

The fungal endophyte *Metarhizium*  
*brunneum*: Possible multifunctional  
plant promoter in sustainable agricul-  
ture

BACHELOR THESIS

Emmerich, 20.03.2022

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## Abstract

The overuse of mineral fertilizers and chemical pesticides in the world's agricultural sectors continues to be exorbitant, and is associated with a number of serious consequences for the entire complex environment. The reasons for this include the steadily growing world population and the accompanying degradation of the soil. As an alternative to chemical agents, biological plant protection/strengthening stimulants based on effective microorganisms have become established in the last decades. Research in recent years has focused on entomopathogenic fungal endophytes. The fungal endophyte *Metarhizium brunneum*, has long been proving its value as a mycoinsecticide in crop protection and there are indications of various plant-strengthening properties. The aim of this thesis is therefore to provide an overview of the current state of knowledge about the endophyte *M. brunneum* and at the same time, based on the literature, to discuss its potential as an alternative multifunctional plant promoter for sustainable agriculture. A literature research method was used to conduct a quantitative analysis of the existing knowledge on *M. brunneum*. The information and data collected as a result were systematically categorized and presented. The analysis has shown that the generalist *M. brunneum* covers a wide host range, has the ability to transfer nutrients in exchange, activates or produces plant hormones, compensates for stressors, and has many possibilities for detection and commercialization. The discussion of these results has led to the evidence that the endophyte can indeed be used as a plant growth promoter. The work also points to gaps in knowledge in several areas and emphasizes that further studies are needed.

## Zusammenfassung

Der übermäßige Einsatz von Mineraldüngern und chemischen Pflanzenschutzmitteln in der weltweiten Landwirtschaft ist nach wie vor exorbitant und mit einer Reihe von schwerwiegenden Folgen für die gesamte komplexe Umwelt verbunden. Die Gründe dafür sind unter anderem die stetig wachsende Weltbevölkerung und die damit einhergehende Degradierung des Bodens. Als Alternative zu chemischen Mitteln haben sich in den letzten Jahrzehnten biologische Pflanzenschutzmittel auf der Basis von effektiven Mikroorganismen durchgesetzt. In den letzten Jahren hat sich die Forschung auf entomopathogene Pilz-Endophyten konzentriert. Der Pilzendophyt *Metarhizium brunneum* hat sich seit langem als Mykoinsektizid im Pflanzenschutz bewährt und es gibt Hinweise auf verschiedene pflanzenstärkende Eigenschaften. Ziel dieser Arbeit ist es daher, einen Überblick über den aktuellen Wissensstand zum Endophyten *M. brunneum* zu geben und gleichzeitig, basierend auf der Literatur, sein Potenzial als alternativer multifunktionaler Pflanzenförderer für eine nachhaltige Landwirtschaft zu diskutieren. Mit Hilfe einer Literaturrecherche wurde das vorhandene Wissen über *M. brunneum* quantitativ analysiert. Die daraufhin gesammelten Informationen und Daten wurden systematisch kategorisiert und dargestellt. Die Analyse hat gezeigt, dass der Generalist *M. brunneum* ein breites Wirtsspektrum abdeckt, die Fähigkeit besitzt, Nährstoffe im Austausch zu übertragen, Pflanzenhormone aktiviert oder produziert, Stressoren kompensiert und viele Möglichkeiten der Detektion und Kommerzialisierung bietet. Die Diskussion dieser Ergebnisse hat zu dem Nachweis geführt, dass der Endophyt tatsächlich als möglicher Pflanzenwachstumsförderer eingesetzt werden kann.

Die Arbeit weist auch auf Wissenslücken in mehreren Bereichen hin und betont, dass weitere Studien erforderlich sind.

## Table of contents

<b>Abstract</b> .....	i
<b>Zusammenfassung</b> .....	ii
<b>Table of contents</b> .....	iv
<b>Table of figures and tables</b> .....	vi
<b>Table of acronyms</b> .....	vii
<b>1. Introduction</b> .....	1
<b>1.1. Background and problem definition</b> .....	1
<b>1.2. Introduction of endophytic fungi</b> .....	3
<b>1.3. <i>Metarhizium</i> spp.</b> .....	5
<b>1.4. Scope of this thesis</b> .....	9
<b>2. Material and methods</b> .....	11
<b>2.1. Research approach</b> .....	11
<b>2.2. Search method and data processing</b> .....	12
<b>2.3. General research – Endophytic fungi and <i>Metarhizium</i> spp</b> .....	13
<b>2.4. Specific research – <i>Metarhizium brunneum</i></b> .....	14
<b>3. Results and discussion</b> .....	18
<b>3.1. Ecology of <i>Metarhizium brunneum</i></b> .....	18
<b>3.1.1. Modus vivendi</b> .....	19
<b>3.1.2. Host Containment</b> .....	22
<b>3.1.3. Nutrient and water management – is there evidence out there?</b> .....	36

3.1.4.	<b>Discussion - room for improvement</b> .....	38
3.2.	<b>Physiology – Response to abiotic factors</b> .....	48
	<b>Abiotic factors – ready to discuss</b> .....	52
3.3.	<b>Process engineering – the commercialization</b> .....	58
3.3.1.	<b>The methods of detection</b> .....	58
	<b>Discussion about the methods</b> .....	61
3.3.2.	<b>Commercialization techniques</b> .....	63
	<b>Discussion of the commercialization procedures</b> .....	67
	<b>Application in practice – options</b> .....	71
3.4.	<b>Overall restrictions</b> .....	76
4.	<b>Conclusions</b> .....	77
	<b>References</b> .....	79
	<b>Declaration</b> .....	98

## Table of figures and tables

<b>Figure 1.</b> Yeast/mould like structure. ....	6
<b>Figure 2.</b> Cadaver of a cockroach. Killed after successful infection with <i>M. anisopliae</i> . Almost completely covered with conidia (Gao et al. 2011). ....	8
<b>Figure 3.</b> The carcass of <i>M. sanguinipes</i> , successfully killed by <i>M. brunneum</i> F52 and coated with its olive colored spores (Dakhel et al. 2019).....	35
<b>Figure 4.</b> Simple model of nutrient transfer between a tripartite association of organisms.....	37
<b>Figure 5.</b> Illustration of the simplified manufacturing process from encapsulated mycelium to application. ....	65
<b>Table 1.</b> Summary of research articles published in the last two decades on potential host plants of <i>M. brunneum</i> , tested under laboratory conditions. For this purpose, strain, host plant, inoculation method, area of the plant from which re-isolation was performed, recording of effects on the host plant (positive or negative) and associated reference are tabulated.....	28
<b>Table 2.</b> Summary of evaluated published titles on the successful entomopathogenicity of <i>M. brunneum</i> . Listed are strain, host, and the respective reference. This table serves as an overview of the results of the last two decades of naturally occurring and in vitro insect hosts on the different host groups of the endophyte.....	32

## Table of acronyms

Abbreviation	Meaning	Abbreviation	Meaning
<b>FEs</b>	Fungal endophytes	<b>dtx A</b>	Destruxin A
<b>Pr1</b>	Subtilisin-like protease	<b>BM</b>	Biomass
<b>PARB clade</b>	Group of species around <i>M. anisopliae</i>	<b>WUE</b>	Water use efficiency
<b>PCR</b>	Polymerase chain reaction	<b>ABA</b>	Abscisic acid
<b>qPCR</b>	Real-time PCR	<b>IAA</b>	Indole-3-acetic acid
<b>RFLP</b>	Restriction fragment length polymorphism	<b>SA</b>	Salicylic acid
<b>Mad2</b>	Mitotic arrest deficient 2 (adhesin gene/protein)	<b>JA</b>	Jasmonic acid
<b>n.s.</b>	No specification	<b>SSR</b>	Simple sequence repeat
<b>dtxs</b>	Destruxins; secondary metabolites	<b>MS</b>	Microsclerotia
<b>FCBK</b>	Fungal colonized barley kernel	<b>pH</b>	pH value
<b>h</b>	Time / hours		



## 1. Introduction

### 1.1. Background and problem definition

The production and use of mineral fertilizers for crops has increased significantly within the last century. In order to cope with the growing population, the associated food production and the accompanying degradation of land, ever greater quantities are being produced synthetically (nitrogen fertilizers) or mined from fossil raw materials e.g. phosphates (Erisman et al. 2008; Paungfoo-Lonhienne et al. 2012; Neset and Cordell 2012; Oelkers and Valsami-Jones 2008). In addition to fertilizers, the market for chemical crop protection products continues to boom. Although many old and new pesticides do not have actual registration certificates (e.g., neonicotinoids in the EU) (The European Commission 2013), the use and quantity of various products in commerce is still very large and of great importance for conventional agriculture, e.g. phosphonates as herbicides (Stehle and Schulz 2015), in view of the constantly growing population of this world.

The global consequences of this type of agricultural management are devastating for the environment and for all life forms on earth. According to a recent review article, direct or indirect exposure to pesticides can pose a serious risk to human health, causing diseases such as cancer, respiratory illnesses and neurological disorders (Rani et al. 2021). In addition, the natural biodiversity that supports many important ecological cycles is increasingly disturbed, and soil chemical processes such as salinization and acidification are driven by excessive residues, promoting erosion and soil infertility (Aktar et al. 2009; Rani et al. 2021).

The leaching of excess fertilizers and pesticides into water bodies leads to eutrophication and can result in massive species extinctions (Neset and Cordell 2012). (Neset and Cordell 2012). In addition to existing methods of use such as integrated pest management (IPM) and integrated crop management (ICM), which prescribe the rational use of pesticides in order to reduce harmful effects to a minimum and to optimize the efficiency and specificity of the products, research is increasingly devoted to the development of biological crop protection stimulants and fertilizers (Poveda et al. 2021; Rani et al. 2021). Research on biological plant strengthening and crop protection products has been ongoing for decades (Dara 2019; La Cruz Quiroz et al. 2019) furthermore, it is estimated that this sector can increase its annual growth in future economics (La Cruz Quiroz et al. 2019; Yakhin et al. 2016). In particular, the properties of beneficial fungi and bacteria that can potentially regulate and modify physiological processes in the plant, and additionally influence the rhizosphere and phyllosphere, have been the subject of much research in recent years, and their usefulness for agriculture has been positively evaluated (Yakhin et al. 2016). The use of biological crop protection and plant strengthening agents can potentially reduce the amount of agricultural chemicals used, which are substantially hazardous to the environment (Kolomazník et al. 2012; Yakhin et al. 2016). In addition to already known beneficial fungi such as arbuscular mycorrhizal fungi, modern detection techniques have identified a number of known and new endophytic fungal species and strains that are already and potentially suitable for use as biostimulants for crop protection and plant strengthening (Tóthné Bogdányi et al. 2019; La Cruz Quiroz et al. 2019).

## 1.2. Introduction of endophytic fungi

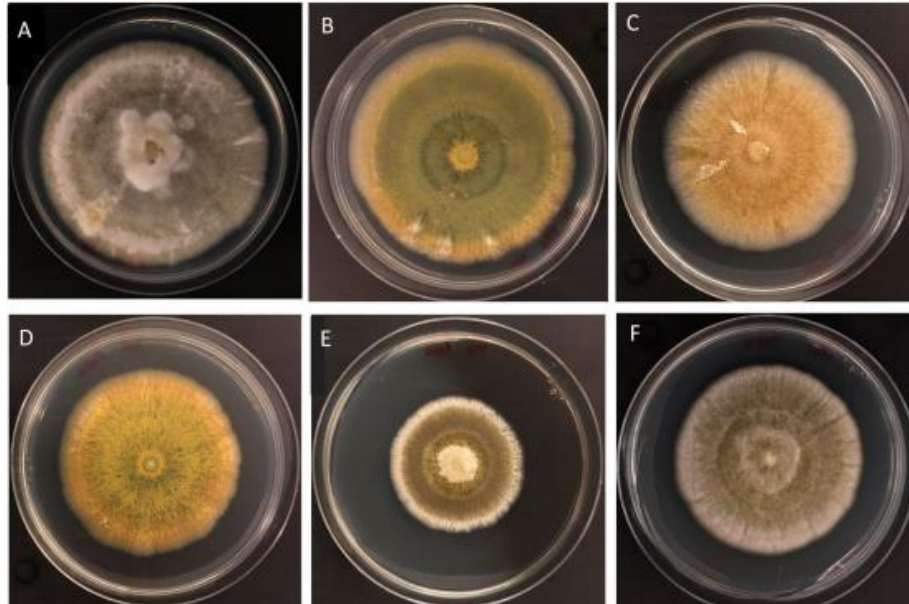
In principle, a distinction is made between epiphytes and endophytes in the co-existence of plants as potential hosts for microorganisms such as bacteria and fungi. Epiphytes live on outside of their respective host (Petrini 1991). They serve only as an introduction in this report and are not explained further. While endophytes, as the name suggests, live inside the plant cell or tissue. The name endophyte is derived from the Greek *endo* meaning 'inside' and *phyte* (*phyton*) meaning 'plant' (Wilson 1995). This term encompasses a diverse range of endosymbionts that are capable of colonizing almost any plant organ (Schulz and Boyle 2005). As already described, all organs within the host that are located above the soil (trunk, leaves, etc.) as well as within the soil (roots) can be colonized. Similarly, there are representatives that colonize only one of the two areas (Aroca 2013). According to Schulz and Boyle (2005) and Junker et al. (2012), it is difficult to judge individual endophytes according to their symbiotic interaction, whether mutualistic, commensalistic, optional saprobic or parasitic. This is attributable to the respective environmental conditions that ultimately influence symbiosis. In this context, the term fungal endophytes (FEs) is used, which is employed by several mycologists for endophytic fungi that do not cause any detectable symptoms of disease in the host plant at any time (Petrini 1991; Wilson 1995; Schulz and Boyle 2005; Aroca 2013). According to the taxonomic classification, the FEs appear in a wide spectrum (Blackwell 2011). A large proportion of the FEs belong to the taxon Dikarya, which is subdivided into the phyla Ascomycota and Basidiomycota (Arnold and Lutzoni 2007; Arnold et al. 2007; Weiss et al. 2011; Andrade-Linares et al. 2011; Schulz and Boyle 2005).

Fungal endophytes are ascribed the same age as arbuscular mycorrhizal associations (Krings et al. 2007). As with arbuscular mycorrhizae, FEs are thought to play similarly important roles in the survival of both individual plants and plant communities and are also responsible for particular alterations (Saikkonen et al. 2004; Torres et al. 2012; Rodriguez and Redman 2008). Based on their specific colonization patterns, fungal endophytes have been divided into two main groups. One consists of Clavicipitaceous endophytes (Ascomycota) and the other of non-Clavicipitaceous endophytes (Aroca 2013). As the name indicates, Clavicipitaceous endophytes belong to the family of Clavicipitaceae, which are classified in the phylum Ascomycota (Clay and Schardl 2002). According to Clay and Schardl (2002), the host range of these FEs is rather limited to cold and warm season grasses (family *Poaceae*) and here to colonization of the shoots and rhizomes. The symbioses tend to be mutualistic and the intercellular spaces of the plant tissue serve as growth habitat for the FEs (Schardl et al. 2004). Transmission occurs vertically via the seeds of the host plant (Schardl et al. 2004). The difference to the non-Clavicipitaceous endophytes mainly relates to horizontal transmission and phylogenetic diversity (Aroca 2013). The FEs in the non-Clavicipitaceous group develop both saprophytic and parasitic forms and are thus also referred to as facultative saprophytes (Schulz and Boyle 2005; Arnold and Lutzoni 2007). However, with the help of molecular methods, obligate biotrophs were also found in this group, suggesting a high level of species diversity (Neubert et al. 2006; LM et al. 2006; Knapp et al. 2012).

