of indole-3-acetamide (IAM) from tryptophan by a Trp mono-oxygenase, leading to formation of IAA through hydrolysis of IAM (Liao et al., 2017). A tryptophan-independent IAA pathway has been hypothesized to be present in plants, but evidence is scarce, and no genes involved in this proposed pathway have been identified so far (Di et al., 2015; Nonhebel, 2015). Auxin production without tryptophan has been reported for some microorganisms, but again, any pathways involved in this process are yet to be further investigated (Ahmad et al., 2020a; Jahn et al., 2021; Goud et al., 2024).

A few species of *Metarhizium* have been reported as potential IAA producers, including *M. robertsii*, *M. anisopliae*, *M. marquandii*, *M. humberi*, and *M. pingshaense* (Liao et al., 2017;

Baron et al., 2020; Siqueira et al., 2020; Chaudhary et al., 2023). In association with *Arabidopsis* roots, *M. robertsii* synthesizes IAA, increases lateral root formation and promotes plant growth in the host (Liao et al., 2017). Interestingly, the IAA biosynthetic pathway is also involved in pathogenicity against arthropod hosts by *Metarhizium*, indicating that auxin production is possibly used by these fungi to establish more than one type of host colonization via an adaptation of previously existing IAA pathways towards entomopathogenicity (Liao et al., 2017).

Despite the relevance of IAA production as a mechanism in both plant- and insect-association, not a lot is understood about its production by *Metarhizium*. A relatively small number of species and strains have been tested for their ability to produce IAA *in vitro* and, to our knowledge, only one study by Liao et al. (2017) has used a state-of-the-art, chromatography-based methodology to determine IAA production with higher precision. In our study, we aim to evaluate several *Metarhizium* strains with varied lifestyles, ranging from specialists to generalists, regarding their ability to produce this phytohormone in liquid media. Production and concentrations of IAA product in the presence or absence of the precursor L-tryptophan, as well as in corn root exudate, were assessed through liquid chromatography with tandem mass spectrometry (LC-MS). Finally, strains of *M. anisopliae* with varying levels of IAA production *in vitro* were selected for plant inoculation assays, in order to compare their effects on corn plant growth and determine rates of endophytic colonization in plant roots and the rhizosphere. The results of these experiments contribute towards our general knowledge about IAA production by *Metarhizium* and raise ideas for further investigations about the role of this hormone in their ecological interactions.

## 3.2 Material and methods

### 3.2.1 Preparation of fungal inoculum for IAA production assays

To obtain fungal biomass for the IAA production assays, 1 ml of conidial suspensions ($1\times10^7$ conidia/ml) were inoculated into 100 ml of YPD broth (Sigma-Aldrich, USA) in flasks and incubated at 27°C and 120 rpm for 4-5 days. After significant biomass growth had been achieved, the broth was filtered using sterilized filter paper (P8, Fisher Scientific, USA) and fresh mycelia was washed with sterile deionized water to remove leftover nutritional medium. The mycelial mass was then weighed and transferred to flasks with new liquid media for IAA production assessment. Three types of media composition were used to test auxin production, namely, 0.2X Murashige-Skoog (MS) medium (Sigma-Aldrich, USA) alone, 0.2X MS medium amended with 0.005% L-tryptophan (Bioshop, Canada), or 0.2X MS medium amended with 1% corn root exudate. Root exudate was prepared by incubating 50 previously sterilized corn seeds in flasks with 100 ml sterile deionized water for four days at 27ºC and 120 rpm. For each treatment, 0.60 g of fresh mycelia was transferred to 120 ml of liquid media in three replicates. Flasks were incubated under agitation (120 rpm) in the dark for 16 hours at 27°C. Prior to extraction, the mycelial mass was removed by filtration using filter paper.

### 3.2.2 Extraction of filtrate for IAA quantification

Prior to extraction, indole-3-propionic acid (IPA, Sigma-Aldrich, USA) was added to each sample as an internal standard, to a final concentration of 0.1 µg/ml. Extraction was performed by vigorously mixing the fungal filtrate with an equal volume of ethyl acetate (Sigma-Aldrich, USA) for about two minutes. The organic layer, carrying the IAA extracted from the filtrate, was carefully removed without disturbing the bottom aqueous layer. The procedure was performed twice for each sample to ensure full extraction of IAA from the

aqueous layer. The total resulting organic layer was then air evaporated to dryness, redissolved in 250 µl of pure methanol (Sigma-Aldrich, USA) per sample, and filtered through a 0.2 µm filter into a dark glass vial for chromatography analysis.

### 3.2.3 Quantification of IAA through LC-MS

IAA concentration in fungal extracts was assessed through liquid chromatography-tandem mass spectrometry (LC-MS/MS) with adaptations from the methodology used by Liao et al. (2017) based on Sirrenberg et al. (2009). For IAA quantification, a calibration curve was prepared with pure IAA (Sigma-Aldrich, USA). Indole-3-propionic acid (IPA, Sigma-Aldrich, USA) was added to all samples at a final concentration of 0.1 µl/ml as an internal standard. The IAA standard curve was prepared at the following concentrations: 5 ppm, 2.5 ppm, 1 ppm, 500 ppb, 250 ppb, 100 ppb, 50 ppb, and 25 ppb. The instrument LOD was calculated using the concentration of IAA that produced a measurable signal with a S/N ratio ≥3. LOQ = 3.3 x LOD. The LOD value was determined as 6 ng/ml (ppb) and the LOQ as 19.8 ng/mL (ppb). The method detection limit, taking into consideration the sample preparations steps, were determined as MDL 0.015 ng/ml and MQL 0.0495 ng/ml.

Samples were analyzed in an Agilent 1290 Infinity II liquid chromatography (LC) system coupled with an Agilent 6470B triple quadruple mass spectrometer (QQQMS; Agilent Technologies, Santa Clara, CA, USA). Instrument control was performed using the MassHunter LC/MS Data Acquisition Software v10.1 (Agilent Technologies). Chromatographic separations were performed on a ZORBAX RRHT Extend-C18 LC column (2.1mm × 50mm, 1.8µm, Agilent Technologies). Mobile phases were LC-MS grade water with 0.1% formic acid (A) and LC-MS grade acetonitrile (B), with a flow rate of 0.4 ml/min and an injection volume of 5 µl. The column compartment temperature was maintained at 35 °C. The

following gradient elution program was used: 0 min, 5 % B; 5 min, 50 % B; 5.5 min, 100 % B. Total run time was 8 minutes, with a post-run time of 3 minutes for re-equilibration.

The QQQMS was equipped with an Agilent jet stream electrospray ionization source and MS data was acquired using multiple reaction monitoring (MRM). The ESI source conditions were as follows: drying gas temperature, 325°C; drying gas flow of 10 l/min; nebulizer pressure of 45 psi; sheath gas temperature of 325°C, sheath gas flow of 11 l/min; VCap setting of 2500V; nozzle voltage of 1000V. The MRM method parameters are listed in Table 3 (Appendix). All samples were analyzed in a randomized order with an injection blank and QC calibration standard (IAA, 500 ppb) analyzed every 10 samples. The QC standard was within 5% of its concentration throughout the sequence. No carryover was detected.

### 3.2.4 Assessment of plant colonization and plant growth promotion by fungal strains

Corn seedlings were inoculated with four fungal strains selected based on their levels of IAA production *in vitro*. Following surface sterilization as previously described in this work, corn seeds were germinated on water agar plates for four days and subsequently transferred to individual pots (460 ml) with sterilized all-purpose soil (Lambert, Canada). Seedlings were inoculated following the soil drench method by pipetting 5 ml of conidial suspensions at a concentration of $1\times10^7$ conidia/ml around the base of the seedling stem. In the control group, 5 ml of water plus 0.01% triton-X were added to the pots instead. Five biological replicates were prepared for each treatment. Pots were placed for 15 days in a greenhouse chamber with about 14:10 h of natural light:dark cycle, daytime temperature set to 27ºC, and humidity levels between 60-80%. Plants were watered every other day with 50 ml sterile deionized water throughout the duration of the experiment.

Plants were removed from the pots 15 days after inoculation and excess soil attached to the roots was collected for assessment of rhizoplane colonization. The soil was weighed, mixed with sterile deionized water plus 0.01% triton-X and diluted before plating in modified selective CTC medium (PDA with 0.5 g/l chloramphenicol, 0.5 g/l cycloheximide, and 0.004 g/l thiabendazole). Colonization rate was calculated as colony forming units (CFU) per gram of rhizoplane soil. To assess endophytic colonization, root sections were weighed, surface sterilized by immersion in 2% NaClO for 10 s and washed in sterile deionized water to remove excess bleach. Root sections were then cut into 2-5 mm pieces and homogenized in sterile water before plating in CTC medium. Colonization rate was measured in CFU per gram of root. The same procedure was used to assess rhizoplane colonization, with exception of the surface sterilization step which was skipped to maintain viable *Metarhizium* growing in the root surface. To investigate whether plant growth parameters were influenced by fungal inoculations, shoot (aerial) and root lengths were measured, and their fresh weight was collected. Shoots were then placed in a drying oven at 60ºC until complete removal of moisture to obtain the dry shoot biomass. All growth parameters were compared between fungal treatments and the control group.

