Biological control of wireworms (*Agriotes* spp.) in potato cultivation using the entomopathogenic fungus *Metarhizium brunneum*: Factors that influence the effectiveness of mycoinsecticide formulations

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Summary

Agricultural production of field crops is one of the most important tasks to feed the growing human population. Since suitable cultivation areas are limited, crop protection plays a key role in the efficient use of existing fields by controlling weeds, microbial pathogens, viruses and animal pests. Among animal pests, insects cause by far the greatest economic losses by crop damage through infestation of above- or belowground plant parts. An example of soil-dwelling pest insects are wireworms, the larvae of click beetles (Coleoptera: Elateridae), which are considered important pests in potato cultivation worldwide. In Germany, wireworms of genus *Agriotes*, with the most widespread species *A. obscurus*, *A. lineatus* and *A. sputator*, pose a serious threat to farmers producing fresh potatoes. Since the European Commission has banned effective agrochemicals for plant protection due to their harmful effects on the environment, wireworm control in potato cultivation is limited. In search for sustainable alternatives in the integrated pest management, biological control of wireworms using entomopathogenic fungi seems promising. However, although pathogenicity of *Metarhizium brunneum* isolates against wireworms of the genus *Agriotes* has been confirmed and various mycoinsecticide formulations have been developed, the effectiveness in field use varied considerably and the exact causes are poorly understood. In the present dissertation, the wireworm control potential of two new formulations based on the entomopathogenic fungus *M. brunneum* was investigated in more detail. A granule (AgriMet-Granule) and a wettable powder (AgriMet-Dry Product) were tested for soil application during potato planting to reduce the respective wireworm population as part of an inoculative biocontrol strategy. Prior to the field trials, laboratory assays with the AgriMet-Granule showed that the required contact between infectious conidia and the larvae was likely to occur, since an attracting effect of the formulation on *A. obscurus*, *A. sputator* and *A. lineatus* was observed using a Y-Olfactometer. In addition to the olfactory preference to the AgriMet-Granule, the larvae accepted the carrier as food source, which emphasised the control potential of the granule formulation. The AgriMet-Dry Product did not contain any additional attractants and was expected to be effective by being evenly sprayed in the furrow of the potato ridge with the potato seed dressing. However, application of the AgriMet formulations during potato planting was limited by the compatibility of the formulated *M. brunneum* isolate with only one active ingredient of the fungicide seed dressings commonly used in potato cultivation. Another

shortcoming of the formulation properties of the mycoinsecticides tested was the loss of fungal viability after storage and shipment, which was observed in quality control tests. Despite these technical obstacles, the effectiveness of the AgriMet-Granule and the AgriMet-Dry Product was tested in a multi-year field trial (2018-2020) and a significant reduction of wireworm tuber damage was observed. However, the effectiveness was relatively low and varied between years. Adequate effectiveness of the desired inoculative biocontrol strategy required fungal proliferation at the target site, but this was not observed by determining the colony-forming units of *Metarhizium* spp. after application of the AgriMet formulations in the potato ridge. An important factor affecting fungal proliferation was the low soil temperature (< 17 °C) during application in spring. The insufficient rhizosphere competence of *M. brunneum* at low soil temperatures poses great challenges to the intended use of the mycoinsecticides at potato planting in April or May. In addition to this ecological problem, an intrinsic factor influencing the effectiveness was identified when testing the appropriate application rates of the formulations in standardised greenhouse and laboratory bioassays. Besides the high application rates required to protect potato tubers, it was found that the used *M. brunneum* isolate exhibited differences in virulence against *A. obscurus, A. sputator* and *A. lineatus*, with *A. obscurus* being the most sensitive. Considering mixed wireworm populations on a given infested area, the application of mycoinsecticides based on a single *M. brunneum* isolate with differences in virulence against the most important wireworm species seems unsuitable. Therefore, the combined treatment of two *Metarhizium* spp. isolates, each with a specific virulence profile, were tested to enhance the effectiveness in field use by targeting multiple wireworm species to the same extent. Although additive effectiveness against *A. obscurus* and *A. sputator* was observed with almost equal survival probabilities after the combined treatment, differences in the proliferation performance of the tested isolates pose challenges for subsequent formulations. The identified technical, ecological and intrinsic factors demonstrate the complexity of using the entomopathogenic fungus *M. brunneum* against wireworms in potato cultivation and must be considered in the future development of marketable mycoinsecticides. The commercial success of mycoinsecticides depends on the creation of a lethal potential at the target site against the most important wireworm species, which is why the application should be embedded in the holistic agronomic system to create suitable conditions for the fungal mode of action and thus effective wireworm control.

Zusammenfassung

Die landwirtschaftliche Produktion von Ackerbaukulturen ist eine der wichtigsten Aufgaben, um die wachsende Weltbevölkerung zu ernähren. Da die für den Ackerbau geeigneten Flächen begrenzt sind, spielt der Pflanzenschutz durch die Bekämpfung von Unkräutern, mikrobiellen Krankheitserregern, Viren und tierischen Schädlingen eine Schlüsselrolle bei der effizienten Nutzung der vorhandenen Felder. Unter den tierischen Schädlingen verursachen Insekten bei weitem die größten wirtschaftlichen Verluste, indem sie durch den Befall ober- oder unterirdischer Pflanzenteile den Ertrag oder die Ertragsqualität mindern. Ein Beispiel für bodenbewohnende Schadinsekten sind Drahtwürmer, die Larven von Schnellkäfern (Coleoptera: Elateridae), die weltweit als wichtige Schädlinge im Kartoffelanbau gelten. In Deutschland stellen Drahtwürmer der Gattung *Agriotes*, mit den am weitesten verbreiteten Arten *A. obscurus*, *A. lineatus* und *A. sputator*, eine ernsthafte Bedrohung für Landwirte dar, die Speisekartoffeln erzeugen. Da die Europäische Kommission wirksame Agrochemikalien für den Pflanzenschutz aufgrund ihrer schädlichen Auswirkungen auf die Umwelt verboten hat, ist die Drahtwurmbekämpfung im Kartoffelanbau nur begrenzt möglich. Auf der Suche nach nachhaltigen Alternativen im Rahmen des integrierten Pflanzenschutzes ist die biologische Bekämpfung von Drahtwürmern mit entomopathogenen Pilzen in der aktuellen Forschung sehr populär. Obwohl die Pathogenität von *Metarhizium brunneum*-Isolaten gegen Drahtwürmer der Gattung *Agriotes* bestätigt und verschiedene Mykoinsektizid-Formulierungen entwickelt wurden, ist die Wirksamkeit im Feldeinsatz sehr unterschiedlich und die genauen Ursachen sind kaum bekannt. In der vorliegenden Dissertation wurde das Drahtwurmbekämpfungspotenzial von zwei neuen Formulierungen auf der Grundlage des entomopathogenen Pilzes *M. brunneum* eingehender untersucht. Ein Granulat (AgriMet-Granulat) und ein lösbares Pulver (AgriMet-Trockenprodukt) wurden für die Ausbringung in den Boden während der Kartoffelpflanzung getestet, um die jeweilige Drahtwurmpopulation als Teil einer inokulativen Biokontrollstrategie zu reduzieren. Vor der Feldtestung zeigten Laborversuche mit dem AgriMet-Granulat, dass der erforderliche Kontakt zwischen den infektiösen Konidien und den Larven wahrscheinlich zustande kommt, da mit Hilfe eines Y-Olfactometer eine anziehende Wirkung der Formulierung auf *A. obscurus*, *A. sputator* und *A. lineatus* beobachtet wurde. Neben der olfaktorischen Präferenz für das AgriMet-Granulat akzeptierten die Larven den Träger als Nahrungsquelle, was das

Bekämpfungspotenzial der Granulat-Formulierung unterstrich. Das AgriMet-Trockenprodukt enthielt keine zusätzlichen Lockstoffe und sollte seine Wirkung über die gleichmäßige Verteilung in die Furche des Kartoffeldaches mit der Spritzung der Kartoffelbeize entfalten. Die Anwendung der AgriMet-Formulierungen während der Kartoffelpflanzung wurde jedoch durch die Kompatibilität des formulierten *M. brunneum*-Isolats mit nur einem Wirkstoff der im Kartoffelanbau üblichen Fungizid-Saatgutbeizmittel eingeschränkt. Ein weiteres Defizit in Bezug auf die Formulierungseigenschaften der getesteten Mykoinsektizide war ein Verlust der Lebensfähigkeit der Pilze nach der Lagerung oder dem Versand, der bei Qualitätskontrolltests festgestellt wurde. Trotz dieser technischen Hindernisse wurde die Wirksamkeit des AgriMet-Granulats und des AgriMet-Trockenprodukt in einem mehrjährigen Feldversuch (2018-2020) getestet, und es wurde eine signifikante Reduzierung der Drahtwurmknollenschäden beobachtet. Die Wirksamkeit war jedoch relativ gering und variierte zwischen den Versuchsjahren. Als Teil der angestrebten inokulativen Biokontrollstrategie war eine Pilzvermehrung am Zielort erforderlich, um das letale Potenzial für eine angemessene Wirksamkeit aufzubauen. Die Pilzvermehrung wurde durch die Bestimmung der koloniebildenden Einheiten von *Metarhizium* spp. nach der Anwendung der AgriMet-Formulierungen im Kartoffeldamm untersucht, jedoch nicht im ausreichendne Maß beobachte. Ein wichtiger Faktor, der die Pilzvermehrung und damit die Wirksamkeit beeinflusste, war die niedrige Bodentemperatur (< 17 °C) während der Ausbringung im Frühjahr. Die unzureichende Rhizosphären-Kompetenz von *M. brunneum* bei niedrigen Bodentemperaturen stellt eine große Herausforderung für den beabsichtigten Einsatz der Mykoinsektizide bei der Kartoffelpflanzung im April oder Mai dar. Zusätzlich zu diesem ökologischen Problem wurde bei der Prüfung der geeigneten Aufwandmengen der Formulierungen in standardisierten Gewächshaus- und Labor-Bioassays ein intrinsischer Faktor identifiziert, der die Wirksamkeit beeinflusst. Neben den hohen Aufwandmengen, die zum Schutz der Kartoffelknollen erforderlich sind, wurde festgestellt, dass das verwendete *M. brunneum*-Isolat Unterschiede in der Virulenz gegenüber *A. obscurus*, *A. sputator* und *A. lineatus* aufwies, wobei *A. obscurus* am empfindlichsten war. In Anbetracht gemischter Drahtwurmpopulationen auf landwirtschaftlich genutzten Flächen scheint die Anwendung von Mykoinsektiziden auf der Grundlage eines einzigen *M. brunneum*-Isolats mit Unterschieden in der Virulenz gegen die wichtigsten Drahtwurmarten ungeeignet zu sein. Daher wurde die kombinierte Behandlung von zwei

Metarhizium spp.-Isolaten, die jeweils ein spezifisches Virulenzprofil aufwiesen, getestet, um die Wirksamkeit im Feldeinsatz zu erhöhen, indem mehrere Drahtwurmarten in gleichem Maße abgetötet werden. Obwohl eine additive Wirksamkeit gegen *A. obscurus* und *A. sputator* mit nahezu gleichen Überlebenswahrscheinlichkeiten nach der kombinierten Behandlung beobachtet wurde, stellen Unterschiede in der Vermehrungsleistung der getesteten Isolate eine Herausforderung für die nachfolgende Formulierung dar. Die identifizierten technischen, ökologischen und intrinsischen Faktoren zeigen die Komplexität des Einsatzes des entomopathogenen Pilzes *M. brunneum* gegen Drahtwürmer im Kartoffelanbau und müssen bei der künftigen Entwicklung marktfähiger Mykoinsektizide berücksichtigt werden. Der kommerzielle Erfolg von Mykoinsektiziden hängt von der Schaffung eines letalen Potenzials am Zielort gegen die wichtigsten Drahtwurmarten ab, weshalb die Anwendung in das ganzheitliche agronomische System eingebettet sein sollte, um geeignete Bedingungen für die pilzliche Wirkungsweise und damit eine effektive Drahtwurmbekämpfung zu schaffen.

General Introduction

Wireworm (Coleoptera: Elateridae) damage in potato cultivation

Insects are the most ubiquitous and species-rich group of animals on this planet (Grimaldi et al. 2005). The majority of known insect species (> 600,000) feed on living plant material referred to as phytophagous, with almost every plant species being attacked (Oerke & Dehne 2004). Thus, phytophagous insects compete with humans for agricultural resources, and resulting crop damage is an important economic problem worldwide, as it severely affects agronomic productivity, e.g., in potato cultivation (Douglas 2018).

The Food and Agriculture Organization (FAO) highlighted the potato (*Solanum tuberosum*) as the most important non-grain food crop for the growing human population. Germany ranked 7th in world potato production in 2007 with an output of 11.6 million tons (Mackay 2009). These potatoes are cultivated for a variety of uses, with the largest share of approximate 5 million tons for direct consumption as fresh potato (Hambloch & Rampold 2020). However, Willersinn et al. (2015) estimated that approximately one quarter of the fresh potato production is lost because they do not meet the high quality standards demanded by consumers. They revealed that 75 percent of these quality losses are caused by storage properties and stringent potato quality requirements from retailers based on meticulous consumer preferences. Therefore, the feeding damage to potato tubers by phytophagous generalist insects, like wireworms, has become an increasing problem for potato growers (Parker & Howard 2001).

Wireworms are the larval stage of click beetles (Coleoptera: Elateridae) and a serious agricultural pest, especially in potato cultivation (Traugott et al. 2008). The holes produced by their feeding activity are shown in Figure 1 and reduce potato quality rather than yield (Vernon & Van Herk 2013b). Approximately 39 wireworm species from 21 genera of Elateridae are known as pests in potatoes worldwide (Jansson & Seal 1994). In Germany, the genus *Agriotes*, with the most widespread species *A. obscurus*, *A. sputator* and *A. lineatus*, is especially important in potato cultivation, where different species can occur individually or coexist (Ritter & Richter 2013). The respective level of infestation on a given field is variable and the distribution of wireworms is often patchy, based on food availability and oviposition (Seal et al. 1997, Blackshaw & Vernon 2008, Benefer et al. 2010). During their 3-5 years of development in the soil, the

shiny, golden-brown *Agriotes* larvae reach a maximum length of 30 mm, depending on the species, while their width does not exceed 6 mm (Subklew 1935, Miles 1942, Klausnitzer 1994, Furlan 1998). Due to their long larval development, wireworms can cause a problem for other crops such as wheat (Vernon 2005), which is included in the crop rotation of potatoes (Myers et al. 2008). Under the mentioned circumstances, the control of wireworms on agricultural land is of crucial importance to increase the agronomic productivity.

Figure 1: Potato tuber damage (A, B) caused by the wireworm species *Agriotes obscurus* (C).

An established method in agricultural practice to control or rather reduce harmful organisms is the use of pesticides (Oerke 2006). The European Commission defines a pesticide as any substance or a mixture of substances that prevents, destroys, or controls a harmful organism (pest) or disease, or protect plants or plant products during production, storage and transport. Pesticides intended exclusively for the protection of crops are referred to as plant protection products (EC 2021). Plant protection products based on the neonicotinoid Thiamethoxam or the pyrazole Fipronil were effective tools to control wireworms in the past (Vernon et al. 2013a). The insecticidal bait Goldor Bait® (BASF) contained the neurotoxin Fipronil causing a lethal over-excitation of the larvae after direct contact (Heger et al. 2010). However, the European Food Safety Authority assessed high risks for bees by Fipronil in several insecticide indications (EFSA 2013a). This prompted the European Commission to restrict the use of Fipronil as pesticide against wireworms through Regulation (EU) No 781/2013 in 2013. The harmful effect on honeybees was also the rationale for the European Union Regulation (EU) No 485/2013 in 2013, which restricted the use of neonicotinoids like Thiamethoxam (EFSA 2013b). Due to the ban of these effective, but ecological harmful

agrochemicals for the control of wireworms in potato cultivation, research focused on integrated pest management strategies against the soil-dwelling larvae (Furlan 2005).

Box 1 Glossary

The purpose of a plant protection product is the control of one or more pests including insects, fungi or weeds to increase agronomic productivity. The control success of pests or rather the adequate protection of yield/yield quality is the justification for the use of any pesticide (Anonymous 2017). However, the terminology used for describing the control success is sometimes ambiguous and misleading, because specific terms are often used synonymously in a wrong context. Uniform terminology is important for comparing the results of different studies since it includes the underlying evaluation criteria of a given pesticide. In the present thesis, the control success related terms (i) effectiveness, (ii) efficiency and (iii) efficacy are defined based on the approval-relevant EPPO Guideline PP 1/214 (4) as follows:

(i) **Effectiveness**: The capability of successfully controlling a certain pest. It provides information on the relation of the achieved to the desired goal.

(ii) **Efficiency**: The quality of successfully controlling a certain pest. It provides information on the relation of the required resources used achieving the desired goal.

(iii) **Efficacy**: The net result of positive (effectiveness) and negative effects (phytotoxicity, development of resistance, non-target organism and human safety, ease of use, compatibility with cultural practices) resulting in an overall agricultural benefit.

Integrated pest management in wireworm control

Integrated pest management (IPM) is an ecosystem-based strategy with the goal to suppress pest populations by combining multiple management approaches for greater efficacy. In this context, selective measures should always be the first choice (Ehler 2006). The main foundation of IPM is based on crop-specific cultivation measures such as crop rotation or the choice of a resistant variety to prevent pest infestation. The crop rotation in potato cultivation consists of a grass clover mixture (Paffrath et al. 2003). Since click beetles prefer a dense vegetation cover for oviposition to avoid dehydration of their 20-200 eggs (Traugott et al. 2015), it seems obvious that grass cover promotes pest reproduction (Keiser et al. 2012). Therefore, Schepl & Paffrath (2005) investigated the control potential of the crop rotation to prevent wireworm infestation in advance. However, a longer period between grass clover and potatoes only slightly reduced tuber damage. Neuhoff et al. (2007) suggested that an early harvest may reduce the harm to tubers as the wireworm damage increases with the progression of the vegetation period.

Since only certain potato varieties are able to realise maximum yield also with early harvest conditions (EUROPLANT Pflanzenzucht GmbH 2020), this approach is not suitable for practical use. Avoiding planting of potatoes in an infested area might be the most effective cultivation method to prevent tuber damage, but reliable methods for wireworm risk assessment are still under investigation (Furlan et al. 2020). The IPM's second line of defence are mechanical or physical measures, e.g., soil tillage. Although a frequent and high intensity of deep ploughing showed an effect against sensitive larval stages of wireworms (Seal et al. 1992), this mechanical measure has a negative impact on the entire cultivation management (Derpsch 2008, Triplett & Dick 2008, Alcántara et al. 2016). As a further physical control measure, the so-called "flooding" was investigated under laboratory conditions by Van Herk & Vernon (2006) with promising results depending on soil salinity and soil temperature. The aim was to cover the wireworm habitat completely with water in order to drown the larvae (Lane & Jones 1936). However, the practical implementation is often problematic, not only because of the high cost for the required water (Sauer et al. 2010), but also because of the induced negative impact of anaerobic conditions on flora, fauna (Kozlowski 1984) and soil microbiota (Unger et al. 2009). Another idea, tested by Furlan et al. (2004), was based on the biofumigant potential of *Brassicaceae* against wireworms. The incorporation of formulated defatted seed meals of mustard (*Brassica carinata*) into the soil led to an enzymatic degradation of glucosinolates and the formation of toxic isothiocyanates (Fahey et al. 2001). This method is not practiced because successful reduction of wireworms requires a high application rate and complex environmental factors must be considered (Furlan et al. 2010). Since agrochemicals are not available as a last inevitable alternative in IPM, biological control or rather biocontrol could serve as intervention measure using living organisms to suppress specific pest populations reducing their damage potential (Eilenberg et al. 2001). The benefits of biological control are justified based on the reduction of pesticide residues, human and non-target organism safety and preservation of biodiversity in managed ecosystems (Elliott et al. 1995). Since the aforementioned IPM measures in potato cultivation have failed in terms of effectiveness and/or practicability, research has shown great interest in the biological control of wireworms using biopesticides (Parker & Howard 2001). Biopesticides are defined as pest controlling agents containing living microbials (bacteria, fungi, and viruses), entomopathogenic nematodes, insect predators, insect pheromones or plant derived products (Kiewnick 2007). Especially biopesticides based on entomopathogenic fungi

are considered promising for the use against wireworms in potato cultivation (Parker & Howard 2001).

Entomopathogenic fungi as biocontrol agents against wireworms

Entomopathogenic fungi have the ability to attack, kill and digest living stages of insects. These soil-borne microorganisms occur naturally and are characterised by their filamentous growth and production of infectious spores, here called conidia (Samson et al. 2013). Entomopathogenic fungi have been applied as biocontrol agents across the globe and in various crops since the 1880s (Steinhaus 1956, De Faria & Wraight 2007). The most common used genera of entomopathogenic fungi to date are *Beauveria* and *Metarhizium* (Ascomycota: Hypocreales: Clavicipitaceae), which have already been successfully formulated as fungal biopesticide against insects, called mycoinsecticide (Meyling & Eilenberg 2007, De Faria & Wraight 2007). For instance, the mycoinsecticide Metapro (Andermatt Biocontrol Suisse AG) is registered for the control of garden chafer larvae (*Phyllopertha horticola*) in Switzerland and the Met52 granule (Novozymes France SAS) achieves the control of black vine weevil larvae (*Otiorhynchus sulcatus*) in Germany. Both mycoinsecticide formulations are based on *Metarhizium anisopliae* (Metchnikoff) Sorokin. In vitro studies confirmed the control potential of *Metarhizium brunneum* (Petch) against species of the wireworm genus *Agriotes* (Ansari et al. 2009, Eckard et al. 2014), which were killed through the initialisation of lethal infection (Goettel & Glare 2010). The infection cycle represents the mode of action and thus dictates the objectives developing control strategies based on entomopathogenic fungi (Senthil-Nathan 2015). The infection starts with the adhesion of hydrophobic fungal conidia to the insect cuticle (Holder & Keyhani 2005). For this purpose, conidia recognise specific proteins (Wang & St Leger 2007) and topographical signals on the cuticle surface (St Leger et al. 1991). Afterwards, conidia germinate on the cuticle under suitable temperatures (Luz & Fargues 1997) and form an appressorium, which initiates the physical penetration of epicuticle by an infection peg (Pekrul & Grula 1979). At the same time, cuticle-degrading enzymes like proteases, lipases and chitinases help to breach the multi-layered barrier and the penetration proceeds until the hemocoel is reached (Xiao et al. 2012). The fungal presence in the hemocoel triggers the humoral (enzymes, proteins) and/or cellular (phagocytosis, encapsulation) immune response of the insect (Neumann 2008). However, the regulatory host mechanisms are interfered due to a complex interaction with fungal metabolites

(Wang & St Leger 2006). In the further process, the fungus consumes nutrients from the host hemocoel for hyphal growth and sporulation as well as for the production of toxins like destruxins (Hajek & St Leger 1994). The interplay of tissue damaged through infection, consumption of nutrients, dehydration of cells and toxicity led to final insect death (Gillespie & Claydon 1989). Last, hyphae use less sclerotic regions of the integument to emerge on the outside of the insect to sporulate again under suitable conditions (Shah & Pell 2003). Although the basic of the infection cycle is understood, research on the potential use of *Metarhizium* spp. against wireworms is still under investigation due to the lack of a suitable formulation as mycoinsecticide (Aregger 1992, Humbert al. 2017, La Forgia & Verheggen 2019, Sharma et al. 2020).

Mycoinsecticide formulations of *Metarhizium* spp. against wireworms

Basically, mycoinsecticide formulations are intended to be an effective tool that preserve the organism, deliver it to the target site, and enhance biological activity once there. As Box 3 shows, the formulation of *Metarhizium* spp. as mycoinsecticide for the application in potato cultivation is a complex process where multiple requirements from the production to the desired action in the soil have to be met (Jones & Burges 1998). First, the fungus has to be stabilised during production, distribution and storage. Solid or liquid fermentation processes are often used for mass-production of entomopathogenic fungi, while the respective processes influence the viability of fungal propagules (Bradley et al. 1992). Second, a simple and user-friendly handling and application of the formulation is desirable. An application with commonly used devices, e.g., field sprayers or planting machines, makes a formulation accessible to a wider range of farmers (Bateman 2004). Third, the biocontrol agent must be protected from harmful environmental factors at the target site to ensure pathogenicity after the belowground application. Fourth, fungal infection of the pest must take place considering three distinct routes: (i) direct contact with conidia by granules or spray droplets; (ii) secondary pick-up of conidia based on fungal proliferation in the soil after application; (iii) horizontal transmission of the fungi from infected larvae (Bateman & Chapple 2001). Enhanced efficiency of the organism at the target site is conceivable through the reproduction of the biocontrol agent and/or a forced contact with the formulation by attraction (Burges 1998). Finally, the production/formulation costs and the associated price for a mycoinsecticide should be in relation to the control success, while being competitive with other insecticides. A successful introduction of *Metarhizium* spp., formulated as mycoinsecticide for the use

in potato cultivation, requires a constant effectiveness against wireworms with a significant reduction of tuber damage (Zaki et al. 2020). In contrast to chemical active ingredients, the use of a living microorganism, e.g., an entomopathogenic fungus, with its own metabolism, growth and reproduction cycle is very complex and might be influenced by several soil ecosystem factors (Carlile et al. 2001, Nicot 2001). Moreover, the unique interaction between the fungal control agent and the soil-dwelling larvae could determine critical formulation properties.

Box 2 Glossary

In the development of a *Metarhizium*-based mycoinsecticide for the use against wireworms in potato cultivation, several stages with specific criteria must be considered to ensure satisfactory control success in field use.

Order	Factor	Stage	Criterion	Motivation
1	Technical	Production and Formulation	Stabilisation of *Metarhizium*	Preservation of fungal viability
2	Technical	Storage	Shelf life of fungal propagules in the formulated product	Flexible application timing without loss of biological activity
3	Technical	Application	Suitability for application with common devices	Acceptance of farmers
4	Technical	Application	Compatibility with fungicide seed dressings	Integration into agricultural practice
5	Ecological	Application	Protection of *Metarhizium* from harmful environmental factors	Creation of a lethal potential at the target site
6	Ecological	Post-Application	Contact and interaction with wireworms	Enhanced activity of *Metarhizium*
7	Intrinsic	Post-Application	Pathogenicity of *Metarhizium* against different wireworm species	Germany-wide use regardless of location
8	Intrinsic	Post-Application	High virulence of *Metarhizium* against different wireworm species	Fast control for timely protection of potato daughter tubers

Previous field trials by Kabaluk et al. (2005) and Brandl et al. (2017) with *Metarhizium*-based mycoinsecticides against wireworms in potato cultivation resulted in low and/or fluctuating effectiveness depending on the respective trial. To enable the use of *Metarhizium*-based mycoinsecticides as a biological control component in IPM strategies, further research is needed to identify the key factors that influence its effectiveness under field conditions.

Research Objectives

German potato growers currently have no effective tool or strategy within the IPM to protect their harvest from feeding damage by the agronomical important larval pest wireworms. The biological control approach using the entomopathogenic fungus *Metarhizium brunneum* seems to be promising. However, previous mycoinsecticide formulations achieved only insufficient control success and possible influencing factors have hardly been researched.

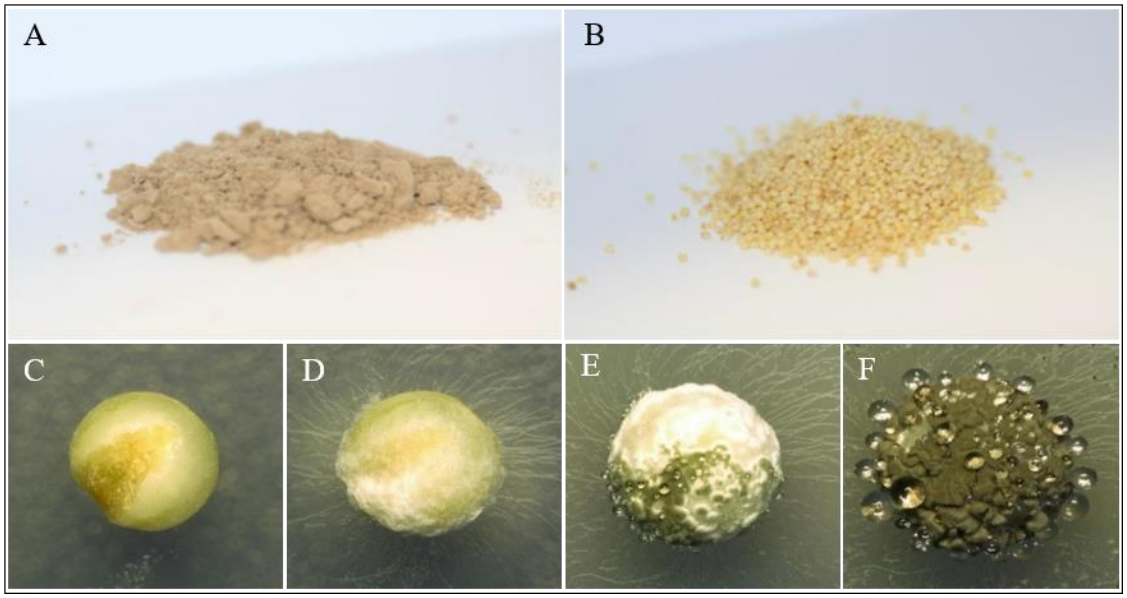

Figure 2: The mycoinsecticide formulations AgriMet-Dry Product (A) and AgriMet-Granule (C) based on the *Metarhizium brunneum* isolate JKI-BI-1450 (Photos from Lars Kretschmer). The AgriMet-Granule after 1 day (C), 4 days (D), 7 days (E) and 14 days (F) of incubation on 1.5 % water agar at 25 °C.

The main objective of my thesis was to evaluate the potential of two new mycoinsecticide formulations based on the entomopathogenic fungus *Metarhizium brunneum*, for a sustainable control of wireworms in potato cultivation in Germany. The focus of my research was on a soil granule and a wettable powder, both intended for the application during potato planting and shown in Figure 2. The effectiveness of the formulations was tested in practice-oriented field trials. In addition, potential influencing factors for the development of an effective control strategy were investigated in laboratory, greenhouse and field investigations. My thesis is organised into the following three chapters:

Chapter I - Interaction of the wireworm species *Agriotes obscurus*, *A. sputator* and *A. lineatus* with a new granule formulation of *Metarhizium brunneum*

To better understand the interactions of wireworms with the AgriMet-Granule after the application in the potato ridge, their larval olfactory perception and foraging behaviour were investigated in response to the granulated mycoinsecticide in a standardised laboratory bioassay. The attractiveness of autoclaved millet as substrate was examined using a Y-Olfactometer. Furthermore, the effect of *M. brunneum* colonisation on the attractiveness of the autoclaved millet was tested. The acceptance of wireworms for the coated millet as nutritive food source was assessed during a feed-choice bioassay considering different *M. brunneum* conidia concentrations.

Chapter II - The effectiveness of two new *Metarhizium brunneum* formulations against wireworms in potato cultivation

The effectiveness of the *M. brunneum* formulations AgriMet-Granule (soil granule) and AgriMet-Dry Product (wettable powder) were evaluated during three years of field trials under realistic potato growing conditions in Lower Saxony (Germany) with respect to wireworm tuber damage after harvest. In order to properly interpret the determined effectiveness, soil temperature, soil moisture, fungal concentration in soil, wireworm density and wireworm species composition were measured at the respective field site. In standardised greenhouse and laboratory experiments, the appropriate application rates of both formulations as well as the product qualities were assessed.

Chapter III - Combining two *Metarhizium* spp. isolates to enhance the effectiveness against the wireworm species *Agriotes obscurus*, *A. sputator* and *A. lineatus*

The potential of combining two *Metarhizium* spp. isolates to increase the virulence against wireworms of the genus *Agriotes* were investigated. To this end, the LC_{50} (lethal concentration) and LT_{50} (lethal time) of the single isolates and their mixture were compared with respect to their virulence against three wireworm species to evaluate whether an isolate mixture has the potential of widening the host range at the same virulence levels. Subsequently, the compatibility of the different isolates was analysed in a confrontation test where radial growth and sporulation were determined.