### 1.3. *Metarhizium* spp.

At the end of the 19th century, the genus *Metarhizium* (Ascomycota: Hypocreales) was first described by the scientists Metschinkoff (1879) (*Entomophthora anisopliae*) and shortly afterwards by Sorokin (1883) (*Metarhizium*) (Brunner-Mendoza et al. 2019). At later times, more and more individual species were discovered and most recently classified and described by Kepler et al. (2014). According to Kepler et al. (2014) and Brunner-Mendoza et al. (2019), the number of species is currently over 30. Initially thought to be a facultative insect pathogen (entomopathogen), extensive research over the last few decades has revealed that the genus *Metarhizium* is an exceptionally diverse fungus (Stone and Bidochka 2020). The most diverse genes of the different species enable endophytic, saprotrophic and entomopathogenic forms of nutrient uptake (Stone and Bidochka 2020; Lovett and St Leger 2015; Hu and St Leger 2002). Concurrently, a modulation of the transcriptome takes place, which can reflect the current need and accessibility of individual nutrient sources (Stone and Bidochka 2020). Within *Metarhizium* spp, a wide range of morphological differences can be identified. While the majority of species form branched conidiophores, there are also some that develop simple ones. At the tips of the branches appear one or several phialides, in blunt or elongated form. The chain-forming conidia are either green, brown, lilac or hyaline and vary in their structural shape from cylindrical to spherical to ellipsoidal (Brunner-Mendoza et al. 2019; Kepler et al. 2014). The dimensions can vary between 4.0-14.5 x 2.0-5.0  $\mu\text{m}$ . On artificial growth media such as agar, *Metarhizium* colonies appear predominantly in light and dark green, brown or white (Figure 1).

However, Kepler and Rehner (2013) have found that the morphological characteristics of the *Metarhizium* species are rather inaccurate, as there is often an overlap of characteristics between the different species.



**Figure 1.** Yeast/mould like structure.

Pictured here are the morphological differences of mature (14 days old) *Metarhizium* spp. phyla on potato dextrose agar (PDA), at 27°C. C. *Metarhizium brunneum*. (Bruner-Mendoza et al. 2019).

Classified in the family Clavicipitaceae (Ascomycota; Hypocreales), *Metarhizium* belongs to the category of Clavicipitaceous endophytes according to Clay and Schardl (2002), Rodriguez et al. (2009) and Kepler et al. (2014). Endophytes which mainly prefer species from the Poaceae family as hosts (Clay and Schardl 2002). However, less than a decade later, Wyrebek et al. (2011) have been able to isolate 3 different *Metarhizium* species (*M. robertsii*, *M. brunneum*, *M. guizhouense*) by sampling plant roots, which appear to interact together in the rhizosphere with various plant species (grasses, wildflowers, shrubs and trees). Nevertheless, further research has shown that *Metarhizium* species may prefer colonization of monocotyledonous hosts (e.g., grasses) to dicotyledonous hosts (Moonjely and Bidochka 2019).

*Metarhizium* spp. colonize the host internally within the plant tissue, as is common with endophytic fungi (Stone and Bidochka 2020).

In addition to its endophytic properties, *Metarhizium* spp. also functions as an insect pathogenic fungus. Hence the name *Entomophthora anisopliae* (1879), which refers to the pathogenicity against *Anisopliae austriaca* (scarab beetle) (Stone and Bidochka 2020). A distinction is made between generalists, who have a wide range of insect hosts, and specialists with a small defined host range (St Leger and Wang 2020). *Metarhizium* spp. does not have to be ingested via the insect's digestive tract; the FEs have the ability to penetrate the insect's exoskeleton (cuticle) directly (Brunner-Mendoza et al. 2019; Stone and Bidochka 2020; St Leger and Wang 2020). Several biophysical mechanisms are responsible for these processes. Adhesion and attachment to the outer shell of the insect is achieved through electrostatic or hydrophobic reactions. The first connection to the outer wax layer of the cuticle is established via so-called hydrophobin proteins, which are present on the surface of many fungal spores (Sevim et al. 2012). Special adhesion proteins such as Mad1, enzymes and mucilage play a major role in the adhesion process (Wang and St Leger 2007). Subsequently, with the help of an infection cone and the further secretion of hydrolytic enzymes (lipases, chitinases and proteases), pressure is exerted on the outer skeleton to puncture it (St Leger and Wang 2020). According to Brunner-Mendoza et al. (2019), the most important enzyme, along with a number of others that play a significant role in this process, is subtilisin-like protease (Pr1). Successful penetration of the outer shell (cuticula) allows the fungal hyphae to enter the insect's body cavity (haemocoel), which contains the open circulatory system (hemolymph) consisting a mixture of blood plasma and lymphatic fluid (St Leger and Wang 2020).

Reaching the haemocoel, *Metarhizium* forms so-called blastospores (yeast-like structures) that spread and grow through the hemolymph (Brunner-Mendoza et al. 2019). In most cases, generalists have the ability to kill the host (insect) quickly by producing toxins (Wang et al. 2012; St Leger and Wang 2020). The resulting necrosis usually promotes the degradation of cellular components. In the case of the specialists under *Metarhizium*, it is often the case that they do not have the possibility to develop sufficient enzymes and toxins, which would favor a rapid killing (Gao et al. 2011). In these cases, the death of the host occurs generally after sufficient infected tissue (Figure 2) (St Leger and Wang 2020).



**Figure 2.** Cadaver of a cockroach. Killed after successful infection with *M. anisopliae*. Almost completely covered with conidia (Gao et al. 2011).



## 1.4. Scope of this thesis

The aim of this paper is to provide a review article on *Metarhizium brunneum* (*M. brunneum*), which includes most of the scientific findings to date. Furthermore, the focus is on the discussion of whether the potential of *M. brunneum* in particular represents an alternative to chemical agents as a multifunctional plant promoter for sustainable agriculture.

Research into the use of beneficial microorganisms and biostimulants in agriculture as an alternative to chemical pesticides and fertilizers has increased significantly in recent decades (Miranda-Fuentes et al. 2020; Jaronski 2010). The reasons for this are increased environmental awareness among the population and the fact that the excessive input of chemicals into the environment has serious consequences (Miranda-Fuentes et al. 2020). In addition, foodstuffs are allowed to contain fewer and fewer pesticide residues which has caused many chemical agents to lose their market licenses (Bajwa and Sandhu 2014; Heinrich-Böll-Stiftung 2022). Evidence that the endophyte can be used as a multifunctional plant fortifier has become an increasing focus of research, particularly in the last two decades, as this review article shows. Studies on plant-strengthening and supporting properties of *M. brunneum* are rare, apart from its use as a bioinsecticide, but have increased significantly in the past decade. This work is intended to demonstrate the usefulness of a possible integration of the fungal endophyte *Metarhizium brunneum*, as a multifunctional plant promoter in sustainable agriculture, beyond the known beneficial fungal communities such as arbuscular mycorrhizae.

The research question that accompanies and guides this review article is as follows: Is the fungal endophyte *M. brunneum* suitable as a potential multifunctional plant promoter for sustainable agriculture in the future?

A specific literature review and compilation of current knowledge on the three main areas of ecology, physiology, and chemistry, as well as the respective sub-categories of the fungal endophyte *M. brunneum*, will be used to answer the research question.

Possible relationships, differences and particularities with species, strains and isolates of the own genus or other fungal endophytes are discussed with the help of further literature after each main section, so that the “results and discussion” section is combined into one.

## 2. Material and methods

### 2.1. Research approach

To compile the properties and usability of *Metarhizium brunneum*, a literature research was conducted. In the first steps of my research, and in order to define a problem-solving approach, I first studied the effects of fertilizers and chemical pesticides on the environment, the organisms living in it, and the users. After extensive and informative research, I proceeded to conduct literature searches on entomopathogenic endophyte organisms. This research provided a sufficient overview and the resulting appropriation of knowledge to understand certain correlations and to get suitably introduced into the overall/broader topic. Since this is a large research area, the focus of the search was limited to endophytic (entomopathogenic) fungi to consolidate knowledge. In order to achieve a broader spectrum at the beginning of the study and to establish the necessary basic knowledge, a quantitative research approach was chosen.

The literature study was now restricted to the actual subject area. The focus was now on the genus *Metarhizium* ssp. and in particular the species *M. brunneum*. The following literature analysis now refers to the **ecology** of *Metarhizium brunneum*, the **physiology** and finally the **process engineering**. The approach and methodology to the individual points are described in detail subsequently.

## 2.2. Search method and data processing

To find relevant and useful publications and articles, the following databases were mainly used for the research of this thesis: "Google Scholar", "Web of Science/Knowledge", "PubMed", and "Research Gate". Besides the major databases, the following platforms for peer-reviewed literature were consulted: "ScienceDirect by Elsevier", "SpringerLink", "Taylor & Francis Online", "Wiley Online Library", "The Royal Society Publishing", "MDPI", "frontiers" and "PLOS ONE". In addition, reference books were used in a few cases. In exceptional cases, publications from governments or non-governmental organizations as well as from organizations with vested interests were also considered. The literature study was conducted in two steps, which is described in detail below. The results were evaluated superficially based on the research question and sorted directly for the specific analysis. A bibliography was created using the "Citavi 6" organization program, in which the results found were sorted according to defined subject areas and relevance in relation to the thesis. In order to narrow down the search results and generate the most suitable articles, the keywords/search terms used were combined with "AND". The literature was perceived and evaluated to see whether it provided helpful answers to the questions posed and thus corresponded to the objective of this thesis.

In addition to the general search functions of the databases, the literature (in the text and in the reference list) was searched for references and specific sources that were also relevant. Furthermore, the 'Related research' function of the respective publisher website was used to find further relevant publications.

The highest priority was given to literature dealing exclusively with the defined topic area. Reports in which the necessary information (but to a large extent) was available as secondary information were also rated highly.

### 2.3. General research – Endophytic fungi and *Metarhizium* spp

The first literature search was intended to build up some basic knowledge of entomopathogenic endophytic fungi and was therefore less specific in its approach. Wider search terms were selected to cover a certain spectrum. In addition, the function 'reviews' was predominantly selected in the search engines. The search terms used were “*endophytic fungi*”, “*entomopathogenic fungi*”, “*endophytes*”, “*plant growth promoter*”. This broad search field made it possible to find topic-related and informative literature. In addition, new references were found that were necessary for the subsequent detailed research. In particular, searching the source indexes of these review articles provided further important insight, to specify the research approach.

Once a basic knowledge of fungal endophytes had been established, it was possible to start looking more closely at the specific object of research. The next steps included the specific search for information about the genus *Metarhizium* spp. Again, the function 'review articles' was set in the search engines used in order to initially learn the essentials.

The search terms “*Metarhizium*”, “*Metarhizium spp*”, were used for this purpose. Like the previous search, this compartment now continued to serve to explore a broad, yet more concrete area of research in order to achieve a certain basic framework about the genus *Metarhizium*.

This made it possible to get a rough overview of the extent to which the species *Metarhizium brunneum* is listed in relevant review articles before the specific search.

## 2.4. Specific research – *Metarhizium brunneum*

The second literature review involved a precise and systematic search for specific information needed to answer the research question. With the aim of creating a literature review and thus answering the research question, as already mentioned in the research approach, the search was divided into three major topic areas and a corresponding bibliography was created. Each of these areas was further subdivided into specific elements, which were answered in detail with the help of explicit questions. The topic areas and questions are listed in detail below.

### Ecology of *M. brunneum*

#### Mode of life

- How does *M. brunneum* live when plants are present?
- How does *M. brunneum* live when plants are not present?

#### Interaction – host containment

- With which plants?
- With other organisms (insects)?

### Nutrient management – function of the

- Nutrient uptake/transfer?
- Water uptake/transfer?
- Other substances

For this area, the search terms “*Metarhizium brunneum*”, “*Metarhizium brunneum plant growth promotion*”, “*Metarhizium brunneum review*”, “*Metarhizium brunneum entomopathogenic*”, “*Metarhizium brunneum strain*”, “*Metarhizium brunneum nutrients*”, “*Entomopathogenic fungi*”, “*Endophytic fungi*”, “*Metarhizium*” were used. The aim was to generate articles dealing with specific areas of the listed topics in order to get more specific sources. This topic consisted of designing a detailed overview of the FE's way of life, covering both the natural environment and in vitro conditions, interactions of the host spectrum, as well as its beneficial functions.

### Physiology of *M. brunneum*

#### Response to abiotic factors

- Low and high nutrient levels
- Drought stress / salinity
- UV radiation and temperature
- Other organism populations in direct environment

To get access to data concerning the physiology of *M. brunneum*, the same search terms as for the previous part were used. Due to the fact that relatively little research has been done (explicitly) on the physiology of *M. brunneum*, it was difficult to work with exclusive keywords.

Ultimately, the main method to get data on this topic was to read articles about the endophyte from the previous section 'ecology' very carefully and to analyze text citations and sources more precisely to get suitable media. In the field of *M. brunneum* physiology, the aim was to find out how the endophyte behaves when exposed to various abiotic factors (stressors).

### Process engineering of *M. brunneum*

#### Detection methods – options

- Real time quantitative PCR (qPCR)
- RFLP test
- Raman spectroscopy
- Others

#### Commercialization methods

- Breeding/cultivation
- Encapsulation
- Application in practice

The search terms “*Metarhizium brunneum*”, “*Metarhizium brunneum* plant growth promotion”, “*Metarhizium brunneum* encapsulation”, “*Metarhizium brunneum* shelf life”, “*Metarhizium brunneum* detection”, “*Metarhizium brunneum* PCR” were used to find proper data about this section.



In addition, the previously analyzed sources were consulted. The goal of the research in this section was to generate as much data as possible on the individual processes by which *M. brunneum* and similar fungal endophytes have been detected and commercialized in the past.

### 3. Results and discussion

#### 3.1. Ecology of *Metarhizium brunneum*

According to a study published by Bischoff et al. (2009), the species *M. brunneum* belongs to the species group around *M. anisopliae* var. *anisopliae*. This group of fungal endophytes is composed of a total of four asexual species (*M. pingshaense*, *M. anisopliae*, *M. brunneum*, *M. robertsii* sp. nov.), which form the core complex of the species *M. anisopliae* (St Leger and Wang 2020; Bischoff et al. 2009). Taxonomically, it is a monophyletic group, also referred to as the PARB clade (Bischoff et al. 2009; Mayerhofer et al. 2019a). The common ancestor is *M. anisopliae* as mentioned at the beginning. The evidence of *M. brunneum* was first observed by Thomas Petch in 1930 (Petch 1935). Bischoff et al. (2009) concluded in their study that it is almost impossible to distinguish between *M. brunneum* and *M. anisopliae* purely by morphological criteria. However, modern genetic analysis technology makes it possible to channel and differentiate individual strains, which will be discussed in detail in the following sections. Mature conidia of *M. brunneum* usually show an olive (green) or light yellow to brownish coloration, consequently it belongs to the same color spectrum like many other *Metarhizium* spp. (Figure 1) (Bischoff et al. 2009; Brunner-Mendoza et al. 2019; Roberts and St. Leger 2004). Isolates of the endophyte have already been found in North America, Europe, Asia and Australia (Roberts and St. Leger 2004; Bischoff et al. 2009; Fisher et al. 2011; Keyser et al. 2015; Rehner and Kepler 2017; Kryukov et al. 2017). The ecology section is divided into 3 subsequent sections with a final discussion of the entirety at the end.