### 3.2.5 Statistical analysis of data

All *Metarhizium* strains produced IAA in 0.005% L-tryptophan ("Trp"), but most did not produce detectable amounts of IAA in MS only ("MS") nor in 1% corn root exudate ("RE"). Therefore, data from the Trp treatment only was used to perform a comparison between all strains using one-way analysis of variance (ANOVA) with Tukey's Honest Significant Difference (HSD) post hoc for statistical difference between groups with a p-value of $<0.05$ for significance. ANOVA results and compact letter display (CLD) showing groups with no statistical difference in IAA production based on Tukey's HSD is presented in the Appendix

(Table 5). For those strains which also produced IAA in MS or RE, an unpaired t-test assuming equal variances ($p<0.01$) was used to compare IAA production with Trp medium (Appendix, Table 4). Data from plant growth parameters (root length, aerial length, root weight, fresh and dry aerial weight, and total plant length and weight) and fungal colonization (rhizosphere, rhizoplane and endophytic) was also analysed for statistical difference through ANOVA with Tukey's Honest Significant Difference (HSD) post hoc test between groups. Groups with no significant statistical difference between them are displayed in Fig 12 and Fig. 13 with same letters above bars. All analyses were performed using JASP software (version 0.19) (JASP Team, 2024).

## 3.3 Results

### 3.3.1 Generalist and specialist strains of *Metarhizium* produce IAA in liquid medium

All strains of *Metarhizium* tested in this experiment were able to produce IAA in 0.2X MS medium amended with 0.005% L-tryptophan (Trp). Detectable amounts of IAA are presented as mean (ng/ml) ± standard error in both Fig. 12 and Table 2 (Appendix). In Table 2, samples with no detectable IAA are presented as <DL (below detection limit by LC/MS-MS). Statistical differences between groups according to Tukey HSD are provided in the Appendix (Table 5). Most concentrations of IAA in the Trp treatment fell within the 0.1 – 2.5ng/ml range, with considerable intraspecific variation between strains. The Mexican *M. anisopliae* strain EH-473 stood out as the highest IAA producer in Trp, with a yield of 9.528±0.774 ng/ml, significantly higher than all other *Metarhizium* ($p<0.001$, Tukey HSD). It was followed by *M. robertsii* EH-809 with 2.462±0.219 ng/ml, and *M. brunneum* ARSEF 8680 with 2.269±0.163 ng/ml. The lowest producer was the generalist *M. pingshaense* EH-802, with a mean IAA yield of 0.148±0.03 ng/ml. The specialist *M. acridum* ARSEF 7486 produced

1.482±0.271 ng/ml in Trp, a significantly higher amount than generalists *M. pingshaense* EH-802 and *M. brunneum* ARSEF 2974 ($p<0.01$, Tukey HSD) and similar to some generalists such as *M. anisopliae* EH-849 (1.865±0.100 ng/ml) and *M. robertsii* EH-798 (2.118±0.152 ng/ml). This indicates that there might not be a correlation between lifestyle and level of IAA production *in vitro*, although analysing more specialist strains would be necessary to support this observation. The transitional species *M. guizhouense* strain EH-869 produced 0.972±0.165 ng/ml, only statistically lower than three other strains, namely EH-473 (Tukey HSD, $p<0.001$), *M. robertsii* EH-809 (2.462±0.219 ng/ml) ($p<0.01$), and *M. brunneum* ARSEF 8680 (2.269±0.163 ng/ml) ($p<0.05$).

Two strains of *M. robertsii,* BRK 18-12 and HAB-5, produced detectable amounts of IAA in 0.2X MS without the addition of tryptophan (Appendix, Table 2). However, concentration of IAA was significantly lower in the absence of the precursor compared to the Trp medium for both strains (t-test, $p<0.01$). BRK 18-12 produced 1.00±0.106 ng/ml of IAA with tryptophan and 0.142±0.015 ng/ml in MS only, which represents a 7-fold decrease, while HAB-5 produced 1.098±0.137 ng/ml in Trp and 0.214±0.047 ng/ml in MS only, a 5-fold decrease. Two strains produced detectable amounts of IAA in 0.2X MS medium amended with 1% corn root exudate: *M. robertsii* BRK 18-12, with a production of 0.217±0.021 ng/ml, and *M. brunneum* ARSEF 8680, with 0.176±0.022 ng/ml (mean of two samples, as the third replicate had no detectable IAA). Again, IAA production was significantly lower in RE when compared to Trp for both strains (t-test, $p<0.01$), with a 5- and 10-fold decrease in final IAA concentrations for BRK 18-12 and ARSEF 8680, respectively.

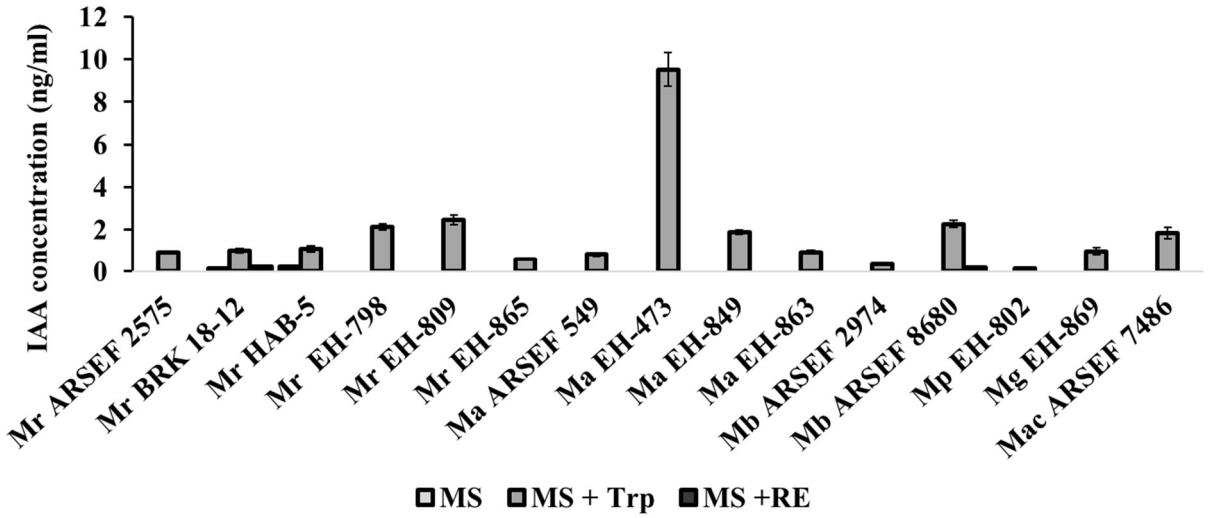


**Figure 12.** *Quantification of IAA production by Metarhizium strains, shown in nanograms per milliliter of liquid media*. Mycelial mass was inoculated into 0.2X MS medium (MS) supplemented or not with either 0.005% L-tryptophan (MS + Trp) or 1% corn root exudate (MS + RE). Samples were collected after 16 hours and IAA was quantified through LC/MS. Bars show mean concentration ± SE calculated from three biological replicates (with exception of EH-802 Trp and ARSEF 8680 RE, with two replicates each).

### 3.3.2 IAA production *in vitro* does not correlate with endophytic colonization rates by strains of *M. anisopliae*

Four strains of *M. anisopliae* were assessed in the previous experiment resulting in varying IAA yields in Trp medium. EH-473 was the highest IAA producer among all *Metarhizium*, with a final concentration of 9.528±0.774 ng/ml — significantly higher than other strains at the $p<0.001$ level according to Tukey HSD (Appendix, Table 5). In contrast, ARSEF 549 was the lowest producer among *M. anisopliae* strains and yielded 0.829±0.078 ng/ml of IAA. The other two *M. anisopliae*, EH-863 and EH-849, produced 0.945±0.085 ng/ml and 1.865±0.100 ng/ml, respectively. This variation in IAA production *in vitro*, particularly between EH-473 and the other strains, prompted the selection of *M. anisopliae* for plant

inoculation experiments in order to compare rhizosphere, rhizoplane and endophytic colonization competencies between these strains. Corn plants were grown in a greenhouse for 15 days following soil drench inoculation with 5 ml of $1\times10^7$/ml conidial suspensions in 0.01% triton-X. Control plants were inoculated with 0.01% triton-X only. After harvesting, plant growth parameters were measured and rates of rhizosphere, rhizoplane and endophytic colonization were analysed by plating soil and root samples in selective agar medium as described in the methodology.

Inoculation with *M. anisopliae* did not have a significant effect in most plant growth parameters in 20-day-old corn seedlings (Fig. 13). No statistically significant differences were observed for aerial height between corn plants inoculated with any of the strains (20.0±0.9 cm for EH-473, 18.5±2.4 cm for ARSEF 549, 18.5±2.4 cm EH-849, and 23.8±2.4 cm for EH-863) compared to each other or to the controls (19.6±1.0 cm) (Tukey HSD, $p<0.05$). While plants inoculated with EH-849 had the shortest mean measurements of aerial fresh weight (0.52±1.4 g), the difference was not statistically different to the controls nor to other groups. Root length was the only parameter in which some effect was recorded for *Metarhizium* inoculated plants: control plants (11.8±0.6 cm) were not statistically different from plants inoculated with EH-473 (15.4±1.0 cm), EH-849 (11.4±1.3 cm), and EH-863 (15.1±1.4 cm), but those inoculated with ARSEF 549 had significantly longer roots (18.7±2.3 cm) than the control group (Tukey HSD, $p<0.05$). EH-473 and EH-863 were also not statistically different from ARSEF 549 (Tukey HSD, $p<0.05$). Root fresh weight, total plant height and total plant weight were not significantly affected by inoculation with any *Metarhizium* strains in this experiment (Fig. 13).