Chapter I

Interaction of the wireworm species *Agriotes obscurus*, *A. sputator* and *A. lineatus* with a new granule formulation of *Metarhizium brunneum*

Abstract

The attract-and-kill strategy is a promising approach in integrated pest management to increase the efficiency of plant protection products by pushing the required contact between target pest and active ingredient. Within the project AgriMet, a new soil granule of the entomopathogenic fungus *Metarhizium brunneum* was developed to control wireworms of the genus *Agriotes* (Coleoptera: Elateridae) in potato cultivation. The formulation is based on autoclaved millet as a substrate to lure the larvae to the infectious conidia on its surface. However, no data existed about the interactions of wireworms with the AgriMet-Granule. Here, I examined the behavioural response of the wireworm species *A. obscurus*, *A. sputator* and *A. lineatus* to the AgriMet-Granule using an olfactometric bioassay in the laboratory. The foraging behaviour of *A. obscurus* and *A. sputator*, with regard to the AgriMet-Granule, was tested with a feed-choice-experiment using a horizontal arena set-up. The tested wireworms significantly preferred autoclaved millet compared to the control. Fungal colonisation on the surface of the AgriMet-Granule did not influence their preference in general. However, differences were observed depending on the respective wireworm species and the two *Metarhizium* spp. isolates used. The feeding bioassay showed that the acceptance of wireworms for the AgriMet-Granule, with a low conidia concentration on its surface, was high. A high conidia concentration on the surface of the AgriMet-Granule led to a decreased acceptance that differed between the wireworm species. However, the survival analysis of wireworms isolated after the feed choice experiment indicated that the wireworms came in contact with the infectious conidia. Overall, the data suggests that autoclaved millet is a suitable substrate for the AgriMet-Granule. It attracts larvae of the genus *Agriotes* to the biocontrol agent using the formulated *M. brunneum* isolate.

Introduction

Volatile semiochemicals are organic metabolites and play an important role for ecological interactions of very different types of organisms at several trophic levels. Semiochemicals may convey intraspecific (pheromones) or interspecific (allelochemicals) information between microbials, plants and animals, modifying the behaviour of the recipient (Nordlund & Lewis 1976, Agelopoulos et al. 1999, Wyatt 2014). Insects use semiochemicals at all stages of development. For instance, to locate nutrient sources, to find sexual partners (Johnson & Gregory 2006) or to avoid environmental hazards caused by natural antagonists (Davis et al. 2013). In agricultural systems, the release of plant semiochemicals mediates attractive or repellent information for host plant identification by pest insects (Bruce et al. 2005). While attracting semiochemicals may promote the damage by phytophagous insects (Visser 1986, Johnson & Gregory 2006), repellent semiochemicals have been shown to interrupt the identification of their host plants (Hayes et al. 1994). Investigations on the attractive or repellent effects of semiochemicals to pest insects have provided crucial knowledge for effective control strategies in integrated pest management (Noldus 1989). One basic control concept is the behavioural manipulation of target pests using an artificial stimulus to lure it to an attractive source (Cook et al. 2007, Witzgall et al. 2010, Smart et al. 2014). For example, synthetic sex pheromones are used in selective traps to detect and monitor populations of click beetles for risk assessment (Tóth 2013). However, attractants can also be used in combination with plant protection products for pest control. The technique of combining an attractive source with a killing agent is known as “attract and kill” and has been regarded a promising approach for the integrated pest management (El-Sayed et al. 2009).

The “attract and kill” technique has the potential to increase the efficiency of plant protection products by pushing the required contact between active ingredient and target pest (Schumann et al. 2013). In particular, the application of plant protection products against soil-dwelling pests may benefit, as targeted control of phytophagous larvae hidden in the soil is very challenging (Hossain et al. 2007). Soil-dwelling pests in numerous agricultural crops are wireworms, the larval stage of click beetles (Coleoptera: Elaterida) (Parker & Howard 2001, Vernon & Van Herk 2013b). The genus *Agriotes* is the most widespread in Germany with regard to potato cultivation and their typical feeding damage reduces potato quality (Ritter & Richter 2013). Klingler (1957) postulated that the general orientation of wireworm towards the host plant is based on

carbon dioxide (CO_2). In this context, Brandl et al. (2017) evaluated an "attract and kill" strategy against wireworms using CO_2 emitting capsules. The capsule formulation contained the entomopathogenic fungi *Metarhizium brunneum* (Petch) as biocontrol agent. Brandl et al. (2017) tested the formulation in field trials with varying effectiveness depending on the application technique used. Based on the study of Rastogi et al. (2002), the effectiveness of the capsule formulation could be influenced by microbial, root and faunal respiration of CO_2 masking the gradient by the capsules to attract wireworms. Nevertheless, biological control of wireworms using *M. brunneum* seems promising and behavioural manipulations through attraction might be beneficial (La Forgia & Verheggen 2019). Since cereal-baited traps were found to be effective for wireworm monitoring (Parker 1996), raw baits should be considered for an "attract and kill" strategy against them.

For this purpose, a new granule formulation of *M. brunneum* was developed to control wireworms as pests in potato cultivation. The idea is that an autoclaved millet grain is coated with liquid fermented biomass of *M. brunneum* by fluidised-bed drying (Bernhardt et al. 2019). The use of millet is justified by its optimal size and stability for common application technology in potato cultivation. The biological activity of the granule should be provided by growth and sporulation out of the thin fungal layer on the surface of the millet grain after application in the soil (Stephan et al. 2020). There, contact of wireworms with the conidia should initialise the infection cycle of *M. brunneum* (Ortiz-Urquiza & Keyhani 2013), which may ultimately result in wireworm reduction (Shah & Pell 2003). Since *Agriotes* larvae are predominately herbivorous (Traugott et al. 2008), an attraction of wireworms by semiochemicals of the substrate millet is conceivable and could increase the likelihood of contact with the infectious conidia on the surface of the granule (El-Sayed et al. 2009). However, no information about an acceptance of wireworms for millet exists. Furthermore, it is unknown if wireworms can olfactorily perceive the natural antagonist *Metarhizium* spp. due to emission of typical semiochemicals. A repellent effect of *Metarhizium* against wireworms would greatly reduce the efficiency of the coated millet control strategy, because the larvae would avoid contact with the active ingredient.

In this study, the interaction of the wireworm species *Agriotes obscurus, A. sputator* and *A. lineatus* with a new granule formulation of the entomopathogenic fungus *M. brunneum* was investigated. First, the behavioural response of the mentioned wireworm species to autoclaved millet was assessed using a Y-Olfactometer. Additionally, the

fungal coating on the autoclaved millet were taken into account comparing two different isolates (*M. brunneum* isolate JKI-BI-1450, *M. robertsii* isolate JKI-BI-1441) to exclude a repellent effect of the used fungi. Second, the wireworms' urge to feed on the coated millet grains was examined after different incubation times. Last, the lethal potential of the granule was determined by assessing the mortality of the tested larvae after the feed choice experiment. This investigation aims to fill the gap on the behavioural response and the feeding behaviour of different wireworm species to a millet-based mycoinsecticide, which is crucial for the optimization of future control strategies against them.

Material and Methods

Wireworm breeding for the bioassays

Larvae of the species *Agriotes obscurus*, *A. sputator* and *A. lineatus* emerged from the laboratory breeding at the JKI Institute for Plant Protection in Field Crops and Grassland (Braunschweig, Germany). Click beetles of the respective species were collected in Wohld (52°18’11.0"N 10°41’11.6"E, Germany) and determined to species level using the identification key of Lohse (1979). Afterwards, beetles were transferred to buckets with soil and wheat (*Triticum aestivum*, Cultivar: Primus, Deutsche Saatgutveredelung AG, Lippstadt, Germany) at 20 °C for laying eggs following the protocol of Kölliker et al. (2009) with minor modifications. After about eight months, larvae originated from the breeding were removed and stored in plastic boxes (18.3 x 13.6 x 6.4 cm, Baumann Saatzuchtbedarf, Waldenburg, Germany) with a moist paper towel at 5 °C until use. To ensure healthy and vital wireworms, the larvae were transferred into soil at 15 °C for acclimatisation and fed with *Triticum aestivum* seeds (Cultivar: Primus, Deutsche Saatgutveredelung AG, Lippstadt, Germany) fourteen days before the start of the experiment.

AgriMet-Granule

The AgriMet-Granule is an autoclaved millet grain coated with the *Metarhizium brunneum* isolate JKI-BI-1450, isolated in 2016 from an infected *A. lineatus* beetle in Germany. For mass production of the fungus material, a liquid fermentation was conducted and the biomass including submerged spores, hyphae and secondary metabolites was coated with the help of a fluidised bed dryer on autoclaved millet grains of the species *Setaria italica* (Alnatura Produktions- und Handels GmbH, Darmstadt, Germany). To investigate the influence of the formulated *Metarhizium* isolate, a comparable granule was produced using the *M. robertsii* isolate JKI-BI-1441 originated from an *Agriotes* sp. larva gathered during field surveys in Italy. Both granules were provided by Tanja Bernhardt (JKI Institute for Biological Control, Darmstadt, Germany) and completely dry, while 1 kg contained 4.5 g of dry fungal biomass. To ensure the functionality, storage at 5 °C and darkness was no longer than twenty weeks.

Olfactometric bioassay with the AgriMet-Granule

Y-glass tube for the olfactometric bioassay

The olfactory perception of the *Metarhizium brunneum* isolate JKI-BI-1450 and *M. robertsii* isolate JKI-BI-1441 grown on millet by the wireworm species *A. obscurus*, *A. sputator* and *A. lineatus* were examined using a Y-glass tube (W.O. Schmidt GmbH Laboratoriumsbedarf, Braunschweig, Germany) shown in Figure 3. The total length of the tube was 150 mm with an internal diameter of 35 mm. Parafilm was placed on each of the three openings to prevent the escape of the wireworms and the volatile organic compounds. The glass tube was filled with vermiculite (Floragard, Oldenburg, Germany) with a grain size of 2-3 mm. In order to obtain a homogenous substrate and to avoid clumping, the vermiculite was additionally sieved (1 mm mesh) before use. After that, 40 g vermiculite were moistened with 25 ml tap water for each glass tube to ensure adequate conditions for wireworm movement.

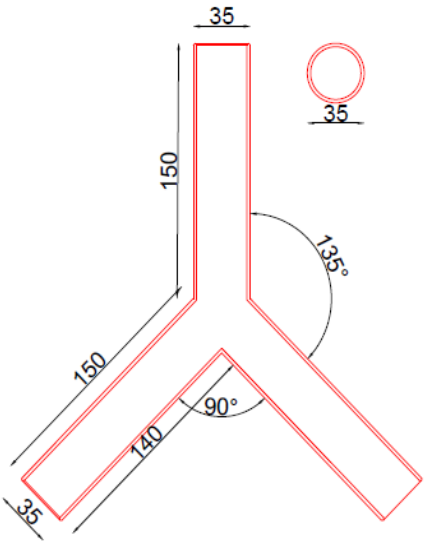

Figure 3: Schematic illustration of the Y-glass tube for the olfactrometric bioassay about the behavioural response of wireworms to the AgriMet-Granule.

Experimental set-up of the olfactometric bioassay

The dual-choice bioassay consisted of the following treatments: (A) autoclaved millet – autoclaved millet, (B) JKI-BI-1450 – autoclaved millet, (C) JKI-BI-1450 – nothing, (D) JKI-BI-1441 – autoclaved millet, (E) JKI-BI-1441 – nothing. Treatments were carried out simultaneously with the wireworm species *A. obscurus*, *A. sputator and A. lineatus* which led to a total of fifteen glass tubes. Each glass tube was filled with the AgriMet-Granule of the corresponding fungal isolate, autoclaved millet or nothing opposite against each other in the two openings at the top. Prior to use, the AgriMet-Granule was incubated for 14 days at 25 °C on 1.5 % water agar to ensure fungal colonisation on the surface. The conidia concentration was determined as in the experimental set-up of the feeding bioassay and reached 8.41×10^7 conidia grain^{-1} (±SD 5.43×10^6) for Isolate JKI-BI-1450 and 2.35×10^8 conidia grain^{-1} (±SD 9.23×10^6) for isolate JKI-BI-1441. Aliquots weighing 1 g of the respective AgriMet-Granule or autoclaved millet were prepared and moistened with 500 µl autoclaved tap water 24 h before the experiment in order to

simulate the swelling in the soil. Wireworms were inserted at the opening at the bottom 20 min after bait insertion to ensure the distribution of volatile organic compounds. After 2 h at 20 °C in the dark, the location of wireworms was recorded. Only those larvae that reached at least the bottom end of one of the two sides connected to the openings at the top were used for the evaluation. No airflow was installed and the experiment was repeated until twenty-five larvae of each treatment had moved and made a decision.

Feeding bioassay with the AgriMet-Granule

Trial arena of the feeding bioassay

The urge of the wireworm species *A. obscurus* and *A. sputator* to feed on millet grains coated with the fungal isolate JKI-BI-1450 was examined by a choice experiment. For this purpose, Petri dish lids of two different manufacturers (Greiner Bio-One GmbH, Kremsmünster, Austria; Brand GmbH & Co. KG, Wertheim, Germany) were used in order to create a completely closed arena with a diameter of 15 cm and a height of 1 cm. The low height was necessary to be able to observe the movement of the wireworms during trials. As substrate 1 kg of a 5:1 mixture of potting soil (Einheitserde Classic Pikiererde CL P, Gebrüder Patzer GmbH & Co. KG, Sinntal, Germany) and sand moistened with 300 ml tap water (49.2 % residual moisture) was used.

Experimental set-up of the feeding bioassay

The feed choice experiment was carried out placing the same amount (g) of the AgriMet-Granule and *Triticum aestivum* seeds (Cultivar: Primus, Deutsche Saatgutveredelung AG, Lippstadt, Germany) opposite against each other in the trial arena and releasing one wireworm in the middle for 48 h. After 24, 25, 26, 27, 28, 29 and 48 h the location of the wireworm was observed and assessed as follows: (W) wireworm in contact with wheat, (G) wireworm in contact with the AgriMet-Granule, (N) wireworm in no contact with wheat or the AgriMet-Granule. At the end, the presence of feeding marks on the AgriMet-Granule (A), wheat (B), both (C) or none (D) was examined with the help of a binocular (Carl Zeiss AG, Oberkochen, Germany). Wheat was used because wireworms accept it very well, while potatoes were not suitable for the test system due to the large size. The AgriMet-Granule was offered after five different incubation times to investigate the influence of the fungal colonisation on the surface of the millet. The coated millet was incubated for 1, 4, 7, 11 or 14 days at 25 °C on 1.5 % water agar and fungal growth was defined by the number of conidia grain^{-1} millet. Therefore, ten millet

grains of the respective incubation time were washed with 0.1 % Tween80® and conidia were removed by vortexing to determine the concentration of conidia grain^{-1} with a haemocytometer. The examination of the conidia concentration was repeated five times. Twenty trial arenas were used at the same time and stored at 20 °C in darkness. At the end of the experiment, tested wireworms were isolated in small cans with moist paper towel and incubated at 25 °C and darkness for six weeks to assess a possible infection with the *M. brunneum* isolate JKI-BI-1450. The larvae were feed with wheat (Cultivar: Primus, Deutsche Saatgutveredelung AG, Lippstadt, Germany) to avoid starving. Infected wireworms were identified based on fungal outgrowth from the cadaver and on morphological criteria of *Metarhizium* spp. (Zimmermann 2007).

Statistical analyses

Statistical analyses were done using the software R Studio (Version 1.4.1106) (RStudio Team 2020). Selection of the best-fitted model was based on the Akaike Information Criterion (AIC) (Burnham & Anderson 2002) after backward elimination of the full model. Subsequent model diagnostic was carried out by visual inspection of the QQ-Plot (sample quantile-theoretical quantile) and Residuals-Prediction-Plot to confirm normal distribution and variance homogeneity.

The influence of the incubation time (explanatory variable) on the conidia concentration on the surface of the AgriMet-Granule (target variable) was analysed with a Linear Model (LM). After log transformation of the target variable, normal distribution and variance homogeneity were met. If significant effects were identified in the analysis of variance (ANOVA, $\alpha = 0.05$), differences between the respective incubation times were examined using the post hoc Tukey HSD test ($\alpha = 0.05$) included in the *emmeans* R package (Lenth et al. 2018).

$$y = log(Conidia\ per\ Grain\ Granule) \sim Incubation\ Time + Repetition$$

To analyse the preference of the tested wireworm species for autoclaved millet or the AgriMet-Granule, a two-tailed Exact Binomial test ($\alpha = 0.05$) was performed for each comparison individually.

The feeding activity of the tested wireworm species was visualised with a three-way mosaic plot using the *vcd* R package (Meyer et al. 2020). Height and width of the boxes correspond to the frequency of feeding marks of the respective wireworm species in each variant. The colours shown in the mosaic plot refer to the Pearson residuals and thus

reflect patterns of dependency. Blue means that there are more observations in the box than would be expected under the null model, and red means that there are fewer observations than would have been expected. The intensity of the colour corresponds to the size of the Pearson residuals.

The visits of the tested wireworm species to the AgriMet-Granule or wheat were expressed as proportion of the seven possible observation time points. The influence of the incubation time of the AgriMet-Granule (explanatory variable) on the visits (target variable) was analysed by using a Generalised Linear Model (GLM, binominal distribution). Global effects were determined with an analysis of deviance. The pairwise comparisons were conducted as described above.

$$y = Proportion\ Visit \sim Incubation\ Time\ Granule + Repetition$$

Kaplan-Meier-Analysis was used to determine the survival probability of the tested *A. obscurus* and *A. sputator* larvae over time subsequent to the feed-choice-experiment. Survival curves over time were created using the *survival* R package (Therneau 2021) and compared with the log-rank test for global differences. To detect significant differences between the five variants within a wireworm species a pairwise comparison of survival curves based on the Bonferroni method was carried out using the *survminer* R package (Kassambara et al. 2021). Mycosis of wireworms was recorded as an "event". If no event was observed by the end of the study, the total survival time could not be accurately determined and was censored.

All graphs except the mosaic plot were created with the R packages *ggplot2* (Wickham 2016), *ggpubr* (Kassambara 2020), *RColorBrewer* (Neuwirth 2014), and *multcompView* (Graves et al. 2019).

Results

Conidia per grain AgriMet-Granule

The conidia concentration on the surface of the AgriMet-Granule coated with *Metarhizium brunneum* isolate JKI-BI-1450 was determined by the number of conidia grain^{-1} AgriMet-Granule and is presented in Table 1. After one day of incubation, no fungal outgrowth was observed and 5.89x10^3 conidia grain^{-1} (±SD 5.06x10^2 conidia grain^{-1}) were detached. Three days later, the number of conidia grain^{-1} increased only slightly to 8.11x10^3 (±SD 6.98x10^2 conidia grain^{-1}), but *M. brunneum* started to grow and very fine hyphae could be observed. After seven days of incubation, the conidia concentration increased significant (Tukey HSD test, $p < 0.0001$) to 1.96x10^7 conidia grain^{-1} (±SD 1.68x10^6 conidia grain^{-1}). The fungal growth of *M. brunneum* on the surface of the AgriMet-Granule was clearly visible based to the white mycelium and the green conidia. The fungus continued to significantly grow (7-11 days: Tukey HSD test, $p < 0.0001$) on the surface of the AgriMet-Granule and reached and maximum of 7.42x10^7 conidia grain^{-1} (±SD 6.39x10^6 conidia grain^{-1}) after 14 days. At this point, the grain was completely covered with conidia of *M. brunneum*.

Table 1: Adjusted mean values of *M. brunneum* conida per grain AgriMet-Granule and standard deviation (±SD) after 1, 4, 7, 11 and 14 days of incubation on 1.5 % water agar at 25 °C and darkness. Adjusted mean values with the same letters are not significantly different based on LM (y = log(Conidia per Grain Granule) ~ Incubation Time + Repetition) and Tukey HSD test ($\alpha = 0.05$).

Incubation time [days]	**Number of conidia grain^{-1} AgriMet-Granule**		
	adjusted mean	±SD	sig
1	5.89x10^3	5.06x10^2	a
4	8.11x10^3	6.98x10^2	a
7	1.96x10^7	1.68x10^6	b
11	5.16x10^7	4.44x10^6	c
14	7.42x10^7	6.39x10^6	c

Olfactometric bioassay with the AgriMet-Granules

The behavioural response to autoclaved millet and/or the AgriMet-Granule examined with a Y-glass tube did not differ between *Agriotes obscurus*, *A. sputator* and *A. lineatus*. Figure 4 illustrates that neither the *Metarhizium brunneum* isolate JKI-BI-1450 nor the *M. robertsii* isolate JKI-BI-1441 had a repellent effect on the tested wireworm species. On the contrary, *A. obscurus* and *A. lineatus* were significantly attracted to the isolate JKI-BI-1441 compared to autoclaved millet (Exact-Binomial-Test, $p = 0.04$). In addition, both isolates were significantly preferred by all wireworm species when the

isolates were offered in comparison to nothing (Exact-Binomial-Test, $p < 0.05$). The simultaneous offer of the isolate JKI-BI-1450 and autoclaved millet resulted in a more or less even split between the twenty-five wireworms of the respective species tested (Exact-Binomial-Test, $p > 0.1$). Similar results were observed with autoclaved millet at both ends of the Y-glass tube. All tested wireworm species were significantly attracted to autoclaved millet compared to nothing (Exact-Binomial-Test, $p < 0.001$). Although the behavioural response was comparable between the species, they differed in their activity. The proportion of all tested wireworms who moved and made a decision was highest by *A. obscurus* in each comparison and ranged between 66-86 %. Larvae of *A. sputator* showed the lowest activity with a maximum of 60 %. The comparison between the isolate JKI-BI-1441 and nothing with *A. sputator* resulted in the lowest proportion of 41 %.

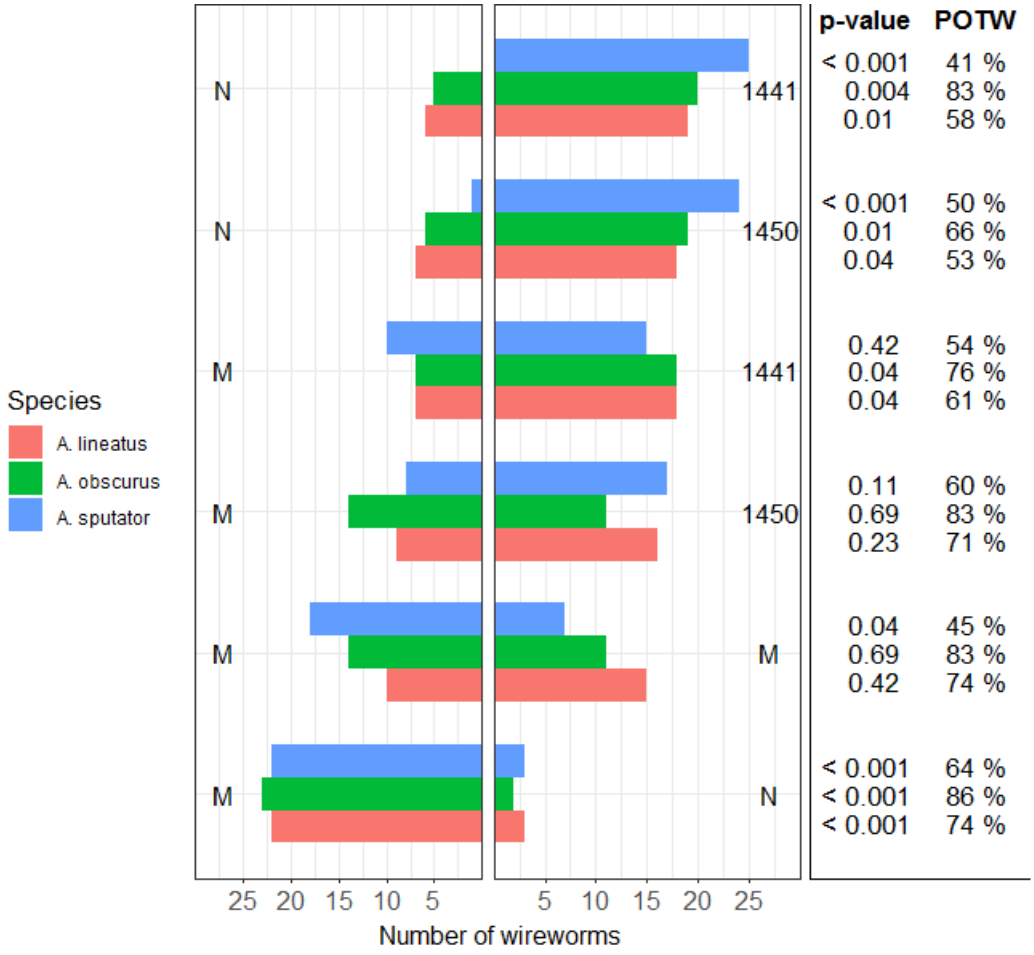

Figure 4: Number of wireworms reaching one side of the Y-glass tube after 2 h. The Y-glass tube was filled with different offers at the two ends (M = autoclaved millet, 1450 = *M. brunneum* isolate JKI-BI-1450 grown on autoclaved millet, 1441 = *M. robertsii* isolate JKI-BI-1441 grown on autoclaved millet, N = nothing). The colours of bars indicate the wireworm species *Agriotes lineatus* (red), *A. obscurus* (green) and *A. sputator* (blue). The *p*-values are based on a two-tailed Exact Binomial test ($\alpha = 0.05$) and are shown on the right as well as the proportion of all tested wireworms who moved and made a decision (POTW).

Feeding bioassay with the AgriMet-Granule

A feed choice experiment was carried out to investigate the urge of *Agriotes obscurus* and *A. sputator* to feed on the AgriMet-Granule coated with *Metarhizium brunneum* isolate JKI-BI-1450. The results of the feeding assay are shown in a mosaic plot (Figure 5). The AgriMet-Granule was well accpeted by both wireworm species after one day of incubation and the frequency of feeding marks on the AgriMet-Granule (A and C) was higher than expected. Compared to the other variants, wheat (B) was the least prefered in the 1 day variant. After 4 days of incubation of the AgriMet-Granule, the frequency of feeding marks on the AgriMet-Granule by *A. obscurus* decreased from 26 to 5 larvae, wherase the amount of *A. sputator* larvae remained constant at 19. In addition, the acceptance of wheat increased due to the raising amount of feeding marks. After 7 days of incubation, the AgriMet-Granule was no longer accpeted as food source by *A. obscurus* and the frequency of feeding marks on wheat was higher than expected with 35 larvae. However, *A. sputator* was still feeding on the AgriMet-Granule (18 larvae) and the frequency of feeding marks on wheat was smaler compared to *A. obscurus*. After an incubation time of 11 days, no feeding marks on the AgriMet-Granule could be assessed by both wireworm species. The frequency of no feeding marks on both offers (D) increased and was higher than expected with 16 larvae by *A. sputator*. After 14 days of incubation, wheat was the only food source that was accepted by both wireworm species, but the share of no feeding marks decreased again.

The assessment of visits at the AgriMet-Granule or wheat 24, 25, 26, 27, 28, 29 and 48 h after the wireworms were released in the trial area is shown in Figure 6. *Agriotes obscurus* and *A. sputator* moved between the offers and were observed at the AgriMet-Granule and wheat in the 1_day variant. After 4 days of incubation, the visits at the AgriMet-Granule of *A. obscurus* decreased significantly, whereas *A. sputator* was still monitored. With a few exceptions, *A. obscurus* was no longer seen at the AgriMet-Granule incubated for 7, 11 and 14 days. However, *A. sputator* was observed at the AgriMet-Granule incubated for 7 days, but only marginally after 11 or 14 days. The wireworms which could not be observed at the AgriMet-Granule in the variants mentioned were active due to the observation at wheat during all incubation times.

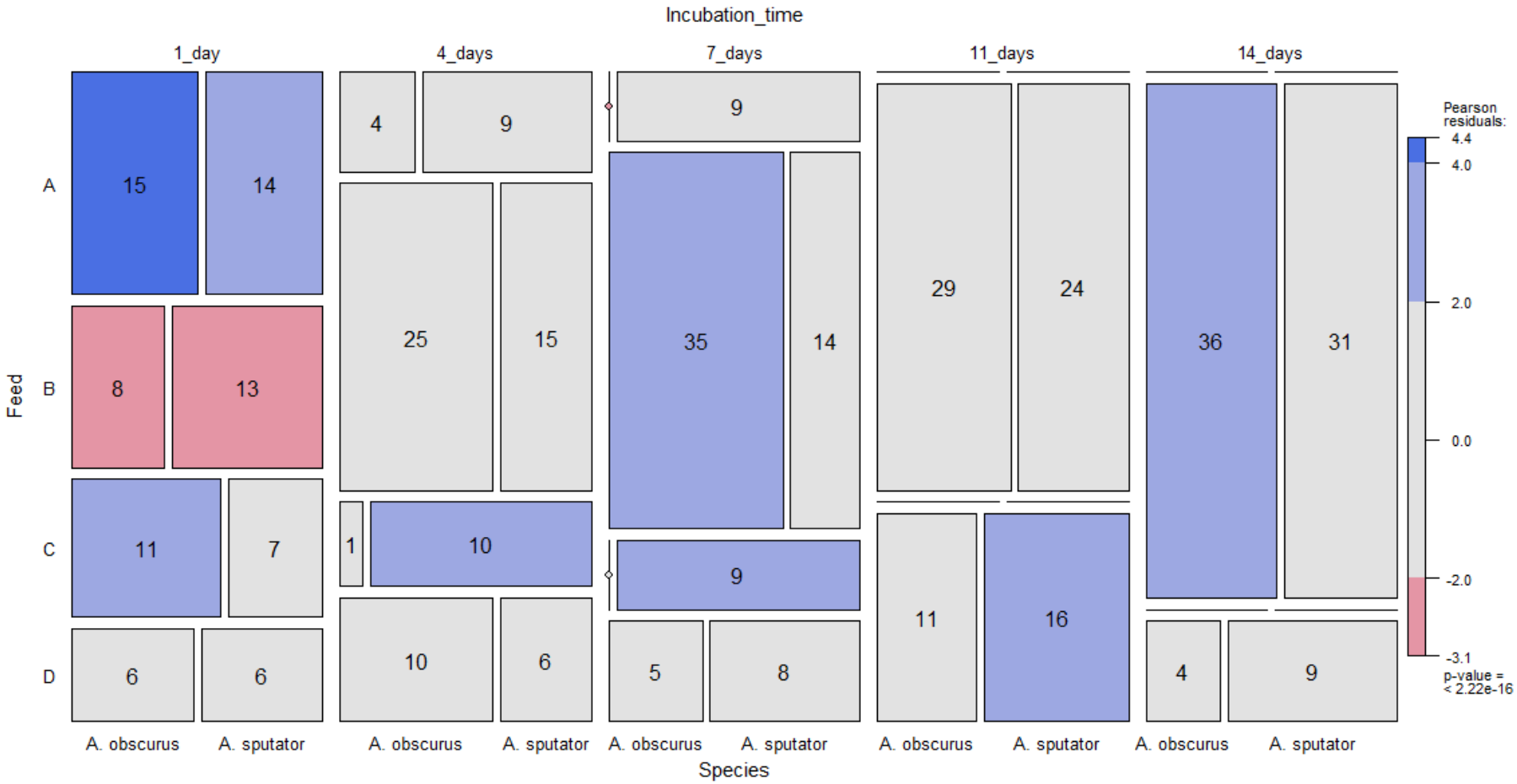

Figure 5: Three-way mosaic plot reflecting the presence of feeding marks (A: AgriMet-Granule, B: wheat , C: AgriMet-Granule and wheat , D: none) of the tested wireworm species (*A. obscurus*, *A. sputator*) based on different incubation times of the AgriMet-Granule (1_day, 4_days, 7_days, 11_days, 14_days). Height and width of the boxes correspond to the frequency of feeding marks of the respective wireworm species in each variant. The colours correspond to the size of the Pearson residuals. High deviations are presented deep blue or red (> 4, corresponds to $\alpha = 0.0001$), medium deviations light blue or red (< 4 and > 2, corresponds to $\alpha = 0.05$) and smal deviations grey (< 2).