### 3.1.1. Modus vivendi

In this section, the results of the literature research on the mode of life of *M. brunneum* are presented. The section is divided into the mode of life of the endophyte when plants are present, and the mode of life when plants are not present.

#### Life in the presence of plants / with plants

Currently, there is little evidence on the interaction between the endophyte *M. brunneum* and plants. Hypotheses and discussions abound, ultimately pointing to the fact that research is needed in this area in particular to gain an accurate understanding of the chemical relationships. Unfortunately, there are currently no studies or attempts that address the detailed mechanisms, tools, and chemical response of the endophyte during colonization of a plant.

According to the findings of many scientists, studies and experiments, the habitat of the endophyte *M. brunneum* is primarily associated with the rhizosphere rather than the phyllosphere (Behie et al. 2015; Liao et al. 2014; Fisher et al. 2011). This means primarily, as Wyrebek et al. (2011) and Steinwender et al. (2015) have found, that the FE, occurring in nature, is considered a plant root sympathizer (Behie et al. 2015). Nevertheless, there is sufficient evidence that the endophyte also occurs in the phyllosphere of the plant, usually after artificial inoculation with a foliar spray technique or via encapsulated conidia or mycelium spread in the rhizosphere (Raya-Díaz et al. 2017a; Jaber and Enkerli 2017; Resquín-Romero et al. 2016; Razinger et al. 2018; Krell et al. 2018c).

According to recent findings by Lahey et al. (2020) and Razinger et al. (2018) colonization of plants with *M. brunneum* occurs both rhizoplane and endophytic.

In their experiment, the scientists found that in both experimental plants (*Phaseolus vulgaris*, *Zea mays*) the highest rhizoplane concentration of *M. brunneum* occurred in the root region proximal to the hypocotyl, whereas the highest endophytic concentration occurred in the hypocotyl (Lahey et al. 2020). Nevertheless, they also found that the colonization behavior varied due to the time period and the type of inoculation; furthermore, *Z. mays* was colonized to a greater extent and more frequently than *P. vulgaris* (Lahey et al. 2020). There is also sufficient evidence that *M. brunneum* is not plant-specific in nature or under laboratory conditions (Steinwender et al. 2015; Resquín-Romero et al. 2016; Lahey et al. 2020), however, it happens that *M. brunneum* colonized certain wildflowers only in combination with *M. robertsii* and never solely (Wyrebek et al. 2011). For more on host specificity, see section host containment<sup>1</sup>. According to Hu and Bidochka (2021), *Metarhizium* spp. possess a so-called adhesin gene (Mad2), which facilitates the adhesion of the conidia of the endophyte to the root surface. *M. brunneum* is also able to produce and utilize the phytohormone indole-3-acetic acid (IAA) in the plant, which in turn can lead to plant growth (Liao et al. 2017; Hu and Bidochka 2021; St Leger and Wang 2020). Tripartite interactions were also observed in the habit of *M. brunneum*. The entomopathogenic endophyte is able to infest soil-borne insect larvae located in the rhizosphere of plant roots and transfer nutrients (preferentially N) from the insect cadavers in symbiosis with the plant roots (Figure 4) in exchange for photosynthetic products (Behie and Bidochka 2014; Behie et al. 2017; Hu and Bidochka 2021).

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<sup>1</sup> See chapter 3.1.2. for further detailed information

### Survival when no plants are present – soil as a habitat

Apart from the fact that *M. brunneum*, due to its genus (*Metarhizium*; Clavicipitaceae), has saprotrophic properties and can therefore survive on organic matter in the soil without interaction with plants or insects, there are currently no studies that specifically address the survival of the endophyte apart from microorganisms and plants. Soil, assuming organic matter, can potentially serve as habitat for *M. brunneum*. Kepler et al. (2015) studied long-term agricultural soils in the mid-Atlantic region of the USA and found that *M. brunneum* populations predominated in areas where soybean (*Glycine max*) was the predominant crop. Steinwender et al. (2014) were able to isolate a *Metarhizium* community from both agricultural soils and adjacent hedgerows in an experiment, located in Denmark. Other species identified were *M. robertsii* and to a lesser extent *M. majus* and *M. flavoviride* (Steinwender et al. 2014). Steinwender et al. (2014) interpret the predominance of the isolated *M. brunneum* genotype as an adaptation of the endophyte to agricultural soil conditions.

### 3.1.2. Host Containment

“Understanding habitat selection of fungal entomopathogens is critical to improve the efficacy, persistence and cost of these fungi as microbial insecticides” (Fisher et al. 2011, 289). The scientific identification and subdivision of the host range is of utmost importance for the subsequent use as a biological plant protection or strengthening agent. Initially, this is the only way to determine with which plants a symbiotic association is generally possible. The natural conditions should be considered first. If *M. brunneum* establishes a connection with a certain plant (host) in a natural way, it can be assumed that this connection can also be established artificially. However, this is usually not so easy to generalize. As the following results show, the natural hosts tend to be (as might be expected) wild shrubs, grasses, bushes and trees, with some exceptions in which the endophyte could be isolated from agricultural land and thus also from economically valuable crops. Under optimal laboratory conditions with very few influencing factors, but also in the greenhouse and partly in the open field, it is possible to realize a variety of economic crops as potential hosts. Since the endophyte is an entomopathogenic fungus, insects and especially the larval and nymph stages are also part of the host spectrum. Not only the discovery of possible hosts is important in this context, but also the determination which strain of *M. brunneum* is involved. In order to differentiate the range of the host spectrum, I have divided the part into specific sections as follows: plants as hosts in a natural environment, in vitro plant hosts, soil-borne insects as hosts and in vitro insect hosts.

### Plants that function as hosts for *M. brunneum* in natural environment

In a field experiment in Ontario, Canada, Wyrebek et al. (2011) collected samples from two independently existing sites, both consisting of a forest with mature trees and an open field with various grasses and wildflowers. The endophyte *M. brunneum* could be isolated mainly from tree roots as well as shrubs and a few wildflowers (Wyrebek et al. 2011). Grasses could not be identified as hosts of *M. brunneum* in this trial (Wyrebek et al. 2011). The trees were *Ostrya virginiana* (American hophornbeam), *Acer nigrum* (black maple), *Fraxinus americana* (white ash), *Thuja occidentalis* (northern white cedar), *Betula alleghaniensis* (golden birch), *Prunus nigra* (Canadian plum), *Abies balsamea* (balsam fir) and *Fagus grandifolia* (American beech), whereas *B. alleghaniensis* was additionally colonized by *M. robertsii* (Wyrebek et al. 2011). The shrubs colonized were *Cornus alternifolia* (pagoda dogwood), *Aronia melanocarpa* (black chokeberry) and *Vitis riparia* (frost grape), whereby *C. alternifolia* was mentioned twice, once also colonized with *M. robertsii* (Wyrebek et al. 2011). The two wildflowers were *Trifolium hybridum* (alsike clover) and *Barbarea vulgaris* (wintercress) (Wyrebek et al. 2011). In the case of wildflowers, it should be mentioned that colonization by *M. brunneum* only took place in simultaneous combination with *M. robertsii*. (Wyrebek et al. 2011). The strain isolates obtained in this experiment are B35-avi, ARSEF 4179, G55-ai and ARSEF 8680 (43A 2i). In another field trial, held in the Willamette Valley in Oregon, United States, Fisher et al. (2011) identified four additional host plants of *M. brunneum*. The root samples from which *M. brunneum* could be isolated were taken from agricultural soil.

The root samples were *Vaccinium* L. (blueberry), *Fragaria* L. (strawberry), *Vitis* L. (grape), and the Christmas tree species *Picea engelmannii* (engelmann spruce), *Abies procera* (noble fir) and *Pseudotsuga menziesii* (douglas fir) (Fisher et al. 2011). In this experiment it was noticed that *M. brunneum* colonizes, by far, *Fragaria* and *Vaccinium* to a particularly high degree, compared to the other three *Metarhizium* species that could be also isolated (Fisher et al. 2011). The strains were as follows ARSEF 2107, ARSEF 3828, ARSEF 6392, ARSEF 4179, ARSEF 4152 and ARSEF 5625. Whereas the previous results were concentrated on the North American continent, *M. brunneum* was also isolated in Europe. In a study by Keyser et al. (2015) on entomopathogenic fungi in agricultural crops in Denmark, *M. brunneum* was isolated from roots of two important economic crops. The two crops sampled in two geographically different fields on the Danish island of Zealand in which *M. brunneum* could be isolated were *Brassica napus* (oilseed rape) and *Triticum aestivum* (winter wheat) (Keyser et al. 2015). In addition, *M. brunneum* has also been isolated from grass pastures that have not been managed for two decades (Keyser et al. 2015). The isolated strains are KVL 12-30, 12-42, 12-37, 12-41 (origin: Denmark) as well as ARSEF 2107 (origin: USA). The *M. brunneum* strain EAMa 01/58-Su was first isolated in 2001 from the soil of wheat plants in Cordoba, Spain (Ríos-Moreno et al. 2017). *M. brunneum* has also been detected in Russia, western Siberia (Tyurin et al. 2021). Here, Tyurin et al. (2021) took root, stem and leaf samples of potato plants from three different locations (1x conventionally farmed, 2x kitchen garden). Five *M. brunneum* strains (DAOM 241817, ARSEF:2107 T, KVL 12-43 and DAOM 241868) were isolated from root fragments (sterilized) and soil (Tyurin et al. 2021).



The endophyte could only be isolated from one of the three sites (kitchen garden, highest N content, neutral pH) where potatoes have been cultivated for at least 10 years.

#### Host plants which are colonized under laboratory conditions

The endophyte has already been brought into contact with economically important plants in several experiments under laboratory and greenhouse conditions over the last two decades (Table 1). Successful colonization with *M. brunneum* (43a-2i) in the rhizosphere as well as the endosphere of two important crops was demonstrated in an experiment by Lahey et al. (2020). The two host plants were *Zea mays* (sweet corn) and *Phaseolus vulgaris* (common bean). Three different conidial inoculation methods were used (infected insect, sucrose and a mixture of both) (Lahey et al. 2020). It should be noted, however, that the colonization of the monocot, maize, plant by *M. brunneum*, in all three application methods, turned out to be much greater than the colonization of the dicot, bean (Lahey et al. 2020). Six years earlier, Liao et al. (2014) successfully inoculated maize (*Zea mays*) plants with *M. brunneum* conidia, significantly improving general physical growth parameters compared to plants inoculated with killed conidia. Further attempts to improve the endophytism of *Solanum tuberosum* (potato) by *M. brunneum* (CB15) by adding cellulase during encapsulation were successful (Krell et al. 2018b; Krell et al. 2018e). Two years before Krell et al. (2018b) conducted the trials with encapsulated *M. brunneum* mycelium on potato plants (*S. tuberosum*),

Ríos-Moreno et al. (2016) had already successfully inoculated potato plants with two strains of *M. brunneum* (EAMa 01/58-Su and BIPESCO5). In this experiment, the endophyte was also reclaimed from all parts of the plant (stem, leaves, tubers, roots) (Ríos-Moreno et al. 2016). This means that *M. brunneum* can establish endophytism with one of the most important food crops in the world.

Another success of Krell et al. (2018c) was the colonization of another solanaceous plant (Solanaceae) with the endophyte *M. brunneum* (BIPESCO5). By testing an unconventional new method of encapsulating the mycelium of *M. brunneum* in calcium alginate/starch granules, the rhizosphere of tomato plants (*Solanum lycopersicum*) was effectively inoculated (Krell et al. 2018c). Thus, evidence of successful endophytism was found even in the stems (and not only roots) of *S. lycopersicum* (Krell et al. 2018c). As another monocotyledonous plant from the Poaceae family, the roots of *Triticum aestivum* (wheat) could also be treated with *M. brunneum* (KVL 04-57 and KVL 12-37) conidia, leading to successful colonization, especially of the rhizosphere (Keyser et al. 2014). In addition, Keyser et al. (2014) noted that the endophyte spreads better through the roots in non-sterile soil than in an artificial soil preparation. The successful colonization of wheat, in particular durum wheat (*Triticum durum*), by *M. brunneum* was demonstrated by further experiments; the strains used were BIPESCO5 and EAMa 01/58-Su (Jaber 2018b; González-Guzmán et al. 2020a; González-Guzmán et al. 2020b; Gonzalez-Guzman et al. 2021). Furthermore, in their experiments on improved Fe supply of sorghum (*Sorghum bicolor*) in calcareous and sandy soils by the application of EF, Raya-Díaz et al. (2017b) and Raya-Díaz et al. (2017a) successfully managed to colonize *S. bicolor* with the *M. brunneum* strain EAMa 01/58-Su.

Evidence of successful cooperation between Fabaceae and *M. brunneum* was provided in an experiment by Jaber and Enkerli (2017) using broad bean (*Vicia faba*). The strain used for this purpose was also BIPESCO5. The seed inoculation method enabled *M. brunneum* to spread systematically in the bean plant and enhance all growth parameters (Jaber and Enkerli 2016; Jaber and Enkerli 2017).

As another representative of the Fabaceae, the soybean (*Glycine max*) was successfully colonized with *M. brunneum* strain F52, although only to a small extent in the stem (Clifton et al. 2018). A superficial (leaf spraying) treatment with the *M. brunneum* strain EAMb 09/01-Su also enabled the isolate to colonize the crops used in the trial, alfalfa (*Medicago sativa*), tomato (*S. lycopersicum*) and melon (*Cucumis melo* var. *reticulatus*) (Resquín-Romero et al. 2016). However, Resquín-Romero et al. (2016) noted that this type of application can lead to faster disappearance of the endophyte within the plant. The leaves and stems were colonized first in this experiment, followed by the root zones (Resquín-Romero et al. 2016). As another representative of the Solanaceae, in the experiment of Jaber and Alananbeh (2018), successful colonization with the EF *M. brunneum* (BIPESCO5) was detected in roots, stems as well as leaves of sweet pepper (*Cap-sicum* spp.). The successful colonization of sunflower (*Helianthus annuus*), another important economic plant, by *M. brunneum* was demonstrated by Miranda-Fuentes et al. (2020) with strains EAMa 01/58-Su and EAMb 09/01-Su as well as Raya-Díaz et al. (2017a) with strain EAMa 01/58-Su in their in vitro assays.

**Table 1.** Summary of research articles published in the last two decades on potential host plants of *M. brunneum*, tested under laboratory conditions. For this purpose, strain, host plant, inoculation method, area of the plant from which re-isolation was performed, recording of effects on the host plant (positive or negative) and associated reference are tabulated.

