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Development of a soil granule based on the entomopathogenic fungus *Metarhizium brunneum* to control *Agriotes* spp.

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„Das Wichtigste ist, dass man nie aufhört Fragen zu stellen“ – Albert Einstein

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Summary

Crop protection faces particularly great challenges for the food production of a growing world population and in view of climate change. However, agriculture always has to contend with crop losses caused by plant pests and diseases. Pest insects of particular importance are wireworms, the larvae of click beetles, which cause reduced crop quality or crop losses in many cultured plants, such as potatoes, maize, cereals, asparagus or lettuce, by feeding on roots, tubers and seedlings. In Europe, larvae of the genus *Agriotes*, including the species *Agriotes lineatus*, *Agriotes obscurus* and *Agriotes sputator*, are considered a particularly severe problem. Because of their development in the soil, lasting two to five years, depending on the species and environmental conditions, they are difficult to control. Biological control strategies are advantageous, because, unlike chemical ones, they usually pose a low risk to the environment, applicators and consumers. Especially the effect of entomopathogenic fungi (EPF) against wireworms has been well documented. For these reasons, the aim of this dissertation was to develop a soil granule based on EPF to control larvae of the species *A. lineatus*, *A. obscurus* and *A. sputator*.

Four steps were taken to achieve this goal. In the first step, an effective fungal strain against *Agriotes* larvae was determined. Then, the producibility of the fungal strain in liquid medium was optimized and the ability to formulate it by using a fluid-bed dryer was investigated. In the fourth step, the influence of the production temperature on different quality factors of the developed granule as well as on virulence factors of the fungal strain were investigated.

To identify an effective fungal strain against the three *Agriotes* species, the virulence of six *Metarhizium* spp. and two *Cordyceps* spp. strains was compared (Chapter I). The *Cordyceps* strains proved to have no pathogenic effect on the larvae, whereas all *Metarhizium* strains were

able to infect and kill the larvae and led to fungal growth on the cadaver. However, the effect of the individual *Metarhizium* strains on the three *Agriotes* species differed. Treatment with the *Metarhizium brunneum* strain JKI-BI-1450 resulted in the highest mortality against larvae of *A. lineatus* with 58% and *A. obscurus* with 94% and intermediate mortality of *A. sputator* with 56%, compared to the other fungal strains. The highest mortal effect against *A. sputator* larvae was caused by the *Metarhizium robertsii* strain JKI-BI-1442 with 94%. In order to explain the different mortality, the speed of germination, the germination after 96 h and the conidial size of the *Metarhizium* strains were determined. Conidia of the three *M. brunneum* strains (JKI-BI-1339, JKI-BI-1450 and LRC112) germinated between 96.8-98.5% after 96 h, whereas the conidia of the *M. robertsii* strains (JKI-BI-1441, JKI-BI-1442 and JKI-BI-1448) only germinated between 90.7-93%. τ, the time point where 50% of the maximal germinated conidia are germinated, was reached significantly faster by JKI-BI-1339 and JKI-BI-1450 after 14.5 h and 15.1 h, respectively. The longest conidia were formed by LRC112 and the widest by JKI-BI-1442. Subsequently, it was investigated whether these factors correlate with final mortality; however, this was not the case. Furthermore, it was investigated whether the effect of the fungal strains varies not only between the different *Agriotes* species, but also within larvae of one species between various populations obtained from different locations. Therefore, the effect of the two *M. brunneum* strains JKI-BI-1450 and ART2825 on different larval populations was investigated, as these fungal strains are already proven to be highly effective against *Agriotes* larvae. The mortality and mycosis effect of the two investigated fungal strains on the larvae of *A. obscurus* differed between the populations. The final mortality of *A. obscurus* larvae lay between 44-94% when treated with JKI-BI-1450 and between 30-95.6% when treated with ART2825. There was no difference observed between the populations of *A. lineatus* larvae and the final mortality was 38% for the JKI-BI-1450 treatments and 48% and 53.5% for the ART2825 treatments. Although the final mortality did not differ significantly between the

different populations of *A. sputator* larvae and lay between 26-36% for JKI-BI-1450 and between 10.7-41.8% for ART2528, there were significant differences found in the time to larval death. Restricted mean time lost (RMTL) differed significantly between the populations and was between 3.6-18.5 for JKI-BI-1450 and between 6-21.5 for ART2825. The *M. brunneum* strain JKI-BI-1450 turned out to be a promising candidate for wireworm control, but the different efficacy against the *Agriotes* species and, in the case of *A. obscurus*, also against different populations, is problematic because so its field efficacy is difficult to predict. The development of a control strategy based on several fungal strains would be a possible solution.

In order to determine the producibility of the fungi, in chapter II all six *Metarhizium* strains were cultivated in three standard liquid media and the formation of submerged spores was evaluated. All fungal strains could be proliferated in liquid cultures and achieved spore concentrations of around 10^7 submerged spores ml^{-1} except for LRC112 with a maximum of 6×10^4 submerged spores ml^{-1}. All further experiments were exclusively carried out with JKI-BI-1450 based on its efficacy. The submerged spore and biomass formation of JKI-BI-1450 was studied at different temperatures and after different incubation periods. The highest submerged spore formation was achieved after 48 h at 25 °C with 6.5×10^5 submerged spores ml^{-1}, as was the highest biomass formation also after 48 h, but at 30 °C with 3.2%. Afterwards, the growth of JKI-BI-1450 in a 3-Liter bioreactor at 25 °C was studied. It was found that submerged spore formation had its optimum between 42.5-48 h after inoculation with spore concentrations between 1.43-2×10^6 submerged spores ml^{-1}, whereas the biomass formation increased to 1.43% throughout 72 h duration of fermentation. To increase fungal production in liquid culture, the influence of the concentration of the media components (glucose, corn steep solid, sodium chloride) was evaluated. The increase of glucose and corn steep solid in the medium resulted in a steady rise of biomass production up to 28.0 g l^{-1} and 23.0 g l^{-1},

respectively. In contrast, changing the sodium chloride concentration in the medium produced inconsistent results, although the highest concentration resulted in the greatest biomass formation with 14.7 g l^{-1}.

After production in liquid culture, the fungal broth must be formulated to make it suitable for storage and application. In chapter III, fluid-bed drying was chosen for the formulation of JKI-BI-1450, in which a thin layer of fungal biomass was sprayed on millet and simultaneously dried. This formulation method proved to be well suited for the production of granule, requiring 4.5 mg of fungal biomass per gram of granule to achieve a granule colonization of nearly 100%. To increase or accelerate the fungal growth on the granule, chitin and various sugars were added to the fungal biomass before spraying and drying. The supplementation of chitin showed no positive effect, whereas the addition of the sugars led to an acceleration of colonization as well as of the formation of conidia, with the best results obtained for fructose. The addition of fructose increased granule colonization from 5.6 to 62.2% after one day of incubation and conidia formation from 3.35×10^5 to 1.13×10^6 conidia per granule grain after 5 days of incubation. The storage stability of the granule was also investigated, as this is important to provide sufficient shelf-life of the final product. After 12 weeks of storage at 5 °C, the granule colonization was still over 90%. The examination of granule quality under simulated field conditions showed that at the optimum residual soil moisture contents of 30% and 45%, the highest colonization was found at 25 °C and 30 °C with 98.9-100% and the most conidia per granule grain at 25 °C with $3.0\text{-}3.5 \times 10^6$. Furthermore, it was demonstrated that the reduction of colonization and formation of conidia was lower when only one environmental parameter (temperature and humidity) was unfavourable compared to both factors being disadvantageous. In addition, a fungicidal seed treatment (Moncut®) was identified as compatible with the developed granule without significant loss of granule colonization.

Summary

In chapter IV, it was investigated how the production temperature of the fungus affects granule colonization and conidia formation on the granule, as well as on germination, conidial size and virulence of the conidia. For this purpose, the biomass required for granule preparation was produced at different temperatures and the produced granules were also incubated at different temperatures. The fungus on the granules demonstrated the highest colonization with 87.17% and conidia formation with 2.77×10^7 conidia per granule grain when the biomass was produced at 25 °C. Furthermore, conidia were produced at different temperatures and then also incubated at different temperatures. Afterwards, their size, germination and virulence were investigated. The conidia produced at 20 °C were significantly wider with 2.21 µm compared to those produced at 25 °C and 30 °C with 2.19 µm. Furthermore, conidia produced at 15 °C reached τ significantly later (15.42 h) than conidia produced at 30 °C (12.31 h). The production temperature of the conidia had no effect on the virulence against *Galleria mellonella* larvae. The mortality ranged between 95.63-100% for all production temperatures with a survival time of 50% (ST_{50}) between 3.97-4.54 days. With regard to the incubation temperature, it was found that at 30 °C smaller conidia were formed with 6.54 µm, germination was faster with τ of 11.40 h and the fungal treatment led to a faster mortality with a ST_{50} of 3.16 days.

In summary, this dissertation identified *M. brunneum* JKI-BI-1450 as pathogenic against larvae of all three *Agriotes* species examined. This fungal strain can be produced in liquid medium and formulated into granule by fluid-bed drying. Both, production and formulation were improved by adjusting the process parameters or media composition and supplementing additives. Furthermore, it was shown that the quality of a fungus-based soil granule can already be influenced by the production conditions of the biomass used for it.

Zusammenfassung

Pflanzenschutz steht für die Nahrungsproduktion einer wachsenden Weltbevölkerung und angesichts des Klimawandels vor besonders großen Herausforderungen. Allerdings hat die Landwirtschaft seit jeher mit Ernteausfällen zu kämpfen, welche von Schädlingen und Pflanzenkrankheiten verursacht werden. Ein besonders wichtiges Schadinsekt ist der Drahtwurm, die Larven der Schnellkäfer, der durch seinen Fraß an Wurzeln, Knollen und Sämlingen bei vielen Kulturpflanzen wie Kartoffeln, Mais, Getreide, Spargel oder Salat zu Qualitätseinbußen oder Ernteausfällen führt. In Europa gelten die Larven der Gattung *Agriotes*, einschließlich der Arten *Agriotes lineatus*, *Agriotes obscurus* und *Agriotes sputator*, als besonders großes Problem. Aufgrund ihrer Entwicklung im Boden, die je nach Art und Umweltbedingungen zwei bis fünf Jahre dauert, sind sie schwer zu bekämpfen. Eine Biologische Bekämpfung ist von Vorteil, da sie im Gegensatz zur Anwendung chemischer Pflanzenschutzmittel in der Regel geringe oder keine Risiken für die Umwelt, die Anwender und die Verbraucher darstellen. Insbesondere die Wirkung entomopathogener Pilze gegen Drahtwürmer ist vielfach dokumentiert. Aus diesen Gründen war das Ziel der vorliegenden Dissertation die Entwicklung eines biologischen Bodengranulats auf Basis eines entomopathogenen Pilzes zur Bekämpfung von Larven der Arten *A. lineatus*, *A. obscurus* und *A. sputator*.

Um dieses Ziel zu erreichen, wurden vier Schritte unternommen. Im ersten Schritt wurde ein gegen *Agriotes*-Larven wirksamer Pilzstamm ermittelt. Anschließend wurde die Produzierbarkeit des Pilzstammes in flüssigem Medium optimiert und die Möglichkeit der Formulierung mittels Wirbelschichttrocknung untersucht. Im vierten Schritt wurde der Einfluss der Produktionstemperatur auf verschiedene Qualitätsfaktoren des entwickelten Granulats sowie auf Virulenzfaktoren des Pilzstammes untersucht.

Zusammenfassung

In Chapter I wurde die Virulenz von sechs *Metarhizium*- und zwei *Cordyceps*-Stämmen verglichen, um einen wirksamen Pilzstamm gegen die drei genannten *Agriotes*-Arten zu identifizieren. Die *Cordyceps*-Stämme zeigten keine pathogene Wirkung auf die Larven, während alle *Metarhizium*-Stämme in der Lage waren, die Larven zu infizieren, zu töten, und zu Pilzwachstum auf den Kadavern zu führen. Die Wirkung der Pilzstämme auf die Larven der drei *Agriotes*-Arten war jedoch unterschiedlich. Die Behandlung mit *Metarhizium brunneum* JKI-BI-1450 führte im Vergleich zu den anderen Pilzstämmen zur höchsten Mortalität der Larven von *A. lineatus* mit 58 % und *A. obscurus* mit 94 % und zu einer mittleren Mortalität bei *A. sputator* mit 56 %. Die beste Wirkung gegen *A. sputator*-Larven hatte der *Metarhizium robertsii*-Stamm JKI-BI-1442 mit 94 %. Um eine Erklärung für die unterschiedlichen Wirkungen zu erhalten, wurden die Keimungsgeschwindigkeit, die Keimung nach 96 h und die Konidiengröße der *Metarhizium*-Stämme bestimmt. Die Konidien der drei *M. brunneum*-Stämme (JKI-BI-1339, JKI-BI-1450 und LRC112) keimten nach 96 h zu 96,8-98,5 %, die Konidien der *M. robertsii*-Stämme (JKI-BI-1441, JKI-BI-1442 und JKI-BI-1448) hingegen nur zu 90,7-93 %. τ, der Zeitpunkt, an dem 50 % der maximal gekeimten Konidien gekeimt sind, wurde von JKI-BI-1339 und JKI-BI-1450 nach 14,5 h bzw. 15,1 h signifikant schneller erreicht. Die längsten Konidien wurden von LRC112 gebildet und die breitesten von JKI-BI-1442. Daraufhin wurde untersucht ob diese Parameter mit der finalen Mortalität korrelieren, was jedoch nicht der Fall war. Weiterhin wurde untersucht, ob die Wirkung der Pilzstämme nicht nur zwischen den verschiedenen *Agriotes*-Arten, sondern auch innerhalb einer Art zwischen verschiedenen Populationen von unterschiedlichen Standorten variiert. Daher wurde die Wirkung der beiden Stämme *M. brunneum* JKI-BI-1450 und ART2825 auf verschiedene Larvenpopulationen untersucht, da diese Pilzstämme nachweislich eine gute Wirkung gegen *Agriotes*-Larven haben. Die Wirkung der beiden Pilzstämme auf die Larven von *A. obscurus* unterschied sich zwischen den Populationen. Die finale Mortalität der

Zusammenfassung

A. obscurus-Larven lag bei der Behandlung mit JKI-BI-1450 zwischen 44-94 % und bei der Behandlung mit ART2825 zwischen 30-95,6 %. Zwischen den Populationen der *A. lineatus*-Larven gab es keine Unterschiede, wobei die finale Mortalität bei den Behandlungen mit JKI-BI-1450 bei 38 % und bei den Behandlungen mit ART2825 bei 48 % und 53,5 % lag. Obwohl sich die finale Mortalität zwischen den verschiedenen Populationen der *A. sputator*-Larven nicht signifikant unterschied und zwischen 26-36 % für JKI-BI-1450 und zwischen 10,7-41,8 % für ART2528 lag, gab es signifikante Unterschiede bei der Zeit bis zum Tod der Larven. Die Restricted Mean Time Lost (RMTL) unterschied sich signifikant zwischen den Populationen und lag zwischen 3,6-18,5 für JKI-BI-1450 und zwischen 6-21,5 für ART2825. Der Pilzstamm *M. brunneum* JKI-BI-1450 erwies sich als vielversprechender Kandidat für die Drahtwurmbekämpfung, aber die unterschiedliche Wirksamkeit gegen die *Agriotes*-Arten und im Fall von *A. obscurus* auch gegen verschiedene Populationen ist problematisch, da seine Wirksamkeit im Feld dadurch schwer vorherzusagen ist. Die Entwicklung einer Kontrollstrategie, die auf mehreren Pilzstämmen basiert, wäre eine mögliche Lösung.

Um die Produzierbarkeit der Pilze zu bestimmen, wurden in Chapter II alle sechs *Metarhizium*-Stämme in drei flüssigen Standardmedien kultiviert und die Bildung von Submerssporen bestimmt. Alle Pilzstämme ließen sich in Flüssigkulturen produzieren und erreichten eine Sporenkonzentration von ca. 10^7 Submerssporen ml^{-1} außer LRC112 mit einer maximalen Sporenkonzentration von 6×10^4 Submerssporen ml^{-1}. Alle weiteren Versuche wurden aufgrund der Wirksamkeit ausschließlich mit JKI-BI-1450 durchgeführt. Die Bildung von Submerssporen und Biomasse dieses Pilzstammes wurde bei verschiedenen Temperaturen und nach unterschiedlichen Inkubationszeiten untersucht. Die maximale Bildung von Submerssporen wurde nach 48 h bei 25 °C mit $6,5 \times 10^5$ Submerssporen ml^{-1} erreicht, die

maximale Biomassebildung ebenfalls nach 48 h, aber bei 30 °C mit 3,2 %. Anschließend wurde das Wachstum von JKI-BI-1450 in einem 3-Liter-Bioreaktor bei 25 °C untersucht. Es wurde ermittelt, dass die Bildung von Submerssporen ihr Optimum zwischen 42,5-48 h nach der Inokulation mit Sporenkonzentrationen zwischen 1,43-2 × 10^6 Submerssporen ml^{-1} hatte, wohingegen die Biomassebildung während der gesamten 72-stündigen Fermentationsdauer auf 1,43 % anstieg. Um die Pilzproduktion in Flüssigkultur zu steigern, wurde der Einfluss der Konzentration der Medienkomponenten (Glukose, Corn steep solid, Natriumchlorid) untersucht. Die Erhöhung der Glukose sowie des Corn steep solid im Medium führten zu einer stetigen Zunahme der Biomasse auf 28,0 g l^{-1} bzw. 23,0 g l^{-1}. Im Gegensatz dazu führte die Änderung des Natriumchlorid-Gehaltes im Medium zu uneinheitlichen Ergebnissen, wobei auch hier die höchste Konzentration zur größten Biomassebildung mit 14,7 g l^{-1} führte.

Nach der Produktion in Flüssigkultur ist es notwendig, die Biomasse zu formulieren, um ein ausbring- und lagerfähiges Produkt zu erhalten. In Chapter III wurde die Wirbelschichttrocknung zur Formulierung von JKI-BI-1450 gewählt, bei der eine dünne Schicht Pilzbiomasse auf Hirse gesprüht und gleichzeitig getrocknet wurde. Diese Formulierungsmethode erwies sich als gut geeignet für die Herstellung eines Granulates, wobei 4,5 mg Pilzbiomasse pro Gramm Granulat benötigt wurden, um eine Granulatkolonisierung von nahezu 100 % zu erreichen. Um das Pilzwachstum auf dem Granulat zu steigern bzw. zu beschleunigen, wurden der Pilzbiomasse vor dem Sprühen und Trocknen Chitin und verschiedene Zucker zugesetzt. Die Zugabe von Chitin zeigte keine positive Wirkung, während die Zugabe der Zucker zu einer Beschleunigung der Kolonisierung sowie der Konidienbildung führte, wobei die besten Ergebnisse mit Fruktose erzielt wurden. Die Zugabe von Fruktose erhöhte die Granulatkolonisierung von 5,6 % auf 62,2 % nach einem Tag Inkubation und die Konidienbildung von 3,35 × 10^5 auf 1,13 × 10^6 Konidien pro Granulatkorn nach 5 Tagen

Inkubation. Auch die Lagerstabilität des Granulats wurde untersucht, da dies wichtig ist, um eine ausreichende Haltbarkeit des Endprodukts zu gewährleisten. Nach 12 Wochen Lagerung bei 5 °C lag die Kolonisierung des Granulats immer noch bei über 90 %. Die Untersuchung der Granulatqualität unter simulierten Feldbedingungen zeigte, dass bei den optimalen Bodenrestfeuchten von 30 % und 45 % die höchste Kolonisierung mit 98,9-100 % bei 25 °C und 30 °C erreicht wurde und die meisten Konidien pro Granulatkorn mit 3,0-3,5 × 10^6 bei 25 °C gebildet wurden. Weiterhin wurde nachgewiesen, dass die durch einen ungünstigen Umweltparameter (Temperatur und Feuchtigkeit) verursachte Verringerung der Kolonisierung und Konidienbildung geringer war, wenn der andere Umweltparameter im optimalen Bereich lag. Außerdem wurde ein fungizides Saatgutbehandlungsmittel (Moncut®) als kompatibel mit dem entwickelten Granulat identifiziert, ohne dass es zu einer signifikanten Verringerung der Granulatkolonisierung kam.

In Chapter IV wurde untersucht, wie sich die Produktionstemperatur des Pilzstammes auf die Granulatkolonisierung und die Konidienbildung auf dem Granulat sowie die Keimung, Konidiengröße und Virulenz der Konidien auswirkt. Zu diesem Zweck wurde die für die Granulatherstellung benötigte Biomasse bei unterschiedlichen Temperaturen erzeugt und die daraus hergestellten Granulate ebenfalls bei unterschiedlichen Temperaturen inkubiert. Der Pilz auf dem Granulat zeigte die höchste Kolonisierung mit 87,17 % und Konidienbildung mit 2,77 × 10^7 Konidien pro Granulatkorn, wenn die Biomasse bei 25 °C hergestellt wurde. Darüber hinaus wurden Konidien bei verschiedenen Temperaturen produziert und dann ebenfalls bei verschiedenen Temperaturen inkubiert und deren Größe, Keimung und Virulenz untersucht. Die bei 20 °C produzierten Konidien waren mit 2,21 µm signifikant breiter als die bei 25 °C und 30 °C produzierten mit 2,19 µm. Außerdem erreichten die bei 15 °C produzierten Konidien τ signifikant später (15,42 h) als die bei 30 °C produzierten Konidien (12,31 h). Die

Zusammenfassung

Produktionstemperatur der Konidien hatte keinen Einfluss auf die Virulenz gegenüber *Galleria mellonella*-Larven. Die Mortalität lag bei allen Produktionstemperaturen zwischen 95,63- 100 % mit einer Überlebenszeit von 50 % (ST_{50}) zwischen 3,97-4,54 Tagen. In Bezug auf die Inkubationstemperatur wurde festgestellt, dass bei 30 °C kürzere Konidien mit 6,54 µm gebildet wurden, die Keimung schneller erfolgte mit τ von 11,40 h und die Pilzbehandlung zu einer schneller eintretenden Mortalität mit einer ST_{50} von 3,16 Tagen führte.

Zusammenfassend lässt sich sagen, dass in dieser Dissertation *M. brunneum* JKI-BI-1450 als Pathogen für die Larven aller drei untersuchten *Agriotes*-Arten identifiziert wurde. Dieser Pilzstamm kann in Flüssigmedium produziert und mittels Wirbelschichttrocknung zu einem Granulat formuliert werden. Sowohl die Produktion als auch die Formulierung konnten durch die Anpassung der Prozessparameter oder der Medienzusammensetzung sowie durch die Zugabe von Zusatzstoffen verbessert werden. Außerdem wurde gezeigt, dass die Qualität eines Bodengranulats auf Pilzbasis bereits durch die Produktionsbedingungen der dafür verwendeten Biomasse beeinflusst werden kann.

General Introduction

Wireworms as pest insects in agriculture

The cultivation of crops is put at risk from damage by micro- and macroorganisms, such as insects, weeds, nematodes and diseases caused by fungi, bacteria and viruses. One of the insects that cause severe damage in many crop species are the larvae of click beetle (Coleoptera: Elateridae), called wireworms (Parker and Howard 2001; Veres et al. 2020). Click beetle, and thus wireworm, is a descriptive term, which comprises 10000 different species from 400 genera (Costa et al. 2010). In addition to some carnivorous wireworms (Traugott et al. 2008; Samoylova et al. 2017), there are many genera of click beetles whose larvae live in soil and feed on roots and seedlings of various plants and can thus lead to enormous crop losses. Crops consumed by wireworms include corn, lettuce, carrot, asparagus and potato, among others (Parker and Howard 2001; van Herk and Vernon 2013). For potato alone, 39 species of from 21 genera of click beetles where reported as damaging the tubers (Jansson and Seal 1994). The wireworms damage the potato by feeding at or through it and leaving typical tunnels in the potato resulting in a loss of quality and crop losses. Furthermore, the feeding tunnels of wireworms in potatoes can lead to a secondary infection with pathogens, such as *Rhizoctonia solani* (Ester and Huiting 2007; Keiser et al. 2012b; Traugott et al. 2015). Infection with *R. solani* can be further associated with drycore on potatoes (Keiser et al. 2012a). This damage results in delayed emergence, lesions on stems and stolons as well as sclerotial formation, deformation and unsaleability of the tubers and a decline of the seed potato quality (Banville 1989; Carling et al. 1989; Scholte 1989; Jager et al. 1991; Brandl et al. 2017).

The most widespread and damaging wireworms in Germany are of the genus *Agriotes* including *Agriotes lineatus*, *Agriotes sputator*, *Agriotes obscurus*, *Agriotes sordidus* and

Figure 1: *Agriotes* larvae.

Agriotes ustulatus (Figure 1). *Agriotes* species pass through up to 13 larval stages over the course of two to five years, ending as short-lived adults that hatch to feed and reproduce from March to June, whereby the number of larval stages and the duration of development depend on the species, geographical location and climate (Jossi and Bigler 1997; Furlan 1998; Furlan 2004; Jossi et al. 2008). The differentiation of the individual species of click beetles in the larval stage is very difficult, but the genera can usually be identified by the abdominal end of the larvae. *Agriotes* for example have a tapered abdomen end. Wireworms already presented a problem to agriculture at the beginning of the 20th century and they were controlled by the use of organochlorines, organophosphates and carbamates since the 1950s. Since the 1990s, it has become increasingly important to find other control strategies as these substances have been banned in Europe (Parker and Howard 2001; van Herk and Vernon 2013) because of their ecotoxicity (Costa 2015), damage of non-target organisms (Desneux et al. 2007; Mitra et al. 2011) and the persistence of the substances in the soil over years (Wilkinson et al. 1976). Subsequently, new types of chemical insecticides included pyrethroids, phenylpyrazoles, and neonicotinoids were used (Jeschke et al. 2011) but their effectiveness is variable and unreliable and/or are also a risk to the health of humans, plants, the environment, bees and other pollinators as well as beneficial spiders and mites (Barsics et al. 2013; Ritter and Richter 2013; Zhang and Nieh 2015; Douglas and Tooker 2016). Therefore, many of these insecticides have been banned in the European Union as well, especially the neonicotinoids and no registered products are available today. In recent years as well as the current one, ATTRACAP® (BIOCARE Biologische Schutzmittel GmbH, Einbeck, Germany),

which is based on *Metarhizium brunneum* strain CB15-III, has been available under an emergency authorisation. Another control strategy used against wireworms is biofumigation, whereby crucifers with insecticidal properties are cultivated. Especially *Brassica* species, which produce a high amount of glucosinolates, show promising effects (Furlan et al. 2010; Noronha 2011). Removal of weeds, mechanic soil cultivation and specific preceding crops can contribute to the reduction of wireworms (Willis et al. 2010). The cultivation of selected potato varieties could also be useful, as not all varieties are equally susceptible to wireworm damage (Kwon et al. 1999; Johnson et al. 2008). However, as these methods alone are not sufficient, the utilization of natural antagonists as biological control agents is an interesting alternative that should be further explored.

Biological control of insects with entomopathogenic fungi

Biological plant protection is based on the application of natural antagonists, plant extracts or pheromones to control pests. Natural antagonists can be viruses or bacteria, fungi, mites, nematodes or insects. According to Eilenberg et al. (2001), biological control can be divided into four sub-areas: conservative (preservation and promotion of naturally occurring beneficial organisms), inoculative (use of starter populations without permanent establishment of the antagonist), inundative (mass application of beneficial organisms against pests) and classical (involves the establishment of non-native species). In contrast to chemical pesticides, biological control agents often have a more selective effect against pests and thus pose less risks to humans, non-target organisms and the environment (Whipps and Lumsden 2001). As a result, they contribute to the conservation of biodiversity and have a low risk that the target pests develop resistance (Ambethgar 2009). Especially entomopathogenic fungi (EPF) of the genus *Metarhizium* are promising candidates for wireworm control (Ladurner et al. 2009; Razinger et al. 2013; Reddy et al. 2014; Sufyan et al. 2017; Sharma et al. 2020) (Figure 2).

Figure 2 : Larvae of *Agriotes obscurus* infected with *Metarhizium brunneum* JKI-BI-1450.

EPF do not systematically form a taxon of their own, but are a collective designation of organisms with similar properties and include all fungi that have a harmful or lethal effect on insects (Roberts and Humber 1981). Over 750 species with this property are known to date (Ramanujam et al. 2014; Sujeetha and Sahayaraj 2014; Kaczmarek and Boguś 2021). EPF are particularly represented in the divisions of the Zoopagomycota and the Ascomycota, but also among the Chytridiomycota (Sinha et al. 2016). They are natural antagonists of arthropods and thus exert a natural control over insect populations (Butt and Goettel 2000; Goettel et al. 2001). EPF can infect insects from nearly all orders; the most common are Hemiptera, Diptera, Coleoptera, Lepidoptera, Orthoptera and Hymenoptera (Ramanujam et al. 2014).

Unlike viruses and bacteria, the infection of an insect with EPF does not occur orally but in most case via the cuticle (Zimmermann 2007a; Zimmermann 2007b). In addition to that infections can also occur peroral via the mouth, perstigmal via the respiratory tract or pertraumatic via wounds (Müller-Kögler 1965; Zimmermann 2007a; Zimmermann 2007b). However, as these infection pathways occurs very rarely, only the percuticular infection is described in detail below (Figure 3). Generally, two phases of the infection of an insect with an

EPF can be distinguished, the parasitic phase (Figure 3 A) and the saprophytic phase (Figure 3 B).

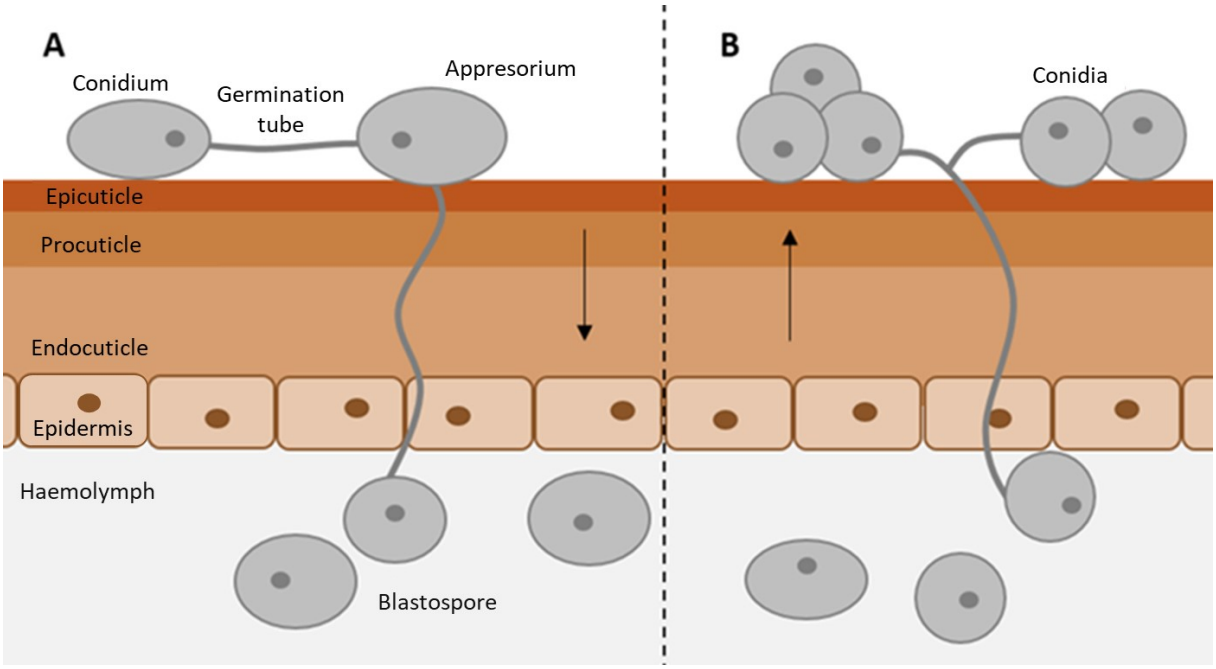

Figure 3: Infection by entomopathogenic fungi; A: parasitic phase, B: saprophytic phase.

Whereas the parasitic phase is defined as the period from the attachment of the spore to the insect until its death, the saprophytic phase includes the outgrowth of the fungal mycelium and the formation of new conidia. The infection begins with the contact of the conidia with the cuticle (Boucias et al. 1988; Thomas and Read 2007). The binding of the conidia to the host cuticle occurs initially via nonspecific hydrophobic interaction and is influenced by the charge and the texture of the cuticle (Boucias et al. 1988; Sosa-Gomez et al. 1997; Ortiz-Urquiza and Keyhani 2013). Thereupon, specific adhesins are formed, such as metarhizium adhesin 1 (MAD1) in the case of *Metarhizium* (Wang and St Leger 2007). Afterwards, the conidium forms a germ tube and at its end often an appressorium, which further strengthens the connection with the target surface. Due to mechanical pressure (Zimmermann 2007a; Zimmermann 2007b; Maina et al. 2018) and the formation of hydrolytic enzymes (e.g. lipases, proteases, chitinase) the cuticle is penetrated, preferably through thinner, weak or

non-sclerotised areas such as the intersegmental membranes (Jarrold et al. 2007; Zimmermann 2007a; Zimmermann 2007b; Schrank and Vainstein 2010; Maina et al. 2018). Following the cuticle penetration, the hyphae grow into the haemocoel of the host and form blastospores, which passively disperse in the host's haemolymph. The proliferating hyphae consume the nutrients present in the haemolymph and the fat body. In addition to the mechanical pressure created by the proliferating hyphae and the consumption of oxygen and nutrients, some fungi are able to produce toxins (Maina et al. 2018), such as cyclic depsipeptides (destruxins) in case of *Metarhizium* (Samuels et al. 1988a; Dumas et al. 1994; Vey et al. 2002; Wang et al. 2012). Samuels et al. (1988b) related the lethal effect of destruxins in lepidopterans to the direct opening of Ca^{2+} channels in cellular membranes, which induces a muscle depolarization in the insect, causing tetanic paralysis. With the death of the host, the parasitic phase ends and the saprophytic phase begins. The fungal hyphae grow from the dead insect, which often mummifies. At appropriate environmental temperature and humidity, the EPF form conidia on the hardened insect body (Butt et al. 2016). These conidia serve to spread the fungus in the environment.

Insects have an innate immune system and are not completely unprotected against the attack of an EPF. The cuticle is the first line of defence of an insect. It consists of germicidal fat molecules such as short-chain fatty acids, aldehydes, wax esters, ketones and alcohols, which all have an antimicrobial effect (Zimmermann 2007a; Zimmermann 2007b). Furthermore, various immune reactions are triggered to prevent the invasion and spread of a fungus. These reactions include the activation of phenoloxidase and various haemocytes, production of antifungal components, melanisation and the production of proteins for detoxification of fungal toxins. Esterases in particular are important for detoxifying destruxins (Pedras et al. 2002). Melanisation can often be recognised by brown spots on the surface of the insect (Zimmermann

2007a; Zimmermann 2007b); however, all these reactions require that the insect immune system recognises the EPF as a pathogen. Receptors like peptide glycans and β-glucan-binding proteins are needed, to recognise fungal pathogen-associated molecular patterns (PAMP'S), such as fungal β-glucans (Butt et al. 2016). On the other hand, fungi have mechanisms to avoid the immune response of the insect. These factors include for example an altered surface of blastospores, which makes them less antigenic, as well as the interruption of the cascade to activate phenoloxidase. Overall, in many cases the host is unable to resist mycosis once the cuticle is penetrated (Hou and Chang 1985; Wang and St Leger 2005; Chouvenc et al. 2009). In addition to the immune response, an insect can also defend itself against infection by EPF through its behaviour. One example is behavioural fever. Infected insects seek warmer places to increase their body temperature, causing the pathogen to die or being suppressed. This behaviour has been observed in reptiles (Vaughn et al. 1974), fish (Reynolds et al. 1976), amphibians (Casterlin and Reynolds 1977) and invertebrates (Casterlin and Reynolds 1979; Casterlin and Reynolds 1980). Ouedraogo et al. (2003) demonstrated that the locust *Locusta migratoria* can develop a behavioural fever that reduces fungal infection by *Metarhizium anisopliae* var. *acridum*. Seeking warm places was not observed in wireworms infected with *Metarhizium*. However, they are able to recognize the presence of *Metarhizium* in the soil and to avoid it (Kabaluk and Ericsson 2007). Another behaviour of insects to prevent successful infection with EPF is grooming though such behaviour was not observed when wireworms were exposed to *Metarhizium* in soil (Kabaluk and Ericsson 2007).

Since the 1950s, numerous plant protection products based on EPF have been developed. Today, EPF are registered in many countries worldwide to replace chemical insecticides (de Faria and Wraight 2007; Li et al. 2010). These plant protection products include for example

Naturalis® based on *Beauveria bassiana* (strain ATCC 74040), Lalguard M52^{GR} based on *M. brunneum* (strain F52) and No Fly® based on *Cordyceps fumosorosea* (strain FE 9901).

Metarhizium

Metarhizium, along with *Beauveria* and *Cordyceps* (formerly *Paecilomyces* and *Isaria*, respectively), is one of the most important fungal genera in biological plant protection and belongs to the division Ascomycota, the order Hypercreales and the family Clavicipitaceae. *Metarhizium* was first described in 1879 by Metschnikoff, who named it *Entomophthora anisopliae* Metchnikoff. In 1880, he renamed the species *Isaria destructor* Metschnikoff. Then in 1883, the species was transferred to the new genus *Metarhizium* by Sorokin and subsequently named *Metarhizium anisopliae* (Met(s)chnikoff) Sorokin after its discoverer Metschnikoff and its host *Anisoplia austriaca*, where it was isolated from (Zimmermann et al. 1995). At that time, the genus consisted of only three species, *M. anisopliae*, *Metarhizium album*, and *Metarhizium flavoviride,* classified on the basis of morphological characteristics of the conidia (Brunner-Mendoza et al. 2019). New molecular biology methods have led to the identification of additional species and today the genus comprises 30 species (Kepler et al. 2014). Fungi of the genus *Metarhizium* are naturally found in soils and on insects worldwide, except the Antarctica (Roberts and St Leger 2004; Zimmermann 2007b). Besides saprophytes, rhizosphere colonizers, and root endophytes, this genus comprises many entomopathogenic species that can infest over 200 insect pest species (Hu and St Leger 2002; Zimmermann 2007b; Wyrebek et al. 2011; Sasan and Bidochka 2012; Brunner-Mendoza et al. 2019). However, host specificity differs greatly between species. *Metarhizium robertsii* and *M. anisopliae* have a broad host range, whereas others show specificity for certain insect groups, e.g. *Metarhizium acridum* and *M. album* for Orthopteran

and Hemiptera or *Metarhizium majus* for beetles (Amiri-Besheli et al. 2000; Brunner-Mendoza et al. 2019).

Fungi are divided into two reproductive stages, the anamorph form with asexual reproduction and the teleomorph form with sexual reproduction. When both are present, it is referred to as holomorphic form. For many EPF only the anamorphic form is known, as it is the case for the *Metarhizium* species investigated in the present study. *Metacordyceps*-like sexual stages of *Metarhizium* were described by Sung et al. (2007). During asexual reproduction, *Metarhizium* forms differently branched and only rarely simple conidiophores. One to several phialides are located at the tips of the conidiophores, on which the conidia are formed. The conidia are hyaline, brown or green and usually form chains (Kepler et al. 2014). The conidia germinate and develop mycelium, on which conidiophores can form again. When the conidium encounters an insect, it attaches to the cuticle. After penetrating the insect´s cuticle into the haemolymph, blastospores are formed by budding of hyphae or by yeast-like growth (Adámek 1965; van Winkelhoff and McCoy 1984; Jenkins and Goettel 1997; Vestergaard et al. 1999).

Aim and objectives of the thesis

At present, there is no chemical or biological plant protection product or other reliable control strategies to control wireworms. There are promising approaches towards the use of EPF against wireworms. Therefore, the aim of this dissertation was to develop a soil granule based on EPF for the control of the larvae of click beetle (*Agriotes*) in potato cultivation.

To achieve this goal, the following objectives were defined for each chapter:

Chapter I: Finding a pathogenic fungal strain with widespread efficacy against *Agriotes* larvae, to ensure the effectiveness of the later plant protection agent in the field.

Chapter II: Optimization of the producibility of the fungal strain in liquid medium and to develop a process for mass production.

