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Developing and optimising the effectiveness of entomopathogenic fungi for biological control of *Cydia pomonella*: A multifaceted approach

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Determination is the key to turn obstacles into opportunities, and challenges into stepping-stones.

Contents

Summary

The food industry, a cornerstone of global health and economy, faces many challenges in meeting the ever-increasing demand for nutritious and accessible products. Central to the success of this industry is the agricultural sector, particularly the cultivation of crops such as pome fruits, which are essential for diversifying diets and providing essential nutrients. However, the journey from farm to fork is not straightforward, with the limited availability of arable land and the relentless threat of insects, microbial pathogens, and pests such as the codling moth. The codling moth (*Cydia pomonella*, Lepidoptera: Tortricidae) is a major threat to pome fruit production, causing considerable losses to fruit farmers not only in Europe but globally. In response to the drawbacks of synthetic insecticides, many farmers are turning to biocontrol strategies, including the use of entomopathogenic fungi (EPF), as sustainable alternatives for pest management. Consequently, research efforts focused on the screening and application of EPF have increased significantly in recent years, reflecting the growing interest in environmentally friendly pest management practices worldwide. This work, which involved the isolation of 32 EPF strains from soil samples using the insect bait method and one strain from strain collection of Julius Kühn Institute (JKI), aimed not only to assess the occurrence and prevalence of these fungi, but also to investigate their virulence and the key role of chitinase production in enhancing their effectiveness to control the pest insect. Following identification procedures by microscopic examination and molecular sequencing of the ITS region, the initial screening phase focused on assessing the virulence of these strains, setting a high benchmark of over 80 % mortality against *C. pomonella*. This rigorous selection criterion led to the identification of 13 strains, with promising virulence. Among these, four strains i.e. JKI-BI-1496 (*Cordyceps fumosorosea*), two strains of JKI-BI-2620 (*Beauveria bassiana*), JKI-BI-2642 (*Beauveria bassiana*), and JKI-BI-2647 (*Metarhizium robertsii*), stood out for their higher chitinase activity, a crucial enzyme that facilitates the degradation of chitin, a key component of the insect exoskeleton, thereby enhancing the fungal invasion and infection process. The research also investigated the environmental adaptability of these strains, subjecting them to a range of temperature conditions and simulated sunlight exposure to assess their resilience and effectiveness under different environmental stressors. This phase was critical in determining the potential field applicability of the

selected EPF strains, given the diverse and often challenging conditions found in natural agricultural environments. The study also investigated the growth and virulence potential of these fungi in four different media types — malt peptone, malt peptone with 1 % chitin, potato dextrose, and potato dextrose with 1 % chitin — to determine the optimal conditions for their proliferation and virulence. The aim of this approach was to enhance the production potential of the strains, with a particular focus on the use of chitin-amended media to simulate the natural infection process and thereby improve the efficacy of the fungi against *C. pomonella*. Particular attention was paid to *C. fumosorosea*, which showed promising properties in preliminary tests. One of the most significant findings of the study was the marked improvement in fungal virulence and effectiveness when grown in a medium containing 1 % colloidal chitin (CC), which not only facilitated a higher mortality in bioassays against *C. pomonella* larvae but also influenced various growth parameters, including spore and biomass production, germination and higher stability as freeze-dried spores. However, the addition of 1 % CC also presented challenges. It notably affected the fungi's resilience to prolonged sunlight exposure, which is a critical factor to consider for the field application of these biocontrol agents. To address the limitations posed by sunlight sensitivity, the research explored the effectiveness of nine water-soluble sunlight protectants. Black tea was identified as a particularly effective agent in preserving the viability and germination of submerged spores under simulated sunlight conditions. Additionally, spores coated with calcofluor white resulted higher shelf life than other additives. This approach to enhance the environmental stability of *C. fumosorosea* spores could be a promising strategy for the application of fungal biocontrol agents against *C. pomonella* in organic apple orchards. The findings emphasise the complex interplay between fungal virulence, environmental adaptability, and innovative formulation strategies in the development of effective biological control agents (BCAs). This research highlights the potential of chitin-amended media and sunlight protectants to enhance the effectiveness and stability of EPF. The findings offer valuable insights into the optimization of biocontrol strategies, paving the way for more sustainable and environmentally friendly pest management approaches in agriculture.

Zusammenfassung

Die Lebensmittelindustrie, ein Eckpfeiler der globalen Gesundheit und Wirtschaft, steht vor vielen Herausforderungen, um die stetig wachsende Nachfrage nach nahrhaften und zugänglichen Produkten zu erfüllen. Zentral für den Erfolg dieser Branche ist der landwirtschaftliche Sektor, insbesondere der Anbau von Kulturen wie Kernobst, die für die Diversifizierung der Ernährung und die Bereitstellung wesentlicher Nährstoffe unerlässlich sind. Der Weg vom Acker bis zur Gabel ist jedoch nicht einfach, mit der begrenzten Verfügbarkeit von Ackerland und der unerbittlichen Bedrohung durch Insekten, mikrobielle Pathogene und Schädlinge wie den Apfelwickler. Der Apfelwickler (*Cydia pomonella*, Lepidoptera: Tortricidae) stellt eine erhebliche Bedrohung für die Produktion von Kernobst dar und führt zu beträchtlichen Verlusten für Obstbauern nicht nur in Europa, sondern auch weltweit. Als Reaktion auf die Nachteile synthetischer Insektizide wenden sich viele Landwirte biologischen Bekämpfungsstrategien zu, einschließlich der Nutzung von entomopathogenen Pilzen (EPF) als nachhaltige Alternativen zur Schädlingsbekämpfung. Folglich haben Forschungsbemühungen, die sich auf das Screening und die Anwendung von EPF konzentrieren, in den letzten Jahren deutlich zugenommen, was das wachsende Interesse an umweltfreundlichen Schädlingsbekämpfungspraktiken weltweit widerspiegelt. Diese Dissertation, bei der 32 Stämme von entomopathogenen Pilzen (EPF) aus Bodenproben mittels der Insektenködermethode isoliert und ein Stamm aus der Stammsammlung des Julius Kühn-Institut (JKI) gewonnen wurde, zielte nicht nur darauf ab, das Vorkommen und die Verbreitung dieser Pilze zu bewerten, sondern auch ihre Virulenz zu untersuchen und die entscheidende Rolle der Chitinaseproduktion bei der Steigerung ihrer Wirksamkeit gegen den Zielorganismus zu erforschen. Nach sorgfältigen Identifikationsprozessen durch mikroskopische Untersuchung und molekulare Sequenzierung der ITS-Region konzentrierte sich die erste Screening-Phase auf die Bewertung der Virulenz dieser Stämme, wobei ein Maßstab von über 80 % Mortalität gegenüber *C. pomonella* gesetzt wurde. Dieses strenge Auswahlkriterium führte zur Identifizierung von 13 Stämmen, die aufgrund ihrer Virulenz ein Potential für die Schädlingsbekämpfung zeigten. Unter diesen ragten vier Stämme, nämlich JKI-BI-1496 (*Cordyceps fumosorosea*), zwei Stämme von JKI-BI-2620 (*Beauveria bassiana*), JKI-BI-2642 (*Beauveria bassiana*) und JKI-BI-2647 (*Metarhizium robertsii*), aufgrund

ihrer höheren Chitinasenaktivität heraus. Die Chitinasenaktivität wird dabei als ein wichtiges Element betrachtet, da es den Abbau von Chitin, einer Schlüsselkomponente des Insektenexoskeletts, erleichtert und so den Pilzbefall und den Infektionsprozess verbessert. Des Weiteren wurde die Umweltanpassungsfähigkeit dieser Stämme untersucht, indem sie einer Reihe von Temperaturbedingungen und simulierter Sonnenlichtexposition ausgesetzt wurden, um ihre Resilienz und Wirksamkeit unter verschiedenen Umweltstressoren zu bewerten. Diese Phase war entscheidend für die Bestimmung der potenziellen Feldanwendbarkeit der ausgewählten EPF-Stämme, angesichts der vielfältigen und oft herausfordernden Bedingungen in natürlichen landwirtschaftlichen Umgebungen. Darüber hinaus untersuchte die Studie das Wachstums- und Virulenzpotenzial dieser Pilze in vier verschiedenen Medientypen – Malzpepton, Malzpepton mit 1 % Chitin, Kartoffeldextrose und Kartoffeldextrose mit 1 % Chitin – um die optimalen Bedingungen für ihre Vermehrung und Virulenz zu ermitteln. Dieser Ansatz zielte darauf ab, dass Produktionspotenzial der Stämme zu erhöhen, mit einem besonderen Fokus auf die Verwendung von Chitin-angereicherten Medien, um den natürlichen Infektionsprozess zu simulieren und so die Wirksamkeit der Pilze gegen *C. pomonella* zu verbessern. Besondere Aufmerksamkeit galt *C. fumosorosea*, das in vorläufigen Tests vielversprechende Eigenschaften zeigte. Einer der bedeutendsten Befunde der Studie war die deutliche Verbesserung der Pilzvirulenz und -Wirksamkeit, wenn sie in Medien mit 1 % kolloidalem Chitin (CC) produziert wurden, was nicht nur mit einer höheren Mortalitätsrate in Bioassays gegen *C. pomonella*-Larven einherging, sondern auch verschiedene Wachstumsparameter beeinflusste, einschließlich Sporen-, Biomasseproduktion, Keimungsgeschwindigkeit sowie eine höhere Stabilität gefriergetrockneter Sporen. Die Zugabe von 1 % CC stellte jedoch auch Herausforderungen dar, insbesondere beeinflusste sie die Resilienz der Pilze gegenüber längerer Sonnenlichtexposition – ein kritischer Faktor bei der Anwednung im Feld. Um die durch Sonnenlichtempfindlichkeit bedingten Einschränkungen zu adressieren, erforschte die Studie die Wirksamkeit von neun wasserlöslichen Sonnenschutzmitteln und identifizierte schließlich schwarzen Tee als besonders wirksames Mittel zur Erhaltung der Lebensfähigkeit und Keimraten von Sporen unter simulierten Sonnenlichtbedingungen. Darüber hinaus führten mit Calcofluor-Weiß beschichtete Sporen zu einer höheren Haltbarkeit als andere Zusatzstoffe. Dieser Ansatz zur Verbesserung der Umweltstabilität von *C. fumosorosea*-

Sporen stellt eine vielversprechende Strategie zur Verbesserung der Feldanwendbarkeit von pilzlichen Biokontrollmitteln dar und könnte die Akzeptanz einer Anwendung von EPF zum Management von *C. pomonella* nicht nur im ökologischen Apfelanbau verbessern. Die Befunde der Studie unterstreichen das komplexe Zusammenspiel zwischen Pilzvirulenz, Umweltanpassungsfähigkeit und innovativen Formulierungsstrategien bei der Entwicklung effektiver biologischer Bekämpfungsmittel. Indem sie das Potenzial von Chitin-angereicherten Medien und Sonnenschutzmitteln zur Steigerung der Wirksamkeit und Stabilität von EPF hervorheben, bietet diese Forschung wertvolle Einblicke in die Optimierung von Biokontrollstrategien und ebnet den Weg für nachhaltigere und umweltfreundlichere Schädlingsbekämpfungsansätze in der Landwirtschaft.

General introduction

At the heart of a thriving global agriculture, apples not only serve as a staple of nutrition but also as architects of economic resilience, providing essential support to communities worldwide. Known scientifically as *Malus domestica*, apples are part of the pome fruit family Rosaceae and have their origins in Asia. Today, apples are cultivated globally for a variety of uses, including desserts, culinary dishes, and cider production (Alford, 2014). Reflecting their role in agriculture, global apple production soared to 95.8 million tons in 2022 (Figure 1), according to the Food and Agriculture Organization (UNFAO, 2024). This makes the apple the premier fruit crop in Europe by harvest volume, with Europe contributing 18.7 million tons to this impressive total (UNFAO, 2024).

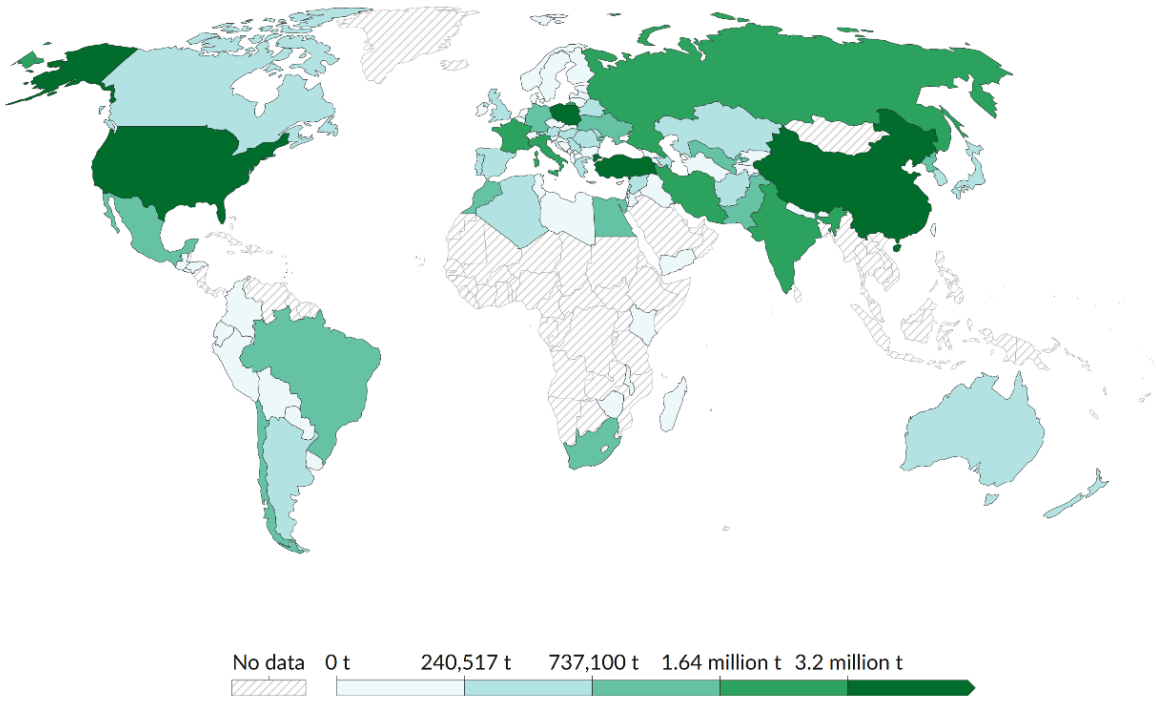

Figure 1. Worldwide apple production in 2022 (Data source: UN Food and Agriculture (FAO), 2024).