<i>M. brunneum</i> strain	Plant host (common name)(family)	Inoculation method	Re-isolated from	Effect on plant? <sup>a</sup>	Observed effects	Reference
43a-2i	<i>Zea mays</i> (maize) (Poaceae)	infected insect, sucrose medium, infected insect + sucrose	hypocotyl, roots	n.s. <sup>b</sup>	- n.s.	(Lahey et al. 2020)
	<i>Phaseolus vulgaris</i> (common bean) (Fabaceae)	infected insect, sucrose medium, infected insect + sucrose	hypocotyl, roots	n.s.	- n.s.	(Lahey et al. 2020)
F52 / BI-PESCO5	<i>Solanum tuberosum</i> (potato) (Solanaceae)	encapsulated mycelium	leaves, stems, roots, tubers	Yes (+)(-)	+ production of secondary metabolite (dtx A) <sup>c</sup>	(Ríos-Moreno et al. 2016)
	<i>Solanum lycopersicum</i> (tomato) (Solanaceae)	encapsulated mycelium in calcium alginate/starch granules	stems, roots	n.s.	- n.s. but strong colonization was measured	(Krell et al. 2018b)
	<i>Triticum durum</i> (durum wheat) (Poaceae)	seed treatment	stems, roots	Yes (+)(-)	+ roots primary colonized + all growth parameters <sup>d</sup> enhanced + significant higher BM <sup>e</sup> production + increased seedling germination - lower WUE <sup>f</sup>	(Jaber 2018a; González-Guzmán et al. 2020a; 2020b; González-Guzmán et al. 2021)
	<i>Vicia faba</i> (broad bean) (Fabaceae)	foliar spray, seed treatment	leaves, stems, roots	Yes (+)	+ all growth parameters enhanced + higher BM production + Increased seedling emergence	(Jaber and Enkerli 2016; 2017)

	<i>Glycine max</i> (soybean) (Fabaceae)	seed treatment	stems	Yes (-)	- possible host-plant improvement for parasitoids - possible reduction of plant defense	(Clifton et al. 2018)
	<i>Capsicum annum.</i> (sweet pepper) (Solanaceae)	drenched roots	leave, stems, roots	Yes (+)	+ all growth parameters enhanced + successful co-colonization with endoparasitoid <i>Aphidius colemani</i>	(Jaber and Alananbeh 2018)
<b>CB15</b>	<i>Solanum tuberosum</i> (potato) (Solanaceae)	encapsulated mycelium + cellulase or inactivated baker's yeast	stems, roots, tubers	Yes (+)	+ significant higher BM production + increased leaf surface area + increased yield + increased WUE under nutrient poor conditions + increased N and P content under nutrient poor and nutrient rich conditions	(Krell et al. 2018b; Krell et al. 2018e; Krell et al. 2018d)
<b>EAMa 01/58-Su</b>	<i>Solanum tuberosum</i> (potato) (Solanaceae)	encapsulated mycelium	leaves, stems, roots, tubers	Yes (+)	+ production of secondary metabolite (dtx A)	(Ríos-Moreno et al. 2016)
	<i>Triticum durum</i> (durum wheat) (Poaceae) <i>Sorghum bicolor</i> (sorghum) (Poaceae)	seed treatment	stems, roots	Yes (+)(-)	+ roots primary colonized + all growth parameters enhanced + significant higher BM production + increased seedling germination - lower WUE	(Jaber 2018a; González-Guzmán et al. 2020a; 2020b; Gonzalez-Guzman et al. 2021)
	<i>Sorghum bicolor</i> (sorghum) (Poaceae)	seed treatment, soil treatment, foliar spray	leaves, stems, roots	Yes (+)	+ greater root length + strong growth despite Fe and P deficiency symptoms + higher Fe and Mn content + increased Fe availability due to lowered pH in calcareous medium	(Raya-Díaz et al. 2017b; 2017a)

	<i>Helianthus annuus</i> (sunflower) (Asteraceae)	soil treatment	rhizosphere	Yes (+)	+ increased protection against pathogenic fungi + strong growth despite Fe and P deficiency symptoms	(Raya–Díaz et al. 2017a; 2017b; Miranda-Fuentes et al. 2020)
<b>EAMb 09/01-Su</b>	<i>Medicago sativa</i> (alfalfa) (Fabaceae)	foliar spray	leaves, stems, roots	No	- n.s.	
	<i>Solanum lycopersicum</i> (tomato) (Solanaceae)	foliar spray	leaves, stems, roots	No	- n.s.	(Resquín-Romero et al. 2016)
	<i>Cucumis melo</i> var. <i>reticulatus</i> (melon) (Cucurbitaceae)	foliar spray	leaves, stems, roots	No	- n.s.	
	<i>Helianthus annuus</i> (sunflower) (Asteraceae)	soil treatment	rhizosphere	Yes (+)	+ increased protection against pathogenic fungi	(Miranda-Fuentes et al. 2020)
<b>KVL 04-57 and KVL 12-37</b>	<i>Triticum aestivum</i> (wheat) (Poaceae)	conidiated wheat roots	roots	n.s.	- n.s.	(Keyser et al. 2014)
<b>n.s.</b>	<i>Zea mays</i> (maize) (Poaceae)	seed treatment, killed conidia	n.s.	Yes (+)	+ all growth parameters enhanced + greater BM production	(Liao et al. 2014)

<sup>a</sup> = an effect on the plant was evaluated. (+) = at least one positive effect was observed. (-) = at least one negative effect was found.

<sup>b</sup>n.s. = no specification; The strains were not specified in the article, the article could not be viewed completely or no details were provided.

<sup>c</sup> dtx A = destruxin A

<sup>d</sup> = including height, length, amount and strength of all plant parts

<sup>e</sup> BM = biomass

<sup>f</sup> WUE = water use efficiency

### Naturally occurring soil-dwelling insects as hosts

As an entomopathogenic endophyte that resides mainly in the rhizosphere of plant roots, soil-borne insects in various stages of growth have been identified as hosts on several occasions (Table 2).

The *M. brunneum* strain BIPESCO5 could originally be isolated from *Cydia pomonella* (codling moth) (Lepidoptera) in Austria, 1967 (Ansari and Butt 2012; Ríos-Moreno et al. 2017). In a study, Kabaluk et al. (2017) sampled wireworms, Coleoptera, (*Agriotes obscurus* and *Agriotes lineatus*) from the soil of agricultural fields, British Columbia, Canada and found asymptomatic infection with the FE *M. brunneum* in 13-100% of live specimens. They point out in their experiment that this could be an enzootic presence of the endophyte (Kabaluk et al. 2017). Also in Germany (Lower Saxony), *Agriotes* spp. were successfully infected with *M. brunneum* (strain: ART2825) in an attract-and-kill method field experiment in potato cultivation (Brandl et al. 2017). Further results (in vivo as well as in vitro) on wireworms and the antagonist *M. brunneum* were obtained by Eckard et al. (2014), Reinbacher et al. (2021), Ansari et al. (2009) and Mayerhofer et al. (2017). In addition to the two *Agriote* spp. previously mentioned, *M. brunneum* was also able to colonize the species *A. sputator*. The in vivo as well as in vitro experiments were performed with *M. brunneum* strains V1002, ART2825 and BIPESCO5/F52, all of which proved useful (Eckard et al. 2014).

## Soil borne insect hosts under laboratory conditions

In particular, the finding that *M. brunneum* is entomopathogenic has motivated many scientists in the field of biological control and plant growth promoters to use and test the FE as an antagonist against many known soil-borne insects (pests) of crops (Table 2). Behie and Bidochka (2014) also refer to the endophyte *M. brunneum* as a widespread (broad-range) insect pathogen.

**Table 2.** Summary of evaluated published titles on the successful entomopathogenicity of *M. brunneum*. Listed are strain, host, and the respective reference. This table serves as an overview of the results of the last two decades of naturally occurring and in vitro insect hosts on the different host groups of the endophyte.

<b><i>M. brunneum</i> strain</b>	<b>Host insect (common name)(family)</b>	<b>Reference</b>
<b>ART2825</b>	<i>Agriotes</i> spp. (wireworms) (Coleoptera)	(Brandl et al. 2017)
	<i>Agriote sputator</i> (wireworm) (Coleoptera)	(Eckard et al. 2014)
<b>ARSEF 3297</b>	<i>Tipula paludosa</i> (crane fly) (Diptera)	(Ansari and Butt 2012)
<b>BIPESCO5 / F52</b>	<i>Agriote sputator</i> (wireworm) (Coleoptera)	(Eckard et al. 2014)
	<i>Cydia pomonella</i> (codling moth) (Lepidoptera)	(Ansari and Butt 2012)
	<i>Galleria mellonella</i> (greater wax moth) (Lepidoptera)	(Ríos-Moreno et al. 2017)
	<i>Popillia japonica</i> (japanese beetle) (Coleoptera)	(Behle et al. 2015)
	<i>Melanoplus sanguinipes</i> (migratory grasshopper) (Orthoptera)	(Dakhel et al. 2019)
<b>EAMa 01/58-Su</b>	<i>Tenebrio molitor</i> (mealworm larvae) (Coleoptera)	(Krell et al. 2018a)
	<i>Alphitobius diaperinus</i> (lesser mealworm) (Coleoptera)	(Behle et al. 2013)
	<i>Galleria mellonella</i> (greater wax moth) (Lepidoptera)	(Ríos-Moreno et al. 2017)
	<i>Bemisia tabaci</i> (sweet potato fly) (Hemiptera)	(Garrido-Jurado et al. 2017)
	<i>Spodoptera littoralis</i> (African cotton leafworm) (Lepidoptera)	(Miranda-Fuentes et al. 2019)
<b>EAMb 09/01-Su</b>	<i>Bactrocera olea</i> (olive fruit fly) (Diptera)	(Yousef et al. 2013)
	<i>Spodoptera littoralis</i> (African cotton leafworm) (Lepidoptera)	(Resquín-Romero et al. 2016)
	<i>Bemisia tabaci</i> (sweet potato fly) (Hemiptera)	(Garrido-Jurado et al. 2017)



<b>H.J.S 1154 and 1868</b>	<i>Delia radicum</i> (cabbage root fly) (Diptera)	(Razinger et al. 2018)
<b>KVL 04-57 and 12-37</b>	<i>Tenebrio molitor</i> (mealworm beetle) (Coleoptera)	(Keyser et al. 2014)
<b>V1002</b>	<i>Agriotes sputator</i> (wireworm) (Coleoptera)	(Eckard et al. 2014)
<b>n.s.<sup>a</sup></b>	<i>Agriotes obscurus</i> (wireworm) (Coleoptera)	(Kabaluk et al. 2017)
	<i>Agriotes lineatus</i> (wireworm) (Coleoptera)	(Eckard et al. 2014; Reinbacher et al. 2021; Ansari et al. 2009; Mayerhofer et al. 2017)
<b>n.s.</b>	<i>Agriotes</i> spp. <sup>b</sup> (wireworms) (Coleoptera)	

<sup>a</sup>n.s. = no specification; The strains were not specified in the article or the article could not be viewed completely.

<sup>b</sup> = only *Agriotes* spp. but no specific species was given by the authors or the article could not be viewed completely.

*M. brunneum* strain EAMb 09/01-Su (origin: meadow forest system, Spain) has been shown in trials to be a very useful antagonist against *Bactrocera olea* (olive fruit fly) (Diptera) (Yousef et al. 2013). The endophyte was extracted, applied in different concentrations and successfully used against both the adult *B. olea* and the larvae and pupae (Yousef et al. 2013). Razinger et al. (2018) successfully tested the efficacy of two *M. brunneum* strains (H.J.S 1154 and 1868) in their experiment on the control of *Delia radicum* (cabbage root fly) (Diptera) by FEs. Mycelium was detected on adult specimens of *D. radicum* as well as on their eggs (Razinger et al. 2018). As another representative of the Diptera order, Ansari and Butt (2012) were able to successfully infect *Tipula paludosa* (crane fly) (Diptera) in the larval stage, both in vitro and under greenhouse conditions, with *M. brunneum* strain ARSEF 3297 in their experiment. Representatives of the insect order Coleoptera also fall into the host scheme of *M. brunneum*.

The strain F52 was used both in vitro and in a field trial in turfgrass plots against *Popilla japonica* (japanese beetle) (Coleoptera) (Behle et al. 2015). The FE colonized larvae of *P. japonica* under laboratory conditions as well as in turfgrass pots (Behle et al. 2015). In a successful experiment by Keyser et al. (2014), larvae of *Tenebrio molitor* (mealworm beetle) (Coleoptera) could be infected with the FE by ingesting inoculated wheat plants (*Triticum aestivum*) with conidia of *M. brunneum* strains KVL 04-57 and KVL 12-37. The endophyte was able to spread through the root system of the wheat plant, thus infecting the *T. molitor* larvae (Keyser et al. 2014). The experiment was tested both under laboratory conditions and in the greenhouse. In an in vivo experiment investigating the effect of destruxin A on organisms, *Galleria mellonella* (greater wax moth) (Lepidoptera) larvae were successfully infected with the *M. brunneum* strains BIPESOC5 and EAMa 01/58-Su (Ríos-Moreno et al. 2017). Another member of the order Lepidoptera, *Spodoptera littoralis* (African cotton leafworm) larvae, could be colonized by *M. brunneum* strain EAMb 09/01-Su both by spray application and ingestion via the digestive system of leaves infected with the endophyte (Resquín-Romero et al. 2016). Species from the order Hemiptera have also emerged as possible hosts of *M. brunneum* in a laboratory experiment. Leaves of the melon plant (*Cucumis melo* var. *reticulatus*) were infested with sweet potato whitefly (*Bemisia tabaci*) nymphs and subsequently treated with a fungal spray treatment (Garrido-Jurado et al. 2017). The treatment contained conidia of the *M. brunneum* strain EAMb 09/01-Su or EAMa 01/58-Su (Garrido-Jurado et al. 2017). *B. tabaci* was successfully colonized by both strains. Insects from the order Orthoptera also fall into the host scheme of *M. brunneum*.

Nymphs in the third instar of the migratory grasshopper (*Melanoplus sanguinipes*) (Orthoptera) were successfully colonized by *M. brunneum* strain F52 (Figure 3) in an in vitro as well as greenhouse experiment conducted in Wyoming, USA (Dakhel et al. 2019).



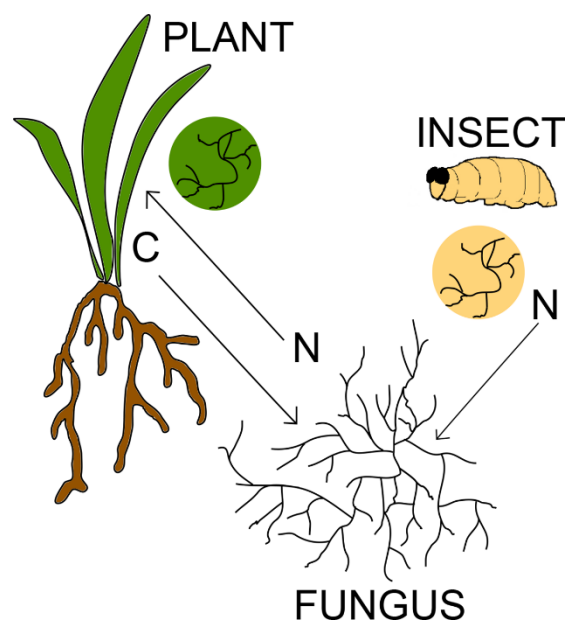
**Figure 3.** The carcass of *M. sanguinipes*, successfully killed by *M. brunneum* F52 and coated with its olive colored spores (Dakhel et al. 2019).

### 3.1.3. Nutrient and water management – is there evidence out there?

In the area of nutrient management, the literature was compiled on whether and how *M. brunneum* is able to take up nutrients from different sources and transfer these nutrients to a host plant in exchange with other products. In the literature search I found exact three articles dealing with nutrient uptake and transfer in *M. brunneum* and its host plants, but I only referred to studies in which nutrient uptake and transfer between the two symbionts took place and could be documented, and less to such studies in which positive effects were found and thus a possible nutrient transfer was hypothesized. Articles in which colonization of a plant with *M. brunneum* has been shown to lead to positive effects are discussed at the end of the 'ecology' section.