Chapter III: Production and optimization of a soil granule by fluid-bed drying and the examination of the influence of field conditions on this granule.

Chapter IV: Determining whether the production temperature of the fungal biomass can influence the granule quality and whether the temperatures at which the conidia are formed influence their size, germination and virulence.

Chapter I

Pathogenicity of *Metarhizium* and *Cordyceps* isolates against larvae of different *Agriotes* species and populations in correlation with conidial size and germination

Abstract

The use of entomopathogenic fungi offers a promising approach to control *Agriotes* larvae. However, the effect against various *Agriotes* species is very different. Therefore, the first step was to look for a fungus that has a good effect against different *Agriotes* species. To achieve this, six *Metarhizium* and two *Cordyceps* strains were studied. Several *Metarhizium* strains were highly efficient and caused over 90% mortality. The most promising strain to control *Agriotes lineatus* and *Agriotes obscurus* was the *Metarhizium brunneum* strain JKI-BI-1450, while *Metarhizium robertsii* strain JKI-BI-1442 was most efficient against *Agriotes sputator* larvae. The *Cordyceps* strains had no pathogenic effect. In a second step, the conidial size and the germination of the effective strains were determined in order to examine whether the parameters were related to their effect, which could not be confirmed for *A. lineatus* and *A. obscurus*. Shorter and wider conidia as well as those that germinate later and show a lower germination after 96 h are more effective against larvae of *A. sputator*. In a third step, it was investigated whether populations of the same *Agriotes* species, differ in their susceptibility to entomopathogenic fungi. Different populations of *A. obscurus* larvae showed variable susceptibility. Significant differences in the Restricted Mean Time Lost (RMTL) but not in the final mortality were determined for different populations of *A. sputator* larvae. In contrast, the

efficacy on *A. lineatus* was similar among the various populations tested. The present study indicates highly specific and complex interactions between the *Agriotes* species and the *Metarhizium* strains.

Introduction

Wireworms are the polyphagous subterranean larvae of click beetles (Coleoptera: Elateridae) and one of the most widespread and detrimental soil pests. They feed on the roots and seedlings of many crops, such as potatoes, wheat and corn (Horton 2006; Johnson et al. 2008; Ansari et al. 2009; Reddy et al. 2014; Milosavljević et al. 2017). The typical damage pattern of wireworms is tunnel-shaped feeding holes in the roots and seedlings of plants (Traugott et al. 2015). This damage causes a reduction in crop quality, which in turn leads to a reduced market value (Johnson et al. 2008; Benjamin et al. 2018) or crop loss (Parker and Howard 2001; Barsics et al. 2013; Ritter and Richter 2013; van Herk and Vernon 2013). In Germany and other European countries, larvae of the genus *Agriotes* cause major damage, especially the species *Agriotes obscurus*, *Agriotes lineatus* and *Agriotes sputator* (Parker and Howard 2001; Sufyan et al. 2007; Burghause and Schmitt 2011; Vidal and Petersen 2011; Lehmhus 2012; Lehmhus and Niepold 2013; Lehmhus 2017). Moreover, these three *Agriotes* species were accidentally introduced to North America and are becoming an increasing problem there (Wilkinson et al. 1976; Vernon et al. 2001; Vernon et al. 2005). The control of wireworms is difficult, due to their long and subterranean life cycle, overlapping generations, polyphagous nutrition and their occurrence in many different arable and grassland crops (Furlan 1998; Furlan 2004; Ritter and Richter 2013; Traugott et al. 2013; Vernon et al. 2013; Sonnemann et al. 2014; Sufyan et al. 2014; Traugott et al. 2015). At present, there are no chemical or biological pesticides with approved efficacy available in Europe (Veres et al. 2020). Other control strategies like crop rotation (Willis et al. 2010), weed control, mechanical tillage and the cultivation of selected

potato varieties help reducing wireworm damage, but are not efficient in controlling wireworm populations. Therefore, studies on potential natural antagonists as biocontrol agents are of great importance. Several natural antagonists, e.g. bacteria, fungi and nematodes have been repeatedly isolated from wireworm populations collected in the field (Kleespies et al. 2013; Leclerque et al. 2013). Furthermore, the efficacy of entomopathogenic fungi (EPF) against wireworms and the resulting protection of the crop has often been studied (Ladurner et al. 2009; Razinger et al. 2013; Reddy et al. 2014; Sufyan et al. 2017; Sharma et al. 2020). Especially fungi of the genus *Metarhizium* have already been successfully used against wireworms (Kabaluk et al. 2005; Kabaluk and Ericsson 2007; Eckard et al. 2017; Razinger et al. 2018). Through these studies, many *Metarhizium* strains are known to have good activity against larvae of the *Agriotes* genus, but the effectiveness of one fungus against different *Agriotes* species varies (Eckard et al. 2013; Eckard et al. 2014).

For this reason, the first aim of the present chapter was to find a fungus with good activity against the three main *Agriotes* species. The effect of six *Metarhizium* spp. and two *Cordyceps fumosorosea* (formerly *Paecilomyces* and *Isaria*, respectively) strains was investigated. Three of the six selected *Metarhizium* strains have already been tested against wireworms in previous studies, like the *Metarhizium brunneum* strain JKI-BI-1339 (well known as M.a. 43, BIPESCO 5 or F52) (Eckard et al. 2014; Reddy et al. 2014; Antwi et al. 2018). Reddy et al. (2014) demonstrated a reduction of wireworms and an increase in standing plants and yield through the application of F52. Also Antwi et al. (2018) demonstrated that F52 combined with other biopesticides protected seedlings against wireworms. The effect of the *M. brunneum* strain LRC112 against *Agriotes* beetles was examined in the laboratory and in the field by Kabaluk (2014) and Kabaluk et al. (2015). Furthermore, a soil granule based on *M. brunneum* strain JKI-BI-1450 was investigated for its ability to reduce wireworm damage

to potatoes in the field (Paluch 2021). The other three *Metarhizium* strains were investigated, as they were isolated from *Agriotes*. In contrast to *Metarhizium*, the effect of *Cordyceps* on *Agriotes* larvae is much less studied although fungi of this species have previously been isolated from *Agriotes* larvae (Kleespies et al. 2013; Ritter and Richter 2013). The *Cordyceps* strains examined in the present study were also isolated from *Agriotes*.

As such efficacy studies are very laborious and time-consuming, the second aim of the present chapter was to identify strain specific characteristics that influence the pathogenicity against the target host, which would be an important selection criterion for a biological agent. In this way, a pre-selection from many fungi could already be made before efficacy experiments. Germination is an important point in the infection of an insect with a fungus. The infection begins with the contact of the conidia with the cuticle (Boucias et al. 1988). Thereupon the conidia attach, germinate and form a germ tubes, which penetrate the cuticle through mechanical pressure and the secretion of hydrolytic enzymes (Jarrold et al. 2007; Zimmermann 2007a; Zimmermann 2007b; Schrank and Vainstein 2010). Rapid germination can benefit an infection, because the more time elapses between attachment and host penetration, the higher the risk that conidia may dry out, be inhibited by cuticular peptides or removed through moulting or other insect behaviours (Hassan et al. 1989; Samuels et al. 1989). Furthermore, a high germination is advantageous, as a positive dose-response relationship has already been described for *Metarhizium* by Cherry et al. (2005) and Ekesi et al. (2002). Jackson et al. (1985) investigated the correlation of the germination and spore length of 18 strains of *Verticillium lecanii* and their virulence against *Macrosiphoniella sanborni*. Fast germination could be associated with virulence, but not spore length. In contrast, Altre et al. (1999) showed that strains of *Paecilomyces fumosoroseus* with longer conidia and faster germination were more virulent against *Plutella xylostella.* In order to achieve the second aim, the germination and conidial size of the potent fungal strains from the efficacy experiment were determined.

The third aim of the present chapter was to find out if pathogenicity of selected *Metarhizium* strains is specific only on host species level, or differs even intra-specifically, among different population of a given host species. The latter would obviously impede successful commercialisation of a given strain, while a promising biocontrol agent would exhibit high efficacy across a wide range of target host populations. This hypothesis was established because there are various studies in which the same fungal strain is pathogenic in different ways against the same *Agriotes* species. In the study of Eckard et al. (2014), *M. brunneum* V1002 achieved a mortality of about 40% after 8 weeks against larvae of *A. lineatus*, whereas the same fungus achieved a mortality of about 90% after already 3 weeks in the study of Ansari et al. (2009). A difference in the effect of *M. brunneum* ART2825 on *A. lineatus* larvae was also reported by Eckard et al. (2014) with 70% mortality after 8 weeks and by Kölliker et al. (2011) with 50% mortality after 9 weeks, even if the difference is less clear than in the preceding example. The effect of the fungus on larvae of *A. obscurus* with 80% mortality and *A. sputator* with 40-45% mortality was very similar in both studies. To investigate this hypothesis, the effect of two *M. brunneum* strains with proven efficacy against different populations of the three main *Agriotes* species was examined. These two fungal strains tested were JKI-BI-1450, the best from the present efficacy experiments and the strain ART2825. Eckard et al. (2014), Kölliker et al. (2011), Mayerhofer et al. (2017), Reinbacher et al. (2021) and Rogge et al. (2017) explored the impact of *M. brunneum* strain ART2825 on the mortality of *Agriotes* larvae, the decrease of their number in the field, damage on potatoes and the establishment of the fungus in the soil.

Material and Methods

Agriotes rearing

Adult *Agriotes* were collected from the following six locations in Germany and Switzerland: near Wohld (52°18’11.0"N, 10°41’11.6"E, 89 m asl (above sea level)), Beienrode

(52°17'36.6"N, 10°49'53.7"E 92 m asl), Geinsheim am Rhein (49°53'16.8"N, 8°25'10.7"E, 84 m asl) and Mühlheim am Main (50°07'48.1"N, 8°49'41.3"E, 103 m asl) in Germany and Wallestalden (46°56'41.6"N, 7°50'17.4"E, 921 m asl) near Langnau in Emmental and Zurich (47°25'3.0"N, 8°31'26.9"E 453 m asl) in Switzerland. Adult click beetles were caught using plastic sheets that were spread out on the ground, covered with cut grass under which the beetles aggregated Kölliker et al. (2009). With these individuals, laboratory rearings were set up following the protocol of Lehmhus and Niepold (2015) in Germany and Kölliker et al. (2009) in Switzerland.

Fungal strains

The fungi investigated in the present study are summarized in Table 1.

Table 1: Origin of entomopathogenic fungi species, strains ID and origin.

Species	Strain ID	Host/Origin	Geographic origin
Metarhizium brunneum	JKI-BI-1339	*Cydia pomonella*	Austria
Metarhizium robertsii	JKI-BI-1441	*Agriotes* sp.	Italy
Metarhizium robertsii	JKI-BI-1442	*Agriotes* sp.	Italy
Metarhizium robertsii	JKI-BI-1448	*Agriotes ustulatus*	Italy
Metarhizium brunneum	JKI-BI-1450	*Agriotes lineatus*	Germany
Metarhizium brunneum	LRC112*	*Agriotes obscurus*	Canada
Metarhizium brunneum	ART2825	*Agriotes obscurus*	Switzerland
Cordyceps fumosorosea	JKI-BI-1513	*Agriotes ustulatus*	Germany
Cordyceps fumosorosea	JKI-BI-1514	*Agriotes ustulatus*	Italy

*was provided by Todd Kabaluk from Agriculture and Agri-Food Canada / Agriculture et Agroalimentaire Canada.

The fungi were cultured on malt peptone agar (MPA) containing 3% (w/v) malt extract (Merck, Darmstadt, Germany), 0.5% (w/v) peptone from soybean (Merck) and 1.8% (w/v) agar-agar (Roth, Karlsruhe, Germany) for 14 days at 25 °C in the dark. After incubation, the conidia were removed from the plates with sterile 0.5% (v/v) Tween® 80 (Merck) by using a sterile spatula. The suspension was filtered through four layers of gauze to remove mycelium. The filtrate was placed in an ultrasonic bath (Sonorex RK 52 35 kHz, Bandelin electronic GmbH & Co. KG,

Berlin, Germany) for 15 min to separate the conidia from each other. Afterwards, the conidia concentration was determined using a haemocytometer and a 5 ml suspension with a concentration of 10^8 conidia ml^{-1} was prepared by diluting with 0.5% (v/v) Tween® 80 for each fungal strain.

Efficacy study on entomopathogenic fungi against *Agriotes*

The effect of the *M. brunneum* strains the *C. fumosorosea* strains given in Table 1 were tested against the larvae of *A. lineatus*, *A. obscurus* and *A. sputator*, offspring from beetles collected at Wohld. The larvae were dipped in the fungal suspensions for 2-3 s and placed individually in plastic boxes (diameter = 7 cm; height = 2.5 cm, with lid, not perforated). In addition to the fungal treatments, an untreated control and a control with 0.5% (v/v) Tween® 80 was included. The boxes were filled 2/3 with soil (Archut, Fruhstorfer Erde Typ Nullerde) (Nitrogen (N) = 20-40 mg l^{-1}, Phosphate (P_2O_5) = 20-40 mg l^{-1}, Potassium (K_2O) = 40-60 mg l^{-1}, pH value = 5.9, Salt content KCl = 0.2 g l^{-1}) which was autoclaved beforehand at 121 °C for 20 min. 500 g of the soil was mixed with 100 ml of autoclaved deionised water. Ten larvae of each *Agriotes* species were used per fungal treatment. Each larva was individually placed in a box and incubated at 25 °C in the dark for four months. The soil was kept moist by spraying sterile deionised water on the surface of the soil. Weekly, the number of alive, dead or dead and mycosed larvae was monitored. For all six *Metarhizium* strains the experiment was repeated five times and for the two *Cordyceps* strains three independent replicates were applied.

Determination of the conidial size

Suspensions of all six *Metarhizium* strains were prepared as described above. Fifty conidia of each fungal strain were photographed, and the length and width were measured using the program cellSens Standard (Olympus, Tokio, Japan). The conidia index was calculated by

dividing the length by the width.

Determination of the germination over time

All six *Metarhizium* strains were incubated for 14 days at 25 °C in dark. After the incubation a small amount of conidia of each fungal strain was transferred into an 1.5-ml Eppendorf tube filled with 0.5% (v/v) Tween® 80 using an inoculation loop. The 1.5-ml Eppendorf tubes were placed on a vortexer for 10 s and afterwards into an ultrasonic bath for 15 min. The conidia concentration was determined using a haemocytometer. For each fungal strain, a 1 ml suspension with a concentration of 10^6 conidia ml^{-1} was prepared by diluting with 0.5% (v/v) Tween® 80. From each suspension, three drops (10 µl each) were dropped onto eight MPA plates, corresponding to the number of evaluation time points. The plates were incubated at 25 °C in the dark and the germination was determined after 9, 12, 15, 18, 21, 24, 27 and 33 h. For each drop, 100 conidia were observed under a light microscope (× 400) and the percentage of germinated conidia was determined. Conidia were rated as germinated when the germ tube was longer than the width of the conidia. This experiment was repeated six times.

Determination of the germination after 96 h

For determination of the overall germination after 96 h, another experiment was set up as described above and 25 mg l^{-1} benomyl (Sigma Aldrich, Buchs, Switzerland) was added to the MPA. The germination was only determined after 96 h. Benomyl does not affect germination but inhibits mycelial growth. The experiment was repeated six times.

Investigation of the efficacy of two *M. brunneum* strains against *Agriotes* larvae from different populations

In this experiment, *M. brunneum* strains JKI-BI-1450 and ART2825 were tested against the larvae of different populations of *A. lineatus*, *A. obscurus* and *A. sputator* from Germany and

Switzerland. The efficacy of JKI-BI-1450 was tested against larvae of *A. lineatus* from Geinsheim and Wohld, *A. obscurus* from Beienrode, Mühlheim and Wallestalden and *A. sputator* from Mühlheim, Zurich and Wohld. The strain ART2825 were tested against larvae of *A. lineatus* from Zurich and Wohld, *A. obscurus* from Beienrode, Wallestalden, Wohld and Zurich and *A. sputator* from Beienrode, Mühlheim and Zurich. Larvae and fungal treatments of the experiment in Germany from the location Wohld were the same as in the present study described above, evaluated 70 days after inoculation. The experiment in Switzerland was set up exclusively for the population test. The strain JKI-BI-1450 was selected because it showed the best effect on two of the three *Agriotes* species in the preceding experiments of the present study. The strain ART2825 has shown high virulence in laboratory assays against larvae of the three *Agriotes* species (Kölliker et al. 2011; Eckard et al. 2014). To determine the larval stages for all larvae used in the experiments, they were photographed prior to use in the bioassay and the head capsule width was measured using the program cellSens Standard, for the *Agriotes* larvae from Germany and the program VHX 6000 (Keyence, Osaka, Japan) for the larvae from Switzerland. Following the description of Klausnitzer (1994), the larval stage was determined by the size of the head capsules. The preparation of the fungal suspensions and the dipping of the larvae were carried out as described above. In addition to the fungal treatments, controls with 0.5% (v/v) Tween® 80 were prepared. The boxes in which the larvae were placed after treatment were filled with approx. 12 g of sterile soil with 45% residual moisture. The moisture content of the soil was determined using a moisture determination balance (Ma30, Sartorius, Göttingen, Germany). The boxes were incubated at 25 °C in the dark and evaluated weekly for 70 days. It was determined whether the larvae were alive, dead or dead and mycosed. To maintain the moisture content during the experiment, control boxes were filled with soil only and weighed at regular intervals throughout incubation. Water was added to every box according to the determined corresponding weight discrepancies observed from the control

boxes. In the treatments with *M. brunneum* JKI-BI-1450, ten individuals were tested per repetition, except for the Wallestalden population, where 20 individuals per repetition were available. Five repetitions were carried out at independent times. In the treatments with *M. brunneum* ART2825, five repetitions with ten individual each were carried out for *A. lineatus* larvae from Wohld and Zurich, *A. obscurus* larvae from Beienrode and *A. sputator* larvae from Beienrode. *A. obscurus* larvae from Wallestalden and *A. sputator* larvae from Zurich were examined with four repetitions with ten individuals each. Owing to a low number of available larvae from the Zurich population, only three repetitions of *A. obscurus* larvae from Zurich with five individuals each and of *A. sputator* larvae from Zurich with ten individuals were examined. The experiment with *A. sputator* larvae from Mühlheim was carried out in four repetitions with ten individuals and a fifth repetition with four individuals only.

Statistical analysis

Data were analysed with the software SAS Studio 3.8. The efficacy of fungi against *Agriotes* larvae was compared based on a survival analysis (Kaplan-Meier-Wilcoxon). Because the mortality was less than 50% for multiple treatments, the Restricted Mean Time Lost (RMTL) was calculated for all larvae that have died (RMTL mortality) and for all larvae that have died and were mycosed (RMTL mycoses) and is defined as the area above the Kaplan-Meier survival curve. RMTL is zero when no experimental animal dies within the experimental period. High and rapid mortality is indicated by a high RMTL. The final mortality and the final mycoses, as well as the larval stage, conidial length, width and index were compared by using a generalized linear model with Wald statistics for type 3 analysis and multiple comparison according to Tukey (GLMM, $p < 0.05$). For the experiment on the efficacy of *Metarhizium* and *Cordyceps* against three species of *Agriotes* larvae, all of the above comparisons were made for all treatments within one *Agriotes* species. To compare the germination over time of the different

fungal strains, τ (time point where 50% of the maximal germinated conidia are germinated) and the slope at the inflection points were calculated for each replication of all repetitions by Seib et al. (2023) according to Dantigny et al. (2011) (Chapter IV). The germination after 96 h, τ and the slope at the inflection point of all fungal strains were investigated and compared using a generalized linear model with Wald statistics for type 3 analysis and multiple comparison according to Tukey (GLMM, $p < 0.05$). The correlation between final mortality and pathogenicity factors was determined using Pearsons's Correlation Test ($p < 0.05$). The mean value was always used for the pathogenicity factors. At the experiment on the efficacy of *Metarhizium* against three species of *Agriotes* larvae from different populations, all parameters were compared within one *Agriotes* species and one fungal treatment across all associated populations. Furthermore, the RMTL mortality, the RMTL mycoses, the final mycoses and the final mortality of all populations from Switzerland were compared with all populations from Germany by using a generalized linear model with Wald statistics for type 3 analysis and multiple comparison according to Tukey with underlying normal distribution (GLMM, $p < 0.05$).

Results

Efficacy study on entomopathogenic fungi against *Agriotes* larvae

The mortality of *Agriotes* larvae differed both between the *Agriotes* species and the fungal strain used. The mortality of *A. lineatus* larvae increased steadily for all fungal treatments at the beginning of the experiment and only after day 91 did the effect of JKI-BI-1450 increase more steeply and stand out more clearly from the other strains (Figure 4).

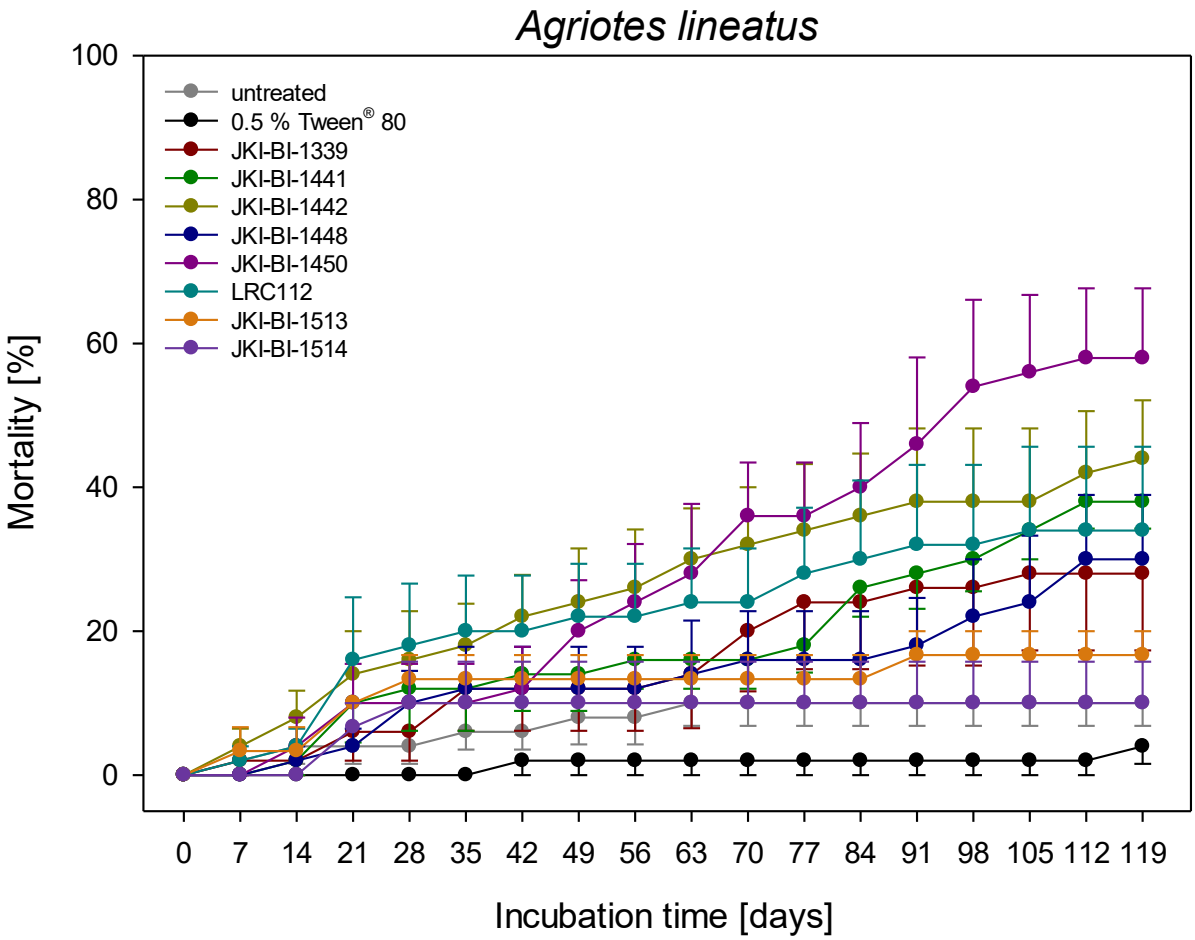

Figure 4: Mortality of larvae of *Agriotes lineatus* treated with six *Metarhizium* strains (*M. brunneum*: JKI-BI-1339, JKI-BI-1450, LRC112; *M. robertsii*: JKI-BI-1441, JKI-BI-1442, JKI-BI-1448) and two *Cordyceps fumosorosea* strains (JKI-BI-1513, JKI-BI-1514). Mean and standard error are shown.

Treatment with JKI-BI-1450 and LRC112 led to a clearly higher mortality of *A. obscurus* larvae at the beginning of the experiment compared to the other fungal strains investigated (Figure 5). After 35 days, the mortality caused by LRC112 increased less than that of JKI-BI-1450 and approached that of JKI-BI-1441 and JKI-BI-1442 at the end of the experiment.

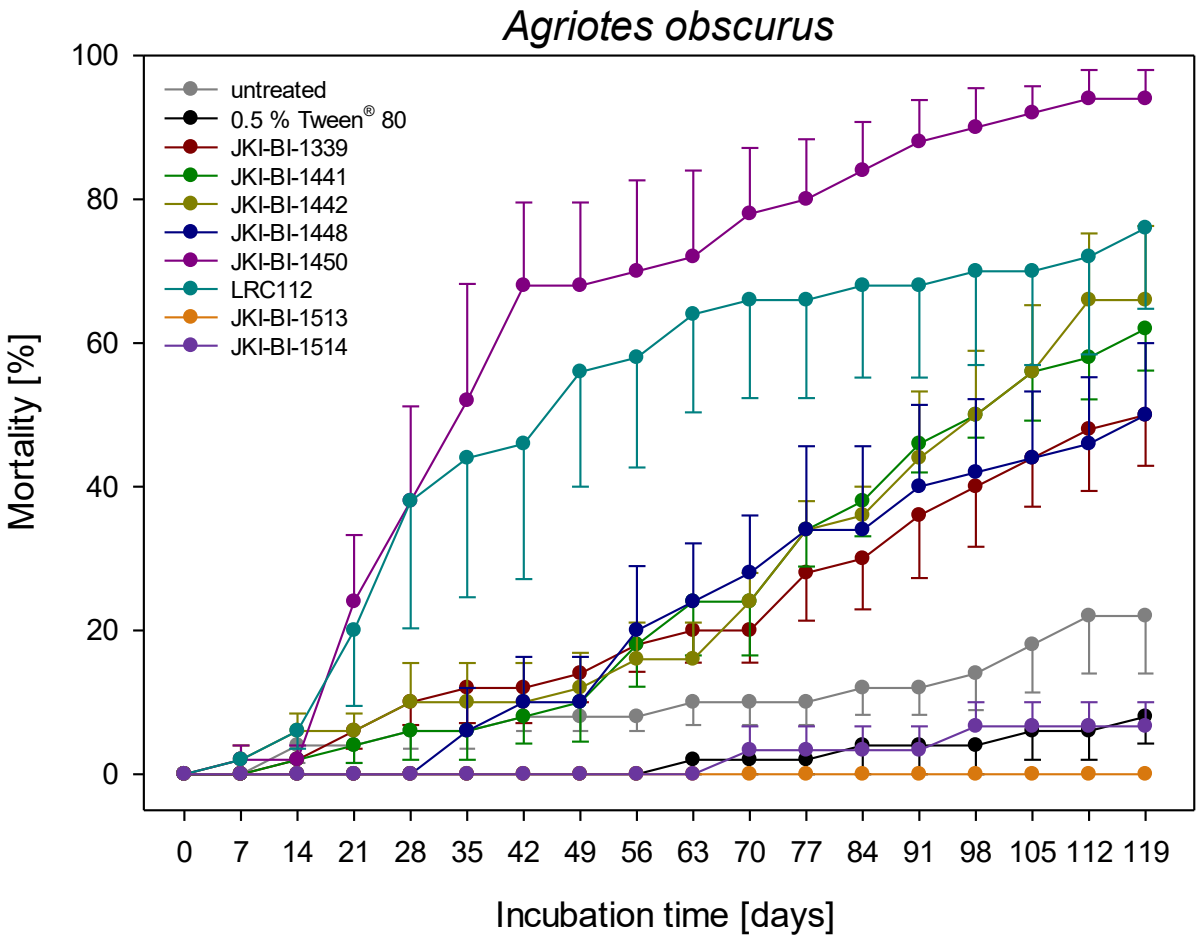

Figure 5: Mortality of larvae of *Agriotes obscurus* treated with six *Metarhizium* strains (*M. brunneum*: JKI-BI-1339, JKI-BI-1450, LRC112; *M. robertsii*: JKI-BI-1441, JKI-BI-1442, JKI-BI-1448) and two *Cordyceps fumosorosea* strains (JKI-BI-1513, JKI-BI-1514). Mean and standard error are shown.

The mortality of *A. sputator* larvae induced by JKI-BI-1339, JKI-BI-1441, JKI-BI-1442 and JKI-BI-1448 increased faster at the beginning of the experiment compared to the other fungal strains (Figure 6). At a later stage of the experiment (after 91 days), the effect of JKI-BI-1450 increased more strongly, but still remained lower than that of the previously mentioned fungal strains.

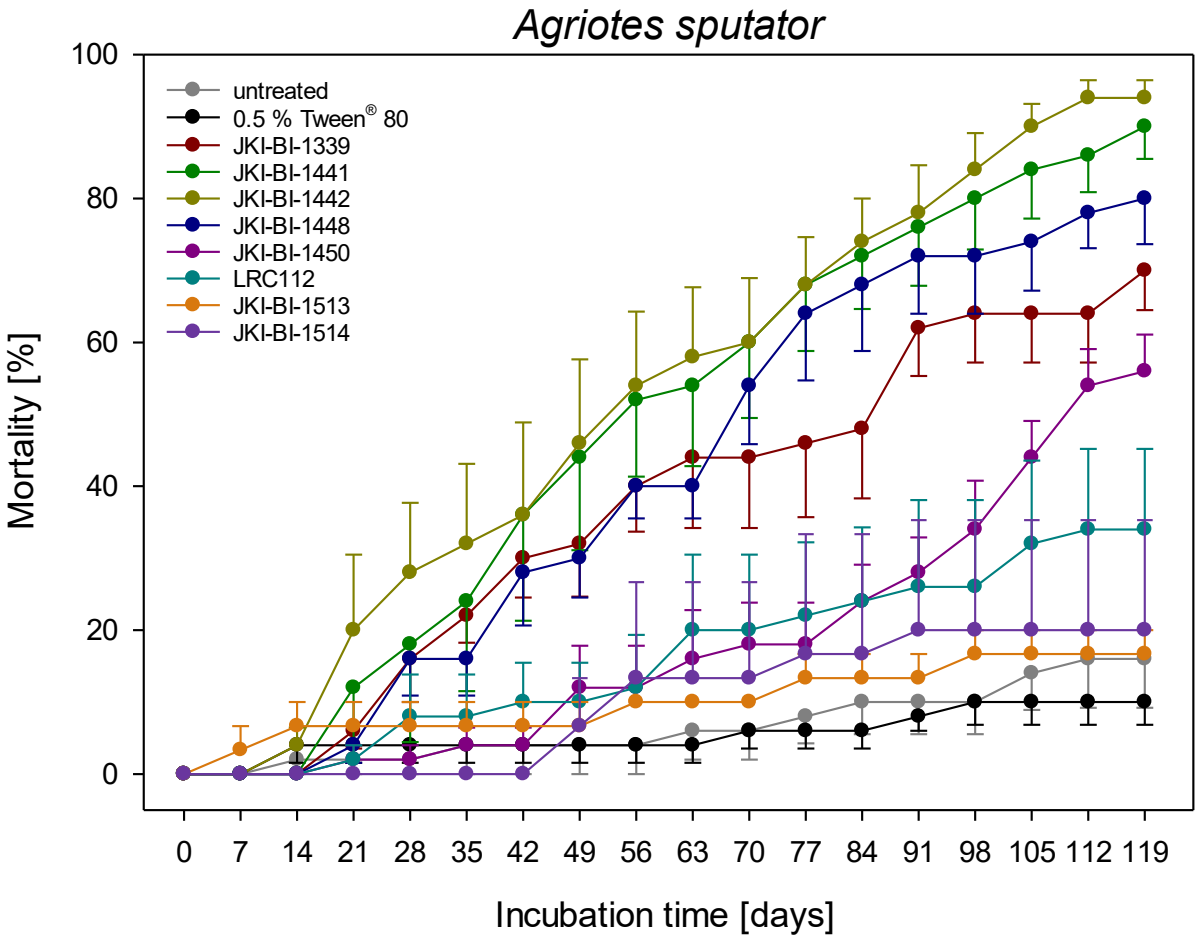

Figure 6: Mortality of larvae of *Agriotes sputator* treated with six *Metarhizium* strains (*M. brunneum*: JKI-BI-1339, JKI-BI-1450, LRC112; *M. robertsii*: JKI-BI-1441, JKI-BI-1442, JKI-BI-1448) and two *Cordyceps fumosorosea* strains (JKI-BI-1513, JKI-BI-1514). Mean and standard error are shown.

RMTL mortality and final mortality of *Cordyceps* treatments were lower than *Metarhizium* treatments in all three *Agriotes* species and did not differ significantly from the controls (Table 2). Furthermore, the treatment with *Cordyceps* never led to fungal growth (mycosis) on the cadaver. However, *Metarhizium* treatments had an effect on survival of all *Agriotes* species, and also lead to mycoses. The final mortality caused by *Metarhizium* infection was generally much lower for *A. lineatus* larvae (28-58%) than for larvae of *A. obscurus* (50-94%) and *A. sputator* (34-94%). There were significant differences in the efficacy parameters of the various fungal strains investigated within the species *A. lineatus* (RMTL mortality: $\chi^2 = 75.97$; df = 9; $p < 0.0001$; RMTL mycoses: $\chi^2 = 88.86$; df = 6; $p < 0.0001$; final mortality: $\chi^2 = 55.23$;

df = 9; $p < 0.0001$; final mycoses: $\chi^2 = 34.86$; df = 7; $p < 0.0001$). Among all strains tested, *M. brunneum* strain JKI-BI-1450 showed the best effect with the highest RMTL mortality of 31.1 and RMTL mycoses of 22, as well as the highest final mortality with 58% and final mycoses with 46%. *M. robertsii* strain JKI-BI-1442 followed with a final mortality of 44%. JKI-BI-1442 had the same RMTL mortality on larvae of *A. lineatus* and *A. obscurus* with 27.6. However, the final mortality on *A. obscurus* larvae was higher with 66%, which indicates a reduced infection time for JKI-BI-1442 on *A. lineatus* larvae compared to *A. obscurus* larvae. As with *A. lineatus*, the efficacy parameters of the different fungal strains showed significant differences on *A. obscurus* larvae (RMTL mortality: $\chi^2 = 505.70$; df = 9; $p < 0.0001$; RMTL mycoses: $\chi^2 = 384.51$; df = 6; $p < 0.0001$; final mortality: $\chi^2 = 181.49$; df = 9; $p < 0.0001$; final mycoses: $\chi^2 = 79.32$; df = 7; $p < 0.0001$). The fungal strain JKI-BI-1450 had the best effect on *A. obscurus* larvae with the highest RMTL mortality (70.1), RMTL mycoses (66.9), final mortality (94%) and final mycoses (90%). The second best efficacy was observed with *M. brunneum* strain LRC112 with a final mortality of 76%. Efficacy parameters of the different fungal strains also revealed significant differences in tests with *A. sputator* (RMTL mortality: $\chi^2 = 211.81$; df = 9; $p < 0.0001$; RMTL mycoses: $\chi^2 = 410.40$; df = 6; $p < 0.0001$; final mortality: $\chi^2 = 276.58$; df = 9; $p < 0.0001$; final mycoses: $\chi^2 = 301.67$; df = 7; $p < 0.0001$). *M. robertsii* strain JKI-BI-1442 was most effective with the highest RMTL mortality (57.7), RMTL mycoses (55.4), final mortality (94%) and final mycoses (88%), closely followed by *M. robertsii* strain JKI-BI-1441 with a final mortality of 90%.

Table 2: Efficacy of six *Metarhizium* strains (*M. brunneum*: JKI-BI-1339, JKI-BI-1450, LRC112; *M. robertsii*: JKI-BI-1441, JKI-BI-1442, JKI-BI-1448) and two *Cordyceps fumosorosea* strains (JKI-BI-1513, JKI-BI-1514) against larvae of *Agriotes lineatus*, *Agriotes obscurus* and *Agriotes sputator*, over 4 months. Different letters represent significant differences within columns and *Agriotes* species. *Metarhizium*: $n = 5$ with ten individuals each, *Cordyceps*: $n = 3$ with ten individuals each.

	RMTL mortality *	**	**RMTL mycoses** *	**	**Final mortality [%]** ***	****	**Final mycoses [%]***	****
A. lineatus								
JKI-BI-1339	17.8±4.6	AB	12.7±4.1	A	28.0±10.7	BCD	18.0±6.6	BC
JKI-BI-1441	20.0±4.7	AB	11.3±3.8	A	38.0±3.7	ABC	18.0±3.7	BC
JKI-BI-1442	27.6±5.6	AB	24.5±5.3	A	44.0±6.8	AB	38.0±7.3	AB
JKI-BI-1448	15.4±4.4	ABC	13.2±4.1	A	30.0±8.9	ABCD	24.0±7.5	ABC
JKI-BI-1450	31.1±4.8	A	22.0±4.1	A	58.0±9.7	A	46.0±6.8	A
LRC112	24.5±5.4	AB	14.3±4.3	A	34.0±11.7	ABC	20.0±12.6	ABC
JKI-BI-1513	14.2±6.2	ABC	0.0±0.0	B	16.7±3.3	BCD	0.0±0.0	C
JKI-BI-1514	9.6±5.2	ABC	0.0±0.0	B	10.0±5.8	BCD	0.0±0.0	C
Untreated	8.5±3.7	BC	n.m.		10.0±3.2	CD	n.m.	
0.5% Tween® 80	1.5±1.5	C	n.m.		4.0±2.4	D	n.m.	
A. obscurus								
JKI-BI-1339	23.7±4.6	B	18.2±4.5	B	50.0±7.1	BC	36.0±5.1	BC
JKI-BI-1441	27.0±4.2	B	24.8±4.2	B	62.0±5.8	B	56.0±4.0	AB
JKI-BI-1442	27.6±4.4	B	22.0±3.9	B	66.0±10.3	AB	58.0±13.9	AB
JKI-BI-1448	23.7±4.2	B	23.5±4.2	B	50.0±10.0	BC	46.0±10.8	B
JKI-BI-1450	70.1±4.4	A	66.9±4.7	A	94.0±4.0	A	90.0±7.7	A
LRC112	55.6±5.6	A	48.3±5.9	A	76.0±11.2	AB	64.0±12.9	AB
JKI-BI-1513	0.0±0.0	C	0.0±0.0	C	0.0±0.0	D	0.0±0.0	C
JKI-BI-1514	2.8±2.0	C	0.0±0.0	C	6.7±3.3	D	0.0±0.0	C
Untreated	10.6±3.8	BC	n.m.		22.0±8.0	CD	n.m.	
0.5% Tween® 80	2.1±1.3	C	n.m.		8.0±3.7	D	n.m.	
A. sputator								
JKI-BI-1339	40.1±5.2	A	37.0±5.1	A	70.0±5.5	AB	66.0±5.1	BC
JKI-BI-1441	53.8±4.6	A	51.2±4.7	A	90.0±4.5	A	86.0±4.0	AB
JKI-BI-1442	57.7±4.6	A	55.4±4.9	A	94.0±2.4	A	88.0±3.7	A
JKI-BI-1448	45.9±4.6	A	44.4±4.8	A	80.0±6.3	AB	76.0±8.1	AB
JKI-BI-1450	19.0±3.7	B	17.2±3.8	B	56.0±5.1	BC	48.0±5.8	C
LRC112	17.8±4.3	B	11.0±4.0	BC	34.0±11.2	CD	16.0±8.7	D
JKI-BI-1513	11.4±5.4	B	0.0±0.0	C	16.7±3.3	D	0.0±0.0	D
JKI-BI-1514	11.0±4.2	B	0.0±0.0	C	20.0±15.3	D	0.0±0.0	D
Untreated	7.1±3.0	B	n.m.		16.0±6.8	D	n.m.	
0.5% Tween® 80	6.0±3.0	B	n.m.		10.0±3.2	D	n.m.	