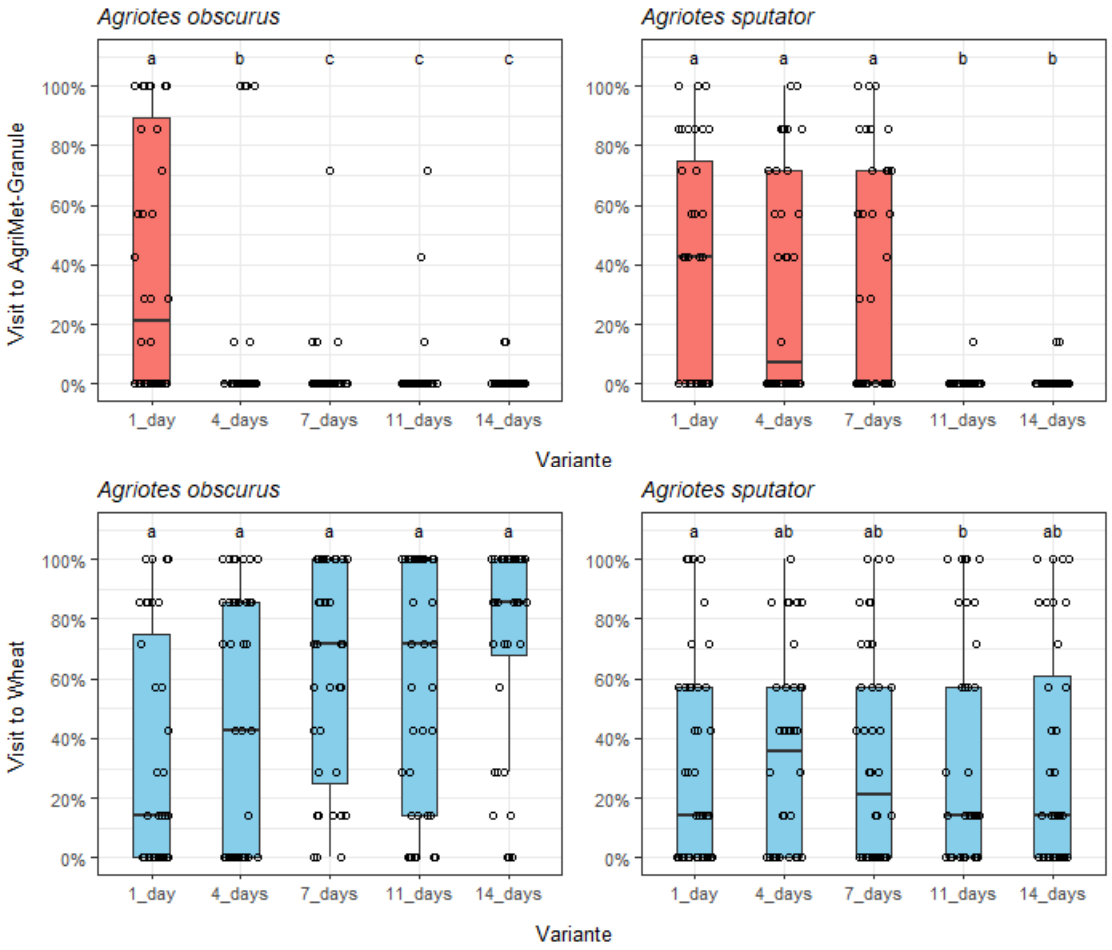

Figure 6: Boxplots of the visits (proportion of 7 observations in percent) to the AgriMet-Granule in red or wheat in blue of the wireworm species *Agriotes obscurus* and *A. sputator* in five different variants (1_day = 1 day incubated AgriMet-Granule against wheat, 4_days = 4 days incubated AgriMet-Granule against wheat, 7_days = 7 days incubated AgriMet-Granule against wheat, 11_days = 11 days incubated AgriMet-Granule against wheat, 14_days = 14 days incubated AgriMet-Granule against wheat). The boxes illustrate the median, 25 % and 75 % quantile. Jittered points represent each larva. Variants with the same letters within the respective species and food source are not significantly different. GLM (y = Proportion Visit ~ Incubation Time Granule + Repetition, family=binomial), Tukey HSD test (α = 0.05).

Wireworms that were not seen at the AgriMet-Granule at higher incubation times must have been there due to the overall survival probability shown in Figure 7. The incubation time of the AgriMet-Granule had a significant influence on the survival probability of *A. obscurus* isolated after the feeding assay (log-rank test, $p < 0.0001$). 20 of the 40 tested wireworms exposed to the AgriMet-Granule incubated for 7 days were killed by the *M. brunneum* isolate JKI-BI-1450. The survival probability was under 50 % after the entire observation time of 42 days. The incubation time of 11 and 14 days of the AgriMet-Granule led to 17 and 14 larvae with a mycosis with no significant difference to the 7 days variant (Bonferroni, $p > 0.05$). Four days of incubation of the AgriMet-Granule resulted in a significantly higher survival probability of approximate 75 % compared to seven days of incubation (Bonferroni, $p = 0.0096$). No mycosis was

observed at both wireworm species after 1 day of incubation of the AgriMet-Granule. However, the survival probability of *A. sputator* was not influenced by the incubation time of the AgriMet-Granule (log-rank test, $p = 0.083$). Only 3-6 larvae of *A. sputator* were killed by the *M. brunneum* Isolate JKI-BI-1450 42 days after the feeding assay and the survival probability was not under 80 % in any variant.

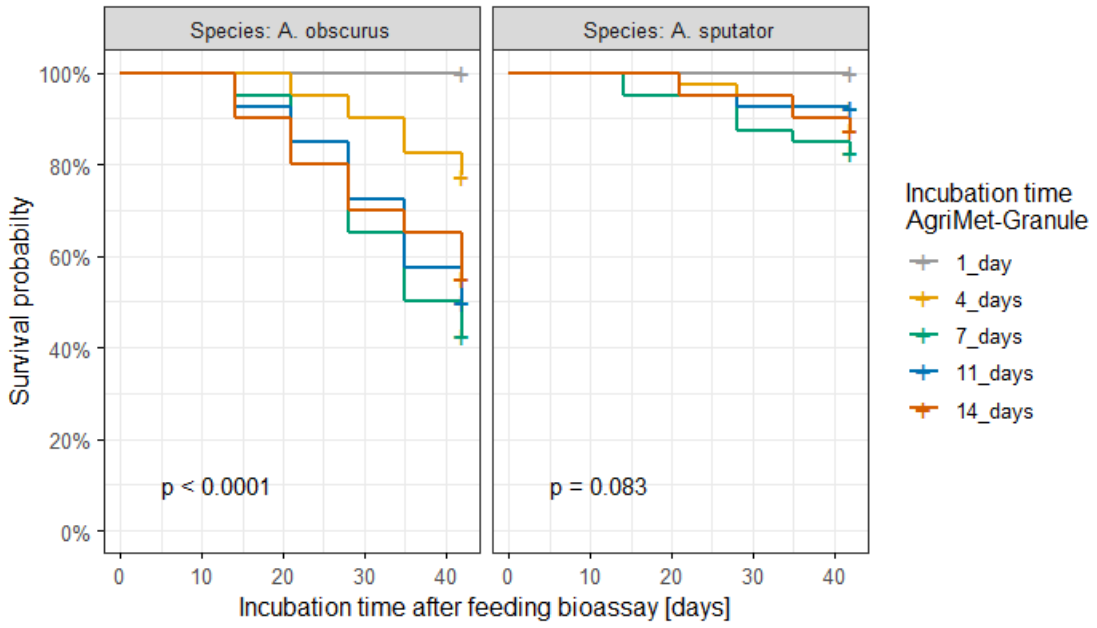

Figure 7: Overall survival probability in percent (Kaplan-Meier-Analysis) of the wireworm species *Agriotes obscurus* and *A. sputator* isolated after the feed-choice bioassay with wheat and the AgriMet-Granule with different incubation times and conidia concentrations (1_day = 5.89×10^3 conidia grain^{-1}; 4_days = 8.11×10^3 conidia grain^{-1}; 7_days = 1.96×10^7 conidia grain^{-1}; 11_days = 5.16×10^7 conidia grain^{-1}; 14_days = 7.42×10^7 conidia grain^{-1}). Larvae were incubated individually for 42 days under laboratory conditions at 25 °C and darkness in small cans filled with moist paper towel. The statistic of the shown *p*-values is based on the log-rank test ($\alpha = 0.05$) and indicates differences between the variants within the respective wireworm species.

Discussion

In the development of an efficient mycoinsecticide, a higher probability of contact between target pest and fungal biocontrol agent can be achieved by the "attract-and-kill" technique (Schumann et al. 2013, Vemmer et al. 2016). Using a bait as substrate for the formulation that is well accepted as nutrient source may serve this purpose. Autoclaved millet was used for the AgriMet-Granule intended for the control of wireworms in potato cultivation. The attractive effect of autoclaved millet was confirmed by a clear preference of the tested wireworm species *A. obscurus*, *A. sputator* and *A. lineatus* in the olfactometric bioassay compared to the control. Semiochemicals emitted by the autoclaved millet grains might be responsible for the preference of the larvae. The perception and orientation of soil-dwelling pests towards a host plant by a CO_2 gradient is widely reported for belowground arthropods (Johnson & Gregory 2006; Guerenstein & Hildebrand 2008). For example, larvae of the western corn rootworm (*Diabrotica virgifera*) rely mainly on CO_2 as attractive semiochemical during foraging (Bernklau & Bjostad 1998). Behavioural studies of Doane & Klingler (1978) showed that *A. obscurus* and *A. lineatus* perceive CO_2. Using electron microscopy, they found that clusters of sensilla on the labial and maxillary palps serve this perception. Since germination and associated mitochondrial metabolism of the millet grains is absent due to autoclaving, the production of CO_2 was reduced (Bewley & Black 2012). Thus, an important attractive volatile might have been missing. However, the exact role of CO_2 for arthropods is being discussed and probably only important for the initialisation of foraging behaviour (Johnson & Nielsen 2012). Even without the stimulus of CO_2, wireworms are able to orient themselves towards specific volatile semiochemicals. Root produced volatile aldehydes including Hexanal and (E)-Hex-2-enal extracted from barley attracted *A. sordidus* in a comparable olfactometric assay to the one used here (Barsics et al. 2017). A gas chromatography–mass spectrometry analysis (GCMS) of milled millet revealed, that the proportion of aldehydes was the highest with 21.81–34.21 % and derivates of Hexanal were the most abundant (Liu et al. 2012). Similar volatile aldehydes were also found in autoclaved seeds (Stotzky & Schenck 1976). Thus, Hexanal derivates are very likely the reason for the preference of wireworms to autoclaved millet in each comparison of the olfactometric bioassay performed in this study.

However, the AgriMet-Granule is coated with a thin layer of the entomopathogenic *Metarhizium brunneum*, which is intended to proliferate under suitable conditions

providing lethal potential against wireworms. Therefore, the wireworm acceptance with respect to autoclaved millet colonised with the natural antagonist *Metarhizium* spp. on the surface was investigated in a parallel bioassay. Comparing autoclaved millet with autoclaved millet completely covered with conidia of *M. brunneum* isolate JKI-BI-1450 or *M. robertsii* isolate JKI-BI-1441 clarified that the preference for autoclaved millet was not negatively influenced by fungal growth. These results are in contrast to the those of Mburu et al. (2009), who investigated the relationship between the virulence and repellency of *M. anisopliae* to the termite species *Macrotermes michaelseni* in a glass Y-tube. They reported a dose-related repellency of virulent isolates. In addition, they identified differences in the volatile blends of two *Metarhizium* isolates, which influenced the behavioural response of the tested termites (Mburu et al. 2011). Bojke et al. (2018) also confirmed the unique volatile blends of different *Metarhizium* spp. isolates. The olfactometric bioassay in this study showed an increased preference for the *M. robertsii* isolate JKI-BI-1441 over the *M. brunneum* isolate JKI-BI-1450. The occurrence of unique volatile blends of the isolates might be an explanation for this. The tested wireworms might have perceived the different blends, which resulted in a varied behavioural response towards the isolates. The exact volatile profile of the used isolates is yet unknown. Mburu et al. (2011) identified qualitative and quantitative differences in the volatile profile of two *Metarhizium* spp. isolates investigated. Although Furanone was rated as the most repellent substance against termites, they pointed out the importance of the composition and the relative amounts of substances of volatile blends. Degradation of millet during the assimilation of entomopathogenic fungi could lead to a quantitative increase of volatile aldehydes like Hexanal derivatives, thereby altering the relative amounts of the compounds. Nevertheless, the AgriMet-Granule can attract *A. obscurus, A. sputator* and *A. lineatus*. However, attraction is only the first part of the intended "attract-and-kill" strategy to control wireworms using the AgriMet-Granule. Prerequisite for the initialisation of the lethal infection cycle is the direct contact between infectious conidia of *Metarhizium* spp. and the larvae (Goettel & Glare 2010). The urge of wireworms to feed on the AgriMet-Granule can fulfil the required contact and is discussed in the following section.

Rizzo & Lehmhus (2014) demonstrated that wireworms are generalist herbivores. Therefore, the acceptance of autoclaved millet as food source was expected and confirmed in the feeding bioassay with *A. obscurus* and *A. sputator*. The larvae bite into the AgriMet-Granule with almost no fungal growth or with $8.11x10^3$ conidia $grain^{-1}$ on

the surface. However, the urge to feed on the AgriMet-Granule was no longer observed at concentrations of $5.16x10^7$ conidia $grain^{-1}$. Again, the olfactory perception of volatile blends could have influenced the behavioural response. Since wireworms can make use of a variety of different nutrient resources, specific volatiles are not required for host identification. Rather, the right composition and ratio of volatiles might be responsible for host recognition (Bruce & Pickett 2011). Thorpe et al. (1947) showed that plant juices or solutions containing carbohydrate, fatty or protein substances elicit the biting response of wireworms. In this context, the initial stimulus to bite into the millet grain might be masked by the volatile blend of the *M. brunneum* isolate used. Consequently, direct contact to the AgriMet-Granule through food ingestion by wireworms might only be ensured until proliferation of *M. brunneum* on the autoclaved millet reaches a certain conidia concentration.

The conidia concentration is of great importance for the intended control strategy, because it is closely linked to the lethal potential of *Metarhizium* spp. (Gabarty et al. 2014). The AgriMet-Granule with a concentration of $1.96x10^7$ conidia $grain^{-1}$ was accepted as food source by *A. obscures* and the survival probability was under 50 %. In contrast, mortality of *A. sputator* was relatively low with a survival probability of 83 %, indicating a selective effectiveness of *M. brunneum* isolate JKI-BI-1450 against different wireworm species. Surprisingly, the survival probability of *A. obscurus* also decreased significantly in the bioassay where no feeding scars on the AgriMet-Granule were observed. Larval mortality is based on contact with the AgriMet-Granule. Although no feeding scars were found, contact alone, and not necessarily feeding, appears to be sufficient for the desired lethal effect. Overall, under laboratory conditions, the AgriMet-Granule showed potential for wireworm control through its attracting effect, acceptance as nutrient source, and formation of a lethal conidia concentration of *M. brunneum* on the surface.

The results suggest the control potential of wireworms by the AgriMet-Granule based on laboratory set ups, but no further environmentally influencing factors were taken into account. However, potatoes are grown in different regions with different soil types, so the emission of further interfering semiochemicals is to be expected. The diffusivity of volatile semiochemicals in terms of distance and intensity depends on the physical properties of soil (Rolston & Møldrup 2012), which get influenced by the proportion of silt, clay, sand and organic matter (Aochi & Farmer 2005). Sand and organic matter increase the formation of soil pores for an optimised diffusion of gases, whereas a high

proportion of clay and loam compacts the soil (Boyle et al. 1989). The soil structure of a potato ridge is related to the region and sandy soils are preferred in Germany because of the capacity to warm up well and their loose structure (Paffrath et al. 2004). In this context, the volatile semiochemicals of the AgriMet-Granule may attract wireworms over a greater distance, which should be investigated in further experiments. However, there are ubiquitous bacteria and fungi in the soil, as well as roots or other plants that also emit volatile semiochemicals. The complex ecosystem in the soil influences the transport of intraspecific or interspecific information over a greater distance by changing or rather masking the individual volatile blend (Peñuelas et al. 2014). In future, several possible interfering semiochemicals should be tested at the same time with a four-way olfactometer arena. For this, autoclaved millet and the *Metarhizium* spp. isolates should be analysed for the exact composition of the volatile blends including the relative amounts of individual substances by GCMS analysis. Accurate information on the volatile semiochemicals involved in the interaction between wireworms and *Metarhizium* spp. can improve the control strategy by selecting highly virulent isolates with attractive blends. In addition, the AgriMet-Granule could be optimised. A special coating associated with volatile semiochemicals could enhance the attractiveness to ensure contact even under influence of other volatile stimuli.

In conclusion, the contact between the AgriMet-Granule and wireworms is assured even when there is no urge to feed at high conidia concentrations. Millet represents an attractive bait for the AgriMet-Granule formulation of *M. brunneum* intended for wireworm control. However, wireworms' preference is influenced by the respective *Metarhizum* spp. isolate. In addition, factors influencing the application environment were not considered during the standardised laboratory tests. It is unclear, to what extent the attracting effect of the AgriMet-Granule is still present among numerous other stimuli in the soil. Greenhouse and field application could provide further information to assess the full control potential of the AgriMet-Granule.

Chapter II

The effectiveness of two new *Metarhizium brunneum* formulations against wireworms in potato cultivation

Abstract

In the absence of officially approved agrochemicals for the use against wireworms (*Agriotes* spp.) in German potato cultivation, the entomopathogenic fungus genus *Metarhizium* has been regarded as promising biocontrol agent. However, previous mycoinsecticide formulations of *M. brunneum* could not achieve consistent effectiveness in field use. Within the scope of the project AgriMet, the effectiveness of two new formulations of *M. brunneum* was evaluated over three years in the field, greenhouse and laboratory. The application of a soil granule and/or a wettable powder (dry product) of *M. brunneum* during potato planting aimed to reduce potato tuber damage by controlling wireworms. During field trials in the Lower Saxony (Germany), the combination of both formulations led to an effectiveness between 1 % and 13.6 % compared to the untreated control. The single application of the AgriMet-Granule and the AgriMet-Dry Product was less successful. The standardised greenhouse set-up in pots with *A. obscurus* indicated that an application rate of at least 300 kg ha^{-1} of the AgriMet-Granule, respectively $1x10^{13}$ conidia ha^{-1} of the AgriMet-Dry Product was necessary to effectively reduce tuber damage (44-54 %), while surprisingly no larval mortality was recorded. The laboratory experiments showed, that only the AgriMet-Dry Product provided a lethal potential against wireworms, whereas the AgriMet-Granule showed deficits in terms of product quality. However, the effectiveness of the AgriMet-Dry Product against *A. obscurus*, *A. sputator* and *A. lineatus* resulted in significant differences in mortality indicating a species-specific virulence of the *M. brunneum* isolate used. Additional influencing factors became apparent through analysing multiple soil parameters at the respective field site such as temperature, moisture, colony-forming units of *Metarhizium* spp. and wireworm density, underlining the complexity of wireworm control using a microbial control agent. Especially the low soil temperatures during spring application pose a serious challenge to the development of an effective control strategy against wireworms.

Introduction

Wireworms are the larvae of click beetles (Coleoptera: Elateridae) and a major pest of a variety of crops including sugar beet, cereals and potatoes. Potatoes (*Solanum tuberosum*) are particularly affected by wireworms, as the soil-dwelling larvae reduce quality rather than yield and tuber damage is usually not detected until harvest (Parker & Howard 2001). Tuber damage consists of narrow tunnels in the tuber flesh as wireworms feed their way into potato tubers in search of food and moisture (Miles 1942, Parker & Howard 2001, Vernon & Van Herk 2013b, Traugott et al. 2015). The typical small holes on the surface provide entry points for the fungal plant pathogen *Rhizoctonia solani* and resulting "drycore" symptoms at the tubers cause serious economic losses (Keiser et al. 2012). Potato lots with significant wireworm damage are not marketable as fresh potatoes because consumers demand high quality without any blemish (Willersinn et al. 2015). Vernon & Van Herk (2013a) suggested that a threshold of less than 5 % of total yield damaged through wireworms would generally meet most industry standards, making it challenging to grow potatoes for direct food consumption. In Germany, especially the species *Agriotes obscurus*, *A. sputator* and *A. lineatus* are a problem to potato cultivation (Lehmhus 2019). Larvae of the genus *Agriotes* spend 3-5 years in soil before pupating (Schepl & Paffrath 2010) posing a harm to the entire crop rotation (Myers et al. 2008). Due to the threat of wireworms to agronomic productivity, the control of these larval pests is of economic importance (Parker & Howard 2001).

Efficient control of wireworms has historically been achieved by the use of insecticidal chemicals such as Fipronil or Thiametoxam (Ritter & Richter 2013). However, the European Commission restricted the approval of these agrochemicals in most indications due to harmful effects on bees assessed by the European Food Safety Authority (EFSA 2013a, EFSA 2013b). These regulations left German potato growers without fast-acting tools against the soil-dwelling pests (BVL 2021). Limited wireworm control options enhance the research of sustainable and environmentally friendly alternatives to profit from the socioeconomic benefits of reducing wireworms in potato cultivation (Benjamin et al. 2018). Different authors reviewed various approaches to reduce wireworms within the integrated pest management (IPM), but stated fluctuating control success (Parker & Howards 2001; Barsics et al. 2013). The authors postulated that entomopathogenic fungi are most promising for developing efficient control strategies against wireworms and efforts to use the species *Metarhizium brunneum* have been intensively pursued (Ericsson et al. 2007, Kabaluk & Ericsson 2007, Reddy et al.

2014, Brandl et al. 2017). The entomopathogenic fungal genus *Metarhizium* is well-studied and active against several agricultural pests such as locust or termites. The mode of action is based on the adhesion of the infectious units, the conidia, to the host cuticle initiating the lethal infection cycle (Zimmermann 2007). In vitro studies confirmed the lethal potential of *Metarhizium* spp. against wireworms of the genus *Agriotes*. The most efficient strains caused up to 73-100 % mortality approximately 8 weeks after exposure, depending on the wireworm species tested (Ansari et al. 2009, Eckard et al. 2014). In light of the pathogenicity of fungal isolates against the larval pests, formulation and commercialisation as biopesticides, more precisely mycoinsecticides, for the use in potato cultivation seems conceivable. However, the challenging task developing an effective mycoinsecticides is to mass-produce stable fungal propagules and to formulate them in most appropriate manner for soil application (Zaki et al. 2020).

The formulation of the biocontrol agent *M. brunneum* as mycoinsecticide is one of the most important factors for its commercial success, as it preserves the organism, deliver it to the target site while enhancing its biological activity (Jones & Burges 1998). Two types of formulations are suitable for the practical implementation: suspensions and dry products (Rhodes 1993). Since the moisture content of fungal conidia affects their storage characteristics and thus important product requirements (Moore et al. 1996), formulation of entomopathogenic fungi as dry products is preferred. Dry products include wettable powders and granules (Jones & Burges 1998). Latter contain a synthetic or organic carrier in which the fungus is incorporated or attached on the outer surface. The carrier can serve as nutrient source for proliferation and increase its persistence after application, while the distribution at the target site is determined by the size of the carrier in combination with the application rate (Goss et al. 1996). Wettable powders consist of powdered fungal propagules miscible in water, which acts as a carrier. The spray mist containing the fungal propagules is distributed evenly, able to hit target pests directly by droplets or colonise organic material in the application area (Silva et al. 2015). Both granules and wettable powders are suitable mycoinsecticide formulations for the application during potato planting, as the technical requirements at common potato-planting machines are given. Since the granule spreader and the spray device used for this purpose function independently of each other, individual and combined use of a granule and wettable powder is conceivable (GRIMME Landmaschinenfabrik GmBH & Co. KG, Damme, Germany).

Within the project AgriMet, a granule and a wettable powder based on the entomopathogenic fungus *M. brunneum* were developed for the application during potato planting. Both formulations are supposed to reduce tuber damage by the control of wireworms. Although the pathogenicity of the used isolate has been tested in the laboratory, no results about the effectiveness of the AgriMet formulations against wireworms exists so far. The AgriMet-Granule consists of an autoclaved millet grain as carrier, which provides the required hardness and grain size for production and application (Stephan et al. 2020). Furthermore, autoclaved millet attracted wireworms of the genus *Agriotes* in an olfactrometric bioassay and is therefore suitable as bait for the "attract-and-kill" technique (Chapter I). The AgriMet-Dry Product is a wettable powder of *M. brunneum* that is soluble in tap water for a spray application of a defined amount of conidia directly into the potato ridge. The biological activity of both the AgriMet-Granule and the AgriMet Dry product should be provided by the formation of infectious conidia after application in soil. Since the release of the entomopathogenic fungus aims at its proliferation and establishment for an extended period, but not permanently, the control strategy represents inoculative biocontrol (Eilenberg et al. 2001). The desired control strategy assumes that fungal reproduction is possible under the abiotic and biotic soil conditions in the potato ridge. However, although comparable granule mycoinsecticide formulations against wireworms have been tested in the field, there are no data relating soil temperature and moisture to the control success (Humbert et al. 2017, Brandl et al. 2017, Sharma et al. 2020). Thus, an important aspect for the interpretation of inoculative control strategies against wireworms is missing and may explain the fluctuating effectiveness in field use.

In this study, the effectiveness of two new formulations of *M. brunneum* against wireworms for the application in potato cultivation was evaluated in more detail. First, the reduction of tuber damage through the application of the AgriMet-Granule and AgriMet-Dry Product was investigated in field trials from 2018-2020. Standardised bioassays in the laboratory and greenhouse provided information on the required application rate and the host spectrum of the mycoinsecticide formulations. Additionally, parameters like soil temperature and water content, wireworm species composition and distribution at each field site were recorded. To verify fungal proliferation of the inoculative control strategy, the *Metarhizium* spp. concentration in the field soil (colony-forming units) was determined throughout the vegetation period after application of the formulations. In the end, the detailed evaluation of the tested

AgriMet formulations for wireworm control in potato cultivation revealed important factors influencing their effectiveness.

Material and Methods

Metarhizium brunneum formulations

AgriMet-Granule

The *Metarhizium brunneum* isolate JKI-BI-1450, isolated in 2016 from an infected *Agriotes lineatus* beetle in Germany, was produced by liquid fermentation. The biomass, including submerged spores, hyphae and metabolites, was coated on autoclaved millet of the species *Seteria italic* (Alnatura Produktions- und Handels GmbH, Darmstadt, Germany) using a fluidised bed dryer. 1 kg AgriMet-Granule contained 4.5 g of the dry fungal biomass. The AgriMet-Granule was dry and dust-free indicated by the Heubach test of Immenroth, which resulted in 0.1-0.2 mg of abrasion per 100 g AgriMet-Granule. Comparable thresholds of the Heubach test for seed dressings are 2 mg abrasion for wheat or 9 mg abrasion for sugar beet (personal communication, Immenroth, 2020, JKI Institute for Application Techniques in Plant Protection, Braunschweig, Germany). The fungus was still active due to the low thermal load during the production process. The AgriMet-Granule was provided by Tanja Bernhardt (JKI Institute for Biological Control, Darmstadt, Germany). To ensure the functionality, the granule was stored at 5 °C and in darkness for no longer than eight weeks.

AgriMet-Dry Product

The AgriMet-Dry Product was a powder soluble in tap water for a spray application. Mass-production of the *M. brunneum* isolate JKI-BI-1450 was carried out by liquid fermentation. The fungal biomass was dried by lyophilisation and ground with a hand mortar to receive a *M. brunneum* powder with a concentration of $1.25x10^9$ spores g^{-1} according to the manufacturer. The AgriMet-Dry Product was provided by Marianne Buschke (ABiTEP GmBH, Berlin, Germany).

ATTRACAP®

ATTRACAP® was commercially available under an emergency authorisation (2018, 2019, 2020) and used as reference product. The recommended application rate of 30 kg ha^{-1} contained $1.2x10^{10}$ conidia. For the production of ATTRACAP® (BIOCARE Biologische Schutzmittel GmbH, Einbeck, Germany), aerial conidia of the *M. brunneum* isolate CB15-III are encapsulated with *Saccharomyces cerevisiae* and maize starch in wet spherical calcium alginate beads. The beads are dried using fluidised-bed drying.

Under moist conditions the beads swell in the potato row and the yeast convert the encapsulated starch to CO_2 (Humbert et al. 2017).

Quality control of the Metarhizium brunneum formulations

With each experiment, a simultaneous quality control was carried out to ensure the functionality of the formulations. The AgriMet-Granule and ATTRACAP® was examined by placing twenty grains or capsules on each of five Petri dishes with 1.5 % water agar. After 14 days at 25 °C in the dark, the number of grains or capsules with fungal growth of *Metarhizium brunneum* on the surface were determined to calculate the rate of outgrowth in percent. In addition, the sporulation of the *M. brunneum* isolate JKI-BI-1450 coated on the AgriMet-Granule was tested in different field soils at 10°C, 15 °C, 20 °C and 25 °C. Therefore, twenty grains of the AgriMet-Granule were placed on each of three Petri dishes with sterile 1.5 % water agar (Control) or untreated soil from the experimental field sites Borg and Suettdorf. The field soil was the same as that used for the analysis of nutritive substances. It was previously sieved (1.6 mm mesh) and moistened to ensure a homogenous substrate and comparability to the control. After 14 days of incubation at the respective temperature, ten grains per Petri Dish were washed with 0.1 % Tween80® and conidia were removed by vortexing to determine the concentration of conidia grain^{-1} with a haemocytometer. This part of the quality control was repeated three times. The AgriMet-Dry Product was verified by spreading 100 µl of a dilution in tap water on each of five Petri dishes filled with 1.5 % malt peptone agar. The plates were incubated for 14 days at 25 °C in darkness and the number of colony-forming units (CFUs) was determined to calculate the CFUs g^{-1} AgriMet-Dry Product. The identification of *M. brunneum* for each formulation was based on morphological criteria (Zimmermann 2007).

Assessment of wireworm potato damage

Wireworm damage during field and greenhouse experiments was assessed following the European and Mediterranean Plant Protection Organization (EPPO) standard PP 1/46 on the conduct and evaluation of wireworm field trials. At harvest, 100 (field) or rather 10 (greenhouse) potato tubers per plot or pot were randomly sampled and categorised by classes based on the number of feeding holes (class 1: 0 holes, class 2: 1-2 holes, class 3: 3-5 holes, class 3: >5 holes). Feeding holes were defined as > 5 mm tunnels into the tuber. Assessment of wireworm potato damage in the field trials was conducted by the Lower Saxony Chamber of Agriculture.

Wireworm breeding for the greenhouse and laboratory experiments

For a species-specific distinction, wireworms used in the greenhouse and laboratory experiments were taken from a laboratory breeding. *Agriotes obscurus, A. sputator* and *A. lineatus* beetles were caught in Wohld (52°18’11.0"N 10°41’11.6"E, Germany) and determined to species level using the identification key of Lohse (1979). Following the protocol of Kölliker et al. (2009) with minor modifications, larvae emerged from the breeding established at the JKI Institute for Plant Protection in Field Crops and Grassland (Braunschweig, Germany) and were stored dark at 5 °C in plastic boxes (18.3 x 13.6 x 6.4 cm, Baumann Saatzuchtbedarf, Waldenburg, Germany) with moist paper towel. 14 days before the start of an experiment wireworms were transferred into soil and stored dark at 15 °C for acclimatisation and nutrition with *Triticum aestivum* seeds (Cultivar: Primus, Deutsche Saatgutveredelung AG, Lippstadt, Germany). The larval stages were determined by measurement of the head width and varied between 83-154 mm by *A. obscurus*, 68-120 mm by *A. sputator* and 74-163 mm by *A. lineatus*.

Effectiveness of the *Metarhizium brunneum* formulations in the field

Field sites

Field trials were conducted near Uelzen, Lower Saxony, the region with the highest potato cultivation in Germany (Suettdorf 53°00’39.7″N, 10°41’26.3″E; Borg 53°00’34.6″N, 10°44’33.0″E; 53°00’35.3″N, 10°44’30.2″E). Field sites were fallows next to potato growing areas and selected based on the occurrence of wireworms to ensure high pest infestation. Four weeks before the start of the experiments, the fallows were prepared by mechanical tillage.

Experimental design of field trials

The effectiveness of the *Metarhizium brunneum* formulations in the field was studied during three years (Suettdorf 2018, Borg 2019 and 2020) using a randomised complete block design with four replicates and an untreated control for each block. The tested treatments with the respective application rates are shown in Table 2. Individual plot size was four 10 m rows (30 m²) and edge effects were avoided by assessing potatoes from the middle two rows. Potatoes were cultivated according to agricultural practice and growth stages were determined with the BBCH code of Hack et al. (1993). The potato (*Solanum tuberosum*) variety Princess (Solana GmbH & Co.KG, Hamburg, Germany) was used in all trials with a planting density of 25 dt ha^{-1} and 0.33 m planting

distance within the row. Field trials were carried out in cooperation with the Lower Saxony Chamber of Agriculture.