However, trends vary by region; for instance, apple production in Germany has adjusted to newer challenges and opportunities, achieving a total of 1.8 million tons in 2022 (UNFAO, 2024; Eurostat 2024). These production figures not only highlight the fruit's

economic and nutritional importance but also underscore the dynamic nature of apple cultivation across different landscapes.

Apple orchards are a prominent feature in the European landscape, covering 0.5 million hectares and accounting for about 13 % of the total fruit area in the EU (Eurostat, 2024). Moreover, in 2022 alone, 980,065 hectares of EU cropland were dedicated to apple cultivation, with Germany accounting for 31,110 hectares of this total (UNFAO, 2024). This cultivation highlights the strategic importance of apples in European agriculture and underscores the dynamic interplay between agricultural practices and regional crop production.

Effective orchard management demands a deep understanding of pests and diseases, their life cycles, and the ecological factors that promote infestations. Growers must navigate varietal susceptibility, potential alternative hosts, and natural predators using a mix of cultural, biotechnological, biological, and chemical controls, coupled with vigilant monitoring to prevent pesticide resistance (NIAB, accessed on 25.04.2024). In Europe, while growers utilize a range of insecticides and acaricides, accounting for about 40 % of production costs, these can be partly offset by biological control methods (Yilmaz et al., 2015; Happe et al., 2019).

Ensuring the sustainability of apple production also requires enhancing public awareness of these practices and the needs of BCAs, safeguarding both current productivity and the future of apple cultivation. This heightened awareness is particularly crucial as the global shift towards environmentally sustainable agriculture intensifies the focus on reducing the use of harmful pesticides. The increasing demand for eco-friendly pest control methods is driven by longstanding battles against pests like the codling moth, *Cydia pomonella* (Linn) (Lepidoptera: Tortricidae), which have historically caused significant damage to the fruit industry by reducing yield and compromising fruit quality. This pest, which has a significant impact on orchard fruits such as pears, apples, and other pome fruits, is a global challenge (Guo et al. 2021; Maggi and Chreil 2023). Recognised for its economic impact, the codling moth has been identified as a high-priority agricultural pest (Beers et al 2003; Wearing et al. 2012; Jiang et al. 2018), requiring detailed research and the development of effective environmentally friendly management strategies to limit its widespread damage.

Entomopathogenic fungi, with their wide distribution and presence in diverse habitats including arable soils, represent a promising avenue in this regard (Beers et al 2003; Blümel et al. 2023).

Cydia pomonella, a pest insect in perennial fruit production: distribution and biology

As shown in Figure 2, *Cydia pomonella* is a highly destructive pest of pome fruit (Lacey et al. 2008; Joshi 2011; Kaisoon 2021). While it primarily attacks apples, it also affects pears, peaches, and apricots, causing significant damage, particularly in apple orchards (Guo et al. 2021). If left untreated, it can cause complete crop losses (Beers et al. 2003; Jiang et al. 2018; Wan et al. 2019; Maggi and Chreil 2023). Poor management of this pest control can lead to ecological degradation and economic setbacks (Jiang et al. 2018). Therefore, it is crucial to implement proactive measures to mitigate these risks and protect biodiversity and agricultural production.

Figure 2. *Cydia pomonella* damaging apples; (A) Areas where apples touch are the entry points for *C. pomonella* (Photo: Whitney Cranshaw, Colorado State University, Bugwood.org), (B) Matured *C. pomonella* (Photo: EPPO, accessed on 28.02.2024), (C) *C. pomonella* L_5 larvae inside apple (Photo: Stephan, D., JKI).

Originally from Eurasia, *C. pomonella* has spread throughout the northern and southern hemispheres, with the exception of Japan and Western Australia, where eradication efforts have been successful (van der Geest and Evenhuis 1991). This pest has expanded its range over the last two centuries and is now almost globally distributed. It is found in almost all countries where apples are grown, making it one of the most successful and invasive pest species worldwide (Jiang et al. 2018). Its distribution is influenced by food availability and climatic conditions in the surrounding regions (Abaajeh 2014). In orchards employing conventional plant protection methods, this pest has traditionally been managed with chemical insecticides. However, this approach poses challenges,

including damage to beneficial organisms, adverse environmental effects, and safety concerns for both applicators and consumers (Lacey and Shapiro-Ilan 2008). As a result, there is a growing need for environmentally friendly control agents suitable for integrated and organic farming practices to effectively control this pest (Lacey and Shapiro-Ilan 2008). In addition, *C. pomonella* is multivoltine and exhibits adaptive behaviours such as facultative diapause and multiple breeding seasons per year, allowing it to thrive in various climatic conditions (Jiang et al. 2018).

Originating in Europe, this pest has now invaded nearly 80 countries on six continents (Figure 3). Currently, *C. pomonella* is found in temperate zones of both the Northern and Southern hemispheres, covering all major apple-growing regions of the world (CABI 2022). It is known to occur in Europe, Mediterranean countries, Central Asia, North America, Argentina, Eastern Australia, New Zealand, and South Africa. In addition, local occurrences have been observed in Brazil, India, China, and other regions (CABI 2022).

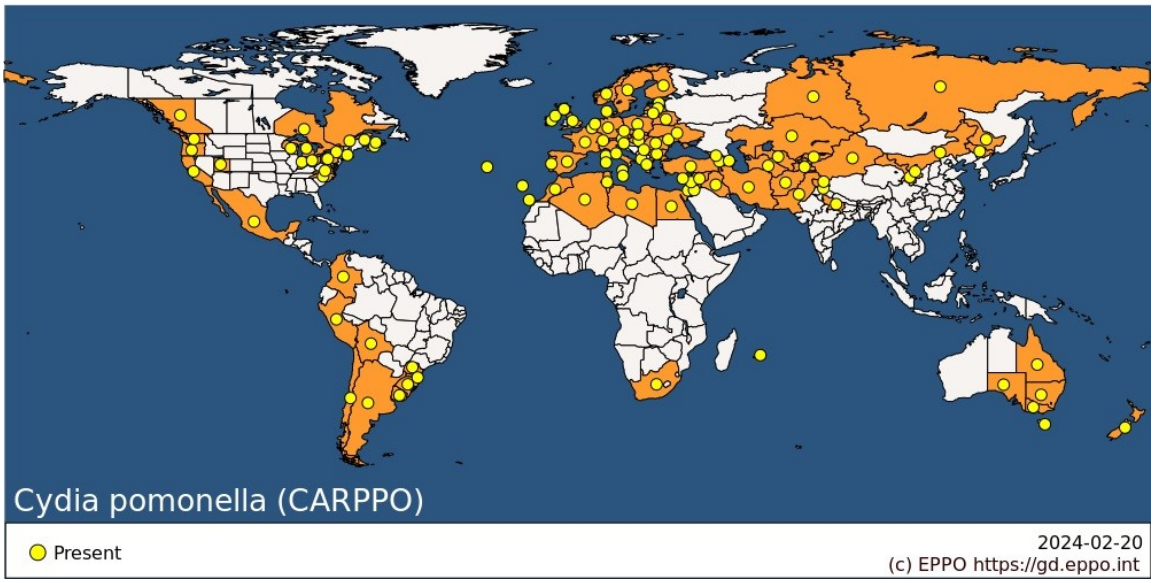

Figure 3. Global distribution of *Cydia pomonella* (EPPO Global database 2024).

Listed as a quarantine pest in several countries, including China, Japan, the UK, and Greece, strict measures are enforced to prevent the introduction and spread of *C. pomonella* (Guo et al. 2021). Despite its limited flight capacity, the spread of this pest to other regions is supported by human migration and transport, as well as the transport of planting material, infested fruits and packaging materials (Jiang et al. 2018).

Cydia pomonella neonates bore into premature apples and develop into fifth instars. Some may transfer to adjacent apples, while others mature to the fifth larval stage (Barnes 1991). Mature larvae leave the fruit to pupate for the next generation or become overwintering diapause larvae, that hide in cocoons under tree bark (Abaajeh 2014). Eggs hatch in 7 - 15 days, with neonate larvae infesting fruit for 3 - 4 weeks. One to four generations occur in apple-growing regions, with females laying 30 - 60 eggs primarily on leaves or fruit (Odendaal et al. 2016; Pickel et al. 2020). Pupation and adult emergence are triggered by spring temperature conditions, typically requiring a degree-day accumulation of around 222.2 °C and a minimum ambient temperature above 11.1 °C (Pickel et al. 2020).

Biological control tools for *C. pomonella*

According to the European Commission (EC), Integrated Pest Management (IPM) is defined as the careful consideration of all available methods to protect plants. It integrates appropriate measures to control populations of harmful organisms while maintaining economically and ecologically justified levels of plant protection (EU Regulation 2009). IPM involves the combination of different plant protection methods, one of which is the use of microbial antagonists to biologically control plant pathogens, a practice categorized as biopesticides (Chandler et al. 2011; Tiwari and Tripathi 2014). The rationale for using biopesticides in IPM lies in their effectiveness in safeguarding crop yield and quality (Kumar et al. 2021). However, the terminology used to describe control success is often unclear and prone to misinterpretation, with terms frequently used interchangeably and inaccurately. Consistent and precise language is crucial for comparing the results of various studies, as it defines the evaluation criteria for biopesticides. According to the EPPO Guideline PP 1/214 (4), this thesis defines three key terms related to control success, namely effectiveness, efficiency, and efficacy. Effectiveness refers to the ability to effectively control a pest, providing insights into the relationship between the achieved and desired outcomes. Efficiency assesses the quality of control, emphasizing the resources needed to accomplish the goal. Efficacy takes into account the overall impact of both positive (effectiveness) and negative effects (such as phytotoxicity, resistance development, effects on non-target organisms,

human safety, ease of use, and compatibility with cultural practices), resulting in a net agricultural benefit.

Biological control, a key element of IPM (EU Regulation 2009), involves the use of living organisms, botanicals and semiochemicals to reduce the population density or impact of specific pest organisms, thereby reducing their abundance or damage potential, highlighting the use of living organisms, including entomopathogenic fungi, in pest management strategies (Deguine et al. 2021). These control methods are applicable to both organic and integrated production systems. Various methods have been developed for controlling *C. pomonella*, including the Sterile Insect Technique (SIT), augmentative release of parasitoids and the application of the baculovirus *Cydia pomonella* granulovirus (CpGV), *Bacillus thuringiensis* (Bt), entomopathogenic nematodes, and entomopathogenic fungi (Cross et al. 1999; Agnello 2004; Kienzle et al. 2008; Pérez-Staples et al. 2021; Mantzoukas et al. 2022).

The Sterile Insect Technique (SIT) is a biological approach used for targeted pest management by introducing a significant number of sterile male organisms into the ecosystem (Balaško et al. 2020). The sterile males are rendered incapable of reproduction through exposure to ionising radiation, chemical agents, or genetic modification techniques (Robinson 2005). The method was first applied in 1994 to reduce *C. pomonella* populations in the Okanagan Valley and Similkameen Valley within southern British Columbia, Canada. The implementation of an area-wide Sterile Insect Release (SIR) programme resulted in a notable reduction in local *C. pomonella* populations. Subsequently, SIT has been employed for *C. pomonella* management endeavors in regions such as South Africa and New Zealand (Horner et al. 2016). Nevertheless, challenges such as economic implications and the efficacy of SIT in high pest density environments have posed ongoing hurdles to the sustained success of this initiative (Balaško et al. 2020).

For augmentative biological control of *C. pomonella*, CpGV, parasoid wasps, *Bacillus thuringiensis* (Bt) and entomopathogenic nematodes (EPNs) have been used as microbial agents (Lacey and Unruh 2005). CpGV is registered in almost all countries where pome fruits are grown. It is extensively utilized and highly effective as a biocontrol agent in integrated and organic apple, pear, and walnut farming (Huber 1998;

Lacey et al. 2008). It is an excellent alternative to nonselective chemical insecticides with no impact on beneficial organisms (Lacey et al. 2008). CpGV offers several advantages as a biocontrol agent: it can be easily formulated, visualised by dark-field light microscopy, and stored for long periods, making it a versatile and practical solution (Lacey et al. 2015). The main disadvantages of CpGV are its sensitivity to solar radiation (Lacey and Arthurs 2005) and the need for frequent reapplication (Balaško et al. 2020).

Parasitoid wasps, including *Ascogaster quadridentata*, *Microdes rufipes*, *Mastrus ridibundus*, *Liotryphon caudatus*, and *Trichogramma*, are key to the control of *C. pomonella*. They lay their eggs on or inside *C. pomonella* larvae, adults, or eggs, leading to the eventual death of the host (Eggleton and Gaston 1990). In recent research, Mátray and Herz (2022) found that suitable food sources like sugar solutions significantly enhance the survival and reproduction of the parasitoid *A. quadridentata* compared to *C. pomonella*. *A. quadridentata* also shows improved survival and effectiveness against codling moths when fed on specific flowers such as buckwheat and coriander (Mátray and Herz 2021; Mátray and Herz 2022). On other hand, *C. pomonella* showed relatively lower reproduction rate by sugar feeding, suggesting that diverse flowering plants could enhance this parasitoid's natural pest control role in orchards (Mátray and Herz 2021). Successful applications of parasitism by species such as *M. ridibundus* and *A. quadradentata* have been also observed in some US states (Lacey and Unruh 2005). Experimental releases of *Trichogramma* species in German apple orchards resulted in a notable reduction of *C. pomonella* populations by 53 – 84 % (Lacey and Unruh 2005). Moreover, the introduction of parasitoids can simultaneously control other orchard pests. However, while beneficial, their sole application may not be sufficient for comprehensive *C. pomonella* control in economically important orchards (Balaško et al. 2020).