*RMTL = Restricted mean time lost (area above the Kaplan-Meier survival curve in the interval 0 to t_{max}) Mean ± standard error; ** Survival analysis (Kaplan-Meier-Wilcoxon); *** Mean ± standard error; **** GLMM, $p < 0.05$; n.m. = no mycoses.

Determination of the conidial sizes

The length, width and index of the conidia differed significantly between the various *Metarhizium* strains (length: χ^2 = 343.44; df = 5; $p < 0.0001$; width: χ^2 = 647.74; df = 5; $p < 0.0001$; index: χ^2 = 480.37; df = 5; $p < 0.0001$). In comparison with all investigated fungal strains, *M. brunneum* strain LRC112 formed the significantly longest conidia with a length of 8.62 µm (Table 3), whereas *M. robertsii* strain JKI-BI-1442 had the significantly widest conidia with 2.76 µm, except *M. robertsii* strain JKI-BI-1448. However, JKI-BI-1448 and JKI-BI-1442 had the significantly lowest conidial index, which becomes manifested in the most round-shaped conidia.

Table 3: Sizes of conidia produced by six strains of *Metarhizium* (*M. brunneum*: JKI-BI-1339, JKI BI 1450, LRC112; *M. robertsii*: JKI-BI-1441, JKI-BI-1442, JKI-BI-1448) on MPA after 14 days at 25 °C. Significant differences within columns are represented by different letters. n = 50.

Fungi	Conidial size					
	Conidial length [µm]**	***	Conidial width [µm]**	***	Conidial index *,**	***
JKI-BI-1339	6.80±0.06	C	2.19±0.02	D	3.13±0.04	B
JKI-BI-1441	7.70±0.06	B	2.65±0.02	B	2.92±0.03	C
JKI-BI-1442	7.39±0.06	B	2.76±0.02	A	2.68±0.03	D
JKI-BI-1448	7.03±0.05	C	2.68±0.02	AB	2.63±0.03	D
JKI-BI-1450	7.01±0.06	C	2.27±0.02	CD	3.11±0.04	B
LRC112	8.62±0.15	A	2.32±0.03	C	3.74±0.06	A

* The index is calculated by dividing the length by the width; ** Mean ± standard error; *** GLMM, $p < 0.05$.

A correlation between the size parameters of the conidia and their effect against larvae of *A. lineatus* (length: r = -0.05077, p = 0.7899; width: r = -0.01980, p = 0.9173; index: r = -0.02584, p = 0.8922) and *A. obscurus* (length: r = 0.18677, p = 0.3230; width: r = -0.21762, p = 0.2480; index: r = 0.28743, p = 0.1235) could not be established. The effect on larvae of *A. sputator* was negative correlated with the conidial length (r = -0.40944, p = 0.0246) and index (r = -0.76672, $p < 0.0001$) and positive correlated with the width (r = 0.63655, p = 0.0002).

Determination of the germination over time and germination after 96 h

The germination of the conidia differed between the various *Metarhizium* strains (Figure 7). *M. brunneum* strains JKI-BI-1339 and JKI-BI-1450 germinated faster and achieved a germination of over 90% after only 24 h. The germination of the *M. robertsii* strains occurred more slowly and was similar within the first 24 h.

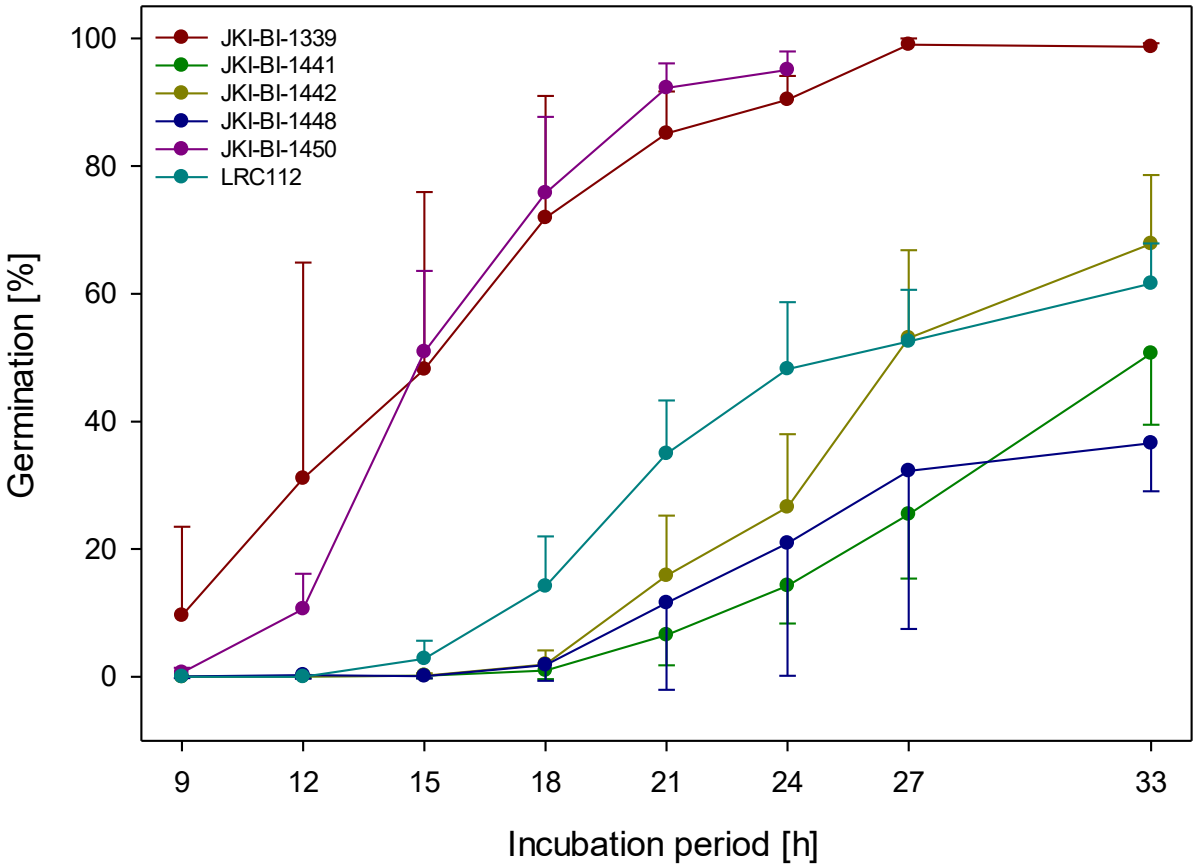

Figure 7: Germination [%] over 33 h incubation of conidia of the *Metarhizium brunneum* strain JKI BI 1339, JKI-BI-1450 and LRC112 and of the *Metarhizium robertsii* strains JKI-BI-1441, JKI BI 1442 and JKI-BI-1448. Mean and standard deviation are shown. After incubation for more than 24 h, the conidia of JKI-BI-1450 formed such long germ tubes that the germination could not be determined. $n = 18$.

The conidia of *M. brunneum* strain LRC112 germinated slower than the other *M. brunneum* strains, but achieved a germination after 96 h that was just as high as the others (Table 4). The germination after 96 h and the two factors of the germination process (τ and the slope at the inflection point) differed significantly between the different fungi (germination after 96 h: $\chi^2 = 71.87$; df = 5; $p < 0.0001$; τ: $\chi^2 = 526.19$; df = 5; $p < 0.0001$; slope inflection: $\chi^2 = 238.74$; df = 5; $p < 0.0001$). The germination after 96 h of all three *M. brunneum* strains was over 96% and significantly higher compared to those of the three *M. robertsii* strains with a germination after 96 h of 90 to 93%. τ, the time point where 50% of the total germinated conidia were germinated, was reached significantly faster by *M. brunneum* strains JKI-BI-1339 and JKI-BI-1450 after 15 h, followed by LRC112 (*M. brunneum*) and JKI-BI-1442 (*M. robertsii*) after 26 h. The two *M. robertsii* strains JKI-BI-1441 and JKI-BI-1448 took significantly longer to reach τ (32 h). The results of τ and slope at the inflection point are similar. The strains JKI-BI-1339 and JKI-BI-1450 that germinated fastest, also showed the significantly steepest slope at the inflection point.

Table 4: Germination [%] after 96 h, τ (time point when 50% of the maximal germinated conidia are germinated) and the slope inflection (slope of the inflectional tangent at the inflection point) over 33 h incubation. *Metarhizium brunneum*: JKI-BI-1339, JKI-BI-1450, LRC112; *Metarhizium robertsii*: JKI-BI-1441, JKI-BI-1442, JKI-BI-1448. Significant differences within columns are represented by different letters. $n = 18$.

Fungi	Germination after 96 h [%]*	**	τ [h]*	**	Slope inflection*	**
JKI-BI-1339	98.3±0.41	A	14.5±0.79	A	13.3±0.80	A
JKI-BI-1441	92.7±0.98	B	32.2±0.66	C	4.8±0.26	C
JKI-BI-1442	93.0±1.36	B	26.6±0.50	B	8.3±0.65	B
JKI-BI-1448	90.7±1.04	B	31.7±1.49	C	5.6±0.55	BC
JKI-BI-1450	96.8±0.80	A	15.1±0.17	A	16±1.33	A
LRC112	98.5±0.31	A	25.9±0.37	B	4.1±0.13	C

* Mean ± standard error; ** GLMM, $p < 0.05$.

A correlation between the germination of the conidia and their effect against larvae of *A. lineatus* (τ: r = -0.06943, $p = 0.7154$; slope at the inflection point: r = 0.12885, $p = 0.4974$; germination after 96 h: r = 0.02805, $p = 0.8830$) and *A. obscurus* (τ: r = -0.31957, $p = 0.0852$;

slope at the inflection point: r = 0.18723, p = 0.3218; germination after 96 h: r = 0.26467, p = 0.1575) could not be established. The effect on larvae of *A. sputator* was positive correlated with τ (r = 0.70567, $p < 0.0001$) and negative with the germination after 96 h (r = -0.65536, $p < 0.0001$). A correlation with the slop could not be proven (r = -0.30013, p = 0.1071).

Investigation of the efficacy of two *M. brunneum* strains against *Agriotes* larvae from different populations

There were no significant differences in susceptibility between the various populations of larvae of *A. lineatus* against the two strains of *M. brunneum* strains examined (Table 5). The efficacy parameters of the fungal treatments differed significantly from the controls, but showed no difference in efficacy between populations (JKI-BI-1450: RMTL mortality: $\chi^2 = 30.87$; df = 3; $p < 0.0001$; RMTL mycoses: $\chi^2 = 6.15$; df = 1; $p = 0.0132$; final mortality: $\chi^2 = 78.65$; df = 3; $p < 0.0001$; final mycoses: $\chi^2 = 1.82$; df = 1; $p = 0.1775$; ART2825: RMTL mortality: $\chi^2 = 44.15$; df = 3; $p < 0.0001$; RMTL mycoses: $\chi^2 = 0.09$; df = 1; $p = 0.7684$; final mortality: $\chi^2 = 79.37$; df = 3; $p < 0.0001$; final mycoses: $\chi^2 = 0.54$; df = 1; $p = 0.4611$). The RMTL mortality of larvae from Geinsheim was 12.6 and of larvae from Wohld 7.4, respectively, when treated with JKI-BI-1450. The final mortality, however, was 38% at both populations. When *A. lineatus* larvae were treated with ART2825 the RMTL mortality was 19.5 for larvae from Zurich and 23.1 for larvae from Wohld and the final mortality 53.5% and 48.0%, respectively. It is important to note that in treatments with ART2825, the average larval stage of 4.5 of larvae from Wohld was significant lower compared to the average larval stage of 6.2 of larvae from Zurich ($\chi^2 = 122.03$; df = 3; $p < 0.0001$). In contrast, the effect of the fungi on the larvae of *A. obscurus* differed depending on the population (JKI-BI-1450: RMTL mortality: $\chi^2 = 476.29$; df = 7; $p < 0.0001$; RMTL mycoses: $\chi^2 = 34.93$; df = 3; $p < 0.0001$; final mortality: $\chi^2 = 507.94$; df = 7; $p < 0.0001$; final mycoses: $\chi^2 = 46.36$; df = 3; $p < 0.0001$; ART 2825: RMTL mortality:

χ^2 = 1467.19; df = 7; p < 0.0001; RMTL mycoses: χ^2 = 61.13; df = 3; p < 0.0001; final mortality: χ^2 = 978.03; df = 7; p < 0.0001; final mycoses: χ^2 = 58.63; df = 3; p < 0.0001). When treated with JKI-BI-1450, larval populations from Mühlheim were much more susceptible with a significantly higher final mortality of 94% compared to populations from Wohld with 74% and Wallestalden with 44%. Again, larvae of the population from Wallestalden were significantly younger with a larval stage of 5 compared to 6.4 in the other populations (χ^2 = 227.04; df = 5; p < 0.0001). ART2825 treatments on *A. obscurus* larvae resulted in significantly lower final mortality of larval populations from Zurich with 30% compared to the other three larval populations with between 94.7-95.6% final mortality. Contrary to the results from the JKI-BI-1450 treatments, on average older larvae exhibited the lowest final mortality (χ^2 = 24.33; df = 7; p = 0.0010). Fungal treatments on *A. sputator* larvae showed that the RMTL mortality and RMTL mycoses were significantly dependent on the populations (JKI-BI-1450: RMTL mortality: χ^2 = 43.66; df = 5; p < 0.0001; RMTL mycoses: χ^2 = 11.35; df = 2; p = 0.0034 ART2825: RMTL mortality: χ^2 = 60.53; df = 5; p < 0.0001; RMTL mycoses: χ^2 = 7.68; df = 2; p = 0.0215). However, final mortality and final mycosis in the fungal treatments showed no significant difference between population, but in some cases to the controls (JKI-BI-1450: final mortality: χ^2 = 15.56; df = 5; p = 0.0082; final mycoses: χ^2 = 1.58; df = 2; p = 0.4536; ART2825: final mortality: χ^2 = 31.11; df = 5; p < 0.0001; final mycoses: χ^2 = 4.40.; df = 2; p = 0.1108). When treated with JKI-BI-1450, the RMTL mortality was significantly higher on the larvae from Zurich with 18.5 compared to the other two populations with 4.6 for larvae from Mühlheim and 3.6 for larvae from Wohld. The final mortality ranged from 18 to 36% and the final mycoses from 18 to 32%. The larvae from Zurich were significantly older (7.2-7.3) than the larvae from Mühlheim (5.7-6.0) at the beginning of the experiment (χ^2 = 134.52; df = 3; p < 0.0001). The RMTL mortality of the treatments with ART2825 was significantly higher on larvae from Beienrode with 21.5 compared to the larvae of Zurich with 6.0. The final mortality

of these treatments was not significantly different from each other and ranged between 10.7-42%. The larval stage of *A. sputator* larvae (6.8-7.2) treated with ART2825 did not differ between the different populations ($\chi^2 = 13.00$; df = 5; $p = 0.0234$).

Finally, RMTL mortality, the RMTL mycoses, the final mortality and the final mycoses were compared across all populations from Switzerland and Germany. The mean RMTL mortality for Germany was 23.5 ± 4.5 and 19.6 ± 5.8 for Switzerland and did not differ significantly from each other ($\chi^2 = 0.24$; df = 1; $p = 0.6235$). The same was true for RMTL mycoses of populations from Germany (22.1 ± 4.6) and Switzerland (18.6 ± 5.6) ($\chi^2 = 0.22$; df = 1; $p = 0.6366$). The mean final mortality and mean final mycoses of the German populations were 58 ± 8% and 53.6 ± 7.8%, and 44.1 ± 10.6% and 40.1 ± 9.6% for Swiss populations, respectively. Neither of these two parameters differed significantly between the populations of the two countries (final mortality $\chi^2 = 1.06$; df = 1; $p = 0.3037$ and final mycoses ($\chi^2 = 1.08$; df = 1; $p = 0.2985$).

Table 5: Efficacy of two *Metarhizium brunneum* strains against different populations of *Agriotes* larvae over 70 days. Different letters represent significant differences within columns and *Agriotes* species and one fungal treatment across all associated populations. JKI-BI-1450: *n* = 5 with ten individuals expect Wallestalden with 20 individuals; ART2825: *Agriotes lineatus* Wohld and Zurich, *Agriotes obscurus* Beienrode, *Agriotes sputator* Beienrode: *n* = 5 with ten individuals, *A. obscurus* Wallestalden and *A. sputator* Zurich: *n* = 4 with ten individuals, *A. obscurus* Zurich: *n* = 3 with five individuals, *A. sputator* Zurich: *n* = 3 with ten individuals, *A. sputator* Mühlheim: *n* = 4 with ten individuals and 1 with four individuals.

Population	Treatment	RMTL mortality *	**	RMTL mycoses *	**	Final mortality [%] ***	****	Final mycoses [%] ***	****	Larval stage	****
A. lineatus											
Geinsheim	JKI-BI-1450	12.6±2.6	A	12.6±2.6	A	38.0±5.8	A	38.0±5.8	A	5.8±0.2	A
Wohld	JKI-BI-1450	7.4±2.1	A	4.9±1.7	A	38.0±5.8	A	30.0±3.2	A	n.d.	
Geinsheim	Control	0.6±0.4	B	n.m.		4.0±2.2	B	n.m.		5.9± 0.2	A
Wohld	Control	0.6±0.6	B	n.m.		2.0±2.0	B	n.m.		n.d.	
Wohld	ART2825	23.1±4.2	A	17.9±4.1	A	53.5±6.3	A	41.3±3.7	A	4.5±0.1	B
Zurich	ART2825	19.5±3.3	A	19.5±3.3	A	48.0±8.6	A	48.0±8.6	A	6.2±0.2	A
Wohld	Control	1.8±1.8	B	n.m.		3.6±3.6	B	n.m.		4.3±0.1	B
Zurich	Control	2.1±1.5	B	n.m.		4.0±2.4	B	n.m.		6.0±0.2	A
A. obscurus											
Beienrode	JKI-BI-1450	27.4±2.7	AB	26.5±2.8	A	86.0±6.8	AB	80.0±7.7	A	6.3±0.1	AB
Mühlheim	JKI-BI-1450	35.3±2.4	A	33.1±2.6	A	94.0±4.0	A	84.0±2.4	A	6.4±0.1	AB
Wallestalden	JKI-BI-1450	17.0±2.3	BC	13.7±2.2	B	44.0±2.4	C	33.0±3.0	B	5.0±0.1	C
Wohld	JKI-BI-1450	25.8±2.8	AB	25.0±2.7	A	74.0±7.5	B	70.0±10.0	A	n.d.	
Beienrode	Control	0.7±0.7	D	n.m.		2.0±2.0	D	n.m.		5.9±0.1	B
Mühlheim	Control	0.4±0.3	D	n.m.		4.0±2.4	D	n.m.		6.5±0.1	A
Wallestalden	Control	9.5±2.1	C	n.m.		21.0±8.6	D	n.m.		5.0±0.1	C
Wohld	Control	0.1±0.1	D	n.m.		2.0±2.0	D	n.m.		n.d.	
Beienrode	ART2825	52.1±2.2	A	51.6±2.4	A	94.7±3.1	A	92.2±4.8	A	5.3±0.2	AB
Wallestalden	ART2825	49.3±2.2	A	47.3±2.6	A	95.6±4.4	A	86.7±8.2	A	5.6±0.2	AB
Wohld	ART2825	51.5±2.5	A	51.5±2.5	A	95.0±5.0	A	95.0±5.0	A	5.0±0.2	B
Zurich	ART2825	8.2±5.3	B	8.2±5.3	B	30.0±10.0	B	30.0±10.0	B	6.2±0.3	A
Beienrode	Control	3.2±2.2	B	n.m.		5.6±5.6	C	n.m.		5.3±0.2	AB
Wallestalden	Control	1.2±1.2	B	n.m.		2.9±2.9	C	n.m.		5.6±0.2	AB
Wohld	Control	0.2±0.2	B	n.m.		2.5±2.5	C	n.m.		4.9±0.2	B

Zurich	Control	0±0	B	n.m.		0±0	C	n.m.		5.8±0.3	AB
A. sputator											
Mühlheim	JKI-BI-1450	4.6±1.4	B	4.1±1.3	B	26.0±8.1	AB	24.0±8.7	A	6.0±0.1	B
Wohld	JKI-BI-1450	3.6±1.4	BC	3.6±1.4	B	18.0±5.8	AB	18.0±5.8	A	n.d.	
Zurich	JKI-BI-1450	18.5±3.8	A	16.7±3.7	A	36.0±11.7	A	32.0±11.1	A	7.3±0.1	A
Mühlheim	Control	0.3±0.3	C	n.m.		4.0±2.4	B	n.m.		5.7±0.1	B
Wohld	Control	0.6±0.6	BC	n.m.		4.0±2.4	B	n.m.		n.d.	
Zurich	Control	7.7±2.7	ABC	n.m.		20.0±11.4	AB	n.m.		7.2±0.1	A
Beienrode	ART2825	21.5±3.9	A	19.3±3.8	A	41.8±12.3	A	37.6±11.9	A	7.0±0.1	AB
Mühlheim	ART2825	16.8±3.9	AB	15.6±3.8	AB	37.0±10.4	AB	33.0±7.7	A	7.2±0.1	AB
Zurich	ART2825	6.0±3.3	BC	6.0±3.3	B	10.7±6.4	ABC	10.7±6.4	A	6.8±0.2	B
Beienrode	Control	5.6±2.2	BC	n.m.		14.4±4.2	BC	n.m.		7.3±0.1	A
Mühlheim	Control	1.5±1.4	C	n.m.		2.0±2.0	C	n.m.		7.2±0.1	AB
Zurich	Control	0.0±0.0	C	n.m.		0.0±0.0	C	n.m.		7.3±0.1	A

* RMTL = Restricted mean time lost (area above the Kaplan-Meier survival curve in the interval 0 to t_{max}) Mean ± standard error; ** Survival analysis (Kaplan-Meier-Wilcoxon); *** Mean ± standard error; **** GLMM, $p < 0.05$; n.m.= no mycoses, n.d.= not determined.

Discussion

The first aim of the present study was to find a fungal strain with a good efficacy against larvae of *A. lineatus*, *A. obscurus* and *A. sputator*. The results confirmed that all investigated *Metarhizium* strains were pathogenic to the larvae of the three *Agriotes* species and led to mycoses on the cadavers regardless of the host from which it was isolated or originated. Similar results to the effectiveness from *Metarhizium* against *Agriotes* larvae were shown by Kabaluk et al. (2005). In their study, 14 *Metarhizium* strains were tested against larvae of *A. obscurus* and *A. lineatus* and several showed promising biocontrol effects. On average, the *Metarhizium* strains in the present study showed a weaker effect on *A. lineatus* larvae compared to the larvae of the other two *Agriotes* species. A lower susceptibility of *A. lineatus* larvae than of *A. obscurus* and *A. sputator* larvae was also reported by Eckard et al. (2014) for two of the three *M. brunneum* strains studied. Moreover, the present study showed that the effect of one fungal strain can differ between *Agriotes* species. The different susceptibility of the larvae of various *Agriotes* species was also shown by Ansari et al. (2009), Eckard et al. (2013) and Eckard et al. (2014) and is problematic for the development of a control strategy based on EPF as a mixed population of *Agriotes* larvae is often found in the field (Lehmhus and Niepold 2015). Thus, a strategy based on a single fungal strain will, most likely, not be effective against all *Agriotes* species. However, a fungal strain with a broader host range was found that showed the best activity against two of the three *Agriotes* species studied. The *M. brunneum* strain JKI-BI-1450 showed the best efficacy against larvae of *A. lineatus* and *A. obscurus* and an intermediate efficacy against *A. sputator* larvae. Therefore, this strain is a very promising candidate for the development of a biocontrol agent. Furthermore, a fungal strain was identified that has a good effect against larvae of *A. sputator*, the *M. robertsii* strain JKI-BI-1442. Therefore, one possible solution would be a soil granule based on two or more fungal strains; however, this would make

the registration more difficult and more expensive. The two investigated *Cordyceps* strains proved to have no pathogenic effect against the larvae of *A. lineatus*, *A. obscurus* and *A. sputator*, even though they were isolated from another *Agriotes* species, *Agriotes ustulatus*. *Cordyceps* strains showing no effects against *Agriotes* larvae was also demonstrated by Ansari et al. (2009). Even though, many studies have found that fungi are very effective against the host from which they were isolated (Chandler 1992; Altre et al. 1999; Pilz et al. 2007). This finding could not be confirmed for the tested *Cordyceps* strains. It can be assumed that these *Cordyceps* strains have either been accidentally isolated from *A. ustulatus*, and their main host is not an *Agriotes* wireworm but another soil dwelling insect, or they may exhibit species specific virulence against *A. ustulatus*. Further investigations including *A. ustulatus* larvae are necessary to answer this question.

The second aim of the present study was to identify strain-specific characteristics that influence pathogenicity of the fungal strains to the three major *Agriotes* species. Therefore, the conidial size and the germination of the fungal strains were examined. However, for *A. lineatus* and *A. obscurus* no correlation between the conidia size and their virulence could be proven, but for *A. sputator*. The negative correlation show that shorter and wider spores are more pathogenic against the larvae of *A. sputator*. In the study of the effect of *Metarhizium anisopliae* against *Nilaparvata lugens* by Samuels et al. (1989), strains with small spores were also more pathogenic. In contrast, Altre et al. (1999) found that *P. fumosoroseus* strains with longer conidia were more effective against *P. xylostella*. It should be noted that the most effective strains in both of the above-mentioned studies also showed a faster germination of the conidia. These results contrast with those in the present study. Slower germinating fungal strains as well as those with lower germination after 96 h are more pathogenic to these larvae of *A. sputator*. Even though a positive correlation between rapid germination and high virulence of EPF has often been described (Al-Aidroos and Seifert 1980; Dillon and Charnley 1985; Samuels et al.

1989; Dillon and Charnley 1990), contradictory results were also observed. According to Boucias and Pendland (1984), a slow-germinating strain of the entomopathogenic fungus *Nomuraea rileyi* showed higher virulence against noctuid caterpillars than faster-germinating strains. A correlation between the germination and the virulence of the fungal strains against *A. lineatus* and *A. obscurus* could not be determined. These results indicate that conidial size and germination are exclusive criteria for preselecting strains for the control of *A. sputator* larvae, but not for the other two species, underlining the complexity of the infection process of EPF. Jackson et al. (1985) reached a similar conclusion for the virulence of 18 strains of the EPF *Verticillium lecanii* to the aphid *Macrosiphoniella sanborni*. The relationship between virulence and fast germination, high sporulation, absence of extracellular amylase activity, high extracellular chitinase activity and spore size were examined; however, the virulence could not be attributed to any single virulence factor. Also Talaei-Hassanloui et al. (2006) could not establish a correlation between conidial size, germination and mycelial growth with virulence of ten *Beauveria bassiana* strains against *P. xylostella* and *Leptinotarsa decemlineata*. The identification of parameters that could help indicate the efficacy of an entomopathogenic fungus would be very helpful for the development of microbial-based biocontrol agents. However, the present research and the available literature do not reveal such general parameters of effectiveness.

The third aim was to determine whether there are variations in the efficacy of one fungal strain against different populations of one *Agriotes* species. For this purpose, the effect of two *M. brunneum* strains against different populations of the three main *Agriotes* species was investigated. A population-dependent efficacy related for all could be confirmed by the present experiments for the species *A. obscurus*. RMTL mortality and mycoses as well as final mortality and mycoses differed between the populations. For *A. sputator* no difference could only be confirmed for RMTL mortality and mycoses, which means that there is no difference in the

final effect, but in the duration until death or mycoses of the larvae. No difference was found in the examination of *A. lineatus*, whereby only two populations could be examined here. Therefore, the hypothesis of a population-dependent effect can be confirmed for *A. obscurus*, for the other two species, especially *A. lineatus*, further experiments are necessary. The population-dependent effect did not differ essentially between the two *M. brunneum* strains studied. The diverse effects of one entomopathogenic fungal strain against different populations of one pest were already noted by Keller et al. (1999) for the effects of *Beauveria brongniartii* against *Melolontha melolontha*. In their study, the insects from Italy were generally less susceptible compared to insects from Switzerland. A difference in susceptibility between the populations from the two countries studied could not be confirmed in the present study. The susceptibility of the populations from Germany did not differ from the populations from Switzerland. Furthermore, younger larvae are often more susceptible to pathogens than older larvae (Butt et al. 2016). To exclude the possibility that a varying susceptibility of the populations was due to differing larval stages, this aspect was investigated in the present study before the experiment, but the results showed no relationship.

The present results show that fungi of the genus *Metarhizium* can be effective in controlling wireworms of different *Agriotes* species. Final mortality of the strains tested in the present study varied considerably and ranged from 30 to 94%. None of the strains tested was most effective against all tested species. However, the *M. brunneum* strain JKI-BI-1450 showed less species-specificity and may exhibit a broader host range. This strain showed a population-dependent effect only in the species *A. obscurus* just like the *M. brunneum* strain ART2825. These strains are more promising candidates for an area-wide control of wireworms. Further testing of these strains is recommended, especially under field conditions in different areas in Europe or even worldwide.

Chapter I

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Notes on other contributors: Reinbacher, L. and Grabenweger, G.: Coordinated the experiment "Investigation of the efficacy of two *M. brunneum* strains against *Agriotes* larvae from different populations", bred the wireworms, provided them for the experiments and collected the data from treatments with ART2825, Paluch, M. and Lehmhus, J.: bred the wireworms and provided them for the experiments, Nikolei, R. M.: Collected part of the data of the experiments: "Determination of the germination over time" and "Determination of the germination after 96 h", Nieszporek U. B.: Collected part of the data of the experiment: "Efficacy study on entomopathogenic fungi against *Agriotes"*.

Chapter II

Effect of media composition and temperature on the growth of *Metarhizium* spp. in liquid culture

Abstract

To develop entomopathogenic fungi as a plant protection agent, the producibility of the fungal strain is crucial. Growth in liquid culture depends on many factors such as nutrient composition, temperature and fermentation time, therefore these factors were investigated to develop an optimized fermentation process. First, the growth of six *Metarhizium* strains in three liquid media was studied, where five strains formed the most spores in Samsi 8 and Catroux media (10^6-10^7 ml^{-1}) and fewer or none in Adamek. During the fermentation of *Metarhizium brunneum* strain JKI-BI-1450 in Samsi 8 in flasks, the fungus produced the most spores at 25 °C after 48 h and 72 h and the most biomass at 30 °C after 48 h closely followed of the biomass production at 25 °C after 48 h. The examination of the growth of this fungal strain in a bioreactor at 25 °C in Samsi 8 demonstrated maximum sporulation between 42.5-48 h, whereas the biomass increased constantly until the end of the fermentation after 72 h. These results indicate a temporal difference in reaching the stationary phase of sporulation between fermentation in a bioreactor and in a flask and further that the production parameters must be adapted not only to the fungal strain but also to the desired produced fungal morphology. Furthermore, biomass production could be increased by raising the concentration of the carbon and nitrogen source as well as sodium chloride of the liquid medium Samsi 8. In summary, the optimal production conditions for *M. brunneum* JKI-BI-1450 are a medium composition of 5% (w/v) glucose, 4.5%

(w/v) corn steep solid, and 1.5% sodium chloride (w/v), an incubation temperature of 25 °C and an incubation duration of 48 h.

Introduction

To employ an entomopathogenic fungi (EPF) as a biocontrol agent, sufficient efficacy against the target pest as well as good producibility are essential (Jenkins and Goettel 1997). EPF can be produced either in solid state or in liquid fermentation (Kleespies and Zimmermann 1992; Kleespies and Zimmermann 1998; Fargues et al. 2002; Ye et al. 2006; Bawin et al. 2016; Nascimento Silva et al. 2018; Iwanicki et al. 2020). These processes are modelled according to the natural growth of a fungus in and on a host insect. After contact of the fungal spore with the insect´s surface, it penetrates the insect cuticle and grows in its haemolymph (Vestergaard et al. 1999; Pedrini 2018). This growth behaviour is used in liquid fermentation. In contrast, solid-state fermentation mimicks fungal growth out of the insect again and formation of conidia on the cuticle after successful infection. EPF of the genus *Metarhizium* are suitable for both production processes. During solid-state fermentation, fungi of the genus *Metarhizium* could form mycelia, conidia, and/or microsclerotia (Yousef-Yousef et al. 2022). Microsclerotia are dehydration-tolerant, compact and melanic solid hyphae aggregated into particles that function as a survival structure (Jaronski and Jackson 2008). Conidia are uniformly shaped and hydrophobic spores for survival and multiplication that are produced by conidiophore cells called phialides (Glare et al. 1996). In liquid medium, *Metarhizium* could form mycelium, mycelium pellets, microsclerotia and differed kinds of spores (Adámek 1965; Kleespies and Zimmermann 1992; Jenkins and Prior 1993; Vega et al. 2003; Ypsilos and Magan 2005). Blastospores are thin-walled, variously shaped, hydrophilic and formed by budding and septation of mycelium (Fargues et al. 2002). Furthermore, Jenkins and Prior (1993) and Leland et al. (2005) reported that spores of *Metarhizium anisopliae* var. *acridum* (strain IM1330189;

formally: *Metarhizium flavoviride* (Driver et al. 2000)) are formed by phialides in liquid medium. Due to their similarity to conidia, they call this form of spores submerged conidia. These submerged conidia differ morphologically and ultrastructurally from conidia formed on solid substrate. They miss a layer of the spore wall and differ in some physical properties (Hegedus et al. 1990). In the present study, no distinction was made between different forms of spores and all spores produced in liquid culture were designated as submerged spores. The focus was on liquid fermentation, as this has several advantages compared to solid-state fermentation. Firstly, the production time for liquid fermentation is much shorter (Ypsilos and Magan 2005; Téllez-Martínez et al. 2016). Furthermore, a liquid medium can be adapted much more precisely to the growth requirements of different fungi and the physiochemical parameters of a fermentation like oxygen supply and temperature can be controlled more accurately (Humphreys et al. 1989). Both the nutrient composition of the liquid medium and the physicochemical parameters, like fermentation temperature and time, have a great influence on growth, sporulation, stability and virulence of submerged propagation of entomopathogenic hyphomycetes (Campbell et al. 1983; Hegedus et al. 1990; Jackson 1997; Fargues et al. 2002; Ibrahim et al. 2002; Mascarin et al. 2015a; Mascarin et al. 2015b; Jaronski and Mascarin 2017). Furthermore, the medium can even influence whether the fungus forms more mycelium or more spores, which was demonstrated by Barnes et al. (1975) for *Metarhizium anisopliae* by using different types of peptone in liquid medium. In addition, the size of the spores also depends on the medium in which it was produced (Fargues et al. 2002).

In this chapter, the growth in liquid culture of the *Metarhizium* strains studied in chapter I was investigated. Furthermore, the growth of the fungal strain *Metarhizium brunneum* JKI-BI-1450, which had shown the best efficacy against *Agriotes* larvae, was determined in a bioreactor and the production parameters were optimized regarding the concentration of the media components

and the fermentation temperature and time. All experiments were carried out considering the subsequent use of the fungal biomass for production of a soil granule by use of fluid-bed drying, which requires a biomass mixture of mycelium and spores (Chapter III).

Materials and Methods

Sporulation of six *Metarhizium* strains in three liquid media

The *M. brunneum* strains JKI-BI-1339, JKI-BI-1450 and LRC112 and the *Metarhizium robertsii* strains JKI-BI-1441, JKI-BI-1442 and JKI-BI-1448 were incubated on malt peptone agar (MPA) containing 3% (w/v) malt extract (Merck, Darmstadt, Germany), 0.5% (w/v) peptone from soybean (Merck) and 1.8% (w/v) agar-agar (Roth, Karlsruhe, Germany) at 25 °C for 14 days in the dark. After incubation, a small amount of conidia was added to autoclaved 0.5% (v/v) Tween® 80 (Merck). These suspensions were placed in an ultrasonic bath (Sonorex RK 52 35 kHz., Bandelin electronic GmbH & Co. KG, Berlin, Germany) for 3 min. The conidia concentration was determined using a haemocytometer and a suspension with a concentration of 5×10^5 conidia ml^{-1} for each fungal strain was prepared by diluting with autoclaved 0.5% (v/v) Tween® 80. For the liquid fermentation of the fungal strain, 50 ml of three different media was autoclaved at 121 °C for 20 min in 100-ml Erlenmeyer flasks. The three tested media were: (i) “Samsi 8” (according to Samšiňáková (Samšináková 1966), adapted for the cultivation of *Cordyceps fumosorosea* (personal communication D. Stephan, Julius Kühn Institute, Federal Research Centre for Cultivated Plants, Institute for Biological Control)) containing 2.5% (w/v) D(+)-glucose (Merck), 2% (w/v) corn steep solid (CSS) (Sigma Aldrich, Buchs, Switzerland) and 0.5% (w/v) sodium chloride (NaCl) (Merck), (ii) “Adamek” (Adámek 1965) containing 4% (w/v) D(+)-glucose, 4% (w/v) granulated yeast extract (Merck) and 3% (w/v) CSS and (iii) “Catroux” (Catroux et al. 1970) containing 3% (w/v) D(+)-sucrose (Roth), 2% (w/v) CSS, 0.5% (w/v) potassium nitrate (KNO_3) (Merck), 6.8%

(w/v) potassium dihydrogen phosphate (KH_2PO_4) (Merck), 0.2% (w/v) calcium carbonate ($CaCO_3$) (Merck) and 0.1% (w/v) magnesium sulphate heptahydrate ($MgSO_4x7H_2O$) (Merck). Each flask was inoculated with 1 ml of the conidial suspensions and incubated at 150 rpm on a horizontal shaker (Novotron, 50 mm deflection, Infors, Bottmingen, Switzerland) for 72 h. After the fermentation time, the submerged spore concentration was determined using a haemocytometer. The experiment was repeated independently six times.

Determination of the optimal fermentation temperature

M. brunneum JKI-BI-1450 was found to be highly promising in the experiments on the efficacy of eight EPF strains against *Agriotes* larvae (Chapter I), so the further experiments on production optimization were carried out only with this strain.

Three 100-ml Erlenmeyer flasks filled with 50 ml Samsi 8 and autoclaved at 121 °C for 20 min were inoculated with an undefined amount of JKI-BI-1450, which was incubated for 14 days at 25 °C in the dark on MPA plates, using an inoculation loop. One flask each was incubated at 20 °C, 25 °C and 30 °C at 150 rpm on a horizontal shaker for 72 h. Thereafter, the submerged spore concentration was determined using a haemocytometer. For each incubation temperature, a suspension with the concentration of 5×10^5 submerged spores ml^{-1} was prepared by dilution with Samsi 8. For each temperature, five 100-ml Erlenmeyer flasks filled with 50 ml Samsi 8 and autoclaved at 121 °C for 20 min were inoculated with 1 ml of the corresponding fungal suspension. For 96 h the flasks were incubated at 150 rpm and the corresponding temperatures on a horizontal shaker. Every 24 h, samples were taken and the submerged spore concentration and dry biomass were determined. To determine the dry biomass, the samples were centrifuged for 20 min at 25 °C and 15,334 × *g* to remove the remaining media components. The supernatant was discarded and replaced with deionised water and the dry biomass was

determined by a moisture determination balance (Ma30, Sartorius, Göttingen, Germany). The experiment was repeated independently three times.

Investigation of the growth of JKI-BI-1450 in a bioreactor

The investigation was conducted in a Minifors 1 bioreactor (Infors HT AG, Bottmingen, Switzerland). The baffle was placed in the culture vessel and the vessel was filled with 3 l Samsi 8 medium. A tube for the oxygen supply, two sampling tubes, a pH and the O_2 electrode and a stirrer with two rushton impellers were placed in the cover. The bioreactor was autoclaved at 121 °C for 20 min and inoculated with a pre-culture. To prepare the pre-culture, a 100-ml Erlenmeyer flask filled with 50 ml Samsi 8 and autoclaved at 121 °C for 20 min was inoculated with an undefined amount of JKI-BI-1450, which was incubated for 14 days at 25 °C in the dark on MPA plates, using an inoculation loop. The flask was incubated for 72 h at 25 °C on a horizontal shaker. After the incubation, the submerged spore concentration of the pre-culture was determined using a haemocytometer and the fermenter was inoculated with 3×10^7 submerged spores. Fermentation was carried out at 25 °C, 600 rpm, without pH control and with an air flow of 6 l h^{-1} for 72 h. During fermentation, the pH and the oxygen saturation were recorded. The oxygen saturation was not measured as an absolute, but as a relative value, where the initially amount was set as 100%. To prevent the medium from overflowing due to excessive foaming during fermentation, silicon anti-foaming emulsion (Roth) was added when the medium foam rised high enough to contact an antifoam electrode. During fermentation, every 4 h samples were taken and the submerged spore concentration, dry biomass and glucose content were determined. The submerged spore concentration and the dry biomass were determined as described above. The glucose concentration was measured by a blood glucose meter (FreeStyle lite ART 12005, Abbott Diabetes Care Inc., Alameda, USA). The experiment was repeated independently three times.