Table 2: *Metarhizium brunneum* formulations, application rates, abbreviations and seed dressing in field trials near Uelzen from 2018 to 2020.

Year	Treatment	Application rate	Abbr.	Seed dressing
2018	ATTRACAP® (*Reference*)	30 kg ha^{-1}	A30	Moncut®
	Granule	30 kg ha^{-1}	G30	Moncut®
	Dry Product	$1x10^{11}$ spores ha^{-1}	DP1	Moncut®
	Granule + Dry Product	30 kg ha^{-1} + $1x10^{11}$ spores ha^{-1}	G30+DP1	Moncut®
	Granule + Dry Product	15 kg ha^{-1} + $0.5x10^{11}$ spores ha^{-1}	G15+DP0.5	Moncut®
2019	ATTRACAP® (*Reference*)	30 kg ha^{-1}	A30	Moncut®
	Granule	30 kg ha^{-1}	G30	Moncut®
	Granule	60 kg ha^{-1}	G60	Moncut®
	Dry Product	$1x10^{11}$ spores ha^{-1}	DP1	Moncut®
	Dry Product	$2x10^{11}$ spores ha^{-1}	DP2	Moncut®
	Granule + Dry Product	30 kg ha^{-1} + $1x10^{11}$ spores ha^{-1}	G30+DP1	Moncut®
2020	ATTRACAP® (*Reference*)	30 kg ha^{-1}	G30	Moncut®
	Granule	30 kg ha^{-1}	G30	Moncut®
	Granule	60 kg ha^{-1}	G60	Moncut®
	Dry Product	$1x10^{11}$ spores ha^{-1}	DP1	Moncut®
	Granule + Dry Product	30 kg ha^{-1} + $1x10^{11}$ spores ha^{-1}	G30+DP1	Moncut®

Application of Metarhizium brunneum formulations in the field

The AgriMet-Granule and ATTRACAP® were applied during potato planting with the AgroDos® 12-volt spreader (LEHNER Maschinenbau GmbH, Westerstetten, Germany) connected to a potato planting machine. The distributor triangle of the spreader was attached behind the planting machine blade to applicate in the furrow under the potato. To ensure protection against phytopathogenic fungi the potato seed dressing Moncut® 460 SC (Belchim Crop Protecion Deutschland GmbH, Burgdorf, Germany) was applied with an application rate of 0.2 L t^{-1} in 80 L ha^{-1} with the hollow cone nozzle ALBUZ® ATR brown (agrotop GmbH, Obertraubling, Germany). For the application of the AgriMet-Dry Product the corresponding amount was dissolved in tap water and a tank mixture with Moncut® 460 SC was prepared. The activity of the *Metarhizium brunneum* isolate JKI-BI-1450 in combination with Moncut® 460 SC was confirmed in a laboratory test system (Bernhardt et al. 2019).

Sampling of wireworms, CFUs and soil temperature/moisture/properties

Wireworms were sampled at four different dates to investigate the predominant species and field distribution. Eight samples per plot were taken randomly at 15 cm depth with a cylindrical (10 cm diameter) auger. Wireworms were extracted by hand and

determined to species level based on their morphology following Klausnitzer (1994) and Cocquempot et al. (1999).

To investigate the abundance and persistence of *Metarhizium* spp., the colony-forming units (CFUs) were analysed 0, 27, 48, 83 and 111 days after application. Five samples per plot were randomly taken in the middle of the potato ridge at 5-15 cm depth with a cylindrical (2.5 cm diameter) auger (Baumann Saatzuchtbedarf, Waldenburg, Germany). Soil samples were transferred in plastic bags (ISTAD, IKEA) and stored at 5 °C until processing. For the isolation of vital fungal cells, 20 g of each sieved (1 mm mesh) soil sample was transferred into a 300 ml flask (Schott AG, Mainz, Germany) and 100 ml 0.1 % Tween80® (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) was added. After 2 h on a shaker (CERTOMAT® R, B. Braun Melsungen AG, Melsungen, Germany) at room temperature, 100 µl supernatant was spread on each of three Petri dishes with semi-selective media (Strasser et al. 1996). The product Syllit® (Arysta LifeScience Germany GmbH, Düsseldorf, Germany) was used for the addition of Dodine. The plates were incubated at 25 °C in the dark. After 14 days, the CFUs of *Metarhizium* spp. were identified by morphological criteria and counted to calculate the CFUs g^{-1} soil (Zimmermann 2007).

Soil temperature and soil moisture were measured every hour over the entire trial period placing three HOBO Micro Station H21-USB datalogger (Onset Computer Cooperation, Bourne, USA) randomly on the field. Each datalogger was equipped with a 12-Bit Temperature Smart Sensor S-TMB-MOxx (Onset Computer Cooperation, Bourne, USA) and a Soil Moisture Smart Sensor S-SMx-M005 (Onset Computer Cooperation, Bourne, USA) which were installed in the potato ridge at seed potato level (~ 15 cm depth). The data were collected with the program HOBOware version 3.7.12 (Onset Computer Cooperation, Bourne, USA) and processed in Excel (Microsoft Office 2016) to calculate the daily mean, minimum and maximum temperature (°C) and water content (m^3 m^{3-1}).

The analysis of the soil properties of the three field sites was carried out by the LUFA Nord-West (Oldenburg, Germany). For this purpose, twenty samples were taken evenly distributed over the respective field site using a cylindrical (2.5 cm diameter) auger (Baumann Saatzuchtbedarf, Waldenbrug, Germany) at a depth of 30 cm. Samples were collected in a bucket and 500 g of composite samples of each field site were sent to the

LUFA Nord-West in sealed bags for the analysis of copper (Cu), the humus content, the carbon-nitrogen ratio (C/N), the pH-value and the soil composition.

Effectiveness of the *Metarhizium brunneum* formulations in the greenhouse

Experimental design in the greenhouse

A greenhouse experiment was carried out over three years (2018-2020) to test the effectiveness of the *Metarhizium brunneum* formulations under standardised conditions. Pots (625 cm² surface, 28 cm height) were filled with 7.5 kg of a soil (Einheitserde Classic Pikiererde CL P, Gebrüder Patzer GmbH & Co. KG, Sinntal, Germany) and sand mixture (5:1). Five *Agriotes obscurus* larvae, one seed potato (*Solanum tuberosum*) of the variety Princess (Kartoffel-Müller, Nersingen, Germany) and the respective treatment were added for one replicate. To adapt the distribution of the potato seed dressing Moncut® 460 SC with an application rate of 0.2 L t^{-1} in 80 L ha^{-1}, the same nozzle as used in the field was attached to a pressure vessel. Each bucket was treated with a pressure of 6 bar for 3 sec on the soil surface at a height of 12 cm. As in the field, the corresponding amount of AgriMet-Dry Product was dissolved in tap water and applied in combination with Moncut® 460 SC. The AgriMet-Granule and ATTRACAP® were applied by hand at the same height as the AgriMet-Dry Product. Based on the research of Eckhard Immenroth regarding to the distribution of the AgriMet-Granule in the potato ridge (personal communication, 2019, JKI, Institute for Application Techniques in Plant Protection, Braunschweig, Germany), the application rate in the field could be transferred to the pots. The calculation of the application rate for the AgriMet-Dry Product was based on the pot surface. After the application, one plant potato was placed in the middle of the bucket and filled up with 12 cm of the soil-sand mixture. The pots were set up in a randomised complete block design with six replicates and stored at 20 °C and 60 % RH from April to July in the greenhouse with a daily exposure of 16 h light and 8 h darkness. The tested treatments are shown in Table 3. After planting, no plant protection product was used and the pots were watered as needed.

Assessment of wireworm survival in the greenhouse

At the end of the experiment, mortality of the released *Agriotes obscurus* larvae was examined by thoroughly searching the substrate of each pot. Wireworms that were not recaptured were assessed as dead, as an escape could be excluded.

Table 3: *Metarhizium brunneum* formulations, application rates, abbreviations and seed dressing in greenhouse experiments from 2018 to 2020.

Year	Treatment	Application rate	Abbr.	Seed dressing
2018	ATTRACAP® (*Reference*)	30 kg ha^{-1}	A30	Moncut®
	Granule	10 kg ha^{-1}	G10	Moncut®
	Granule	30 kg ha^{-1}	G30	Moncut®
	Granule	50 kg ha^{-1}	G50	Moncut®
	Granule	70 kg ha^{-1}	G70	Moncut®
2019	Granule	30 kg ha^{-1}	G30	Moncut®
	Granule	60 kg ha^{-1}	G60	Moncut®
	Dry Product	$1x10^{11}$ spores ha^{-1}	DP1	Moncut®
	Dry Product	$2x10^{11}$ spores ha^{-1}	DP2	Moncut®
	Granule + Dry Product	30 kg ha^{-1} + $1x10^{11}$ spores ha^{-1}	G30+DP1	Moncut®
2020	ATTRACAP® (*Reference*)	30 kg ha^{-1}	A30	Moncut®
	Granule	30 kg ha^{-1}	G30	Moncut®
	Granule	60 kg ha^{-1}	G60	Moncut®
	Granule	300 kg ha^{-1}	G300	Moncut®
	Dry Product	$1x10^{11}$ spores ha^{-1}	DP1	Moncut®
	Dry Product	$2x10^{11}$ spores ha^{-1}	DP2	Moncut®
	Dry Product	$5x10^{12}$ spores ha^{-1}	DP50	Moncut®
	Granule + Dry Product	30 kg ha^{-1} + $1x10^{11}$ spores ha^{-1}	G30+DP1	Moncut®

Effectiveness of the *Metarhizium brunneum* formulations in the laboratory

Bioassay for the LC_{50} and LT_{50} determination

The lethal concentration (LC_{50}) of the AgriMet-Granule and the AgriMet-Dry Product against *Agriotes obscurus*, *A. sputator* and *A. lineatus* was examined in a laboratory experiment using four different application rates shown in Table 4, in comparison with an untreated control.

Table 4: *Metarhizium brunneum* formulations, application rates and abbreviations in the laboratory bioassay.

Treatment	Application rate	Abbr.
Granule	30 kg ha^{-1}	G30
Granule	300 kg ha^{-1}	G300
Granule	3000 kg ha^{-1}	G3000
Granule	15000 kg ha^{-1}	G15000
Dry Product	$1x10^{11}$ spores ha^{-1}	S1
Dry Product	$1x10^{12}$ spores ha^{-1}	S10
Dry Product	$5x10^{12}$ spores ha^{-1}	S50
Dry Product	$1x10^{13}$ spores ha^{-1}	S100

Small plastic cans (50 ml, OPTIMAX Packaging GmbH & Co KG, Norderstedt, Germany) were filled with 35 g of a 5:1 mixture of soil (Einheitserde Classic Pikiererde CL P, Gebrüder Patzer GmbH & Co. KG, Sinntal, Germany) and sand. After the appropriate amount of AgriMet-Granule was mixed in evenly or the dissolved AgriMet-

Dry Product was applied with a pipette respectively, one wireworm was added. As in the greenhouse, the application rate was calculated based on previous research and the surface of the cans used. Here, no potato seed dressing was applied. Plastic cans were maintained under controlled conditions (25 °C; 60 RH) for 105 days and wireworms were fed with *Triticum aestivum* seeds (Cultivar: Primus, Deutsche Saatgutveredelung AG, Lippstadt, Germany) to avoid starving. Larval mortality caused by *M. brunneum* was assessed two times a week by the examination of fungal outgrowth of the cadaver to determine the lethal time of 50 % mortality (LT_{50}). Each treatment contained six wireworms of the respective wireworm species and the bioassay was repeated five times time independent. Thus, a total of 810 larvae were tested.

Statistical analyses

Statistical analyses were carried out using the software R Studio (Version 1.4.1106) (RStudio Team 2020). Data are presented as the arithmetic mean (mean) and standard deviation (±SD) or adjusted mean (adjusted mean) and standard error (±SE), depending on whether a model was used for calculation. To meet the ANOVA's underlying assumptions of normal distribution and variance homogeneity, residuals of the respective model were visually inspected. Normal distribution was checked with the QQ-Plot (sample quantile-theoretical quantile) and variance homogeneity with the Residuals-Prediction-Plot. Selection of the best-fitted model was based on the Akaike Information Criterion (AIC) (Burnham & Anderson 2002) after backward elimination of the full model. Unless otherwise noted, the post hoc Tukey HSD test ($\alpha = 0.05$) was performed with the R package *emmeans* (Lenth et al. 2018) to examine differences between the respective factor levels in the context on an ANOVA.

The influence of the temperature and field soil (explanatory variables) on the fungal growth on the surface of the AgriMet-Granule (target variable) was determined by comparing the number of conidia per grain within a Linear Model (LM). Data were log transformed to ensure normal distribution and variance homogeneity.

$$y = log\ (Conidia\ per\ Grain) \sim Temperature + Soil + Temperature{:}Soil$$

The effect of the tested *Metarhizium* formulations (explanatory variable) on the wireworm tuber damage (target variable) was analysed with a Generalized Linear Model (GLM, binominal distribution) comparing the proportion of undamaged potatoes of each

plot (field) or pot (greenhouse). Global effects were assessed by performing an analysis of deviance.

$$\%\ Undamaged\ Potatoes\ (Field) \sim Treatment + Block + Treatment{:}Block$$

$$\%\ Undamaged\ Potatoes\ (Greenhouse) \sim Treatment + Block$$

Percent treatment effectiveness was calculated relative to the untreated control for each block using Abbott's formula (Abbott 1925).

$$\frac{\%\ Damaged\ Potatoes\ Control - \%\ Damaged\ Potatoes\ Treatment}{\%\ \ Damaged\ Potatoes\ Control} * 100 = \%\ Effectiveness$$

The abundance and persistence of *Metarhizium* spp. in the soil (target variable) after application of the different *M. brunneum* formulations (explanatory variable) was described as number of CFU per g soil. After square-root transformation of the CFU data, normal distribution and variance homogeneity were confirmed. Subsequent analysis of treatment effect was performed for each experimental year by setting up a Linear Mixed Model (LMM) with the *lme4* R package (Bates et al. 2015) and running an analysis of variance. A random effect was included to account for the subsamples within each plot.

$$y = sqrt(CFU) \sim Treatment + Block + (1|Block{:}Treatment))$$

Kaplan-Meier-Analysis was used to determine the survival probability of the tested *A. obscurus*, *A. sputator* and *A. lineatus* larvae over time after incubation with different application rates of the AgriMet-Granule and AgriMet-Dry Product. Survival curves over time were created using the *survival* R package (Therneau 2021) and compared with the log-rank test to determine global differences. To detect significant differences between the application rates within a wireworm species a pairwise comparison of survival curves based on the Bonferroni method was carried out by using the *survminer* R package (Kassambara et al. 2021). To determine the time at which 50 % of the tested individuals died due to the fungal treatment (LT_{50}), the "surv_median()" command of the *survival* R package was used. Mycosis of wireworms was recorded as an "event". If no event was observed by the end of the study, the total survival time could not be accurately determined and was censored. To estimate the LC_{50} values of the AgriMet formulations, a probit analysis was performed using the *ecotox* R package (Hlina et al. 2019)

The density of wireworms on the respective field site was plotted using the R package *desplot* (Wright 2020). Recorded soil temperature, soil water content and precipitation during the field trials were visualised using Microsoft Excel (Version 2016). All other graphs were created with the R packages *ggplot2* (Wickham 2016), *ggpubr* (Kassambara 2020), *RColorBrewer* (Neuwirth 2014), and *multcompView* (Graves et al. 2019).

Results

Quality control of the *Metarhizium brunneum* formulations

The functionality of the different *Metarhizium brunneum* formulations was examined to ensure comparability and verify the manufactures specifications. The manufactures of the AgriMet formulations determined an outgrowth rate for the Granule between 81-100 % and $1.25x10^9$ CFU g^{-1} for the AgriMet-Dry Product. Since ATTRACAP® is commercially available, a 100 % outgrowth rate was expected. Table 5 shows that the outgrowth rate of the used AgriMet-Granule fluctuated and ranged between 69-82 % for all trials, whereas ATTRACAP® achieved an outgrowth rate between 97-100 %. The range of the AgriMet-Dry Product was between $5.6x10^8$-$9.6 x10^8$ CFUs g^{-1}.

Table 5: Quality control of the *Metarhizium brunneum* formulations tested. Mean outgrowth rate in percent of the AgriMet-Granule and ATTRACAP® and mean number of colony-forming unit's (CFUs) of *M. brunneum* per g AgriMet-Dry Product for the application in field, greenhouse and laboratory experiments in 2018 to 2020. The standart deviation is shown as ±SD.

Year	**Experiment**	**AgriMet-Granule**		**ATTRACAP®**		**AgriMet-Dry Product**	
		Outgrowth rate in %				CFUs g^{-1}	
		mean	±SD	mean	±SD	mean	±SD
2018	Field trial	79	6.63	100	0.00	$6.3x10^8$	$5.46x10^7$
	Greenhouse	81	7.35	99	2.0	/	/
2019	Field trial	78	5.10	98	2.5	$8.9x10^8$	$6.4x10^7$
	Greenhouse	69	3.74	99	2.0	$8.3x10^8$	$6.0x10^7$
	Laboratory	70	3.16	/	/	$5.6x10^8$	$8.5x10^7$
2020	Field trial	80	3.16	97	4.0	$7.2x10^8$	$6.2x10^7$
	Greenhouse	82	4.00	98	2.5	$9.6x10^8$	$7.7x10^7$

The quality control of the AgriMet-Granule in field soil revealed that the initial conidia concentration of the *M. brunneum* isolate JKI-BI-1450 coated on the surface of the tested batch was $5.89x10^4$ conidia $grain^{-1}$ (±SD $5.05x10^3$ conidia $grain^{-1}$). The sporulation after 14 days of incubation was significant influenced by the tested temperatures and respective substrate (ANOVA, $p < 0.0001$). Figure 8 shows that notable sporulation of the AgriMet-Granule was only visible on the sterile control substrate 1.5 % water agar. The incubation temperature of 10 °C increased the conidia concentration only slightly to $1x10^5$ conidia $grain^{-1}$. In comparison, a significant increase of the *M. brunneum* concentration was determined at 15 °C with $5.6x10^5$ conidia $grain^{-1}$ (Tukey HSD test, $p < 0.0001$). After an exponential increase to $1.1x10^7$ conidia $grain^{-1}$ at 20 °C (15-20°C: Tukey HSD test, $p < 0.0001$), sporulation reached its maximum at 25 °C with $2.3x10^7$ conidia $grain^{-1}$. In contrast, the incubation of the AgriMet-Granule on the soil from Suettdorf or Borg led to no significant increase in conidia formation up to a temperature

of 20 °C. Only the incubation at 25 °C resulted in a significant but small increase to $3.9x10^5$ (Suettdorf) and $7.1x10^5$ (Borg) conidia grain^{-1}. Numerous grains incubated in the respective soil were found to be colonised by other fungi and bacteria.

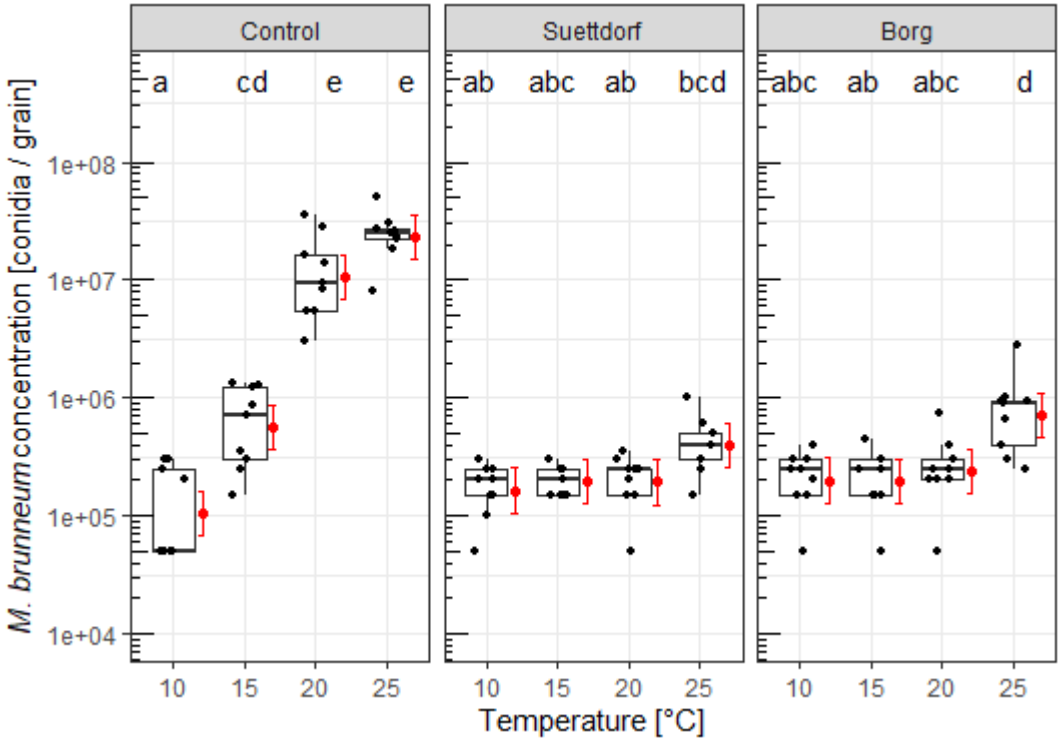

Figure 8: *Metarhizium brunneum* concentration (conidia grain^{-1}) on the AgriMet-Granule after incubation at 10 °C, 15 °C, 20 °C and 25 °C on 1.5 % water agar (Control) or field soil from Suettdorf and Borg for 14 days. Black dots per temperature and substrate represent the mean number of conida grain^{-1} out of ten grains per Petri Dish. Boxplots consist of the median and the 25 % and 75 % quantile. Adjusted mean of conidia grain^{-1} and 95 % confidence interval of the replicates per treatment are illustrated in red and treatments with the same letters are not significantly different. LM (y = log(Conidia per Grain) ~ Temperature + Soil + Temperature:Soil), Tukey HSD test (α = 0.05).

Effectiveness of the *Metarhizium brunneum* formulations in the field

Assessment of wireworm potato damage

The effectiveness of the tested *Metarhizium brunneum* formulations in field trials over three years was assessed by the proportion of undamaged potatoes after harvest and the results are shown in Figure 9. A very high infestation of wireworms in each year was reflected by very small proportions of undamaged potatoes in the untreated controls that ranged between 2.3 % (2018), 13.1 % (2019) and 0.3 % (2020). The tuber damage was significantly reduced by the application of individual *M. brunneum* formulations in each year with strong block effects (Treatment: ANOVA, $p < 0.0001$, Block: ANOVA, $p < 0.0001$). However, the effectiveness of the treatments was very low and neither the reference product ATTRACAP® nor the AgriMet formulations had a constant effect over all years. In 2018, the application of ATTRACAP® reduced the tuber damage

significantly to a proportion of 12 % undamaged potatoes compared to the untreated control (Tukey HSD test, $p < 0.001$). A similar effect was achieved by the combination of the AgriMet-Granule with 30 kg ha^{-1} plus the AgriMet-Dry Product with 1x10^{11} conidia ha^{-1} (10 %) as well as with the AgriMet-Dry Product with 1x10^{11} conidia ha^{-1} individually (9 %), with no difference between treatments (A30-G30_DP1: Tukey HSD test, $p = 0.99$; A30-DP1: Tukey HSD test, $p = 0.88$, G30_DP1-DP1: Tukey HSD test, $p = 0.99$). In 2019, the increased application rate of the AgriMet-Granule to 60 kg ha^{-1} resulted in the highest proportion of undamaged potatoes of 27 % with a significant difference of the untreated control (Tukey HSD test, $p < 0.0001$). As in the previous year, the potato damage was comparable within the treatments ATTRACAP® (23 %) and the combination of the AgriMet-Granule with 30 kg ha^{-1} plus AgriMet-Dry Product with 1x10^{11} conidia ha^{-1} (26 %) (G60-A30: Tukey HSD test, $p = 0.78$; G60-G30_DP1: Tukey HSD test, $p = 0.99$; A30-G30_DP1: Tukey HSD test, $p = 0.96$). Although the AgriMet-Dry Product with 1x10^{11} conidia ha^{-1} individually increased the proportion of undamaged potatoes, there was no significant difference to the control (Tukey HSD test, $p = 0.09$). In addition, a strong block effect was obvious with a large scattering within the treatments (ANOVA, $p < 0.0001$). The majority of plots in block A showed the lowest proportion of undamaged potatoes regardless of the treatment (1-21 %), whereas the proportion in plots from block B was highest (25-56 %). In 2020, wireworm damage was extremely high with 0.3 % undamaged potatoes in the untreated control. The application of ATTRACAP® resulted in 1 % undamaged potatoes with no significant difference to the control (Tukey HSD test, $p = 0.40$). The *Metarhizium* formulation with the best effectiveness was the AgriMet-Granule with 60 kg ha^{-1} and a proportion of 4 % undamaged potatoes (G60-C: Tukey HSD test, $p = 0.0003$; G60-A30: Tukey HSD test, $p = 0.0049$). However, the scattering within this treatment was relatively wide and ranged from 0 % (Block A) to 34 % (Block C) undamaged potatoes. A similar scattering between 0 % (Block C) and 23 % (Block D) of the AgriMet-Dry Product with 1x10^{11} conidia ha^{-1} resulted in 2.8 % undamaged potatoes (DP1-G60: Tukey HSD test, $p = 0.82$). The AgriMet-Dry Product as well as the AgriMet-Granule with 30 kg ha^{-1} (3.1 %) led a small but significant increase in the proportion of undamaged potatoes compared to the control with no difference between the treatments (DP1-C: Tukey HSD test, $p = 0.0045$, G30-C: Tukey HSD test, $p = 0.0019$, DP1-G30: Tukey HSD test, $p = 0.99$). The application of the AgriMet-Dry Product with 2x10^{11} conidia ha^{-1} as well as the

combination of the AgriMet-Granule with 15 kg ha^{-1} plus the AgriMet-Dry Product with $0.5x10^{11}$ conidia ha^{-1} did never reduce the tuber damage significantly.

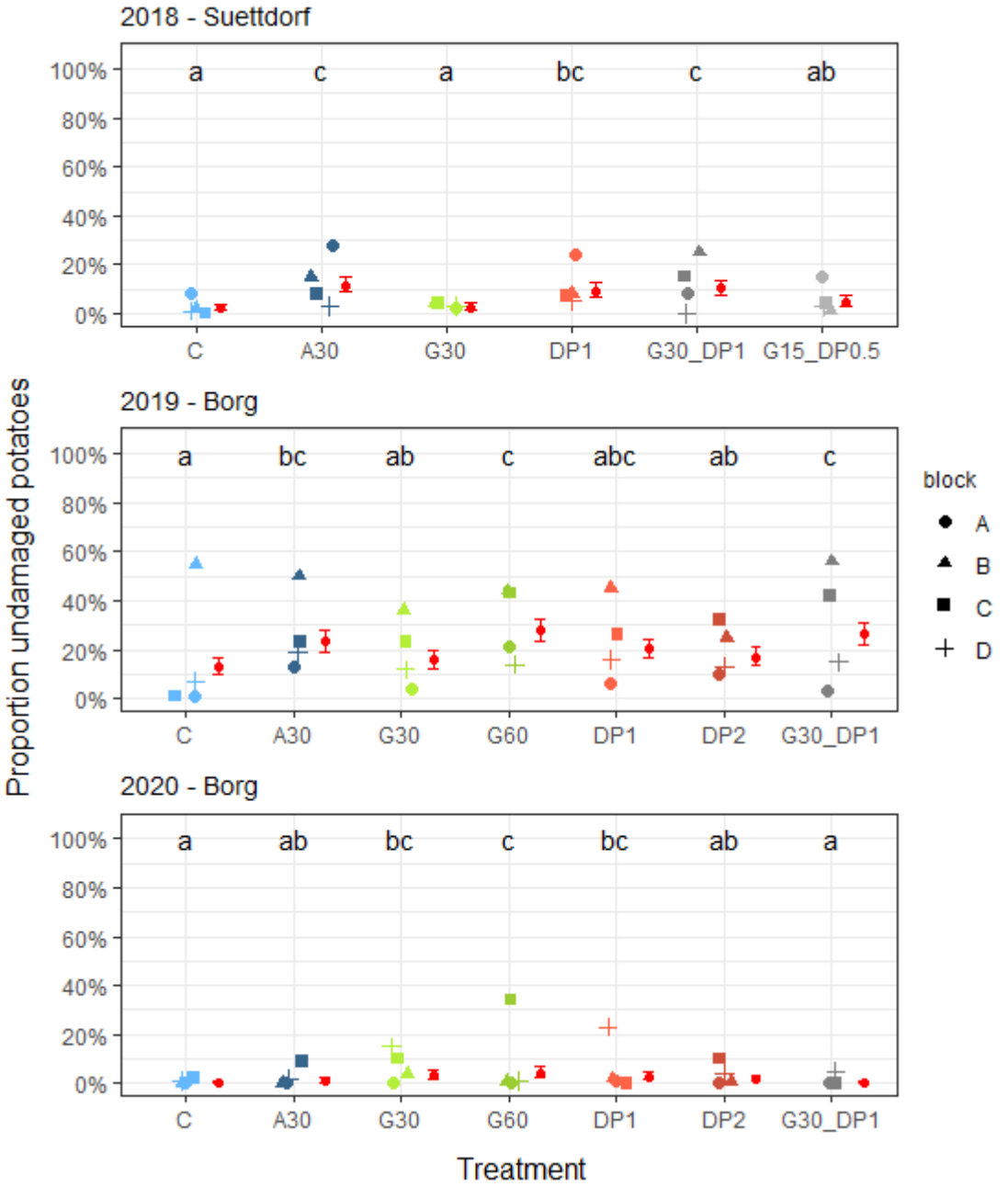

Figure 9: Proportion of undamaged potatoes after harvest of field trials with different formulations of *Metarhizium brunneum* from 2018 to 2020 near Uelzen. The four symbols per treatment (C = untreated control; A30 = ATTRACAP® 30 kg h^{-1}; G30 = AgriMet-Granule 30 kg ha^{-1}; G60 = AgriMet-Granule 60 kg ha^{-1}; DP0.5 = AgriMet-Dry Product $0.5x10^{11}$ conidia ha^{-1}; DP1= AgriMet-Dry Product $1x10^{11}$ conidia ha^{-1}; DP2 = AgriMet-Dry Product $2x10^{11}$ conidia ha^{-1}) represent the percentage of undamaged potatoes out of 100 randomly sampled tubers per plot within each block in a randomized complete block design. Adjusted mean and 95 % confidence interval of the four plots per treatment are illustrated in red and treatments with the same letters within a year are not significantly different. GLM (y = Percentage Undamaged Potatoes ~ Treatment + Block + Treatment:Block, family=binominal), Tukey HSD test (α = 0.05).

The effectiveness of the *M. brunneum* formulations compared to the untreated control according to Abbott (1925) is shown in Table 6 and underlines the statistically analysed low tuber damage reduction. The reference product ATTRACAP® (30 kg ha^{-1}) reached a three-year-average effectiveness of 7.5 %, whereas the same application rate of AgriMet-Granule resulted in only 1.9 %. The increased application rate of the AgriMet-Granule with 60 kg ha^{-1} led to 10.5 % effectiveness in average. Although the AgriMet-Dry Product with $1x10^{11}$ conidia ha^{-1} already reached 7.5 % effectiveness, the doubled application rate did not lead to any improvement. The combined application of the AgriMet-Granule (30 kg ha^{-1}) and the AgriMet-Dry Product ($1x10^{11}$ conidia ha^{-1}) resulted in the highest effectiveness of 13.6 % compared to the untreated control in 2019 and an average of 8.0 % over all years.

Table 6: Effectiveness of the *Metarhizium brunneum* formulations as the percentage of reduced potato damage compared to the untreated control during field trials in 2018 to 2020 (A30 = ATTRACAP® 30 kg h^{-1}; G30 = AgriMet-Granule 30 kg ha^{-1}; G60 = AgriMet-Granule 60 kg ha^{-1}; DP0.5 = AgriMet-Dry Product $0.5x10^{11}$ conidia ha^{-1}; DP1= AgriMet-Dry Product $1x10^{11}$ conidia ha^{-1}; DP2 = AgriMet-Dry Product $2x10^{11}$ conidia ha^{-1}). Mean and standart deviation (±SD) per year was calculated from the effectiveness for each treatment within a block according to Abbott (1925).