Recent research over the last two decades has shown that the FE is able to transfer the macronutrient N (nitrogen) from a colonized insect larval carcass to a host plant in a symbiotic association to obtain in exchange photosynthetic products (C source) from the plant (Behie and Bidochka 2014). The researchers set up an experiment in which they injected *G. mellonella* larvae with a <sup>15</sup>N-labeled nitrogen solution (ammonium sulfate) (Behie and Bidochka 2014). Subsequently, the larvae were infected with *M. brunneum* conidia and placed in a microcosm setup containing soil, different plant species (*P. vulgaris*, *G. max*, *Panicum virgatum* and *T. aestivum*) and fertilized with a N-containing nutrient solution. Later evaluation showed that <sup>15</sup>N was detected in all plants, which, according to Behie and Bidochka (2014), indicates that the FE successfully infected and killed the insect larvae and delivered the nutrient in a symbiotic association with the plant (Figure 4).

That *M. brunneum* is able to increase the bioavailability of the micronutrient Fe (iron) (ferrihydrite, hematite and goethite), in calcareous as well as non-calcareous media, for sorghum plants (*S. bicolor*) was proven by Raya-Díaz et al. (2017b; 2017a) in their studies. The endophyte was able to lower the pH of the CaCO<sub>3</sub> calcareous medium (which reduces the Fe availability for plants) by releasing organic acids, thus providing the sorghum plants with sufficient amounts of the micronutrient (Raya-Díaz et al. 2017b).



**Figure 4.** Simple model of nutrient transfer between a tripartite association of organisms.

In this basic illustration, the FE colonizes both the insect and the plant roots with its hyphae (the circles indicate the mycelium within the tissue of the respective organisms). Shown is how the FE takes up the nutrient N by consuming the insect tissue and transfers it to the plant. In exchange, the plant releases photosynthetic products in the form of C to the endophyte (Branine et al. 2019).

A cornerstone that continues to advance the possibilities of using *M. brunneum* as a plant fortifier has been recently demonstrated by Krell et al. (2018e) in their study on P (phosphorus) supply through FE in potato plants (*S. tuberosum*).

After successful inoculation and colonization of potato plants with encapsulated *M. brunneum*, a significantly high increase in P content within the plant could be measured compared to the control plants (not inoculated), whereas only a rather insignificant increase in N content in the plant could be documented (Krell et al. 2018e).

In a recent study, González-Guzmán et al. (2020b) found that *M. brunneum* is responsible for increased K uptake in durum wheat, but at the same time results in reduced water use efficiency (WUE). In contrast, an improved WUE was indicated by Krell et al. (2018d) in their experiment. Interestingly, however, the WUE was only improved under nutrient poor conditions.

#### 3.1.4. Discussion - room for improvement

The results of the literature research on the topic of ecology in this article have led to varying degrees of success, which is probably largely due to the fact that this area of research has only increasingly become the focus of large biotechnology companies in the last two decades. The results of the three sections are analyzed and discussed below.

##### Modus vivendi

Referring to the ecology of an organism to learn more about it, the habitat in which this organism lives is probably one of the most important points to understand certain correlations that are significant for later processes. The results of the literature research on the habitat of *M. brunneum* were rather meager, which may be due to the fact that this is one of hundreds of FEs, which has also gained popularity only in the last twenty years.

The vast majority of scientists agree on the fact that the endophyte *Metarhizium* preferentially and effectively colonizes the rhizosphere/rhizoplane region of the plants and causes endophytism there, which is most likely why the greatest potential of the FE is to be obtained in the soil (Meyling et al. 2011).

However, endophytism can also occur in the phyllosphere of plants, especially after artificial inoculation by superficial applications as well as by soil application of mycelium or conidia. Nevertheless, the foliar application of Jaber and Enkerli (2017) showed that even large parts of the leaves were colonized, but this endophytic colonization decreased steadily over the period of the experiment (in leaves and stem tissue). This fact was also established by Russo et al. (2015) in their experiment with the endophyte *B. bassiana*. In Resquín-Romero et al. (2016) this behavior could not be observed, the colonization of the leaf and stem tissue remained unchanged or increased over the course of the experiment. Basically, however, it can be stated that *M. brunneum* prefers to colonize roots and the hypocotyl region, although this may be largely related to the type of inoculation and the strain isolate as well as the respective host (Lahey et al. 2020). Interestingly, *M. brunneum* could only be isolated sporadically from stem and leaves of cauliflower plants in the experiment of Razinger et al. (2018) and was not present in roots, whereas the endophyte *Trichoderma* spp. could successfully colonize all plant parts endophytically. The authors speculate that the *M. brunneum* strains used do not possess the necessary properties to colonize cauliflower.

Nevertheless, Resquín-Romero et al. (2016) believe that FEs colonize the plant predominantly at the site where they encounter it (whether phyllosphere or rhizosphere), from where further (transient) dissemination can take place.

So far, only Liao et al. (2017) have found evidence that the FE *M. robertsii* is able to promote or even produce the auxin IAA, or influences the mechanisms that are able to generate this hormone. However, scientists still disagree on whether phytohormones such as abscisic acid (ABA), IAA, salicylic acid (SA) or jasmonic acid (JA) are released as an immune response of the plant to endophyte invasion or whether the endophyte produces them itself (Hu and Bidochka 2021; St Leger and Wang 2020). So-called tripartite interactions have already been observed in mycorrhizal fungus (Bougoure et al. 2014; Kuga et al. 2014; Smith and Smith 2012) and in other endophytes like *Beauveria bassiana*, *Lecanicillium lecanii*, *Aspergillus flavus* (Behie and Bidochka 2014) and *Trimmatostroma* sp. (Leroy et al. 2017). For *Metarhizium* spp., there is only one study so far dealing with the interaction and nutrient exchange between colonized insect, endophyte and plant host, a fact which requires further studies (Behie and Bidochka 2014; Leroy et al. 2017). Evidence that the FEs in the transfer of nutrients to the plant also receive nutrients in return from the plant was provided by Behie et al. (2017) using the endophyte *M. robertsii* and the tracker  $^{13}\text{C}$  ( $^{13}\text{CO}_2$ ). In their study, Wyrebek et al. (2011) were able to discuss possible relationships between co-occurring *Metarhizium* strains that function together in one area and thus form a community with the ecosystem. Tyurin et al. (2021) also note that the occurrence of FEs is in competition with other, possibly pathogenic, endophytes and thus there is a contest for a certain dominance.

Interestingly, the abundance of *M. brunneum* is significantly higher in the rhizosphere of plants than in bare soil (Klingen et al. 2015). Furthermore, Klingen et al. (2015) found that hemic isolates have a significantly higher abundance than exotic strains.



The study by Kepler et al. (2015), which may contain further information, could not be read in its entirety due to a paywall. Unfortunately, during my research I have hardly found any study that deals with the possible saprotrophic properties of *M. brunneum*. But interestingly, I could find several evidence of saprophytic lifestyle especially for the species *M. anisopliae*. According to Freimoser et al. (2003), *M. anisopliae* is a facultative saprophyte that is both saprophytic (free-living) and pathogenic in a variety of species. Lovett and St Leger (2015) refer to the entire genus *Metarhizium* as saprophytes, but give no evidence for this, which may not apply to every species of this genus. *M. robertsii* was also found to be a saprophyte since it remained in the rhizoplane of a plant even after its death and offered access to nutrients (Lovett and St Leger 2015). However, the fact that *M. brunneum* is entomopathogenic may suggest that it can also survive as a saprophyte, as the FE is able to infest and kill insects in order to feed on them. But, due to the descent from the family of Clavicipitaceae, which prefer to live on plants or insects, it is difficult to draw conclusions.

#### Host containment

Looking at the plant host range of *M. brunneum*, it quickly becomes clear how versatile this endophyte is. Interestingly, although it is a Clavicipitaceous endophyte (Clay and Schardl 2002), the vast majority of experiments I have conducted on the natural occurrence of *M. brunneum* have shown little relationship to grasses. In the study by Wyrebek et al. (2011), *M. robertsii* was found to be the only one of three isolated species to associate with grasses, and furthermore, *M. brunneum* was only found in combination with *M. robertsii* in shrubs but not solely (Wyrebek et al. 2011).

Nevertheless, *M. brunneum* is naturally associated with Poaceae species (Rodríguez et al. 2009), as shown by Keyser et al. (2015) and Ríos-Moreno et al. (2017), who isolated different strains of the FE from uncultivated grass pastures and soils where wheat (*T. aestivum*) was cultivated. Shrubs and trees were surprisingly found to be hosts of *M. brunneum* (Wyrebek et al. 2011; Fisher et al. 2011), which is in contrast to the studies of Clay and Schardl (2002) and Moonjely and Bidochka (2019), which reported that *Metarhizium* preferentially colonizes monocotyledonous plants. The finding that *M. brunneum* could be isolated from several agricultural fields may be related either to the fact that the microorganism composition plays a role, that there are certain insect larvae in the soil that serve as hosts, or that the endophyte lives specifically or not in mutualistic symbiosis with plants cultivated there. The fact that *M. brunneum* could be isolated from potato fields (without having been applied there before) may be related to the fact that the entomopathogenic endophyte is virulent against the potato beetle and has therefore settled in the soil of the potato fields (Tyurin et al. 2021).

During the research and analysis of the twenty studies and bioassays on host plants of *M. brunneum* that could be colonized under laboratory and field conditions, it was found that especially the three strains BIBESCO5/F52, EAMa01/58-Su and EAMb09/01-Su had attracted the greatest attention of the scientists. In the case of F52 in particular, this may be due to the fact that this isolate has been used for some time in various bio-based crop protection products.

Many important crops worldwide have been found to be endophytically colonizable by *M. brunneum* (Table 1), which strongly suggests that the endophyte is a generalist rather than a specialist.

The many trials have indicated, among other things, the variety of different inoculation methods that can be used and that may or may not prove useful<sup>2</sup>. However, in some cases it has also been found that monocot plants such as *Z. mays* (Poaceae) are preferentially colonized compared to dicots such as *P. vulgaris* (Fabaceae) (Lahey et al. 2020). That species from the Fabaceae family are less suitable as host plants for *M. brunneum* was also shown in the trial by Clifton et al. (2018) in which the endophyte could only be detected on a very small scale in the stems of *G. max* after inoculation. In the same experiment, it was proven that *B. bassiana* was able to produce a significantly more successful endophytism with *G. max*, and it is also suspected that other *Metarhizium* species are better suited as *G. max* endophytes (Clifton et al. 2018). Khan et al. (2012) were able to significantly improve the growth and quality of soybean (*G. max*) under salt stress using the strain *M. anisopliae* (higher shoot length, fresh and dry biomass). Jaber and Enkerli (2016) also managed to successfully propagate and spread *M. brunneum* and improved growth parameters in *V. faba* in their study, however, the rate of colonization of the leaves and stem decreased during the trial.

In the vast majority of studies, a positive effect on the respective plant was found (Table 1). The positive effects were in several cases improvements in growth parameters and biomass production under normal growth conditions, without stress. Nevertheless, especially under stress conditions such as various nutrient deficiencies, infestation with pathogenic fungi or high pH, positive effects on plant physiology could be observed.

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<sup>2</sup> See chapter 3.2.2. for further detailed information

In particular, the improved uptake of P, N and Fe under nutrient poor but also nutrient rich conditions is of great importance when considering *M. brunneum* as a plant fortifier and not only as an insecticide. More about nutrient management can be found in the last paragraph of this section.

However, negative effects were also observed, albeit in much smaller numbers. Ríos-Moreno et al. (2016) demonstrated increased production of the secondary metabolite dtx A (but only over a short period of time), which can be useful for use as a biological insecticide but can be potentially hazardous to humans if residues remain in food. *Metarhizium* spp. are capable of producing a variety of secondary metabolites (dtxs) (Wang et al. 2012). In a second trial conducted by Ríos-Moreno et al. in 2017, dtx A production by *M. brunneum* was found to be safe for humans and the environment. Another potentially negative phenomenon was observed by Clifton et al. (2018). Here, based on the results, it is hypothesized that the FE *M. brunneum* weakens the protective mechanisms of the host plant (soybean) and increases the acceptability for certain parasites by inducing certain plant hormones. Interestingly, in the same trial with *B. bassiana*, a positive effect was obtained, suggesting that *M. brunneum* or the strain used is not particularly suitable for use in soybean (Clifton et al. 2018). However, whether *Metarhizium* is less suitable for use in *G. max* requires further study, especially since Kepler et al. (2015) were able to isolate *M. brunneum* from a long-term agricultural field on which mainly soybean was cultivated. Improved growth parameters in soybean were also observed for *M. robertsii* as well as *M. anisopliae* (Moonjely et al. 2019; Garcia et al. 2011). The potency and broad plant host range of *M. brunneum* supports the hypothesis of its efficacy as a multifunctional plant strengthening agent.

However, it should not be underestimated that the majority of the studies analyzed were carried out in vitro or in closed systems and thus under perfect conditions without interfering factors. If *M. brunneum* meets or occurs in nature, a so-called rhizosphere specificity can occur as described by Wyrebek et al. (2011). This can have the consequence that the actual application purpose is missed or even ineffective, but the possibility of the emergence of other useful communities or properties also exists.

*Metarhizium* and many strains under this lineage have been considered entomopathogenic fungi since their discovery, however the literature assumes that the original mode of life relates to endophytism with plants (St Leger and Wang 2020). Because of its rather broad host range not only in flora but also in insects, FE *M. brunneum* is also a generalist in this area and is referred to as a multi-host generalist along with *M. robertsii* by St Leger and Wang (2020) (Roberts and St. Leger 2004). However, this does not apply to all *Metarhizium* strains, as there are also specialists from this lineage that have a very limited host range (e.g. *M. acridum* and *M. album*) (St Leger and Wang 2020). According to Rehner and Kepler (2017), the insect host spectrum of the various *M. brunneum* isolates includes species from the order of Coleoptera, Lepidoptera, and Diptera (Table 2). Two strains were also able to successfully colonize the larvae of the sweet potato fly from the order Hemiptera (Garrido-Jurado et al. 2017) and one strain was able to colonize the migratory grasshopper from the order Orthoptera (Dakhel et al. 2019).

My research has shown that, similar to the host plants, the most commonly used *M. brunneum* isolate is strain F52, which has also been able to successfully infect species from three of the five recognized orders of insects and is thus considered to be particularly diverse. Several bioinsecticides containing *M. brunneum* strain F52 are already on the world market (EFSA 2012). In addition to isolate F52, the strains EAMa 01/58-Su and EAMb 09/01-Su have again proven to be versatile. According to studies by Zimmermann (1993) and Driver et al. (2000), the various strains of *M. brunneum* are pathogenic to more than 200 insect species.

#### Nutrient and water management

The fact that *M. brunneum* is able to provide nutrients under various conditions has already been proven by a few studies (Behie and Bidochka 2014; Raya-Díaz et al. 2017b; 2017a; Krell et al. 2018e). Unfortunately, the availability of such studies on *M. brunneum* is still limited and requires further research. Of particular interest was the experiment by Behie and Bidochka (2014) in which they indicated the positive effects of tripartite relationships between endophyte, infected insect and host plant. The ability to take up the macronutrient N from a dead insect and transfer it to the host plant clearly indicates how useful a *Metarhizium*-based crop protection/strengthening agent can be (Barelli et al. 2019; Behie and Bidochka 2014; Moonjely et al. 2019). In further experiments, Behie et al. (2017) were able to indicate how *M. robertsii* obtains photosynthates as a food source in nutrient exchange with the host plant using  $^{13}\text{CO}_2$  labeled with  $^{13}\text{C}$ . Usuki and Narisawa (2007) were also able to demonstrate, using  $^{13}\text{C}$  labeled  $^{13}\text{CO}_2$ , that the FE *Heteroconium chaetospora* receives photosynthetic products in exchange for N to the host plant.