Bacillus thuringiensis (Bt) is also widely used as biopesticide (Lacey and Unruh 2005). Although Bt strains have been shown to be highly pathogenic to *C. pomonella* larvae in laboratory tests, control efficacy in orchards appears to be low. The reason for this is that a lethal dose of Bt toxin is unlikely to be ingested by newborn larvae when they are feeding (Balaško et al. 2020).

Steinernematidae and *Heterorhabditidae* are the families of entomopathogenic nematodes (EPNs) extensively researched for controlling cocooned *C. pomonella* larvae (Odendaal et al. 2016). Commercialization of *Steinernema carpocapsae* has occurred, while cold-tolerant *Heterorhabditis* species are utilized in temperatures below 15° C (Lacey and Unruh 2005). The main obstacles for successful *C. pomonella* control with EPNs are low fall temperatures and desiccation of the infective juvenile stage of EPNs before they have penetrated the host's cocoon (Balaško et al. 2020). Application of infective juveniles of *Steinernema feltiae*, along with adjuvants like wood flour foam or Barricade II fire retardant gel, significantly increased *C. pomonella* larval mortality to levels between 85 % and 97 % (Lacey et al. 2010).

Since the mid-20th century, significant advancements have been made in developing entomopathogenic fungi (EPF) as BCAs in IPM. The phylum Ascomycota, especially the order Hypocreales, is prominent in this area. This order includes genera like *Beauveria*, *Metarhizium*, and *Cordyceps*, which are widely used due to their natural ability to infect and manage pest populations. Most EPF originate from soil environments and employ a similar mode of action. (Figure 4). When fungal conidia encounter a host, conidia (or in some cases blastospores) attach to the insect cuticle by electrostatic and chemical forces. Upon rehydration and chemical stimuli they germinate and form a germ tube that can form a specialised structure, the appressorium (i.e., an enlarged cell extension containing hydrolytic cuticle-degrading enzymes) or penetration peg, which allows the growing hyphae to penetrate the host integument (Inglis et al. 2001; Ortiz-Urquiza and Keyhani 2013). The infection process of certain fungi involves an complex mechanism, starting with the appressorium or penetration peg, which allows the fungus to penetrate the outer layers of the host through a combination of mechanical pressure, hydrolytic enzymes (such as proteases, chitinases, lipases) and other factors (such as oxalate) (Charnley 2003; Ortiz-Urquiza and Keyhani 2013). This penetration process facilitates the entry of the fungus into the nutrient-rich haemolymph. In this environment, the fungus undergoes a significant morphogenetic transformation from filamentous growth to a yeast-like form, known as hyphal bodies or blastospores. This transformation enables the fungus to efficiently exploit nutrients, colonise internal tissues, and evade the host's immune defences. During this phase, the fungus can also produce toxic metabolites that suppress the host's immune response,

increasing its ability to colonise and ultimately leading to the host's death (Inglis et al. 2001; Reddy et al. 2013; Abaajeh 2014; Sharma et al. 2020).

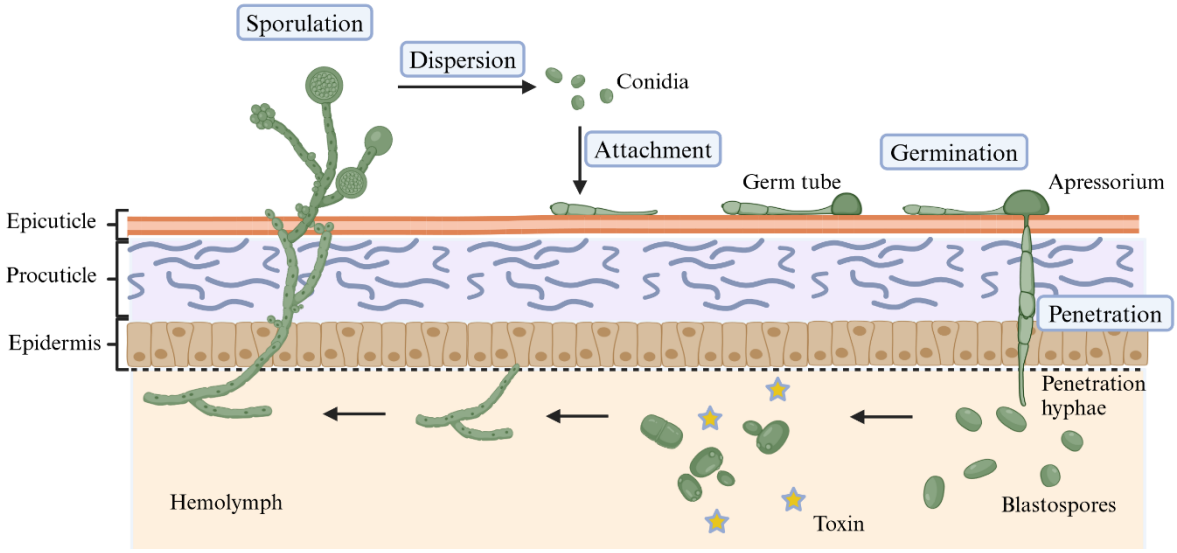

Figure 4. Overview of the infection process and life cycle of entomopathogenic (Hypocreales) fungi in the insect cuticle. First, conidia attach to the insect epicuticle; second, spore germination results in an appressorium that penetrates the host cuticle, followed by hyphae that penetrate into the insect epidermis; next, the fungus multiplies in the host and produces toxins; to complete the infection, the fungal cells differentiate into yeast-like cells called blastospores, and later mycelium grows, sporulation occurs and by dispersal produces conidia which, under suitable environmental conditions, emerge from the insect and spread to other insects. The arrows indicate the direction of fungal growth. The image was generated using BioRender.com.

Beauveria, Metarhizium, and *Cordyceps* account for the largest share of the mycopesticide market (Lacey et al. 2015). EPF are now registered in many countries worldwide and provide alternatives to chemical insecticides (Faria and Wraight 2007). For example, EPF approved as plant protection products in the EU include *Beauveria bassiana* (strain ATCC 74040), *Metarhizium brunneum* (strain Ma 43) and *Cordyceps fumosorosea* (strain FE 9901) (European Commission, accessed on 17.02.2024). On the other hand, Montesinos (2003) highlighted that bureaucracy and high costs are major obstacles to the registration of biopesticides in Europe. For instance, it took seven years for *C. fumosorosea* to be approved (1994-2001). Biological control systems face challenges due to their inherent specificity, where success hinges on interactions among the pest, BCA, and host plant (Montesinos 2003).

Beauveria

Beauveria are filamentous fungi belongs to the taxonomic order Hypocreales, family Cordycipitaceae (Humber 2012). Important species within this genus include *Beauveria bassiana*, *Beauveria brongniartii*, *Beauveria amorpha* and *Beauveria caledonica*. Microscopic examination reveals conidiogenous cells with globose bases, elongated and globose conidia, denticulate rachis, and globose conidia (< 3.5 μm diameters). Spore balls are dense clusters of numerous conidiogenous cells with unicellular, hyaline conidia (Sinha et al. 2016). *Beauveria bassiana*, occurs naturally in soil worldwide, is known to cause white muscardine disease in various insect species, making it an entomopathogenic fungus (Sinha et al. 2016). Many isolates of *Beauveria* show high host specificity. On the host, the fungal mycelium emerges through the exoskeleton and forms a dense white coating on the surface, sometimes forming synnemata (An et al. 2021). In the case of *C. pomonella* control, field investivgations showed that 23.6 % of *C. pomonella* specimens examined in Germany and Austria were infected by *B. bassiana* (Zimmermann et al. 2013). It was reported that *C. pomonella* larvae exposed to commercial preparations of *B. bassiana* strains induced up to 96 % mortality (Gürlek et al. 2018).

Metarhizium

Metarhizium, a genus within the phylum Ascomycota of the order Hypocreales, family Clavicipitaceae, comprises a diverse group of soil-borne EPF (Humber 2012; Sinha et al. 2016; Iwanicki et al. 2019). For example, *M. anisopliae*, *M. brunneum*, *M. robertsii*, *M. guizhouense*, and *M. pingshaense* are the best known EPF (Zimmermann 2007; Iwanicki et al. 2019; Stone and Bidochka 2020). This genus is characterised by a branching pattern of the conidiophores, dense interwinding, and alignment of conidiogenous cells with rounded to conical apices, arranged in a dense hymenium. Conidia are aseptate, cylindrical or ovoid, forming chains, usually aggregated in prismatic or cylindrical columns, or a solid mass of parallel chains, pale to bright green to yellow-green in colour (Zimmermann 2007; Sinha et al. 2016). *Metarhizium robertsii*, a member of the filamentous ascomycetes known as pyrenomycete fungi, includes both insect and plant pathogens (Stone and Bidochka 2020). *Metarhizium robertsii* has been extensively studied and utilised as an insect pathogen for biocontrol, and has significant potential in pest management strategies (Zimmermann 2007; Sinha

et al. 2016; Iwanicki et al. 2019; Stone and Bidochka 2020). Abaajeh and Nchu (2015) conducted an isolation study of different EPF from soil samples, using *C. pomonella* larvae as an insect baits. Subsequently, they evaluated some of these fungi against the larvae of the pest. According to their field study, *M. robertsii* could be isolated in 51 % of soil samples in South Africa and they revealed that two strains of *M. robertsii* (MTL151 and GW461) caused 85 % larval mortality (Abaajeh and Nchu 2015; Gürlek et al. 2018).

Cordyceps

The genus *Cordyceps* belongs to the taxonomic order Hypocreales and family Cordycipitaceae and includes important species such as *C. fumosorosea*, *C. farinosa*, *C. javanica* and *C. lilacinus* (Mascarin et al. 2010; Humber 2012; Sinha et al. 2016; Mascarin et al. 2018). Conidiophores of many species are typically well-developed, synnematous, septate structures with whorls of divergent branches and conidiogenous cells (phialides). The conidiogenous cells are flask to oval-shaped or subglobose with distinct necks, borne singly or in whorls, and the conidia are unicellular and typically hyaline to pale in colour (Sinha et al. 2016). *Cordyceps* exhibit strong epizootic activity against *Bemisia tabaci* and *Trialeurodes* under both greenhouse and field conditions. Tropical and subtropical agricultural soils have been identified as having the greatest potential for its application as a biocontrol agent (Mascarin et al. 2010; Sinha et al. 2016; Mascarin et al. 2018). *Cordyceps farinosus*, which is commonly found on overwintering *C. pomonella* larvae, exhibits high mortality, particularly when combined with *B. bassiana* (Cross et al. 1999). A study by Lacey and Unruh (2005) using *B. bassiana* to manage codling moth populations in the former Soviet Union highlighted the effectiveness of *C. farinosus* and *B. bassiana* in combination with chemical pesticides. Although efforts to employ *B. bassiana* as a microbial control agent for various insects have had moderate success (Goettel et al. 2005), the combination of these two EPF, particularly for the control of *C. pomonella,* is not widely used in North America or Western Europe (Lacey and Unruh 2005). A study conducted in the southern region of Sweden on the diapause of larvae and pupae of the codling moth revealed that 29.5 % of the species were identified as *C. farinosa* (Subinprasert 1987; Zimmermann et al. 2013). A strain of *C. fumosorosea* (Apopka-97), isolated from a

mealybug in Florida, has been commercially developed as a BCA (tradenamed PreFeRal in Europe and PFR-97 in the USA) by Biobest N.V., Belgium, and is recommended for the control of greenhouse whiteflies (Sterk et al. 1996; Faria and Wraight 2001). Field evaluation of *Cordyceps* against pests such as *Cacopsylla pyricola* is crucial for further investigation, especially if formulations can enhance activity under lower humidity conditions (Cross et al. 1999).

Exploring EPF: A collaborative solution to control *C. pomonella*

It is evident that each of the techniques employed to control *C. pomonella*, has its own set of advantages and disadvantages. For instance, in 2004, the first case of resistance against CpGV was detected in two organic orchards in Germany (Fritsch et al. 2020). This has led to the realization that resistance management has become an important factor not only for organic farming, but also for the development of new tools for combination strategies in *C. pomonella* control. In the orchards, where the population of *C. pomonella* has developed resistance to CpGV, the level of infestation was found to be extremely high, reaching a level that could not be controlled (Kienzle et al. 2008). Another limitation of CpGV is its sensitivity to solar radiation (Lacey et al. 2004; Lacey and Arthurs 2005). Consequently, it is necessary to apply the treatment frequently (Balaško et al. 2020). If the combination of resistance management to control *C. pomonella* is being considered, EPF can be used as a new tool. As they have different ways of infection, they can act as natural regulators of insect pests. They produce secondary metabolites with antibacterial, insecticidal, and antioxidant properties, which enable them to adapt readily to environmental changes (Isaka et al. 2005; Wan et al. 2019; Ahmad et al. 2024). Moreover, following epizootics, they frequently inhabit the soil, serving as the origin of fatal diseases in their hosts, thus playing a vital role in environmental sustainable biocontrol initiatives against insect pests in agriculture and forestry (Goettel et al. 2005; Lacey et al. 2015; Ahmad et al. 2024). EPF can be applied to the system before the *C. pomonella* larvae enter to the pupae stage. This could be a potential solution for the control of *C. pomonella* in orchards. While numerous mycoinsecticides have been developed for commercial purposes worldwide, their effectiveness as biocontrol agents, especially against *C. pomonella*, is still in the early stages of development (Ahmad et al. 2024).