Treatment	**Effectiveness [%]**					
	2018		**2019**		**2020**	
	mean	±SD	mean	±SD	mean	±SD
A30	11.3	7.2	9.0	12.2	2.1	2.9
G30	2.0	1.4	-2.9	23.9	6.6	5.2
G60	/	/	11.4	24.1	8.7	13.9
DP1	11.9	5.4	4.4	17.1	6.3	9.2
DP2	/	/	-4.9	36.9	3.1	3.2
G30_DP1	9.6	10.1	13.6	16.3	1.0	1.8
G15_DP0.5	3.4	2.8	/	/	/	/

Wireworm sampling during field trials

Wireworms were extracted from eight soil samples per plot at four sampling dates to determine the species composition and density over time. The results of the wireworm species composition are shown in Figure 10. The dominant species during each field trial was *Agriotes lineatus* with a share between 60-70 % of the sampled wireworms. *Agriotes obscurus* was the second dominant species in every year with a share between 25-35 %. In 2018 and 2020, a small number of the species *Hemicrepidius niger*, *Agrypnus murinus* and *Oedostethus quadripustulatus* was determined. However, the wireworms were not evenly distributed over each field site. Figure 11 shows that the agglomeration of wireworms in 2020 was highest in block A and lowest in block C. In 2019, block B was the area with the fewest wireworms, whereas the most were in block

D. There was a clear difference between block A and B to C and D in 2018. Overall, the distribution of wireworms was always nested.

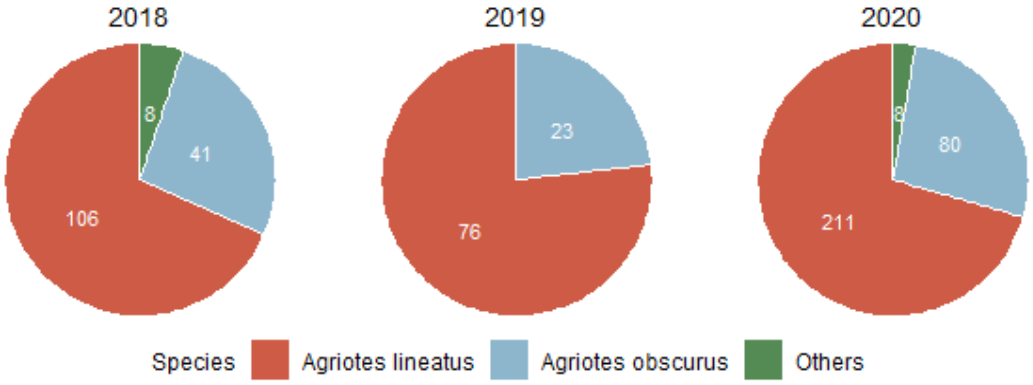

Figure 10: Wireworm species composition of the field trials in potatoes near Uelzen in three years. The colours represent the different wireworm species (red: *Agriotes lineatus*, blue: *A. obscurus*, green: others) and the respective total number is illustrated in white.

Persistence of Metarhizium spp. during field trials

The abundance and persistence of *Metarhizium* spp. was determined by the number of CFUs g^{-1} soil and the results as well as the associated statistics are shown in Table 8. There was no significant difference in the number of CFUs g^{-1} soil before the application of the formulations of *M. brunneum* (ANOVA, $p > 0.05$) in each of the three field trials. However, the initial concentration of *Metarhizium* spp. was twenty times higher in 2018 compared to 2019 and 2020. In 2018, the application of G30+DP1 was the only treatment which increased the concentration of *Metarhizium* spp. significantly 48 days after application (DAA) to a maximum of 959.9 CFUs g^{-1} soil (ANOVA, $p = 0.03$). 83 DAA the number von CFUs g^{-1} soil decreased and equalled to the initial value. In 2019, the application of G60, DP2 and G30+DP1 led to a significant increase of the CFUs g^{-1} soil repeatedly 48 DAA (ANOVA, $p < 0.001$). G60 and G30+DP1 reached an equal level of 138.8 CFUs g^{-1} soil and 154.1 CFUs g^{-1} soil, whereas DP2 achieved 87.4 CFUs g^{-1} soil. The abundance of *Metarhizium* spp. in the treatment G60 increased further to a maximum of 191.8 CFUs g^{-1} soil after 111 DAA, whereas the CFUs g^{-1} soil of the treatment G30+DP1 decreased 111 DAA. The treatment DP2 reached a maximum of 139.1 CFUs g^{-1} soil after 83 days and decreased slightly 111 DAA. In 2020, no treatment was able to increase the CFUs g^{-1} soil significant. However, the application of A30 resulted in a slight increase up to 86.1 CFUs g^{-1} soil after 83 days (ANOVA, $p = 0.54$). In addition, G60 reached 42.1 CFUs g^{-1} soil and G30+DP1 36.6 CFUs g^{-1} soil after 83 days. 111 DAA the CFUs g^{-1} soil equalled to the *Metarhizium* spp. concentration before the application of the formulations.

Soil temperature and water content during field trials

The soil temperature and water content in the potato ridge at a depth of ~15 cm was measured to consider the effect of environmental factors on the effectiveness of the *Metarhizium brunneum* formulations during field trials (see Figure 12). At the beginning of the vegetation period, the temperature in the potato ridge was 16.5 °C in 2018, 9.8 °C in 2019 and 14.7 °C in 2020. The temperature increased slowly and reached 20 °C after 25 days in 2018, 40 days in 2019 and 42 days in 2020. Afterwards, the temperature was at approximately 20 °C with small fluctuations during June and July. However, the temperature in 2020 decreased after 42 days and remained below 20 °C until 101 days after the application. This resulted in the lowest average temperature of the entire vegetation period of 17.1 °C. The average temperature in 2018 was 19.4 °C and 18.7 °C in 2019. The highest temperature per day was between 23.6 °C (2018) and 25.3 °C (2019), with an absolute maximum of 29.2 °C measured in 2019 after 94 days. The increasing temperature over the vegetation period resulted in a decreasing water content in the potato ridge. The water content differed between the years at the beginning of the vegetation period and was between 0.12 m^3/m^3 (2018) and 0.18 m^3/m^3 (2019). Over time, the water content dropped due to the low total precipitation (2018: 77.6 mm; 2020: 172.7 mm) to a minimum of 0.07 m^3/m^3 in 2018 and 0.05 m^3/m^3 in 2020. However, the total precipitation in 2019 was higher (216.2 mm) and increased the falling water content from 0.07 m^3/m^3 to an average of 0.14 m^3/m^3 until harvest. In addition to the temperature and the precipitation, the water consumption of the potato plant has an influence on the water content in the potato ridge. After 60 to 80 days after planting the potato reached BBCH stage 60 and the water content in the potato ridge decreased due to tuber formation and the additional water consumption.

Soil properties during field trials

The soil properties of the three field sites used for testing the *M. brunneum* formulations were analysed by the LUFA Nord-West, and the results are shown in Table 7. The ratio of the soil components clay, silt and sand was nearly the same at all field sites and corresponded to that of light loamy-sandy soils. The pH-value of all soils was 5.9 and the carbon-to-nitrogen ratio (C/N ratio) varied between 12-14. The humus content was lowest in Suettdorf (2018) at 3.2 % and highest in Borg (2020) at 4.8 %. The concentration of copper ranged between 1.1-1.3 mg kg^{-1} in Borg and 3.8 mg kg^{-1} in

Suettdorf. Overall, the soil properties of the different field sties were very similar and did not show any noticeable values in the analysed parameters.

Table 7: Soil properties of the three field sites in Suettdorf and Borg during the testing of the *M. brunneum* formulations from 2018 to 2020.

Year	**Field site**	**Soil analysis**						
		Copper [mg kg^{-1}]	Humus content [%]	C/N ratio	pH value	Soil components [%]		
						clay	silt	sand
2018	Suettdorf	3.8	3.2	12	5.9	6.1	13.8	80.1
2019	Borg	1.3	4.6	14	5.9	6.0	15.2	78.8
2020	Borg	1.1	4.8	14	5.9	6.1	16.0	77.9

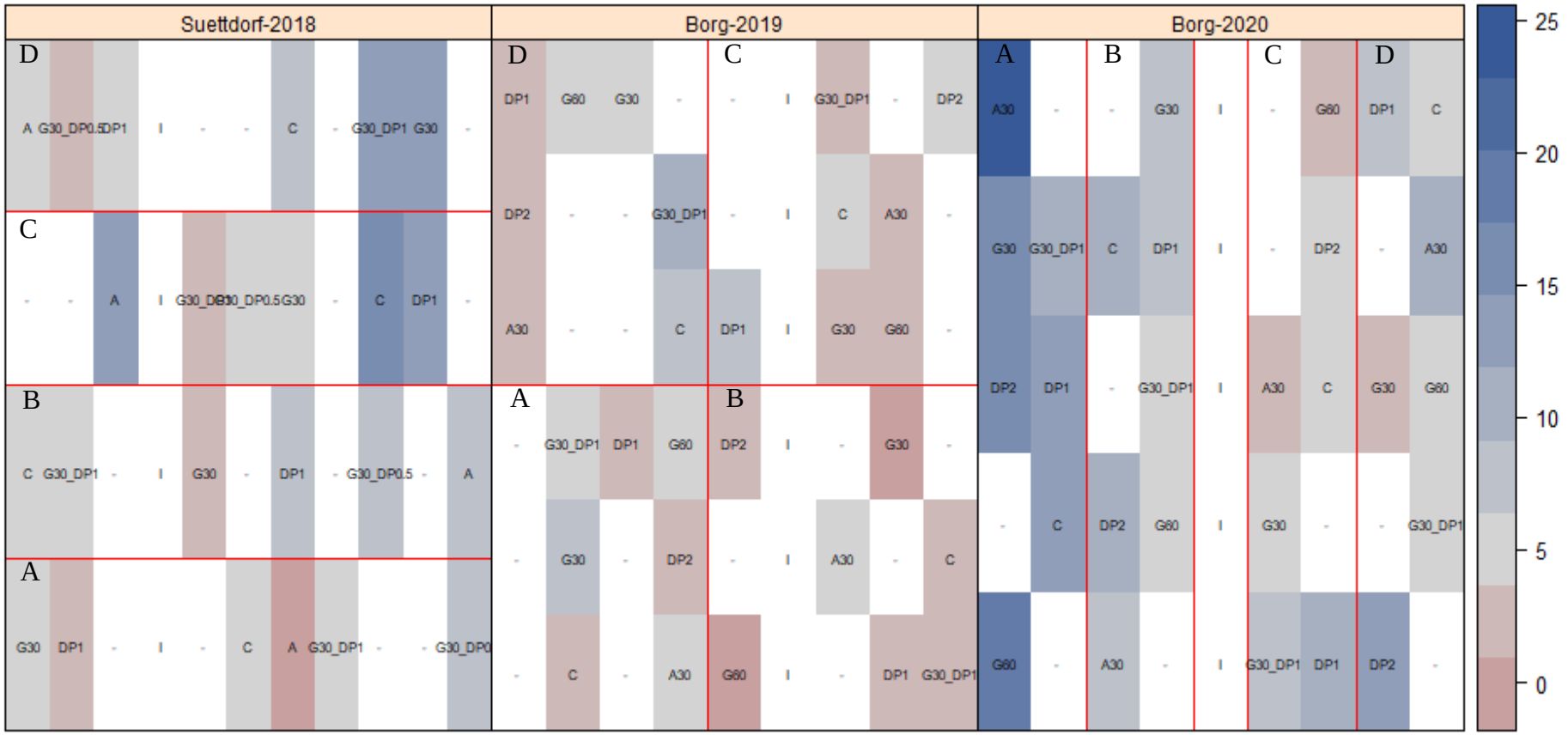

Figure 11: Wireworm density of the field trials in 2018 (Suettdorf), 2019 (Borg) and 2020 (Borg) based on the sum of four samplings with a cylindric auger with eigth samples per plot. The colour of each plot corresponds with the number of wireworms and is illustrated in red (no wireworms) to blue (many wireworms). The abbreviations in each plot represent the treatments (C = untreated control; A30 = ATTRACAP® 30 kg h^{-1}; G30 = AgriMet-Granule 30 kg ha^{-1}; G60 = AgriMet-Granule 60 kg ha^{-1}; DP0.5 = AgriMet-Dry Product 0.5x10^{11} conidia ha^{-1}; DP1= AgriMet-Dry Product 1x10^{11} conidia ha^{-1}; DP2 = AgriMet-Dry Product 2x10^{11} conidia ha^{-1}). The four blocks (A, B, C, D) per trial are separated with red lines. Plots that have not been sampled are displayed with "-" and tramlines with "I".

Table 8: Adjusted mean number of colony-forming units (CFUs) g^{-1} soil of *Metarhizium* spp. and standard error (±SE) during field trials near Uelzen in three years before (0) and 27, 43, 83 and 111 days after the application (DAA) of different formulations von *M. brunneum* (A30 = ATTRACAP® 30 kg h^{-1}; G30 = AgriMet-Granule 30 kg ha^{-1}; G60 = AgriMet-Granule 60 kg ha^{-1}; DP0.5 = AgriMet-Dry Product $0.5x10^{11}$ conidia ha^{-1}; DP1= AgriMet-Dry Product $1x10^{11}$ conidia ha^{-1}; DP2 = AgriMet-Dry Product $2x10^{11}$ conidia ha^{-1}) and an untreated control (C). Adjusted mean values of the treatments with the same letters within a sampling date are not significantly different. LMM (y = sqrt(CFU) ~ Treatment + Block + (1|Block:Treatment)) , Tukey HSD test (α = 0.05).

Year	DAA	Treatment (adjusted mean number of CFUs g^{-1} soil ±SE)											
		C		**A30**		**G30**		**DP1**		**G30+DP1**		**G15+DP0.5**	
2018	0	363.8 ± 95.4	a	379.6 ± 97.4	a	489.7 ± 110.6	a	460.2 ± 107.2	a	467.9 ± 108.2	a	515.4 ± 113.5	a
	27	480.7 ± 56.5	a	548.5 ± 60.4	a	573.7 ± 61.8	a	664.6 ± 66.5	a	749.5 ± 70.6	a	677.8 ± 67.1	a
	48	371.5 ± 82.0	a	570.9 ± 101.7	ab	538.0 ± 98.7	ab	611.5 ± 105.2	ab	959.9 ± 131.8	b	706.8 ± 113.1	ab
	83	491.5 ± 78.8	a	550.0 ± 83.3	a	565.4 ± 84.5	a	649.4 ± 90.6	a	589.8 ± 86.3	a	665.0 ± 91.6	a
	111	367.1 ± 67.1	a	323.7 ± 63.0	a	407.4 ± 70.7	a	390.9 ± 69.2	a	534.2 ± 81.0	a	591.6 ± 85.2	a

		C		**A30**		**G30**		**G60**		**DP1**		**DP2**		**G30+DP1**	
2019	0	7.9 ± 6.6	a	17.1 ± 9.7	a	14.8 ± 9.0	a	13.0 ± 8.5	a	7.9 ± 6.6	a	25.2 ± 11.8	a	23.2 ± 11.3	a
	27	34.2 ± 16.5	a	37.8 ± 17.4	a	26.8 ± 14.6	a	61.2 ± 22.1	a	30.6 ± 15.6	a	52.5 ± 20.5	a	73.3 ± 14.2	a
	48	33.8 ± 13.2	ab	30.9 ± 12.6	ab	30.9 ± 12.6	ab	138.8 ± 26.8	c	13.4 ± 8.3	a	87.4 ± 21.3	bc	154.1 ± 28.2	c
	83	25.6 ± 9.2	a	34.1 ± 10.7	a	29.2 ± 9.9	a	176.1 ± 24.2	b	13.4 ± 6.7	a	139.1 ± 21.6	b	133.3 ± 21.1	b
	111	17.0 ± 6.3	a	24.9 ± 7.6	a	22.5 ± 7.2	a	191.8 ± 21.1	c	3.8 ± 3.0	a	109.2 ± 16.0	bc	85.8 ± 14.1	b

		C		**A30**		**G30**		**G60**		**DP1**		**DP2**		**G30+DP1**	
2020	0	11.8 ± 6.7	a	24.2 ± 9.6	a	25.7 ± 9.9	a	19.2 ± 8.5	a	49.9 ± 13.7	a	22.3 ± 9.2	a	27.3 ± 10.2	a
	27	19.7 ± 9.3	a	12.6 ± 7.4	a	24.4 ± 10.3	a	17.2 ± 8.7	a	20.7 ± 9.5	a	9.3 ± 6.4	a	17.7 ± 8.8	a
	48	16.6 ± 10.7	a	35.6 ± 15.7	a	15.0 ± 10.2	a	35.0 ± 15.6	a	19.6 ± 11.6	a	25.4 ± 13.2	a	13.6 ± 9.7	a
	83	7.8 ± 7.7	a	86.1 ± 25.6	a	11.4 ± 9.3	a	42.1 ± 17.9	a	7.7 ± 7.7	a	28.9 ± 14.8	a	36.6 ± 16.7	a
	111	18.0 ± 9.7	a	27.5 ± 12.0	a	35.3 ± 13.6	a	10.7 ± 7.5	a	31.3 ± 12.8	a	21.3 ± 10.6	a	26.4 ± 11.8	a

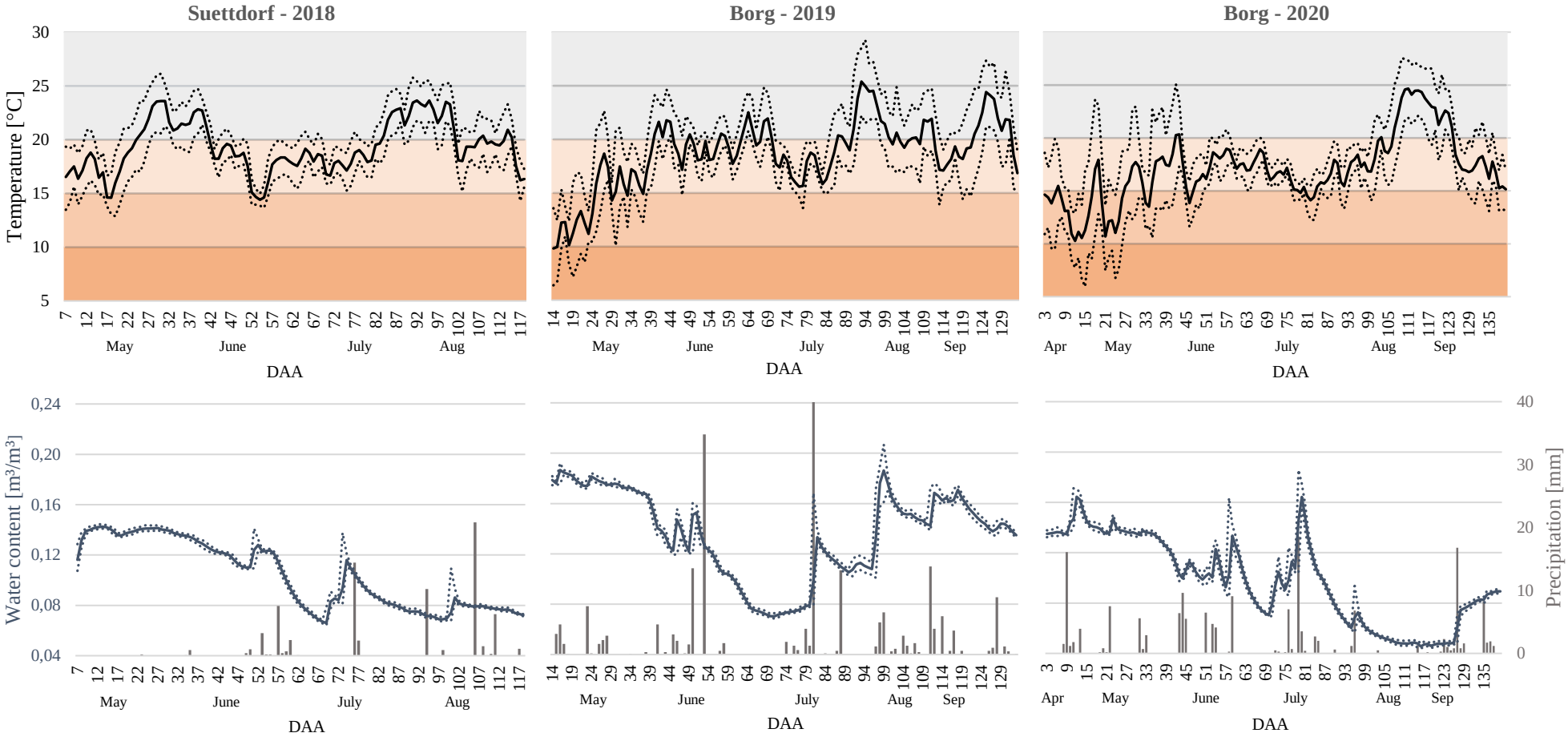

Figure 12: Temperature [°C] and water content [m³/m³] in the potato ridged at a depth of 15 cm after potato planting during field trials with *Metarhizium brunneum* formulations in Suettdorf (2018) and Borg (2019, 2020). Measurement was carried out by placing three HOBO-Datalogger randomly on the field site equipped with a temperature and a moisture sensor each. Daily means are presented as full lines and the maximum and minimum per day are illustrated with dotted lines. Data of the total precipitation per day was obtained from a metrological measurement station of the Deutsche Wetterdienst (DWD) in Uelzen and are shown as grey bars.

Effectiveness of the *Metarhizium brunneum* formulations in the greenhouse

For the assessment of the appropriate application rate of the *Metarhizium brunneum* formulations AgriMet-Granule and AgriMet-Dry Product, a standardised greenhouse experiment with pots was carried out and the results are shown in Figure 13. The number of recaptured *Agriotes obscurus* larvae was not significantly influenced by any of the tested formulations. Even up to 100 times of the field application rate of the AgriMet-Granule (3000 kg ha^{-1}) and the AgriMet-Dry Product ($1x10^{13}$ conidia ha^{-1}) decreased the number of wireworms only slightly compared to the control. The reference product ATTRACAP® also had no influence on the number of wireworms. Overall, the mean number of recaptured larvae was between 2.33 ± 0.36 (DP100) to 3.6 ± 0.26 (G30). However, the assessment of the tuber damage revealed differences between the treatments. The proportion of undamaged potatoes in the untreated control was 13.8 % (±SE 2.6 %) and 37.5 % (±SE 4.6 %) for the reference product ATTRACAP® (Tukey HSD test, $p = 0.0003$). The application of the AgriMet-Granule and AgriMet-Dry Product increased the proportion of undamaged potatoes significantly compared to the untreated control (ANOVA, $p < 0.0001$) with a significant impact of the application rates. While the field application rate of 30 kg ha^{-1} of the AgriMet-Granule was not able to reduce the tuber damage (Tukey HSD test, $p = 0.98$), an increased application rate of 60 kg ha^{-1} and 3000 kg ha^{-1} resulted in 42.5 % (G60) and 58.2 % (G3000) undamaged potatoes with a significant difference to the untreated control (Tukey HSD test, $p < 0.0001$). A similar result was assessed for the AgriMet-Dry Product. The application rates of $1x10^{11}$ conidia ha^{-1} and $2x10^{11}$ conidia ha^{-1} led to slightly increased proportions of 24.8 % (±SE 4.2 %) and 28.2 % (±SE 4.4 %) undamaged potatoes compared to the untreated control with no significant difference. However, $1x10^{13}$ conidia ha^{-1} of the AgriMet-Dry Product reduced the potato damage significant (Tukey HSD test, $p < 0.0001$) with 58.3 % (±SE 6.9 %) undamaged potatoes. The results of the high application rates of both formulations were comparable and slightly higher than the reference product ATTRACAP®. The combination of the AgriMet-Granule and the AgriMet-Dry Product resulted in 41.7 % (G30_DP1) and 55.2 % (G60_DP2) undamaged potatoes.

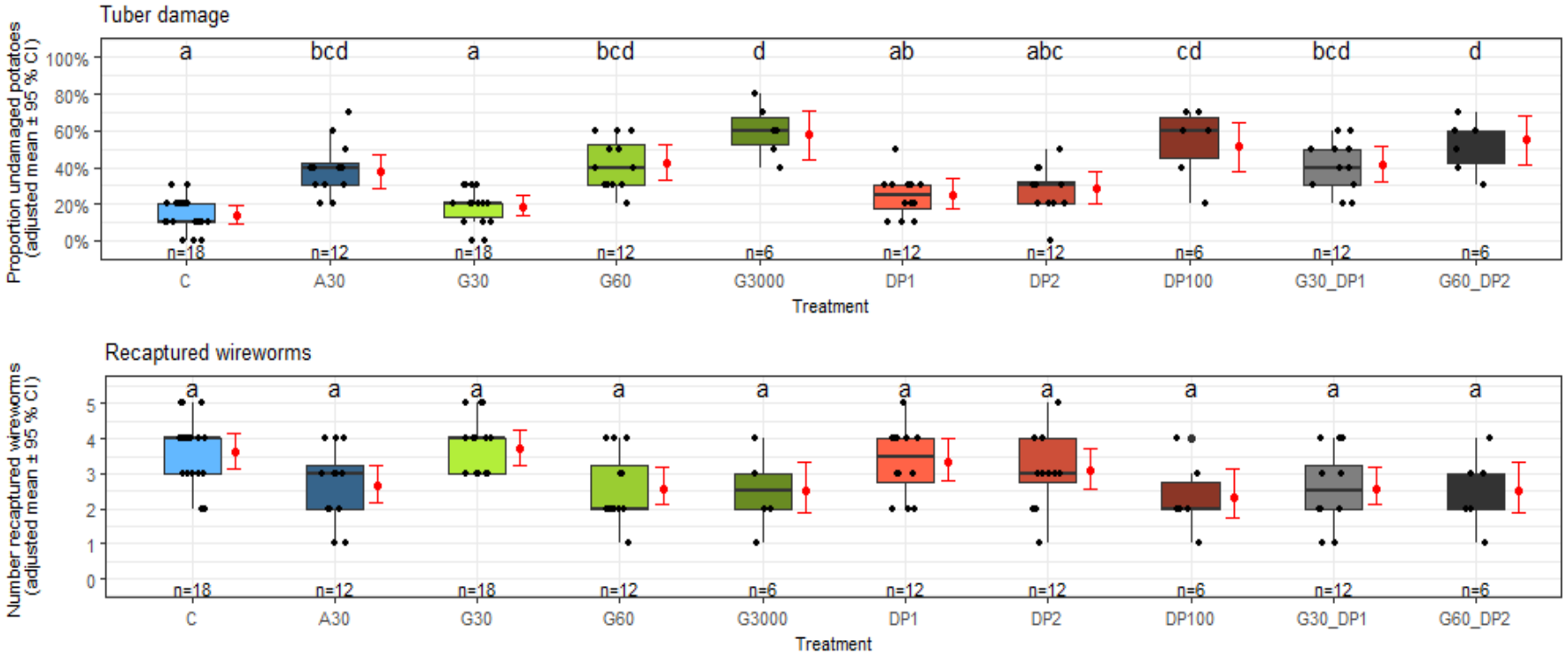

Figure 13: Recaptured *Agriotes obscurus* larvae and percentage of undamaged potatoes after harvest of a full potato growing season in the greenhouse experiments from 2018 to 2020. Different formulations of *Metarhizium brunneum* were applied during planting with six replicates (pots) per treatment and repetition in a randomized complete block design (A30 = ATTRACAP® 30 kg h^{-1}; G30 = AgriMet-Granule 30 kg ha^{-1}; G60 = AgriMet-Granule 60 kg ha^{-1}; DP0.5 = AgriMet-Dry Product $0.5x10^{11}$ conidia ha^{-1}; DP1= AgriMet-Dry Product $1x10^{11}$ conidia ha^{-1}; DP2 = AgriMet-Dry Product $2x10^{11}$ conidia ha^{-1}). **Top:** Dots per treatment represent the percentage of undamaged potatoes out of ten potatoes per pot. Boxplots consist of the median and the 25 % and 75 % quantile. Adjusted mean and 95 % confidence interval of n pots per treatment are illustrated in red and treatments with the same letters are not significantly different. GLM (y = Percentage Undamaged Potatoes ~ Treatment + Year, family=binominal), Tukey HSD test α = (0.05). **Bottom:** Jittered boxplot of recaptured *A. obscurus* larvae per pot. Five larvae per pot were released at the beginning. Boxplots consist of the median and the 25 % and 75 % quantile. Adjusted mean of recaptured *A. obscurus* larvae and 95 % confidence interval of the replicates per treatment are illustrated in red and treatments with the same letters are not significantly different. GLM (y = Recaptured Wireworms ~ Treatment + Year, family=poisson), Tukey HSD test (α = 0.05).

The effectiveness of the treatments based on the calculation according to Abbott (1925) is shown in Table 9 and indicated a dose-related reduction of tuber damage by the AgriMet formulations. The field application rates of the respective formulations resulted in an effectiveness of approximate 22 %. A 100-fold increase in the application rate of the AgriMet-Granule and AgriMet-Dry Product doubled the effectiveness to a maximum of 54.0 % and 44.1 %, respectively.

Table 9: Effectiveness of the *Metarhizium brunneum* formulations as the percentage of reduced potato damage compared to the untreated control during greenhouse experiments. Mean of all replicates (n) and standart deviation (±SD) was calculated from the effectiveness for each treatment within a block according to Abbott (1925).

Treatment	**Application rate**	**n**	**Effectiveness [%]**	
			mean	±SD
ATTRACAP ®	30 kg ha^{-1}	12	19.9	22.1
AgriMet-Granule	30 kg ha^{-1}	18	18.8	23.34
AgriMet-Granule	60 kg ha^{-1}	12	22.0	22.91
AgriMet-Granule	300 kg ha^{-1}	6	54.0	10.66
AgriMet-Dry Product	$1x10^{11}$ conidia ha^{-1}	12	24.1	23.43
AgriMet-Dry Product	$2x10^{11}$ conidia ha^{-1}	12	25.4	22.19
AgriMet-Dry Product	$1x10^{13}$ conidia ha^{-1}	6	44.1	23.71
AgriMet-Granule + Dry Product	30 kg ha^{-1} + $1x10^{11}$ conidia ha^{-1}	18	29.5	22.41
AgriMet-Granule + Dry Product	60 kg ha^{-1} + $2x10^{11}$ conidia ha^{-1}	6	42.8	18.29

Effectiveness of the *Metarhizium brunneum* formulations in the laboratory

A laboratory bioassay with four different application rates of the *Metarhizium brunneum* formulations AgriMet-Granule and AgriMet-Dry Product was performed to define the required amount for a reduction of the wireworm species *Agriotes obscurus*, *A. sputator* and *A. lineatus*. The field application rate of 30 kg ha^{-1} for the AgriMet-Granule and $1x10^{11}$ conidia ha^{-1} for the AgriMet-Dry Product was increased exponentially because no information on the lethal effects was previously available. The Kaplan-Meier survival curves in Figure 14 show, that the mortality caused by the *M. brunneum* formulations was very low, although the field application rates have been increased up to 100 times. Only the AgriMet-Dry Product resulted in a noteworthy reduction of survival probability of *A. obscurus* with significant differences between the application rates (log-rank test, $p < 0.0001$). The field application rate of $1x10^{11}$ conidia ha^{-1} killed almost no larva of *A. obscurus* and reduced the survival probability only to 98 % after 59 days. A tenfold increase of the application rate to $1x10^{12}$ conidia ha^{-1} reduced the survival probability to 63 % after 77 days, which differed significantly from the field application rate (Bonferroni, $p < 0.01$). After 66 days of incubation with an application rate of $5x10^{12}$ conidia ha^{-1} of the AgriMet-Dry Product the survival probability of *A. obscurus* was at

28 % and differed significantly to the lower ones (Bonferroni, $1x10^{11}$ conidia ha^{-1} = p < 0.0001, $1x10^{12}$ conidia ha^{-1} = p < 0.01). The LT_{50} of this concentration was 54 days. The highest application rate of $1x10^{13}$ conidia ha^{-1} led to the lowest survival probability of 18 % after 63 days with a LT_{50} of 45.5 days.