Currently, Krell et al. (2018e) provide the only evidence for increased P and simultaneous low N transfer between host plant and *M. brunneum*. A similar phenomenon is observed in arbuscular mycorrhiza (Unger et al. 2016). However, there is ample evidence that fungal endophytes are able to transfer essential nutrients such as P and Fe in exchange with host plants (Chhabra and Dowling 2017).

An important property of *M. brunneum* was studied by Raya-Díaz et al. (2017b; 2017a), in their experiments with different calcareous mediums, the FE showed that it could lower the pH of the very alkaline milieu in the rhizosphere by releasing organic acids to make the nutrient Fe available to the plant. Raya-Díaz et al. (2017b) also suggest that endophytic fungi are capable of releasing siderophores, substances of very low molecular weight, which facilitate chelation and thus increase the availability of iron. In a similar experiment by Sánchez-Rodríguez et al. (2016), *B. bassiana* was able to make Fe available to plants from calcareous substrate after successful inoculation. There is little evidence to date on the uptake of K by *M. brunneum*. González-Guzmán et al. (2020b) hypothesize that the increased uptake of K in their experiment is related to the increased presence of this nutrient in the substrate, and they also assume that the increased K uptake significantly reduced the WUE of the host plant. Interestingly, the other FE tested in this experiment, *B. bassiana*, indicated significantly more benefits, in addition to improved WUE and better mobilization of Zn and K (González-Guzmán et al. 2020b). The experiment by Krell et al. (2018d) showed that *M. brunneum* significantly promotes WUE of the host plant under nutrient deficient conditions and not under balanced nutrient conditions, which in turn strengthens the hypothesis of using the FE as a multifunctional plant promoter.

### 3.2. Physiology – Response to abiotic factors

The physiology of *M. brunneum*, the second part of this literature research, deals with the abiotic factors that have an influence on the physiology of the endophyte, as well as the reaction and behavior when confronted with them. This section is particularly important when considering the use of the FE as a plant fortifier or protectant, as environmental factors such as UV radiation, temperature, nutrient deficiencies or surplus, behavior towards and with other organisms, and further stress factors are particularly important.

#### Nutrient fluctuations, water stress, temperature and other stress factors

As already described in the ecology section of this article, Krell et al. (2018d) were able to conduct tests with *M. brunneum* and *S. tuberosum* in nutrient-poor and nutrient-rich substrates. It turned out that especially under the nutrient-poor conditions an increased endophytism took place and additionally the nutrient transfer of the important macronutrients P and N increased significantly, compared to the control without FE (Krell et al. 2018d). In addition, a significantly improved nutrient uptake was also observed in the nutrient-rich milieu. Crops grown on calcareous soils often suffer from Fe deficiency (del Campillo and Torrent 1992). As an essential micronutrient, Fe is of paramount importance for many important processes within the plant (enzymatic, photosynthetic). Raya-Díaz et al. (2017b) proved in their experiments that *M. brunneum* is able to supply plants growing in calcareous soils with sufficient iron after successful colonization by lowering the soil pH value.



By releasing organic acids, the FE is able to alter its immediate environment in the rhizosphere, consequently increasing the concentrations of e.g., Fe, Zn or P (Liaud et al. 2014). Dara et al. (2017) were able to provide initial evidence that *M. brunneum* is able to supply plants growing under high water stress with sufficient nutrients and water and thus improve growth conditions. An improved WUE of potato plants under nutrient-poor conditions was observed by Krell et al. (2018b; 2018e) in their trial after successful inoculation with *M. brunneum*.

I have found data on the handling and reaction of *M. brunneum* with different temperatures mainly in articles dealing with different methods of commercialization. The optimal germination and growth temperature for the endophyte was determined in several studies (in vitro as well as in situ) to be ~22-30°C (Behle and Jackson 2014; Lozano-Tovar et al. 2017; EFSA 2012; Gonzalez-Guzman et al. 2021; Jaber and Araj 2018; Kryukov et al. 2017; Steinwender et al. 2015). According to the EFSA (2012), isolate F52 only grows up to a temperature of 37°C. The results of Kryukov et al. (2017) also state that *M. brunneum* is less heat tolerant, as at temperatures of 35-40°C the mycelial growth is largely stopped, but is able to actively develop at temperatures of 10-20°C. Even at temperatures of 7-18°C a relatively high conidia germination was observed (Nishi et al. 2013; Klingen et al. 2015). *M. brunneum* is thus considered a rather mesophilic organism, which displays limited virulence under drought conditions (Tyurin et al. 2021). Lozano-Tovar et al. (2017) indicated in their experiment that at least the fungicidal activity of the isolate used was resistant to 60°C for 2h and 120°C for 20 min. Storage of *M. brunneum* stock cultures on agar is also possible in a 10% glycerol solution at -80°C (Behle and Jackson 2014; Gonzalez-Guzman et al. 2021).

The influence of UV radiation can only be explained for the genus *Metarhizium* (Roberts and St. Leger 2004; Braga et al. 2001a) or the species *M. anisopliae* (Braga et al. 2001a; 2001b; Chelico et al. 2006; Alves et al. 1998), *M. robertsii* (Lovett and St Leger 2015) and *M. acridum* (Rangel et al. 2005) since only one result is available for the FE *M. brunneum* (Lozano-Tovar et al. 2017). Exposure to solar ultraviolet radiation is usually divided into UV-A (380-315 nm), UV-B (315-280 nm), and UV-C (280-100 nm) radiation and expressed in wavelengths in nm. Lovett and St Leger (2015) claim that DNA, as the main target, suffers from UV radiation, resulting in mutagenesis and cell death. According to one study, *Metarhizium* is able to show some tolerance to UV radiation through the formation of its dark green pigmented conidia. The study by Chelico et al. (2006) have shown that photoreactivation is the main mechanism for repairing UV-induced damage to DNA in *Metarhizium*, the radiation used was UV-C radiation of 200-280 nm. The results of Braga et al. (2001b) have once again shown how differently individual strains of a species react to stressors. Three *M. anisopliae* isolates were tested under natural conditions and one strain was found to have a high tolerance to UV-A (320-400 nm) radiation and a low tolerance to UV-B (280-320 nm), while another showed exactly the opposite (Braga et al. 2001b). UV-A exposure for 4 h reduced the cultivability of the strains from 10-60% (depending on the strain) and surviving conidia showed a delayed germination rate in all strains (Braga et al. 2001b). One *M. brunneum* (EAMb 09/01-Su) strain could be classified as UV resistant by Lozano-Tovar et al. (2017). After 2, 4 and 6 h of UV irradiation, active metabolites of the FE were still detected, which inhibited mycelial growth of the target fungal pathogen (Lozano-Tovar et al. 2017).

The pH value of the soil or the medium in which the FE is located or applied is an important abiotic factor that must be taken into account, as the pH value can have a significant influence on the activity of fungi (Lozano-Tovar et al. 2017). *M. brunneum* strain F52 (formerly *M. anisopliae*) can grow in a pH range of 3.3 - 8.5 (EFSA 2012). However, Lozano-Tovar et al. (2017) found that the fungicidal activity of *M. brunneum* metabolites against *V. dahliae* was significantly negative for 24 h at pH values of 7.5 and 8.5.

#### From the reaction to organisms to the reaction of organisms

As described in the first section, *M. brunneum* is an entomopathogenic endophytic fungi that is pathogenic to a variety of soil-borne insects (especially larvae). However, some insects have also developed prophylactic behaviors that can trigger an increased immune defense against *M. brunneum* (Lu and St Leger 2016). According to the results of the study by Kabaluk et al. (2017), there is evidence that wireworms (*Agriotes* spp.) in particular can form a symbiotic association with various bacterial strains in order to protect themselves against the FE *M. brunneum*.

Jaber and Araj (2018) provide the first evidence that effective coexistence between *M. brunneum* and the parasitoid *Aphidius colemani* is possible under greenhouse conditions against the aphid *Myzus persicae*. Interestingly, the exact opposite was found in the experiment by Clifton et al. (2018) although only *M. brunneum* was used as the only active ingredient against the aphid *Aphis glycines*. The colonization with the FE favored the aphid infestation of the test plant (Clifton et al. 2018).

Evidence for interaction and coexistence of individual *Metarhizium* strains in nature was provided by Wyrebek et al. (2011) in their experiment, as previously reported in the host containment section<sup>3</sup>. There is also evidence that isolates of the FE *M. brunneum* are capable of releasing antifungal secondary metabolites that are particularly effective against the plant fungal pathogens *Verticillium dahliae* and *Phytophthora megasperma* (Lozano-Tovar et al. 2017; Miranda-Fuentes et al. 2020). The efficacy of *M. brunneum* was also demonstrated against various species of the fungal pathogen *Fusarium* spp (Jaber and Alananbeh 2018; Jaber 2018a). Gupta et al. (2021) conclude in their study that FEs are able to induce a certain immunity and disease resistance in plants by activating the defense mechanisms of the plant host. In an experiment to evaluate the effects of FCBK on prokaryotic and fungal communities in soil, Mayerhofer et al. (2019b) did not detect structural changes in soil-borne fungi or changes in prokaryotic communities.

### Abiotic factors – ready to discuss

Interestingly, a positive growth and colonization effect was recorded for the endophyte *B. bassiana* under high nutrient density in the soil, but under nutrient-poor conditions exactly the opposite occurred (Tall and Meyling 2018). The presence of the FE led to restricted growth and to a further possible nutrient deficit (Tall and Meyling 2018). However, Paungfoo-Lonhienne et al. (2015) claim to have found in their experiment that *B. bassiana* is positively affected in soils where there is an N deficit, in the same comparison with *Metarhizium* spp. which were very negatively affected under N deficiency.

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<sup>3</sup> See chapter 3.1.2.

This again may be evidence that the different strains of *Metarhizium* spp., *Beauveria* spp. or other fungal endophytes process various influences and conditions differently. It is also interesting to note that under nutrient-poor conditions, the appearance of adhesive Mad2 (it is suspected that it serves to bond with the plant epidermis) was increased in *M. robertsii* (Barelli et al. 2011). An improved Fe uptake of the experimental plants in calcareous soil could also be achieved by the FE *B. bassiana* (Sánchez-Rodríguez et al. 2015). In addition, *B. bassiana* was able to modify the mineral K in such a way that an uptake by the plant was favored (Sánchez-Rodríguez et al. 2015). Gonzalez-Guzman et al. (2021) also conducted trials with *M. brunneum* and *B. bassiana* on calcareous soils, showing improved uptake of mineral elements such as Mn and Zn from the soil. This in turn may be due to the ability of the endophytes to release organic acids into the soil. Dara et al. (2017) obtained even better results with *B. bassiana* under water stress conditions than with *M. brunneum*. This may be due to the fact that *B. bassiana* associates more successfully with Brassicaceae plants (Yerukala 2019).

The temperature behavior of *M. brunneum* strongly resembles that of *M. anisopliae*, which may be due to the commonality from the PARB clade. Interestingly, at a temperature of 10°C, *M. anisopliae* shows a strong decrease in conidial growth (Yeo et al. 2003; Kryukov et al. 2017) whereas *M. brunneum* shows a widely active conidial growth between 7 and 10°C (Nishi et al. 2013; Klingen et al. 2015). Adding other *Metarhizium* species it becomes clear that the optimal temperature for this species ranges from 22-30°C and other fungal endophytes such as *B. bassiana* and *Verticillium lecanii* also show optimal growth at temperatures of 20-25°C (Yeo et al. 2003; Jaronski 2010).

If the temperatures go to the extreme, however, there are clear differences. *M. robertsii* e.g., is able to grow at temperatures of 35-37.5°C, but hardly grows at 10°C, in comparison *M. pemphigum* and *M. brunneum* can grow actively up to a maximum temperature of 30°C (Kryukov et al. 2017). However, *M. brunneum* can also withstand very extreme temperatures of 60 and 120 °C, even if only for a very limited period of time (Lozano-Tovar et al. 2017). The experiment by Jaronski (2010) has also shown that temperatures around 40°C can be tolerated by the *M. brunneum* isolate F52 for a few hours and that it starts to grow again only a few hours after exposure to heat. The cold tolerance of *M. brunneum* makes the FE particularly interesting for use in temperate regions of the world, both as a plant protection and as a plant strengthening agent, since temperatures below 10°C at night occur here on many days of the year. However, the study by Bidochka et al. (2001) again shows that even individual isolates of a species can have different temperature tolerances, possibly depending on the origin of the isolate, for instance whether an isolate was obtained from the soil of a forest or from the soil of an agricultural area. The fact that there is too little or no research on temperature response mechanisms of *M. brunneum* or other endophytic fungi, which are necessary for a better understanding of processes during temperature fluctuations and extreme temperatures, must lead to more research in this area. Especially under the current threat of climate change and the associated temperature changes in many regions of the world.

Particularly for the development of mycoinsecticides to be used in the phyllosphere, research on the effects of solar UV radiation on the fungal endophyte is important (Shang et al. 2012).

The results of the literature have shown how differently individual species of a genus and also the individual strains of a species react to UV-A, UV-B and UV-C radiation. *M. acridum* has been found to be particularly tolerant to UV-B, possibly due to its origin in the Australian desert (Rangel et al. 2005). For *M. brunneum* only two experiments could be found. First, in which the FE is described as UV resistant, but further information on radiation intensity and type of UV radiation is missing (Lozano-Tovar et al. 2017). In the other experiment by Yousef et al. (2013), the efficacy of *M. brunneum* strain EAMb 09/01-Su was significantly weakened by exposure to UV-B radiation at 2, 4, and 6 h. Interestingly, it has been possible to equip *B. bassiana* with an exogenous tyrosinase gene (from *Agrobacterium*), which promoted the production of yellowish pigments, which in turn showed a significantly higher UV resistance (Shang et al. 2012). This technique may be transferable to other FEs. Alves et al. (1998) formulated *M. anisopliae* conidia suspensions with different oils (vegetable, emulsifiable and mineral) and showed improved tolerance to UV radiation for up to 6 h. Braga et al. (2001b) have shown in their experiment that full spectrum sunlight has little effect on the conidial cultivability of *M. anisopliae* conidia, but UV-A radiation has significant limitations in two of the three strains tested, again indicating that strain selection of a species and its site of action should be carefully considered.

That the genus *Metarhizium* is able to change the pH value of its environment, in favor of itself and others, has already been proven in several studies. According to St Leger et al. (1999), *M. anisopliae* is able to change the pH of its microenvironment through various metabolic activities in order to improve the performance of certain enzymes.

As well as *M. brunneum* is able to release organic acids that lower the pH value in its immediate environment, in favor of the dissolution of mineral elements (Raya-Díaz et al. 2017b; Sánchez-Rodríguez et al. 2016). The reason that the FE *M. anisopliae* can grow at a pH value of 8 is, according to St Leger et al. (1999), that acid overproducing mutants may have formed next to the wild type, which are able to regulate the environmental pH value by producing altered oxalic acid. Interestingly, Bidochka et al. (1998) did not detect any significant effects of pH values of 4.07 - 8.54 on the recovery of the entomopathogenic FE *M. anisopliae* and *B. bassiana* in peat and loam soil. Rath et al. (1992) also found that pH has little effect on *M. anisopliae*, which is capable of germinating in a wide pH range. In relation to pH as an abiotic factor, there are unfortunately few studies that relate to the influence of pH values on *M. brunneum*, but certainly on *M. anisopliae*. Since pH is an important factor, especially in view of the climatic changes that also affect soil pH, it is advisable to conduct more sustained research in this area (Rengel 2011).