All four *M. anisopliae* strains successfully colonized corn plants at the rhizosphere, rhizoplane and endophytic levels (Fig. 14). Strain EH-849 had the lowest rate of reisolation from rhizosphere soil ($0.69\times10^3$ CFU/g), followed by EH-473 ($3.3\times10^3$ CFU/g) and ARSEF 549 ($2.43\times10^4$ CFU/g). EH-863 had the highest rate of rhizosphere colonization at $1.14\times10^5$

CFU/g, significantly higher than EH-849 and EH-473 but not ARSEF 549 (Tukey HSD, $p<0.05$). In the rhizoplane, ARSEF 549 was the most competent colonizer with $3.13\times10^4$ CFU per gram of homogenized corn roots, significantly higher than EH-863 ($4.95\times10^3$ CFU/g) but not statistically different from EH-849 ($9.60\times10^3$ CFU/g) and EH-473 ($1.47\times10^4$ CFU/g) (Tukey HSD, $p<0.05$). Since it is not possible to eliminate endophytic fungi when assessing rhizoplane colonization, a small part of the reported reisolation rates is likely due to fungi growing internally in the roots instead of the superficial level; however, endophytic colonization is considerably lower and so should not present a major influence in the reported rates of rhizoplane colonization.

Homogenized corn roots with previously sterilized surfaces were used to assess the level of endophytic colonization by *M. anisopliae*. Following a similar pattern to that observed for the rhizoplane, ARSEF 549 was again the most competent colonizer inside plant root tissues, with a mean count of $7.41\times10^2$ CFU per gram of root. Endophytic colonization by ARSEF 549 was significantly higher than all other strains ($p<0.001$, Tukey HSD). Strain EH-863 had a colonization rate of $0.41\times10^2$ CFU/g, while EH-849, at only $0.17\times10^2$ CFU/g, was the poorest colonizer of all strains. Interestingly, EH-473, which was the highest IAA producer *in vitro*, was not the most competent endophyte. With a colonization rate of $0.81\times10^2$ CFU/g, EH-473 was not statistically different from either EH-849 nor EH-863 and was a significantly lower colonizer than ARSEF 549 ($p<0.001$, Tukey HSD).

Overall, strain EH-849 had the lowest rates of CFU reisolation in all tested conditions, possibly indicating poorer competence towards plant colonization from this strain compared to others. Comparatively, ARSEF 549 was by far the most competent colonizer as an endophyte and had also the highest rate of rhizoplane colonization. Its presence in rhizosphere soil was only lower than that of EH-863, but the difference between them was not statistically significant (Tukey HSD, $p<0.05$).

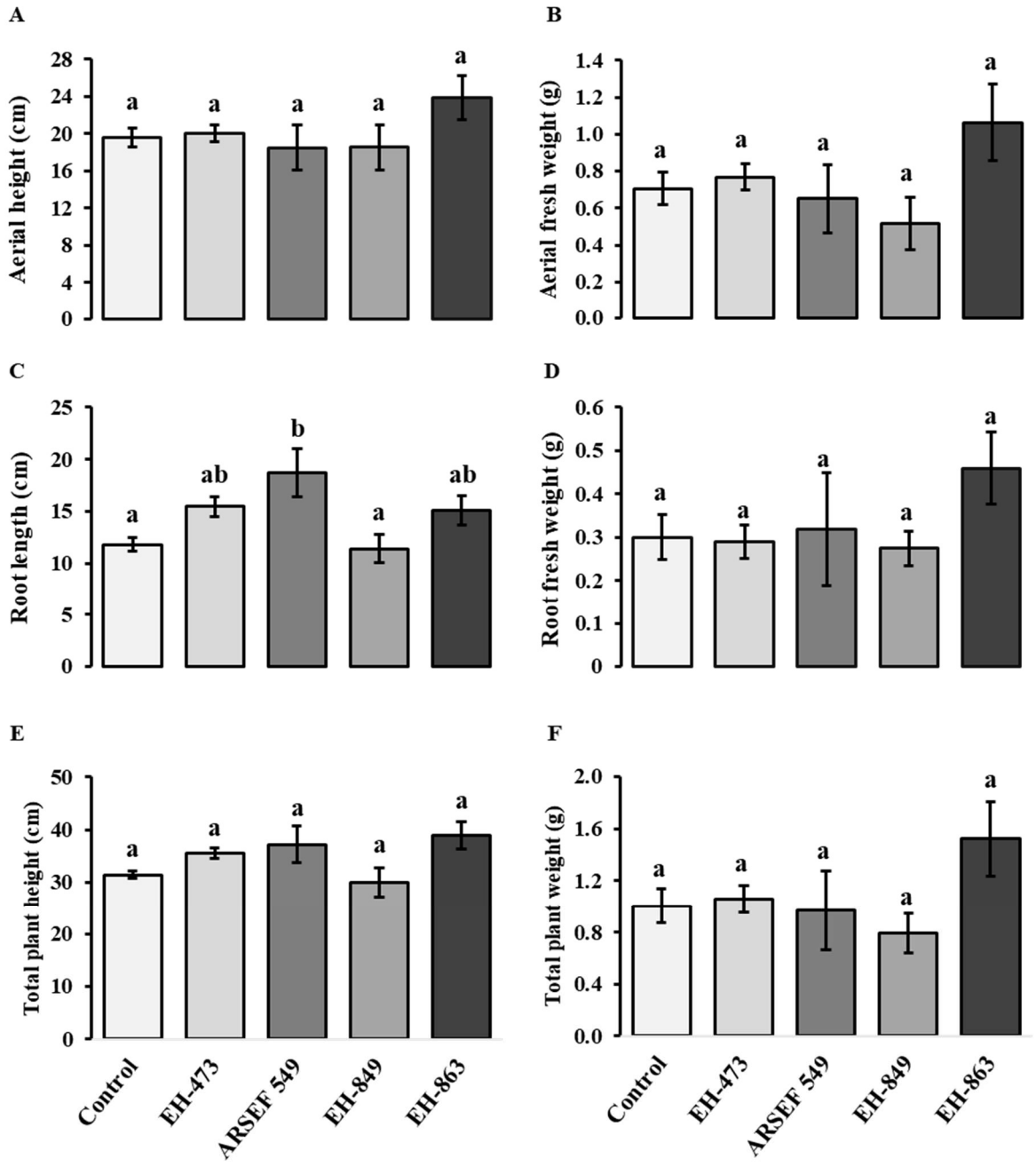


**Figure 13.** *Plant growth parameters in corn plants 15 days after inoculation with M. anisopliae strains*. Five-day-old seedling roots were inoculated with 5ml of $1\times10^7$/ml conidial suspensions of *M. anisopliae* EH-473, ARSEF 549, EH-849, and EH-863. Water plus 0.01% triton-X was added to the control plants. Graphs show measurements of plant aerial height (A), aerial fresh weight (B), root length (C), and root fresh weight (D). Bars represent means of five biological replicates ± standard error. Groups with the same letter on top of bars are not significantly different from each other (Tukey HSD, $p<0.05$).