Developing EPF as biocontrol agent

Biological control agents (BCAs), comprising beneficial microorganisms as EPF and represent a new generation of plant protection products. A number of factors must be considered for a successful BCA. These include compatibility with field routine practice, storable nature, adaptability to different field conditions and soil types, has to provide reproducible results and safety for humans, animals and plants (Blümel et al. 2023). This implies that following the identification of the optimal strain for the desired effect on a target crop, its production is initiated in accordance with the design of a specific formulation (Bashan et al. 2014). The development of these EPF to BCA involves multiple steps, including the isolating of the microorganisms in pure culture, the screening of their efficacy through various bioassays, and the conduct of trials under real application conditions (Montesinos 2003). The entire process can be depicted in Figure 5.

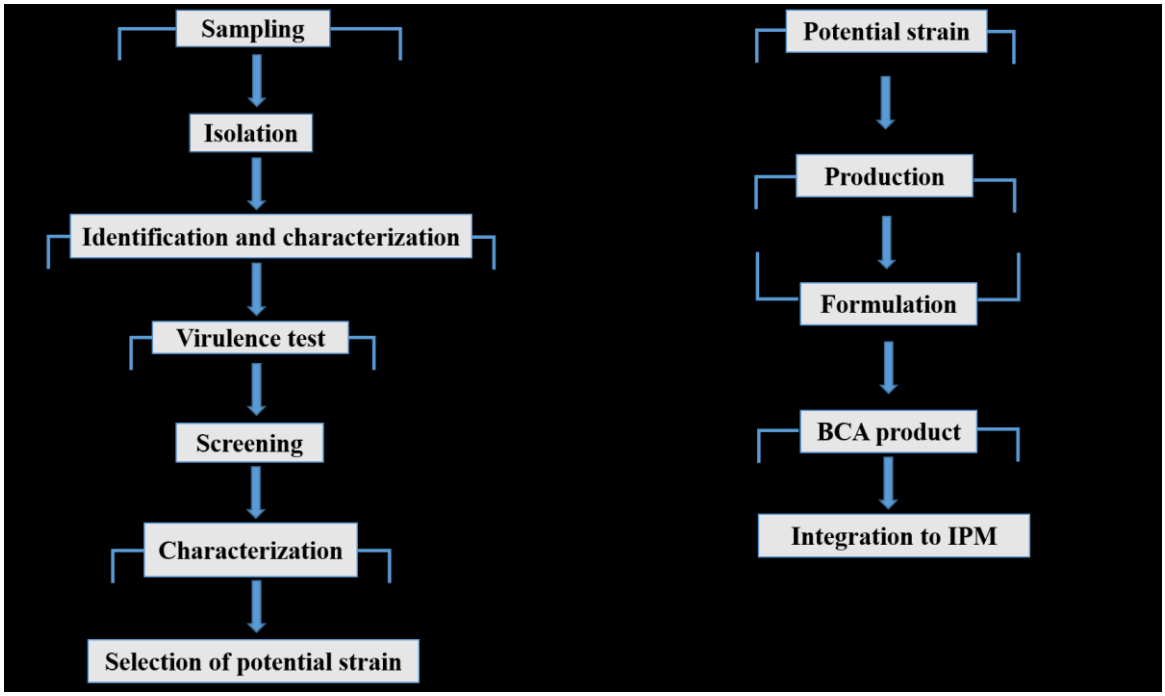

Figure 5. The procedure for screening and developing entomopathogenic fungi (EPF) to biological control agent (BCA) (modified from Montesinos (2003) and Köhl et al. (2011)).

The sampling and isolation of the EPF represents the most crucial aspect of the entire process. The success of this endeavour is contingent upon the selection of an appropriate location. Soil is a significant reservoir for EPF, and these soil fungi play a

pivotal role in biological control (Lacey et al. 2015). EPF are capable of adapting to their environmental conditions including specific climatic conditions and habitat types. Therefore, it is important to isolate and identify local fungal strains for the purpose of controlling insect pests in related areas (Gürlek et al. 2018). The presence of EPF is contingent upon a number of factors, including soil type, plant species, and cultivation methods. Soil provides protection against UV light, high temperatures, and other environmental factors that could impede their dispersal (Mantzoukas et al. 2022). Following the isolation of the sample, the critical step is the screening for antagonistic activity against the target pest or pathogen (Montesinos 2003). Typically, during this stage, a collection of isolated microorganisms is considered for efficacy bioassay towards the pest in a small-scale with controlled environment (*in vivo*, *in vitro* or planta tests). This process is suitable for the selection of potential BCA (Montesinos 2003). The subsequent step is to identify and characterise the isolated microorganisms. For identification, it is advisable to examine their DNA or physical characteristics in order to attain species-level accuracy (Köhl et al. 2011). Furthermore, there are certain criteria for characterising the selected microorganisms. Moreover, characterisation of potential EPF is also important for virulence against targeted pests (Montesinos 2003). According to Mantzoukas et al. (2022), a set of morphological, physiological, and behavioural parameters determine insect susceptibility to potential EPF, including stress tolerance against environmental factors (pH, UV light, temperature, and humidity).

Formulation can improve the characteristics of fungal preparations or simplify their application. However, their effectiveness ultimately depends on the inclusion of strong biological material that is suitable for the intended purpose (Lacey et al. 2015). The choice of production method depends on the type of inoculum needed, and different isolates may exhibit varied growth characteristics on various production media (Charnley 2003). Microorganism production typically occurs through liquid or solid fermentation processes, although exceptions exist such as viruses. It is evident that fermentation parameters play an important role in determining the efficacy and shelf life of BCAs in formulations (Köhl et al. 2011; Lacey et al. 2015). The nature of the agent (bacteria, fungi or yeast, nematodes, or viruses) determines the industrial scale-up method, which typically involves *in vivo* or *in vitro*, solid-state, submerged liquid fermentation or biphasic fermentation. These methods benefit from advancements in the

pharmaceutical and food industries (Mascarin 2015). Regardless of the fermentation method employed, the objective is to achieve a high yield at minimal cost. This is often achieved by using molasses, peptones or industrial-grade protein hydrolysates as culture medium (Montesinos 2003; Mascarin et al. 2010). Furthermore, submerged fermentation is used as a cost-effective and easy technology for the mass production of secondary metabolites, antibiotics and/or organic acids (García-Estrada et al. 2016). Following the mass production of the product through fermentation process, it is necessary to stabilise the product for long-term preservation and to make the product suitable for tolerating environmental stress factors (Montesinos 2003; Mascarin et al. 2010; Mascarin 2015). In the formulation process, it's crucial to account for stabilisation methods, which entail selecting appropriate drying technology and compatible additives to enhance the formulation's stability (Mascarin 2015; Stephan et al. 2021).

Regardless of the preservation method used for microbial cells, the final product should undergo formulation prior to use. This process involves incorporating biocompatible additives that enhance the survival, application and stability of the product, or attract and stimulate feeding in the target pest. These additives may include wetting and dispersal agents, nutrients, and ultraviolet light- or osmotic-protection agents (Boyetchko et al. 1998). Some additives assist microbial cells in resisting environmental stressors, such as damage from ultraviolet light, limited water availability, nutrient scarcity, or other forms of stress. In many cases, this formulation technology resembles that used for chemical pesticides and pharmaceutical products (Montesinos 2003).

In terms of both preservation and stability enhancement, the drying process represents the most suitable method for EPF (Mascarin 2015). Furthermore, an understanding of the interactions between fungal propagules and their hosts and their environment is of vital importance for the formulation development process. Factors such as resistance to desiccation stress and storage stability guide the formulation design process. Components added during growth or drying can enhance desiccation tolerance. The drying method selected can affect fungal viability and storage stability, as dehydration damage can harm cell integrity and metabolism, which in turn impacts viability (Montesinos 2003; Jaronski 2010; Jackson et al. 2010; Mascarin 2015). At the industrial level, cost-effective drying methods such as spray- or fluidised bed-drying are preferred

(Montesinos 2003). Spray-drying employs high temperatures for the rapid drying of large quantities of culture (Droby et al. 2016), rendering it an appropriate method for heat-resistant endospore-producing BCAs. However, there has been limited research into the preservation of blastospores or submerged conidia of EPF. In this context, the term “submerged spores” is used to refer to both blastospores and submerged conidia. Stephan and Zimmermann (1998) were the first to utilise the spray-drying process to investigate the preservation of submerged spores (including blastospores) of *M. anisopliae*. Another study on the use of spray-drying for *B. bassiana* was conducted by Mascarin et al. (2016). During the spray-drying process, a blastospore suspension was enhanced with skim milk, with or without ascorbic acid (Mascarin et al. 2016). This process generates microparticles of blastospores and a supportive matrix under conditions that are relatively harsh for drying sensitive microbes (Dietsch et al. 2021). Moreover, viability showed an upward trend with higher skimmed milk powder concentrations and lower inlet temperatures. In addition, no significant difference in viability was observed between spray-dried submerged spores of *B. bassiana* and *M. flavoviride* (Stephan and Zimmermann 1998).

Freeze-drying remains a traditional method of extending the shelf life of BCAs, and there have been no recent studies on freeze-dried blastospores (Dietsch et al. 2021). This process is commonly used to preserve bacterial cultures in research and industry (Morgan et al. 2006; Stephan et al. 2016). However, notable research has shown that the survival of blastospores of *C. fumosorosea* during freeze-drying can be enhanced by including whole milk, glucose, or casamino acids in the suspension (Cliquet and Jackson 1999; Cliquet and Jackson 2005). While freeze-drying maintains high cell viability for bacteria, yeast and EPF, it is more costly than the other drying processes (Montesinos 2003; Droby et al. 2016).

Another type of drying that has become popular in biocontrol is fluid-bed drying. This method is a suitable option for heat-sensitive microorganisms due to its relatively low drying temperatures (Droby et al. 2016). Previously developed by Stephan et al. (2021) and subsequently utilized by Seib et al. (2023), a granule formulation technique employing entomopathogenic fungi was evaluated for its effectiveness with the *M. brunneum* (JKI-BI-1450). The process involved cultivating the fungus in liquid medium

and then coating autoclaved millet with a thin layer of biomass using fluid-bed drying (Stephan et al. 2021; Seib et al. 2023). Regardless of the preservation method, EPF must be formulated before use, typically with biocompatible additives to enhance survival, application, and stability. These additives include wetting agents, dispersal agents, nutrients, and UV or osmotic protection agents (Montesinos 2003). Stephan et al. (2001) formulated granules using fluid-bed coated biomass of three entomopathogenic fungi genera (*B. bassiana*, *M. brunneum* and *C. fumosorosea*). Autoclaved millet served as the core particle, with a thin layer of fungal biomass applied as coating for controlling soil- dwelling pests.

The final step involves testing the most promising formulation of the selected candidate on crops in different locations and seasons, and to incorporate it into current or developing crop protection strategies for comprehensive evaluation (Köhl et al. 2011).

Objectives of this study:

This thesis aims to develop effective biological control agent (BCA) using entomopathogenic fungi (EPF) to control *C. pomonella.* The research is divided into three focused chapters.

In Chapter I, the potential of EPF as BCAs against *C. pomonella* was assessed by investigating the occurrence, prevalence, and effectiveness of EPF strains from an orchard. This involved isolating 32 EPF strains from soil and one more strain from strain collection of Julius Kühn Institute (JKI) identifying them by molecular sequencing of the ITS region, and assessing their virulence against *C. pomonella* larvae. The study was further narrowed down to selected strains exhibiting chitinase activity, an indicator of potential virulence, to assess their resilience to environmental stress. From these, promising strains were selected for a detailed study of their growth behavior in different media compositions, with the aim of optimising their application in controlling *C. pomonella* in agricultural settings.

In Chapter II, the research shifted to improving the effectiveness of the entomopathogenic fungus JKI-BI-1496 (*Cordyceps fumosorosea*) against *C. pomonella* by amending the media with chitin. The effects of chitin, chitosan and colloidal chitin

on spore virulence, spore growth, biomass production, germination, and resistance to sunlight and processing conditions such as freeze-drying, were investigated to optimise the effectiveness of *C. fumosorosea*.

In Chapter III, the challenge of the simulated sunlight reduction in spore germination, observed when chitin derivatives were added to the media, was addressed. Therefore, the use of water-soluble sunlight protectants was investigated to improve the environmental resistance of *C. fumosorosea* spores during spray-drying, with the aim of reducing sunlight sensitivity, extending shelf life and promoting sustainable pest management practices. Overall, this thesis contributes to the field of agricultural pest management by investigating and improving the utility of EPF, particularly *C. fumosorosea*, as a sustainable and effective method for controlling *C. pomonella*.

Chapter I

Isolation, characterisation and variation of growth parameters of entomopathogenic fungi from soil samples of an apple orchard

Abstract

Entomopathogenic fungi (EPF) have demonstrated their potential as important biological control agents (BCAs) against pest species. Here, the investigation was done to not only study the occurrence and prevalence of EPF from an organic apple orchard in Germany against *Cydia pomonella*, but also the differences in their virulence and the role of chitinase production as a factor influencing their effectiveness. To this end, 32 EPF strains were isolated from soil samples using the insect bait method and one strain was taken from the strain collection from JKI. The EPF were identified using microscopic examination and molecular sequencing of the ITS region, BLOC region and elongation factor-1-alpha. Initial screening was based on mortality (≥ 80 % mortality) against *C. pomonella* using a virulence test. Later, 13 selected strains were tested for chitinase activity using an agar-chitin plate assay, with four potential strains showing higher (≥ 1) chitinase activity. These four strains, JKI-BI-1496 (*Cordyceps fumosorosea*), JKI-BI-2620 (*Beauveria bassiana*), JKI-BI-2642 (*Beauveria bassiana*) and JKI-BI-2647 (*Metarhizium robertsii*) were selected for experiments with environmental stress factors, such as temperature (5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C) and simulated sunlight. In addition, various growth parameters such as spore productivity, production of dry biomass and optical density of fungi were evaluated in four different media (malt peptone, malt peptone with 1 % chitin, potato dextrose and potato dextrose with 1 % chitin) to observe the production potential of the different strains. JKI-BI-1496 showed higher tolerance for environmental stress factors (higher radial growth at 25 °C and 20.4 % germination after 3 hours of sunlight simulation) compared to other three strains. On other hand, adding 1 % chitin to different media did not influence spore productivity, but influence dry biomass and optical density of all strains.