For a more detailed examination of the growth, the period of the maximum sporulation was monitored more closely. The fermentation was carried out as described above. Samples were taken every 4 h in the period 0-42 h after inoculation and every 0.5 h in the period 42-50 h after inoculation. In the period 52-72 h after incubation, samples were taken again every 4 h. The samples were taken automatically by means of pumps and a fraction collector inside a refrigerator and stored there until evaluation. The submerged spore concentration, dry biomass and the glucose content of the samples were determined as described above. The experiment was repeated independently three times.

Optimization of the medium Samsi 8 to increase the biomass production of JKI-BI-1450

Biomass production of JKI-BI-1450 in Samsi 8 medium with altered D(+)-glucose-, CCS- and NaCl-concentration was investigated (Table 6).

Table 6: Modified concentrations of the components of the medium Samsi 8.

	Concentration [%]											
D(+)-Glucose	0	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5
CCS	0	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5
NaCl	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1	1.5

A total of 1990 µl of each media were transferred in four cavity of a 24-well plate (Greiner BioOne GmbH, Kremsmünster, Austria). Each cavity was inoculated with 10 µl of a pre-culture of JKI-BI-1450. To prepare the pre-culture, a 100-ml Erlenmeyer flask filled with 50 ml Samsi 8 and autoclaved at 121 °C for 20 min was inoculated with an undefined amount of JKI-BI-1450, which was incubated for 14 days at 25 °C in the dark on MPA plates, using an inoculation loop. The flask was incubated for 72 h at 25 °C on a horizontal shaker. The inoculated 24-well plates were incubated at 150 rpm on a horizontal shaker for 72 h at 25 °C and 85% humidity. After incubation, the samples were centrifuged at 25 °C and 15,334 × *g* for 20 min to remove remaining media components. The supernatant was discarded and replaced

with deionised water. For dry biomass determination the samples were dried in dishes weighed beforehand at 80 °C until no weight loss occurred anymore. The dry biomass was determined by subtracting the weight of the empty dish from the weight of the dish with dried biomass. The experiment was repeated independently three times.

Statistical analysis

Data were statistically analysed with the software SAS Studio 3.8. All experiments were analysed by using a generalized linear model with Wald statistics for type 3 analysis and multiple comparison according to Tukey (GLMM, $p < 0.05$), expect the experiment "Comparison of sporulation of six *Metarhizium* strains in three liquid media". The comparison of the sporulation was carried out by using a Kruskal-Wallis test, posthoc: Wilcoxon test ($p < 0.05$).

Results

Comparison of the sporulation of six *Metarhizium* strains in three liquid media

The investigation of the producibility of six *Metarhizium* strains in liquid culture demonstrated that all fungal strain grew and sporulated in artificial medium. The strains JKI-BI-1339, JKI-BI-1441, JKI-BI-1442, JKI-BI-1448 and JKI-BI-1450 formed significantly most submerged spores in the media Catroux and Samsi 8 with 10^6-10^7 submerged spores ml^{-1} compared to the sporulation in the medium Adamek with less than 10^5 submerged spores ml^{-1} (Figure 8) (JKI-BI-1339: ($\chi^2 = 18.9062$; df = 2; $p < 0.0001$); JKI-BI-1441: ($\chi^2 = 15.7667$; df = 2; $p = 0.0004$); JKI-BI-1442: ($\chi^2 = 15.4599$; df = 2; $p = 0.0004$); JKI-BI-1448: ($\chi^2 = 14.0473$; df = 2; $p = 0.0009$); JKI-BI-1450: ($\chi^2 = 17.9969$; df = 2; $p = 0.0001$)). In the Adamek medium, JKI-BI-1448 did not form any submerged spores at all. The strain LRC112 formed less than 10^5 submerged spores ml^{-1} in all three media, which did not differ significantly from each other

($\chi^2 = 0.0118$; df = 2; $p = 0.9941$). Comparing the sporulation of all fungal strains in one medium, there was no difference in fermentation in the Adamek medium ($\chi^2 = 3.9632$; df =5; $p = 0.5547$). In the medium Catroux, LRC112 formed significantly less spores than the other fungal strains ($\chi^2 = 20.5963$; df = 5; $p = 0.0010$). The greatest differences were seen in the medium Samsi 8 ($\chi^2 = 29.9822$; df = 5; $p < 0.0001$). JKI-BI-1339 formed the most spores in the Samsi 8 medium, with significant difference to JKI-BI-1441 and LRC112. The sporulation of the fungal strains JKI-BI-1442, JKI-BI-1448 and JKI-BI-1450 was between those mentioned above. LRC112 had the lowest spore concentration, which was significantly different from all other fungal strains, expect JKI-BI-1448. The comparison of the sporulation by Kruskal-Wallis test ($p < 0.05$) of the two examined fungal species *M. brunneum* ($3.05 \pm 5.37 \times 10^6$ submerged spores ml^{-1}) and *M. robertsii* ($5.26 \pm 0.10 \times 10^6$ submerged spores ml^{-1}) showed no difference over all media ($\chi^2 = 0.9133$; df = 1; $p = 0.3393$).

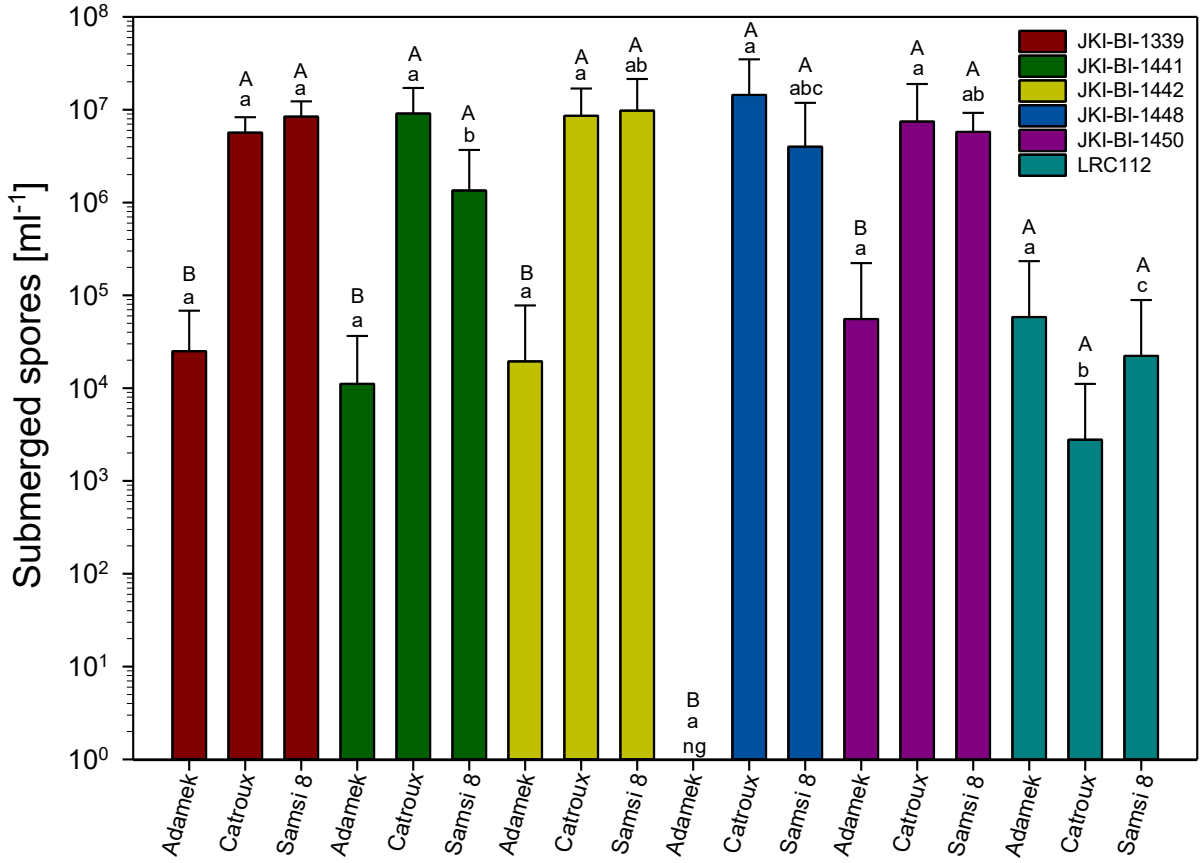


Figure 8: Submerged spore concentration of the *Metarhizium brunneum* strains JKI-BI-1339, JKI-BI-1450 and LRC112 and of the *Metarhizium robertsii* strains JKI-BI-1441, JKI-BI-1442 and JKI-BI-1448 in three different liquid media after fermentation for 72 h at 25 °C. Mean and standard deviation are shown. ng = no growth. Different letters show significant differences, upper case letter: Comparison of one fungal strain in all media, lower case letters: Comparison of all fungal stains in one medium. Kruskal-Wallis test, posthoc: Wilcoxon test ($p < 0.05$). $n = 9$.

Determination of the optimum fermentation temperature

The sporulation from JKI-BI-1450 in Sami 8 was significantly highest at an incubation at 25 °C after 48 h and 72 h with 6.5 resp. 6.0 × 10^5 submerged spores ml^{-1} (χ^2 = 965.73; df = 11; $p < 0.0001$), followed by the sporulation at 20 °C after the same incubation times with 3.1 resp. 3.5 × 10^5 submerged spores ml^{-1} (Figure 9). After a longer incubation period of 96 h, the spore concentration decreased again at the two temperatures mentioned and was no longer significantly different from the spore formation at 30 °C. A significant increase in spore concentration could not be determined during incubation at 30 °C.

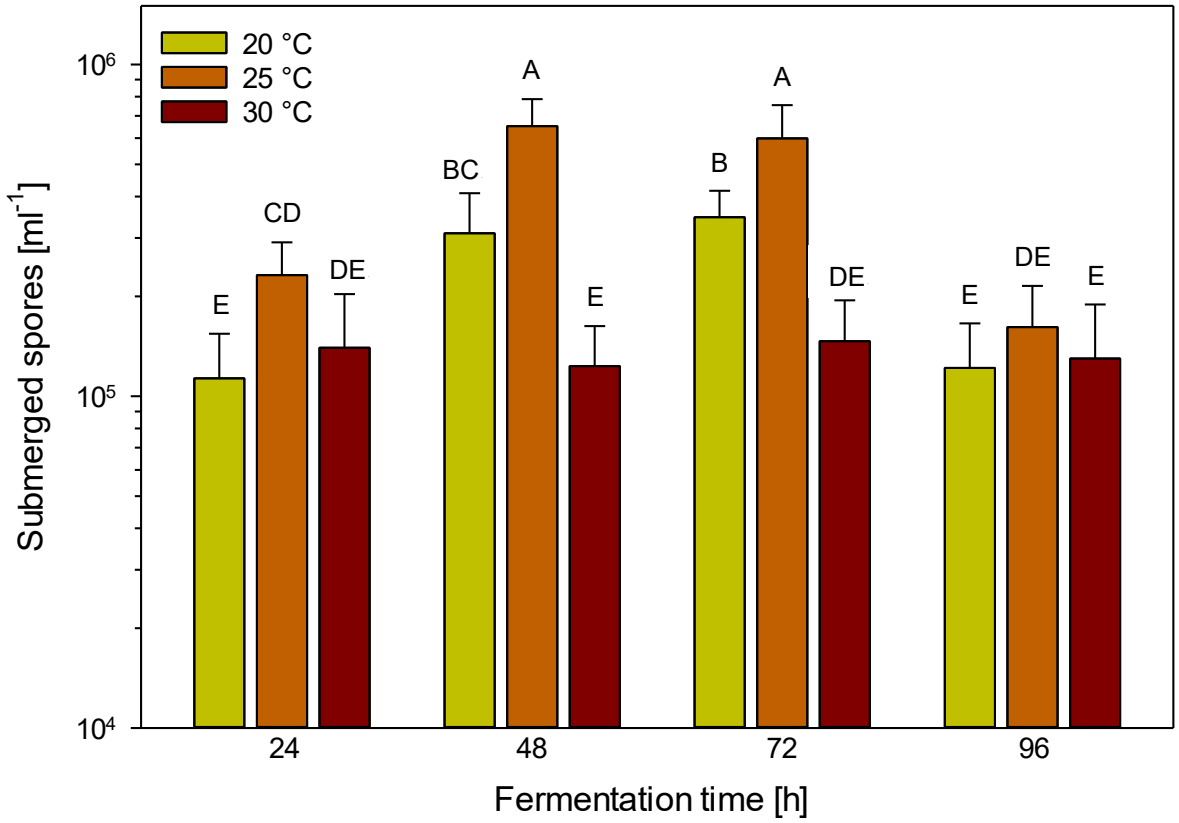


Figure 9: Influence of fermentation time and temperature on submerged spore formation of *Metarhizium brunneum* strain JKI-BI-1450 in liquid medium. Mean and the standard deviation are shown. Different letters show significant differences. GLMM ($p < 0.05$). $n = 15$.

Most biomass was yielded after 48 h at 30 °C with 3.2%, but this was not significantly different from production after 48 h at 25 °C and after 72 h and 96 h at 25 °C and 30 °C ($\chi^2 = 584.37$; df = 11; $p < 0.0001$) (Figure 10). After 24 h and 48 h, JKI-BI-1450 formed more biomass at 30 °C than at the other temperatures, whereas after 72 h and 96 h incubation at 25 °C resulted in most biomass. Incubation at 20 °C resulted in lower biomass production than at the two higher temperatures on each evaluation time.

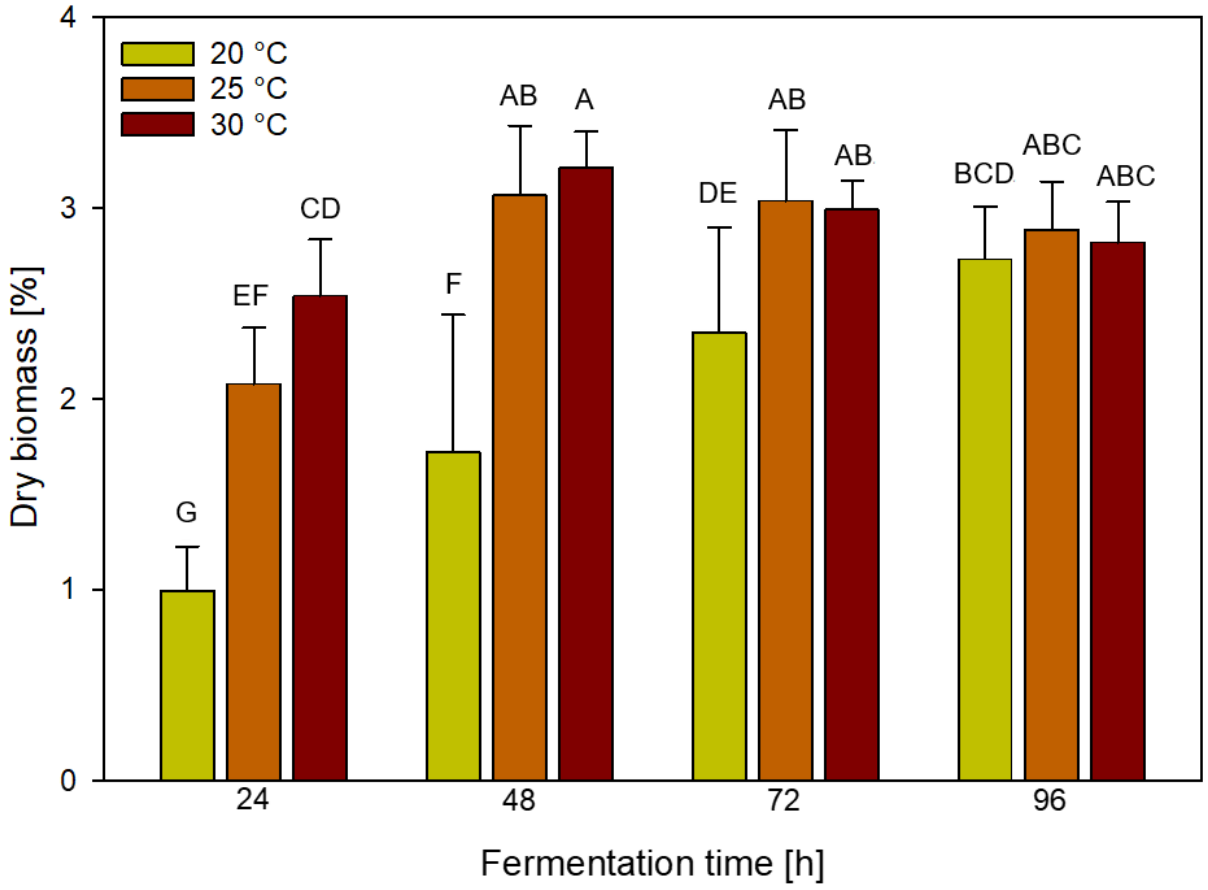


Figure 10: Influence of fermentation time and temperature on biomass production of *Metarhizium brunneum* strain JKI-BI-1450 in liquid medium. Mean and the standard deviation are shown. Different letters show significant differences. GLMM ($p < 0.05$). $n = 15$.

Investigation of the growth of JKI-BI-1450 in a bioreactor

The fermentation started with 10^5 submerged spores ml^{-1} and reached the maximum sporulation after 44 h with 6×10^6 submerged spores ml^{-1} (Figure 11). After that, the concentration decreased again to 3.75×10^5 submerged spores ml^{-1} after 72 h. The biomass concentration started at 0.6%, increased until the end of the fermentation, and reached a value of 1.76%. The relative value of the dissolved O_2 increased after inoculation and fell steadily from 33 h to 0% after 62 h. The glucose concentration started at 25 g l^{-1} and began to decrease after 24 h. The pH value was 4 at the beginning of the fermentation, decreased slightly between 20 and 40 h and increased after 44 h and reached 4.8 after 72 h.

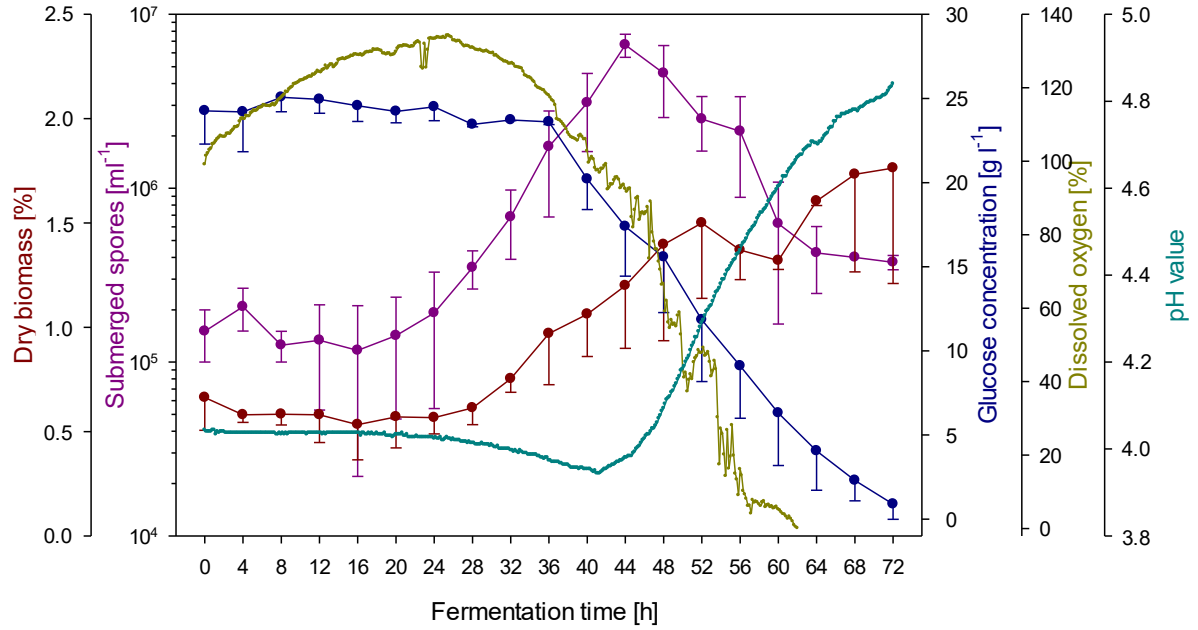


Figure 11: Time profile of batch fermentation experiments of *Metarhizium brunneum* strain JKI-BI-1450 in the bioreactor in the liquid medium Samsi 8 over 72 h. Mean and standard deviation of submerged spore concentration, dry biomass and glucose content are shown. The O_2 level and the pH value are shown as mean without any measure of variation. These parameters were measured every 10 min during fermentation and a representation of the variation has a negative impact on the clarity of the graph. $n = 3$.

The half-hour sampling demonstrated a stationary phase of sporulation between 42.5-48 h after the exponential phase between 32-42 h (Figure 11 and Figure 12). Thereafter, the concentration of the submerged spores in the medium decreased.

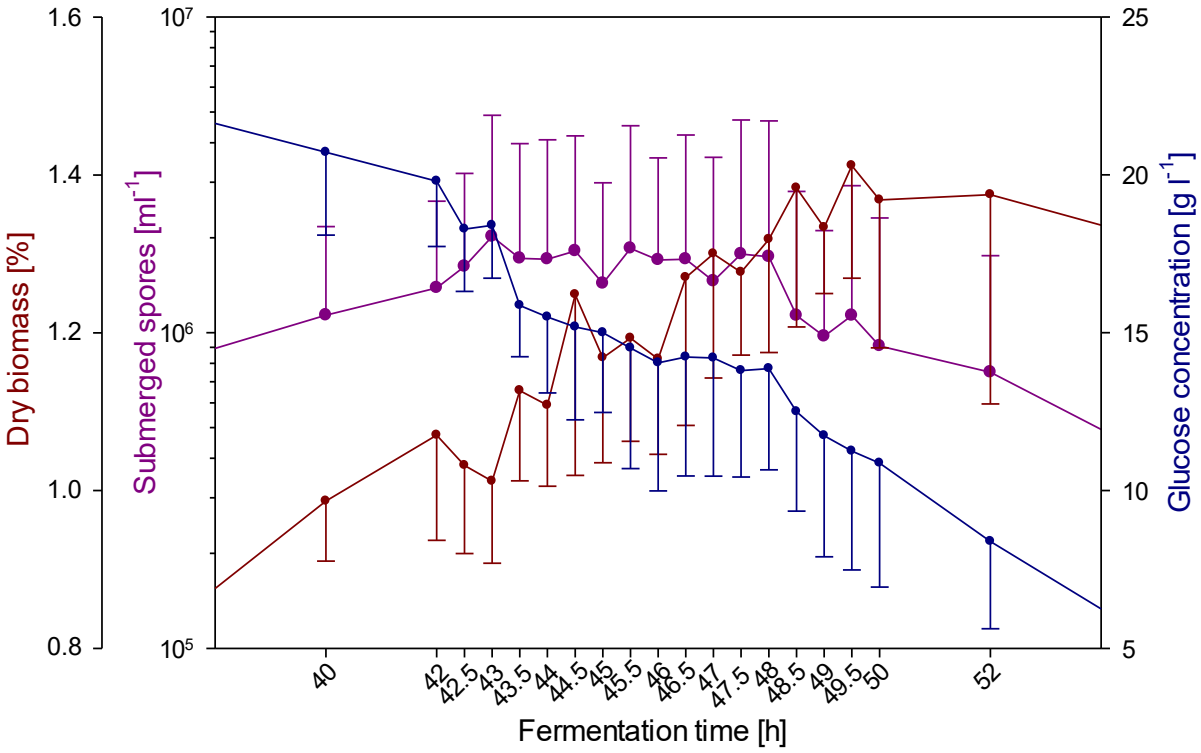


Figure 12: Time profile of batch fermentation experiments of *Metarhizium brunneum* strain JKI-BI-1450 in the bioreactor in the liquid medium Samsi 8 over 72 h. Only 36 to 56 h post inoculation is shown to better represent the period of maximum fungal sporulation. Mean and standard deviation are shown. $n = 6$.

Optimization of the medium Samsi 8 to increase the biomass production of JKI-BI-1450

The biomass production could be increased by raising the concentration of all components of Samsi 8, where glucose had the major influence on the growth of JKI-BI-1450 ($\chi^2 = 157.61$; df = 11; $p < 0.0001$) (Figure 13). Although the fungal strain was also able to grow without glucose (7.2 g l^{-1} dry biomass), dry biomass was highest at 5% glucose concentration with 28 g l^{-1} and significantly different from all other concentrations except 5.5%.

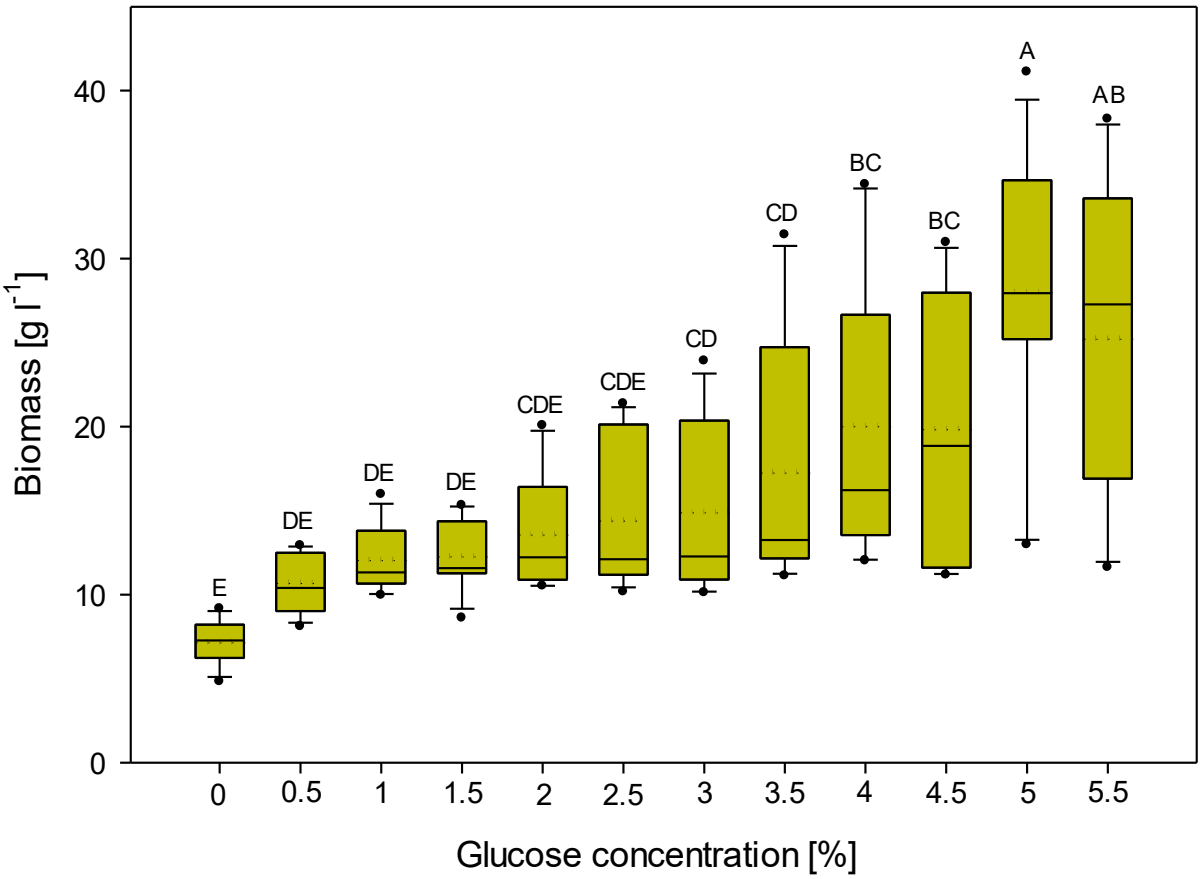


Figure 13: Biomass production of *Metarhizium brunneum* strain JKI-BI-1450 in Samsi 8 medium with different glucose concentrations. The upper and lower limits of the boxes correspond to the 25% and 75% percentile. The median is shown as a solid line and the mean as a dotted line. The whiskers mark the 10th and 90th percentiles, black dots mark outlier values. Different letters show significant differences. GLMM ($p < 0.05$). $n = 12$.

As in the case of glucose, JKI-BI-1450 was able to grow in a medium without CSS (Figure 14), while growth in this medium was very low with 3 g l^{-1} dry biomass. The biomass production increased significantly with increasing CSS content in the medium and reached a maximum of 23 g l^{-1} dry biomass with 4.5% CSS ($\chi^2 = 314.40$; df = 11; $p < 0.0001$), although no significant differences in biomass production were found in media with CSS concentrations ranging from 3 to 5.5%.

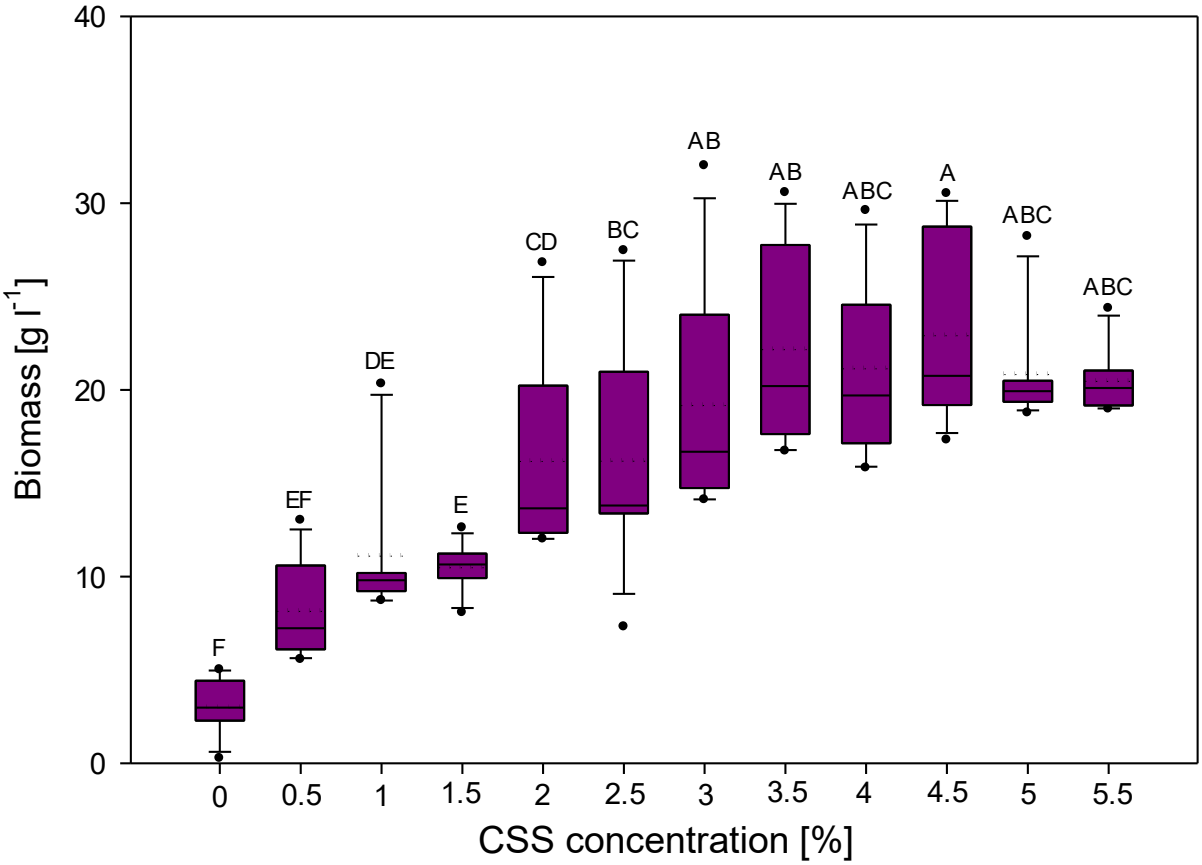


Figure 14: Biomass production of *Metarhizium brunneum* strain JKI-BI-1450 in Samsi 8 medium with different corn steep solid (CSS) concentrations. The upper and lower limits of the boxes correspond to the 25% and 75% percentile. The median is shown as a solid line and the mean as a dotted line. The whiskers mark the 10th and 90th percentiles, black dots mark outlier values. Different letters show significant differences. GLMM ($p < 0.05$). $n = 12$.

The concentration of NaCl had also a significant effect on the growth of JKI-BI-1450 ($\chi^2 = 76.55$; df = 11; $p < 0.0001$) (Figure 15). With 0% NaCl in the medium, fungal growth can also be observed with 13 g l^{-1}. In contrast to glucose and CSS, biomass did not increase almost uniformly with higher NaCl concentrations, but increased and decreased several times. However, the fungal strain reached the highest biomass concentration with 14.7 g l^{-1} at the highest NaCl concentration of 1.5%.

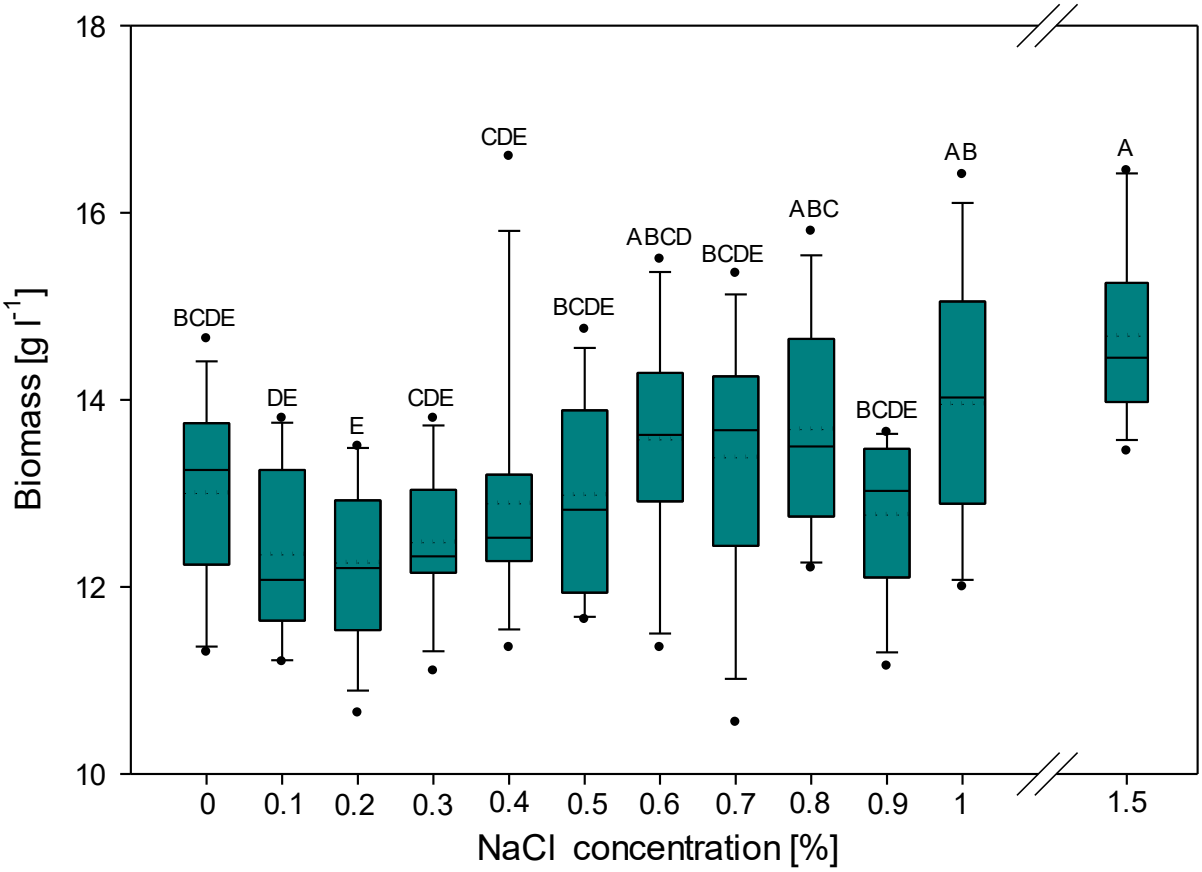


Figure 15: Biomass production of *Metarhizium brunneum* strain JKI-BI-1450 in Samsi 8 medium with different sodium chloride (NaCl) concentrations. The upper and lower limits of the boxes correspond to the 25% and 75% percentile. The median is shown as a solid line and the mean as a dotted line. The whiskers mark the 10th and 90th percentiles, black dots mark outlier values. Different letters show significant differences. GLMM ($p < 0.05$). $n = 12$.

Discussion

The analyses of the sporulation of six *Metarhizium* strains in three different media demonstrated that all fungal strains are able to grow and form submerged spores in liquid medium. Sporulation strongly depends on the medium composition as well as the fungi and ranges between 10^3-10^7 submerged spores ml^{-1}, apart from JKI-BI-1448, which did not sporulate in the medium Adamek at all. A comparable production of 10^6 blastospores ml^{-1} were demonstrated by Mslim and Kamarudin (2014) for the submerged cultivation of *M. anisopliae*. A slightly higher sporulation with 10^5-10^8 blastospores ml^{-1} for ten strains of *M. anisopliae* in four liquid

media was shown by Adib et al. (2013). Similar results were also reported by Kleespies and Zimmermann (1992) with 10^6-10^8 spores ml^{-1}. The relevance of the medium composition for the growth and sporulation of *Metarhizium* was also demonstrated by Barnes et al. (1975), Campbell et al. (1983), Kleespies and Zimmermann (1992), Jackson (1997), Fargues et al. (2002), Adib et al. (2013) and Iwanicki et al. (2018). Iwanicki et al. (2020), for example, found a doubling of blastospore formation in liquid medium with corn steep liquor as the nitrogen source compared to yeast extract. However, medium-dependent growth is not only the case for *Metarhizium*. Similar findings were described for other entomopathogenic hyphomycetes such as *Beauveria* and *Cordyceps* (formerly *Paecilomyces* and *Isaria*, respectively) (Samšináková 1966; Barnes et al. 1975; Campbell et al. 1983; Inch et al. 1986; Rombach 1989; Vidal et al. 1998; Jackson et al. 2003). Except for the strain LRC112, all investigated strains sporulated the least in the medium Adamek with 0-10^4 submerged spores ml^{-1}. This sporulation is less than in other studies. Iwanicki et al. (2018) observed a sporulation of over 10^8 blastospores ml^{-1} for eleven *Metarhizium* strains in the medium Adamek. However, it must be taken into account that there were differences in the preparation of this medium, which included additional 4% Tween® 80 as well as the fermentation parameters such as temperature, shaking speed and inoculum, which could also influence the sporulation of the fungi in liquid media (Kleespies and Zimmermann 1992). In contrast, a lower and very strain-specific sporulation of *M. anisopliae* strains was demonstrated by Bitencourt et al. (2023). In their study, three *M. anisopliae* strains were examined that produced 10^4, 10^6 and 10^8 spores ml^{-1} in Adamek medium. Therefore, the lower sporulation in the present experiments could be attributed to the specificity of the strain. Nevertheless, it would be interesting to examine in further experiments what causes the difference between the sporulation in the medium Adamek and the other two. The added sugar cannot be the reason, as the media Samsi 8 and Adamek contain glucose and the medium Catroux contains sucrose. CSS is also included in a similar concentration in all

three media. In order to increase sporulation, attempts could be made in further experiments to add Tween® 80 or polyethylene glycol 200 in the medium (Kleespies and Zimmermann 1992).

Besides nutrient availability, temperature is also crucial for the growth of EPF. The optimum growth temperature is highly dependent of the fungus genus, species and strain. Kleespies and Zimmermann (1992) proved that the temperature at which three strains of *M. anisopliae* sporulated most differed from each other. Two strains produced most spores at the highest investigated temperature (30 °C) and one at the lowest (25 °C). Therefore, this parameter should be studied for each strain to optimize the production process. For *M. brunneum* JKI-BI-1450, the highest sporulation occurred at 25 °C at all evaluation time points at the fermentation in flask. The sporulation was highest at 48 h and 72 h after inoculation. The maximum biomass formation was achieved after 48 h at 30 °C. After 24 h and 48 h the biomass concentration was higher at 30 °C compared to lower temperatures. In the later fermentation process, the fungus formed the most biomass at 25 °C. Jackson et al. (2011) also proved that the optimum fermentation temperature for biomass of *Mycoleptodiscus terrestris* depends on the time of evaluation. After 1 and 2 days, the biomass was highest at a fermentation at 30 °C, followed by 26 °C. Other ascomycetes have the same or a comparable temperature optimum for biomass production. Jin et al. (1996) determined that *Trichoderma harzianum* forms most biomass at 32 °C and Kim et al. (1999) identified the temperature optimum of *Aspergillus* sp. at 30 °C.