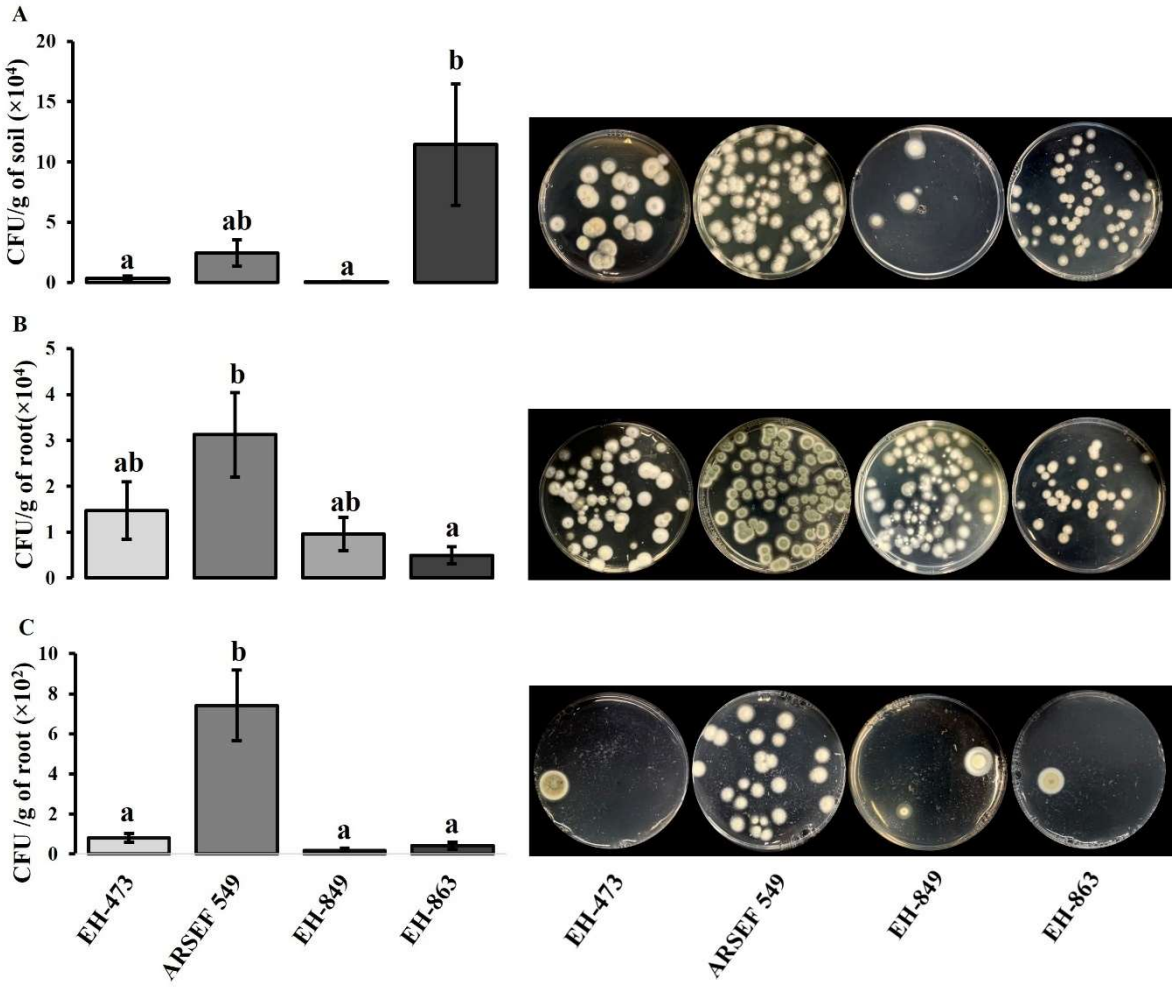


**Figure 14.** *Rates of colonization of rhizosphere, rhizoplane and endophytic colonization by M. anisopliae strains.* Roots and rhizosphere soil from 20-day-old corn plants were harvested and plated in selective agar medium to analyse the rate of colonization by four *M. anisopliae* strains. Rhizosphere (A) colonization is shown in CFU per gram of soil. Rhizoplane (B) and endophytic (C) colonization is shown in CFU per gram of root. Bars represent means of five biological replicates ± standard error. Same letters on top of bar indicates no statistically significant difference between groups (Tukey's HSD, $p<0.05$). Pictures on the right are representative and show strains germinating in selective CTC medium following reisolation from rhizosphere (A), rhizoplane (B), and plant roots as endophytes (C) as described previously.

### 3.4 Discussion

Our analysis of IAA production showed that both generalist and specialist strains of *Metarhizium* are able to produce IAA during liquid fermentation in a tryptophan-rich medium and, interestingly, there was no clear disparity between lifestyles in terms of volume of IAA produced. The strains with the highest and the lowest average IAA yield were both generalists (*M. anisopliae* EH-473 and *M. pingshaense* EH-802, respectively) (Table 2). Apart from EH-473, which produced exceptionally high amounts of the compound, the final IAA concentration after 16 hours of inoculation in Trp medium fell within the 0.1 - 2.5 ng/ml range for all other strains. The specialist strain *M. acridum* strain ARSEF 7486 produced 1.824±0.271 ng/ml, in par with some generalist strains such as *M. anisopliae* and *M. robertsii* (Table 2). Overall, we observed a high level of intraspecific variation of IAA production in *Metarhizium*. We also report that *B. bassiana* and *T. harzianum* produced IAA in the presence of tryptophan (Appendix, Table 6), confirming reports of auxin production by these species in previous studies (Contreras-Cornejo et al., 2009; Liao et al., 2017; Nieto-Jacobo et al., 2017).

L-tryptophan is recognized as the major precursor for IAA production both in plants and fungi (Di et al., 2015). In almost all studies that have analyzed IAA production by plant-associated fungi, significantly higher titers of IAA are achieved in liquid culture when the fungal inoculum is added to tryptophan-rich media, and most fungal strains do not produce any IAA without the presence of an exogenous source of tryptophan. The vast majority of the *Metarhizium* strains tested in our study only produced IAA when L-tryptophan was added to the medium (Table 2). However, two strains of *M. robertsii*, namely HAB-5 and BRK 18-12, did produce IAA in the tryptophan-free medium, a possible indication of the involvement of other biosynthetic pathways in IAA production by these fungi. However, IAA production remained higher in the presence of exogenous Trp; BRK 18-12 had a 7-fold higher production of IAA in tryptophan (1.008±0.106 ng/ml) than in tryptophan-free medium (0.142±0.015

ng/ml), while HAB-5 had a 5-fold higher production in tryptophan (1.098±0.137 ng/ml) than in tryptophan-free medium (0.214±0.047 ng/ml). Other fungi who are able to produce IAA without additional exogenous tryptophan also tend to exhibit higher production when tryptophan is available. In an apparent exception to this pattern, Siqueira et al. (2020) detected a significantly higher concentration of indolic compounds produced by *M. anisopliae* strain ESALQ 1669 in the absence of tryptophan. Tryptophan-independent IAA production has been reported in fungi and other microorganisms, but only tryptophan-dependent biosynthetic pathways have been described and are generally better understood (as reviewed by Tang et al., 2023). The most detailed investigation into IAA synthesis by *Metarhizium* is limited to tryptophan-dependent pathways only: *M. robertsii* ARSEF 2575 produces IAA through two possible biosynthetic pathways, as both tryptamine (TAM) and indole-3-acetamide (IAM) intermediates have been proposed to be present in this fungus through substrate feeding assays (Liao et al., 2017). Our experiment confirmed the lack of IAA production by ARSEF 2575 in the absence of exogenous tryptophan as reported by Liao et al. (2017).

*M. robertsii* BRK 18-12 produced IAA when incubated in a liquid medium amended with corn root exudate (Table 2), with a mean IAA concentration (0.217±0.021 ng/ml) about five times lower than that recorded in the tryptophan medium (1.008±0.106 ng/ml). This strain also produced IAA without tryptophan (0.142±0.015 ng/ml), at about half the rate of production in root exudate. It is possible that some compounds released in the exudate were used by the fungus to produce IAA, or that the exudate composition triggered further tryptophan-independent production by BRK 18-12 through a yet unidentified tryptophan-independent pathway in this strain. Interestingly, *M. robertsii* HAB-5 produced IAA in the absence of tryptophan, but not in the presence of corn root exudate. The contrary was observed for *M. brunneum* ARSEF 8680, which produced some IAA in corn root exudate (0.176±0.022 ng/ml), but no detectable amounts were present in MS medium. The lack of IAA detection in Trp-free

medium indicates that the fungus is unable or was not triggered to synthesize the molecule through a tryptophan-independent pathway. Positive detection of IAA in the root exudate medium therefore raises the hypothesis that compounds in the root exudate might have been utilized by the fungus for auxin production. While we did not test the corn root exudate for its composition, it is known that corn plants do release tryptophan as one of the major organic compounds in its exudate (Pereira et al., 2020). However, root exudate composition varies depending on the environmental condition plants are exposed to, and it is hard to determine whether seeds germinated in a sterile, liquid environment would release tryptophan into its exudate. If any tryptophan was indeed present in the corn root exudate, it remains a question whether those fungal strains used the exudate-derived tryptophan to synthesize IAA, and, if so, why the same process was not observed for other *Metarhizium* strains.

Overall, five main Trp-dependent biosynthetic pathways for IAA production have been identified in microorganisms, namely involving the major intermediate metabolites tryptamine (TAM), indole-3-acetamide (IAM), indole-3-pyruvic acid (IPA/IPyA), indole-3-acetonitrile (IAN), and tryptophan side-chain oxidase (TSO) (Tang et al., 2023). The first two, TAM and IAM, are proposed to be present in *M. robertsii*, with the specific gene *Metarhizium* gene *Mrtdc*, a homologous of the tryptophan decarboxylase gene found in plants, being involved in the conversion of Trp into TAM (Liao et al., 2017). So far, there is no indication that *Metarhizium* is able to produce IAA through any of the other pathways, but they have been proposed to be present in other fungal species. A study with six ectomycorrhizal fungal species indicated that all could produce IAA through the IPA/IPyA, through the observation of tryptophan aminotransferase activity and the presence of intermediate metabolites indole-3-ethanol and indole-3-lactic acid in culture extracts (Kumla et al., 2020). The phytopathogen rice blast fungus *Magnoporthe oryzae* also produces IAA through the IPA pathway, as was described by Dong et al. (2022) who identified the tryptophan aminotransferase (*MoTam1*) and

indole-3-pyruvate decarboxylase (*MoIpd1*) genes, orthologs to TAMs and IPDs originally identified in another phytopathogen, the corn smut fungus *Ustilago maydis.*

Contrary to the pathways mentioned previously, studies on the indole-3-acetonitrile (IAN) and tryptophan side-chain oxidase (TSO) pathways in fungi are very limited or inexistent. In the IAN pathway, which has been identified on bacteria, Trp is first converted into indole-3-acetaldoxime (IAOx), then into either indole-3-acetyl glucosinolate as an intermediate or directly into IAN, which is then finally converted into IAA by nitrilases (Tang et al., 2023). Several details about this pathway still require further investigation in microorganisms, including fungi, which include determining the key enzyme gene that allows the initial conversion of Trp into IAOx (Tang et al., 2023). The TSO pathway, which has only been identified in the bacterium *Pseudomonas fluorescens* amongst microorganisms, produces less IAA than other pathways, could possibly be only a supplementary pathway for auxin production (Tang et al., 2023).