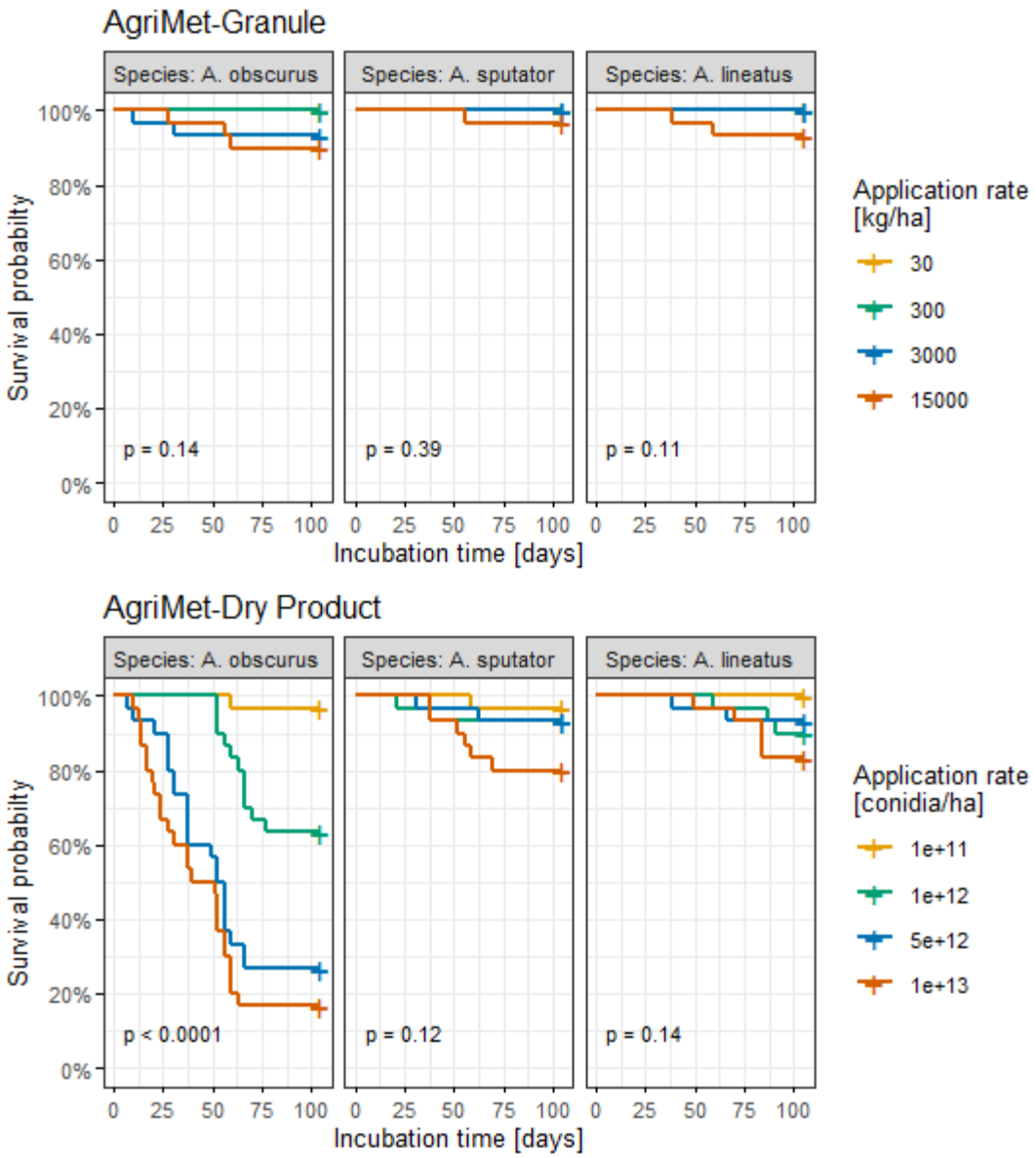

Figure 14: Overall survival probability in percent (Kaplan-Meier-Analysis) of the wireworm species *Agriotes obscurus*, *A. sputator* and *A. lineatus* incubated with four different application rates of the *Metarhizium brunneum* formulations AgriMet-Granule and AgriMet-Dry Product over 105 days under laboratory conditions in small cans filled with soil at 25 °C and darkness. The statistic of the shown *p*-values is based on the log-rank test (α = 0.05) and indicates differences between the application rates within a formulation and the respective wireworm species.

However, there was no significant difference between the two highest application rates of the AgriMet-Dry Product. The lethal effect of the AgriMet-Dry Product against *A. sputator* and *A. lineatus* was much weaker and only the highest application rate of $1x10^{13}$ conidia ha^{-1} was able to reduce the survival probability to a maximum of 80 % after 70 days for *A. sputator* and 82 % after 84 days for *A. lineatus*. The lower application rates resulted in only a few infected larvae of *A. sputator* and *A. lineatus*. The bioassay with the AgriMet-Granule against *A. obscurus*, *A. sputator* and *A. lineatus* resulted in no killed larva at the field application rate of 30 kg ha^{-1}. Only very few larvae with a mycosis at the highest application rate of 15000 kg ha^{-1} were examined, but no differences to the lower application rates could be calculated. Because of the very low mortality caused by the *M. brunneum* formulations the calculation of the lethal concentration, at which 50 % of the individuals died, was only possible for the AgriMet-Dry Product and *A. obscurus*. The LC_{50} value after 105 days of incubation was $2.86x10^{12}$ conidia ha^{-1} (FI 95 % $1.68x10^{12}$-$4.96x10^{12}$ conidia ha^{-1}) and is shown in Table 10.

Table 10: Effectiveness of the *Metarhizium brunneum* formulations AgriMet-Granule and AgriMet-Dry Product against the wireworm species *Agriotes obscurus*, *A. sputator* and *A. lineatus* after 105 days of incubation under laboratory conditions at 25 °C and darkness in small cans. Probit analysis was used to calculate the values of the lethal concentration (LC_{50}) for each wireworm species exposed to the respective *M. brunneum* formulation individually.

Wireworm species	**No. of larvae**	***M. brunneum* formulation**	**LC_{50} (Conidia ml^{-1})**	**95 % Fiducial interval**		**Slope**	**± SE_a**	**X^2_b**	**df_c**	**$pgof_d$**
				Lower	**Upper**					
Agriotes	150	AgriMet-Dry Product	$2.86x10^{12}$	$1.68x10^{12}$	$4.96x10^{12}$	1.09	0.195	13.6	18	0.754
obscurus	150	AgriMet-Granule				*				
Agriotes	150	AgriMet-Dry Product				*				
sputator	150	AgriMet-Granule				*				
Agriotes	150	AgriMet-Dry Product				*				
lineatus	150	AgriMet-Granule				*				

*Unable to estimate because mortality caused by any concentration of the respective formulation was < 50 %

a Standard Error of the slope

b Chi-square goodness-of-fit on the probit model

c degree of freedom

d Pearson's chi square goodness-of-fit test ($\alpha = 0.05$)

Discussion

In this study, the effectiveness of the AgriMet-Granule and the AgriMet-Dry Product against wireworms for the use in potato cultivation were tested in field experiments. A prerequisite for a reliable investigation of pesticide effectiveness in the field is the presence of an adequate pest infestation to ensure realistic conditions (Anonymous 2012a). Across all field trials, the proportion of undamaged potatoes in the untreated controls ranged between 0.3-13.1 % indicating a very high degree of wireworm infestation. The reason for this is probably on the cultivation history, as all field sites used for the experiments were uncultivated fallows that were prepared by mechanical tillage shortly before potato planting. Since the previous dense green was suitable for oviposition of click beetles and larval development was not disturbed by any agricultural measures, a large wireworm population was able to establish (Gough & Evans 1942, Seal et al. 1992, Furlan 1996, Furlan et al. 2009). Biocontrol agents often show lower effectiveness in case of a very high pest incidence and are recommended for low or medium infestation, such as the tested reference product ATTRACAP® (BIOCARE GmbH 2020). In the field trials performed in this study, ATTRACAP® with an application rate of 30 kg ha^{-1} reduced the tuber damage only by 2.1-11.3 % compared to the untreated control. The effectiveness of the AgriMet-Granule (30-60 kg ha^{-1}) and the AgriMet-Dry Product (1-2x10^{11} conidia ha^{-1}) was comparable to ATTRACAP® depending on the respective year, but overall too low for a worthwhile control strategy. Neither the single application of the AgriMet formulations nor the combined application of both could increase the effectiveness above 14 % compared to the untreated control. Previously approved chemical insecticides, such as Fipronil, applied in potatoes achieved an effectiveness of 50-85 %, which would also be desirable for the AgriMet formulations (Kuhar & Alvarez 2008, Ladurner et al. 2009). The low effectiveness is probably due to a complex combination of various factors, which influence the control potential of *M. brunneum* in the soil. The following discussion focuses on these factors and suggestions on how a successful wireworm control strategy based on *M. brunneum* might be implemented.

In general, the AgriMet formulations were intended to ensure pest reduction by inoculative biocontrol. For this, the biocontrol agent *Metarhizium brunneum* should proliferate after application, form conidia in the soil and kill wireworms by initiating the lethal infection cycle (Hajek & St Leger 1994, Roy et al. 2006, Vega et al. 2012). The

level of wireworm susceptibility is closely related to the conidia concentration (Quesada-Moraga et al. 2006, Gabarty et al. 2014). The first factor in this context was the appropriate application rate of the AgriMet-Granule and AgriMet-Dry Product, which was investigated under standardised conditions in the greenhouse and laboratory. Using the field application rates in the greenhouse experiment resulted in a slightly better effectiveness of 21.9-25.4 %. In addition to the constant temperature in the pots, regular moistening by watering might have favoured fungal proliferation and thereby enhanced the effectiveness (Dimbi et al. 2004, Ekesi et al. 2003, Arzumanov et al. 2005). An acceptable effectiveness in the greenhouse of up to 44.1-53.9 % was achieved by increasing the application rate of each formulation by a factor of one hundred. However, the reduced tuber damage in the greenhouse was not the result of killed wireworms, since no significant differences in the number of recaptured larvae compared to the untreated control were observed. Therefore, the laboratory experiment was designed to provide information on the lethal potential of the formulations, because reducing wireworms was the overall goal of the control strategy.

The testing of the AgriMet formulations in the laboratory revealed that even an application rate of 15,000 kg ha^{-1} of the AgriMet-Granule resulted only in a 2-10 % decrease in survival probability for all tested wireworm species. This indicated a lack of sufficient conidia formation and related lethal effectiveness. In contrast, the AgriMet-Dry Product with application rates of $5x10^{12}$-$1x10^{13}$ conidia ha^{-1} reduced the survival probability of *A. obscurus* by 82 %, which underlined its control potential. However, in contrast to the greenhouse and field experiments, larvae in the laboratory bioassay were exposed to the AgriMet-Dry Product in very narrow space without the possibility to avoid fungal infested areas. Based on the inoculative biocontrol approach, extensive proliferation of *M. brunneum* in the potato ridge is required to provide the lethal potential examined in the laboratory experiments. The abundance of *Metarhizium* spp. in the potato ridge during field trials, described as the colony-forming units (CFU) per g soil, reached a maximum of 959.9 CFU g^{-1} by the combined application of the AgriMet-Dry Product ($1x10^{11}$ conidia ha^{-1}) and the AgriMet-Granule (30 kg ha^{-1}) in 2018. None treatment surpassed 200 CFU g^{-1} soil in 2019 and 2020. Since the desired proliferation of *Metarhizium* spp. in the potato ridge could only be detected to a limited extent, the AgriMet formulations do not seem to be suited for the desired inoculative control strategy and several reasons are worth considering. First, the area of soil sampling may not match with the area in which the formulations were applied. Immenroth (not

published) verified the distribution of the AgriMet-Granule evenly and close around the seed potatoes. The simple spray application of the AgriMet-Dry Product with the potato seed dressing during planting also accomplished a surface distribution of infectious *M. brunneum* conidia in the area of the seed potatoes (Matthews 2008). As a result, an error in soil sampling can be excluded for the low *Metarhizium* spp. concentration in the potato ridge. Second, the soil properties may have had an impact on the fungal proliferation after mycoinsecticide application. Since the analysed properties of the light loamy-sandy soils of the different field sites were comparable and did not show any striking values, this aspect can also be excluded. It is, however, more likely that the application rate might be responsible for the low CFU's g^{-1} soil recovered during the field trials. Consistent with the results presented here, a previous experiment by Rogge et al. (2017), using *M. brunneum* formulated as fungal colonised barley kernels, showed that the application of $1x10^{12}$ conidia ha^{-1} resulted in 153-456 CFU g^{-1} substrate. With this treatment, most of the tested wireworms survived. However, the authors reported that an application rate of $1x10^{15}$ conidia ha^{-1} led to 182,945 CFU g^{-1} substrate. This concentration of approximately $1x10^{6}$ CFU g^{-1} soil significantly reduced the number of tested wireworms and should therefore be used as benchmark for inoculative biocontrol strategies. Thus, the application rate may have had an influence on the low effectiveness of the AgriMet formulations during the field trials due to the insufficient proliferation of *M. brunneum* and related lack of infectious conidia. Despite the promising results of the AgriMet-Granule and AgriMet-Dry Product at very high application rates in the greenhouse, 3000 kg ha^{-1} or rather $1x10^{13}$ conidia ha^{-1} are not feasible for particle use, since the application technology has limitations (personal communication, Lehner Maschinenbau GmbH, 2020). Moreover, the cost for the formulations would exceed the additional revenue from more marketable potatoes. However, the evaluation of the application rate has to be considered with caution, as no consistent product stability was given.

Product stability is an important formulation property that determines, among other factors, storability. *M. brunneum* needs to be remain viable during storage without loss of activity and degradation of the desired formulations properties (Jones & Burges 1998). However, both the AgriMet-Granule and the AgriMet-Dry Product show deficits in terms of product stability during storage. For the AgriMet-Dry Product, a concentration of $1.25x10^{9}$ CFU g^{-1} was reported by the manufactures and $5.6\text{-}9.6x10^{8}$ CFU g^{-1} was finally assessed. The small loss of vitality could be due to the heterogeneous

particle size of the powder and the small quantity used for the quality control. The AgriMet-Granule also showed clear deficits in storage stability four weeks after shipment. A vitality loss of up to 20 % in individual granule batches was assessed, compared to the manufacture specifications, indicating a critical factor influencing the effectiveness. Based on the results of the quality control, biological activity was expected on average only at 75 % of the respective quantities applied, which might be another explanation for the low CFU values in the field trials. Since the outgrowth rate of the reference product ATTRACAP® ranged between 97-100 %, encapsulation of the fungal biocontrol agent seems to provide better protection during storage compared to the outer coating of the AgriMet-Granule. Additional coatings on the AgriMet-Granule could be helpful to protect the fungus from physical or chemical influences and thus improve product stability (Jones & Burges 1998). Besides the preserved vitality of the organism during storage and shipment, the fungus must be able to proliferate under the environmental conditions at the target site. Therefore, abiotic factors must also be considered when evaluating the effectiveness to account for the ecological requirements of *M. brunneum*.

Granule formulations of entomopathogenic fungi with cereal grains are advantageous in principle because they provide a suitable substrate for fungal growth and prolong persistence in the soil (Burges 1998). Since only 5.89×10^4 conidia grain^{-1} were determined on the surface of the AgriMet-Granule, the thin *M. brunneum* layer on the surface of the autoclaved millet must first proliferate for the formation of sufficient fungal material after application. Growth, germination and related virulence of *Metarhizium* spp. are temperature-dependent with an optimum of 25-30 °C, whereas lower (15 °C) or higher (35 °C) temperatures influence the development negatively (Dimbi et al. 2004, Ekesi et al. 1999, Bugeme et al. 2009, Tumuhaise et al. 2018). Bernhardt (not published) verified the mentioned temperature conditions for the *M. brunneum* isolate JKI-BI-1450 used for the AgriMet formulations. At the beginning of all field trials, the soil temperature in the potato ridge showed a daily mean of 9.8-14.7 °C. Soil warmed up very slowly, reaching an average temperature of 17.1 °C. A daily mean temperature of 25 °C was determined on a single day in the end of July 2019. Overall, the soil temperature in the potato ridge was too low for optimal growth and germination of isolate JKI-BI-1450, especially during the application in April/May. This was confirmed by the quality control of the AgriMet-Granule in field soil, which resulted in no visible fungal growth or sporulation after incubation at 15 °C for 14 days. Crucial

problems arise from the very slow growth of the *M. brunneum* isolate JKI-BI-1450 on the AgriMet-Granule at low soil temperatures. First, seasonal activity of wireworms requires lethal potential by *M. brunneum* shortly after application. The wireworms move into upper soil layers in early spring and autumn preferring foraging at suitable soil moisture and temperature (Langenbuch 1932, Brian et al. 1947, Burrage et al. 1963, Parker & Howard 2001). Summer drought as well as moulting can decrease wireworm activity in the upper soil layer in between these periods, reducing the likelihood of contact with formulated *M. brunneum* (Evans & Gough 1942, Furlan 1998, Staley et al. 2007). Second, larval death can take 41-80 days after contact with fungal conidia, depending on the respective isolate and wireworm species (Chapter III, Brandl et al. 2017). Therefore, infection by *M. brunneum* later in the potato growing season would not prevent damage to daughter tubers (Hack et al. 1993). Third, until *M. brunneum* proliferation on the AgriMet-Granule occurs, the autoclaved millet grain serves as nutrient-rich substrate for ubiquitous soil-borne fungi using accessible carbon sources for metabolism and growth (Geervani & Eggum 1989, Dighton 2007, Kennedy 1994, Deacon 2013). Especially opportunistic saprophytic fungi from agricultural soils are adapted to low temperatures and might compete for the autoclaved millet after application (Hajek 1997, Pietikäinen et al. 2005, Bárcena-Morena et al. 2009). The decomposition of the coated autoclaved millet grain might prevent the development of the isolate JKI-BI-1450. Thus, wireworm control with the AgriMet-Granule cannot be successful at the recorded temperatures. However, even the incubation of the AgriMet-Granule at 25 °C for 14 days only led to a minimal increase in conidia concentration to $7.1x10^5$ conidia $grain^{-1}$. This might explain the lack of lethal potential in the greenhouse and laboratory tests, since insufficient amounts of conidia are formed in soil to kill wireworms (Chapter III). However, the AgriMet-Dry Product is also negatively affected by the low temperatures during application. The spray application should directly hit the wireworms through droplets or colonise organic material, but fungal germination should be unlikely under the conditions recorded. Thus, temperature in the potato ridge is another critical factor influencing the effectiveness of the AgriMet formulations in the field. Effectiveness of mycoinsecticide formulations against wireworms may be improved by using isolates of the genus *Metarhizium* from other geographic regions that show greater thermotolerance for low temperatures. For example, Isolate ARSEF 4343 originating from Macquarie Island close to the Antarctica was cold-active even at 5 °C and might be better suited for the use in potato cultivation (Fernandes et al. 2008).

However, the thermotolerance of an isolate must be in proportion to its virulence, which leads to the next point of discussion.

Usually, several wireworm species occur simultaneously in one field because larval development takes 3-5 years and the populations overlap (Subklew 1935, Miles 1942, Klausnitzer 1994, Furlan 1998). Thus, a universal and effective application of the AgriMet formulations in potato cultivation throughout Germany requires a high virulence of formulated *M. brunneum* isolate JKI-BI-1450 against the most important wireworm species. However, *A. obscurus*, *A. sputator* and *A. lineatus*, exposed to the AgriMet-Dry Product in the laboratory bioassay, showed significant differences in mortality. While the survival probability of *A. obscurus* was reduced by 80 %, the survival probability of *A. sputator* and *A. lineatus* decreased by only 20 %, indicating selectivity of isolate JKI-BI-1450. Bernhardt et al. (2019) confirmed the selectivity of isolate JKI-BI-1450 against *A. obscurus* in a standardised laboratory dip test. As *A. lineatus* was the most abundant wireworm species during the performed field trials and isolate JKI-BI-1450 showed only low virulence against it, no relevant control potential could be expected by the application of the AgriMet formulations in the field. The specific virulence of *M. brunneum* isolates intended for wireworm control poses a crucial problem in practical use. Lehmhus (2019) reported a diverse wireworm species composition on German potato fields varying by region. The most common species are *A. obscurus*, *A. sputator* and *A. lineatus*, but further species of agronomic importance like *A. ustulatus* and *A. sordidus* occurred in the south/southwest of Germany. Lehmhus (2019) postulated that the change to a warmer climate might advance the distribution of new, economic important species. It is therefore unlikely that a mycoinsecticide containing a single biocontrol agent is capable to control the diverse pest complex of wireworms.

Several factors such as the application rate, soil temperature and isolate selectivity might explain the low or missing effectiveness of the AgriMet formulations in the field. Consequently, the recorded reduction of tuber damage during the field and greenhouse experiments cannot be attributed to the reduction of wireworms alone. The effect of the AgriMet formulations in the field and greenhouse might probably be the result of behavioural changes of wireworms after fungal infection. Previous studies with the desert locust *Schistocerca gregaria* and the grasshopper *Zonocerus variegatus* demonstrated a reduction of feeding after infection with *M. flavoviride* (Moore et al. 1992, Thomas et al. 1997). The pollen consumption of the flower thrips *Megalurothrips*

sjostedti was also reduced by an infection with *M. anisopliae* (Ekesi et al. 1999). A sublethal infection of wireworms may have influenced the foraging behaviour and thereby resulted in a reduction of tuber damage. Another partial reason for the field trial results could be the spatial distribution of wireworms at the field site and the associated experimental design. Because click beetles can lay eggs in multiple clusters during oviposition, the occurrence of larvae in the field is not uniform (Furlan 1996, Sufyan et al. 2014). Wireworm sampling in each plot confirmed a patchy distribution, and minor tuber damage, regardless of treatment, was observed primarily in plots where very few or no wireworms were found. These individual plots may have influenced the significance of the statistical comparison of the mean proportion of undamaged potatoes. Reliable data could be generated by using more blocks/repetitions in the experimental design, reducing the weighting of each plot. Another option could be the systematic placement of blocks at the field site, taking into account the distribution of wireworms. The use of excluded or adjacent untreated controls could be useful to evaluate the effectiveness of treatments without bias from wireworm distribution (Anonymous 2012a).

In conclusion, none of the AgriMet formulations based on the biocontrol agent *M. brunneum* improved tuber protection compared to ATTRACAP® after field application. Insufficient product stability, low soil temperatures during spring application and the limited virulence of the formulated isolate JKI-BI-1450 against *A. lineatus* and *A. sputator* may have had a decisive influence on the control potential of wireworms. The AgriMet-Granule and the AgriMet-Dry Product need be optimised based on these influencing factors to ensure a meaningful field use. Future field research on the effectiveness should be based on knowledge of the detailed composition and spatial distribution of wireworm species to avoid bias due to the experimental design. Only reliable effectiveness data of field trials can convince agricultural practitioners to use *M. brunneum* against wireworms in potato cultivation and promote sustainable agriculture through biocontrol.

Chapter III

Combining two *Metarhizium* spp. isolates to enhance the effectiveness against the wireworm species *Agriotes obscurus*, *A. sputator* and *A. lineatus*

Abstract

Although numerous *Metarhizium*-based mycoinsecticides were tested for the use against wireworms (Coleoptera: Elateridae) in potato cultivation, the effectiveness was low and/or fluctuating. Since different wireworm species may be present on a given potato field, host specificity may be the reason for inadequate control success when using *Metarhizium* spp. as biocontrol agent. For this reason, a combined treatment using two *Metarhizium* spp. isolates (*M. brunneum* isolate JKI-BI-1450, *M. robertsii* isolate JKI-BI-1441) for effective wireworm control was tested. Therefore, the LC_{50} and LT_{50} of the single *Metarhizium spp.* isolates and their combination were compared after a standardised bioassay in the laboratory with the wireworm species *A. obscurus*, *A. lineatus* and *A. sputator*. In addition, a confrontation test in petri dishes was carried out to evaluate the tolerance of the *Metarhizium spp.* isolates in terms of growth and sporulation for a desirable formulation. The bioassay indicated that a high conidia concentration between $1x10^6$-$1x10^7$ conidia ml^{-1} was necessary for lethal wireworm infection, while host specificity was confirmed. *Agriotes obscurus* and *A. sputator* showed significant differences in survival probability exposed to the single isolates. The combined application of the two isolates resulted in an additive effectiveness and survival probability of the two wireworm species exposed to $1x10^7$ conidia ml^{-1} was comparably reduced to 18-38 %. However, neither the solo nor the combined treatment of the tested *Metarhizium* spp. isolates resulted in a significant decrease in survival probability of *A. lineatus*. Confrontation of the *Metarhizium* spp. isolates on agar plates revealed differences in their proliferation performance, which may be problematic for the production of a granule formulation. Nevertheless, the data suggest that the combined application of two *Metarhizium* spp. isolates is promising and the next step could be to test a two isolate-based wettable powder in the field.

Introduction

Entomopathogenic fungi have been used for biological control of insect pests since the 1880s (Steinhaus 1956). The most important representatives are from the genera *Beauveria* and *Metarhizium*, which are successfully applied against pest insects in many agriculture crops, like sugarcane in Brazil or coffee in Colombia (Van Lenteren et al. 2018). The use of these soil-borne fungi permits pest control with minimal impact on the natural biodiversity of the respective ecosystem while ensuring human safety (Zimmermann 2007, Skinner et al. 2014). Since the fungal mode of action is based on contact between infectious conidia and target pest cuticle, pest control using entomopathogenic fungi includes the application of fungal propagules that aims to increase the likelihood of required contact (Hajek & St Leger 1994). Based on the occurrence as natural antagonist of numerous agricultural pests, entomopathogenic fungi are of great importance for future control strategies of the integrated pest management (Shah & Pell 2003).

The entomopathogenic fungus genus *Metarhizium* provides the opportunity for a sustainable and environmentally friendly control strategy against wireworms in potato cultivation (Barsics et al. 2013). Wireworms are the larval stage of click beetles (Coleoptera: Elateridae). The feeding damage to potatoes (*Solanum tuberosum*) by the soil-dwelling pest led to significant quality losses emphasising the economic importance (Parker & Howard 2001, Vernon & Van Herk 2013b). Due to the 3-5-year life cycle in the soil, various wireworm species can occur at one field site at the same time (Klausnitzer 1994, Furlan 1998). With regard to potato cultivation in Germany, the genus *Agriotes* is the most widespread including the species *A. obscurus*, *A. lineatus* and *A. sputator* (Ritter & Richter 2013). Due to several successful in-vitro studies, the use of *Metarhizium* spp. to control wireworms has been considered a promising alternative to the application of insecticides (Ansari et al. 2009, Kleespies et al. 2013, Eckard et al. 2014). In recent years, possible *Metarhizium* formulations like fungus colonised cereal kernels (Kabaluk et al. 2007, Rogge et al. 2017), complex alginate beads (Brandl et al. 2017) or fungus coated millet grains (Chapter II) were investigated for the application against the soil-dwelling pest. Kabaluk et al. (2007) and Rogge et al. (2017) demonstrated satisfactory results with their *Metarhizium* spp. formulations, while testing an individual sensitive wireworm species. In contrast, the presence of various wireworm species at one field site, as in the field trials of Brandl et al. (2017) and Chapter II, resulted in fluctuating effectiveness of the *M. brunneum* formulations used.

Controlling various wireworms at one given field site might be problematic due to a limited virulence of a given *Metarhizium* spp. isolate (Ansari et al. 2009, Kabaluk et al. 2013, Eckard et al. 2014). A screening for an appropriate *Metarhizium* spp. isolate against *Agriotes obscurus*, *A. lineatus* and *A. sputator* by Bernhardt et al. (2019) revealed that the tested isolates were able to infect larvae of the three mentioned species, but not to the same extent. This indicated that a single *Metarhizium* spp. isolate might not be suitable for an efficient control of wireworms considering an unknown species composition in practical field use.

In the past, the combined treatment of *Metarhizium* spp. with entomopathogenic nematodes, or bacterial metabolites obtained an enhanced control potential for agricultural pests. A study by Ansari et al. (2004) showed that the mixed application of *M. anisopliae* and nematodes of the species *Steinernema glaseri* increased larval mortality of white grubs (*Hoplia philanthus* Füssly) compared to the individual treatment with the biocontrol agents. Ericsson et al. (2007) reported that the synergistically interaction of Spinosad (bacterial metabolites) and *M. anisopliae* led to a significantly higher mortality of wireworms compared to the treatments alone. However, both studies did not consider the host range and mainly focused on measuring the effectiveness rate against target pests. So far, the combination of two or more *Metarhizium* spp. isolates with the goal to have generic control against wireworms has not been investigated.

Here, the effectiveness of a combined application of two *Metarhizium* spp. isolates against wireworms for an enhanced control of the most important wireworm species, with regard to potato cultivation in Germany, was tested. For this purpose, the tolerance of the *M. brunneum* isolate JKI-BI-1450 and the *M. robertsii* isolate JKI-BI-1441 in terms of growth and sporulation was tested by a confrontation test in Petri dishes. Furthermore, larvae of *Agriotes obscurus*, *A. lineatus* and *A. sputator* were exposed to different concentrations of single and combined *Metarhizium* spp. isolates (JKI-BI-1450, JKI-BI-1441) in a standardised laboratory bioassay. Comparing the effectiveness of the single and combined treatment in terms of survival probability, the LC_{50} and the LT_{50} indicated a promising possibility for the control of the most important *Agriotes* species using two *Metarhizium* spp. isolates for future mycoinsecticide formulations.

Material and Methods

Wireworm breeding for the bioassay

Wireworm larvae of the species *Agriotes obscurus*, *A. sputator* and *A. lineatus* originated from the laboratory breeding for a species-specific examination. Following the protocol of Kölliker et al. (2009) with minor modification, click beetles of the respective species were collected in Wohld (52°18’11.0"N 10°41’11.6"E) and determined to species level using the identification key of Lohse (1979). Beetles were kept in buckets with soil and wheat at 20 °C for laying eggs. After eight months, emerged larvae were removed and stored at 5 °C in plastic boxes (18.3 x 13.6 x 6.4 cm, Baumann Saatzuchtbedarf, Waldenburg, Germany) with moist paper towel. Fourteen days before the start of an experiment, wireworms were transferred into soil at 15 °C for acclimatisation and fed with *Triticum aestivum* seeds (Cultivar: Primus, Deutsche Saatgutveredelung AG, Lippstadt, Germany) to provide vital larvae.

Metarhizium spp. isolates for the confrontation test and bioassay

The *Metarhizium brunneum* isolate JKI-BI-1450 was isolated from an adult of *Agriotes* sp. collected from a meadow in Wohld (52°18’11.0"N 10°41’11.6"E, Germany). The *M. robertsii* isolate JKI-BI-1441 originated from an *Agriotes* sp. larva gathered during field surveys in Italy. Both isolates were provided by Tanja Bernhardt (JKI, Institute for Biological Plant Protection, Darmstadt, Germany) and stored dark at 5 °C until use.

Effectiveness of the *Metarhizium* spp. isolates against wireworms in the laboratory

Fungal inoculum of the Metarhizium spp. isolates

The *Metarhizium* spp. isolates JKI-BI-1450 and JKI-BI-1441 were cultured on malt peptone agar for 14 days at 25 °C in darkness. Fully overgrown plates were washed with 3 ml of 0.1 % Tween80® (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and scraped with a single use spatula (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) to detach aerial conidia. The suspensions were filtered to remove mycelium and homogenised by vortexing. Multiple concentration of the conidia suspensions of the two isolates were prepared by dilution with 0.1 % Tween80® using a haemocytometer.

Bioassay for the LC_{50} and LT_{50} determination

The determination of the lethal concentrations that caused 50 % mortality (LC_{50}) by the individual *Metarhizium* spp. isolates and the combination of both were carried out using five concentrations: $1x10^3$ conidia ml^{-1}, $1x10^4$ conidia ml^{-1}, $1x10^5$ conidia ml^{-1}, $1x10^6$ conidia ml^{-1}, $1x10^7$ conidia ml^{-1}. Larvae of *Agriotes obscurus, A. sputator* and *A. lineatus* were dipped singly in 10 ml fungal suspension for 10 s. For the combination of the isolates, 5 ml of the respective concentration were mixed. Control larvae were dipped in 0.1 % Tween80®. After dipping, each larva was transferred into a plastic can (50 ml, OPTIMAX Packaging GmbH & Co KG, Norderstedt, Germany) filled with 30 g soil (Einheitserde Classic Pikiererde CL P, Gebrüder Patzer GmbH & Co. KG, Sinntal, Germany) and stored in darkness at 25 °C. During the trial, wireworms were fed every two weeks with *Triticum aestivum* seeds (Cultivar: Primus, Deutsche Saatgutveredelung AG, Lippstadt, Germany) to avoid starving. To define mortality caused by *Metarhizium* spp., infected wireworms were identified based on fungal outgrowth from the cadaver and on morphological criteria of *Metarhizium* spp. (Zimmermann 2007). This assessment was performed twice a week for 130 days to determine the lethal time. Each isolate-concentration combination contained six larvae of the respective wireworm species and the bioassay was repeated five times. Thus, a total of 1620 larvae were investigated.