The investigation of the growth of JKI-BI-1450 in the bioreactor showed a typical growth curve. In the first 20 h after inoculation with submerged spores they germinate and form mycelium. From 24 h onwards submerged spore concentration increases, which can be explained by the fact that the fungus enters its exponential growth phase, in which new submerged spores are formed on the before formed mycelium. The biomass also increases with a slight delay after

28 h. Between 42-48 h the fungus reaches the stationary phase of sporulation. In this phase the fungus establishes a plateau of maximum sporulation, because newly formed and germinating spores are present in the same number. After that, the submerged spore concentration decreases, which could be caused either by their germination or their lysis. Similar observations were made by Iwanicki et al. (2020) for *Metarhizium robertsii*, although the decrease in spore concentration was observed after 96 h of fermentation. The maximum spore concentration and the point of the stationary phase is strongly dependent on the fungus, the medium and the fermentation parameters (such as temperature and oxygen supply) (Bidochka et al. 1987; Braga et al. 1999; Pham et al. 2009). Adib et al. (2013) demonstrated that the *M. anisopliae* strain 4556 formed more spores after 72 h in media with CSS, yeast extract, glucose and Tween® 80, whereas the same fungal strain formed more spores after 48 h in media with yeast and malt extracts. A variation of the time of maximum spore production in different media was also determined for *M. flavoviride* by Issaly et al. (2005). In contrast to the production of the submerged spores, the biomass increases during the whole fermentation time. Similar results were also obtained by Adámek (1965) for *M. anisopliae* up to a fermentation period of 140 h. During the fermentation process, autolysis can also set in, resulting in a decrease in biomass content after the exogenous carbon source has been consumed (Braga et al. 1999). The fact that the biomass level in the medium decreases with prolonged fermentation time was also shown by Arumugam et al. (2015) for *Nigrospora* sp.. In their study, the decrease in biomass temporally coincided with the depletion of the carbon source. The comparison of the fermentation of JKI-BI-1450 in the flask and in the bioreactor demonstrated that the sporulation in flasks was highest after 48 h and 72 h, whereas in the bioreactor the stationary phase already occurred between 42.5-48 h. This acceleration of sporulation could be due to the better oxygen supply in the bioreactor compared to the flask. There were also differences in biomass formation. In the bioreactor, the biomass concentration increased steadily between 28-72 h (end

of fermentation), whereas the biomass concentration in the flask stayed the same after 48 h. These results demonstrate that when optimizing one production parameter, the other parameters must also be taken into account and the transfer of optimized settings from the system flask to bioreactor must be verified.

Besides the formation of submerged spores and biomass, the growth of a filamentous fungus can also be detected indirectly via oxygen consumption, glucose decrease in the medium or pH value changes (Taber and Taber 1982; Braga et al. 1999). During its growth, the fungus metabolises the glucose in the medium, which can be observed in the experiment for JKI-BI-1450 after 36 h by the drop in the glucose level in the medium. Braga et al. (1999) also demonstrated a glucose decrease in the medium at the time of the biomass increase. In addition to the decrease of glucose in the medium, the pH value also changes during fermentation. In the present experiment, the pH value degraded slightly at first and rose steadily from 44 h after inoculation until the end of the experiment. The decrease of the pH value during fermentation has already been described many times for different fungi and can be explained by the formation of acid substances during the consumption of carbons such as glucose (Tamerler 1998; Issaly et al. 2005; Arumugam et al. 2015; Iwanicki et al. 2020). Iwanicki et al. (2020) was able to detect organic acids and carbonic acid at the end of an aerobic fermentation of *M. robertsii* due to the respiration process. The subsequent increase in pH observed in the present study has already been demonstrated in other studies (Tamerler 1998; Issaly et al. 2005; Arumugam et al. 2015). Arumugam et al. (2015) attribute the increase in the pH value of the medium during the fermentation of *Nigrospora* sp. to the release of ammonia from the degradation of primary and secondary metabolites. Beside the decrease of glucose and pH value, the growth of JKI-BI-1450 leads to an increased oxygen consumption of the fungus, which lowers the measurable oxygen content in the medium after 24 h. Braga et al. (1999) also demonstrated an exponential increase

in oxygen consumption after 36 h in the liquid fermentation of *M. anisopliae*. In the present experiment, the oxygen content in the medium dropped steadily after 24 h and was at 0% after 62 h. Although the same amount of oxygen was added constantly throughout the fermentation, the consumption by the fungus was so high that no more oxygen was measurable in the medium. The results of the present fermentation experiments provide information on the growth of the fungus and the optimum harvest time for the production of a soil granule (Chapter III). Based solely on the sporulation, a harvest after 42-48 h would be optimal. In terms of biomass yield, a later harvest date would be advantageous.

To optimize the production of JKI-BI-1450 in Samsi 8, the growth of the fungus was investigated in the medium with varying concentrations of nutrients. The experiment shows that biomass production can be increased by raising the glucose concentration, which is the major carbon source in this medium. With 28 g l^{-1} most biomass was produced in the medium with 5% glucose, which represents a doubling of biomass at a doubling of the glucose content. Jenkins and Prior (1993) observed similar results and were able to increase the biomass production of *M. flavoviride* by raising the sucrose level in the medium. The optimum carbon concentration for biomass production depends on the type of carbon. Mehta et al. (2012) determined in their study that *M. anisopliae* produced most biomass in medium with the highest lactose concentration (30 g l^{-1}), the lowest dextrose concentration (10 g l^{-1}) and the middle fructose concentration (20 g l^{-1}). Not only biomass production is influenced by the glucose content in the medium, but also the sporulation. Increased sporulation of *M. anisopliae* and *M. robertsii* in modified Adamek medium with increased glucose content was observed by Iwanicki et al. (2018). Even at a glucose concentration of 0%, low fungal growth was observed. CSS contains a small amount of sugar, which is apparently sufficient for the fungi to grow. CSS is a by-product from the large-scale extraction of starch from corn and is very well suitable for

the production of EPF in liquid medium (Kleespies and Zimmermann 1992; Iwanicki et al. 2020). The CSS used in the present study consists of 14-20% lactic acid, < 2% reducing sugars, approx. 2% amino nitrogen, 7-8% total nitrogen, 44-50% protein content and approx. 3% phosphorus (personal communication with Merck, Darmstadt, Germany). The biomass formation could be increased by raising the CSS concentration up to 3%. A further increase no longer resulted in a significant increase of the biomass. At a concentration of 4.5%, the biomass reached the maximum of 23 g l^{-1}. Iwanicki et al. (2020) also investigated the influence of the corn steep liquor (CSL) concentration (4-10%) on the growth of *M. robertsii*. However, they examined blastospore formation and not biomass production. Blastospore formation was highest in media with 8 or 10% CSL, depending on the time of evaluation.

Besides the concentration of the carbon and nitrogen components, the C/N ratio is important for the growth of EPF. Behle and Jackson (2014) showed that *M. brunneum* formed more biomass in media with a C/N ratio of 30:1 than with a ratio of 50:1. Issaly et al. (2005) also demonstrated a higher blastospore production in media with a low C/N ratio (1.6:1) compared to a high C/N ratio (52:1). In the present study, most biomass was produced in media with 5% glucose and 2% CSS (C/N 13:1) and with 2.5% glucose and 4.5% CSS (C/N 3:1). As both C/N ratios are low compared to other studies, further experiments should be carried out with higher C/N ratios in order to examine the influence of this factor on the growth of JKI-BI-1450. The concentration of the medium component NaCl had the least influence on biomass formation. The rise in biomass was only 19.5% compared to 288% for CSS and 666% for glucose, by increasing the concentration in the medium of the respective optimum compositions. Lowest and highest values were compared, not the highest values with the standard recipe. Biomass production of 15-28 g l^{-1} is similar to other studies e.g. Braga et al. (1999) with 25 g l^{-1} or higher compared to Mascarin et al. (2014) with 7-14 g l^{-1} and to Adámek (1965) with 9 g l^{-1}.

The optimization of liquid fermentation of EPF is very complex, as many factors influence the growth of the fungus. The effects of one growth factor such as temperature or nutrient availability are not independent and influence each other. For example, Bidochka et al. (1987) demonstrated for *Beauveria bassiana* the dependence of the fermentation period for the maximum sporulation and biomass formation from the medium. Based on the results obtained, a medium with 5% glucose, 4.5% CSS and 1.5% NaCl, a fermentation temperature of 25 °C and a fermentation time of 48 h would be best for the production of biomass and submerged spores of JKI-BI-1450. However, these factors were examined individually. Whether a combination of these optimized factors would also lead to better results would have to be tested in further experiments. The study of Issaly et al. (2005) demonstrated an increase in the formation of blastospores of *M. flavoviride*, which was achieved by the combination of the formerly optimized fermentation parameters.

In summary, the results of the present study demonstrated the great influence of production parameters on the growth of EPF and that both sporulation and biomass formation of JKI-BI-1450 can be significantly improved by the choice of the right medium, as well as incubation temperature and time.

Notes on other contributors: Sturm née. Ruppenthal, A. M.: Collected the data of experiment: “Optimization of the medium Samsi 8 to increase the biomass production of JKI-BI-1450”. Nieszporek, U. B.: Collected the data of the experiment: “Sporulation of six *Metarhizium* strains in three liquid media”.

Chapter III

Optimization of a *Metarhizium* soil granule formulation by fluid-bed drying

Abstract

To develop a strategy for soil application, fungal biomass produced in liquid form was sprayed onto millet grains by means of fluid-bed drying, thus producing a granule. To optimize this formulation process, the amount of biomass required to produce the granules was investigated and whether the growth of the fungus on the granules could be improved or accelerated by supplementing additives. For the *Metarhizium brunneum* strain JKI-BI-1450, a biomass input of 4.5 mg per g granule was necessary to achieve sufficient colonization of the granule. The present results indicate that fungal growth and conidia formation on the granule could be accelerated by the addition of sugars during fluid-bed drying. Furthermore, the storability of the granules was investigated by different temperatures. When stored at 5 and 20 °C the mean survival time (ST_{50}) of the colonization was 84 and 19 weeks, respectively. These results indicate that storage at lower temperatures prolongs the survival of the granules. The colonization and conidia formation of the fungus on the granules was also investigated at different temperatures and moistures and after contact with fungicidal seed treatments. The best growth was achieved at 25 °C and 30-45% residual moisture, and the effect of the granules depended on the fungicidal seed treatment. However, fungicides could be found that can be applied at the approved concentration together with the granules based on entomopathogenic fungi with very little loss of fungal vitality.

Introduction

The effective use of microorganisms as plant protection agents depends among others on the possibility of mass production and an effective formulation. Formulation is particularly difficult for the control of soil-dwelling pests with entomopathogenic fungi (EPF) because successful infection requires contact between the insect and a living infectious unit of the fungus (Zimmermann 2007a; Zimmermann 2007b). Thus, for control of soil pests, the fungus must be applied to the soil or the seed must be treated, as direct application of the fungus to the insect is not possible. In addition to the application of EPF into the soil as a spray application, another successful method is the use of soil granules (Kim et al. 2020; Shah et al. 2023). There are two categories of soil granules or more precisely of application strategies; (i) conidia or other infectious units are applied directly to the soil; (ii) the fungus is introduced into the soil in dried form. In this case, the fungus only grows in the soil after contact with moisture and forms the infectious units there. A common method for the production of a soil granule for the direct application of infection units is the solid-state fermentation of EPF on solid substrates such as grain or rice (Pham et al. 2010; Mar and Lumyong 2012; Xie et al. 2016; Jaronski 2023; Sala et al. 2023). In this case, the fungus grows on the substrate and forms conidia before it is introduced into the soil. The advantage of this process is that this granule can be produced easily and cheaply in large quantities and the fungus is present as an infectious unit in the soil immediately after application and can infect insects (Kim et al. 2020). The disadvantages of this type of granule production are the relatively long production time in comparison with other production strategies, such as liquid fermentation, high risk of contamination and a dusty product, which can be dangerous to the user (Pham et al. 2010; Mar and Lumyong 2012). Nevertheless, this method is frequently applied and used for the production of biocontrol agents (Bhargava et al. 2008; Mattedi et al. 2023).

However, the granule developed in the present study follows the other application strategy. Accordingly, the fungus was produced in liquid culture, which has the advantages of a short fermentation time, a low risk of contamination and a specific adjustability of the production parameters adapted to the fungus (Bidochka et al. 1987; Braga et al. 1999; Adib et al. 2013). However, one disadvantage is that the resulting fermentation broth has to be formulated after production. Formulation is important to turn the fermentation broth into an applicable and storable form (Awan et al. 2021). Various methods are available for the formulation of microorganisms after liquid cultivation. One of those is the encapsulation in calcium alginate granule (Quimby et al. 2004; Peters and Fiege 2017). Furthermore, the formulation through various drying methods is well suitable. The appropriateness of freeze-, spray- and fluid-bed drying for the formulation of EPF was already demonstrated by Stephan and Zimmermann (2001). The survival strongly depends on the fungus and the parameters such as drying temperature and duration (Horaczek and Viernstein 2004). In the present study, the fluid-bed drying was used to produce the soil granule. The suitability of this method for the production of soil granules based on EPF has already been described by Stephan et al. (2020). For fluid-bed drying, gas flows through a layer of solid particles from below. This flow causes the particles to move and transforms them from a solid bed into a fluidized bed. In the process, the particles are mixed intensively and get dried evenly. In addition to the drying of particles, fluid-bed drying can be used for coating (Srivastava and Mishra 2010; Stephan et al. 2020). Coating is the covering of a carrier substance with a film-forming liquid. A coating liquid is sprayed onto a carrier substance. The liquid evaporates during the drying process and only the coating substance remains as a thin layer around the carrier substance. In the present study, this coating substance was biomass of the *Metarhizium brunneum* strain JKI-BI-1450. The carrier material used was millet (Alnatura Produktions- und Handels GmbH, Darmstadt, Germany). Millet is uniformly small and round and therefore well suited for formulation by

fluid-bed drying (Stephan et al. 2020). Furthermore, fungi of the genus *Metarhizium* are able to grow on millet during solid-state fermentation, which indicates that the fungus can utilize the nutrients in the millet and these are probably beneficial for later growth of the fungus on the granule (Kim et al. 2011; Song et al. 2019; Kim et al. 2020; Sharma et al. 2020; Frank Sullivan et al. 2021). In case of sufficient moisture and temperature in the soil, the fungus grows on the granule and forms conidia. These conidia can then infect insects living in the soil.

The aim of the present study was to examine the applicability of the method established by Stephan et al. (2020) for the formulation of soil granules based on *M. brunneum* JKI-BI-1450 for the control of *Agriotes* larvae in potato and to optimize and accelerate the growth of the fungus and its conidia formation (conidiation) on the granules. Besides the highest possible conidia density in the soil, it is also important for a successful infection that the conidia are formed on the granule as quickly as possible to prevent the granule for drying out, being colonized by other microorganisms or eaten by soil-dwelling insects, which degrades the effect of the granule. It was examined whether the colonization and conidiation of the granule can be increased or accelerated by the addition of different carbon sources. The carbon sources investigated were chitin as well as various sugars, as the usability of these substances by fungi such as *Metarhizium* has already been proven (Campbell et al. 1983; Sun and Liu 2006; Wu et al. 2010). Furthermore, the increase and acceleration of the conidiation of fungal alginate capsules by the addition of these substances has been demonstrated previously (Shah et al. 1999; Gerding-González et al. 2007; Lorenz et al. 2020).

Environmental conditions in the field are often not optimal for the growth of EPF. Therefore, the growth of the fungus on the granule was investigated at simulated soil temperature and different soil moistures. For the application in integrated pest management, it is also crucial that the developed granule can be used in combination with common fungicidal seed treatments in

potato cultivation. It is often not possible to renounce these agents, as otherwise fungal diseases would attack the potatoes in excess. Therefore, the effect of the application of five fungicidal seed treatments at their maximum application rate on the colonization and conidiation of the granule was investigated.

Material and Methods

Production of the soil granule

The *M. brunneum* strain JKI-BI-1450 was fermented in a bioreactor as described in chapter II. For purification, the biomass was centrifuged at 25 °C, and 15,334 × *g* for 10 min. The supernatant was discarded and the pellet resuspended with 0.9% (w/v) sodium chloride (NaCl) (Merck, Darmstadt, Germany). This process was repeated three times. The dry biomass was then determined by using a moisture determination balance (Ma30, Sartorius, Göttingen, Germany). The concentration of the fungal biomass suspensions (depending on the experimental target) was prepared by dilution with 0.9% (w/v) NaCl. The granule was produced by using a fluid-bed dryer as described in Seib et al. (2023) (Chapter IV) according to Stephan et al. (2020).

Determination of biomass concentration for granule production

The biomass of JKI-BI-1450 was produced in the bioreactor and purified as described in chapter II. Suspensions with biomass concentrations of 3, 2, 1, 3×10^{-1}, 3×10^{-2} and 3×10^{-3}% were prepared. Then, granules were prepared for each biomass concentration as described above by coating 15 ml of each suspension onto 100 g of autoclaved millet using a fluid-bed dryer. Thereby, granules with 4.5, 3, 1.5, 4.5^{-1}, 4.5^{-2}, 4.5^{-3} mg biomass per g granule were produced. Ten granule grains of each granule were placed on plates with 1% (v/w) agar-agar and incubated at 25 °C for 7 days in the dark. After this incubation period, the number of colonized granule

grains was determined. Granule grains covered with mycelium or mycelium and conidia of JKI-BI-1450 were counted as colonized. The experiment was repeated three times independently with three replicates each.

Granule production based on conidia

Strain JKI-BI-1450 was incubated on malt peptone agar plates (MPA) containing 3% (w/v) malt extract (Merck, Darmstadt, Germany), 0.5% (w/v) peptone from soybean (Merck) and 1.8% (w/v) agar-agar (Roth, Karlsruhe, Germany) at 25 °C for 14 days in the dark. After that, 1 ml of Tween® 80 (Merck) was added to each plate and the conidia were removed by scraping the surface with a sterile spatula. The conidia concentration was determined by using a haemocytometer. Suspensions with concentrations of 10^3, 10^5, 10^7 and 10^9 conidia ml^{-1} were prepared. Ten ml of the suspensions were sprayed onto 100 g of millet using fluid-bed drying as descripted in chapter IV and (Seib et al. 2023). Through these, granules with 10^2, 10^4, 10^6, and 10^8 conidia per g granule were produced. Granule containing 4.5 mg biomass per g granule was used for control purposes. In the preceding experiment, it was determined that this concentration is necessary to achieve a granule colonization of 100%. This kind of granule contains on average 1.5×10^4 submerged spores per g granule. The biomass was prepared in the bioreactor as described above. Ten granule grains of each granule were placed on plates with 1% (v/w) agar-agar and incubated at 25 °C for 14 days in the dark. After this incubation period, the number of colonized granule grains was determined as descripted above. To determine the conidia concentration, 1 ml of sterile 0.5% (v/v) Tween® 80 was added to each petri plate. The granule grains were removed were scrapped of by using a sterile spatula and the suspension was transferred to a 1.5-ml Eppendorf tube. The Eppendorf tubes were vortexed for 10 s and afterwards in an ultrasonic bath (Sonorex RK 52 35 kHz, Bandelin electronic GmbH & Co. KG, Berlin, Germany) for 15 min. The conidia concentration was determined

using a haemocytometer. The experiment was repeated three times independently with ten replicates each.

Influence of chitin on fungal growth

Influence of different chitin concentrations on fungal growth

The strain JKI-BI-1450 was incubated on MPA plates at 25 °C for 14 days in the dark. Conidia were suspended in 1 ml sterile 0.5% (v/v) Tween® 80 using a sterile loop in a 1.5-ml Eppendorf tube. The Eppendorf tubes were placed in an ultrasonic bath for 3 min. The conidia concentration was determined by using a haemocytometer and a concentration of 10^6 conidia ml^{-1} was prepared by dilution with sterile 0.5% (v/v) Tween® 80. Plates with MPA + 0, 0.1, 0.3, 0.5, and 1% chitin (Roth) were prepared. Two µl of the conidia suspension were added to the centre of each plate, which was then incubated at 25 °C for 14 days in dark. Afterwards, the diameter of the fungal colony was measured twice, perpendicular to one another. To determine the conidiation, 2 ml of sterile 0.5% (v/v) Tween® 80 were added to each plate and the conidia were scraped off with a sterile spatula. The conidia concentration was determined by using a haemocytometer and the conidia mm^{-2} were calculated based on the final colony size. The experiment was repeated three times independently with ten replicates each.

Influence of chitin on fungal growth on granule

Only in this experiment, the biomass for the granule was produced in Erlenmeyer flasks and not in a bioreactor. The fungal strain JKI-BI-1450 was incubated on MPA plates at 25 °C for 14 days in the dark. Fifty ml liquid medium containing 2.5% (w/v) D(+)-glucose (Merck), 2% (w/v) corn steep solid (Sigma Aldrich, Buchs, Switzerland) and 0.5% (w/v) NaCl (Merck) were autoclaved at 121 °C for 20 min in 100-ml Erlenmeyer flasks. The flasks were inoculated with an undefined amount of fungal material from the plates. The flasks were incubated for 72 h at

25 °C and 150 rpm on a horizontal shaker (Novotron, 50 mm deflection, Infors, Bottmingen, Switzerland). The biomass was purified and the granules were produced by fluid-bed drying as described above except that the 15 g biomass suspension (3%, w/v) was mixed with 15 g chitin solution (1%, w/v). Furthermore, a water control was prepared, for which the 15 g biomass was mixed with 15 g sterile deionised water. Ten granule grains of each granule were placed on plates with 1% (w/v) agar-agar and incubated at 25 °C for 14 days. After this incubation period, the number of colonized granule grains and the conidia per granule grain were determined as described above. The experiment was repeated three times independently with three replicates each.

Acceleration of fungal growth on granule through the addition of sugar

Influence of sugar on granule colonization

Biomass of JKI-BI-1450 was produced in the bioreactor and purified and the granules were produced by fluid-bed drying as described above except that the biomass suspension was mixed with sugar solutions. The 15 g biomass suspension (3%, w/v) was mixed with 15 g sugar solution (20%, w/v) and coated onto 100 g millet grains each. The sugars examined were lactose (DocMorris, Heerlen, Niederlande), D(-)-fructose (Roth), D(+)-glucose and D(+)-sucrose (Roth). Ten granule grains of each granule were placed on plates with 1% (v/w) agar-agar and incubated at 25 °C for 1, 2, 3, 7 and 14 days in the dark. After this incubation periods, the number of colonized granule grains was determined as described above. The experiment was repeated three times independently with three replicates each.

Influence of sugar on conidiation

The granules with added sugar were prepared as described above. Ten granule grains of each granule were placed on plates with 1% (v/w) agar-agar and incubated at 25 °C for 3, 5, 7, 10,

12 and 14 days in the dark. After this incubation periods, the conidiation was determined as described above. The experiment was carried out with five replicates.

Vitality stability of the granule

A granule based on strain JKI-BI-1450 was prepared as described above and stored at 5 °C and 20 °C in medical plastic pill bottles. After 4, 8, 12, 26, 52 and 104 weeks the granule colonization was determined as described above. Ten repetitions were performed for each time point. Five replicates were performed for the incubation temperature of 5 °C and two replicates for the one of 20 °C.

Temperature and humidity dependence of fungal growth on granule

Soil (Fruhstorfer Erde Typ Nullerde, Archut GmbH u. Co. KG Industrie-Erdenwerk, Lauterbach, Germany) (Nitrogen (N) = 20-40 mg l^{-1}, Phosphate (P_2O_5) = 20-40 mg l^{-1}, Potassium (K_2O) = 40-60 mg l^{-1}, pH value = 5.9, Salt content KCl = 0.2 g l^{-1}) was autoclaved at 121 °C for 20 min. Then the soil was mixed with sterile deionised water and the residual moisture levels were adjusted to 5, 15, 30, 45 and 60% using a moisture determination balance. Plates (60 × 15 mm) were half-filled with the soil with the different moisture contents. A granule based on the fungal strain JKI-BI-1450 was prepared as described above. Ten granule grains were placed in each plate and weighed. The plates were incubated at 15, 20, 25 and 30 °C for 2 and 4 weeks in the dark. In order to keep the moisture content of the soil constant during the experiment, the plates were weighed twice a week. The loss of weight due to evaporation was compensated by addition of sterile deionised water. After incubation the granule colonization was determined as descripted above. To determine the conidiation, the soil and the granule grains were transferred into a 50-ml reaction vessel. Twenty-five ml of 0.5% Tween® 80 were filled into each reaction vessel and placed on an Intelli Mixer with the settings F-C1,

Rpm: 99, (neoLab, Heidelberg, Germany) for 15 min. The conidia concentrations were determined by using a haemocytometer. The experiment was carried out with three replicates and three repetitions.

Influence of fungicidal seed treatments on fungal growth on the granule

A granule based on JKI-BI-1450 was prepared as described above. Granule grains were placed on 1% (w/v) agar-agar and sprayed with the fungicidal dressings Ortiva®, Moncut®, Risolex®, Emesto® Silver, RhizoVital®42 by using a spraylab (typ 210/120 SPS 01.44.2010, Schachtner Gerätetechnik, Ludwigsburg, Germany). In addition to the fungicidal seed treatments, an untreated and a water control were prepared. The fungicidal seed treatments were sprayed onto the granule at their maximum application rate. The application rate and mixing ratio can be found in Table 7. When planting potatoes, the potato is placed in a furrow. This furrow is sprayed with a width of 20 cm. The ridges have a spacing of 75 cm. Therefore, only 20/75th of the area is treated and the application rate had to be adjusted accordingly. The water control was treated with the same application rate and thus the settings similar to the treatment with Ortiva® were adopted, as this was the highest. The correct application rate was regulated via the application speed.

The plates with the sprayed granule grains were incubated at 25 °C for 14 and 28 days in the dark. After this incubation period, the number of colonized granule grains and the conidia per granule grain were determined as described above. The experiment was carried out with five replicates and five repetitions.

Table 7: Active ingredients, application rate and mixing ratios of the fungicidal seed treatments.

Product	Active ingredient	Product [l ha^{-1}]	Application rate [l ha^{-1}]	Adjusted application rate [l ha^{-1}]
Ortiva®	Flutolanil	3	200	750
Moncut®	Azoxystrobin	0.4	60	225
Risolex®	Tolclofos-methyl	1.2	80	300
Emesto® Silver	Prothioconazol, Penflufen	0.5	60	225
RhizoVital®42	*Bacillus velezensis* strain FZB42	0.5	80	300

Statistical analysis

The data was statistically analysed with the software SAS Studio 3.8. All experiments were analysed by using a generalized linear model with Wald statistics for type 3 analysis and multiple comparison according to Tukey (GLMM, $p < 0.05$), expect the experiment of the storability of the granule by different temperatures, which was compared based on a survival analysis (Kaplan-Meier-Wilcoxon) and expect the experiment on the effect of the fungicidal seed treatments. The comparison of the effect of these agents was carried out by using a Kruskal-Wallis test, posthoc: Wilcoxon test ($p < 0.05$).

Results

Determination of biomass concentration for granule production

The experiment demonstrated that 4.5 mg biomass per g granule was necessary to achieve a sufficient granule colonization of 100% (Figure 16). Less biomass application significantly decreased granule colonization ($\chi^2 = 159.66$; df = 5; $p < 0.0001$). The fungus was not able to colonize the granule when 0.045 mg or less biomass per g granule were used for granule production.

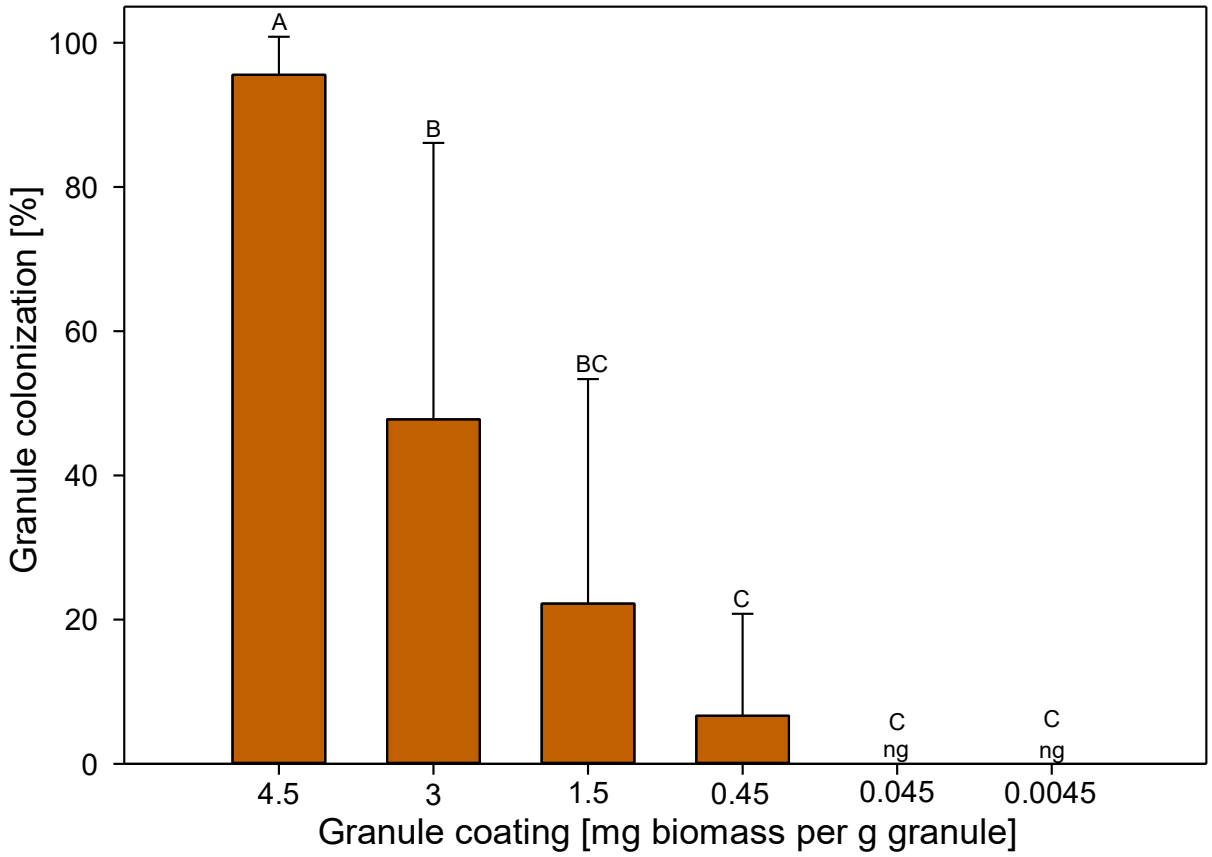


Figure 16: Granule colonization of *Metarhizium brunneum* strain JKI-BI-1450 based granules coated with different biomass concentrations. Mean and the standard deviation are shown. ng = no growth. Different letters show significant differences. GLMM ($p < 0.05$). $n = 9$.

Granule production based on conidia

The different coating of the granules influenced both colonization ($\chi^2 = 1673.73$; df = 4; $p < 0.0001$) and conidiation ($\chi^2 = 180.43$; df = 4; $p < 0.0001$) (Figure 17). Granules coated with 10^6 or 10^8 conidia per g granule or with 4.5 mg biomass per g granule did not differ significantly in granule colonization and conidiation after 2 weeks of incubation. Both growth parameters were significantly lower when granules were coated with 10^4 conidia per g granule. Coating the granule with 10^2 conidia per granule grain resulted in significantly lowest conidiation and granule colonization.

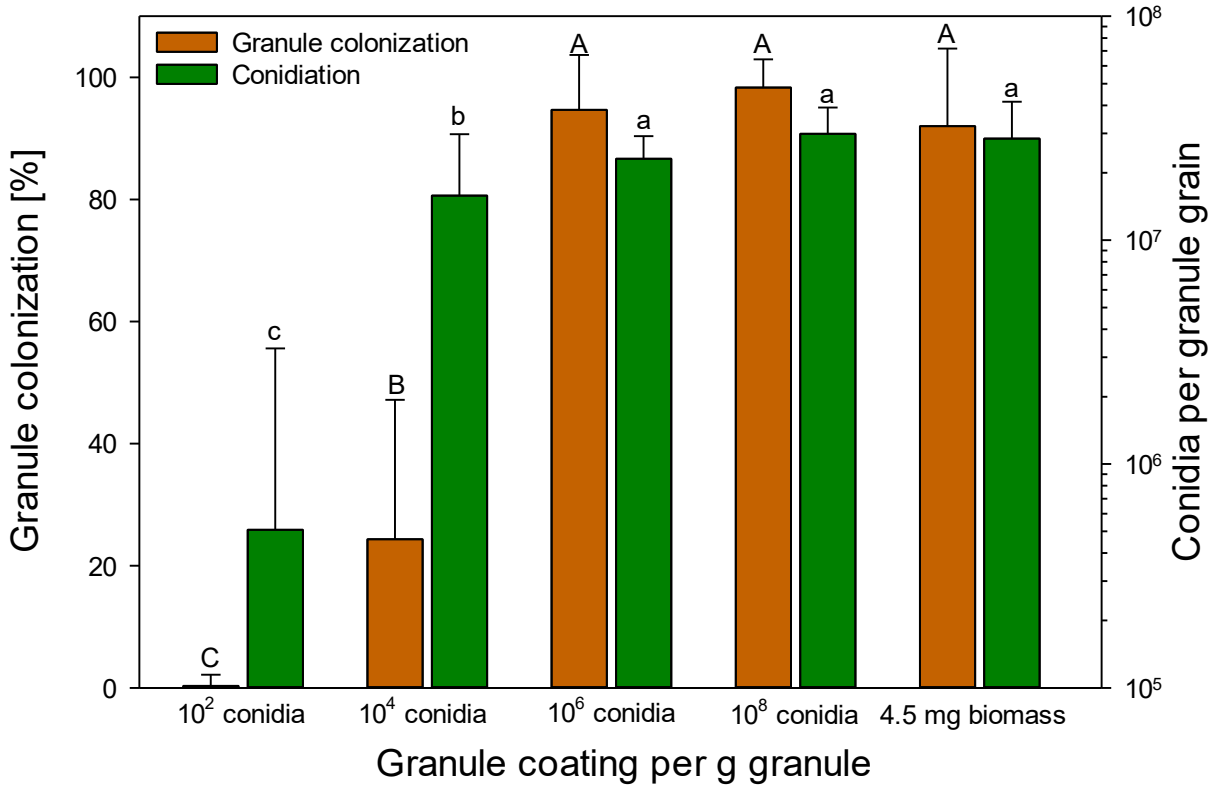


Figure 17: Conidia formation and granule colonization of a *Metarhizium brunneum* strain JKI-BI-1450 based granules coated with different conidia concentrations and 4.5 mg biomass g^{-1}. Mean and the standard deviation are shown. Different upper case resp. lower case letters show significant differences. GLMM ($p < 0.05$). $n = 30$.

Influence of chitin on fungal growth

Influence of different chitin concentrations on fungal growth

Both colony size ($\chi^2 = 47.78$; df = 4; $p < 0.0001$) and conidiation ($\chi^2 = 103.20$; df = 4; $p < 0.0001$) could be increased by adding chitin to malt peptone agar (Figure 18). At the concentration of 1% chitin in the medium, both growth parameters were significantly highest with 1720 mm^2 and 4.09×10^5 conidia mm^{-1}. With decreasing chitin concentration, the colony size and the conidia concentration declined.

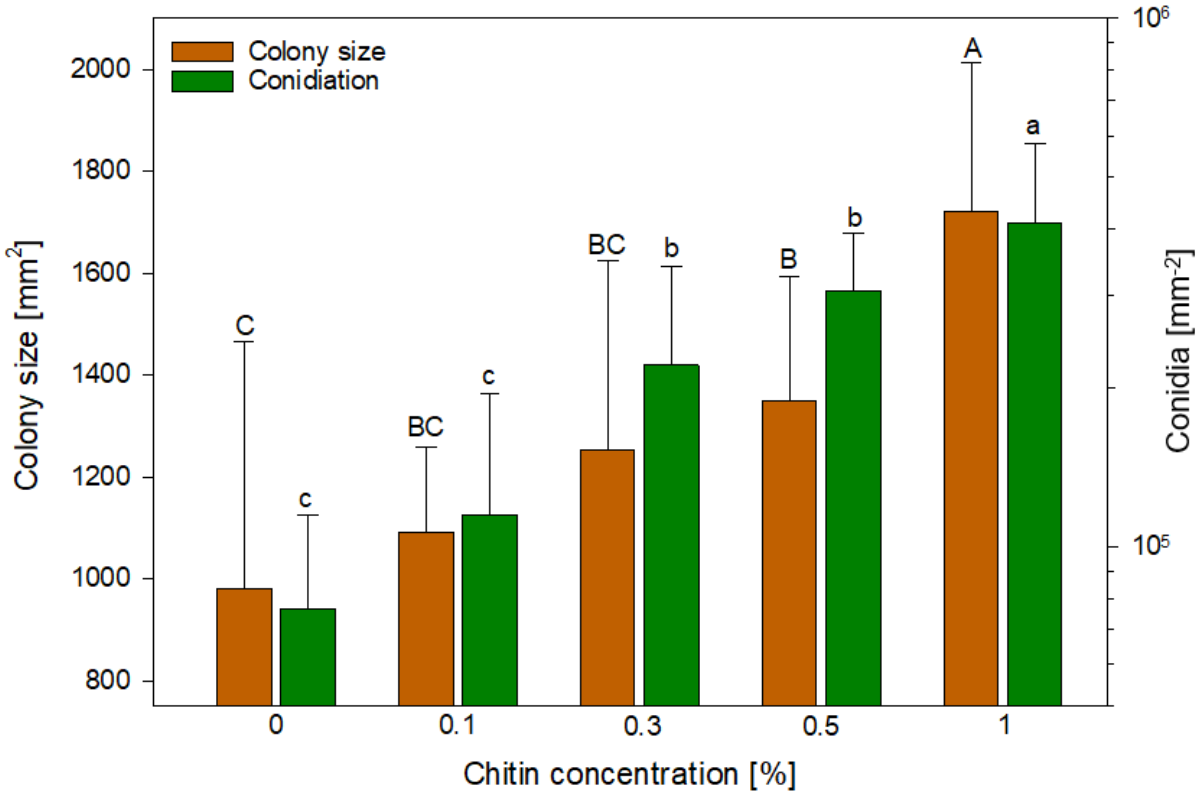


Figure 18: Colony size and conidia formation from *Metarhizium brunneum* strain JKI-BI-1450 on malt peptone agar with different chitin concentrations. Mean and standard deviation are shown. Different upper case resp. lower case letters show significant differences. GLMM ($p < 0.05$). $n = 15$.

Influence of chitin on fungal growth on the granule

The comparison of granule colonization (24 h: ($\chi^2 = 1.13$; df = 1; $p = 0.2888$); 48 h: ($\chi^2 = 0.01$; df = 1; $p = 0.9065$); 72 h: ($\chi^2 = 0.04$; df = 1; $p = 0.8344$); 1 week: ($\chi^2 = 2.57$; df = 1; p = 0.1088)) and conidiation (1 week: ($\chi^2 = 0.06$; df = 1; $p = 0.8066$); 2 weeks: ($\chi^2 = 0.82$; df = 1; $p = 0.3664$)) of the treatments with and without chitin did not show a significant difference at any of the evaluation times (Table 8).

Table 8: Influence of the addition of chitin on the granule colonization and conidia formation on a granule of *Metarhizium brunneum* strain JKI-BI-1450. $n = 9$.