So far, the most detailed observations of auxin production by *Metarhizium* have only suggested the presence of the IAM and TAM pathways in *M. robertsii* (Liao et al., 2017). Apart from IAA itself, other molecules in these biosynthetic pathways have practical implications in the biology and ecology of *Metarhizium* and other fungi. Tryptamine, for example, has been found to increase virulence by the specialist *M. acridum* against locusts, and that inducing accumulation of tryptamine in *M. anisopliae* resulted in this generalist becoming more virulent against locust (Tong et al., 2020). Another intermediate molecule in this pathway, indole-3-acetaldehyde (IAAld) can be used to produce tryptophol (TOL), which is a proposed quorum sensing molecule (QSM) in fungi (Wongsuk et al., 2016). The factors that influence the rate at which *Metarhizium* directs Trp-dependent pathways into IAA synthesis compared to other byproducts is unknown, but it is likely to include multiple environmental cues. Furukawa et al. (1996) found that in the phytopathogenic fungus *Rhizoctonia* sp., the rate of production of

either IAA or tryptophol in the indole-3-pyruvate pathway was influenced by the level of aeration of the culture media. Luo et al. (2017) demonstrated that another plant pathogen, *Fusarium graminearum*, can utilize L-tryptophan as an energy source in detriment of IAA synthesis.

Our findings show a lower level of IAA production, at the nanogram per milliliter of liquid medium, than some previously reported for *Metarhizium*, which sometimes reach a few micrograms per milliliter of liquid medium. The short incubation period used in our experiments (16 hours) possibly did not allow the fungi to reach their maximum levels of IAA production, and it is possible that longer incubation times would result in higher yields of the compound through accumulation over time. In an attempt to achieve optimal conditions for auxin production by *T. harzianum* in liquid culture, Napitupulu et al. (2019) found that IAA concentrations increased daily until reaching a plateau only after the fourth day of incubation. Notably, *T. harzianum* is a much faster growing organism than *Metarhizium*, and substantial IAA yields would probably be reached earlier by *Trichoderma* than *Metarhizium*. Most previous reports of IAA production by *Metarhizium* are taken from cultures incubated for several days or up to a full week in nutrient-rich media such as potato dextrose broth (PDB), allowing for auxin accumulation over time as fungal biomass increases while simultaneously releasing the compound into the liquid culture (Baron et al., 2020; Siqueira et al. 2020). Besides time of incubation, the choice of medium composition used in experiments can also affect final concentrations of IAA, as its production is influenced by multiple environmental conditions. Nieto-Jacobo et al. (2017) report significant variation of IAA production by the same strains of *Trichoderma* when grown in either PDB or MS medium, even when both are amended with the same concentration of L-tryptophan. Other studies on IAA production by *Metarhizium* have used media much richer in carbon and nitrogen, such as PDB and yeast peptone dextrose (YPD), compared to MS (Baron et al., 2020; Siqueira et al. 2020). Napitupulu et al. (2019)

describe how several other parameters also affect IAA titers produced during *T. harzianum* fermentation in LB broth medium, including initial medium pH, incubation temperature and medium salinity. It is likely that *Metarhizium* strains tested in our study would also be affected by such parameters, and thus yield different concentrations of IAA in varying experimental conditions which have not been tested yet.

Different methodologies for IAA detection and quantification can affect the reported final concentrations of auxin in liquid cultures. Prior to our experiments, Liao et al. (2017) conducted the only study using liquid chromatography to quantify IAA production in *Metarhizium*. Reports of IAA production in other species, including *M. anisopliae*, *M. robertsii*, *M. pingshaense*, *M. humberi,* and *M. marquandii*, were based on colorimetric tests of auxin concentration using the indole-binding Salkowski reagent (Baron & Rigobelo, 2020; Siqueira et al., 2020; Chaudhary et al., 2023). The Salkowski reagent, composed of a mixture of iron (III) chloride and sulfuric or perchloric acid, is not IAA-specific and is known to bind to other indolic compounds, which can lead to an overestimation of the actual amount of IAA present in a solution, as was recently investigated by Guardado-Fierros et al. (2024). Chromatography-based methods, such as LC/MS, remain as the most precise ways of detecting and quantifying IAA production by microorganisms in liquid cultures. It remains a question why, in our experiments, ARSEF 2575 produced less IAA than the results reported by Liao et al. (2017), but variation in parameters previously discussed, such as initial medium pH, for example, could be part of the explanation and hindered higher auxin yields in our experiment.

The effects of exogenous IAA on plants vary according to the concentration of IAA that the plant is in contact with. While IAA is the most common auxin phytohormone, a high concentration of IAA in plant tissues can actually counteract the plant growth promotion effects of the molecule due to induction of overproduction of ethylene and abscisic acid (ABA), which can lead to plant senescence, morphological abnormalities, and inhibition of seed germination

(Bunsangiam et al., 2021). Experiments looking at the optimal concentration of exogenous IAA application to plant roots to induce plant growth show that a threshold concentration is reached, after which IAA concentration becomes deleterious to plant morphological development. For example, in rice (*Oryza sativa*), daily application of both synthetic and biosynthetic IAA derived from *Enterobacter* sp. resulted in higher plant growth promotion effects when a 10 µg/l IAA solution was used compared to a 50 µg/l solution (Srisuk et al., 2018). However, the constant reapplication of IAA solution into the soil certainly must be taking into consideration when interpreting these results.

The varied plant species, methods of application, and frequency of IAA application make it hard to establish specific ranges of IAA that are beneficial across different types of plants, and it is hard to extrapolate this information into how much IAA must be produced by a microorganism in relation to its biomass in order for it to exert the same effects. Regardless, it seems that the *in vitro* titters of IAA produced by *Metarhizium* mycelia in our experiment are very low compared to those recorded for known plant growth promoting bacteria, for example. Some hypotheses can be discussed regarding these observations; first, it is possible that *Metarhizium* would be able to produce higher titters of IAA *in vivo*, during interaction with the host roots; and second, it is possible that the later stage of morphological development of *Metarhizium* used in this experimental setup is past its optimal period for IAA synthesis. As has been shown by Moonjely & Trail (2024, in pre-print), transcriptome analysis of *M. anisopliae* during early-stage colonization of barley showed that the appressorial formation stage is correlated with upregulated expression of genes involved in the IAA biosynthetic pathway, so it could be that *Metarhizium* produces more IAA in these early steps *in vivo* compared to older mycelial mass grown *in vitro*. It is also possible that, at this early stage, IAA production plays a bigger role in the morphological development of appressoria than in initiating a signalling cascade in the plant host, although both processes could take place at the

same time. Methanol extracts from the IAA producer *M. robertsii* had the same effects on plant roots as the addition of a 0.1µM solution of synthetic IAA, demonstrating that, *in vivo*, this species does possess the ability to produce enough auxin to exert a biologically relevant response from its host (Liao et al., 2017).

Regarding the precursor L-tryptophan, it is relevant to note that, as part of routine culturing of fungal strains, all *Metarhizium* were initially grown in potato dextrose broth (PDA) agar plates. Moreover, mycelial mass was obtained by culturing in another nutritional medium, yeast peptone broth (YPB). With PDA and YPB both being nutritionally rich media, these could contain tryptophan or residual IAA in their composition. When assessing the production of IAA by *Trichoderma* in PDB medium, Illescas et al. (2021) used the uninoculated medium as a control and found small, but detectable amounts of different phytohormones, including IAA. Through sequentially rinsing the fungal mycelial mass with distilled water prior to transfer into diluted (0.2X) MS medium, a basal medium composed mostly of salts, we expect to minimize the influence of the presence of Trp or IAA not accounted for in the experiment. The lack of IAA detection in Trp-free fungal MS extracts, including those amended with diluted corn root exudates, indicates that, if Trp residues were present in these solutions, they were not present in high enough quantity to significantly influence IAA synthesis by *Metarhizium*. Equally, if any residual IAA was present, its concentration fell below the detection limits, thus not representing a major influence on the detection of *Metarhizium*-derived auxins. Surely, the influence of the initial culturing conditions – fungal growth in PDB and YPB – cannot be ruled out, as we did not assess whether any exogenous Trp present in these culture media were absorbed by *Metarhizium* at these growth stages, and if so, whether this could result in a later influence in the patterns of IAA production *in vitro* even if residual Trp was no longer present at that stage.

Several studies have investigated the effects of *Metarhizium* inoculations in corn plants, often with successful establishment of the fungus in soil and plant roots, as well as positive effects on plant growth promotion on the hosts (Liao et al., 2014; Moonjely & Bidochka, 2019; Ahmad et al., 2020b; Russo et al., 2020; Flonc et al., 2021; Liu et al., 2022). In our study, four strains of *M. anisopliae* with varying abilities to produce IAA in liquid media were tested for their ability to colonize corn plant roots. Following soil drench inoculation of seedlings and 15 days of plant growth in a greenhouse, all four strains had successfully colonized the rhizosphere, rhizoplane, and, at varying degrees, internal root structures of their host plants, but effects in plant growth compared to the control group were limited only to root length (Fig. 12). All *M. anisopliae* strains colonized both rhizosphere and rhizoplane, but ARSEF 549 was notably more competent than the other strains in establishing these symbiotic relationships. Overall, our results confirm the ability of *M. anisopliae* strains to establish associations with the corn root at a superficial level, and reinforce that endophytic competence is a strain-dependent trait within the species. Still, the rate of reisolation of CFU per gram of root was lower in all *M. anisopliae* than what has been previously reported for more competent endophytic fungi such as *P. chlamydosporia* (Moonjely & Bidochka, 2019).