Introduction

The codling moth *Cydia pomonella* L. (Lepidoptera: Tortricidae) is an important pest in perennial apple production worldwide, especially in Europe. As the insects live mostly in apples during larval development, they are well protected from both insecticidal sprays and predators, making control difficult (Losel et al. 2000; Abaajeh and Nchu 2015). Concerns about safety, environmental impact and sustainability of synthetic pesticides have led to the development and use of control methods in the context of integrated pest management (IPM). The use of *Cydia pomonella* granulovirus (CpGV), for example, has increased in recent years due the growing interest in organic farming (Lacey et al. 2008). This approach aligns with IPM strategies in Europe, emphasizing reduced synthetic pesticide reliance. Effectiveness varies by application specifics and existing pest conditions. Despite its importance, there is limited research on natural antagonists against *C. pomonella* in Europe (Lacey et al. 2008; Zimmermann et al. 2013; Belien 2018; Balaško et al. 2020).

Entomopathogenic fungi (EPF) are prevalent in various agricultural soils and are particularly valuable in organic apple orchards for controlling pests like the apple codling moth (Santos et al. 2022). Soil conditions, including pH, moisture and nutrient levels, play a crucial role in the dynamics of EPF, influencing their growth, survival, and dispersal (Lestari et al. 2016; Gürlek et al. 2018; Litwin et al. 2020; Yadav et al. 2021; Islam et al. 2021). EPF can survive in the soil for long periods as spores, which germinate under favorable conditions to infect pests (Islam et al. 2021).

The diversity of EPF within an orchard can vary with the genetic background of different apple varieties, which may attract distinct EPF species or strains. This diversity is further influenced by factors such as microclimates, soil types and orchard management practices (Kasambala Donga et al. 2021). When it comes to isolating EPF from soil samples, the soil type may be also important factor, as it can affect the shape and size of the spores, which are important for efficacy (Zimmermann 2007a). Local EPF strains, which are often more ecologically compatible with specific pests, tend to be more effective than exotic strains (Gürlek et al. 2018). However, the use of chemical pesticides, such as fungicides and herbicides, has been shown to reduce the prevalence

of EPF by directly affecting their survival and indirectly by altering soil properties and microbial communities (Bruck 2010; Kasambala Donga et al. 2021). Therefore, the identification and optimization of local EPF strains are essential for developing sustainable IPM strategies and maintaining the health of soil ecosystems (Litwin et al. 2020; Yadav et al. 2021; Islam et al. 2021).

Several strains of EPF have been marketed as biopesticides for the control of various insect pests. *Beauveria*, *Metarhizium*, and *Cordyceps* are the most important genera of EPF used as biocontrol agents worldwide (Sharma et al. 2020). The main important desirable characteristics of EPF are: low environmental impact, efficacy throughout the insect's life cycle to control the specific pest and mass producibility (Jaronski 2014; Gebremariam et al. 2021). EPF penetrate the cuticle of the insect during infection by an interplay of enzymes and mechanical pressure of the germ tube (Rajula et al. 2020). One of the enzyme classes produced by *Cordyceps fumosorosea* Wize (formerly *Isaria fumosorosea*) (Hypocreales: Cordycipitacea) are the chitinases (Ali et al. 2010b), which can attack the insect exoskeleton composed of chitin. Optimizing chitin utilization may therefore improve fungal growth and survival, which in turn increases EPF virulence. Some fungi have chitin-binding proteins that can help them adhere to the insect cuticle (Ali et al. 2010b). These proteins can be optimized to improve the attachment of the fungi to the insect. In addition, EPF can also use chitin as a carbon source (Lv et al. 2023).

The overall objective of the current study was to isolate and characterize soil-borne EPF from an apple orchard in Hesse, Germany. Soil samples were collected from different areas of an apple orchard, which were then processed and cultured in the laboratory to isolate EPF using the insect bait method. First objective of the current study was to gain insight into the effectiveness of isolated EPF against *C. pomonella*, though virulence test and chitinase activity test for selecting the potential EPF. As a variety of EPF were isolated, the second objective was to characterize the molecular and morphological aspects. Third objective was to study their growth parameters under environmental factors such as temperature and simulated sunlight and different media compositions (influence of chitin on spore productivity, dry biomass and optical density).

Material and Methods

Site specification and soil sample collection

To investigate the natural occurrence of EPF, soil samples were collected in Schaafheim, Germany (49°55'11.5"N, 8°57'57.5"E) in autumn, October 2019. The apple orchard is controlled and certified by both the Kontrollverein ökologischer Landbau e.V. and the Demeter Association. Several apple varieties (Braeburn, Delbarestivale, Elstar, Fuji, Gala, Idared, Jonagold, Pilot, Pinova, Rubinette, Topaz) are grown in this apple orchard (approximately 1 ha in size). To collect the soil samples between the rows, the top 15 cm of the soil was removed with a drill with diameter of 6 cm (Ehlert & Partner GbR, Niederkassel-Rheidt, Germany) made of steel. Two samples were taken from each soil sample for the insect bait method with *Galleria melonella* L. (Lepidoptera: Pyralidae), the greater wax moth and *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), mealworms. A total of 64 soil samples were taken in sealable plastic bags. For disinfection, the drill was wiped with Bacillol® AF each time samples were taken from different rows. The samples were stored at 4 °C until further processing.

Rearing of *Galleria mellonella*

Galleria mellonella larvae used for the *Galleria* bait method came from the Julius Kühn Institute's own rearing. All stages were kept at 20 - 25 °C in the dark. Adult moths and larvae of stage L_3 and higher were kept in plastic boxes (57 cm × 39 cm × 28 cm) with wire gauze for ventilation. The first and second instar larvae were reared in jars (22 cm × 10 cm), in which ventilation was also ensured by wire mesh (0.1 mm^2), but with an additional filter paper underneath to prevent the larvae from escaping. To avoid daylight, boxes and mason jars were placed in cardboard boxes. The diet for the first and second instar larvae consisted of 500 g honey (liquid) (Alnatura GmbH, Darmstadt, Germany), 500 g glycerin (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), 139 g beeswax (Stübener Kräutergarten, Dornbirn, Austria), 722 g whole meal flour (Herrnmühle, Reichelsheim, Germany) and 222 g brewer's yeast powder (Herrnmühle, Reichelsheim, Germany). The beeswax was melted and all the ingredients were mixed together. Flattened balls were formed from this mass, which fit into the preserving jars (approx. 6 cm in diameter). For the older larvae, the diet contained 500 g oat flakes

(Alnatura GmbH), 500 g honey (liquid) (Alnatura GmbH), 500 g glycerin (Carl Roth GmbH + Co. KG), 200 g brewer's yeast powder (Herrnmühle, Reichelsheim, Germany), 200 g skimmed milk powder (J. M. Gabler-Saliter Milchwerk GmbH and Co. KG, Obergünzburg, Germany) and 200 g wheat germ (Herrnmühle, Reichelsheim, Germany). First, the dry ingredients were hand mixed and then honey and glycerin were added. The diets were stored in a refrigerator. Filter paper strips were clamped into the moth boxes for laying eggs. The papers with the eggs were removed twice a week and placed in a mason jar with a food ball. After the larvae reached the L_3 stage, they were transferred to a plastic box with older larvae diet and fed twice a week. About 50 larvae were transferred to a separate box without food once a week. These larvae were allowed to pupate for further rearing.

Isolation of EPF from soil samples

Prior to EPF isolation, any kind of impurities (roots, leaves, seeds and large stones) were removed and all clods of soil were broken by hand and mixed well under sterile condition. The samples were then filled into two 50 mL-Schott-Duran bottles (VWR International GmbH, Darmstadt, Germany), with perforated caps. Zimmermann's (1986) "*Galleria* bait method" was used with slight modifications to isolate the EPF. Besides the larvae of *G. mellonella* (larval stage L_4 - L_5), L_5 larvae of *Teneblio molitor* were also used. The rearing of *G. mellonella* larvae has been described in previous section and the *Tenebrio* L_5 larvae were obtained from commercial suppliers (futterhaus.de). 10 insects of both *G. mellonella* and *T. molitor* were loaded separately in 50 mL-Schott-Duran glass bottle (sterile) and filled with the prepared soil up to the neck of the bottle. The bottles were kept in an incubator (IPP 500, Memmert) at 25 °C without light for 14 days. For the first 5 days, they were rotated to encourage the larvae to move upwards in the soil, increasing the likelihood that they would come in contact with the fungal spores.

After the incubation period, all insects were removed from the soil. All larvae and pupae were checked regularly, maximum twice a week. Insects were sorted out based on the movement, survival and fungal growth (alive, dead with no fungal growth and dead with fungal growth). Alive and dead insects with no evidence of fungal growth were discarded. Dead insects with fungal growth were surface sterilized and placed in

moist chambers. For this purpose, they were immersed in 1 % (w/v) sodium hypochlorite (NaClO) for 30 seconds and then washed for 5 seconds with autoclaved deionized water. They were then placed separately in smaller plastic boxes (10.5 cm × 7.5 cm × 5 cm, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) with moistened dental rolls (so-called "moist chambers") and incubated further for 7 days at 25 °C in the dark. Outgrowing fungi were transferred under sterile condition with disposable inoculation loops to petri plates (94 mm × 15 mm, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) containing malt-peptone-agar (MPA). These MPA petri plates contained 3 % (w/v) malt extract (Merck, Darmstadt, Germany), 0.5 % (w/v) peptone from soybean (Merck) and 1.8 % (w/v) agar-agar (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and incubated for 3 - 5 days at 25 °C.

For further purification, fungal isolates from these plates were transferred via a dilution smear technique onto another MPA-plates to reduce contamination and incubated for 7 days at 25 °C. To ensure the purity of the isolates, two additional dilution smears were prepared on new MPA-plates and incubated for subsequent periods of 7 and 14 days 25 °C. Final pure cultures were examined under a binocular microscope (Carl Zeiss Microscopy Deutschland GmbH, Jena, Germany) for any signs of contamination after 14 days. Upon confirming the purity, cultures were photographed for documentation and re-plated as needed for further experimental use or long-term storage. For the storage period of 6 to 12 months, the pure cultures were maintained as agar slants with the same medium and stored in refrigerators at 8 °C. For long-term storage (over decades) the cultures were deep-frozen at - 80 °C in cryotubes (Microbank, Pro-Lab Diagnostics, Ontario, Canada).

Morphological and molecular identification of EPF

A total of 32 strains were isolated from collected soil samples. Another strain JKI-BI-1496 from Julius Kühn Institute´s own strain collection was included in the experiment as a reference. This strain was isolated from *C. pomonella* in 1971 by Müller-Kögler in Darmstadt (former strain number Pfr 4). The Zeiss Stemi SV 11 binocular microscope (× 0.6/ × 4.0) magnification was used to identify the fungi. All strains were morphologically characterized based on colony morphology, conidial texture, colour and size (size was measured by Axiplan microscope (Carl Zeiss Microscopy

Deutschland GmbH, Jena, Germany) using the program cellSens Standard, Olympus). After morphological characterization, molecular characterization was performed using DNA sequencing, followed by molecular identification of EPF and phylogenetic analyses.

Isolated EPF strains were identified to genus level using the internal transcribed spacer (ITS) locus of fungal ribosomal DNA. For DNA isolation, the DNeasy® Plant Mini Kit 250 (QIAGEN, Hilden, Germany) was used according to the manufacturer's instructions. Fungi were grown on MPA at 25 °C and fungal material (mycelium and conidia) was transferred to a 2-mL tube containing Lysing Matrix A (MP Biomedicals, Illkirch-Graffenstaden, France). The tubes were stored at - 80 ºC for 24 hours, and the frozen fungal material was then ground in a FastPrep24 homogenizer (MP Biomedicals, Illkirch-Graffenstaden, France) for 20 seconds. Buffer and RNase were then added according to the manufacturer's protocol. After DNA isolation, PCRs were carried out with AccuStart II PCR ToughMix (from Quantabio) and primers depending on the fungal genus. To identify the fungi of the genus *Metarhizium* to the species level, part of the translation elongation factor-1-alpha was sequenced (Bischoff et al. 2009; Rehner and Kepler 2017). For *Beauveria* part of the BLOC region (Rehner et al. 2006; Gürlek et al. 2018) and for *Cordyceps* part of the ITS region (Abaajeh 2014) were sequenced. Primers for *Metarhizium* were EF2F (5'-GGAGGACAAGACTCACACATCAACG-3') and EFjR (5'-TGYTCNCGRGTYTGNCCRTCYTTT-3'), for *Beauveria* were B22U (5'-GTCGCAGCCAGAGAGCAACT-3') and B822L (5'-GATTCGCAACGTCAACTT-3') and for *Cordyceps* were ITS 1F (5'-CTTGGTCATTTAGAGGAAGTAA) and 4A (5'-CGCCGTTTACTGGGGGCAATCCCTG-3').