Evaluation time point [d]	Growth parameter			
	Granule colonization [%]		Conidia per granule grain	
	Control*, **	Chitin*, **	Control*, **	Chitin*, **
1	6.67±8.7	3.3±5.0	0.0±0.0	0.0±0.0
2	61.1±20.9	62.2±21.7	0.0±0.0	0.0±0.0
3	90.0±13.2	91.1±10.5	0.0±0.0	0.0±0.0
7	97.8±4.4	100±0.0	$2.64±2.08 \times 10^6$	$2.44±1.59 \times 10^6$
14	100±0.0	100±0.0	$1.29±0.60 \times 10^7$	$1.09±0.38 \times 10^7$

* Means ± standard deviation, ** Compared were the granule colonization and the conidia per granule grain of the control and the chitin treatment at each evaluation time point separately. No difference could be detected at any evaluation time point GLMM ($p < 0.05$).

Acceleration of fungal growth on granule through the addition of sugar

Influence of sugar on granule colonization

The results of this experiment showed that the addition of sugar accelerated the colonization of the granule with the fungus and already after one day the colonization differed significantly ($\chi^2 = 93.90$; df = 4; $p < 0.0001$) (Figure 19). Most colonization occurred in the treatments with fructose (62%) and sucrose (57%). Glucose treatment (37%) followed, which differed significantly from fructose treatment, but not from sucrose treatment. The lowest colonization was found in the treatment with lactose and the control with 12% and 6%. These treatments were not significantly different from each other but from all other treatments. After 2, 3 and 7 days, the colonization of all sugar treatments was around 100%. They were not significantly different from each other but from the control (2 days: ($\chi^2 = 558.61$; df =4; $p < 0.0001$); 3 days: ($\chi^2 = 36.43$; df = 4; $p < 0.0001$); 7 days: ($\chi^2 = 21.59$; df = 4; $p = 0.0002$)). The comparison of a treatment over the entire experiment period showed that all sugar treatments reached their maximum colonization after 2 days (fructose: ($\chi^2 = 114.25$; df = 4; $p < 0.0001$); glucose: ($\chi^2 = 711.12$; df = 4; $p < 0.0001$); lactose: ($\chi^2 = 1888.91$; df = 4; $p < 0.0001$); sucrose: ($\chi^2 = 126.79$; df = 4; $p < 0.0001$)). The colonization of the control increased steadily over the

incubation period, which did not differ significantly from 7 days onwards ($\chi^2 = 160.37$; df = 4; $p < 0.0001$).

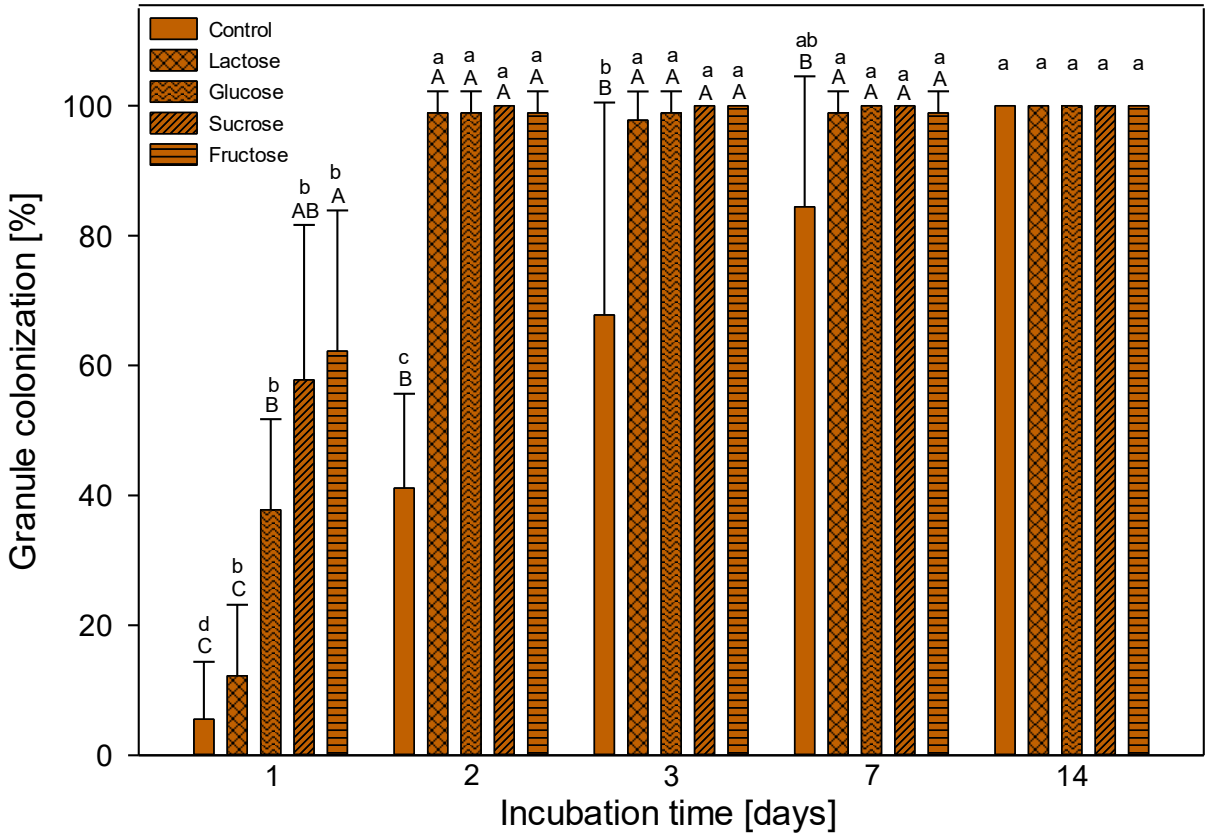


Figure 19: Granule colonization of *Metarhizium brunneum* strain JKI-BI-1450 based granules with different sugars added. Mean and standard deviation are shown. Different upper case letters show significant differences between all treatments within one evaluation time. Different lower case letters show significant differences in one treatment across all evaluation times. GLMM ($p < 0.05$). $n = 9$.

Influence of sugar on conidiation

The addition of sugars to the coating liquid during the production of the granules also had a positive effect on their conidiation (Figure 20). After 5 days of incubation, the conidiation differed between the various treatments ($\chi^2 = 31.50$; df = 4; $p < 0.0001$). In the treatments with fructose, sucrose and lactose the most conidia per granule grain were formed with 1.13×10^6, 1.10×10^6 and 0.93×10^6 conidia per granule grain, respectively. The lowest number of conidia was formed in the control without sugar with 3.35×10^5 conidia per granule grain, which was

significantly different from the treatments just mentioned. 4.41×10^5 conidia per granule grain were formed in the glucose treatment, which was significantly different from the treatment with fructose and sucrose. After 7 days, most conidia were formed in the treatments with fructose and lactose with 7.23 and 5.85×10^6 conidia per granule grain. The conidiation of the control was 2.56×10^6 conidia per granule grain and was significantly different from all sugar treatments except glucose ($\chi^2 = 46.76$; df = 4; $p < 0.0001$). With increasing incubation time, the conidiation of the control and the sugar treatments no longer differed significantly and was between 1.58-2.7 $\times 10^7$ conidia per granule grain (10 days: ($\chi^2 = 7.69$; df = 4; $p = 0.1036$); 12 days: ($\chi^2 = 6.04$; df = 4; $p = 0.1965$); 14 days: ($\chi^2 = 4.45$; df = 4; $p = 0.3487$)). Comparison of one treatment over the experiment period showed that all treatments reached their maximum conidiation after 10 days (fructose: ($\chi^2 = 280.65$; df = 5; $p < 0.0001$); glucose: ($\chi^2 = 248.70$; df = 5; $p < 0.0001$); lactose: ($\chi^2 = 547.93$; df = 5; $p < 0.0001$); sucrose: ($\chi^2 = 523.77$; df = 5; $p < 0.0001$); control: ($\chi^2 = 195.00$; df = 5; $p < 0.0001$)).

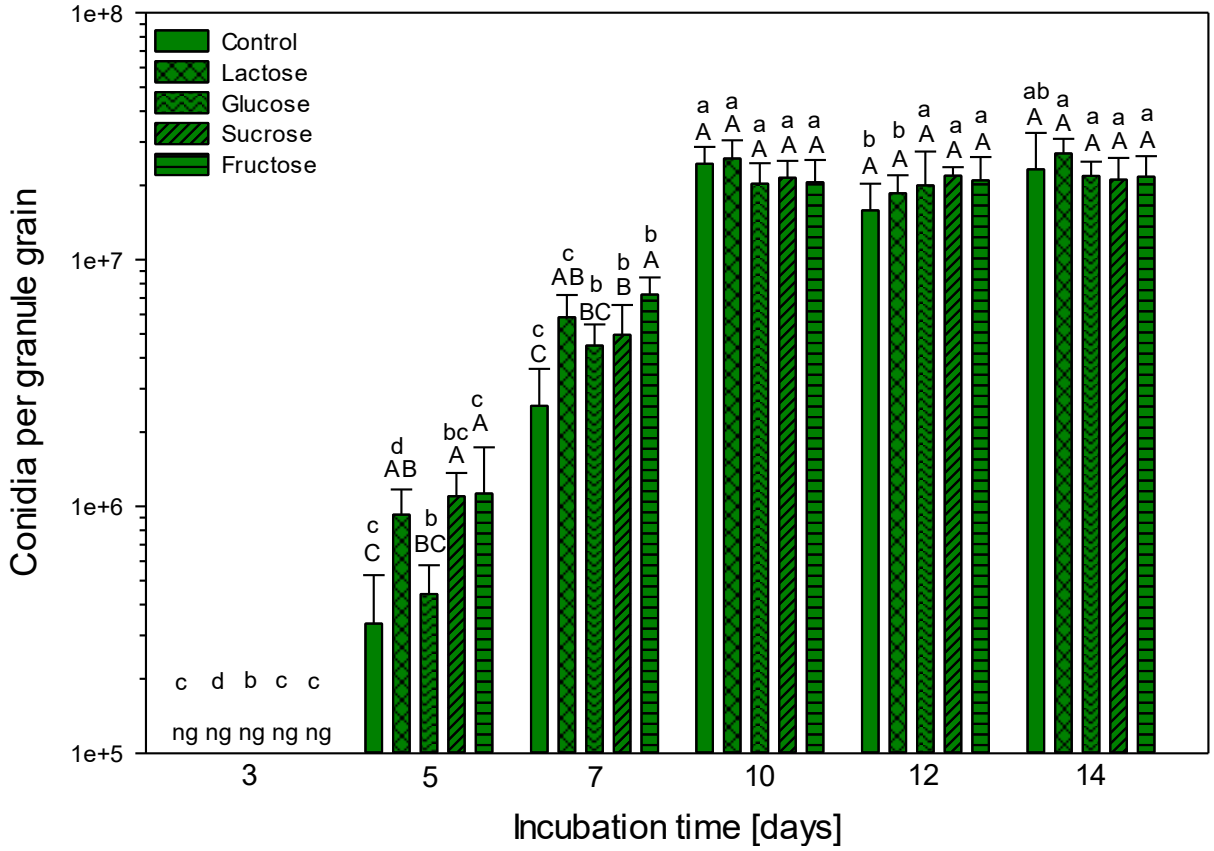


Figure 20: Conidia formation of *Metarhizium brunneum* strain JKI-BI-1450 based granules with different sugars added. Mean and standard deviation are shown. ng = no growth. Different upper case letters show significant differences between all treatments within one evaluation time. Different lower case letters show significant differences in one treatment across all evaluation times. GLMM ($p < 0.05$). $n = 5$.

Vitality stability of the granule

Storage of the granule at different temperatures led to a significant difference in the decrease in colonization ($\chi^2 = 1196.1411$; df = 1; $p < 0.0001$) (Figure 21). Storage at 5 °C resulted in a mean survival time (ST_{50}) of colonization after 84 weeks and at 20 °C after 19 weeks.

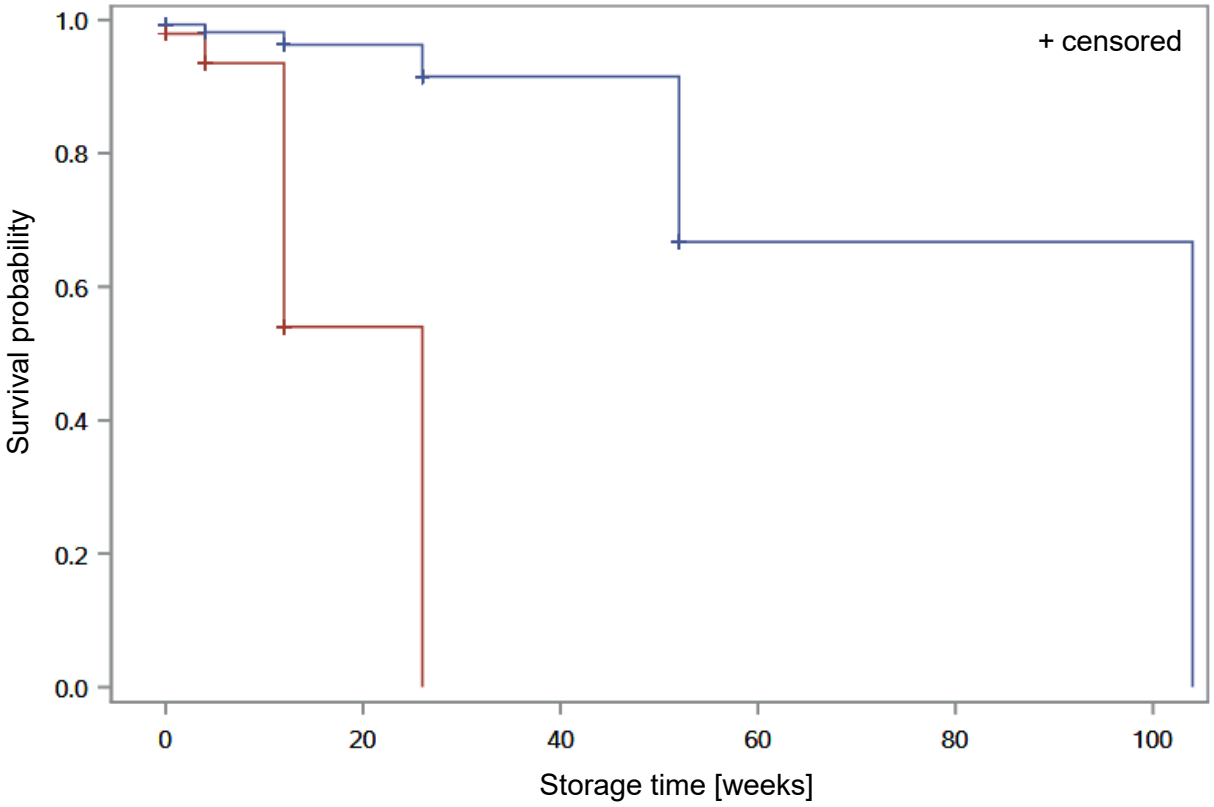


Figure 21: Granule colonization of *Metarhizium brunneum* strain JKI-BI-1450 based granules over two years at 5 °C (blue) and 20 °C (red) incubation temperature.

Influence of temperature and moisture of fungal growth on granule

The residual moisture of the soil of 5% was not sufficient for JKI-BI-1450 to grew on the granule (Table 9). At 15% residual moisture, 15 °C during the test period was not high enough for the fungus to colonized the granule. Colony formation only begun at 20 °C, but with a delay and a significant difference to higher temperatures. At the residual moistures of 30 and 45%, the colonization was 93-100% regardless of the temperature and incubation time. Colonization at 60% residual moisture was dependent on temperature, just as at 15%, although to a lesser extent. At higher temperatures of 25-30 °C, colonization was higher, but there was only a significant difference between the treatments at 15 °C after 2 weeks and the other treatments at this residual moisture.

Table 9. Granule colonization of granules based on *Metarhizium brunneum* strain JKI-BI-1450 at different residual moistures and incubation temperatures. $n = 9$.

Residual moisture [%]	Incubation time [weeks]	Granule colonization [%]								
		Incubation temperature [°C]								
		15*	**	20*	**	25*	**	30*	**	
5	2	0.0±0.0	(C)	0.0±0.0	(C)	0.0±0.0	(B)	0.0±0.0	(C)	
	4	0.0±0.0	(c)	0.0±0.0	(c)	0.0±0.0	(c)	0.0±0.0	(c)	
15	2	0.0±0.0	B (C)	8.9±9.3	B (C)	88.9±19.6	A (A)	93.3±8.7	A (A)	$\chi^2 = 559.55$ df = 3; $p < 0.0001$
	4	0.0±0.0	c (c)	60.0±42.4	b (b)	67.8±35.3	ab (b)	96.7±10.0	a (a)	$\chi^2 = 63.68$; df = 3; $p < 0.0001$
30	2	95.6±7.3	A (A)	93.3±7.1	A (A)	98.9±3.3	A (A)	98.9±3.3	A (A)	$\chi^2 = 7.20$ df = 3; $p = 0.0658$
	4	98.9±3.3	a (a)	98.9±3.3	a (a)	100.0±0.0	a (a)	100.0±0.0	a (a)	$\chi^2 = 2.25$; df = 3; $p = 0.5222$
45	2	100.0±0.0	(A)	100.0±0.0	(A)	100.0±0.0	(A)	100.0±0.0	(A)	
	4	100.0±0.0	a (a)	98.9±3.3	a (a)	98.9±3.3	a (a)	98.9±3.3	a (a)	$\chi^2 = 1.13$; df = 3; $p = 0.7710$
60	2	46.7±19.4	B (B)	82.2±13.0	A (B)	91.1±11.7	A (A)	84.4±12.4	A (B)	$\chi^2 = 58.26$; df = 3; $p < 0.0001$
	4	54.4±15.1	a (b)	62.2±23.3	a (b)	63.3±18.0	a (b)	71.1±29.8	a (b)	$\chi^2 = 2.85$; df = 3; $p = 0.4155$
	2	$\chi^2 = 1133.06$; df = 4; $p < 0.0001$		$\chi^2 = 1552.25$; df = 4; $p < 0.0001$		$\chi^2 = 690.09$; df = 4; $p<0.0001$		$\chi^2 = 1535.44$; df = 4; $p<0.0001$		
	4	$\chi^2 = 2099.62$; df = 4; $p < 0.0001$		$\chi^2 = 140.10$; df = 4; $p < 0.0001$		$\chi^2 = 211.52$; df = 4; $p < 0.0001$		$\chi^2 = 370.15$; df = 4; $p < 0.0001$		

*Means ± standard deviation, ** Means with the same letter are not significantly different (GLMM, $p < 0.05$); letters without brackets: comparison of one residual moisture and one incubation period over all incubation temperatures; letters with brackets: comparison of an incubation temperature and one incubation period over all residual moistures; upper case letters: comparison after 2 weeks of incubation; lower case letters: comparison after 4 weeks of incubation.

The fungus did not grow on the granules in a soil with 5% residual moisture, so it could not form conidia (Table 10). At 15% residual moisture, the fungus was only able to form conidia at 20 °C and after 4 weeks with 2.2×10^4 conidia per granule grain, although it already showed low growth after 2 weeks. Even at higher temperatures, conidiation was very low at this residual moisture and did not differ significantly. At 30, 45 and 60% residual moisture of the soil, the fungus always formed the most spores at 25 °C between 1.9-3.5 × 10^6 conidia per granule grain, followed with decreasing conidiation at 20, 30 and 15 °C. At all temperatures, conidiation was significantly lower at 60% than at 30 and 45%, which were not significantly different.

Table 10. Conidia formation on granule based on *Metarhizium brunneum* strain JKI-BI-1450 at different residual moistures and incubation temperatures. $n = 9$.

Residual moisture [%]	Incubation time [weeks]	Conidia per granule grain								
		Incubation temperature [°C]								
		15*	**	20*	**	25*	**	30*	**	
5	2	0.0±0.0	(C)	0.0±0.0	(C)	0.0±0.0	(C)	0.0±0.0	(C)	
	4	0.0±0.0	(c)	0.0±0.0	(c)	0.0±0.0	(c)	0.0±0.0	(c)	
15	2	0.0±0.0	B (C)	0.0±0.0	B (C)	$2.6±3.2 \times 10^4$	A (C)	$9.3±27.8 \times 10^3$	AB (C)	$\chi^2 = 10.42$; df = 3; $p = 0.0153$
	4	0.0±0.0	a (c)	$2.2±4.4 \times 10^4$	a (c)	$5.7±6.8 \times 10^4$	a (c)	$4.7±5.8 \times 10^4$	a (c)	$\chi^2 = 8.05$; df = 3; $p = 0.0450$
30	2	$6.7±1.4 \times 10^5$	D (A)	$2.2±0.3 \times 10^6$	B (A)	$3.4±0.9 \times 10^6$	A (A)	$1.3±0.2 \times 10^6$	C (A)	$\chi^2 = 189.77$; df = 3; $p < 0.0001$
	4	$1.0±0.1 \times 10^6$	d (a)	$2.3±0.2 \times 10^6$	b (a)	$3.5±0.2 \times 10^6$	a (a)	$1.5±0.1 \times 10^6$	c (a)	$\chi^2 = 1341.31$; df = 3; $p < 0.0001$
45	2	$6.9±1.2 \times 10^5$	D (A)	$2.0±0.2 \times 10^6$	B (A)	$3.0±0.2 \times 10^6$	A (A)	$1.3±0.2 \times 10^6$	C (A)	$\chi^2 = 974.87$; df = 3; $p < 0.0001$
	4	$1.1±0.1 \times 10^6$	d (a)	$2.2±0.1 \times 10^6$	b (a)	$3.3±0.3 \times 10^6$	a (a)	$1.5±0.2 \times 10^6$	c (a)	$\chi^2 = 884.68$; df = 3; $p < 0.0001$
60	2	$3.7±2.8 \times 10^5$	D (B)	$1.4±0.3 \times 10^6$	B (B)	$1.9±0.3 \times 10^6$	A (B)	$8.3±0.9 \times 10^5$	C (B)	$\chi^2 = 203.49$; df = 3; $p < 0.0001$
	4	$4.5±0.5 \times 10^5$	d (b)	$1.6±0.2 \times 10^6$	b (b)	$2.4±0.4 \times 10^6$	a (b)	$1.0±0.4 \times 10^6$	c (b)	$\chi^2 = 232.36$; df = 3; $p < 0.0001$
	2	$\chi^2 = 205.92$; df = 4; $p < 0.0001$		$\chi^2 = 1034.68$; df = 4; $p < 0.0001$		$\chi^2 = 574.10$; df = 4; $p < 0.0001$		$\chi^2 = 1239.40$; df = 4 $p < 0.0001$		
	4	$\chi^2 = 2852.23$; df = 4; $p < 0.0001$		$\chi^2 = 2571.95$; df = 4; $p < 0.0001$		$\chi^2 = 2310.55$; df = 4; $p < 0.0001$		$\chi^2 = 560.62$; df = 4; $p < 0.0001$		

*Means ± standard deviation; ** Means with the same letter are not significantly different (GLMM, $p < 0.05$); letters without brackets: comparison of one residual moisture and one incubation period over all incubation temperatures; letters with brackets: comparison of one incubation temperature and one incubation period over all residual moistures; upper case letters: comparison after 2 weeks of incubation; lower case letters: comparison after 4 weeks of incubation.

Influence of fungicidal seed treatments

The effect of seed treatments on colonization differed significantly from each other ($\chi^2 = 159.8378$; df = 13; $p < 0.0001$). The granule colonization of the Moncut®, Risolex® and Emesto® Silver treatments and the water and untreated control was over 80% and did not differ significantly from each other (Figure 22). A decrease of granule colonization (50-55%) was observed with the Ortiva® treatment. During the treatment with RhizoVital®42, the fungus only grew on a few granule grains. The granule colonization was significantly different from all other treatments.

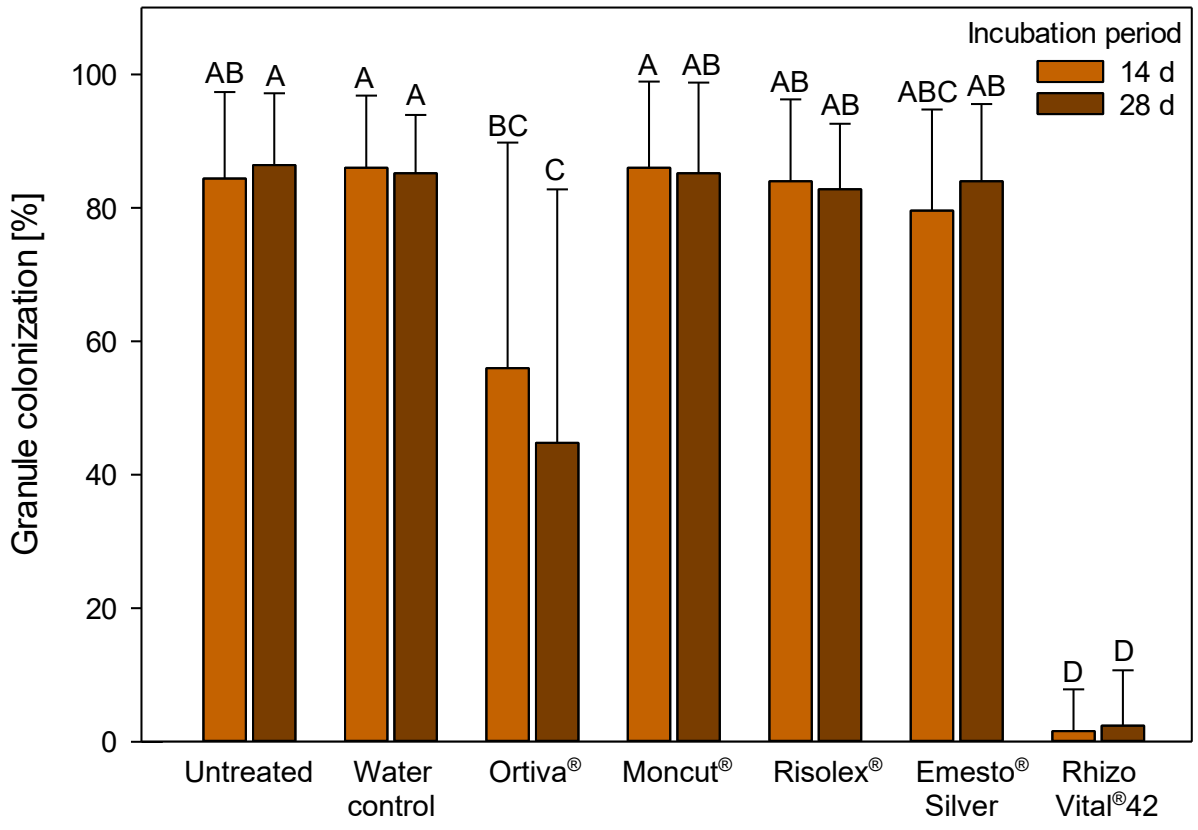


Figure 22: Granule colonization of *Metarhizium brunneum* strain JKI-BI-1450 based granules after application of fungicidal seed treatments. Mean and standard deviation are shown. Different letters show significant differences. Kruskal-Wallis test, posthoc: Wilcoxon test ($p < 0.05$). $n = 25$.

Like colonization, the influence of seed treatments on conidiation differed significantly from each other (χ^2 = 304.7725; df = 13; $p < 0.0001$). The treatment with Moncut® resulted in the lowest reduction of conidiation (Figure 23). A significant difference between the treatment with Moncut® and the untreated and water control was only shown between the water control after 4 weeks and Moncut® after 4 weeks. The conidiation after 2 weeks with these three treatments did not show any difference. Risolex®, Emesto® Silver, Ortiva® and RhizoVital®42 followed with steadily decreasing conidiation where the fungus was not able to form conidia on the granule after treatment with RhizoVital®42.

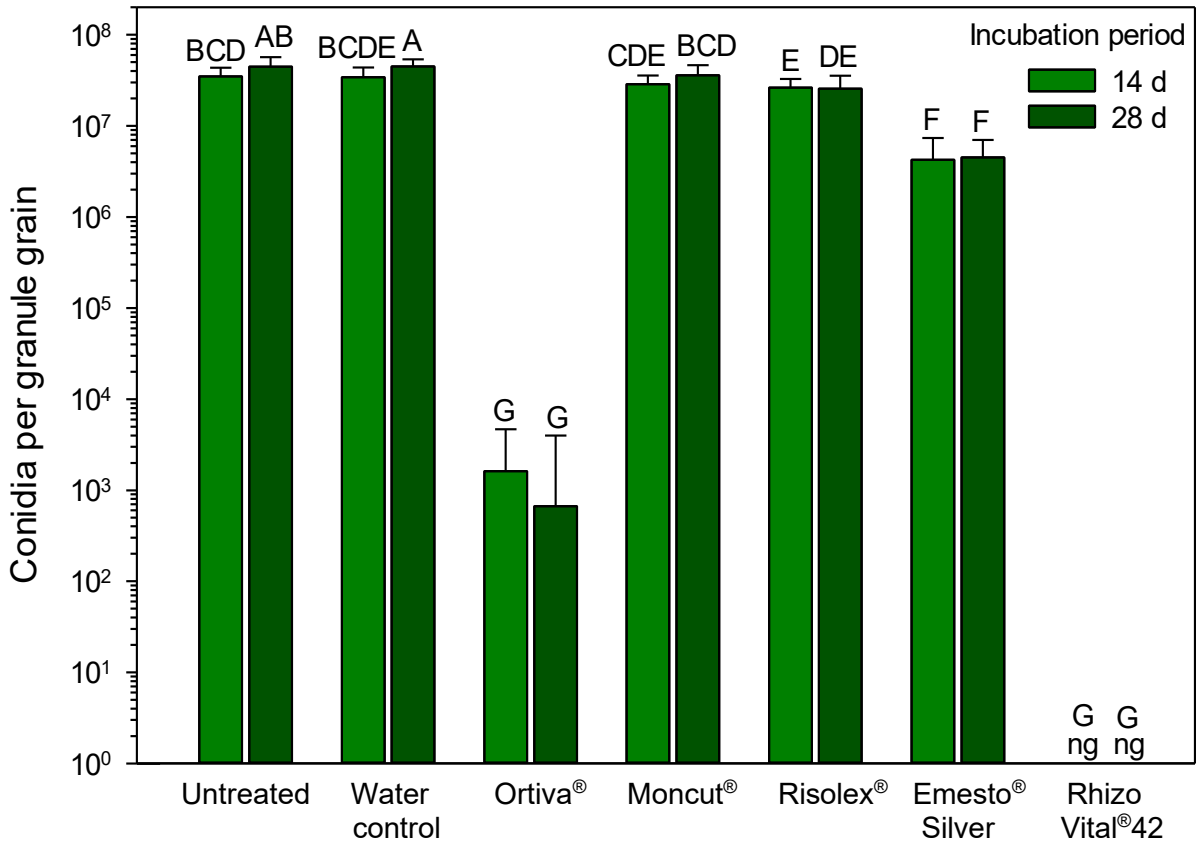


Figure 23: Conidia formation of *Metarhizium brunneum* strain JKI-BI-1450 based granules after application of fungicidal seed treatments. Mean and standard deviation are shown. ng: no growth / no conidiation. Different letters show significant differences. Kruskal-Wallis test, posthoc: Wilcoxon test ($p < 0.05$). $n = 25$.

Discussion

It was shown that biomass of the *M. brunneum* JKI-BI-1450 is suitable for the production for granules using a fluid-bed dryer as established by Stephan et al. (2020). The biomass used was produced in liquid culture and consisted of mycelium and submerged spores. However, the use of 4.5 mg biomass per g granule was necessary to achieve a granule colonization of over 90%. The submerged spores seem to be crucial for the production of a granule with high colonization, as the production of a granule exclusively based on mycelium resulted only very rarely in granule colonization after incubation (Seib, data not shown). Therefore, the input of biomass could perhaps be reduced if the biomass contains a large amount of spores. To achieve this goal, the harvest time of the fermentation could be adjusted. For a high spore yield, a harvest after 42 to 48 h was advantageous compared to 72 h (Chapter II). Another possibility to reduce the use of biomass for the production of granules would be to improve the thermo-tolerance because heat stress occurs during fluid-bed drying. Stephan et al. (2020) demonstrated that *M. brunneum* was more susceptible to heat compared to *Cordyceps fumosorosea* and *Beauveria bassiana*, and that more biomass was required to produce granules based on *Metarhizium* than on the other two EPF. A granule based on a *M. brunneum* strain showed a colonization of less than 50% using 1.4 mg biomass per g granule (Stephan et al. 2020). In contrast, granules based on *C. fumosorosea* or *B. bassiana* with a biomass input of 0.006 mg per g granule already showed a colonization of almost 100%. The thermo-tolerance of *Metarhizium* could be improved by the composition of the production media (Jackson 1997; Mascarin et al. 2018). The present experiments showed that, in addition to biomass, conidia are also suitable for the production of granules by fluid-bed drying, which was also demonstrated by Stephan et al. (2020). This finding is not fully surprising, since conidia are often more tolerant to heat and dehydration than submerged spores (Silman et al. 1993; Wang et al. 2013). However, the results in the

present study demonstrated that 10^6 conidia per g granule were required to achieve the same colonization as when 4.5 mg biomass per g granules was used. As conidia take longer to produce than submerged spores, this approach would only have been pursued if the amount of conidia used had been much lower (Dorta et al. 1996; Jackson 1997; Alkhaibari et al. 2017; Stephan et al. 2020).

An attempt was made to improve and accelerate the growth of the fungus on the granule by addition of a further carbon substance to the biomass during the process of fluid-bed drying. It was first investigated whether the addition of chitin to MPA improves the growth and conidiation of the fungus. By addition of chitin fungal growth on culture media plates but not on the produced granule was increased. In contrast to the present experiments, Lorenz et al. (2020) showed an increase of sporulation of *Metarhizium pemphigi* alginate pellets when chitin was added during production. Also Gerding-González et al. (2007) showed that the conidia formation of *B. bassiana* on alginate pellets could be increased when chitin was added during the formulation process. However, the authors noted that the higher conidia formation due to the addition of chitin could also be the result from a lower pellet contamination by *Penicillium*.

Next to chitin, various sugars were also added to the biomass before fluid-bed drying. On the one hand, the sugars should act as a quickly accessible source of nutrients and on the other hand they could function as heat protection. Besides a large nutrient reserve for growth (Rousseau et al. 1972; Thevelein 1984), trehalose is responsible for the survival of microorganisms of stressful conditions such as heat and desiccation by protecting proteins and cell membranes from inactivation and denaturation (Hottiger et al. 1987; van Laere 1989; Ribeiro et al. 1999; Liu et al. 2009; Rangel and Roberts 2018). This protection can be further enhanced by the addition of sugars (Ball et al. 1943; Donovan 1977; Back et al. 1979) and is based on the binding

of the sugars to the phospholipids of the fungal membrane (Crowe et al. 1984; Crowe et al. 1985; Crowe et al. 1987; Crowe et al. 1990). In the present experiments, the addition of sugars resulted in improving fungal growth on the granule after fluid-bed drying. The addition of all sugars studied (lactose, glucose, sucrose and fructose) accelerated both the colonization of the granule with the fungus and its conidiation. The colonization was particularly accelerated by the addition of sucrose and fructose, the conidiation by glucose, sucrose and fructose. Other studies had demonstrated that the addition of sugars increased the survivability of fungi during drying processes (Li et al. 1993). Pell et al. (1998) showed that mycelium of *Zoophthora radicans* forms more conidia after drying when it was mixed with maltose solution beforehand, which is not the case after 1 week of storage. In contrast, Li et al. (1993) found a positive effect for the addition of maltose on the storage stability of *Z. radicans.* Moreover, the addition of sugar provides the fungus with an easily utilisable carbon source for its growth after drying. Moslim et al. (2009) demonstrated a positive effect on the colonization and conidiation of a *Metarhizium* granule by supplementing additional nutrients to the formulation. Rapid growth of the fungus on the granules is very important when applied in the field, because other microorganisms in the soil also can use millet as a source of nutrients, grow on it and can thus prevent the growth of the coated fungi. Further experiments to clarify whether the addition of sugars does not also accelerate the growth of other microorganisms on the granule are required. To prevent the growth of other microorganisms, the granule could also be coated with a layer of a bactericidal agent.

Besides a high and fast colonization and conidiation of the granule, storage stability is of high importance for a biological control agent. The developed granule demonstrated a good storability at 5 °C, but a rapid decrease in colonization at higher temperatures. The temperature dependence of the storage stability of microorganisms has already been proven in previous

studies (Walstad et al. 1970; Roberts et al. 1987; McClatchie et al. 1994; Moore et al. 1996; Jackson et al. 1997; Stephan et al. 1997; Stephan and Zimmermann 2001; Kim et al. 2019). According to the results in the present study and the literature, a low temperature is preferable for storage. The viability of a formulated fungus after storage at higher temperatures can be improved by appropriate packaging, as demonstrated by Przyklenk et al. (2017) for encapsulated conidia of *M. brunneum* by packaging the dried beads in inert multilayer aluminium/polyethylene-bags.

In addition to formulability and storability, it is also crucial that the selected fungal strain can grow and form conidia under the prevailing environmental conditions. Good growth and conidiation of the fungus on the granule under optimal laboratory conditions is no guarantee that this will be the case in the field, where both the temperature and the soil moisture are often not optimal. Therefore, these quality factors of the granule were analysed at different temperatures and moistures in soil. Residual soil moisture of 30 and 45% was demonstrated to lead to the highest colonization and conidiation, which were reduced at lower and higher residual moisture. Arthurs and Thomas (2001) demonstrated that the higher the residual moisture, the higher also the conidia formation of *Metarhizium anisopliae* var. *acridum* on cadavers of *Schistocerca gregaria*. However, the experimental designs of the present study and by Arthurs and Thomas (2001) are difficult to compare. The present experiments were conducted in soil and in soil with high moisture the air supply could be reduced, which again reduces the growth of JKI-BI-1450 (Seib, personal observation). Furthermore, it was shown that the two factors influence each other. The fungus was able to grow at low residual soil moisture of 15%, provided the temperature was high enough. The same was observed for the temperature. The fungus is able to grow at 15% residual moisture if it is incubated at minimum 20 °C. It can therefore be assumed that the fungus is able to grow even if one growth factor

(temperature or moisture) is suboptimal, as long as the other growth factors are sufficient. Li et al. (2019) reported similar results for the germination of *Trichothecium roseum* and Xu et al. (2001) for the germination of *Monilinia fructigena*. In contrast, conidiation of *M. anisopliae* var. *acridum* on cadavers of *S. gregaria* could not be increased at lower temperatures due to higher moisture (Arthurs and Thomas 2001). Furthermore, it is important that the selected fungal strain can be applied together with common fungicidal seed treatments for use in integrated plant protection. Therefore, the effect of fungicidal seed treatments on the developed granule has to be investigated before they are used in the field. Moncut® showed the least effect on the growth of JKI-BI-1450 on the granule, very closely followed by Risolex® and Emesto® Silver. Contact with the product Ortiva® leads to a very strong decrease in colonization and conidiation, whereas RhizoVital®42 prevents the colonization almost completely and the conidia formation completely. The varying susceptibility to the chemical agents could be due to their different active ingredients and thus mechanisms of action, which appear to be different in their effectiveness against JKI-BI-1450 or also due to the different application rate. Samson et al. (2005) and Wu et al. (2020) also proved the agent-dependent effect of fungicides. In Samson et al. (2005) and in the present experiments some fungicides caused only a slight reduction in fungal vitality when treated with the recommended application rate, proving that granules based on EPF can be used together with a fungicidal seed treatment.

In summary, the *M. brunneum* JKI-BI-1450 is suitable for granule production by means of fluid-bed drying. However, for economic reasons, the required biomass quantity should be reduced, which might be achieved by further optimizing the production and the associated increase in sporulation in liquid media, and/or by using additives in the formulation process. Furthermore, the fungus can grow on the granule at a wide range of temperatures and moistures and is suitable for use in combination with fungicidal seed treatments.

Chapter III

Notes on other contributors: Nieszporek, U. B.: Collected the data of the experiment: “Determination of biomass concentration for granule production”, Efremova, I.: Collected the data of the experiments: “Influence of chitin on fungal growth on granule” and “Influence of sugar on granule colonization”. Sturm née. Ruppenthal, A. M.: Collected the data of the experiment: “Influence of different chitin concentrations on fungal growth”.