Kabaluk et al. (2017) speculate that increased competitive ability of soil-borne bacterial strains is responsible for the release of certain metabolites and other compounds, which in turn inhibits the propagation or adhesion of *M. brunneum*. It should be noted, Miranda-Fuentes et al. (2020) hypothesize that the protection offered by the endophyte to plants against, for example, the fungal pathogen *V. dahliae*, is more likely due to competition between the two fungi in the soil community. The trial by Mayerhofer et al. (2019b) was the only one that investigated the application and effects of *M. brunneum* on soil-borne organism communities such as prokaryotes as well as fungi.



However, the fact that a product has to be tested for this issue before it can be approved in the European Union (The European Commission 2011) indicates the importance of further studies in the future and in relation to the use of *M. brunneum* isolates.

### 3.3. Process engineering – the commercialization

In the last section of this thesis, the possible methods and techniques of detection and commercialization of the endophyte are described and discussed. As Hetjens et al. (2021) noted in their recent study, the identification of fungi that have infected an organism is particularly essential for the successful implementation of efficacy studies as well as environmental impact appraisals. When it comes to the use of *M. brunneum* as a product in the agricultural and horticultural sector as a plant fortifier or biological pesticide (mycoinsecticide), this is probably one of the most important areas for future research. In particular, meticulous research has been and is being carried out in the last decade on the production of effective and usable inoculation techniques and products (Vega 2018). Consequently, this section is subdivided into the area around the different detection methods as well as methods for commercialization.

#### 3.3.1. The methods of detection

During the literature research on detection methods of *M. brunneum*, I came across three procedures that are used in science. Firstly, PCR (polymerase chain reaction) method is commonly used for qualitative detection or qPCR (real time quantitative PCR) method for quantitative analysis, RFLP (restriction fragment length polymorphism) analysis was also used as a confirmation method of PCR products. Furthermore, Raman spectroscopy was successfully applied in this field of application, as well as morphological methods and procedures that are explained as other methods.

### PCR, qPCR techniques and RFLP

In the research on detection techniques, PCR analysis emerged as the definitive method. Kortsinoglou et al. (2020) describe molecular identification as a basic requirement for the identification and development of new strains and the resulting importance for agricultural biotechnology. In order to obtain a PCR result that is as free from interference and errors as possible, the DNA of the bio sample must first be extracted (Rådström et al. 2004). For this purpose, isolates of the FE are grown on nutrient media, from which the mycelium can be harvested after a few days and then extracted using a DNA isolation kit (Wyrebek et al. 2011). Based on the DNA, suitable primers, DNA polymerase, nucleotides and a polymerase buffer solution, the PCR amplification can now be continued (Mayerhofer et al. 2019a). In almost all bioassays used for this literature review, a PCR procedure was used to identify *M. brunneum* (Bischoff et al. 2009; Fisher et al. 2011; Sevim et al. 2012; Kabaluk et al. 2017; Kryukov et al. 2017; Mayerhofer et al. 2017; Clifton et al. 2018; Mayerhofer et al. 2019b; Miranda-Fuentes et al. 2020; Tyurin et al. 2021). Some researchers have used special methods such as species-specific PCR with specific primer pairs (molecular markers) (Steinwender et al. 2014; Steinwender et al. 2015; Rehner and Kepler 2017; Mayerhofer et al. 2019a), AFLP (amplified fragment length polymorphisms) for better evaluation of diversity (Keyser et al. 2015), RFLP for verification of PCR products (Behie et al. 2015; Wyrebek et al. 2011), as well as a qPCR assay (Krell et al. 2018e; Ríos-Moreno et al. 2017) for faster and highly specific detection from the environment (cultivation independent) for accurate analysis of habitat preferences (Schneider et al. 2011).

Sequence analysis of the 5' end of the 1- $\alpha$  elongation factor and simple sequence repeat (SSR) typing proved particularly useful in the case of *M. brunneum* (Steinwender et al. 2014).

#### Raman spectroscopy for identification of *M. brunneum*

During the literature search on detection methods of *M. brunneum* I came across a single attempt to identify different *M. brunneum* isolates using Raman spectroscopy (Hetjens et al. 2021). The scientists involved in this experiment succeeded in generating Raman spectra of conidia from the five fungal endophytes used (*M. brunneum* isolates Ca8II, CB15III, CB16III; *M. pemphigi* X1c; *B. bassiana* DSM 62075). With the help of these spectra, models could be developed that indicate information on the species and isolate of a fungal sample taken from a host (insect) (Hetjens et al. 2021). The authors describe this type of detection as particularly cost-effective compared to the PCR method and predict that further research can expand this approach more specifically (Hetjens et al. 2021).

#### Morphological methods

The classical morphological methods, in which primarily external features, conspicuousness, shape and structure are analyzed, are of little use in the detection of fungal endophytes, especially when it comes to taxonomically closely related species, e.g., *M. anisopliae* and *M. brunneum*, which cannot be distinguished in this specific way (Brunner-Mendoza et al. 2019; Jaber and Alananbeh 2018).

## Other methods

Another way to detect the presence of *M. brunneum* is to look for accumulations of ergot alkaloids, less in plants and more in insects and culture. Leadmon et al. (2020) have shown in an experiment that *M. brunneum* produces the ergoline alkaloid d-lyseergic acid amide (ergine) mainly in culture but also in colonized insects.

## Discussion about the methods

Considering the detection methods of *M. brunneum* or other fungal endophytes, it quickly becomes clear that these are mainly molecular identification techniques (Kortsinoglou et al. 2020), only a significantly small part is based on morphological methods (Brunner-Mendoza et al. 2019), which usually rather serve the pre-determination than for taxonomic classification, as already described in the introduction. Only with the aid of molecular detection methods was it possible to identify the PARB clade surrounding the FE *M. anisopliae*, which comprises four cryptic phylogenetic species, including *M. brunneum* (Bischoff et al. 2009). The presence of ergosterol in insects can therefore indicate a colonization with *M. brunneum* but can also mean that it is any other representative of the family Claviceps (Weete et al. 2010; Leadmon et al. 2020). The fact that the ergot alkaloids occur after infestation of the endophyte mainly in insects (cadavers or alive) and less or hardly detectable in plants also strongly limits this method (Leadmon et al. 2020). Thus, further analyses such as a PCR test are necessary.

The use of Raman spectroscopy, despite the fact that it was first used for the detection of fungi in 1995, has interestingly only been used a few times since (Edwards et al. 1995). Strycker et al. (2020) and Hetjens et al. (2021) may have set a milestone in the development of low-cost and less time-consuming detection techniques with their recently published studies on the detection of microfungi using Raman spectroscopy. A portable version of a Raman microspectrometer (Strycker et al. 2020) could thus be used directly in the field to detect an FE or other micro fungi without first isolating and culturing them. The fact that a PCR-based method was chosen for the majority of the literature and experiments reviewed for this section may be due to the fact that this type of molecular detection is simply the most accurate, especially when it comes to detecting different strains and isolates of a species (Motamedi et al. 2011). Nevertheless, this is also the most expensive variant, which requires a lot of equipment, substances and work steps. However, the PCR-based variants are probably the only ones capable of recognizing and assigning newly emerged isolates to a species. In particular, the increasing number of molecular markers such as SSR markers (simple sequence repeats) facilitates the evaluation and detection of *M. anisopliae* strains from environmental samples (Enkerli et al. 2005; Schneider et al. 2011). With the help of the PCR-based RFLP method, it is possible to directly examine samples that have just been taken, e.g., an infected insect, in order to make assumptions about the habits and behavior in nature (Leal et al. 1997).

### 3.3.2. Commercialization techniques

In order to make *M. brunneum* usable and applicable for the agricultural sector, especially the techniques of commercialization play an important role besides the methods of endophyte detection (Murphy et al. 2018). The aim is to explore methods and formulations of transforming the FE into an applicable and affordable product that ensures endophyte survival, shelf life and storage, as well as on-site efficacy (Jackson et al. 2010; Vega 2018; Murphy et al. 2018; La Cruz Quiroz et al. 2019). In particular, the survival and germination of conidia to the point of application is of tremendous importance for efficacy as a tonic or mycoinsecticide and is usually reflected as colony forming units (Behle and Jackson 2014). During the literature review, it became apparent that scientists and industry have been researching formulations of conidia-containing granules or substrates for the past thirty years (5418164; Hall and Barry 1995; Faria and Wraight 2007; Li et al. 2010).

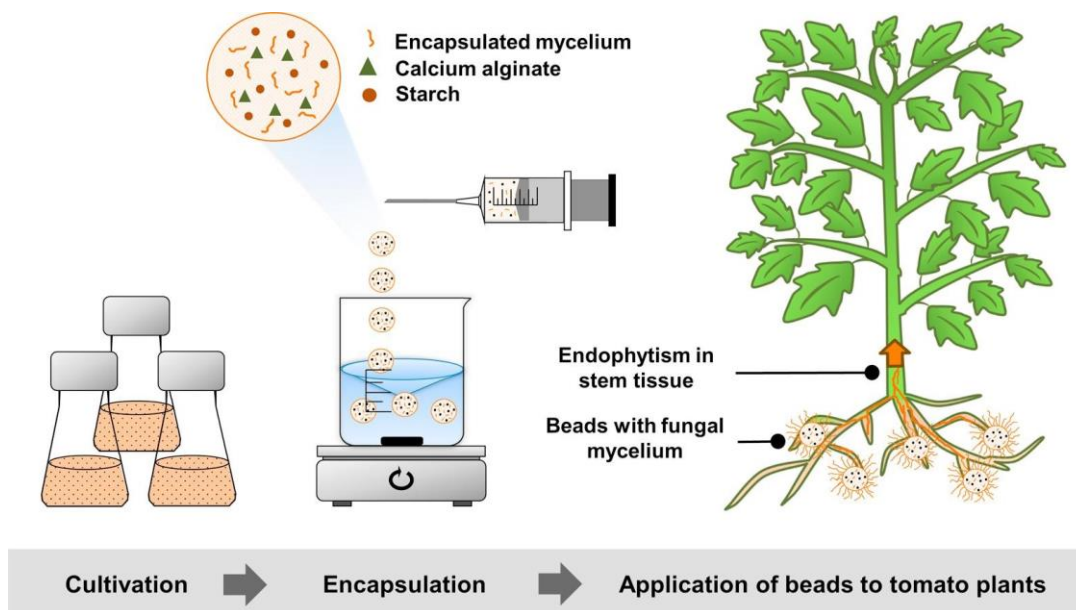
Beyond the known conidia substrates and granules, Behle and Jackson (2014) researched a method for liquid fermentation of *M. brunneum* (strain: F52) and the successful formation of microsclerotia (MS). So-called MS are an aggregate state that the mycelium of some entomopathogenic and phytopathogenic fungi can enter e.g., to overwinter (Song 2018). In terms of structure, MS are similar to sclerotia (compact mycelium containing food reserves) but are significantly smaller (Song 2018). Under optimal growth conditions, the MS can germinate and form effective conidia and spores. In their experiment, Behle and Jackson (2014) tested two different, favorable nitrogen media as a food source for the formation of MS of *M. brunneum*. The N media were soy flour and cottonseed meal in Carbon:Nitrogen ratios of 30:1 and 50:1, respectively (Behle and Jackson 2014).

However, in the experiment, the granules with the higher C:N ratio were found to be significantly more effective, longer lasting and resulted in greater conidia production (soy flour and cottonseed meal performed similarly), furthermore, the experiment indicated the economic advantage due to very low costs of N resources. (Behle and Jackson 2014). The functionality as well as effectiveness of the granules was measured by the death rate of infected individuals of the lesser mealworm (*Alphitobius diaperinus*). In a study conducted one year earlier, Behle et al. (2013) had already gained experience with a formulation of MS granules of *M. brunneum* against *Ixodes scapularis* (deer tick). The most recent study in this area was conducted in 2021 by Stephan et al. Like Behle and Jackson (2014) in the previous paragraph, the scientists have also researched a liquid fermentation process that can be transferred to granules by a fluid-bed coating process (Stephan et al. 2021). For this purpose, biomass containing mycelium and submerged spores of *M. brunneum* strain BIPESCO5 was prepared in liquid culture. After a preliminary nutrient coating consisting of malt extract and/or peptane, the liquid biomass was successfully applied to autoclaved millet grains using the fluid-bed method (Stephan et al. 2021).

In three further studies, the endophyte was incorporated into a calcium alginate/starch solution and solidified into beads by two different crosslink solutions. Humbert et al. conducted an experiment around 2017 in which they exchanged the commonly used crosslinker calcium chloride ( $\text{CaCl}_2$ ) (CC) for calcium gluconate ( $\text{C}_{12}\text{H}_{22}\text{CaO}_{14}$ ) (CG). In their trial, they mixed sodium alginate, corn starch, conidia of *M. brunneum* (strain: ART 2825), and water to form a solution, which was then dripped into the crosslinking solution containing CG or CC to solidify into beads (Humbert et al. 2017).



The CG crosslinker has been shown to significantly improve the survival of encapsulated *M. brunneum* conidia after the drying and rehydration process, compared to the classical CC crosslinker (Humbert et al. 2017). In addition, a nutritive effect on the cells of the endophyte and a reduced hygroscopicity of the granules could be demonstrated.



**Figure 5.** Illustration of the simplified manufacturing process from encapsulated mycelium to application.

**Cultivation:** The mycelium of *M. brunneum* is grown in a nutrient solution and then filtered. **Encapsulation:** The mycelium is mixed with calcium alginate, starch and water and dropped via a dispenser into the CC-containing crosslink solution, in which the beads harden from the outside in. The beads are then sieved and dried. **Application:** The beads are applied in the root zone of tomato plants and form successful endophytism with the plant (Krell et al. 2018b).

In the same year, Przyklenk et al. (2017) also worked on the encapsulation of *M. brunneum* strain BIPESCO5 in Ca-alginate beads including a starch component. However, the focus of this experiment was to reduce the conidia used to a minimum in order to obtain a cost-efficient and effective formulation. The scientists achieved this by preparing a solution consisting of 2% sodium alginate, 20% corn starch and 20% baker's yeast (*Saccharomyces cerevisiae*) and 0.01% spore powder, which was gelled by the crosslinker CC (Przyklenk et al. 2017).

In the other study, conducted by Krell et al. (2018c), instead of conidia, mycelium of *M. brunneum* (strain: BIPESCO5) was mixed with sodium alginate, corn starch and water to form a suspension, which was then dripped into a CC crosslink solution (Figure 5). After drying, the beads were inserted into the root zone of tomato plants and established endophytically in the plant stems (Krell et al. 2018c). Finally, Krell et al. (2018c) also found improved survival of the mycelium during the drying phase of the beads, furthermore they also assume that the calcium alginate/starch mixture leads to improved survival of the endophyte in the encapsulated state. In a subsequent experiment based on the method of encapsulation with increased osmolarity Krell et al. (2018a; 2018c) found that increased endogenous polyol concentrations, especially of arabitol and mannitol, in the mycelium of encapsulated *M. brunneum* resulted in storage of the beads for up to 12 months. Furthermore, virulence against *Tenebrio molitor* larvae was preserved. An experiment, also carried out by Krell et al. (2018b), dealt specifically with the addition of cellulase during the encapsulation process of *M. brunneum* (strain: CB15) mycelium. The incorporation of cellulase resulted in increased enzymatic activity, which improved mycelial growth to the point of sporulation on the beads and can thus lead to increased endophytism in plants (Krell et al. 2018b).

Mayerhofer et al. (2019b) used the process first described by Aregger (1992), in which barley kernels are colonized by a fungus. Whole-grain barley kernels are inoculated in a controlled manner with the *M. brunneum* strain EAMa01/58-Su and incubated over a period of 24-42 days, at 23°C and with the addition of water in polyamide bags, to produce the greatest possible number of conidia (Aregger 1992; Mayerhofer et al. 2019b).