PCR amplifications were performed in a total volume of 25 µl, which included 12.5 µl AccuStart II PCR ToughMix (Quantabio), 1.5 µl 10 pmol of each primer, 4.5 µl of sterile water and 5 µl of template DNA. The amplifications were done for 3 minutes at 95 °C, 30 seconds at 50 °C, and then, 35 cycles were performed as follows: 30 seconds at 95 °C, 1 minute at the annealing temperature of 55 °C, and 1 minute at 72 °C. Reactions were then incubated at 72 °C for another 5 minutes. After performing all amplifications, PCR products were separated on a 1 % (w/v) agarose gel, loaded with 1kb Marker (DNA ladder- Thermofisher) and stained with Midori Green Direct

(Biozym Scientific GmbH). Visualization and caption of the gel images was performed using the ChemoCam Imager ECL UV trans-illuminator and software (ChemoCam, INTAS Science Imaging Instruments GmbH, Göttingen, Germany). The PCR amplification products were purified using the DNA purification kit DNA Clean&Concentrator™-25 (Zymo Research Europe GmbH, Freiburg, Germany) following the protocol of Zymo Research for DNA purification. DNA concentration and quality was determined by measuring 1 µl of the DNA samples in a Nanodrop 2000c Spectrometer (Thermo Scientific, Wilmington, Delaware USA). Subsequently, the purified PCR products were sent to Microsynth Seqlab (Göttingen) for sequencing. The resulting sequences were processed using Geneious R8 software for the evaluation of Microsynth sequence results. Five reference species were selected using NCBI BLAST with an identity similarity range between 100 % and 95 %. The phylogenetic tree was constructed taking into account that a higher percentage identity value is directly proportional to higher similarity of the DNA sequences (Ananda et al. 2020). The phylogenetic trees of the isolated strains were constructed by comparing the obtained sequences with published sequences from NCBI (Table 1) using MEGA11. The algorithm underlying the phylogenetic tree was a combination of the maximum likelihood (ML) method, and a model selected based on the model selection application (Reddy 2011). According to the model selection result, the best fitting model was used for the phylogenetic tree. For example, Kimura's 2-parameter model was selected for *Beauveria* (BIC value: 7423.5) and Tamura's 3-parameter model was selected for *Cordyceps* (BIC value: 21959.6) and *Metarhizium* (BIC value: 4222.6) and validated with 500 bootstrap replicates. The bootstrap value for a clade is the proportion of the replicate trees that recovered that particular clade. These values can be mapped on a bootstrap consensus tree, which is constructed by summing all replicate trees using a consensus method (Russo and Selvatti 2018). For tree interference, ML method was chosen as a statistical analysis, Nearest- Neighbor-Interchange (NNI) as ML heuristic method, and the BIONJ method for the initial tree construction. The experiments of morphological and molecular indentification were individually repeated three times.

Table 1. Reference sequences of *Beauveria, Metarhizium* and *Cordyceps*, used in phylogenetic analyses, strain/ isolate number, GenBank accession number, country of origin and publication.

Fungi name	GenBank Accession number	Country of origin	Reference
B. bassiana ELA-24	MH181847	Turkey	(Gürlek et al. 2018)
B. bassiana SLF-50 [BoteGHA]	MT122124	Turkey	(Clifton et al. 2020)
B. bassiana ELA-36	MH181857	Turkey	(Gürlek et al. 2018)
B. bassiana BCC1446	MN401541	Thailand	(Khonsanit et al. 2020)
B. bassiana BCC1848	MN401540	Thailand	(Khonsanit et al. 2020)
M. guizhouense ARSEF 9951	MK156076	North America	(Rehner 2020)
M. guizhouense ARSEF 9885	MK156075	North America	(Rehner 2020)
M. guizhouense ARSEF 9871	MK156074	North America	(Rehner 2020)
M. guizhouense DAOM 241832	MK391329	Switzerland	(Inglis et al. 2019)
M. guizhouense DAOM 241929	MK391366	Switzerland	(Inglis et al. 2019)
M. brunneum ARSEF 3297	XM_014693123	Mexico	(Hu et al. 2014)
M. brunneum	MH711929	India	(Deependra et al. 2020)
M. brunneum ARSEF 4152	EU248853	Australia	(Bischoff et al. 2009)
M. brunneum ARSEF 4179	EU248854	Australia	(Bischoff et al. 2009)
M. brunneum ARSEF 2107	EU248855	USA	(Bischoff et al. 2009)

Fungi name	GenBank Accession number	Country of origin	Reference
M. robertsii ARSEF 727	DQ463994	Brazil	(Bischoff et al. 2009)
M. robertsii ARSEF 7501	EU248849	Australia	(Bischoff et al. 2009)
M. robertsii ARSEF 4739	EU248848	Australia	(Bischoff et al. 2009)
M. robertsii ARSEF 9884	MK156072	North America	(Rehner 2020)
M. robertsii ARSEF 9779	MK156068	North America	(Rehner 2020)
C. fumosorosea BCC 20180	MH532834	Thailand	(Kuephadungphan et al. 2018)
Isaria fumosorosea	JF792885	Greece	(Beris et al. 2013)
C. fumosorosea	MH855199	USA	(Vu et al. 2019)
C. fumosorosea	MH872372	USA	(Lestari et al. 2016)
C. fumosorosea	MH854970	USA	(Vu et al. 2019)

Rearing of *Cydia pomonella*

Fifth instar larvae (L_5) of *C. pomonella* were used for the virulence test. L_1 larvae were obtained from the Julius Kühn Institute (JKI) and reared with until reaching the L_5 stage. As the larvae of *C. pomonella* are prone to cannibalism, the larvae were kept individually. Rearing took place in individual chambers made of plastic grids. The newly hatched L_1 larvae were collected with a fine brush from the incubation trays in which their eggs were incubated and placed in 140 mm × 70 mm × 36 mm grids (Licefa Kunststoffverarbeitung GmbH and Co. KG, Bad Salzuflen, Germany) with a special feeding medium. Main ingradients of this feeding medium were 0.02 g/mL agar-agar (Gustav Essig GmbH, Mannheim, Germany), 0.08 g/mL corn meal (Herrnmühle, Reichelsheim, Germany), 0.08 g/mL brewer`s yeast (Herrnmühle, Reichelsheim, Germany), 0.08 g/mL wheat germ (Herrnmühle, Reichelsheim, Germany), 0.01 g/mL ascorbic acid (Gustav Essig GmbH, Mannheim, Germany), 0.003 g/mL nipagin (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and 0.003 g/mL benzoic acid (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). A total number of 50 larvae were placed individually in the chambers of a grid and then the entire grid was wrapped in cling film. As the larvae tend to feed through the foil and escape in later stages, the lids of the grids were placed upside down on the foil and fixed with rubber rings. In addition, a folded paper towel was placed between the foil and the lid. With this procedure, the individual chambers were well sealed with folded paper towel and loss of the larvae through cannibalism or escape could be largely avoided. The larvae were incubated at 25 °C until they reached the L_5, the last larval instar before pupation. The L_5 larvae were carefully removed from the grids with spring steel forceps just before they were needed for further experiments.

Screening of EPF

Two different tests were carried out for screening EPF. The first screening based on percentage of mortality against *C. pomonella* and was performed through virulence test. To select the potential EPF, a second screening was performed with an enzymatic activity experiment using an agar-chitin assay, as the media composition with nitrogenous polysaccharides was in the focus.

Virulence of EPF against *C. pomonella*

Virulence of isolated EPF were measured based on the percentage of mortality of L_5 *C. pomonella* larvae. This experiment was conducted to identify virulent strains against *C. pomonella*. 32 isolated fungal strains were tested in this experiment. A fungal strain JKI-BI-1496 (*C. fumosorosea*) from own collection of JKI was used as a reference strain. 20 L_5 larvae of *C. pomonella* were exposed directly to an approximately 14-day-old fungal culture (MPA-plates) and incubated for at least 30 minutes shaking the larvae gently twice during incubation. Each larva was placed in a small flat plastic container (10.5 cm × 7.5 cm × 5.0 cm) with a small piece of cardboard (2 cm long, 2 rows) with sterile forceps and were incubated at 25 °C for 21 days. At the end of the incubation period, the number of dead larvae were counted and the virulence of each fungal strain was evaluated. The experiment was done without any repetition.

Chitinase activity test of EPF against *C. pomonella*

To evaluate the chitinase activity among the potential strains against *C. pomonella*, colloidal chitin (CC) was made from chitin, modified with HCl through demineralization (No and Meyers 1995). To prepare CC, 20 g of chitin powder (Merck, Darmstadt, Germany) was weighed and placed in a 1000-mL beaker. 150 mL of 12 M HCl was added slowly with constant stirring using a magnetic stirrer for 60 minutes in a chemical fume hood at room temperature. The chitin-HCl mixture was filtered through eight layers of gauze to remove the large chitin chunks. The filtrate obtained was treated with 2 L of ice-cold distilled water to allow precipitation of CC and was incubated overnight at room temperature to allow better precipitation of CC. After overnight incubation, the filtrate was passed through three layers of Whatmann filter paper (diameter 130 mm) in a Buchner funnel located in a vacuum filtration flask. Around 3 L of tap water was passed through the CC to raise the pH and this was continued until pH 7 was reached. The CC obtained was pressed between coffee filter paper (to remove additional moisture), and then placed in a 100-mL glass beaker. Then the CC was autoclaved (20 minutes at 121 °C) and stored at 4 °C until further use. For the determination of enzymatic chitinase activities, 1 % (w/v) of CC was used. A 14-days-

old fungal disc (0.5 cm diameter) was inoculated in the agar-chitin plate and incubated for 5 days at 4 °C. The plate was examined after 5 days and a clear zone became visible with Lugol's Iodin. Then the area of the clear zone was calculated according to the following equation:

Index of relative enzymatic activity (RA) = (Total diameter of clear zone - Diameter of colony)/Diameter of colony

According to Dancan *et al.* (2008), it has enzymatic activity if the result of the relative enzymatic activity is ≥ 1. This experiment repeated three times.

Temperature optima of EPF

To evaluate the optimal growth temperature of the different fungal strains on malt-peptone-agar, small disks (0.5 cm diameter) from 14 days old EPF-plates (MPA-medium) were cut out and each disk was placed in the middle of a small MPA-plate with a sterile inoculation needle. All plates were incubated for 4 days at 5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C and 35 °C to determine the growth area. After 4 days, the growth area was photographed and analysed with ImageJ software. This experiment conducted by three repetitions with three replications.

Testing the EPF for their tolerance to simulated sunlight

To test the EPF for their tolerance to simulated sunlight, aerial conidia of EPF were cultivated for 14 days at 25 °C on MPA-plate. 5 mL 0.5 % Tween 80 was pipetted on the petri plates and the conidia were scraped with a Drigalski spatula. After filtering over four layers of cotton gauze the conidial suspension was kept for 3 minutes in the sonicator. After preparing of 3 mL of a conidial suspension of 1×10^6 conidia/mL for each sunlight exposition time, 1 mL was pipetted in one compartment of the 24-well control plate (cell culture multiwell plate 24 well, PS, Greiner BIO-ONE). This plate was irradiated without lid in a SUNTEST XXL + FD sunlight simulator (Atlas Material Testing Technology, Illinois, USA). The lid was removed to avoid light refraction and bundling effects due to evaporation and subsequent condensation. In the sample chamber of the sunlight simulator, the highest possible humidity (95 %) was ensured to prevent the samples from drying out. Other 24-well control plate was wrapped with lid

aluminium foil to expose the conidia to the same temperature conditions but protect them from solar radiation. All the samples were irradiated with 300 - 800 nm at 20 °C. The black standard (BST) served as the temperature sensor and the fan was set to 2000 rpm. The samples were thus exposed to a total of 3780 kJ/m^2 for 3 hours. At the end of the exposure 3 droplets (10 µL) of each sample were pipetted on a MPA-plate. All plates were incubated at 20 °C for 24 hours. Samples were taken from the MPA-plate and the germination of the spore was determined. 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. The experiment was repeated three times with three replications.

Influence of media composition on spore productivity, dry biomass and optical density

Submerged spores from liquid fermentation in flask culture were used for spore productivity, dry biomass and optical density. To prepare the starter culture, previously prepared 100-mL Erlenmeyer flasks containing 50 mL autoclaved malt peptone media were used and the specific fungus was inoculated with a loop of a sterile inoculation needle. The inoculated Erlenmeyer flasks were incubated for 72 hours at 150 rpm and 25 °C on a horizontal shaker (Novotron, 50 mm deflection, Infors, Bottmingen, Switzerland). New starter cultures were established for each experiment, and all media cultures used in subsequent experiments were inoculated with fungal material from these starter cultures.

Spore productivity

To analyse the productivity of the submerged spores through spore counting 50 mL of four liquid media, named as malt peptone (MP) (malt = 30 g/L, peptone = 9 g/L), malt peptone with 1 % (w/v) of chitin (MPC), potato dextrose (PD) (26.5 g/L) (Merck, Darmstadt, Germany) and potato dextrose with 1 % (w/v) of chitin (PDC). These four media were inoculated with a spore concentration of 1 × 10^6 submerged spores/mL (ss/mL) from the starter culture. After incubation for 72 hours at 25 °C and 150 rpm, the dry biomass and number of spores were determined. It was also determined how the

fungal strains behaved during growth in the liquid medium. The cell number was determined using a Thoma counting chamber (Improved Neubauer, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) and adjusted to the cell number required for the respective experiment. The experiment was repeated three times.

Determination of dry biomass

For the measurement of dry biomass, the 50 mL spore suspension was poured into a filter paper and vacuum pump was used for filtration. This filter paper (diameter 150 mm) was then placed in a bowl (the weight of the bowl was measured beforehand) and the wet mass was weighed. This bowl with the wet filter paper was then stored at 60 °C for 72 hours and the dry biomass was weighed. The experiment conducted by three repetitions.