Chapter IV

Investigation on the influence of production and incubation temperature on the growth, virulence, germination, and conidial size of *Metarhizium brunneum* for granule development

Abstract

Important for the infection of an insect with an entomopathogenic fungus and its use as a plant protection agent are its growth, conidia formation (conidiation), germination, and virulence, which all depend on the environmental temperature. Not only the influence of the environmental temperature was investigated, but also of the production temperature of the fungus. For this purpose, *Metarhizium brunneum* JKI-BI-1450 was produced and incubated at different temperatures, and the factors mentioned as well as conidial size were determined. The temperature at which the fungus was produced affects its subsequent growth and conidiation on granule formulation, the speed of germination, and the conidial width, but not its final germination or virulence. The growth and conidiation was at its highest when the fungus was produced at 25 °C. Conidia produced at 20 °C were significantly wider than conidia produced at higher temperatures, whereas conidia produced at 30 °C germinated significantly faster than conidia produced at lower temperatures The incubation temperature optimum of JKI-BI-1450 in relation to growth, speed of germination, and survival time was 25-30 °C and for conidiation 20-25 °C. Conidial length decreased with increasing incubation temperature. Although the fungus could not be adapted to unfavourable conditions by the production temperature, it was

found that the quality of a biological control agent based on entomopathogenic fungi can be positively influenced by its production temperature.

Introduction

The aim of the present study was to investigate the effects of different temperatures on the growth, conidia formation (conidiation), conidial size, germination and virulence of a *Metarhizium*-based soil granule, which was developed to control pest insects in the soil, especially in potato cultivation. Therefore, a formulation technology for granules based on entomopathogenic fungi (EPF) was developed (Stephan et al. 2020), and examined whether this technology can be used for *Metarhizium brunneum* strain JKI-BI-1450. To produce the granule, the fungus was initially produced in liquid medium. Subsequently, a thin layer of biomass was coated on autoclaved millet by fluid-bed drying. The advantage of applying a soil granule produced this way is that only after its application in the soil and the associated contact with moisture does the fungus overgrow the granule (Figure 24) and conidiate on the surface, which in turn leads to the pest insect being infected once contact has been established with the conidia (Ortiz-Urquiza et al. 2013). The direct application of conidia into the soil has the advantage that the infectious unit of the fungus is directly present, however the product is dusting, and thus poses a risk for both the user and bystander.

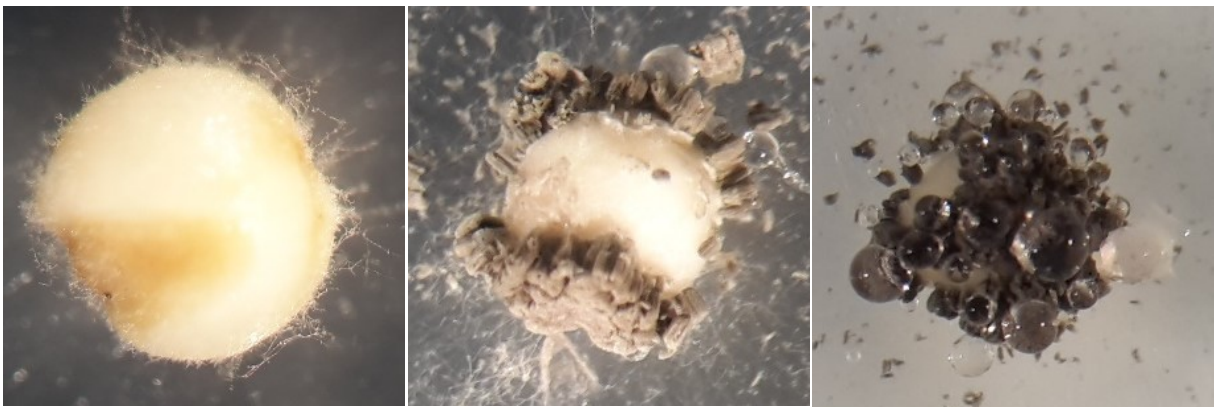


Figure 24: Growth and conidia formation of *Metarhizium brunneum* JKI-BI-1450 on a granule grain.

Therefore, the growth and conidiation of the fungus on the granule in the soil are essential for its effectiveness, and for both of these processes, the temperature of the environment is important. Generally, temperatures between 20-30 °C are optimal for the growth and sporulation of EPF, which is also valid for *Metarhizium* (Fargues et al. 1992; Ouedraogo et al. 1997; Ekesi et al. 1999; Dimbi et al. 2004). However, the temperature optimum varies between different *Metarhizium* species. Kryukov et al. (2017) demonstrated that *M. brunneum* exhibited the best growth at 25 °C, whereas the optimum growth temperature of *Metarhizium robertsii* was 30 °C. Only *M. robertsii* grew at 35 or 37.5 °C, whereas *M. brunneum* was not able to grow at these temperatures. Keyser et al. (2014) showed that the examined *M. brunneum* species exhibited their maximum mycelial growth at 24 °C. Strains of *Metarhizium acridum*, *Metarhizium guizhouense,* and *M. robertsii* had their maximum at 28 °C or even at 32 °C.

One constraint in applying EPF for controlling soil dwelling pests is the difference in soil temperatures and the temperature optimum of the fungi. The granule investigated in the present study should later be used in cultivation at the same time when potato planting, where the soil temperature is lower than the optimum of the fungus (Paluch 2021). An application at lower temperatures can lead to a reduced growth and conidiation of the fungus (Hallsworth and Magan 1996; Ekesi et al. 1999; Hallsworth and Magan 1999). As conidia are the infection units of the fungus (Hajek and St. Leger 1994), lower levels of conidiation may lead to a reduction in efficacy (Bharadwaj and Stafford 2011). The fact that many microorganisms cope better with stress conditions if they have already been exposed to them was taken advantage of (Kapoor et al. 1990; Guan et al. 2012; Andrade-Linares et al. 2016a; Andrade-Linares et al. 2016b). Therefore, it was investigated whether the fungus is adapted to unfavourable temperatures by cultivating the fungus several times at these temperatures. Following growth and conidiation of the fungus on the granule, the next step to successful infection is the contact of the conidia with

the insect cuticle. After adhesion on the cuticle, the conidia form a germ tube, which penetrates the cuticle through enzymatic and mechanical pressure. Moreover, germination is also heavily influenced by temperature (Carruthers and Haynes 1986; Ekesi et al. 1999). Walstad et al. (1970) and Dimbi et al. (2004) demonstrated the highest germination for *Metarhizium* after 24 h at 25 °C. Similar results were determined by Hywel-Jones and Gillespie (1990), with the fastest and highest germination having being observed after an incubation at 25 to 30 °C. Rath et al. (1995) and Skalický et al. (2014) showed that germination up to 100% was even possible at low temperatures, although such values are obtained slower compared to higher temperatures. After adhesion, germination, and penetration, the fungus can multiply in the haemolymph and kill the insect either by nutrient deprivation or toxins, which some fungi are able to produce (Kershaw et al. 1999; Golo et al. 2014). Apart from growth and germination, the virulence of EPF is also temperature-dependent (Stimmann 1968; Ekesi et al. 1999). For *Metarhizium,* many studies showed the highest or fastest mortality at higher temperatures of 30 or 35 °C (Dimbi et al. 2004; Bugeme et al. 2009) or 25 to 30 °C (Fargues et al. 1997; Onsongo et al. 2019), respectively.

Investigations on the influence of temperature on the growth, germination, and virulence of EPF have often been described. The novelty of the present study is that both the influence of the production temperature and the incubation temperature were investigated. Firstly, it was investigated whether the production temperature of the biomass used for the granule-based formulation affects the growth of the fungus on this granule at different incubation temperatures. The purpose of these experiments was to verify whether the quality of the granule, and its ability to grow at sub-optimal temperatures could be improved by the previous production parameters of the biomass. In the application strategy used in the present study, the infectious conidia were only formed after application in soil under moist conditions and at sufficient temperatures. Therefore, in the second step, it was assessed whether the temperature

for conidiation had an influence on their size, germination, and virulence to better understand the potential field conditions.

Materials and Methods

Fungal strain

M. brunneum strain JKI-BI-1450 was isolated in 2013 at the Institute for Biological Control, Julius Kühn Institute, Darmstadt (Germany) from an infected adult *Agriotes lineatus,* which was collected by Jörn Lehmhus (Julius Kühn Institute, Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Field Crops and Grassland, Braunschweig, Germany).

Maintenance of JKI-BI-1450

JKI-BI-1450 was stored at -80 °C in cryo-tubes (Microbank, Pro-Lab Diagnostics, Richmond Hill, Canada) and routinely cultured on malt peptone agar (MPA) containing 3% (w/v) malt extract (Merck, Darmstadt, Germany), 0.5% (w/v) peptone from soybean (Merck), and 1.8% (w/v) agar-agar (Roth, Karlsruhe, Germany).

Colony size and conidiation at different temperatures

JKI-BI-1450 was spread on MPA and incubated for 2 weeks at 25 °C in the dark. A total of 2 µL of a conidial suspension (10^8 conidia ml^{-1} in sterile 0.5% (v/v) Tween® 80 (Merck)) of JKI-BI-1450 was pipetted in the center of a MPA plate. The plates were then incubated for 14 days at 15, 20, 25, 30, and 37 °C in the dark, respectively. The diameter of the fungal colony was measured twice, perpendicular to one another. To determine the conidiation, 2 ml of sterile 0.5% (v/v) Tween® 80 was added to each plate and the conidia were scraped off with a sterile spatula. The conidia concentration was determined using a haemocytometer, and the conidia

per mm^2 was calculated based on the final colony size. The experiment was repeated six times independently with five replicates each.

Temperature adaptation

JKI-BI-1450 was cultivated on MPA for 14 days at 15, 20, 25, and 30 °C in the dark, respectively. New MPA plates were then inoculated with the fungi produced at these temperatures and incubated for 14 days at the same temperatures as before in the dark. This subsequent cultivation was repeated three times. After that, the final adapted fungal cultures of each growth temperature were stored at −80 °C.

Effects of production and incubation temperature on the fungal growth

The granules were produced as described by Stephan et al. (2020). For liquid culture inoculation, the temperature-adapted fungal cultures were taken from the −80 °C deep freezer and cultivated for 21 days on MPA at the corresponding temperature in the dark. For the liquid production of fungal biomass, 50 ml sterile medium containing 2.5% (w/v) D(+)-glucose (Merck), 2% (w/v) corn steep (Sigma Aldrich, Buchs, Switzerland), and 0.5% (w/v) sodium chloride (Merck) in 100 ml flasks was used. Each flask was inoculated with 5×10^6 conidia suspension of the described above 21-day-old cultures in 1 ml of sterile 0.5% (v/v) Tween® 80 and were then incubated at 130 rpm on a horizontal shaker (Novotron, 50 mm deflection, Infors, Bottmingen, Switzerland). In liquid culture, the conidia of JKI-BI-1450 germinate and form mycelium and submerged spores. The duration of the production process is influenced by the production temperature. To determine the end of the exponential growth phase at each production temperature, the formation of the submerged spores was observed microscopically every 24 h. Based on these results, the incubation time at 15 °C was set to 120 h, and at 30 °C to 144 h, respectively. At 20 and 25 °C the fungus was cultivated for 72 h. The biomass

suspension of each cultivation was centrifuged for 10 min at 25 °C and 15,334 × *g*. The supernatant was then discarded, and the pellet was resuspended in 0.9% (w/v) sodium chloride solution and centrifuged again. This process was repeated twice. The dry weight of the biomass was determined using a moisture determination balance (Ma30, Sartorius, Göttingen, Germany), and a suspension of 3% dry biomass was prepared by diluting with a 0.9% (w/v) sodium chloride solution. From each biomass suspension a granule was prepared with a laboratory fluid-bed dryer (Strea-1, Aeromatic-Fielder AG, Bubendorf, Switzerland, nozzle diameter: 1 mm, Container volume: 16.5 l). In contrast to the process described by Stephan et al. (2020), 100 g of autoclaved millet seeds were placed in the container, and 15 g of the 3% biomass suspension was sprayed by a top spray variant on the millet with a nozzle pressure of 2 bar. The granules produced in this way will be henceforth referred to as 15 °C-granule, 20 °C-granule, 25 °C-granule, and 30 °C-granule throughout the present study. For each batch, ten granule grains (millet seeds covered with fungus) were placed on five plates filled with 1% (w/v) agar-agar and were then incubated at 15, 20, 25, and 30 °C for 14 and 28 days in the dark, respectively. After this incubation period, the number of colonized granule grains was determined. Granule grains covered with the mycelium or mycelium and conidia of JKI-BI-1450 were counted as colonized. To determine the conidia concentration, 1 ml of sterile 0.5% (v/v) Tween 80® was added to each plate. The granule grains and the suspension was transferred to a 1.5-ml Eppendorf tube. The Eppendorf tubes were placed on a vortexer for 10 s and afterwards in an ultrasonic bath (Sonorex RK 52 35 kHz., Bandelin electronic GmbH & Co. KG, Berlin, Germany) for 15 min. The conidia concentration was determined using a haemocytometer. Based on the colonization and the determined amount of conidia per plate, the conidia per granule grain were determined by dividing the amount of conidia by the number of colonized granule grains.

This calculation allowed to determine the conidiation independently of colonization. Ten conidia of each plate were photographed, and the length and width was measured using the program cellSens Standard (Figure 25). The conidial index was calculated by dividing the length by the width. The experiment was repeated independently three times.

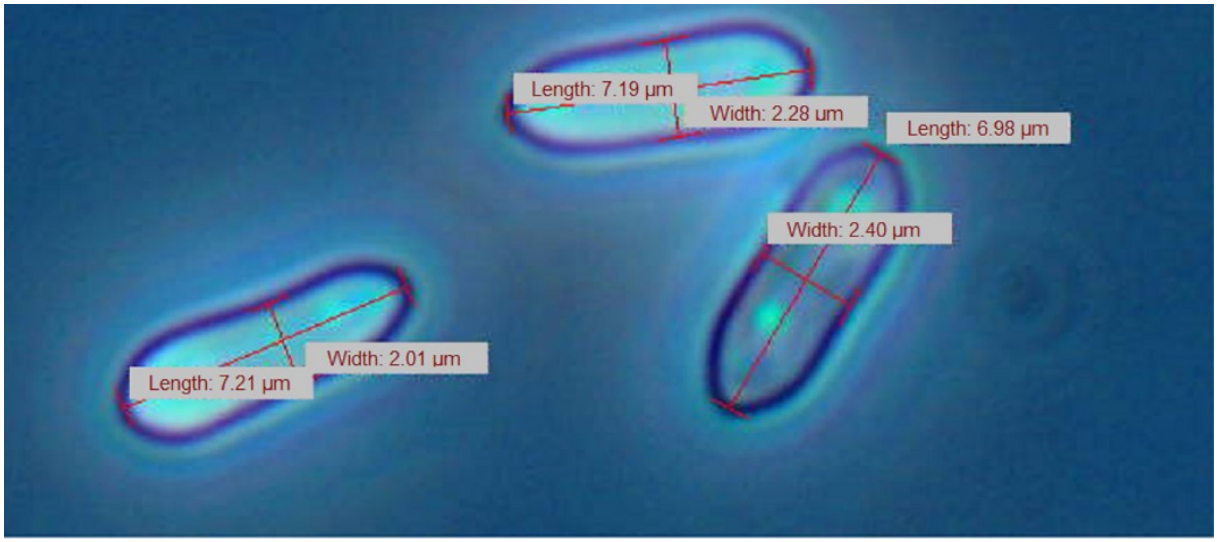


Figure 25: Measurement of conidia.

Effect of more unfavourable incubation temperature

A 25 °C-granule was produced and formulated as described. Ten granule grains were placed on five separate plates filled with 1% (w/v) agar-agar and were incubated at 5, 10, 25, and 35 °C for 14, 28, and 42 days in the dark, respectively. The granule colonization, the conidia per granule grain, and the conidial size were measured as described above. The experiment was repeated independently three times.

Effect of simulated soil temperature

To simulate the soil temperature in the field during potato planting as realistically as possible, soil temperature data from a field trial in Uelzen Niedersachsen, Germany were used for adjusting the temperature profile of the incubator. The data were based on temperatures measured in 2018 and 2019, which were obtained from three points inside the potato mound.

From these six measurements, averages were calculated every 4 h for 6 weeks, starting from the day of potato planting (7 May 2018 and 8 May 2019) (Paluch 2021). A 25 °C-granule was produced as described above. Ten granule grains were placed on plates filled with 1% (w/v) agar-agar. In total, 60 plates were incubated at the simulated soil temperature, and an additional 60 plates were incubated at a constant temperature of 25 °C in the dark. Ten plates of each treatment were evaluated weekly over 6 weeks. The granule colonization, the conidia per granule grain, and the conidial size were measured as described above. The experiment was repeated independently three times.

Effect of temperature on the germination and the virulence

Preparation of fungus suspension

To determine the germination, the temperature-adapted fungal cultures were incubated for 21 days on MPA at the corresponding temperatures in the dark. An undefined number of conidia of each fungus was suspended in a 1.5-ml Eppendorf tube filled with 0.5% (v/v) Tween® 80. The Eppendorf tubes were placed on a vortexer for 10 s and afterwards in an ultrasonic bath for 15 min. The conidia concentration was determined using a haemocytometer, and a conidial suspension of 10^6 conidia ml^{-1} was made for each adapted fungus by diluting with 0.5% (v/v) Tween® 80. The length and width were determined for 100 conidia as described above.

Speed of germination

Twenty-four droplets of each conidial suspension with a volume of 10 µL were placed on MPA plates, and were incubated at 15, 20, 25, and 30 °C in the dark, respectively. After 3, 6, 9, 12, 15, 18, 21, and 24 h, respectively, three droplets each were cut out of the agar and placed on a slide. On each agar piece, one hundred conidia were observed under the light microscope (× 400), and the percentage of germinated conidia was subsequently determined. Conidia were

rated as germinated when the germ tube was longer than the width of the conidia. The experiment was repeated independently three times.

Final germination

To determine the final germination, the experiment was repeated as described above, except that 25 mg/L benomyl (Sigma Aldrich) was added to the MPA. The germination was determined after incubating for 96 h at the corresponding temperatures in the dark. The experiment was repeated independently three times.

Effect of temperature on the virulence

The temperature-adapted fungal cultures were incubated for 21 days on MPA at the corresponding temperatures in the dark. Five ml of 0.5% (v/v) Tween® 80 were added to each plate, following which the suspensions were filtered through four layers of gauze, the supernatant was sonicated (Sonorex RK 52, Bandelin electronic GmbH & Co. KG 35 kHz) for 15 min, and for each adapted fungal culture 5 ml of a conidial suspension with 10^7 conidia m^{-1} with 0.5% (v/v) Tween® 80 was prepared. Afterwards, *Galleria mellonella* larvae (larval stage 5-6) were dipped for 2-3 s into the four fungal suspensions, 0.5% (v/v) Tween® 80, or sterile deionised water. Each larvae was placed individually into a plastic box (7 cm diameter and 2.5 cm height). Ten larvae per treatment were incubated at 15, 20, 25, and 30 °C in the dark, respectively. The number of dead larvae was determined daily for a period of 14 days. The experiment was independently repeated four times.

Statistical analysis

The data was statistically analyzed with the software SAS Studio 3.8. Normality of the data was tested using the Shapiro-Wilk test and the homogeneity of variance was checked by the Levene's test. To compare the colony size and the conidiation at different temperatures, and the

effect of extreme incubation temperatures on the fungal growth, the Kruskal-Wallis test, posthoc: Wilcoxon test ($p < 0.05$) was used. For analyzing the effects of production and incubation temperature, or incubation period on the fungal growth and the conidial size, a generalized linear model with Wald statistics for type 3 analysis and multiple comparison according to the Tukey test (GLMM, $p < 0.05$) was employed. For the analysis of the effect of one parameter (production temperature, incubation temperature, and incubation period), the data of the other two parameters were pooled. The effect of simulated soil temperature on the fungal growth was analyzed for each week by the non-parametric Mann-Whitney U-test ($p < 0.05$). Furthermore, the data of each incubation temperature was compared using a generalized linear model ($p < 0.05$). The final germination was determined to be above 99% in all treatments. Due to the method that was used, a more exact determination of the values was not possible, and therefore a statistical evaluation was not conducted. To compare the germination progress, the results of the germination speed and final germination were assembled, and τ was calculated for each treatment and every replicate. For the calculation of τ, a non-linear regression according to Dantigny et al. (2011) was used (Formula (1)).

Formula (1): Calculation of τ:

$$P = P_{max}[1 - \frac{1}{1} + \left(\frac{t}{\tau}\right)^{\mathrm{d}}] \qquad (1)$$

where P is the percentage of germinated conidia in dependence of the maximum percentage of germination, (P_{max}), t designates the germination time, τ is the point of time when 50% of the maximal germinated conidia have been germinated, and d is the design parameter. The design parameter was selected according to Dantigny et al. (2011).

Furthermore, the slopes at the inflection point for each treatment and every replicate were calculated with Formula (2).

$$Slope = \frac{(\mathrm{d} \times P_{max} \times q\ )}{\tau \times q^{\frac{1}{\mathrm{d}}} \times (q+1)^2} \qquad (2)$$

Formula (2): Calculation of the slope at the inflection point:

With $q = \left(\frac{\mathrm{d}-1}{\mathrm{d}+1}\right)$

For analyzing the effects of the production and incubation temperature on τ and the slope at the infection point a generalized linear model with Wald statistics for type 3 analysis and multiple comparison according to the Tukey test (GLMM, $p < 0.05$) was used. For the analysis of the effect of one parameter, the data of the other parameters were pooled. To analyze the virulence, the mean survival time (ST_{50}) for each replicate was calculated using a survival analysis (Kaplan-Meier) and compared using a generalized linear model ($p < 0.05$). For analyzing the effect of the production temperature and both controls, data of all incubation temperatures, whereas for the effect of the incubation temperature, data of all production temperatures were pooled.

Results

Colony size and conidiation at different temperatures

The incubation temperature had a significant effect on the colony size ($\chi^2 = 137.3667$; df = 4; $p < 0.0001$) and conidiation ($\chi^2 = 125.8045$; df = 4; $p < 0.0001$). Moreover, the results demonstrated a significantly larger colony size of *M. brunneum* strain JKI-BI-1450 after

incubation at 25 or 30 °C than after incubation at 15 or 20 °C (Figure 26). In contrast, the fungus formed significantly more conidia at 20 or 25 °C than at 15 or 30 °C, respectively.

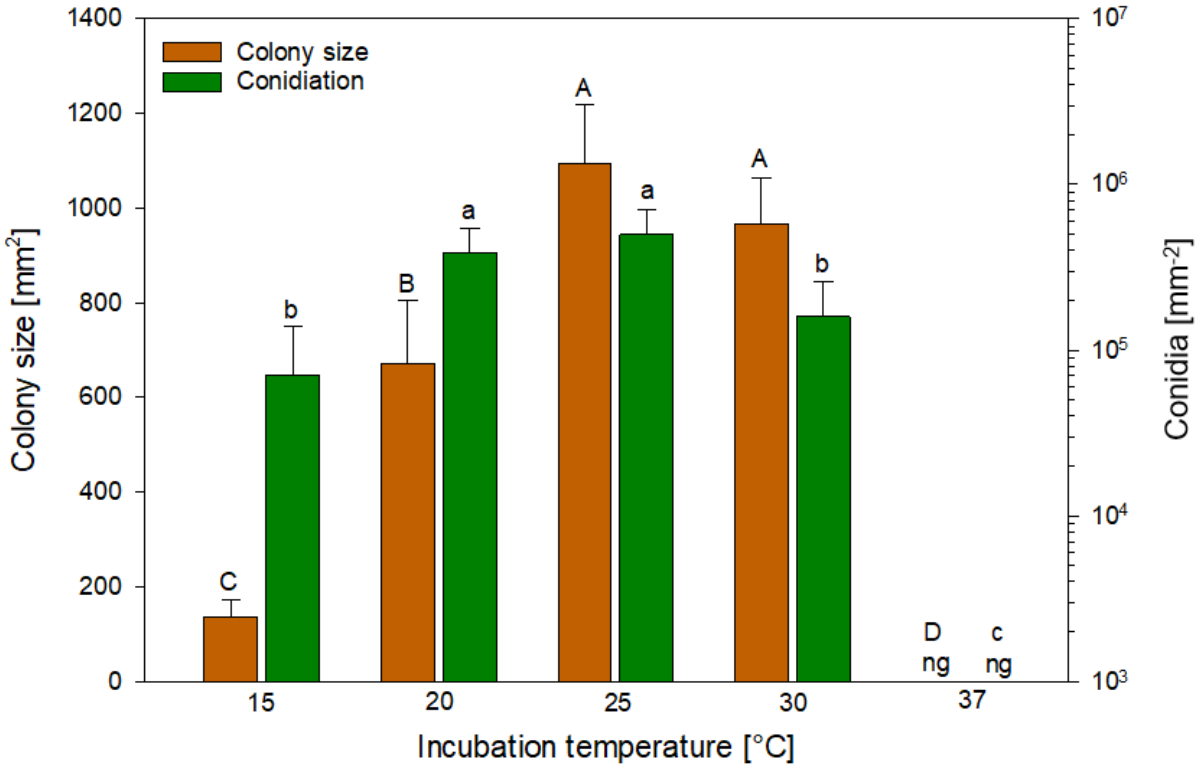


Figure 26: Influence of temperature on colony size and conidia per mm^2 of *Metarhizium brunneum* strain JKI-BI-1450 after 14 days. Means and standard deviation are shown. ng = no growth. Different upper case resp. lower case letters show significant differences. Kruskal-Wallis test, posthoc: Wilcoxon test ($p < 0.05$), $n = 30$.

Effects of production and incubation temperature on the fungal growth on the soil granule

The granule colonization by JKI-BI-1450 was found to have been significantly influenced by the production and incubation temperature of the biomass (production temperature: ($\chi^2 = 2298.94$; df = 3; $p < 0.001$); and incubation temperature ($\chi^2 = 118.70$; df = 3; $p < 0.001$)) (Table 11). In brief, over all incubation temperatures, granule colonization was 87% for the granules prepared with the biomass produced at 25 °C. At a production temperature of 20 and

30 °C, respectively, granule colonization was significantly reduced but still higher than 70%. When the biomass was produced at 15 °C, only 1.7% of granule grains were colonized. In contrast, when incubated at 15 °C across all production temperatures combined, maximum colonization was achieved at an average of 72%. With increasing incubation temperatures, the percentage of colonized granule grains decreased continuously, but did not reach less than 50%. The incubation period was found to exhibit no effects on the granule colonization ($\chi^2 = 0.27$; df = 1; $p = 0.6037$).

Table 11. Effects of the production and incubation conditions on the fungal growth of granules coated with the *Metarhizium brunneum* strain JKI-BI-1450.

Conditions		**Growth parameter**				
		Granule colonization [%]*	**	**Conidia per granule grain***	**	***n***
Production temperature [°C]	15	1.66±4.34	D	$3.85±25.2 \times 10^5$	C	120
	20	81.25±19.98	B	$2.47±1.70 \times 10^7$	AB	
	25	87.17±17.40	A	$2.77±1.66 \times 10^7$	A	
	30	72.25±23.24	C	$2.31±2.00 \times 10^7$	B	
Incubation temperature [°C]	15	72.41±42.21	A	$1.09±1.18 \times 10^7$	C	120
	20	61.58±37.28	B	$2.26±1.74 \times 10^7$	B	
	25	56.92±36.53	B	$3.17±2.45 \times 10^7$	A	
	30	50.92±36.69	C	$1.06±0.97 \times 10^7$	C	
Incubation period [weeks]	2	60.83±39.15	A	$1.32±1.32 \times 10^7$	B	240
	4	60.08±38.80	A	$2.47±2.19 \times 10^7$	A	

* Mean ± standard deviation; ** Means of one condition at one growth parameter with the same letters are not significantly different. (GLMM, $p < 0.05$).

Besides the granule colonization, the conidiation of JKI-BI-1450 on the granule grains was significantly influenced by the production temperature of the biomass ($\chi^2 = 432.21$; df = 3; $p < 0.0001$). The granule-containing biomass produced at 25 °C formed the most conidia with 2.75×10^7 conidia per granule grain, followed by the 20 °C-granule and 30 °C-granule over all incubation temperatures. The conidiation of the 15 °C-granule was a 100times lower than the other granules and differed significantly from granules with the other production temperatures. In addition to the production temperature, the incubation temperature of the granules was found

to significantly influence the conidiation (χ^2 = 285.84; df = 3; p < 0.0001). The conidia concentration on the granules incubated at 25 °C was significantly the highest with 3.17×10^7 conidia per granule grain, followed by incubation at 20 °C. The conidiation was observed to be the lowest at 15 and 30 °C incubation. They differed significantly from the other incubation temperatures, but not from each other. The incubation period also significantly influenced the conidiation (χ^2 = 122.85; df = 1; p < 0.0001). The conidia per granule grain doubled from the second to the 4th week of incubation, and differed significantly from each other, summarized over all production and incubation temperatures.

In addition to colonization and conidiation, the conidial size was also examined. The fungal growth on the 15 °C-granule was extremely low, so the conidial size could not be determined for this granule (Table 12). The production temperature of the biomass did not affect the length (χ^2 = 4.24; df = 2; p = 0.1199) but did significantly impact the width (χ^2 = 11.20; df = 2; p = 0.0037) and the conidial index (χ^2 = 13.21; df = 2; p = 0.0014). Significantly wider and more roundish conidia were formed on the granule-containing biomass produced at 20 °C compared to the higher temperatures. The incubation temperature of the granules also influenced the size of the conidia (length: (χ^2 = 542.28; df = 3; p < 0.0001); width: (χ^2 = 166.61; df = 3; p < 0.0001); and index; (χ^2 = 169.62; df = 3; p < 0.0001)). Conidia were significantly longer and wider when the granules were incubated at 15 °C compared to incubation at higher temperatures. The conidial index also revealed that the conidia were significantly more rounded when the granules were incubated at higher rather than lower temperatures. Furthermore, the conidia were significantly longer after an incubation period of 4 weeks than after 2 weeks (χ^2 = 11.24; df = 1; p = 0.0008). The incubation period was found to have no influence on the conidial width (χ^2 = 1.44; df = 1; p = 0.2306) or the conidial index (χ^2 = 1.86; df = 1; p = 0.1727).

Table 12: Effects of the production and incubation conditions on the conidial size of the *Metarhizium brunneum* strain JKI-BI-1450 formed on the granules.

Conditions		Conidial size						
		Conidial length [µm]**	***	**Conidial width [µm]****	***	**Conidial index*, ****	***	***n***
Production temperature [°C]	20	6.70±0.58	A	2.21±0.22	A	3.05±0.39	B	1200
	25	6.75±0.58	A	2.19±0.20	B	3.10±0.38	A	1200
	30	6.73±0.67	A	2.19±0.22	B	3.11±0.43	A	1166
Incubation temperature [°C]	15	7.06±0.55	A	2.26±0.22	A	3.16±0.42	A	900
	20	6.78±0.50	B	2.15±0.19	C	3.18±0.39	A	900
	25	6.51±0.52	C	2.16±0.19	C	3.05±0.38	B	900
	30	6.54±0.70	C	2.22±0.23	B	2.97±0.37	C	866
Incubation period [weeks]	2	6.69±0.59	B	2.19±0.21	A	3.08±0.39	A	1780
	4	6.76±0.63	A	2.20±0.22	A	3.10±0.41	A	1786

* The index is calculated by dividing the length by the width. ** Mean ± standard deviation. *** Means of one condition at one conidial size parameter with the same letters are not significantly different. (GLMM, $p < 0.05$).

Effect of more unfavourable incubation temperature on the fungal growth

Within the investigation period, the fungus was not able to grow or to form conidia on the granule incubated at 5 or 35 °C (Table 13). The four incubation temperatures and three incubation periods resulted in significant differences in colonization ($\chi^2 = 173.0394$; df = 11; $p < 0.0001$) and conidiation ($\chi^2 = 160.1709$; df = 11; $p < 0.0001$). Incubating for 2 weeks at 10 °C revealed a significantly lower granule colonization, only reaching 80%, whereas 25 °C achieved nearly 100% granule colonization in the same timeframe. No significant differences were observed between 10 and 25 °C for incubation periods of 4 and 6 weeks, respectively. The conidiation was significantly lower after incubation at 10 °C rather than at 25 °C for each evaluation time point. After 2 weeks, JKI-BI-1450 did not form any conidia on the granule. After 4 weeks, 4.67×10^3 conidia per granule grain were formed. The conidia concentration increased a hundred-fold after incubating for an additional 2 weeks. When incubated at 25 °C, the conidia concentration was above 10^7 at all evaluation time points.

Table 13: Effects of the incubation temperature and period on the fungal growth of a granule based on *Metarhizium brunneum* strain JKI-BI-1450 biomass produced at 25 °C. *n*=15.

Incubation Temperature [°C]	Incubation period [weeks]	Growth parameter			
		Granule colonization [%]*	**	Conidia per granule grain*	**
5	2	0.0±0.0	C	0.0±0.0	C
	4	0.0±0.0	C	0.0±0.0	C
	6	0.0±0.0	C	0.0±0.0	C
10	2	80.0±15.49	B	0.0±0.0	C
	4	98.67±3.4	A	$4.67±6.94 \times 10^3$	BC
	6	99.33±2.49	A	$5.99±9.29 \times 10^5$	B
25	2	99.33±2.49	A	$2.41±1.59 \times 10^7$	A
	4	100.0±0	A	$3.74±1.39 \times 10^7$	A
	6	99.33±2.49	A	$3.86±1.03 \times 10^7$	A
35	2	0.0±0.0	C	0.0±0.0	C
	4	0.0±0.0	C	0.0±0.0	C
	6	0.0±0.0	C	0.0±0.0	C

* Mean ± standard deviation. ** Means of one growth parameter with the same letters are not significantly different. (Kruskal-Wallis test, posthoc: Wilcoxon test ($p < 0.05$).

In addition, significantly longer ($\chi^2 = 318.15$; df = 1; $p < 0.0001$) and wider ($\chi^2 = 420.42$; df = 1; $p < 0.0001$) conidia were formed on granules incubated at 10 °C compared to 25 °C (Table 14). The conidial index did not differ between these two incubation temperatures ($\chi^2 = 0.50$; df = 1; $p = 0.4796$). The incubation period had no effect on the conidial size length: ($\chi^2 = 2.57$; df = 1; $p = 0.1089$); width ($\chi^2 = 0.08$; df = 1; $p = 0.7716$); and index ($\chi^2 = 2.54$; df = 1; $p = 0.1107$)).

Table 14: Effects of the incubation conditions on the conidial size of the *Metarhizium brunneum* strain JKI-BI-1450 on the granules.

Conditions		Conidial size						
		Conidial length [µm]**	***	Conidial width [µm]**	***	Conidial index *, **	***	*n*
Incubation temperature [°C]	10	7.96±1.18	A	2.75±0.38	A	2.93±0.44	A	210
	25	6.62±0.50	B	2.24±0.15	B	2.97±0.30	A	300
Incubation period [weeks]	4	7.18±1.12	A	2.43±0.35	A	2.97±0.37	A	240
	6	7.16±1.03	A	2.47±0.37	A	2.92±0.36	A	270

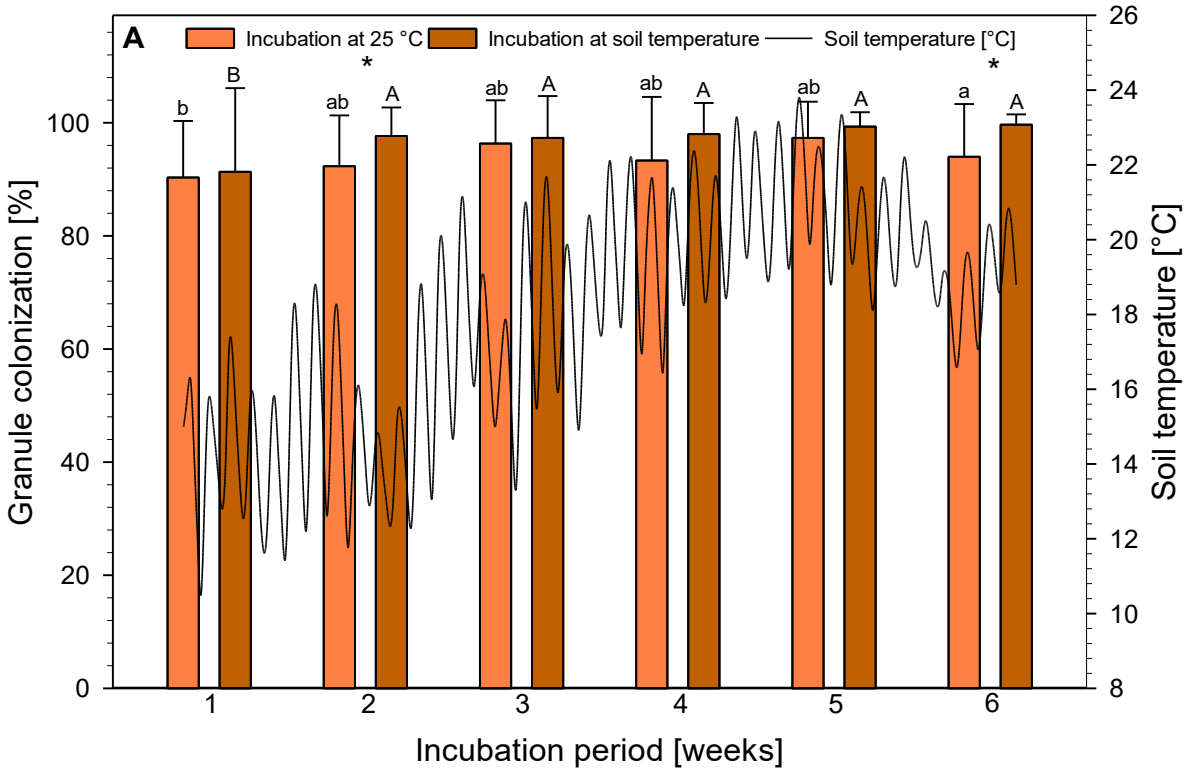
* The index is calculated by dividing length by width. ** Mean ± standard deviation. *** Means of one condition with one conidial size parameter and the same letters are not significantly different. (GLMM, $p < 0.05$).

Chapter IV

Effect of simulated soil temperature on the fungal growth

First, the colonization at 25 °C was compared with the colonization at simulated soil temperature for each week independently. In this comparison of the incubation temperatures, the granule colonization was found to not differ significantly, except for the incubation periods of 2 and 6 weeks (Figure 27 A). Here, the colonization was significantly higher for the granules that were incubated at the simulated soil temperature compared to 25 °C (week 1: (χ^2 = 2.3677; df = 1; p = 0.1239); week 2: (χ^2 = 8.3617; df = 1; p = 0.0038); week 3: (χ^2 = 0.4229; df = 1; p = 0.5155); week 4: (χ^2 = 3.6004; df = 1; p = 0.0578); week 5: (χ^2 = 1.6223; df = 1; p = 0.2028); and week 6: (χ^2 = 50.7817; df = 1; p < 0.0001)). Secondly, the colonization of the fixed and simulated incubation temperature was compared separately over the experimental period. Both the incubation at 25 °C and at the simulated soil temperature showed no significant increases in colonization from the 2nd week onwards and was between 90-100% (25 °C: (χ^2 = 12.54; df = 5; p = 0.0281); and simulated soil temperature (χ^2 = 25.22; df = 5; p < 0.0001)). Conidiation at 25 °C was also compared with the conidiation at simulated soil temperature for each week of the experiment (Figure 27 B). During week 4, the conidia concentration was significantly lower for the granules incubated at the simulated soil temperature rather than at 25 °C (week 1: (χ^2 = 17.1323; df = 1; p < 0.0001); week 2: (χ^2 = 15.5149; df = 1; p < 0.0001); week 3: (χ^2 = 13.6696; df = 1; p = 0.0002); week 4: (χ^2 = 1.3300; df = 1; p = 0.2488); week 5: (χ^2 = 0.2310; df = 1; p = 0.6308); and week 6: (χ^2 = 3.1751; df = 1; p = 0.0748)). Thereafter, the conidia per granule grain no longer differed. The conidiation at one incubation temperature was also compared separately over the duration of the experiment. During incubation at 25 °C the maximum conidia concentration was achieved after 2 weeks with 1.86-2.17 × 10^7 conidia per granule grain (χ^2 = 88.47; df = 5; p < 0.0001), while during incubation at simulated soil

temperature this was not achieved until after 4 weeks with 1.44-1.79 × 10^7 conidia per granule grain (χ^2 = 197.58; df = 5; p < 0.0001).