Plants secrete a variety of metabolites in their root exudate which have a major influence in the microbiome living around their roots, and its composition changes according to the plant's health status and its need to recruit protective microbes to fight pathogens (Rolfe et al., 2019; Seitz et al., 2022; Dhungana et al., 2023). The composition of the root exudate includes major organic compounds such as amino acids, and tryptophan can be detected amongst these molecules (Vives-Peris et al., 2020). Although the relationship between tryptophan exudation by roots and symbiosis has not been extensively studied in fungal symbionts, it is known that rhizospheric bacteria utilize plant-derived tryptophan and likely convert it into IAA, resulting in positive plant growth traits (Kuzmicheva et al., 2017). Corn

plants are known to release tryptophan into their root exudate, which could be a potential source of this precursor for auxin-producing root associated microorganisms; however, as mentioned before, tryptophan release by corn plants into the exudate is not constant and varies according to factors such as plant genotype, nutritional status of the plant, and environmental stresses such as heat and drought, which were found to result in increased exudation of L-tryptophan (Pereira et al., 2020; Tiziani et al., 2022). While corn seedlings in our study might have released tryptophan into the soil, it is impossible to determine without an actual assessment of the exudate's composition in the particular environmental conditions the plants were exposed to in this experimental setup.

Auxin production is often correlated with increased plant root colonization by root-associated microbes, likely due to increased lateral root formation induced by microbial derived auxins (Zhang et al. 2013; Herrera-Jiménez et al., 2018). However, not all studies have reported a strict correlation between fungal IAA production *in vitro* and plant growth promotion *in vivo* (Nieto-Jacobo et al., 2017). In our experiments, no correlation between IAA production in liquid media and root colonization by *Metarhizium* was observed after 15 days of fungal inoculation in corn seedlings. Interestingly, while ARSEF 549 had the lowest mean production of IAA among the four strains *in vitro*, it was a significantly better colonizer in corn plants than the other strains. EH-473, which was an outlier and by far the highest IAA producer among all *Metarhizium* species, showed lower rhizoplane and endophytic colonization rates than ARSEF 549 (Fig. 13). While it is logical to hypothesize that higher ability to produce IAA leads to more efficient endophytic colonization by plant-associated fungi, the complexity of the plant-fungus relationship *in vivo* involves several other factors that are not at play when assessing production of fungal auxins *in vitro*. In our experiment, nearly all strains only produced IAA when exogenous L-tryptophan was added to the liquid medium for uptake and further use in the auxin biosynthetic pathways towards IAA production (Table 2). Therefore, for those plant-associated

fungi who rely exclusively on exogenous tryptophan as a precursor for auxin synthesis, the production and secretion of this compound will be limited by the amount of L-tryptophan available for uptake in the root environment. A study by Herrera-Jiménez et al. (2018) showed that plant growth promotion by IAA-producing strains of *Trichoderma* increased when the fungal inoculum was added to plants simultaneously with a tryptophan solution compared to fungal inoculation alone. This shows that the root exudate does not necessarily supply L-tryptophan in titers that allow fungi to achieve their maximum capacity of IAA synthesis. Other environmental factors not yet investigated, as well as complex signaling between plant and fungi, are also probably involved in inducing or repressing IAA synthesis by root-associated *Metarhizium*. It is possible that, due to such complex interactions, ARSEF 549 produced more IAA than EH-473 in the root environment resulting in higher colonization rates, or that ARSEF 549 utilized mechanisms partially or completely independent of IAA production that resulted in increased endophytic competence. Further research with these strains, particularly ARSEF 549, will be needed to unveil these mechanisms at play during root colonization.

IAA production has evolved independently in multiple microorganisms and is known to be involved in complex physiological responses and regulation of gene expression across kingdoms (Fu et al., 2015; Tang et al., 2023). While investigating the biosynthetic pathways involved in auxin production by *M. robertsii*, Liao et al. (2017) found that, besides being released in contact with host plants, IAA is also involved in pathogenesis of *Metarhizium* towards insect hosts, an indication that the auxin pathway plays further roles in the survival of these fungi besides establishment of root colonization. *M. robertsii* previously grown in PDA plates amended with IAA showed enhanced virulence and higher appressorial formation rates compared to controls. Whether appressorial formation and IAA production are also intertwined during plant root colonization by *Metarhizium* was mostly unknown, until recent research described the involvement of IAA synthesis by *M. anisopliae* during colonization of plant host

barley (*Hordeum vulgare*). Moonjely & Trail (2024, in pre-print) showed that *M. anisopliae* spores germinating and colonizing on barley roots present upregulation of candidate genes associated with IAA synthesis, specifically during the step of appressorial differentiation. In fact, appressorial formation is related to a significantly divergent pattern of gene expression compared to other early stages of *Metarhizium* morphological development. These observations raise the hypothesis that the production of appressoria by *Metarhizium*, coupled with simultaneous biosynthesis of auxins, is not restricted to the insect host infection process, but also plays an important role in the establishment of endophytic relationships in the plant root environment.

Interestingly, IAA is also detected in insect tissues, and it has been shown that different species of insects can synthesize IAA utilising Trp-dependent pathways. A synthetic pathway has been proposed which involves the the formation of indole-3-acetaldoxime (IAOx) as an intermediate metabolite (Yokoyama et al., 2017). Like in microorganisms, involves the formation of indole-3-acetadehyde (IAAld) as a middle product, however, an extra previous step involves the production of indole-3-acetaldoxime (IAOx), which is not identified to be present in bacterial or fungal based IAA production. However, IAAld can also be shifted towards the production of indole-3-ethanol (IEtOH) in insects, affecting the rate of IAA production (Yokoyama et al., 2017). Miyata et al. (2021) proposed an insect IAA pathway that shares plant and bacterial middle products. As has been detected in fungi, a conversion of tryptophan into IAAld through the TAM pathway is also present in insects. A genomic analysis carried out by Natahusada et al. (2024) across multiple arthropod species determined that currently known genes involved in IAA pathways in other organisms are not widespread across this group, but transcripts that encode for several homologous proteins to those involved in these pathways were identified in specific groups of arthropods – for example, homologous of two enzymes involved in the TAM pathway are commonly found across both insects and

hexapods. The extent to which these pathways actually lead to IAA production is unknown, as the authors discuss that one type of enzyme proposed to play a major role in IAA conversion in insects, namely the aromatic aldehyde synthases (AAS) could be homologs of an enzyme mainly responsible for cuticular hardening. Aldehyde synthases and oxidases are also mostly undetected in the transcriptomes of most insects (Natahusada et al., 2024). Still, these genomic analyses demonstrate the capacity of arthropods to potentially synthesize IAA, adding to a long list of organisms that can produce this important signalling molecule, and raising further questions about the roles of fungal derived auxins in the insect infection process. The presence of insect IAA could also pose a challenge in quantifying the production of fungal auxins *in vivo* – methods such as the use of stable isotope labelled molecules to trace the origin of IAA are alternatives to explore in future research on this interaction.

Our study has found that all tested *Metarhizium* species, including specialist *M. acridum* and the transitional *M. guizhouense*, are able to produce IAA *in vitro*. It is possible that the IAA biosynthetic pathway has been conserved in these lineages mostly because it is also involved in the insect infection process, but since specialist *Metarhizium* have retained their competence as plant root colonizers (Moonjely & Bidochka, 2019), we cannot rule out that IAA is one of the mechanisms that allows their establishment as plant colonizers. In fact, Moonjely & Bidochka (2019) show that *M. acridum* ARSEF 7486, the same strain we have demonstrated can produce IAA *in vitro*, has a comparable ability to associate with dicot plant roots as some generalist strains. Hu et al. (2023) also reports that *M. acridum* ARSEF 324 retains to some extent the ability to associate with roots of hemp plants. Although *M. acridum* are not isolated very often in association with roots in natural environments (Wyrebek et al., 2011), these observations in the laboratory challenge hypothesis such as the one presented by St. Leger (2024), which propose that the speciation of specialist *Metarhizium* strains would, in theory, have resulted in a loss of plant colonization abilities. Further investigation into the roles

of IAA in host colonization is necessary to increase our knowledge of how *Metarhizium* utilizes IAA to adapt to multiple lifestyles, and to what extent it redirects this pre-existing plant-association metabolic pathway towards entomopathogenicity.

### 3.5 Conclusion

*Metarhizium* is a genus with a complex evolutionary history and species with varied ranges of both plant and insect hosts. Modern *Metarhizium* strains likely arose from generalist, plant-associated ancestral lineages which developed the ability to infect arthropods by redirecting plant-association genes towards the establishment of insect pathogenicity (Gao et al., 2011; St. Leger, 2024). Investigating key metabolic pathways involved in host colonization processes of both plants and insects is a valuable tool to develop our understanding of the adaptations these fungi have gone through during their evolutionary history to shift from strict plant-associated fungi to their current multiple lifestyles. Our experiments showed that IAA production, often associated with the root colonization process, is ubiquitous across *Metarhizium* and is retained in specialist species such as *M. acridum*. It is also known that specialists retain their ability to successfully colonize the roots of plant hosts, even if they some of them, like *M. acridum*, are seldom isolated from the root environment in nature (Wyrebek et al. 2011; Moonjely & Bidochka, 2019). Auxins also play a role towards successful pathogenicity against insect hosts, indicating that this metabolite could be produced by *Metarhizium* with the purpose of colonizing different kinds of hosts, including specialist strains (Liao et al., 2017).

Our results effectively reveal that IAA production is ubiquitously preserved across several *Metarhizium* species, providing information on this topic of *Metarhizium* ecology which still requires further investigation. The higher endophytic competence by a single strain

of *M. anisopliae*, however, reveals that *in vitro* assessment of auxin production levels by *Metarhizium* mycelial mass does pose limitations into further understanding the full scope of the IAA pathways present in this lineage. It is plausible that the analysis of IAA production *in vivo*, both during plant and insect interactions might present a different scenario than the patterns observed in vitro; i.e. *Metarhizium* strains that are more virulent against insect hosts and/or more competent endophytes could be producing more IAA during host colonization, challenging the observations made *in vitro*. The pathways that are involved in this process still require further investigation. Liao et al. (2017) have identified a *Metarhizium* gene, *Mrtdc*, directly involved in the tryptamine (TAM) pathway in *M. robertsii*, making it an ideal candidate for investigating the pathways utilised by this and other *Metarhizium* strains during auxin production. Looking into the levels of expression of *Mrtdc* or its homologues could provide an interesting insight into whether the TAM pathway is being utilised by other *Metarhizium* in the process of IAA production – more specifically, this is an interesting approach to investigate those strains which managed to produce some IAA even in the absence of added Trp, and/or the addition of corn root exudate into the liquid media.