The resulting fungus colonized barley kernels (FCBKs) were tested in a pot and field experiment to determine whether *M. brunneum* affects native soil microbial communities (Mayerhofer et al. 2019b).

In addition to the methods of producing granules or beads, research is also being conducted in the direction of foliar spray techniques, on the one hand liquid preparations that can be sprayed on directly (Resquín-Romero et al. 2016; Raya-Díaz et al. 2017b; Jaber and Enkerli 2017) and on the other hand powders that are emulsified with liquids (Kassa et al. 2004). The methodology behind this is quite simple, in an in vitro essay *M. brunneum* conidia are grown on petri dishes and mixed with pure water and an emulsifier or wetting agent after a certain maturation period (Resquín-Romero et al. 2016; Jaber and Enkerli 2017). The finished emulsion is sprayed onto the adaxial and abaxial leaf surfaces using a spraying technique (hand sprayer).

## Discussion of the commercialization procedures

Two methods in particular play a major role in the commercialization process of *M. brunneum*. Firstly, the well-known technique of encapsulating conidia, mycelium or spores in Ca-alginates is at the forefront and has already been successfully used in trials specifically for *M. brunneum* (Krell et al. 2018a; 2018b; 2018c; Humbert et al. 2017; Przyklenk et al. 2017), but also for other important FEs such as *Beauveria bassiana* (Faria and Wraight 2007; Vemmer et al. 2016; Marques et al. 1999; Pereira and Roberts 1991), *M. anisopliae* (Faria and Wraight 2007; Marques et al. 1999; Pereira and Roberts 1991) and *Trichoderma* spp. (Jurić et al. 2019; Cumagun 2014).

However, the production of conidia in particular is an extremely costly affair and reduces the economic value (Samson 2013; Jackson et al. 2010). Nevertheless, as the experiments of Krell et al. (2018a; 2018b; 2018c), Przyklenk et al. (2017) and Humbert et al. (2017) have shown, by adding nutrients and using different cross link solutions, the conidia content in the beads can be reduced to a minimum, thus reducing the overall production costs. At the same time, the reduction of the conidia content and the addition of nutrients and baker's yeast in the granules results in an increased shelf life, effectiveness and better survival chances of the endophyte (Przyklenk et al. 2017). Apart from the Ca-alginate process, Krell et al. (2018b; 2018e) have shown in their studies that the use of amidated pectin instead of alginate is also possible and represents an alternative.

The second method is liquid fermentation, in which a basal salt solution containing vitamins, trace elements and purified water is prepared, to which a carbon and a nitrogen source are added (Jackson and Jaronski 2009). This medium serves as a food source for the previously grown fungal conidia. This technique has been successfully applied to *M. brunneum* (Jaronski and Jackson 2008; Behle et al. 2013; Behle and Jackson 2014; Stephan et al. 2021), *M. anisopliae* (Gao et al. 2007; Jaronski and Jackson 2008) and *Tricoderma viride* (Gao et al. 2007) as well as to the FEs *Beauveria bassiana* and *Cordyceps fumosorosea* (Stephan et al. 2021). In particular, Gao et al. (2007) highlighted at the end of their experiment that the C:N ratio varies from species to species and plays an important role during the formulation process. Despite the more or less identical liquid fermentation procedure, the objectives of Stephan et al. and Behle and Jackson are set differently.

While Behle and Jackson (2014) and Jaronski and Jackson (2008) successfully produced MS of *M. brunneum* in granule form (clay base) in their experiment, which proved to be extremely effective and inexpensive, Stephan et al. (2021) produced a conidia solution that was applied to sterilized millet grains together with a nutrient suspension via fluid bed procedure, which did not prove to be very effective in the case of *M. brunneum*. Mascarin et al. (2014) were also able to culture MS of *Metarhizium* species and hypothesize higher efficacy due to more persistence compared to conventional conidia or mycelial preparations. The authors Jaronski and Jackson (2008) demonstrate the exceptional efficacy, in terms of high performance at very low humidity, of MS preparations of *Metarhizium* spp. compared to conventional conidia granules. The method of using cereal grains as a granular base and inoculating them with a solution containing conidia was already tried by Aregger in 1992. Recently, this method was successfully applied by Reinbacher et al. (2021), who planted and established a cover crop inoculated with *M. brunneum* on the site before the main crop. Trials on spraying procedures with *M. brunneum* conidia-containing solutions seem to be few so far. This may be due, among other things, to the fact that *M. brunneum* is more of an endophyte which primarily resides in the rhizosphere (Bruck 2010). Nevertheless, successful trials have been carried out in which the leaves or the trunk were colonized with the endophyte via a foliar spray application (Jaber and Enkerli 2017; Resquín-Romero et al. 2016). But the duration of colonization by foliar application was found to be rather short-lived in Resquín-Romero et al. (2016) attempts.

An important point, that was considered in most of the tests, was the durability of the final product. It turned out that especially the granules, beads or powders which were equipped with a nutrient layer, showed a greater longevity and the associated germination capacity compared to a liquid solution. This is due to the source of nutrition on the one hand and the drying process on the other, which ensures a very low level of residual moisture, which in turn slows down or even prevents the germination process. According to the results of La Cruz Quiroz et al. (2019) study on fungal endophyte-based biopesticides, the liquid products have a slightly longer shelf life, compared to dry products. The method of inoculating cereal grains and using them as granules has proved less successful, at least with regard to storage (Humbert et al. 2017). Over a period of 24 months, at a temperature of 2°C and depending on the strain used, there was a significant loss of germination and overall viability (Aregger 1992). A general statement can hardly be made, since the longevity of the conidia or the mycelium varies depending on the endophyte or even the strain within the species, even under optimal storage conditions (Gao et al. 2007; Przyklenk et al. 2017). This makes the commercialization process somewhat difficult, as each strain has to be researched for its own optimal formulation, and ultimately the site of application also plays an important role. Depending on the site of action and efficacy, whether in the rhizosphere or phyllosphere, whether as a plant fortifier or insecticide, it is important to use the correct application strategy (La Cruz Quiroz et al. 2019). Nevertheless, several products containing *M. brunneum* and other endophytes have already made it onto the global market, but the focus here is on biological plant protection rather than plant strengthening agents (CBC (Europe) S.r.l. 2019; Biocare 2021; Sullivan et al. 2020).

However, according to Murphy et al. (2018), there are still two problems that persist, one being the difficulty in designing a 100% reliable product formulation and the other being the consistency of the product, leading to poor use in agriculture and little confidence in FE inoculation strategies. To counteract this, the authors have developed a detailed master plan conceptually focused on the commercialization of endophytes (Murphy et al. 2018). An important point that can influence the commercialization and therefore must be sufficiently tested during the process is the fact that these are microbial biocontrol agents, which among other things can have a great influence on the microbial community in their place of destination (Köhl et al. 2019). Further information about abiotic factors can be found in the [physiology](#) section of this report. Interestingly, most of the trials looking at *M. brunneum* commercialization methods are aimed at its use as a bioinsecticide, i.e., against pests, rather than formulations related to plant fortification. However, this may also be related to the fact that research on plant fortifying or supporting *M. brunneum* isolates has only recently begun and is still in its infancy but is receiving brisk growth.

### Application in practice – options

As already explained in the previous two sections, there are different ways and techniques of using *M. brunneum* strains in the practical field. These are based on the respective application site (rhizosphere or phyllosphere) and accordingly on the respective mode of action.

As can be seen from the previous sections, the application can be a plant protection measure in which an insect pest is targeted, a plant strengthening application under stress conditions, or even a plant strengthening and plant protection measure at the same time. Basically, the field application (as mentioned in the beginning) is divided into two different application areas, on the one hand, the above-ground area of the plant (phyllosphere) where the foliage (phylloplane), stem and flowers/fruits are located and on the other hand, the subterranean area (rhizosphere) which is composed of the root system (rhizoplane), soil and the lower part of the hypocotyl.

#### Above-ground application

The implementation in the phyllosphere is limited to the type of spray application. As listed in Table 1, *M. brunneum* isolates have already been used several times in trials with foliar spray techniques (Jaber and Enkerli 2016; 2017; Raya-Díaz et al. 2017a; 2017b; Resquín-Romero et al. 2016). Agents already approved on the world market, such as Taerain® Met52EC, TickEx EC® and GranMetWP, which contain *M. brunneum* as the active ingredient, are also used in spray application (EFSA 2012). The use of the spray application is primarily as an insecticide against insect pests on the leaf or stem surface but also possible on soil surface (EFSA 2012; Raya-Díaz et al. 2017a; Miranda-Fuentes et al. 2020). The results of the application may vary. For example, in the study by Resquín-Romero et al. (2016), only transient but effective colonization of the entire plant tissue was observed with a single spray application onto the leaf surface and a conidial suspension of  $1.0 \times 10^8 \text{ ml}^{-1}$ .



Moreover, the mortality rate of the target pest was 33-60%. However, spray application can also have positive effects on plant physiology, with conidia suspension densities of  $5.0 \times 10^6$  and  $5.0 \times 10^8$  conidia  $\text{ml}^{-1}$  showing improved plant growth parameters and reduced Fe chlorosis symptoms (Raya-Díaz et al. 2017a; Raya-Díaz et al. 2017b). The application in this case has taken place once on the soil. Jaber and Enkerli (2017), on the other hand, used a  $1.0 \times 10^7$  conidia  $\text{ml}^{-1}$  suspension as a single spray application, mainly on the leaves, resulting in high colonization by the endophyte in all plant parts (at different percentages). After 7 days, the first positive effects on plant growth parameters could already be observed. As a means of implementation can be used the classic common methods such as application via hand sprayer or via the field sprayer, both as soil directed spray, foliar application or the drenching of the entire plant (EFSA 2012).

#### Below-ground application

The application in the rhizosphere was the more preferred technique when it comes to the application of *M. brunneum*. Nevertheless, the methods that can be used are more diverse than for surface application. Encapsulated mycelium or conidia in granules or beads (Ríos-Moreno et al. 2016; Krell et al. 2018b; 2018e; 2018d) together with the seed treatment variant (Jaber 2018a; Gonzalez-Guzman et al. 2021; González-Guzmán et al. 2020b; 2020a; Clifton et al. 2018; Raya-Díaz et al. 2017b; Liao et al. 2014; Keyser et al. 2014) is the most common way to apply the FE in the rhizosphere. In addition, trials were conducted using infected insects as application media (Lahey et al. 2020) and roots directly drenched with a conidia solution (Jaber and Alananbeh 2018).

In addition, several granule-based products are already available on the European market, such as ATTRACAP® (Biocare 2021), Bio 1020®, Taenure® Met 52® and GranMet GR (EFSA 2012) which are used as plant protection agents. For applications in the rhizosphere, colonization of the rhizoplane of the plants occurred in most studies, as well as subsequent colonization of the hypocotyl and stem; leaves were colonized only in isolated exceptions (Table 1).

The large number of studies on rhizosphere application and the various soil applications on the market indicate that the fungal endophyte *M. brunneum* is a preferential soil-borne organism, which is also entomopathogenic against many soil-dwelling insect larvae. Accordingly, the below ground applications may be the more effective measures that work over a longer period of time and are more likely to be effective (with regard to plant promoting properties). Furthermore, direct application with the seed (seed treatment/coating) or additionally during sowing (granules/beads) may be more effective than spray application, as this would again require the use of additional equipment. The measure can be regarded as a prophylactic approach. In addition, there is less influence of abiotic factors that may affect colonization or survival of the FE. If there is an acute cause, such as an infestation of insect pests in the phyllosphere, spray application may be the more appropriate option as it gets to the site more quickly. The use of infected insects and the soaking of roots may only be useful for research purposes in order to gain further knowledge, but in practice these methods will most likely not be used for logistical and effectiveness reasons.

An interesting fact is that in all the products listed above the *M. brunneum* strain BIBESCO5 / F52 is used, which has already appeared several times in the literature, except for the product ATTRACAP®, in which the *M. brunneum* strain CB15-III was used, which was almost absent from the literature studied.

### 3.4. Overall restrictions

This thesis may be influenced by possible limitations. Research on the fungal endophyte *M. brunneum* is still in its infancy, due to the fact that it has only been discovered for agricultural use for a few decades. It is clear in which areas more research has been done than in others, leading to a large number of results in one area (e.g., host containment) and relatively few results in another (e.g., nutrient transfer, abiotic factors).

Further difficulties were caused by the fact that *M. brunneum* has only been identified as an independent species of the PARB clade for a few years and was previously circulating under the general name *M. anisopliae* F52.

It has been difficult at times to search individual papers, articles and studies for relevant information, as the data available on *M. anisopliae* is many times higher than that on *M. brunneum*. There may be many more studies on, in particular, the F52 isolate, but these have been published under the species *M. anisopliae* and so would require a further specific search, which again is extremely time consuming and labor intensive.

Another limitation is the fact that paywalls do not allow sufficient information to be extracted from a study, which in some respects reduces the results.

## 4. Conclusions

The main objective of this thesis was to summarize and discuss the knowledge available to date on all factors useful for the use of the fungal endophyte *M. brunneum* as a multifunctional plant promoting agent. This article aims to provide an overview of the current state of scientific knowledge on this topic. Furthermore, the paper shall indicate in which areas knowledge gaps exist and further research is necessary to create a detailed portfolio in order to sustainably optimize and commercialize biological crop protection in the future.

The results of this thesis show that the FE *M. brunneum* has great potential to be used in the coming decades as a multifunctional plant promoter, thus as a self-contained biostimulant or as an additive to other biostimulants, both in areas of agriculture and horticulture in many regions of the world. As an entomopathogenic generalist capable of successfully infecting and killing a wide range of soil-borne insect larvae (from the order: Coleoptera, Lepidoptera, Diptera, Hemiptera, Orthoptera), it has long been considered a biological mycoinsecticide. In addition, it has the ability to colonize the rhizosphere, rhizoplane and phylloplane of various economically important crops and to carry out successful endophytism at these sites. This symbiosis results not only in possible protection against plant pathogenic insects but also in plant strengthening properties. There is already initial and verified evidence suggesting that colonization by *M. brunneum* can lead to growth promoting actions under both nutrient-poor and nutrient-rich conditions. In addition, there is evidence of exchange of essential macro and micronutrients and photosynthetic products between the endophyte and host, as well as tripartite associations with involved insect cadavers or other organic matter.

In particular, the ability to induce organic acids in the rhizosphere of plant roots to lower the pH in calcareous soils, thus making minerals such as Fe, Mn, and Zn soluble and available to the plant, may be of tremendous importance for future agriculture.

Due to the fact that *M. brunneum* has been in use as a pesticide for several decades, there is already a lot of knowledge about commercialization methodology and detection. However, in order to develop even more efficient and economically valued products, it is important to conduct intensive research in this area. Explicit research and studies on the influence of abiotic factors have unfortunately only taken place in a few cases, which leads to a lack of knowledge in this area. Since even the individual isolates of a genus act very differently, it is difficult to refer to other endophytic fungal species.

Based on these conclusions, research institutes of science and the plant protection industry should consider this fungal endophyte for further research on its use as a multifunctional plant promoter.

In this context, it should be further investigated how *M. brunneum* reacts to abiotic factors, which mechanisms and functions are behind the nutrient transfer and to what extent the endophyte is able to activate or produce plant hormones to improve plant growth or activate plant defense mechanisms. In order to improve efficiency, it is also necessary to conduct more field experiments to explain and evaluate the relationships and effects to and on existing (esp. soilborne) organism communities in a natural environment.

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Moritz Lasse Brandt