Confrontation test of the *Metarhizium* spp. isolates

Confrontation test of the *Metarhizium* spp. isolates JKI-BI-1450 and JKI-BI-1441 were performed on MPA plates with a diameter of 9 cm (Greiner Bio-One GmbH, Kremsmünster, Austria). Plates were evenly inoculated with 100 µl of a freshly prepared conidia suspension of the respective *Metarhizium* spp. isolate containing $1x10^7$ conidia ml^{-1} and preincubated at 25 °C in the dark. After two days, agar plugs with a diameter of 1 cm carrying actively growing mycelium were punched out from the centre of the plates and transferred to the centre of new MPA plates with a uniform spacing of 2 cm to the outer edge and 3 cm between two plugs. The following variants were prepared and incubated at 25 °C in darkness: (V1) isolate JKI-BI-1450 against no plug, (V2) isolate JKI-BI-1441 against no plug, (V3) isolate JKI-BI-1450 against isolate JKI-BI-1450, (V4) isolate JKI-BI-1441 against isolate JKI-BI-1441, (V5) isolate JKI-BI-1450 against isolate JKI-BI-1441. The comparison of the control variants (V1-4) with the direct confrontation of the isolates used (V5) should provide evidence about their

compatibility. In order to observe a possible mutual interference to the proliferation of the isolates tested, three plates per variant were analysed in terms of conidia formation and radial growth after 8, 10, 12 and 14 days of incubation. Because examined plates were removed, twelve plates of each variant were prepared for each variant. To determine the radial growth of the isolates, comparable photos of each plate were taken using the reflex camera EOS 2000D Kit (Canon, Tokyo, Japan) equipped with a 18-55 mm objective (Canon, Tokyo, Japan). The diffuse exposure, the distance between plate and camera (37 cm) and all camera settings were always the same to ensure comparability of the photos. Fungal growth out of the respective plugs was measured using the image processing software ImageJ (Rasband 1997) and expressed as mm^2. To determine the conidia concentration of the isolates, a plug with a diameter of 3 cm was punched out around the original placed, transferred to a 15 ml falcon (Brand GmbH & Co. Kg, Wertheim, Germany) and vortexed in 2 ml 0.1 % Tween80® (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) for 10 sec to remove attached conidia. The conidia concentration was determined using a haemocytometer and expressed as conidia ml^{-1}. The described experiment was repeated three times resulting in a total of nine replicates per variant.

Statistical analyses

Statistical analyses were carried out using the software R Studio (Version 1.4.1106) (RStudio Team 2020).

Kaplan-Meier-Analysis was used to determine the survival probability of the tested *A. obscurus*, *A. sputator* and *A. lineatus* larvae over time after exposure to the different conidia concentrations of the fungal treatments. Survival curves over time were created with the *survival* R package (Therneau 2021) and compared with the log-rank test to determine global effects. Significant differences between the conidia concentrations within a wireworm species and *Metarhizium* treatment were detected via a pairwise comparison of survival curves based on the Bonferroni method using the *survminer* R package (Kassambara et al. 2021). To determine the time at which 50 % of the tested individuals died due to the fungal treatment (LT_{50}), the "surv_median()" command of the *survival* R package was used. Mycosis of wireworms was recorded as an "event". If no event was observed by the end of the study, the total survival time could not be accurately determined and was censored. To estimate the LC_{50} values for each

combination of wireworm species and *Metarhizium* treatment, a probit analysis was performed using the *ecotox* R package (Hlina et al. 2019)

The effect of confrontation of the *Metarhizium* spp. isolates (explanatory variable) on the conidia concentration (target variable) and radial growth (target variable) over time (explanatory variable) was analysed by running an analysis of variance with a Linear Model. The conidia concentration data were log transformed to meet the assumption of normal distribution and variance homogeneity.

$$y = log(Conidia\ Concentration) \sim Variante + Day + Variante{:}Day$$

$$y = Radial\ Growth \sim Variante + Day + Variante{:}Day$$

Selection of the best-fitted model was based on the Akaike Information Criterion (AIC) (Burnham & Anderson 2002) after backward elimination of the full model. To ensure the assumptions of normal distribution and variance homogeneity, residuals of the respective model were visually inspected with the QQ-Plot (sample quantile-theoretical quantile) and Residuals-Prediction-Plot. Differences between adjusted means were tested with Tukey HSD test and values with $p < 0.05$ were considered significantly different.

All graphs were created with the R packages *ggplot2* (Wickham 2016), *ggpubr* (Kassambara 2020), *RColorBrewer* (Neuwirth 2014), and *multcompView* (Gravesn et al. 2019).

Results

Effectiveness of the *Metarhizium* spp. isolates against wireworms in the laboratory

A laboratory bioassay was performed to investigate the individual and combined effectiveness of the *Metarhizium* spp. isolates JKI-BI-1450 and JKI-BI-1441 against the wireworm species *Agriotes obscurus, A. sputator* and *A. lineatus.* The Kaplan-Meier survival curves in Figure 15 show significant differences between the used concentrations (log-rank test, $p < 0.0001$). Notable larval mortality of *A. obscurus* and *A. sputator* started at a concentration of $1x10^6$ conidia ml^{-1}, whereby $1x10^7$ conidia ml^{-1} always infected most wireworms significantly (Bonferroni, $p < 0.01$). Only a few larvae were found with mycosis at the lower concentrations ($1x10^3$ conidia ml^{-1}, $1x10^4$ conidia ml^{-1}, $1x10^5$ conidia ml^{-1}) and no larvae were infected in the control with 0.1% Tween 80®. The lethal effectiveness against *A. lineatus* was very low in every treatment and just the *M. brunneum* isolate JKI-BI-1450 ($1x10^7$ conidia ml^{-1}) led to a significant decrease of the survival probability. Overall, the mortality was very low and never reached a survival probability of 0 %. Despite the influence of the concentrations, the isolates differed in their individual effectiveness against the tested wireworm species. The *M. brunneum* isolate JKI-BI-1450 killed the most *A. obscurus* larvae at the highest concentration resulting in a survival probability of 12 % after 85 days and a LT_{50} of 41.5 days. The effectiveness of this isolate against the other species was lower with a survival probability of 70 % (*A. sputator*) and 62 % (*A. lineatus*) after 130 days. In contrast, the *M. robertsii* isolate JKI-BI-1441 infected the most *A. sputator* larvae at the highest concentration after 117 days with a survival probability of 22 % and a LT_{50} of 80 days. *Agriotes obscurus* larvae were less effected by the isolate JKI-BI-1441 with a survival probability of 58 % after 106 days and only a few *A. lineatus* showed a mycosis at the end of the experiment. The combination of both isolates differed less in the probability of survival against *A. obscurus* and *A. sputator* compared to the individual treatments. The survival probability of *A. obscurus* was at 18 % after 110 days (LT_{50} 54 days) and the survival probability of *A. sputator* was at 38 % after 117 days (LT_{50} 97.5 days). However, the combination could not reduce the survival probability of *A. lineatus* below 80 %.

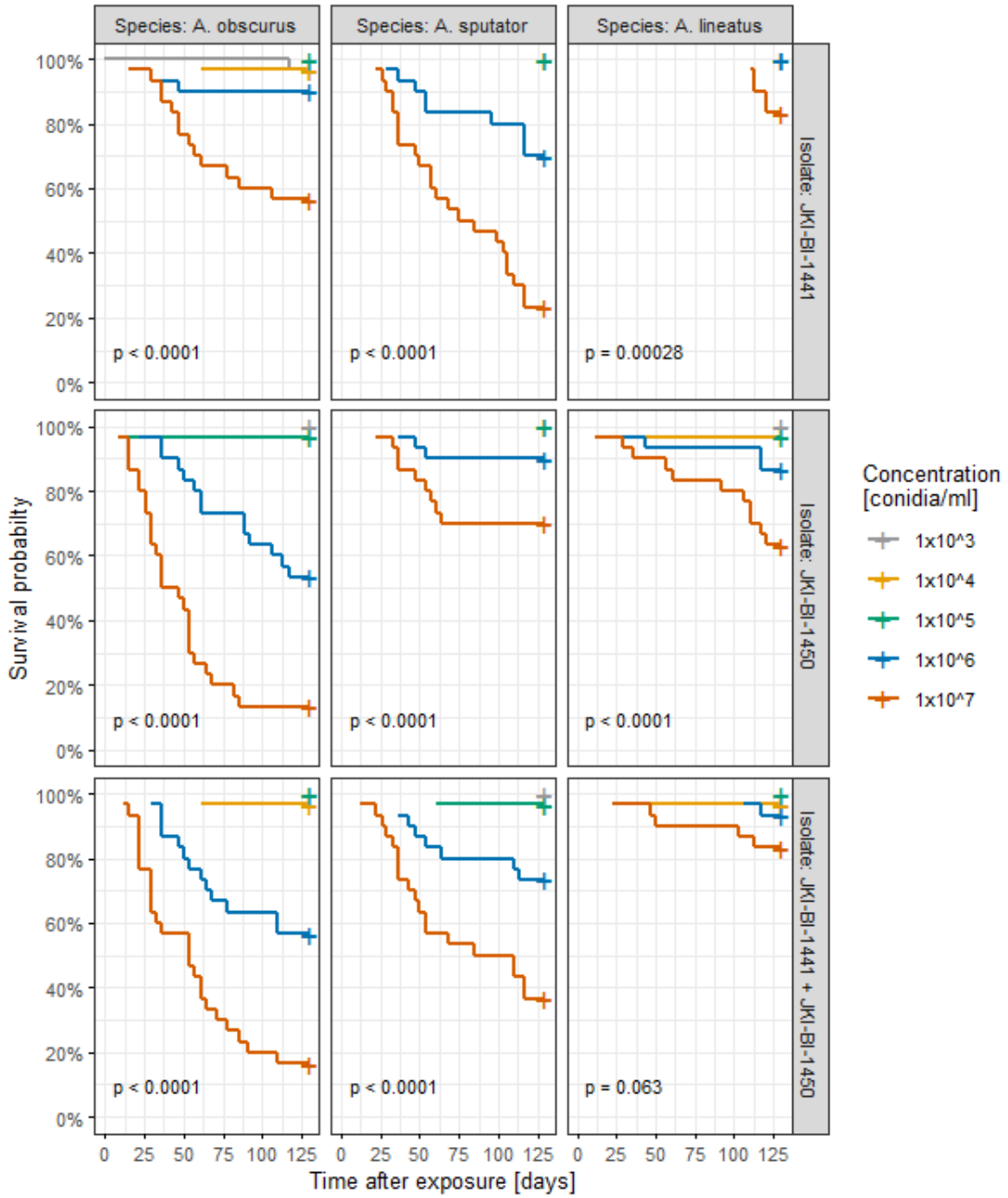

Figure 15: Overall survival probability in percent (Kaplan-Meier-Analysis) of the wireworm species *Agriotes obscurus*, *A. sputator* and *A. lineatus* exposed to five different concentrations (1x10³ conidia ml^{-1}, 1x10^4 conidia ml^{-1}, 1x10^5 conidia ml^{-1}, 1x10^6 conidia ml^{-1}, 1x10^7 conidia ml^{-1}) of the *Metarhizium* spp. isolates JKI-BI-1450, JKI-BI-1441 and a combination of both. Treated larvae were incubated for 130 days under laboratory conditions in small cans filled with soil at 25 °C and darkness. The statistic of the shown *p*-values is based on the log-rank test (α = 0.05) and indicates differences between the concentrations within a treatment and the respective wireworm species.

The values of the LC_{50} calculated by probit analysis are shown in Table 11. Here, the differences in effectiveness of the *Metarhizium* spp. isolates against the tested wireworm species became even more apparent. The conidia concentration of isolate JKI-BI-1450 to cause 50 % mortality to *A. obscurus* was 1.28x10^6 conidia ml^{-1} (FI 95 % 6.04x10^5 -

$2.96x10^6$ conidia ml^{-1}), whereas the LC_{50} of the isolate JKI-BI-1441 could not be calculated due to the low mortality. In contrast, the LC_{50} of isolate JKI-BI-1441 against *A. sputator* was $2.84x10^6$ conidia ml^{-1} (FI 95 % $1.67x10^6$-$5.18x10^6$ conidia ml^{-1}), while the estimation of the LC_{50} of *A. sputator* exposed to JKI-BI-1450 was not possible. The treatment with a combination of the isolates leads to an LC_{50} of $4.78x10^6$ conidia ml^{-1} (FI 95 % $2.17x10^6$-$1.59x10^7$ conidia ml^{-1}) against *A. sputator* and $1.63x10^6$ conidia ml^{-1} (FI 95 % $6.59x10^5$-$4.90x10^6$ conidia ml^{-1}) against *A. obscurus*. Neither the single isolates nor their combination was able to cause 50 % mortality of *A. lineatus.*

Table 11: Effectiveness of the *Metarhizium* spp. isolates JKI-BI-1450 (1450), JKI-BI-1441 (1441) and the combination of both against the wireworm species *Agriotes obscurus*, *A. lineatus* and *A. sputator* after 130 days of incubation under laboratory conditions at 25 °C and darkness in small cans filled with soil. Probit analysis was used to estimate the values of the lethal concentration (LC_{50}) for each wireworm species exposed to the respective *Metarhizium* spp. isolate individually.

Wireworm species	**No. of larvae**	***Metarhizium* spp. isolate**	**LC_{50} (Conidia ml^{-1})**	**95 % Fiducial interval**		**Slope**	**± SE_a**	**X^2_b**	**df_c**	**$pgof_d$**
				Lower	**Upper**					
Agriotes obscurus	150	JKI-BI-1450	$1.28x10^6$	$6.04x10^5$	$2.96x10^6$	1.18	0.18	32.0	23	0.10
	150	JKI-BI-1441				*				
	150	JKI-BI-1441 + JKI-BI-1450	$1.63x10^6$	$6.59x10^5$	$4.90x10^6$	1.18	0.19	45.3	12	0.09
Agriotes sputator	150	JKI-BI-1450				*				
	150	JKI-BI-1441	$2.84x10^6$	$1.67x10^6$	$5.18x10^6$	1.48	0.27	4.9	23	0.34
	150	JKI-BI-1441 + JKI-BI-1450	$4.78x10^6$	$2.17x10^6$	$1.59x10^7$	0.87	0.16	21.1	23	0.57
Agriotes lineatus	150	JKI-BI-1450				*				
	150	JKI-BI-1441				*				
	150	JKI-BI-1441 + JKI-BI-1450				*				

*Unable to estimate because mortality caused by any concentration of *Metarhizium* spp. was < 50 %

[a] Standard Error of the slope

[b] Chi-square goodness-of-fit on the probit model

[c] degree of freedom

[d] Pearson's chi square goodness-of-fit test ($\alpha = 0.05$)

Confrontation test of the *Metarhizium* spp. isolates

The compatibility of the *Metarhizium brunneum* isolate JKI-BI-1450 and *M. robertsii* isolate JKI-BI-1441 in terms of conidia formation and radial growth was investigated by their confrontation on agar plates in comparison with control variants. Looking at the tested isolates individually in Figure 16, the conidia concentrations were statistical comparable at each time of investigation regardless of the variant, indicating the compatibility of both isolates in terms of conidia formation (Tukey HSD test, $p > 0.05$). However, the comparison of isolate JKI-BI-1450 and JKI-BI-1441 revealed significant differences in conidia concentration after 8 days of incubation, determined with $7.9x10^4$-$1.2x10^5$ conidia ml^{-1} for isolate JKI-BI-1450 and $2.6x10^7$-$3.4x10^7$ conidia ml^{-1} for isolate JKI-BI-1441 (Tukey HSD test, $p < 0.001$). After 14 days, the conidia concentration of isolate JKI-BI-1441 increase to its maximum of $5.4x10^7$-$7.5x10^7$ conidia ml^{-1}, while that of isolate JKI-BI-1450, still with significant differences to isolate JKI-BI-1441, reached $6.4x10^6$-$9.9x10^6$ conidia ml^{-1} (Tukey HSD test, $p < 0.001$). The measurement of the radial growth indicated similar differences between the isolates, but less compatibility comparing the individual radial growth between the variants at end of the incubation time. Figure 17 shows that although the radial growth of the respective isolate was comparable between the variants on day 8 and 10 (Tukey HSD test, $p > 0.05$), significant differences were analysed at day 12 and 14 (Tukey HSD test, $p < 0.01$). After 8 days of incubation, the radial growth of isolate JKI-BI-1441 was significant higher with 623-694 mm^2 compared to 454-499 mm^2 of JKI-BI-1450 (Tukey HSD test, $p < 0.01$). The growth rate of both isolates was almost the same reaching 908-938 mm^2 (JKI-BI-1441) or rather 638-732 mm^2 (JKI-BI-1450) after 10 days (Tukey HSD test, $p < 0.001$). The isolates continued to grow consistently and after 12 days of incubation, the mycelium of the isolates almost got in contact with each other in a confrontation but did not touch. Since the isolates could no longer spread unhindered, the radial growth was influenced explaining the differences of isolate JKI-BI-1450 between variant 1 and variant 3 or rather between variant 2 and variant 4 of isolate JKI-BI-1441. Due to the faster radial growth of isolate JKI-BI-1441, the growth of isolate JKI-BI-1450 was suppressed in their direct confrontation from day 12. This resulted in the significant lowest radial growth of isolate JKI-BI-1450 compared to all isolates after 12 days or rather 14 days of incubation.

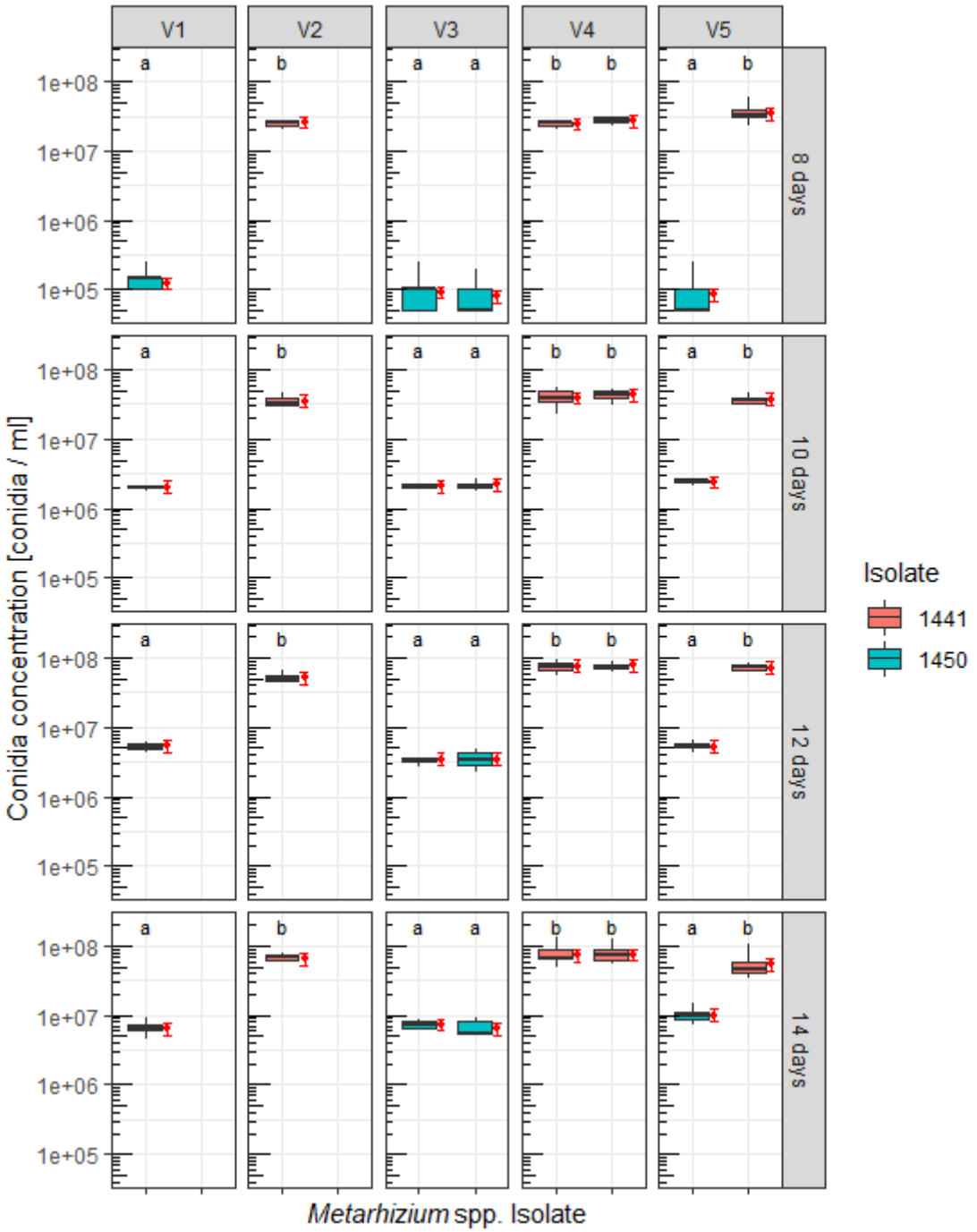

Figure 16: Conidia concentration (conidia ml^{-1}) of the *Metarhizium* isolates JKI-BI-1450 (blue) and JKI-BI-1441 (red) after 8, 10, 12 and 14 days on MPA plates in a confrontation test comparing five variants (V1 = JKI-BI-1450 against nothing, V2 = JKI-BI-1441 against nothing, V3 = JKI-BI-1450 against JKI-BI-1450, V4 = JKI-BI-1441 against JKI-BI-1441, V5 = JKI-BI-1450 against JKI-BI-1441). Adjusted mean and 95 % confidence interval of nine replicates per respective isolate are illustrated in red. The statistical pairwise comparison was performed within a sampling time. Isolates with the same letters are not significantly different based on LM (y = log(Conidia Concentration) ~ Variante + Day + Variante:Day), Tukey HSD test (α = 0.05).

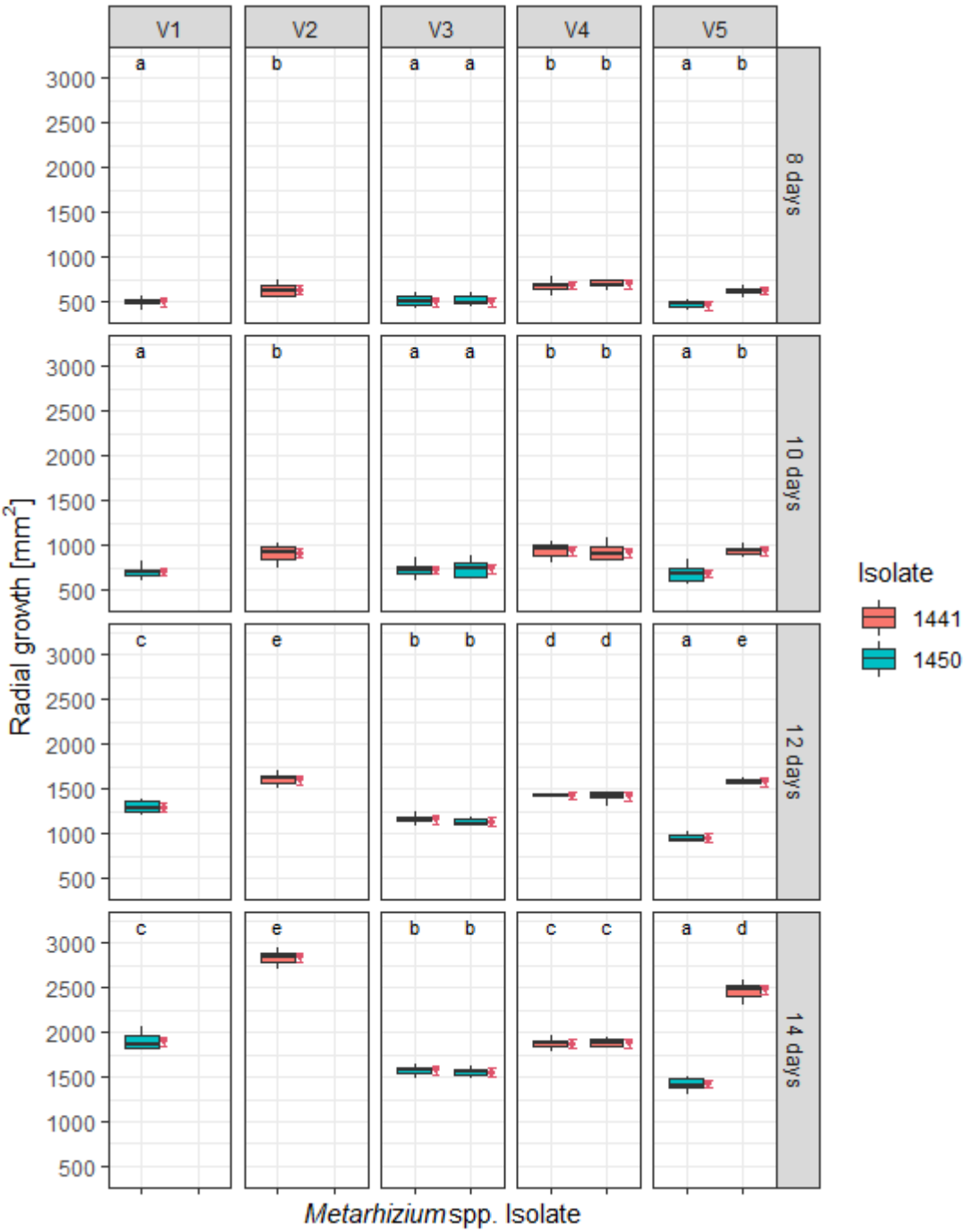

Figure 17: Radial growth (mm²) of the *Metarhizium* isolates JKI-BI-1450 (blue) and JKI-BI-1441 (red) after 8, 10, 12 and 14 days on MPA plates in a confrontation test comparing five variants (V1 = JKI-BI-1450 against nothing, V2 = JKI-BI-1441 against nothing, V3 = JKI-BI-1450 against JKI-BI-1450, V4 = JKI-BI-1441 against JKI-BI-1441, V5 = JKI-BI-1450 against JKI-BI-1441). Adjusted mean and 95 % confidence interval of nine replicates per respective isolate are illustrated in red. The statistical pairwise comparison was performed within a incubation time. Isolates with the same letters are not significantly different based on LM (y = Radial Growth ~ Variante + Day + Variante:Day), Tukey HSD test (α = 0.05).

Discussion

Highly virulent *Metarhizium* spp. isolates are a prerequisite for the development of an efficient biological control strategy against the soil-dwelling potato pest's wireworms (Jaronski 1997, Burges 1998, Shahid et al. 2012). In this study, two *Metarhizium* spp. isolates intended for a mycoinsecticide formulation varied considerably in their virulence against the wireworm species *Agriotes obscurus*, *A. sputator* and *A. lineatus*. *Metarhizium robertsii* isolate JKI-BI-1441 was most virulent with an inoculum concentration of $1x10^7$ conidia ml^{-1} against *A. sputator* reducing the survival probability to 22 %. Larvae of *A. lineatus* were least sensitive to isolate JKI-BI-1441. In contrast, *M. brunneum* isolate JKI-BI-1450 was least effective against *A. sputator* and showed its highest virulence against *A. obscurus*. The treatment with $1x10^7$ conidia ml^{-1} decreased the survival probability of *A. obscurus* to 12 %. These results are in line with the in vitro screening of Eckard et al. (2014), who also searched for virulent *Metarhizium* isolates to control wireworms. They compared the mortality of *A. obscurus*, *A. lineatus* and *A. sputator* after exposure to three different *M. brunneum* strains identifying their individual virulence profiles. In another bioassay of Ansari et al. (2009), eleven *M. anisopliae* strains were tested in vitro against *A. lineatus* and larval mortality ranged between 0 and 100 %, depending on the respective strain. In summary, the *Metarhizium* spp. isolates screened so far and intended for wireworm control showed a narrow host range with high virulence only against individual wireworm species. Thus, the effectiveness of formulated isolates for the use in potato cultivation depends on the presence of sensitive wireworm species in a given infested field. However, multiple wireworm species often co-occur on a given field site and the respective composition varies regionally (Miles 1942, Klausnitzer 1994, Furlan 1998, Ritter & Richter 2013). Moreover, the distinction of wireworm species based on morphological criteria is complex and difficult for practicing potato farmers (Klausnitzer 1994, Cocquempot et al. 1999). Therefore, the application of a mycoinsecticide based on a single *Metarhizium* isolate with a narrow host range seems impractical. Following the idea of Gonzalez et al. (2016), existing formulations could be optimised by combining multiple pathogens to increase the efficiency of wireworm control.

In this context, the approach was tested whether combining two *Metarhizium* spp. isolates would reduce the variability in virulence by controlling multiple wireworm species synergistically. The combined treatment consisting of the *M. brunneum* isolate JKI-BI-1450 and *M. robertsii* isolate JKI-BI-1441 counterbalanced the specific

virulence against *A. obscurus* and *A. sputator* resulting in an additive effectiveness. The combined treatment with an inoculum concentration of $1x10^7$ conidia ml^{-1} reduced the survival probability of *A. obscurus* to 18 % and of *A. sputator* to 38 %. However, the time until larval death prolonged by 14-17 days after exposure to the isolate mixture compared to the individual treatments. The slowdown in effectiveness reduces the efficiency of the desired control strategy using two *Metarhizium* isolates, since the period for controlling wireworms in potato cultivation is limited by the time of daughter tuber formation. Ericsson et al. (2007) investigated the synergistic interaction between Spinosad, a low-risk insecticide produced by the bacterium *Saccharopolyspora spinosa*, and *M. anisopliae* against *A. lineatus* and *A. obscurus*. They indicated, that the time to larval death was shortened by the combined treatment. A study of Ansari et al. (2004) considered the interaction of *M. anisopliae* with entomopathogenic nematodes for the control of third-instar *Hoplia philanthus*. Their combination of biocontrol agents led to an additive or synergistic effectiveness that killed larvae more rapidly compared to individual treatments. In both studies, faster effectiveness against the pests could be due to the combination of different microbial pathogens or nematodes with their individual mode of actions. The mode of actions of entomopathogenic fungi, bacteria and nematodes differ in host uptake or rather host penetration, action site and associated mechanism as well as in temporal progress (Senthil-Nathan 2015). Steinhaus (1958) assessed that stressed insects seems to be more susceptible to pathogens. Accordingly, one pathogen may have acted as a stressor making the pest more susceptible by changing the immune-physiological reaction, while the other pathogen then got the opportunity to develop its virulent potential. In the present study, the mechanism for the observed additive effectiveness of *Metarhizium* isolate JKI-BI-1450 and JKI-BI-1441 against the wireworm species tested remains unclear. However, since the two pathogens from the same fungal genus were combined, the mode of actions may have differed only in certain details. The combination of cuticle-degrading enzymes produced by the isolates may have enhanced interaction with the larval integument and penetration into the haemocoel (Xiao et al. 2012). Assuming that both isolates breached the multi-layered barrier, greater diversity of fungal metabolites in the haemocoel may have decrease cellular immunity of *A. obscurus* and *A. sputator* by influencing haemocyte count or encapsulation response (Neumann 2008). Understanding the exact immunological mechanisms of the additive effectiveness would be helpful for precise optimisation and

should be explored through molecular and enzymatic analysis following the study of Yaroslavtseva et al. (2017).