The use of chromatography-based methodologies could be employed to further investigate IAA production by *Metarhizium* by looking into the *in vivo* presence of the auxin compound during host colonization. While it is known that IAA is involved in virulence by *Metarhizium*, to our knowledge, the *in vivo* production of this compound has not been measured, although Liao et al. (2017) have demonstrated, through immunolocalization, the accumulation of IAA in the mucilaginous layer that surrounds the fungal hyphae during spore germination. Insect virulence bioassays coupled with IAA extraction and measuring through LC-MS could provide further insights into the relationship between *in vivo* IAA production and entomopathogenicity in *Metarhizium*. The fact that insects can produce some IAA could hinder the proper identification of fungal-derived IAA, an issue that is also present when investigating

fungal-derived IAA in the roots during fungal colonization. A possible workaround to ensure the traceability of Trp-derived IAA molecules produced by a single organism would be the implementation of stable labelled isotope molecules, a tool that has been used to investigate biosynthetic pathways in order microorganisms. For example, labelled tryptophan molecules ($^{15}N_2$ L-Trp) detected through High Resolution Mass Spectrometry (HRMS) were used by Gallardo-Fernández et al. (2022) to elucidate one of the two possible biosynthetic pathways employed by *Saccharomyces cerevisiae* yeast to synthesize melatonin (Mel) molecules. The use of isotope labelled molecules would not be a novelty in the research of *Metarhizium* biology, as the use of marked molecules has been employed to investigate the transfer of carbon- and nitrogen-derived nutrients between *Metarhizium* and plant hosts (Behie et al., 2012; Behie et al., 2017; Barelli et al., 2019), but it would be the first use of this methodology in the research of auxin synthesis by *Metarhizium*.

Whether IAA production is ubiquitously employed by generalists and specialists towards the establishment of plant- and insect-associated lifestyles remains a topic for further research. The lack of a direct correlation between *in vitro* IAA production by *Metarhizium* and their competence as endophytic colonizers *in vivo* suggests that this metabolite could be used in other processes, such as insect infection. Assessment of IAA production by generalists and specialists in insect hosts, gene expression assays, and substrate feeding assays are plausible ideas to further investigate the roles of auxin in entomopathogenicity, whether IAA biosynthetic pathways such as TAM and IAM are conserved in the genus, and if other yet unidentified pathways, including tryptophan-independent ones, are present across *Metarhizium* lineages.

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## Appendix

**Table 2.** *Quantification of IAA production by Metarhizium strains, shown in nanograms per milliliter of liquid media.* Mycelial mass was inoculated into 0.2X MS medium supplemented or not with either 0.005% L-tryptophan or 1% corn root exudate. Samples were collected after 16 hours and IAA was quantified through LC/MS. Mean±SE were calculated from three biological replicates (with exception of EH-802 Trp and ARSEF 8680 RE, with two replicates each). <DL indicates no detection of the compound above the equipment's detection limit.

| | | **IAA concentration (ng/ml)** | | |
|---|---|---|---|---|
| | | **MS** | **MS + 0.005% Trp** | **MS + 1% corn RE** |
| *M. robertsii* | ARSEF 2575 | < DL | 0.936±0.050 | < DL |
| | BRK 18-12 | 0.142±0.015 | 1.008±0.106 | 0.217±0.021 |
| | HAB-5 | 0.214±0.047 | 1.098±0.137 | < DL |
| | EH-798 | < DL | 2.118±0.152 | < DL |
| | EH-809 | < DL | 2.462±0.219 | < DL |
| | EH-865 | < DL | 0.610±0.068 | < DL |
| *M. anisopliae* | ARSEF 549 | < DL | 0.829±0.078 | < DL |
| | EH-473 | < DL | 9.528±0.774 | < DL |
| | EH-849 | < DL | 1.865±0.100 | < DL |
| | EH-863 | < DL | 0.945±0.085 | < DL |
| *M. brunneum* | ARSEF 2974 | < DL | 0.330±0.045 | < DL |
| | ARSEF 8680 | < DL | 2.269±0.163 | 0.176±0.022 |
| *M. pingshaense* | EH-802 | < DL | 0.148±0.019 | < DL |
| *M. guizhouense* | EH-869 | < DL | 0.972±0.165 | < DL |
| *M. acridum* | ARSEF 7486 | < DL | 1.842±0.271 | < DL |

**Table 3.** *MRM method parameters for LC/MS analysis of indole-3-acetic acid (IAA) and indole-3-propionic acid (IPA) concentrations in liquid media.* Caffeine was spiked into samples as an injection standard for data normalization, at a final concentration of 100 ppb.

| Chemical | Precursor Ion | MS1 Res | Product Ion | MS2 Res | Dwell | Fragmentor | Collision Energy | Cell Accelerator Voltage | Polarity |
|---|---|---|---|---|---|---|---|---|---|
| IAA | 174 | Unit | 128 | Unit | 200 | 66 | 20 | 4 | Negative |
| IAA | 174 | Unit | 130 | Unit | 200 | 66 | 8 | 4 | Negative |
| IPA | 188 | Unit | 59 | Unit | 200 | 94 | 12 | 4 | Negative |
| IPA | 188 | Unit | 116 | Unit | 200 | 94 | 16 | 4 | Negative |
| Caffeine | 195 | Unit | 110 | Unit | 100 | 119 | 24 | 3 | Positive |
| Caffeine | 195 | Unit | 138 | Unit | 100 | 119 | 20 | 5 | Positive |

**Table 4.** *Comparison of IAA production by selected Metarhizium strains in tryptophan and tryptophan-free media*. BRK 18-12, HAB-5, and ARSEF 8680 were the only strains that produced some IAA in tryptophan-free media. Unpaired Student's t-tests assuming equal variance were performed to determine statistically significant differences between production in MS or RE compared to 0.005% L-tryptophan, at the $p<0.05$ level.

| Strains / treatment | | Mean (IAA in ng/ml) | Variance | df | t Stat | P(T<=t) two-tail |
|---|---|---|---|---|---|---|
| BRK 18-12 | MS | 0.142 | 0.001 | 3 | -8.119 | 0.001 |
| | Trp | 1.008 | 0.033 | | | |
| BRK 18-12 | RE | 0.217 | 0.001 | 3 | -7.346 | 0.002 |
| | Trp | 1.008 | 0.033 | | | |
| HAB-5 | MS | 0.214 | 0.007 | 4 | -6.105 | 0.004 |
| | Trp | 1.098 | 0.056 | | | |
| ARSEF 8680 | Trp | 2.269 | 0.080 | 3 | 9.897 | 0.002 |
| | RE | 0.176 | 0.001 | | | |

**Table 5.** *Statistical analysis of IAA production by Metarhizium strains in 0.005% L-tryptophan*. Statistical difference between groups was assessed through one-way analysis of variance (ANOVA). P<0.001 indicates significant difference between groups. Post hoc comparisons between groups were performed through Tukey's Honest Significant Difference (HSD) test. Compact letter display (CLD) is used to show results; strains with the same letter were not significantly different from each other (p<0.05) regarding IAA production in 0.005% L-tryptophan.

| Cases | Sum of Squares | df | Mean Square | F | p |
|---|---|---|---|---|---|
| Column 2 | 218.181 | 15 | 14.545 | 87.273 | < .001 |
| Residuals | 5.167 | 31 | 0.167 | | |

| CLD based on Post Hoc comparisons (LSD) | |
|---|---|
| Mr ARSEF 2575 | abcd |
| Mr BRK 18-12 | abcd |
| Mr HAB-5 | abcde |
| Mr EH-798 | def |
| Mr EH-809 | f |
| Mr EH-865 | ab |
| Ma ARSEF 549 | abc |
| MaEH-473 | g |
| Ma EH-849 | cdef |
| Ma EH-863 | abcd |
| Mac ARSEF 7486 | bcdef |
| Mb ARSEF 2974 | a |
| Mb ARSEF 8680 | ef |
| Mp EH-869 | abcd |
| Mg EH-802 | a |

**Table 6.** *Quantification of IAA production by B. bassiana and T. harzianum, shown in nanograms per milliliter of liquid media.* Mycelial mass was inoculated into 0.2X MS medium supplemented or not with either 0.005% L-tryptophan or 1% corn root exudate. Samples were collected after 16 hours, and IAA was quantified through LC/MS-MS. Mean±SE were calculated from three biological replicates. <DL indicates no detection of the compound above the equipment's detection limit.

| | | IAA concentration (ng/ml) | | |
|---|---|---|---|---|
| | | **MS** | **MS + 0.005% Trp** | **MS + 1% corn RE** |
| *B. bassiana* | ARSEF 252 | < DL | 1.233±0.180 | < DL |
| *T. harzianum* | UAHM 4162 | < DL | 0.902±0.172 | < DL |