Measuring the optical density of fungal cultures

The optical density of the fungal culture during liquid fermentation was measured using the Cell Growth Quantifier (CGQ) (Aquila biolabs GmbH, Baesweiler, Germany), a measuring device that enables non-invasive, real-time monitoring of organism growth in culture vessels. For this purpose, the flasks were placed on a CGQ sensor plate during the incubation, which repetitively measures the biomass density through the wall of the shake flask. The sensor emits light in the form of light pulses into the culture medium and measures the intensity of the light that is scattered back from the biomass (backscatter measurement). The higher the concentration of microorganisms in the medium, the higher the intensity of the reflected light. The device takes a measurement for every 5 seconds with 150 rpm at 25 °C. The optical density data was recorded every minute for 72 hours. This experiment was done by three repetitions.

Statistical analysis

The statistical analyses were carried out with the software R Studio (Version 1.4.1106) (RStudio Team 2020). Data are presented as arithmetic mean (mean), standard deviation (± SD) and standard error (± SE), depending on whether a model was used for calculation. For relative chitinase activity tests, after square-root transformation of the

chitinase activity value data, normal distribution and variance homogeneity were confirmed. This was done to meet ANOVA`s underlying assumptions of normal distribution. The normal distribution was checked with the QQ-Plot (sample quantile-theoretical quantile) and variance homogeneity with the Residuals-Prediction-Plot. The formula used for linear model (LM) is as follows:

$$LM (sqrt (y) \sim x + repetition)$$

For analysis of influence of different temperature optima and sunlight simulation tolerance, the following formula has been used for LM:

$$LM (y \sim x + strain + x: strain + repetition + replication)$$

The rest of the experiments of effect of media composition on spore count, dry biomass and optical density, the following formula has been executed for LM:

$$LM (y \sim x + strain + x: strain + repetition)$$

Furthermore, post hoc Tukey HSD test ($\alpha = 0.05$) was performed with the R package emmeans (Lenth, 2016) to examine differences between the respective factor levels in the context on an ANOVA. All the data were transformed to ensure normal distribution and variance homogeneity.

Results

Morphological and molecular (at genus level) characterization

The morphological characterization of EPF strains revealed differences in colour, texture and spore length and width (Table 2, Figure 1). Examination of colour and texture with a binocular at 2 to 3 weeks after inoculation revealed that all *Beauveria* showed a whitish and smooth texture, whereas *Cordyceps* showed a yellowish brown/ pinkish colour. The texture of *Cordyceps* were elevated and raised. *Metarhizium* differed in both texture and colour. They had a green or dark green colour and a powdery texture. 100 spores were taken for length and width measurements and the average values are given in the Table 2. It was found that *Beauveria* spores were almost

round to oval in shape, *Cordyceps* spores were also oval, but slightly more elongated compered to *Beauveria* spores. The spores of *Metarhizium* had an elongated shape.

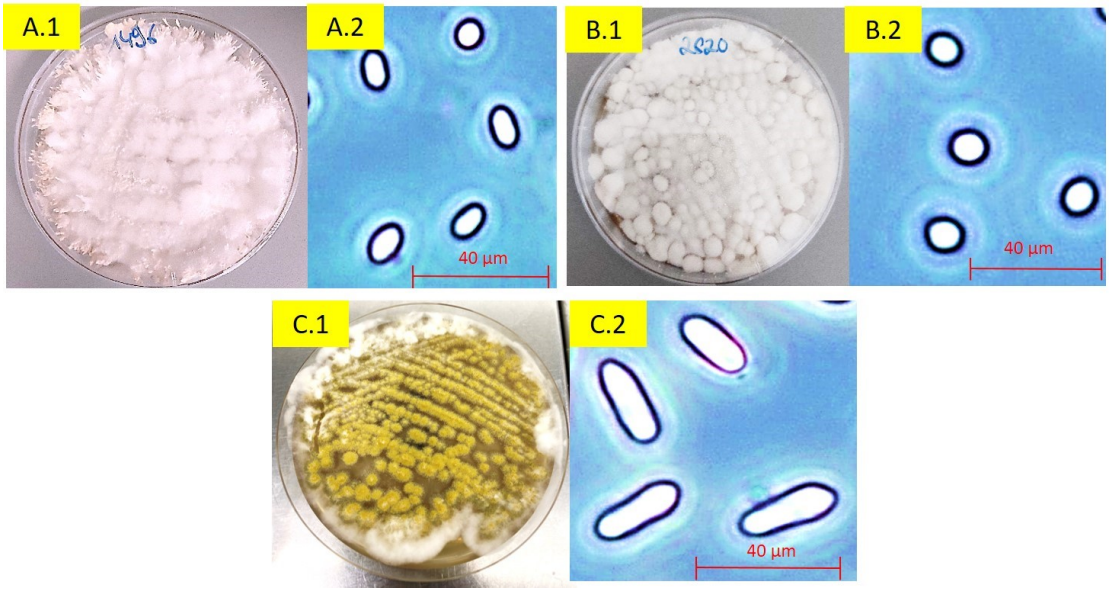

Figure 1. A.1 JKI-BI-1496 (*Cordyceps fumosorosea*), A.2 Spore dimension of JKI-BI-1496; B.1 JKI-BI-2620 (*Beauveria bassiana*), B.2 Spore dimension of JKI-BI-2620; C.1 JKI-BI-2647 (*Metharhizium robertsii*), C.2 Spore dimension of JKI-BI-2647

Table 2. Morphological characteristics (colour, texture, spore length, spore width) of isolated EPF strains and host species.

JKI-BI-Nr.	Genus	Host	Colour	Texture	Spore length (µm) ± SE	Spore width (µm) ± SE
2619	*Beauveria*	*G. melonella*	White	Smooth	2.4 ± 0.02	1.9 ± 0.02
2620	*Beauveria*	*T. molitor*	White	Smooth	2.4 ± 0.02	2.0 ± 0.01
2632	*Beauveria*	*G. melonella*	White	Smooth	1.8 ± 0.03	1.4 ± 0.02
2642	*Beauveria*	*T. molitor*	White	Smooth	2.1 ± 0.01	1.8 ± 0.02
2637	*Cordyceps*	*G. melonella*	White to yellowish brown	Smooth and elevated	5.1 ± 0.1	2.2 ± 0.04
2638	*Cordyceps*	*G. melonella*	White to yellowish brown	Smooth and elevated	5.3 ± 0.1	2.3 ± 0.03
2623	*Cordyceps*	*T. molitor*	White to yellowish brown	Smooth and elevated	5.8 ± 0.1	3.2 ± 0.03
1496	*Cordyceps*	*C. pomonella*	White to yellowish brown	Smooth and elevated	2.9 ± 0.03	1.9 ± 0.02
2618	*Cordyceps*	*G. melonella*	White to yellowish brown	Smooth and elevated	4.6 ± 0.1	2.1 ± 0.02
2636	*Cordyceps*	*G. melonella*	White to yellowish brown	Smooth	5.3 ± 0.2	2.2 ± 0.04
2654	*Cordyceps*	*G. melonella*	White to yellowish brown	Smooth	6.5 ± 0.02	2.3 ± 0.1
2639	*Metarhizium*	*T. molitor*	Greenish	Powdery	6.1 ± 0.1	2.3 ± 0.02
2641	*Metarhizium*	*T. molitor*	Greenish	Powdery	6.2 ± 0.1	2.2 ± 0.01
2631	*Metarhizium*	*T. molitor*	Greenish	Powdery	6.9 ± 0.1	2.5 ± 0.02
2626	*Metarhizium*	*T. molitor*	Greenish	Powdery	6.8 ± 0.1	2.5 ± 0.02
2624	*Metarhizium*	*T. molitor*	Greenish	Powdery	6.7 ± 0.1	2.5 ± 0.02
2633	*Metarhizium*	*G. melonella*	Greenish	Powdery	6.9 ± 0.1	2.5 ± 0.02
2647	*Metarhizium*	*T. molitor*	Greenish	Powdery	7.2 ± 0.1	2.7 ± 0.02

JKI-BI-Nr.	Genus	Host	Colour	Texture	Spore length (µm) ± SE	Spore width (µm) ± SE
2649	*Metarhizium*	*T. molitor*	Greenish	Powdery	7.4 ± 0.1	2.6 ± 0.03
2644	*Metarhizium*	*T. molitor*	Greenish	Powdery	7.4 ± 0.1	2.6 ± 0.02
2625	*Metarhizium*	*T. molitor*	Greenish	Powdery	7.3 ± 0.1	2.6 ± 0.02
2640	*Metarhizium*	*T. molitor*	Greenish	Powdery	7.2 ± 0.1	2.8 ± 0.03
2650	*Metarhizium*	*T. molitor*	Greenish	Powdery	7.3 ± 0.1	2.7 ± 0.03
2628	*Metarhizium*	*T. molitor*	Greenish	Powdery	7.2 ± 0.1	2.8 ± 0.02
2621	*Metarhizium*	*T. molitor*	Greenish	Powdery	7.3 ± 0.1	2.9 ± 0.02
2653	*Metarhizium*	*T. molitor*	Greenish	Powdery	7.5 ± 0.1	2.9 ± 0.02
2622	*Metarhizium*	*T. molitor*	Greenish	Powdery	7.4 ± 0.1	2.9 ± 0.02
2648	*Metarhizium*	*T. molitor*	Greenish	Powdery	7.5 ± 0.1	2.9 ± 0.03
2652	*Metarhizium*	*T. molitor*	Greenish	Powdery	7.6 ± 0.1	2.9 ± 0.02
2651	*Metarhizium*	*T. molitor*	Greenish	Powdery	7.5 ± 0.1	3.0 ± 0.02
2627	*Metarhizium*	*T. molitor*	Greenish	Powdery	9.8 ± 0.1	3.4 ± 0.03
2629	*Metarhizium*	*T. molitor*	Greenish	Powdery	10.9 ± 0.1	3.2 ± 0.02
2646	*Metarhizium*	*T. molitor*	Greenish	Powdery	7.5 ± 0.1	2.6 ± 0.02

The molecular characterization was carried out in two steps. First, all strains were screened with ITS primers (Appendix: Table 1). Of these 33 strains, four were from *Beauveria* (20 %), seven from *Cordyceps* (12 %) and 22 from *Metarhizium* (68 %).

Virulence test of EPF against *C. pomonella*

After molecular identification at genus level, a primary screening with a virulence test was performed with all strains (Figure 2). All strains showed different levels of virulence for *C. pomonella* larvae. Among them, 13 strains showed ≥ 80 % mortality, whereas eleven strains showed 50 - 79 % mortality and the rest nine strains showed < 50 % mortality. Based on the virulence test, 13 most effective strains were selected for further investigations.

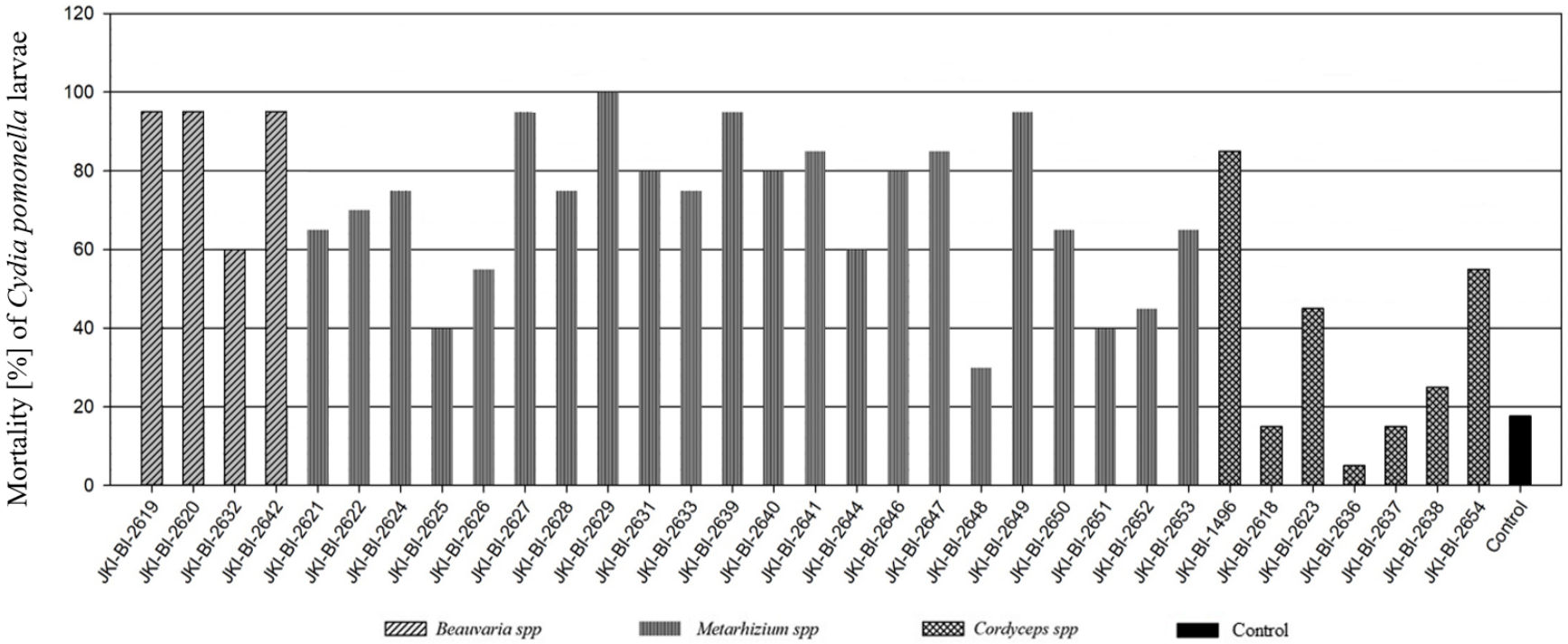

Figure 2. Virulence test based on mortality [%] of isolated entomopathogenic fungi strains against *Cydia pomonella* L_5 larvae.