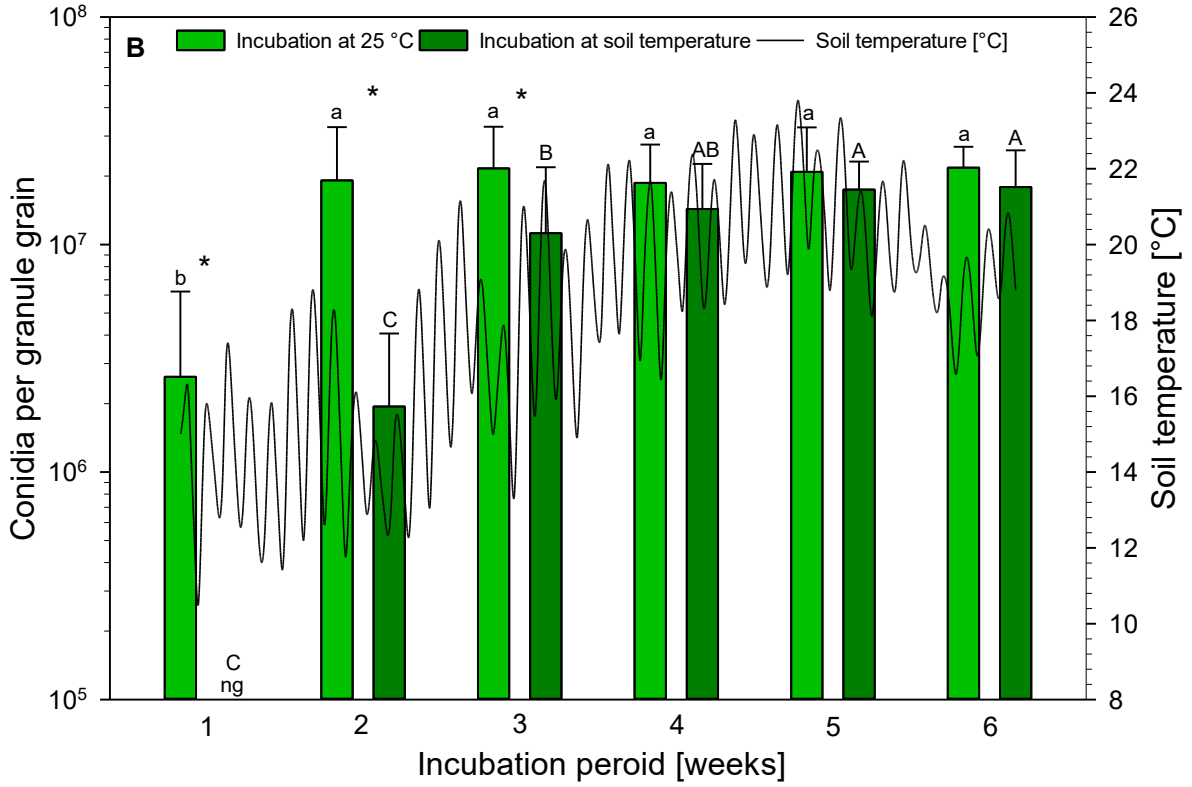


Figure 27: Effect of incubation at 25 °C and at simulated soil temperature on the granule colonization (A), and on the number of conidia per granule grain (B) of *Metarhizium brunneum* strain JKI-BI-1450 based granules. Means and standard deviation are shown. ng = no growth. Means with * at one evaluation time are significantly different. Mann-Whitney U-Test ($p < 0.5$). $n = 15$. The differences between the evaluation times of the incubation at 25 °C are represented by different lower case letters and for the simulated soil temperature by different upper case letters. GLMM ($p < 0.05$). $n = 30$. The black line indicates the simulated soil temperatures from the day the potatoes were planted until 6 weeks thereafter. $n = 3$.

As no conidia were formed after 1 week of incubation at the simulating soil temperature, this time point was not considered when determining the conidial size. The granule formed significantly longer ($\chi^2 = 862.81$; df = 1; $p < 0.0001$) and wider ($\chi^2 = 7.45$; df = 1; $p = 0.0063$) conidia during incubation at the simulated soil temperature rather than at 25 °C, where the granule was significantly rounder ($\chi^2 = 285.16$; df = 1; $p < 0.0001$) (Table 15). The incubation period also influenced the conidial size. After 3 weeks of incubation, the conidia were significantly the longest ($\chi^2 = 14.83$; df = 4; $p = 0.0051$), whereas the conidia were the widest ($\chi^2 = 19.60$; df = 4; $p = 0.0006$) after 4 weeks of incubation. In addition, the conidia were

significantly most elongated after 2 and 3 weeks of incubation and became more round with increasing incubation time ($\chi^2 = 23.93$; df = 4; $p < 0.0001$).

Table 15: Effects of the incubation conditions on the conidial size of the *Metarhizium brunneum* strain JKI-BI-1450 on the granules. Incubation temperature $n = 1500$; Incubation period $n = 600$.

Condition		Conidial size					
		Conidial length [µm]***	****	Conidial width [µm]***	****	Conidial index *, ***	****
Incubation temperature [°C]	Soil temp.**	6.96±0.54	A	2.23±0.22	A	3.15 ±0.40	A
	25	6.38±0.55	B	2.21±0.19	B	2.91±0.38	B
Incubation period [weeks]	2	6.70±0.63	AB	2.19±0.20	B	3.07±0.39	A
	3	6.73±0.64	A	2.22±0.21	AB	3.07±0.43	A
	4	6.63±0.59	B	2.23±0.22	A	3.00±0.43	B
	5	6.66±0.63	AB	2.24±0.21	A	3.00±0.42	B
	6	6.63±0.59	B	2.23±0.19	AB	3.00±0.38	B

* The index is calculated by dividing the length by the width. ** Soil temperature data from a field trial in Uelzen Niedersachsen, Germany (Figure 27). *** Mean ± standard deviation. **** Means of one condition at one conidial size parameter with the same letters are not significantly different. (GLMM, $p < 0.05$).

Effect of temperature on conidial size and germination

Incubation at 15 and 20 °C resulted in significantly longer ($\chi^2 = 234.64$; df = 3; $p < 0.0001$) and rounder ($\chi^2 = 134.10$; df = 3; $p < 0.0001$) conidia compared to higher temperatures (Table 16). However, the incubation temperature had no significant influence on the conidia width ($\chi^2 = 6.53$; df = 3; $p = 0.0887$).

Table 16: Effects of the incubation temperature on the conidial size of the *Metarhizium brunneum* strain JKI-BI-1450 on malt peptone agar plates after 21 days. $n = 300$.

Condition		Conidial size					
		Conidial length [µm]**	***	Conidial width [µm]**	***	Conidial index *, **	***
Incubation temperature [°C]	15	7.04±0.36	A	2.26±0.15	A	3.13±0.26	A
	20	6.97±0.37	A	2.27±0.19	A	3.09±0.30	AB
	25	6.47±0.51	C	2.26±0.15	A	2.87±0.29	C
	30	6.82±0.68	B	2.24±0.17	A	3.06±0.33	B

* The index is calculated by dividing the length by the width. ** Mean ± standard deviation; *** Means of one condition with one conidial size parameter and the same letters are not significantly different. (GLMM, $p < 0.05$).

The germination was found to be faster when the fungus was produced at higher temperatures (Figure 28). This effect was observed across all incubation temperatures.

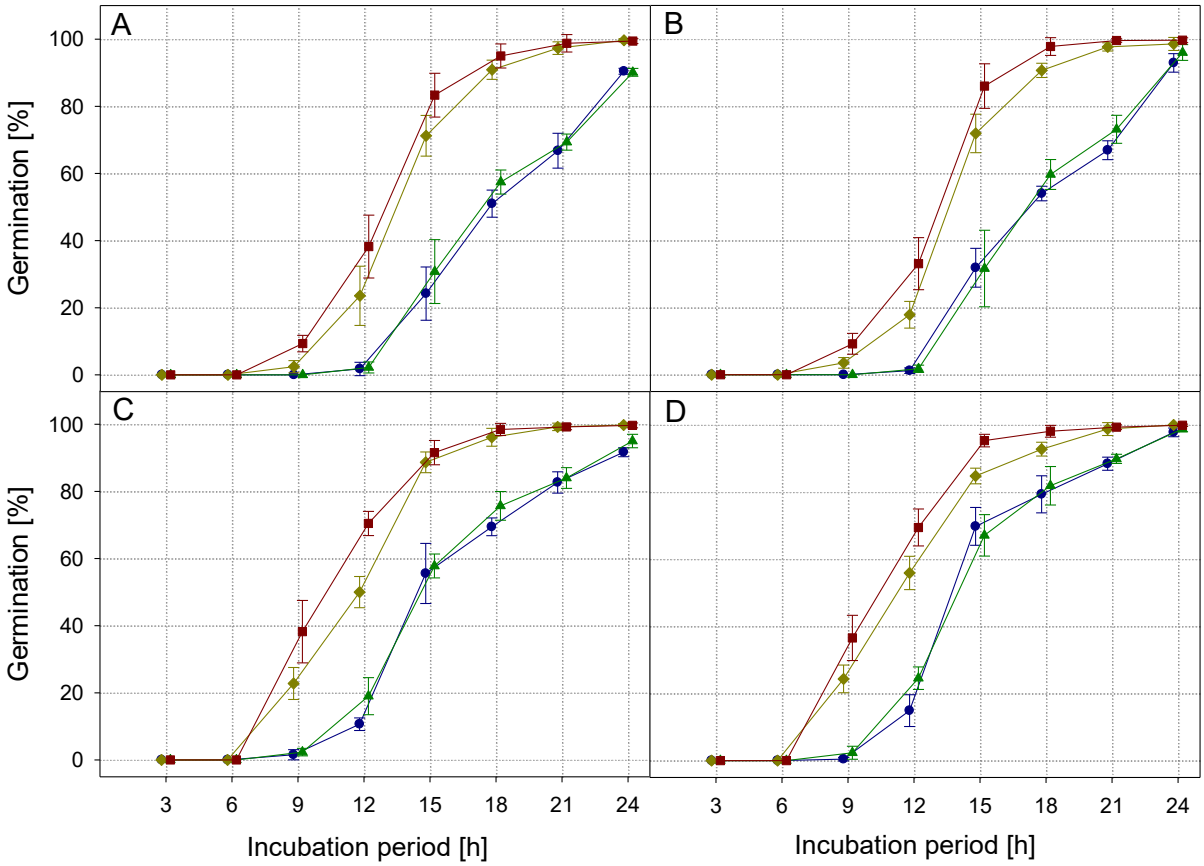


Figure 28: Effects of the production and incubation temperature on the germination [%] of *Metarhizium brunneum* JKI-BI-1450 are shown (mean and standard deviation). The symbols show the different production temperatures (● = 15 °C, ▲ = 20 °C, ◆ = 25 °C, ■ = 30 °C) and the letters correspond to the incubation temperature (A = 15 °C, B = 20 °C, C = 25 °C, and D = 30 °C). The symbols are slightly offset for better visibility. $n = 9$.

The statistical evaluation of this experiment is presented in Table 17. τ was reached significantly fastest with a production temperature of 30 °C (after 12.3 h), closely followed by 25 °C (after 12.8 h) ($\chi^2 = 1376.52$; df = 3; $p < 0.0001$). When the fungus was produced at 15 and 20 °C, τ was reached significantly slower (3 h later). The incubation temperature also exhibited a significant influence on τ ($\chi^2 = 2822.81$; df = 3; $p < 0.0001$). The higher the temperatures at which the fungus was incubated, the faster τ was reached. τ achieved values between 11 h at

30 °C and 17 h at 15 °C, with a significant difference observed among all incubation temperatures. The slope at the inflection point was also significantly influenced by the production temperature (χ^2 = 11.99; df = 3; p = 0.0074). When the fungus was produced at 20 °C, the slope was at its steepest, while at 25 °C, the slope was found to be at its lowest. The slopes of the other two production temperatures were deemed to be in between. When incubated at the higher temperatures (25 or 30 °C, respectively), the slope of the germination was significantly steeper than when incubated at the lower temperatures (15 or 20 °C, respectively) (χ^2 = 92.95; df = 3; p < 0.0001).

Table 17: Effects of the production and incubation temperature of the germination [%] of *Metarhizium brunneum* strain JKI-BI-1450 after 96 h, and of the germination process over 24 h. τ is the time point where 50% of the maximal germinated conidia are germinated. n = 9.

Conditions		Germination after 96 h [%]*	τ*	**	Slope inflection*	**
Production temperature [°C]	15	99.53±0.70	15.42±2.44	C	13.69±4.65	AB
	20	99.00 ±1.12	15.32±2.15	C	15.33±5.95	B
	25	99.67±0.59	12.81±2.14	B	13.00±2.27	A
	30	99.17±1.08	12.31±1.70	A	14.99±3.51	AB
Incubation temperature [°C]	15	99.47±0.70	16.18±1.83	D	12.07±4.48	A
	20	99.19±1.04	15.71±1.64	C	11.32±2.25	A
	25	99.39±0.96	12.57±1.18	B	15.99±3.25	B
	30	99.31±1.01	11.40±1.39	A	17.63±3.74	B

* Mean ± standard deviation; ** Means of τ and slope inflection at one condition with the same letters are not significantly different; (GLMM, p < 0.05).

Effect of temperature on the virulence

The production temperature of the fungus was found to have no influence on the ST_{50} but did differ significantly from the controls (χ^2 = 129.33; df = 5; p < 0.0001) (Table 18). The ST_{50} of the water control was significantly the highest with 10.83 days followed by the Tween® 80 control (7.63 days), and the fungal treatments with 3.94-4.54 days. However, it was found that the survival time significantly decreased with increasing incubation temperature (χ^2 = 102.65; df = 3; p < 0.0001). ST_{50} was highest when the larvae were incubated at 15 °C, with an average of 6.03 days, and lowest at 30 °C, with an average of 3.16 days, respectively. The final mortality

of the fungal treatments in relation to the production temperature did not differ significantly from each other (95-100%) but from the control treatments (χ^2 = 560.82; df = 5; $p < 0.0001$). The final mortality at 15 to 25 °C incubation was between 98.38-100% and differed significantly from the one observed at 30 °C (χ^2 = 17.17; df = 3; $p = 0.0007$).

Table 18: Effects of the production and incubation temperature on the virulence of *Metarhizium brunneum* strain JKI-BI-1450 against *Galleria mellonella*. *n* = 16.

Conditions		**ST_{50} [d]***	**	**Final mortality [%]***	**
Control	Water	10.83±2.85	C	18.75±19.28	C
	0.5% Tween® 80	7.63±5.16	B	55.00±21.6	B
Production temperature [°C]	15	3.97±1.39	A	98.13±5.44	A
	20	4.03±1.35	A	100 ±0.0	A
	25	4.54±1.84	A	95.63±10.31	A
	30	4.04±1.74	A	98.13±7.5	A
Incubation temperature [°C]	15	6.03±1.31	C	99.38±2.5	A
	20	4.06±1.32	B	100±0.0	A
	25	3.33±0.76	AB	100±0.0	A
	30	3.16±0.94	A	92.5±12.38	B

* Mean ± standard deviation. ** Means of ST_{50} and final mortality at one condition with the same letters are not significantly different. (GLMM, $p < 0.05$).

Discussion

The *M. brunneum* strain JKI-BI-1450 can form mycelium and conidia across a wide temperature range. This fungus generated the largest colony size and the most conidia/mm^2 at 25 °C. Similar results for the radial growth of *M. brunneum* were reported by Keyser et al. (2014) and Kryukov et al. (2017), respectively. More specifically, the optimum for mycelial growth of JKI-BI-1450 was 25-30 °C, and for conidiation 20-25 °C, respectively. Thomas and Jenkins (1997), and Onsongo et al. (2019), determined a higher temperature optimum for mycelia growth than for conidia formation for *Metarhizium anisopliae* and *Metarhizium flavoviride* as well. This effect has also been observed for other fungi such as *Mycosphaerella fijiensis* var. *difformis* (Jacome and Schuh 1993), and *Pyrenophora semeniperda* (Campbell et al. 1996). These results suggest that the optima for

mycelial growth and conidiation are slightly different and could indicate that the fungus does not form mycelium and conidia uniformly in the field at fluctuating temperatures (day and night rhythm). As the soil temperature at the time of potato planting in Germany which ranged from 9.6 to 14.7 °C (Paluch 2021) was considerably below the optimum temperature of JKI-BI-1450 (25 °C), attempts were made to improve its growth under sub-optimal conditions. However, the assumption that the growth of the fungus at unfavourable temperatures can be improved by prior exposure to this stress could not be confirmed. No indications were found that JKI-BI-1450 is able to adapt to unfavourable temperatures. Andrade-Linares et al. (2016b) showed that not all of the fungi they assessed were able to increase their growth through priming. Most studies of stress adaptation have been conducted with conidia exposed to the stressor immediately prior to incubation. Therefore, further research on the quality of conidia of JKI-BI-1450 directly exposed to lower temperatures would be essential to clarify whether this fungus is able to adapt to stressful conditions. The results of this experiment show that the growth of the fungus and conidiation on the granules, which is crucial for its effectiveness, are dependent on and can be positively influenced by its previous production temperature. When the biomass, which was produced at the optimal growing temperature of 25 °C, was used for the formulation of the granule, both growth parameters were at their highest regardless of the incubation temperature. Therefore, the fungus is better able to grow at unfavourable temperatures on granules produced at this temperature than on ones produced at higher or lower temperatures.

The temperature during liquid production has an influence on the production of secondary metabolites of *Nigrospora* sp., which was previously shown by Arumugam et al. (2015). Furthermore, Kim et al. (1999) demonstrated that the incubation temperature at liquid production causes the synthesis of phytases from *Aspergillus* sp.. Therefore, it would be

interesting to examine in further experiments whether the production temperature can affect the metabolism of JKI-BI-1450, which might have an impact on the growth of the granules. The production temperature can also influence further formulation steps. Jin et al. (1996) showed that submerged spores of *Trichoderma harzianum* were able to survive vacuum drying better when produced at 32 °C, rather than at higher or lower temperatures. A varying survival of the biomass during fluid-bed drying could be a possible explanation for the diverse results of the different production temperatures. Another possible explanation could be that the different production temperatures resulted in a varying proportion of mycelia and submerged spores in the suspensions used for the production of the granules. It should be noted that the granules were prepared with a defined biomass concentration, rather than a defined spore concentration. Stephan et al. (2020) demonstrated that the mycelium, submerged spores, or conidia of *M. brunneum* JKI-BI-1339 were suitable for the production of a granule by fluid-bed drying. However, an attempt to produce a granule based solely on the mycelium of JKI-BI-1450, was unsuccessful, as the fungus was unable to grow on this granule (Seib, unpublished data). Therefore, further experiments are required to determine the importance of the mycelium/spore proportion on the granule quality. In the present experiments, the submerged spore concentration of the biomass suspensions were determined but the difference in the growth of the fungus on the different granules cannot be due to the amount of submerged spores, but maybe to a difference in the characteristics of the spores themselves. The spore concentration of the biomass suspensions averaged 3.7×10^6 submerged spores ml^{-1}.

The present results show the highest granule colonization occurs following an incubation at 15 °C. This result is, however, at in contrast with both the present and other results from different studies, in which a temperature optimum of *Metarhizium* was observed between 25-30 °C (Walstad et al. 1970; Roberts and Campbell 1977; Alves et al. 1984; Hywel-Jones and

Gillespie 1990; Fargues et al. 1992; Ekesi et al. 1999; Milner et al. 2003; Dimbi et al. 2004), respectively. This higher colonization at low temperature can be attributed to increased contamination at higher incubation temperatures. At elevated temperatures, the granules were more often colonized by bacteria than after incubation at lower temperatures preventing the growth of the fungus as a consequence. During the process of fluid-bed drying, non-sterile air is blown into the cylinder, which could lead to these contaminations. In subsequent experiments, the effects of higher colonization by incubation at lower temperatures were no longer observed. Consequently, and as the germination and virulence tests were conducted exclusively with conidia and not with the granules, the results of all the following experiments were not influenced. The highest conidiation was observed when incubated at 25 °C, which was also confirmed by the other experiments in the present study. Thomas and Jenkins (1997), and Tefera and Pringle (2003), showed a temperature optimum for conidiation at 24-25 °C for *Metarhizium*. On a 25 °C-granule, JKI-BI-1450 was observed to grow between 10-30 °C. A comparison of growth at 10 and 25 °C revealed that after 4 weeks of incubation, there were no significant differences observed concerning granule colonization. However, the conidia concentration was consistently lower at 10 °C than at 25 °C at all evaluation times. The mycelium of *Metarhizium* strains can be formed at 10 °C with no or reduced sporulation, as previously confirmed by Skalický et al. (2014). In order to better understand the growth of the fungus under field conditions, a 25 °C-granule was incubated at a simulated soil temperature as well as at 25 °C, and the growth between the two was then compared. It was found that there was no difference in the colonization, but that conidiation was slower at the simulated soil temperature. The fungus formed conidia on the granule only after 2 weeks at the simulated soil temperature. Consecutively, the pest insects in the soil can only become infected 2 weeks after an application of the granule. The main aim was to protect the progeny tubers and not the seed

potato, so later conidiation would not necessarily present a problem. In fact, it would allow other micro- and macro-organisms in the soil time to colonize or feed on the grains.

The influence of fluctuating temperatures on the colony growth and virulence of *Beauveria bassiana* was investigated by Ghazanfar et al. (2020). The results demonstrated that the more the temperatures fluctuated, the more the growth was reduced compared to the growth at a constant 25 °C. Furthermore, the effect against *Heliothis virescens* was reduced by incubation at fluctuating temperatures. This effect was not seen for *Spodoptera littoralis*, which was also investigated.

The influence of the incubation temperature on the size has been demonstrated in all experiments. For strain JKI-BI-1450, incubation at lower temperatures consistently resulted in the formation of longer and partly wider conidia. Similar results were reported by Glare et al. (2021) for 16 *Metarhizium* strains from seven species. For other fungi, this effect was reported by Phillips (1982) and Phillips (1984) for *Monilinia fructicola*, by Tian and Bertolini (1999) for *Monilinia laxa,* and by Tian and Bertolini (1996) for *Botrytis allii* and *Penicillium hirsutum*, respectively. In contrast, Maitlo et al. (2017) described that *Fusarium oxysporum* f. sp. *ciceris* formed the longest and broadest conidia when incubated at 30 °C, while at lower or higher temperatures the conidia were smaller. These findings strongly suggest that the relationship between the temperature and conidial size differs among the fungi of different genera and must be examined individually for each fungus. Further research must be conducted to understand the meaning of conidial size for biological activity. As infectious conidia are first formed on the granule in the soil, the effects of conidia production temperature, and thus their size, as well as the subsequent incubation temperature on germination and virulence were investigated.

In the present study, it was determined that the higher the temperature at which the fungus was produced and incubated, the faster it reached the time at which 50% of the finally germinated

conidia were germinated (τ). These findings are in contrast to those reported by Phillips (1982), where conidia produced at lower temperatures germinated faster. These different outcomes indicate that the relationship between the temperature and germination is strongly dependent on the fungus, as well as the conidial size. However, the present experiments revealed a germination between 90-100% after 24 h, and over 99% after 96 h at all production and incubation temperatures. At lower temperatures, the conidia germinated more slowly, but the amount of germinated conidia after 96 h was not lower than at higher temperatures. A comparable time delay of germination was also shown by Skalický et al. (2014) for nine *M. anisopliae* strains comparing the germination at 15 and 20 °C after 24 and 48 h, respectively. Dimbi et al. (2004) determined that the incubation temperature had a significantly greater influence on the germination of several *M. anisopliae* strains after 24 h than was observed in the present experiments. All strains showed a maximum germination of about 90% at 25 °C, whereas the germination at 15 °C was below 10%, respectively. Results on the influence of the germination speed on the virulence are inconsistent within the literature. Some studies have indicated that rapidly germinating spores are more virulent (Al-Aidroos and Seifert 1980; Dillon and Charnley 1985; Samuels et al. 1989; Dillon and Charnley 1990; Altre et al. 1999), whereas others have reached the opposite conclusion (Boucias and Pendland 1984).

The influence of the temperature on the virulence of JKI-BI-1450 was therefore examined. The production temperature, and thus the conidial size and the speed of germination had no effect on the ST_{50} or the final mortality of *G. mellonella,* contradicting the results of Altre et al. (1999) on the virulence of different strains of *Paecilomyces fumosoroseus* against *Plutella xylostella*, where larger conidia were found to be more virulent. Furthermore, the production temperature of the conidia affects their germination speed but not the final germination and virulence. Therefore, a successful infection in this context seems to depend on more than just fast

germination. The present experiments demonstrated that the higher the incubation temperature, the lower the ST_{50}. In the case of JKI-BI-1450, a high incubation temperature ranging from 25-30 °C was found to be correlated with a great mycelia growth in terms of colony size, fast germination, and fast virulence. In addition to fast germination, a faster growth of the fungus on the cuticle promotes a successful and faster penetration through the fungus, reaching susceptible areas more rapidly. Dimbi et al. (2004), Bugeme et al. (2009), Fargues et al. (1997), and Onsongo et al. (2019) demonstrated the highest or among the highest mortality of *Metarhizium* when incubated at 30 °C. In contrast, the present results indicate a significantly lower mortality at 30 °C compared to the other incubation temperatures, although with a low ST_{50}. The influence of higher incubation temperatures on the speed of insect development could be a possible explanation, as earlier molting potentially strips off conidia. To verify this, one would need to repeat the experiment with younger larvae.

In summary, it can be demonstrated that the growth of an entomopathogenic fungus on a soil granule can be influenced and improved by its production conditions. Colonization and conidiation of a granule were at their highest when the fungus was produced at its optimum temperature. Furthermore, it was observed that the fungus was also able to grow on the granules and form conidia at lower temperatures and at simulated soil temperatures at the time of potato planting, which was particularly important, as the fungal strain JKI-BI-1450 and formulation process were selected for the control of wireworms in potatoes. However, the growth of the fungus, along with the germination and duration until lethal effect on the insects were found to be prolonged by lower temperatures. If this effect endangers the potential of the granule to control wireworms, an application at higher temperatures, for example in the year preceding potato planting, would provide the fungus with sufficient time to grow, sporulate, and establish in the soil, thereby reducing the population of the pest insect prior to potato cultivation.

Chapter IV

This chapter has been published with few modifications in:

Seib, T.; Fischer, K.; Sturm, A. M.; Stephan, D. (2023): Investigation on the influence of production and incubation temperature on the growth, virulence, germination, and conidial size of *Metarhizium brunneum* for granule development. Journal of Fungi. 9 (6), 668. Doi: 10.3390/jof9060668.

Notes on other contributors: Sturm née. Ruppenthal, A. M.: Collected the data of the experiment: “Effect of temperature on the conidial size, germination and the virulence”, Hamburger F.: Collected the data of the colony sizes of the experiment: “Colony size and conidiation at different temperatures “.

General Discussion

To employ an entomopathogenic fungi (EPF) as a biocontrol agent, several steps have to be taken and requirements have to be carefully examined. First, a fungal strain must be found that is highly virulent against the target pest. Then, appropriate methods to produce and formulate the EPF are needed and the storability of the formulated EPF must be examined. Thereupon, it must be verified whether the developed formulation can be used under field conditions (Montesinos 2003).

Identification of a suitable fungal strain for the control of *Agriotes* larvae

All *Metarhizium* strains tested in the present study were effective against the three *Agriotes* species, though the effect of the fungal strains differed greatly between the *Agriotes* species. *Agriotes lineatus* was less susceptible to infection with *Metarhizium* (Chapter I). One possible explanation would be a difference in the composition of the cuticle of the different *Agriotes* species. The composition of the cuticle of the *Agriotes* larvae may influence the germination of the conidia and thus the virulence of the fungi. Antifungal components on the cuticle, for example fatty acids with a medium chain length (Smith and Grula 1982; Saito and Aoki 1983; Barnes and Moore 1997; Gołębiowski et al. 2015; Gutierrez et al. 2015), can inhibit the germination of conidia (Saito and Aoki 1983; Barnes and Moore 1997; Boguś et al. 2010), which in turn could prevent lethality of an infection with an EPF. Furthermore, the immune response of the different *Agriotes* species might not be identical and some fungal strains could be able to overcome it more effectively than others. Besides the fact that the fungal strains had a weaker effect on *A. lineatus* compared to the other two species, different *Metarhizium* strains are most effective against certain *Agriotes* larvae. The *Metarhizium brunneum* strain JKI-BI-1450 showed the best effect on *A. lineatus* and *Agriotes obscurus* and the

Metarhizium robertsii strain JKI-BI-1442 against the larvae of *Agriotes sputator*. This finding shows how highly specific the effect of EPF can be even against very closely related pest insects. For *A. obscurus* and *A. sputator,* even different effects of one fungal strain could be observed between different populations of the same species.

These results on the different efficacy against different *Agriotes* species should be taken into account for the field application, as *Agriotes* larvae often occur in mixed populations in one field and larvae of other Elateridae genera may also be present (Lehmhus and Niepold 2015). However, a very promising candidate was found in *M. brunneum* strain JKI-BI-1450, which showed the best efficacy against larvae of *A. lineatus* and *A. obscurus* and intermediate efficacy against *A. sputator*. The use of a fungal strain with a wide host range in the Elateridae family would be beneficial. If one fungal strain alone will not show the desired effect, a mixed granule of different strains would be a possible solution.

In addition to a lethal effect, it is also advantageous if the fungus grows out of the dead insect and forms conidia. In this way, the cadaver serves as a bioreactor of its own and the fungus can multiply in the soil over the initially applied amount and infect further pest insects. For the *Metarhizium* strains examined in the present study, the mycosis percentage of dead larvae was 70% for *A. lineatus*, 87% for *A. obscurus*, and 85% for *A. sputator*. It is conceivable that other wireworms could be infected by the conidia formed on the dead insects, which would increase the effect of the fungus.

Another aspect that must be considered before using the *Metarhizium* strains in the field is the time between application of the fungi and the death of the insect. The results of the present study and those of Paluch (2021), Razinger et al. (2018) and Eckard et al. (2013) demonstrated that it may take several weeks until a sufficient effect appears and that the mortality of the

Agriotes larvae increased over the entire duration of the experiments. It should be noted that the present experiments and the experiments of Paluch (2021) were carried out at 25 °C, whereas the experiments by Eckard et al. (2013) and Razinger et al. (2018) were performed at 23 °C and 21 °C, respectively. Presumably, the lethal effect takes even longer at the soil temperatures present in the potato ridge during potato planting, which are typical between 9.8-16.5 °C at the beginning of the vegetation period and between 17.1-19.4 °C over the entire vegetation period (2018-2020) (Paluch 2021). Results from chapter IV showed that the mean survival time (ST_{50}) of *Galleria mellonella* larvae treated with JKI-BI-1450 is higher when incubated at lower temperatures, but not the final mortality. Additionally, Neuhoff et al. (2007) noted that the damage to tubers caused by wireworms is greater the later the potatoes are harvested. As discussed below, the prolonged period between application and onset of the effect could have a negative impact on the suitability of the fungus for the control of *Agriotes* larvae in the field.

Mass production and formulation of an entomopathogenic fungus as a prerequisite for application as a plant protection agent

Good efficacy of an EPF against the target pest is crucial for its use as a biocontrol agent. However, the best efficacy is useless, if the fungus cannot be economically produced in sufficient amounts for the actual field application. To evaluate the most effective production process, it is necessary to clarify how the biological agent will be formulated and applied later on. First of all, it needs to be decided which form of the fungus will be utilized in the field, which dictates the form of production. If conidia are to be applied, production on a solid medium such as cereals should be selected, whereas if submerged spores are to be used, the fungus must be produced in a liquid medium (Jaronski 2023). Both production methods have advantages and disadvantages. Solid-state fermentation takes longer and is more susceptible to

contamination but conidia are considered to be environmentally more stable than submerged spores (Pham et al. 2010; Mar and Lumyong 2012; Bernardo et al. 2020). Furthermore, conidia of Hyphomycetes like *Metarhizium* are hydrophobic and propagate passively from infected cadavers (Shah and Pell 2003). Submerged spores can be produced more quickly, but are environmentally less stable than conidia (Ypsilos and Magan 2005; Mascarin et al. 2019; Bernardo et al. 2020). However, they may germinate more quickly, which can have a positive impact on the infection speed (Jackson 1997; Vega et al. 1999). The optimal form of production is also depending on the fungus, as some are better suited for solid-state fermentation and others for liquid fermentation. The choice of the formulation method is also of decisive importance. First of all, it must be clarified how the subsequent biological agent will be applied, which depends, among other things, on the pest insect. Powder formulations or agglomerates, which need to be suspended before application and then sprayed, are suitable for application to plants as well as into the soil, whereas granules are only suitable for application on or in soil. Both powder formulations and granules can be prepared with outcomes from solid-state fermentation as well as liquid fermentation. After solid-state fermentation, cereal grains covered with mycelium and conidia are present. These can be introduced directly into the soil as a granule, which is one of the most common formulations (Maina et al. 2018). To produce a spray able formulation, the conidia can be mechanically detached from the cereal grains and then brought into suspension and sprayed. The formulation of fungal material produced in liquid fermentation is most often done by drying. The different drying methods also have their own advantages and disadvantages. By spray and fluid-bed drying a large amount of product can be obtained in a relatively short time but both methods induce strong thermal stress on the fungus, which not all can survive. The more gentle freeze-drying process requires a lot of energy, and this process is also not suitable for all fungi. All three drying methods can be used to produce

sprayable formulations, while spray and fluid-bed drying can also be used to produce agglomerates and granules. A developed formulation must have a stable shelf life, be easy to use and safe for users and bystanders (Awan et al. 2021).

For the reasons mentioned above, the decision was made in favour of liquid fermentation, but a granule was nevertheless developed in which the environmentally stable conidia represented the infectious unit. In this way, the user does not come into contact with the fungus by dust released during application, as conidia are only formed when the granule comes into contact with moisture in the soil. However, this application strategy can also be seen as a disadvantage, as the infectious units are not immediately available after application. At simulated soil temperature, it takes 2 weeks for conidia to form on the granule (Chapter IV). Only after this time *Agriotes* larvae may become infected with the EPF. The granule to be applied itself is not dusty with an abrasion of 0.1-0.2 mg per 100 g, which was proven by Immenroth (Julius Kühn Institute, Federal Research Centre for Cultivated Plants, Institute for Application Techniques in Plant Protection, Braunschweig, Germany) using the Heubach test and can be applied with already existing fertilizer carriers, whereby the application rate is technically limited (Paluch 2021).

Challenge of the time between application and onset of the lethal effect of the fungi

Wireworm control in potato cultivation is primarily about protecting the daughter tubers rather than the seed tuber. According to Allen and Scott (1980), it takes 12 weeks from planting potatoes to the formation of the daughter tubers. During this time, the fungus would have to grow on the granules, form conidia, infect the insect and lead to its death of feeding stop. However, one aspect that occurs in many of the experiments in the present study is the long time period it takes for the fungus to grow on the granules (Chapter III and IV) and to kill the

Agriotes larvae (Chapter I). Even under optimal conditions, the fungus on the granules needs five days to form conidia (Chapter III). Environmental conditions in the field, such as suboptimal soil moisture and temperature or the parallel application of fungicides, can further delay conidiation on the granule and thus the lethal effect of the fungus on the larvae (Chapters III and IV). During this time, it is possible that the millet is colonized by other microorganisms and thus the fungus is prevented from further growing on the granule. Such potential negative interaction with other microorganisms is indicated by the treatment of the granule with RhizoVital®42 (Chapter III), which prevented the fungus from growing. Paluch (2021) investigated in laboratory experiments the growth of JKI-BI-1450 from granules developed in the present study grew in field soils. Incubation in field soil had a slightly reducing effect on colonization but a major decrease in conidia formation compared to water agar. As the colonization was slightly reduced at incubation in field soil, it would be quite conceivable that it also forms more conidia on the granule after longer incubation. Similar results were observed at Shah et al. (2023) for a *Metarhizium anisopliae* granule. Also in their study, the sporulation on a granule was significantly lower on non-sterile soil compared to sterile soil.

The results in chapter II have demonstrated that the addition of supplements during fluid-bed drying can accelerate the conidiation on the granule. The addition of additives such as sugars could be a way of compensating for the reduced or delayed growth of the fungus in non-sterile soil. Furthermore, application of the granule in late summer of the previous year when the catch crop is sown (soil temperature in August: 20-25 °C) could perhaps overcome the issue of the slow growth of the fungus at lower temperatures and the long time between contact of the fungus and the *Agriotes* larvae until their death (Paluch 2021). Then the fungus could grow better as it prefers higher temperatures, establish itself in the soil and reduce the *Agriotes* population over time. The ability of *M. brunneum* to persist in the soil for a long time after

recent application has been demonstrated by Hernández et al. (2023). In their study, the persistence of an applied strain of *M. brunneum* (colony forming units) in soil was demonstrated over a period of 250 days with two applications, with the level of remaining fungus depending on the formulation. Also in the study of Yang et al. (2019), different *Metarhizium* strains were detected in the soil over a longer period after application but the colony forming units of the fungus decreased steadily over the duration of the experiment. To further optimize the application strategy it will be important to determine to which extent JKI-BI-1450 persists in the soil after application of the developed granule.

Direct introduction of infectious units into the soil could be another way to achieve a faster infection. Paluch (2021) investigated the effect of freeze-dried submerged spores of JKI-BI-1450 as well as the granule developed in the present study against wireworm damage in potato cultivation. Both formulations showed a slight reduction of tuber damage, which proves the potential of this control strategy but also the problem of transferring experimental results from the laboratory to the field and the need to optimize the granule and its application.

Conclusion and Outlook

In order to develop a soil granule based on an entomopathogenic fungi (EPF) for the control of *Agriotes* larvae, it was searched for an effective fungal strain, the producibility and formulability of the fungal strain were tested, and the viability of the formulation under simulated field conditions was investigated.

Firstly, the *Metarhizium brunneum* strain JKI-BI-1450 with good efficiency against the three economically most important *Agriotes* species was determined. JKI-BI-1450 can be produced in artificial medium and forms both mycelium and submerged spores. The biomass produced in liquid media is well suited for the production of a granule where it is coated onto millet grains using fluid-bed drying. In the present laboratory experiments, a relatively large amount of biomass was required to produce a granule with sufficient colonization. The formulation developed is non-dusting and can be applied with conventional fertilizer spreaders. The examination of the viability of the fungus on the granule showed that it is influenced by many factors. Though several fungicides used in potato cultivation have a negative impact on the growth of the fungus on the granules, it has been demonstrated that the product Moncut® can be used together with the developed granule with only a slight loss of vitality.

In summary, the fungal strain *M. brunneum* JKI-BI-1450 complies with many requirements that have are decisive for its successful application to control wireworms, including efficacy, the producibility in artificial medium and the formulation by fluid-bed drying. However, some additional topics need to be further clarified based on the results of the present study. First, the effect of JKI-BI-1450 on other *Agriotes* species and wireworms of other genera should be investigated. To reduce the high amount of biomass required for granule production, it needs to be investigated whether an increase in spores in the biomass would actually contribute to its

colonization and conidiation. If so, further attempts should be made to increase the sporulation of the fungus during liquid fermentation. The influence of a constant pH value and the oxygen content in the medium during fermentation on sporulation still holds some potential for optimization. Issaly et al. (2005) has proven that sporulation of *Metarhizium flavoviride* can be increased by regulating the pH value to 6 or 7, compared to fermentation with an unregulated pH value, and by a constant high level of dissolved oxygen in the medium compared to a low level or no regulation of dissolved oxygen.

Though it was demonstrated that the conidiation of JKI-BI-1450 on the granules can be accelerated by the addition of sugars, it should be investigated whether this beneficial effect can be further increased by other additives or higher sugar concentrations and how this variation may affect the growth of the fungus on the granules in non-sterile soil or whether the required biomass for granule production can be reduced this way. The loss of vitality due to the simultaneous application together with fungicidal seed treatments used in potato planting could perhaps be prevented by an additional protective layer around the granules applied during formulation or by timely separating the application of a fungicide from the granules.

Finally, although several important technical questions toward the production and formulation of *M. brunneum* JKI-BI-1450 as a biocontrol agent of wireworms could be achieved in the present study, there are still unsolved ecological issues regarding host range and specificity, competitiveness in non-sterile soil and speed of action which need to be further addressed.

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Ehrenwörtliche Erklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter angefertigt habe. Sämtliche aus fremden Quellen direkt oder indirekt übernommenen Gedanken sowie sämtliche von Anderen direkt oder indirekt übernommenen Daten, Techniken und Materialien sind als solche kenntlich gemacht.

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Curriculum vitae

Abstracts and Conference Contributions

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