So far, the discussion of this study on the virulence of the isolates JKI-BI-1450 and JKI-BI-1441 against *A. obscurus*, *A. sputator* and *A. lineatus* referred to the highest tested inoculum concentration of $1x10^7$ conidia ml^{-1}. However, my dose-response bioassay showed that the isolates individually or combined were non-virulent to the larvae tested at $1x10^3$, $1x10^4$ and $1x10^5$ conidia ml^{-1}. Fungal treatment concentrations of at least $1.28x10^6$ - $4.78x10^6$ conidia ml^{-1} were necessary to kill fifty percent of the sensitive wireworm species *A. obscurus* and *A. sputator*. Consistent with my results, Rogge et al. (2017) observed more likely mycosis of *A. obscurus* with increasing conidia concentration of *M. brunneum* in a semi-field pot experiment. Field trials of Kabaluk et al. (2007) showed that very high doses of *Metarhizium* conidia were required to reduce the number of wireworms significant. According to the mentioned results, a high conidia concentration of *Metarhizium* at the respective target site results in improved wireworm control effectiveness. Consequently, fast growth and high sporulation of an applied *Metarhizium* isolate, hereafter referred to as proliferation performance, are further prerequisites for developing an efficient inoculative biocontrol strategy against wireworms.

The incubation of the tested isolates on MPA plates revealed that *Metarhizium robertsii* isolate JKI-BI-1441 forms overall more conidia in less time than *M. brunneum* isolate JKI-BI-1450. Furthermore, the mycelium of isolate JKI-BI-1441 spread more rabidly resulting in mutual interference between the tested isolates during their direct confrontation. Following my approach to combine the *Metarhizium* spp. isolates, the restricted proliferation performance of isolate JKI-BI-1450 poses a serious problem in optimising existing granule formulations. The granule formulation of Stephan et al. (2020) is based on autoclaved millet grain coated with fungal biomass consisting of mycelium and submerged spores produced by liquid fermentation. For the targeted optimisation, a mixture of two isolates would be coated onto the millet grain. Since the biological activity is caused by fungal colonisation on the granule, the superior proliferation performance of isolate JKI-BI-1441 would suppress the spread of isolate JKI-BI-1450. As a result, re-sporulation of isolate JKI-BI-1450 would be minimised and the desired wireworm control potential by additive effectiveness limited. Because a spray application of an isolate mixture can directly hit wireworms by droplets,

formulation as wettable powder may be better suited for the combined use of two *Metarhizium* isolates (Burges 1998).

In conclusion, the use of a single *Metarhizium* isolate as biocontrol agent is not sufficient for efficient wireworm control in potato cultivation. Isolates of *Metarhizium* spp. tested against a variety of wireworm species have specific virulence, which limits their suitability for field use. Although combined use of *Metarhizium* spp. isolates may improve the control potential through additive effectiveness, their differential proliferation performance must be considered when developing a formulation. Mutual interference between combined isolates after application must be excluded to ensure unimpeded growth and sporulation of each isolate. Nevertheless, a combined use of biocontrol agents could improve the efficiency of wireworm control by targeting several wireworm species to the same extent.

General discussion

The main objective of fungal-based biocontrol strategies against wireworms in potato cultivation is the protection of potato daughter tubers through the reduction of the soil-dwelling larvae (Shah & Pell 2003, Ritter & Richter 2013, Traugott et al. 2015). In the present study, *Metarhizium brunneum* Isolate JKI-BI-1450 was formulated as granule (AgriMet-Granule) and wettable powder (AgriMet-Dry Product) for soil application during potato planting. Although the AgriMet-Granule and AgriMet-Dry Product are easy to use, technological, ecological and intrinsic factors may critically influence the effectiveness of these mycoinsecticides. To evaluate the idea that mycoinsecticides could play a role as a component of IPM strategies, the mentioned influencing factors are discussed below in terms of the characteristics of a marketable mycoinsecticide, specific entomological aspects of wireworm biology, and potato cultivation practice.

Limitations of mycoinsecticides for the use against wireworms in potato cultivation

A mycoinsecticide formulation consisting of the entomopathogenic fungus *Metarhizium brunneum* serves three goals: first, it is a tool of preserving the microorganism; second, it introduces the microorganism into the potato ridge, and third, it enhances the biological activity of the microorganism in soil to increase the probability of lethal wireworm infection (Burges 1998). Jones & Burges (1998) specified the required properties for successful introduction of a marketable mycoinsecticide as follows: product stability, ease of handling and application with agricultural equipment, low environmental persistence and biodegradability, enhanced activity of the organism through pest interaction, and economically relevant effectiveness. If one of these properties are not met, there are limitations to the practical use of a mycoinsecticide and thus to its successful marketing.

The first limitation of the tested AgriMet mycoinsecticides was a technical obstacle related to product stability. Jaronski (1997) pointed out that product stability is important to ensure vitality and associated biological activity after production until application. The application timing is related to the potato planting, which is weather-dependent and can therefore vary by several weeks (Stark et. al 2020). Since flexible application timing precludes precisely timed production, mycoinsecticides used in potato cultivation must have a shelf life of several weeks. However, the AgriMet-Granule showed clear deficits in storage stability four weeks after shipment. My results showed a vitality loss of up to

20 % in individual granule batches, compared to the manufacture specifications (Chapter II). The biocontrol agent *M. brunneum* appears to be susceptible to degradation on the surface of the millet grain. Since the reference product ATTRACAP® was shipped the same way without a notable loss of vitality, encapsulation of the biocontrol agent may increase the shelf life of mycoinsecticides (Przyklenk et al. 2017). The AgriMet-Dry Product also exhibited a deviation from the viable fungal cells reported by the manufactures (Chapter II). Although Hajek & Eilenberg (2018) postulated that low stability and rapid degradability of biocontrol agents are positive aspects in terms of their environmental impact, these properties could also negatively influence the shelf life of the mycoinsecticides during storage. Because only a portion of the applied amount of both the AgriMet-Granule and AgriMet-Dry Product develops its biological activity, the control efficiency of the mycoinsecticides is negatively affected.

Another technical limitation related to the application during potato planting was identified when Bernhardt et al. (2019) tested the compatibility of the *M. brunneum* isolate JKI-BI-1450 with common potato seed dressings. Potato seed dressings are important pesticides in conventional cropping systems to protect seed potatoes from phytopathogenic fungi, such as *Colletotrichum coccodes, Helminthosporium solani* or *Rhizoctonia solani*. Since these fungi negatively affect the growth of the potato plants and the associated tuber formation, the use of seed dressings is essential for plant protection (Stevenson et al. 2001, Tsror 2010, Fiers et al. 2012). Although several seed dressings are available for the use in conventional potato cultivation (BVL 2021), not all may be suitable for use with entomopathogenic fungi. A possible application of the *M. brunneum* isolate JKI-BI-1450 with Moncut®, containing the active ingredient Flutolanil (Bontenbroich 2010), was confirmed by laboratory tests (Bernhardt et al. 2019). The product Moncut® provides protection only against *R. solani* and is approved for the normal tuber dressing technique (Bontenbroich 2010). In contrast, the seed dressing Ortiva®, with the active ingredient Azoxystrobin, protects against *C. coccodes, H. solani* and *R. solani* and is applied with the new in-furrow application technique (Räder et al. 2010). However, Ortiva® significantly affected growth and sporulation of *M. brunneum* isolate JKI-BI-1450 (Bernhardt et al. 2019). Thus, control options against soil-born phytopathogenic fungi are limited when using *M. brunneum* isolate JKI-BI-1450 for wireworm control. Since a variety of active ingredients should be used to avoid the development of resistance (Baibakova et al. 2019), the limitation to Moncut® could be a problem in the long-term control of phytopathogenic fungi harmful to potatoes.

One of the most important limitations of the tested mycoinsecticides is the impact of the soil temperatures on the fungal proliferation during application in spring. For the intended inoculative control strategy, mycoinsecticides must promote the reproduction of the fungus in the potato ridge to ensure or rather increase biological activity, while protecting the microorganism from harmful environmental factors (Jones & Burges 1998). The biological activity of *M. brunneum* against wireworms is based on the provision of an adequate amount of conidia, required to initialise lethal infection (Kabaluk et al. 2007, Rogge et al. 2017, Chapter III). Since germination, growth and sporulation of *Metarhizium* spp. is negatively affected by low temperatures (< 20 °C) (Ouedraogo et al. 1997, Ekesi et al. 1999, Hallsworth & Magan 1999, Arthurs & Thomas 2001), a mycoinsecticide for the use in potato cultivation should tolerate the ambient soil conditions when applied. In my field trials, measured soil temperatures in the potato ridge at 15 cm depth ranged between 9.8-14.7 °C during potato planting in spring. Four to six weeks passed before soil temperature reached 20 °C (Chapter II). Although application of the AgriMet-Granule and AgriMet-Dry Product hit the active phase of wireworms at potato planting (Langenbuch 1932, Burrage et al. 1963, Furlan 1998), the proliferation conditions for *M. brunneum* seem inappropriate at this time. Moreover, I observed the decomposition of the AgriMet-Granule by other microorganisms (Chapter II). Ubiquitous soil-borne microorganisms appear to be better adapted to low soil temperatures (Pietikäinen et al. 2005), using the millet grain as nutrient source. Colonisation of the AgriMet-Granule by these microorganisms can suppress growth of *M. brunneum* and inactivate its control potential. The AgriMet-Dry Product is also affected by the low soil temperatures, as the activity of the fungus must recover after the drying process during formulation (personal communication, Marianne Buschke, ABiTEP GmbH). In conclusion, low soil temperatures are a major factor influencing the effectiveness of *M. brunneum* for the use against wireworms in potato cultivation. The application of a mycoinsecticide at a later stage, when soil temperatures are higher, is limited by the potato growing season. Allen & Scott (1980) observed that approximate twelve weeks elapse from planting the seed potato to the formation of daughter tubers, which should be protected from wireworms. Since mortality of wireworms occurs five to seven weeks after contact with infectious conidia (Chapter III), biological activity of *Metarhizium* must be provided not later than early summer. At this point, however, the potato has formed lush foliage (Hack et al. 1993), making it difficult to incorporate a mycoinsecticide into the potato ridge. Following the screening of Amritha De Croos &

Bidochka (1999), *Metarhizium* spp. isolates tolerating low temperatures should be considered to optimise the desired inoculative control strategy against the soil-dwelling pests.

The last crucial factor limiting the use of the tested mycoinsecticides is the differential effectiveness of *M. brunneum* Isolate JKI-BI-1450 against the wireworm species *Agriotes obscurus*, *A. sputator* and *A. lineatus*. The development of mycoinsecticides involves the screening for a suitable fungal isolate, which forms the basis of the formulation and must meet certain requirements for the successful introduction of a marketable product (Jones & Burges 1998). Maina et al. (2018) recommended to evaluated fungal candidates with regard to their ecological characteristics, production capabilities, environmental risks and virulence against the target pest(s). Among these intrinsic factors, the virulence of the fungal isolate is the most important requirement, because it determines the control potential. In the case of wireworm control in potato cultivation, the aim is to achieve comparable virulence against the wireworm pest complex, since multiple species can occur simultaneously in an infested field (Ritter & Richter 2013). Lehmhus (2019) observed regional differences in wireworm species compositions, with *A. obscurus*, *A. sputator* and *A. lineatus* as the most abundant species in German potato cultivation. However, mortality of these wireworm species differed markedly after treatment with a conidial suspension of *M. brunneum* Isolate JKI-BI-1450 or its formulation as AgriMet-Dry product (Chapter II, Chapter III). Only the survival probability of *A. obscurus* was significantly reduced at high conidia concentrations or application rates, whereas *A. sputator* and *A. lineatus* were only slightly sensitive to the *M. brunneum* Isolate JKI-BI-1450 (Chapter II, Chapter III). The studies of Eckard et al. (2014) and Ansari et al. (2009) came to similar findings with regard to the virulence of *Metarhizium* spp. isolates against the economically most important wireworm species. However, developing a range of host specific mycoinsecticides, each tailored against different wireworm species, seems not practical, because the morphological identification of wireworm to species level is not feasible for farmers. Even for skilled entomologists, species-specific identification of field-caught larvae is difficult (Klausnitzer 1994, Cocquempot et al. 1999). Thus, a mycoinsecticide containing a single fungal isolate is unlikely to control the diverse pest complex of wireworms in potato cultivation. My laboratory approach of combining two *Metarhizium* spp. isolates overcomes the specific virulence and may increase the efficiency of mycoinsecticides in field use. However, the formulation of two biocontrol

agents of the same genus is difficult due to their individual proliferation performance (Chapter III) and the more expensive approval process. For the registration of a plant protection product, each active ingredient, in this case the fungal isolate, must be approved separately. The registration dossier requires extensive research and is therefore very cost-intensive (Montesinos 2003). If dossiers have to be prepared for two active ingredients, economic considerations come into play. Therefore, further rapid-throughput bioassays with fungal isolates are needed to identify candidates that exhibit adequate virulence against the economic important *Agriotes* species.

Box 3 Glossary

The effectiveness of the tested AgriMet-mycoinsecticides for the use against wireworms in potato cultivation may be influenced by technical, ecological and intrinsic factors.

Order	Factor	Stage	Criterion	Limitation
1	Technical	Production and Formulation	Stabilisation of the *Metarhizium*	*
2	Technical	Storage	Shelf life of fungal propagules in the formulated product	Loss of vitality of the formulated *M. brunneum* isolate JKI-BI-1450 four weeks after shipment
3	Technical	Application	Suitability for application with common devices	*
4	Technical	Application	Compatibility with fungicide seed dressings	Only compatible with the active ingredient Flutolanil
5	Ecological	Application	Protection of *Metarhizium* from harmful environmental factors	Unsuitable soil temperatures (< 17 °C) for proliferation of *M. brunneum* isolate JKI-BI-1450 during application in spring
6	Ecological	Post-Application	Contact and interaction with wireworms	*
7	Intrinsic	Post-Application	Pathogenicity of *Metarhizium* against different wireworm species	*
8	Intrinsic	Post-Application	High virulence of *Metarhizium* against different wireworm species	Significant differences in virulence of *M. brunneum* isolate JKI-BI-1450 against *Agriotes obscurus*, *A. sputator* and *A. lineatus*

*The mycoinsecticides meet the required criterion.

In summary, both the fungal candidate and the environmental conditions at the target site determine the requirements to achieve the criteria for successful introduction of a marketable mycoinsecticide as defined by Jones & Burges (1998). To overcome the ecological and intrinsic limitations of the tested mycoinsecticides for the use in potato cultivation an expanded screening of fungal isolates considering categories such as production, antagonistic effectiveness against all wireworm species, and ecological requirements at the target site are needed. Additional coatings during formulation might solve the technical obstacles in terms of storage stability and application. However, even after an optimisation the wireworm control success might still fluctuate considerably due to environmental factors.

Mycoinsecticides as a component of IPM against wireworms

The limitations of the tested mycoinsecticides against wireworms in potato cultivation reinforce the idea that successful pest reduction will not be achieved by a single control strategy, but by a holistic approach using all available IPM tools. Various methods for controlling wireworms have been discussed by Parker & Howard (2002) and Poggi et al. (2021) that refer to the modifications of the crop rotation or intensive tillage, in addition to the use of entomopathogenic fungi. On their own, these cultural, mechanical and biological control strategies show low effectiveness against the soil-dwelling larvae, but when combined they might achieve sustainable reduction of pest infestation. However, despite the theoretically promising idea to use a combination of several available IPM tools for reducing wireworm populations on arable fields, there are trade-offs with respect to the particular cropping system.

Cropping systems comprise all spatial and temporal aspects of managing arable crops and are subdivided according to different criteria, such as watering, utilisation of crops, the use of chemical pesticides or soil cultivation (Yang et al. 2020). With regard to soil cultivation, tillage with a plough has become an important part of cultivation management, because it creates the physical conditions for crop growth while turning the soil, which has a positive effect on weed and pest control (El Titi 2002). With regard to the control of wireworms, repeated disturbance of the soil by intensive and deep tillage can kill the larvae through direct injuries, exposure to predators, or dehydration on the soil surface (Salt & Hollick 1949, Seal et al. 1992). However, ploughing also leads to adverse effects on the agronomic productivity, as intensely tilled soils are susceptible to erosion and siltation (Papiernik et al. 2007). In addition, soil aeration by

ploughing promotes humus depletion, which negatively affects the soil fertility (Stockfisch et al. 1999). Therefore, tillage in modern agriculture is shifting to ploughless methods with lower working depths limiting the mechanical IPM element for wireworm control.

Another way of categorising cropping systems is the use of pesticides and fertilisers. In contrast to the conventional cropping system, the organic cropping system aims to increase the resilience of the agriculturally ecosystem for environmentally friendly cultivation by excluding synthetic pesticides and mineral fertilisers (Mäder et al. 2002, Reganold & Wachter 2016). The absence of mineral fertilisers in the organic cropping system has a particular impact on the crop rotation, as the nutrient supply to the crops must be ensured in a different way. Therefore, catch crops such as annual to biennial grass-clover is a supporting pillar of a crop rotation in organic cropping systems, because it fixes the available nitrogen, builds up humus, suppresses weeds and leaves a loose soil (Köpke 1995, Watson et al. 2002). Nevertheless, Schepl & Paffrath (2005) suggest avoiding fallows and catch crops like grass-clover or lucerne (*Medicago sativa*) for the prevention of wireworm infestation, as these plants form a dense green cover suitable for oviposition of click beetles. However, because of the benefits of catch crops to the holistic agronomic system, farmers tend to avoid growing potatoes in areas of wireworm infestation rather than avoiding grass-clover. The cultivation of catch crops is also becoming increasingly important for conventional cropping systems due to research on important cultivation aspects (Hansen & Djurhuus 1997, Constantin et al. 2010) and the enactment of political framework conditions in the interest of sustainable agriculture (Michels et al. 2020, Jürging et al. 2021). Under these circumstances, catch crops are essential for crop cultivation and should rather be considered as opportunity to optimise the use of mycoinsecticides.

One of the most important factors influencing the effectiveness of mycoinsecticides against wireworms in potato cultivation in my study was the unsuitable soil temperatures during application at spring potato planting (Chapter II). However, mycoinsecticides could be applied when environmental soil conditions are suited for the proliferation of *M. brunneum*. Optimum soil temperatures for fungal growth of 20-25 °C were observed in potato cultivation in Lower Saxony around August (Chapter II). These soil temperatures are likely to be common in all German arable fields in autumn, at a time when catch crops are sown (Vos & Van der Putten 1997, De Notaris et al. 2018). The application of mycoinsecticides with catch crops in autumn provides, in addition to the

suitable soil temperature for proliferation of *M. brunneum*, an increased likelihood of contact between infectious fungal conidia and wireworms. The larvae are still active in the upper soil layers at this time of the year (Langenbuch 1932, Brian et al. 1947, Burrage et al. 1963) and might feed on the belowground plant parts of the catch crops. The technical implementation of such an application is conceivable. Granules could be applied by installing spreaders for molluscicides (slug pellets) in front of the tractor, and the trailing seeder would incorporate the mycoinsecticide into the soil (Bloch & Bachinger 2012). For the application of wettable powders, the CULTAN method (Controlled Uptake Long Term Ammonium Nutrition) would be possible, where a suspension is injected 7-20 cm into the soil with pressure through several tail wheels. The distance between the tail wheels is flexible, which would allow a uniform distribution of the conidia suspension in a wireworm-infested field (Sommer 2005). The application technique described allows an annual application of mycoinsecticides, which would increase the likelihood of significantly reducing wireworm infestation when growing a sensitive crop such as potatoes.

In summary, the aforementioned mechanical and cultural approaches to reduce wireworm populations on arable fields are not appropriate for the sustainable agriculture we seek. Mycoinsecticides could expand the IPM toolbox of wireworm control if their use is adapted to the ecological requirements discussed. The annual application of mycoinsecticides in catch crops would be an alternative control strategy that should be tested in conventional and organic cropping systems to assess the additional effect of the respective crop rotations and agricultural management activities. Such a control strategy requires long-term planning and patience, as the control success will probably happen very slowly. In order to implement such a strategy into practice, farmers need to be convinced of the long-term control effect, as they are mostly focused on short-term maximisation of crop yield or quality. However, so far, no data exist regarding long-term control strategies against wireworms that combine cultural measures with the proposed biological approach using mycoinsecticides in catch crops. A usual field trial series would not meet the complexity typically found in practice, because the respective crop rotation depends on regional requirements in terms of climate and soil fertility, as well as on the intended utilisation of the respective farm type (Dury et al. 2012, Francis & Clegg 2020). The resulting diversity of options in crop management can only be achieved with a multifactorial monitoring across Germany including the use of mycoinsecticides. This will require farmers with wireworm-infested fields who will

provide detailed reports on their cultivation management and set up wireworm traps on a regular basis to assess the infestation level. In addition, the participating farmers should carry out standardised wireworm control measures. Finally, the impact on wireworms should be evaluated over several years based on the infestation dynamics in combination with the damage to sensitive crops. Such data could provide better indicators for the effective use of mycoinsecticides in combination with cultural measures in the particular cropping system, paving the way for sustainable wireworm control.

Effectiveness calculation of mycoinsecticides for the use against wireworms in potato cultivation: What do we measure and what does it mean?

Mycoinsecticides could play a key role for IPM strategies against wireworms on arable fields. A priority in developing a successful mycoinsecticide is the improvement of its effectiveness (Moore & Prior 1993). Field trials are an important part of this research, as they verify the performance of a plant protection product under realistic conditions. Guidance on how to conduct appropriate field trials is provided by the European and Mediterranean Plant Protection Organization **(**EPPO). Numerous standards specify approval-relevant aspects, under which field trials should be planned, conducted, assessed, recorded and interpreted (Anonymous 2012a, Anonymous 2012c, Anonymous 2019). In this context, the EPPO refers to good experimental practice (GEP). GEP comprise of qualified staff, use of suitable equipment, uniform protocols, reliable operation and the recording and appropriate analysis of data. The overall goal of GEP is to provide high-quality field trials that generate comparable data for different registration authorities (Anonymous 2012b). Furthermore, these data serve as a basis for discussion in the context of scientific exchange between various participants in the plant protection community, such as scientists, companies, consultants and farmers. Only if the presented data of field trials are conclusively analysed, the effectiveness of a plant protection product can be properly judged and the associated control strategy further developed. Thus, GEP is an important pillar for proper assessment of product performance in field use.

The mycoinsecticide formulations investigated in the present study were additionally tested for their effectiveness in reducing wireworm damage to potatoes in a multi-site and multi-year field trial series. Therefore, five specialised experimental stations throughout Germany were commissioned in order to account for regional differences in environment and climate. To collect robust field trial data that address the GEP

requirements listed above, EPPO Standards are typically used. Since the experimental stations are regularly assigned to conduct independent field trials for scientific studies or registration dossiers of plant protection products against wireworms in potatoes, a uniform protocol and appropriate analysis of data were assumed. However, the conduct and analysis of field trials by the experimental stations testing the AgriMet formulations in potato cultivation varied considerably. Regarding the experimental set up, some used a randomised complete block design, while others tested the mycoinsecticides with a split plot design. In rating the potato damage by wireworms, all experimental stages classified the tuber damage according to the amount of holes (class 1 = no holes, class 2 = 1-2 holes, class 3 = 3-5 holes, class 4 > 5 holes), but some rated one hundred tubers per plot and others twenty-four potato plants per plot. Most importantly, the analysis of results varied significantly. The EPPO advises that the analysis of effectiveness should be calculated by using statistical tests based on an appropriate model (Anonymous 2012a). In contrast, the experimental stations calculated the effectiveness as percent control according to Abbott (1925). This is in principle acceptable (Anonymous 2012a), but a closer look at the results revealed that each experimental station has adapted Abbott's formula in a different way.

The Abbott formula was created in 1925 by entomologists of the Insecticide and Fungicide Board to compare the effectiveness of insecticides against the San Jose scale *Quadraspidiotus perniciosus* (Abbott 1925). The percent control is calculated by dividing the percentage of insects killed by the treatment through the percentage of live insects in the untreated control. For wireworm control, the number of damaged potatoes (feeding holes) is used to calculate the percentage effectiveness. The derivation of the Abbott formula from an insect count to potato damage has apparently prompted the experimental stations testing the AgriMet mycoinsecticides to reinterpret the basic elements of the formula. While some excluded only class 1 potatoes (no holes) for calculation, others also counted class 2 potatoes (1-2 holes) as undamaged. Another method was to weight the number of tuber holes before calculating them according to Abbott (1925). Although every calculation has its practical justification, the results are difficult to compare. Different effectiveness percentages might be calculated, even though the mycoinsecticides would have provided the same control performance in the field trials across Germany. The heterogeneous analyses of the trial series may have a greater influence on the assessed effectiveness of the mycoinsecticides tested than the regional differences in environment and climate. In conclusion, the experimental

stations throughout Germany provided field trial data of mycoinsecticides against wireworms in potato cultivation, which were evaluated with different self-developed evaluation procedures while using the identical synonym of effectiveness. In the absence of a detailed disclosure of the respective evaluation procedure, fundamental problems arise in terms of standardising product effectiveness for registration or scientific studies. Widespread misinterpretations about newly developed plant protection products could occur and must be avoided by a uniform and appropriate calculation of effectiveness.

A uniform statistical procedure for calculating the effectiveness of plant protection products against wireworms in potato cultivation is urgently needed for a standardised comparison of product performance that takes into account all the important driving factors. In order to reflect the control success of the fungal control agent in a way that is more comprehensive, a calculation formula that uses a simple percentage value seems to be desirable. However, the existing calculation formulas should be reviewed. Only damaged potatoes are used in Abbott's calculation (1925) to evaluate the effect on wireworms. Thus, the effect on the soil-dwelling pest can only be estimated. It remains unclear, whether the reduced feeding activity of wireworms was due to the control agent or to environmental factors, such as drought or alternative food sources (Lees 1943, Rizzo & Lehmhus 2014). To reasonably calculate the effectiveness of a plant protection product, the prevailing conditions of wireworm infestation should be taken into account. In most infested potato fields, the distribution of soil-dwelling wireworms is patchy and non-uniform (Chapter II). However, Abbott's formula assumes uniform pest infestation in the experimental set-up (Abbott 1925) and produces effectiveness values that do not reflect the real situation in field trials.

Henderson & Tilton (1955) developed a calculation formula that accounts for heterogeneous infestation on a given experimental field. They modified Abbott's formula (1925), while testing acaricides against the brown wheat mite *Petrobia latens*. In addition to a spatial reference of infestation (plots of control and treatment), the authors also considered a temporal reference of infestation (before and after treatment). The latter would account for the level of infestation and put the non-uniform wireworm distribution into perspective (Henderson & Tilton 1955). A prerequisite for the calculation formula of Henderson & Tilton is that the pest numbers can be accurately determined before and after treatment. Existing wireworm monitoring methods are either based on the extraction of larvae from defined soil samples or on the setting of cereal bait traps (Poggi et al. 2021). Nevertheless, accurate field sampling for the

quantitative determination of the respective wireworm population is very difficult. Before treatment, or rather before potato planting, larval activity is very low in the upper soil layers due to unsuitable soil conditions in early spring (Lafrance 1968, Kovacs et al. 2006). After treatment, only a large sample size leads to a meaningful monitoring, as Parker (1996) pointed out that single traps cannot be used to give an estimate of population density per unit area. In addition, the slow action of entomopathogenic fungi against wireworms, which varies between 3-10 weeks (Chapter III), requires multiple samplings to assess the effect of the mycoinsecticides tested. Under the circumstance of inadequate wireworm monitoring tools, neither the Henderson & Tilton (1955) nor the Abbott formula (1925) seems to be suitable for the effectiveness calculation of mycoinsecticides for the use in potato cultivation. A new way of calculating potato damage that includes an approximate estimation of wireworm infestation is needed. To this end, statistical models (Linear Model, Generalized Least Squares, Generalized Linear Model) could be used for evaluating the effectiveness of a given plant protection product.

Conclusion

The withdrawal of chemical pesticides marks a new era of wireworm control. It seems unlikely that the use of biocontrol agents can replace the banned agrochemicals with equal effectiveness in a timely manner. An adequate substitution with mycoinsecticides is only possible, if the identified technical, ecological and intrinsic factors are improved. This requires the optimisation of important formulation properties and the synergistic effect of different biocontrol agents to overcome the specific virulence of certain *Metarhizium* spp. isolates against economically important wireworm species. The application of such an optimised formulation should be embedded in the holistic agronomic system, to provide a suitable time in the crop rotation for the fungal mode of action. Finally, the effectiveness calculation must also be revised and standardised in order to make better predictions based on field trials and to provide reliable data for the registration process. New challenges in wireworm control require new approaches that may initially be daunting due to their complexity. However, to tackle these challenges is the only way to provide effective wireworm control alternatives for sustainable agriculture. My hope is to stimulate further studies on the development of new, optimised control strategies against wireworms as pest in potato cultivation using the entomopathogenic fungi *M. brunneum*.

Outlook and Perspective

More sustainable control strategies are urgently needed to minimise crop damage by insect pests, such as wireworm damage to potatoes. Mycoinsecticides could play a pivotal role, as they can be used in a targeted manner. However, despite the importance of pesticide effectiveness, other aspects, namely the impact on the environment and human health are becoming more important in the development of new pesticides (Aktar et al. 2009, Damalas 2009, Gill & Garg 2014).

In the last decades, plant protection was intended to secure the production of sufficient food for a steadily growing human population (Alexandratos & Bruinsma 2012). The efficient use of chemical pesticides for plant protection has maximised the yield of the required field crops (Stetter & Lieb 2000), however, with the downside of pesticide resistance and harmful residues (Georghiou 2012, Nicolopoulou-Stamati et al. 2016). In recent years, there has been a fundamental shift in the evaluation of the benefits of pesticide use relative to their potential environmental risks. Driven by an ecologically aware society, the preservation of biodiversity in a well-functioning biosphere is gaining increasing importance in food production (Carvalho 2017). The growing evidence of the potential negative impact of agricultural intensification on insect abundance and biodiversity in many regions worldwide (Pettis et al. 2013, Vanbergen & Initiative 2013, Newbold et al. 2015, Seibold et al. 2019), has prompted Germany to launch an "Insect Conservation Action Program" in 2021. In this, Germany postulated to promote the expansion of organic farmed agricultural land in Germany to 20 % by 2030 (Aktionsprogramm Insektenschutz, BMU, 13.04.2021). The area of organic cultivation worldwide increased from 58 million hectares in 2016 to 71 million hectares in 2018 representing an increase of 22 % in two years (FiBL 2021). Van Lenteren et al. (2018) described the socioeconomic opportunities of biological control through numerous applications, including mycoinsecticides. Although biological control is pushed by the stringent risk assessment for the registration of pesticides based on the aforementioned concerns through agrochemicals (Villaverde et al. 2014, Gehen et al. 2019), the availability and use of biopesticides remain a very small percentage in agricultural practice as stated by Kumar & Singh (2015). One reason for this is the relatively low acceptance of biopesticides by farmers, because the effectiveness is often lower than that of their chemical counterparts. However, the long-term benefits of biopesticides through biodiversity conservation may justify their lower effectiveness, but this effect is

currently not measured quantitatively. To obtain a grade or index that reflects the socioeconomic long-term benefits of pesticides on the environment, positive and negative externalities should be monetised and included in their evaluation.

Although simplifying complex ecological interrelationships in agronomic pest control with numbers presents a challenge, scientists are commanded to find new ways to express scientific knowledge. Therefore, following the idea of Leach & Mumford (2008), a method should be developed to calculate an index that reflects the monetised and disclosed positive and negative impacts of pesticides. A modular formula could be created, whose building components consist of the effectiveness and various aspects associated with the pesticide's impacts in the holistic agronomic system in which they are used. As a result, such an index would provide guidance for companies to develop more sustainable products. Harmful effects and associated costs would become apparent. Farmers would be able to better assess the overall environmental impact of their actions, while comparing different pesticides and assessing realistic socioeconomic benefits. To this end, an alliance with stakeholders from the plant protection sector is needed. Collaboration among companies, farmers, advisors, authorities and universities may innovate the evaluation of pesticides and created an index that contribute to sustainable agriculture every participant agrees with.

Plant protection in agriculture has to face the challenging task to reconcile economic productivity and environmental integrity under consideration of constantly changing social, political and ecological requirements (Robertson & Swinton 2005, MacLeod et al. 2010, Lamberth et al. 2013, Lamichhane et al. 2015). A paradigm shift in the evaluation of pest control success and associated benefits is inevitable to ensure acceptance of modern agricultural practice. Extensive monitoring programs and detailed interdisciplinary environmental analyses are therefore required to monetise the ecologic impacts of different agricultural practices. For new sustainable plant protection methods identifying the factors that influence their control success might be the key to provide satisfactory effectiveness in future.

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