**Table 7.** *Statistical analysis of plant growth parameters for plants inoculated with Metarhizium strains*. Statistical difference between groups was assessed through one-way analysis of variance (ANOVA). (*) indicates significant difference between groups at the $p<0.05$ level. Differences in root length between treatments (Tukey HSD, $p<0.05$) are presented as compact display letters (CDL) in Fig. 12 (Chapter 3).

| Parameter | Cases | Sum of Squares | df | Mean Square | F | p |
|---|---|---|---|---|---|---|
| Fresh leaf weight | Column 1 | 0.823 | 4 | 0.206 | 1.874 | 0.154 |
| | Residuals | 2.197 | 20 | 0.11 | | |
| Leaf height | Column 1 | 96.496 | 4 | 24.124 | 1.26 | 0.318 |
| | Residuals | 382.864 | 20 | 19.143 | | |
| Root weight | Column 1 | 0.112 | 4 | 0.028 | 0.943 | 0.459 |
| | Residuals | 0.594 | 20 | 0.03 | | |
| Root length | Column 1 | 178.37 | 4 | 44.593 | 4.278 | 0.012* |
| | Residuals | 208.46 | 20 | 10.423 | | |
| Dry leaf weight | Column 1 | 0.007 | 4 | 0.002 | 1.321 | 0.296 |
| | Residuals | 0.025 | 20 | 0.001 | | |
| Total plant length | Column 1 | 289.49 | 4 | 72.373 | 2.641 | 0.064 |
| | Residuals | 548.02 | 20 | 27.401 | | |
| Total plant weight | Column 1 | 1.487 | 4 | 0.372 | 1.63 | 0.206 |
| | Residuals | 4.561 | 20 | 0.228 | | |

**Table 8.** *Statistical analysis of colonization rates of Metarhizium in plant roots*. Statistical difference between groups was assessed through one-way analysis of variance (ANOVA) test with Tukey's HSD post hoc for significant differences between groups. All parameters had groups with statistically significant difference at the $p<0.05$ level.

| Parameter | Cases | Sum of Squares | df | Mean Square | F | p |
|---|---|---|---|---|---|---|
| Rhizosphere | Column 1 | $4.301\times10^{+10}$ | 3 | $1.434\times10^{+10}$ | 4.306 | 0.021 |
| | Residuals | $5.327\times10^{+10}$ | 16 | $3.329\times10^{+9}$ | | |
| Rhizoplane | Column 1 | $1.969\times10^{+9}$ | 3 | 24.124 | 3.74 | 0.033 |
| | Residuals | $2.808\times10^{+9}$ | 16 | 19.143 | | |
| Endophyte | Column 1 | $1.823\times10^{+6}$ | 3 | 6.08E+05 | 15.03 | < .001 |
| | Residuals | 6.47E+05 | 16 | 4.04E+04 | | |

**Table 9.** *Rates of germination and appressorial formation by Metarhizium strains after 24 hours of germination in two substrates (polystyrene and cellophane) and four nutritional sources (0.01% YE, 0.005% alanine, 1% corn RE and 1% bean RE).* Values are presented as percentages and represent the average of three counts of one hundred conidia for each sample, plus the standard error for each count. "na" indicates that appressorial formation could not be assessed due to lack of germination in that condition.

| Fungal species | Strain | Polystyrene | | | | | | | | Cellophane | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Germination (%) | | | | Appressorial formation (%) | | | | Germination (%) | | | | Appressorial formation (%) | | | |
| | | YE | Ala | Corn RE | Bean RE | YE | Ala | Corn RE | Bean RE | YE | Ala | Corn RE | Bean RE | YE | Ala | Corn RE | Bean RE |
| *M. robertsii* | ARSEF 2575 | 90.7 ±3.2 | 87.3 ±1.8 | 87.7 ±1.7 | 90.3 ±1.2 | 84.7 ±0.9 | 59.7 ±8.1 | 69.7 ±7.3 | 52.3 ±6.1 | 78.3 ±8.7 | 63.0 ±4.0 | 31.0 ±6.7 | 16.7 ±7.4 | 1.7 ±0.3 | 1.3 ±0.7 | 0.3 ±0.3 | 0.0 |
| | BRK 18-12 | 50.0 ±6.4 | 4.3 ±0.9 | 8.3 ±0.3 | 5.7 ±0.7 | 31.7 ±12.3 | 7.3 ±.19 | 2.7 ±0.7 | 18.7 ±2.8 | 6.7± 0.9 | 0.0 | 0.0 | 0.0 | 0.0 | na | na | na |
| | HAB-5 | 0.3 ±0.3 | 0.7 ±0.3 | 1.3 ±0.7 | 0.3 ±0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | na | na | na | na |
| | EH-798 | 93.3 ±0.9 | 85.3 ±0.9 | 51.0 ±12.5 | 34.3 ±6.0 | 72.0 ±6.5 | 88.0 ±1.0 | 48.7 ±6.9 | 76.0 ±2.7 | 92.0 ±1.2 | 82.3 ±3.4 | 39.7 ±8.4 | 23.3 ±6.1 | 36.7 ±6.5 | 19.7 ±1.5 | 0.0 | 3.3± 1.7 |
| | EH-809 | 13.7 ±1.5 | 5.7 ±0.7 | 6.0 ±0.6 | 0.0 | 0.0 | 0.0 | 0.0 | na | 10.0 ±0.7 | 0.0 | 0.0 | 0.0 | 0.0 | na | na | na |
| | EH-865 | 71.0 ±1.7 | 23.0 ±2.1 | 28.3 ±6.4 | 17.3 ±5.0 | 8.3 ±4.4 | 0.0 | 3.0 ±1.2 | 0.0 | 20.7 ±1.8 | 10.7 ±0.7 | 12.0 ±4.4 | 12.3 ±2.7 | 0.0 | 0.0 | 0.0 | 0.0 |
| *M. anisopliae* | ARSEF 549 | 92.3 ±2.0 | 81.0 ±8.5 | 79.7 ±8.9 | 57.7 ±12.2 | 97.0 ±1.5 | 54.3 ±6.7 | 70.3 ±10.7 | 91.7 ±1.7 | 53.7 ±4.7 | 19.7 ±3.5 | 29.3 ±0.9 | 57.3 ±8.3 | 63.0 ±6.8 | 0.0 | 2.3 ±0.9 | 0.0 |
| | EH-473 | 95.7 ±2.0 | 64.7 ±3.3 | 59.3 ±10.4 | 69.0 ±5.1 | 95.0 ±1.5 | 53.3 ±3.9 | 78.0 ±1.0 | 95.0 ±1.0 | 96.0 ±2.3 | 75.7 ±4.2 | 44.0 ±3.1 | 25.3 ±5.8 | 74.7 ±5.0 | 11.7 ±7.7 | 24.3 ±9.2 | 8.3 ±5.5 |
| | EH-849 | 89.0 ±3.1 | 61.3 ±12.7 | 67.0 ±8.7 | 34.0 ±1.0 | 97.3 ±1.2 | 83.7 ±0.7 | 82.3 ±0.9 | 85.3 ±0.7 | 57.0 ±4.9 | 31.7 ±6.2 | 28.7 ±2.2 | 38.3 ±10.9 | 33.0 ±3.5 | 18.0 ±2.0 | 18.7 ±0.9 | 3.7 ±1.2 |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EH-863 | 84.3 ±2.9 | 41.0 ±6.7 | 54.0 ±5.2 | 48.0 ±7.6 | 92.3 ±0.9 | 19.3 ±1.2 | 40.3 ±5.0 | 75.7 ±4.1 | 36.0 ±1.7 | 22.7 ±10.9 | 20.7 ±5.7 | 12.7 ±6.2 | 2.3 ±0.7 | 0.0 | 0.0 | 0.0 |
| *M.brunneum* | ARSEF 2974 | 82.0 ±3.8 | 17.7 ±0.9 | 18.7 ±3.7 | 28.7 ±2.0 | 77.7 ±3.8 | 1.0 ±0.6 | 44.0 ±2.1 | 40.3 ±3.8 | 5.7 ±2.3 | 0.0 | 6.0 ±1.2 | 0.0 | 0.0 | na | 0.0 | na |
| | ARSEF 8680 | 2.7± 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | na | na | na | 0.0 | 0.0 | 0.0 | 0.0 | na | na | na | na |
| *M. pingshaense* | EH-802 | 96.0 ±1.0 | 69.3 ±0.9 | 72.0 ±8.1 | 77.3 ±3.0 | 94.3 ±0.3 | 25.3 ±4.7 | 64.0 ±4.7 | 19.0 ±1.2 | 71.3 ±3.0 | 40.0 ±1.5 | 55.7 ±6.4 | 65.3 ±3.0 | 40.0 ±1.7 | 0.0 | 6.3 ±1.5 | 0.0 |
| *M. guizhouense* | EH-869 | 24.7 ±0.7 | 26.3 ±6.7 | 11.0 ±0.6 | 12.3 ±2.6 | 57.7 ±5.5 | 0.0 | 4.7 ±0.9 | 1.3 ±0.3 | 32.0 ±7.1 | 7.7 ±4.3 | 20.0 ±10.7 | 1.0 ±0.6 | 0.0 | 0.0 | 0.0 | 0.0 |
| *M. acridum* | ARSEF 324 | 0.0 | 0.0 | 0.0 | 0.0 | na | na | na | na | 0.0 | 0.0 | 0.0 | 0.0 | na | na | na | na |
| | ARSEF 7486 | 1.3 ±0.9 | 5.0 ±3.5 | 3.3 ±1.9 | 0.0 | 0.0 | 0.0 | 0.0 | na | 0.0 | 0.0 | 0.0 | 0.0 | na | na | na | na |