Molecular (at species level) characterisation

Among 13 selected strains, three belonged to *Beauveria bassiana* (Balsamo-Crivelli) (Hypocreales: Cordycipitaceae), one strain to *C. fumosorosea*, two strains *to Metarhizium guizhouense* (Chen & Guo) (Hypocreales: Clavicipitaceae), three strains to *Metarhizium brunneum* Petch (Clavicipitaceae: Clavicipitaceae) and four strains to *M. robertsii*. *C. fumosorosea* clustered with its reference sequence BCC 20180 (98 %). Although isolated from different regions, JKI-BI-1496 is also closely related to *C. fumosorosea* CBS: 192.28 (96 %), which was isolated from the USA. The other two reference sequences from MF872372 *C. fumosorosea* isolate W5 and JF792885 *I. fumosorosea* (which is currently known as *C. fumosorosea*) belong to the same clade with an 81 % identity value. Five references from NCBI were compared to the JKI-BI-2619, JKI-BI-2620 and JKI-BI-2642. JKI-BI-2619 and JKI-BI-2642 belonged to the same clade with a bootstrap value of 98 %. These two strains are 100 % identical to JKI-BI-2620. All three strains were closely related to *B. bassiana* ELA-36 (89 %), which was isolated from Turkey. Another *B. bassiana* SLF-50 BoteGHA (MT122124) had a similar sequence (73 %). The elongation factor sequence was analysed for *Metarhizium* strains. A BLAST analysis revealed the presence of three *Metarhizium* species in this strain collection. Of the seven *Metarhizium* strains, two were from *M. guizhouense,* which are closely related to *M. guizhouense* ARSEF 9871. The other two strains of *M. brunneum* (JKI-BI-2631 and JKI-BI-2639) were closely related to *M. brunneum* ARSEF 3297 (86 % and 100 %). The remaining five *Metarhizium* strains belonged to *M. robertsii* (JKI-BI-2640, JKI-BI-2641, JKI-BI-2646, JKI-BI-2647 and JKI-BI-2649), which are closely related to *M. robertsii* ARSEF 9884 (100 %) and belong to the same clade. All the plylogenetic trees are presented in Appendix: Figure 1 (A, B, C.1, C.2 and C.3).

Chitinase activity test of EPF

Among 13 identified strains, four strains (JKI-BI-1496, JKI-BI-2620, JKI-BI-2642 and JKI-BI-2647) had a significantly higher chitinase activity value (≥ 1) compared to the others (ANOVA; $F = 20.5$, $df = 12$, $p < 0.0001$; Tukey HSD test (Table 3)). These four starins had been selected for further experiments.

Table 3. Comparative analysis of relative chitinase activity test of 13 strains through agar-chitin plate assay.

JKI-BI-Nr.	Strain name	Relative chitinase activity (Mean ± SE)
1496	*Cordyceps fumosorosea*	1.3 ± 0.1 (a*)
2619	*Beauveria bassiana*	0.4 ± 0.1 (bc)
2620	*Beauveria bassiana*	1.9 ± 0.2 (a)
2627	*Metarhizium guizhouense*	0.4 ± 0.1 (bc)
2629	*Metarhizium guizhouense*	0.6 ± 0.1 (bc)
2631	*Metarhizium brunneum*	0.2 ± 0.1 (bc)
2639	*Metarhizium brunneum*	0.2 ± 0.1 (b)
2640	*Metarhizium robertsii*	0.3 ± 0.1 (bc)
2641	*Metarhizium brunneum*	0.6 ± 0.1 (cd)
2642	*Beauveria bassiana*	1.3 ± 0.1 (a)
2646	*Metarhizium robertsii*	0.6 ± 0.1 (bc)
2647	*Metarhizium robertsii*	1.2 ± 0.1 (ad)
2649	*Metarhizium robertsii*	0.5 ± 0.1 (bc)

*: Mean ± SD with the same letters after executing linear model are not significantly different (Tukey HSD test, $\alpha = 0.05$, n = 3 × 3)

Temperature optima of EPF

Temperature had an effect on the growth area (ANOVA; $F = 114.00$, $df = 6$, $p < 0.0001$). However, the effect of temperature was strain dependent (ANOVA; $F = 34.1$, $df = 18$, $p < 0.0001$). The effects of seven different temperatures on growth area in [%] of the four EPF strains examined are shown in Figure 3. An increase in temperature from 10 °C to 15 °C had a significant effect on strain JKI-BI-1496 (*C. fumosorosea*) with an increase in growth area from 0.8 % to 3.0 % ($df = 220$, $p < 0.0001$). At a temperature of 20 °C the growth area (6.1 %) of *C. fumosorosea* increased further and was also higher than that of the other three strains (JKI-BI-2620 (3.1 %), JKI-BI-2642 (3.3 %) and JKI-BI-2647 (1.6 %)). There is a general trend that a temperature increase between 5 °C and 25 °C has a positive effect on growth area.

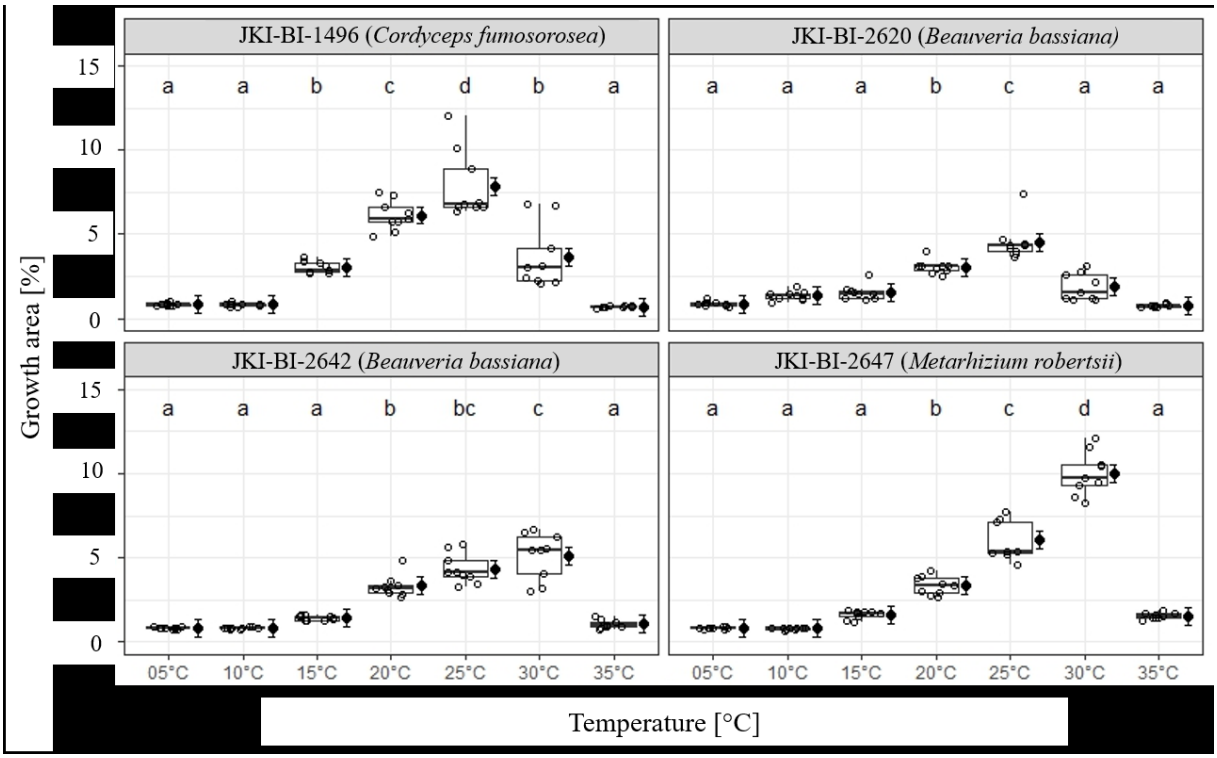

Figure 3. Effect of different temperatures on growth area [%] of four entomopathogenic fungi strains. Jittered boxplots consist of the median and the 25 % and 75 % quantile. Black dots and error bars represent adjusted mean with 95 % confidence limits per temperature optima. Means followed by the same letters are not significantly different (Tukey HSD test, $\alpha = 0.05$, n = 3 × 3).

At 25 °C, the growth area increased in all four strains. For example, JKI-BI-1496 increased significantly (7.9 %, $df = 220$, $p < 0.0001$) and reached its highest value, while JKI-BI-2647 (6.1 %) was smaller, but largest compared to other temperatures. When the temperature was increased to 30 °C, 10.0 % growth area was observed in JKI-BI-2647, while JKI-BI-2620 showed a lowest growth area (1.9 %). A temperature increase to 35 °C reduced the growth area drastically with no significant difference between strains.

Testing the EPF for their tolerance to simulated sunlight

In a sunlight simulation experiment the germination after exposure was significantly affected (ANOVA; $F = 12.1$, $df = 3$, $p < 0.0001$) (Figure 4).

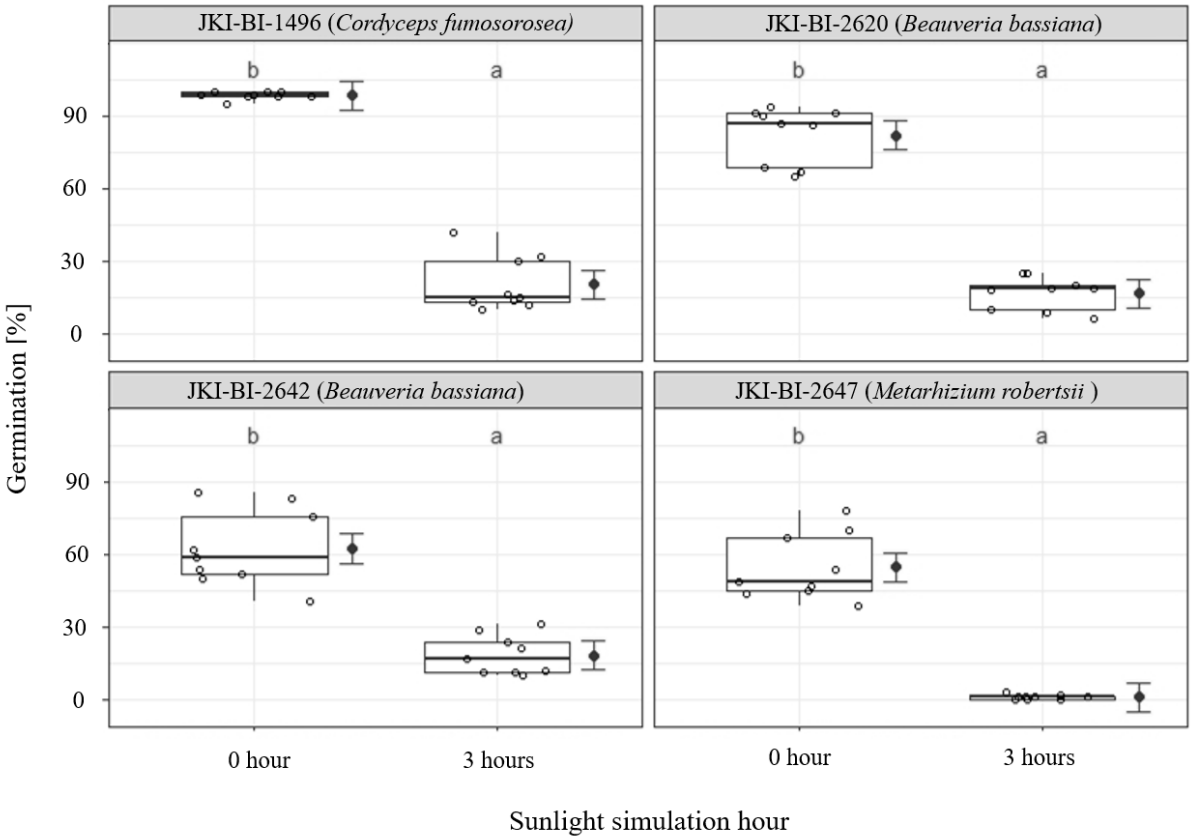

Figure 4. Effect of 3 hours of sunlight simulation and no sunlight simulation (0 hour) on germination [%] of four selected entomopathogenic fungi strains. Jittered boxplots consist of the median and the 25 % and 75 % quantile. Black dots and error bars represent adjusted mean with 95 % confidence limits per sunlight simulation hour. Means followed by the same letters are not significantly different (Tukey HSD test, $\alpha = 0.05$, n = 3 × 3).

Germination of the control plates without sunlight simulation, ranged from 54.8 % to 99.0 %, depending on the strain. After 3 hours exposure to simulated sunlight, a significant lower conidial germination has been observed for all strains. At the end of the experiment, germination was below 21 % for all strains. JKI-BI-1496 (*C. fumosorosea*) was the most sunlight tolerant strain (germination 20.4 %) followed by JKI-BI-2620 (16.8 %), JKI-BI-2642 (18.4 %), and JKI-BI-2647 (1.0 %). Pairwise analyses of the results (without sunlight versus 3 hours of sunlight simulation per strains) showed significant differences in germination. For JKI-BI-1496, the germination decreased from 98.6 % to 20.4 % after 3 hours of sunlight simulation. The two *B. bassiana* strains, JKI-BI-2620 and JKI-BI-2642, showed a significant decrease in germination from 82.2 % to 16.8 % and from 62.6 % to 18.4 % respectively. Surprisingly, *M. robertsii* (JKI-BI-2647) almost reached inactivation (1.0 %) of conidia after 3 hours of sunlight simulation.

Influence of media composition on spore productivity, dry biomass and optical density

Spore productivity through counting spore number

Media type affected spore numbers (ANOVA; $F = 2.3$, $df = 9$, $p = 0.04$). Moreover, the type of medium had a significant effect on growth of submerged spores (ANOVA; $F = 9.5$, $df = 3$, $p < 0.0001$). JKI-BI-1496 (*C. fumosorosea*) and JKI-BI-2620 (*B. bassiana*) showed significantly lower submerged spores/mL (ss/mL) compared to the other two strains, when produced in PD medium (Figure 5).

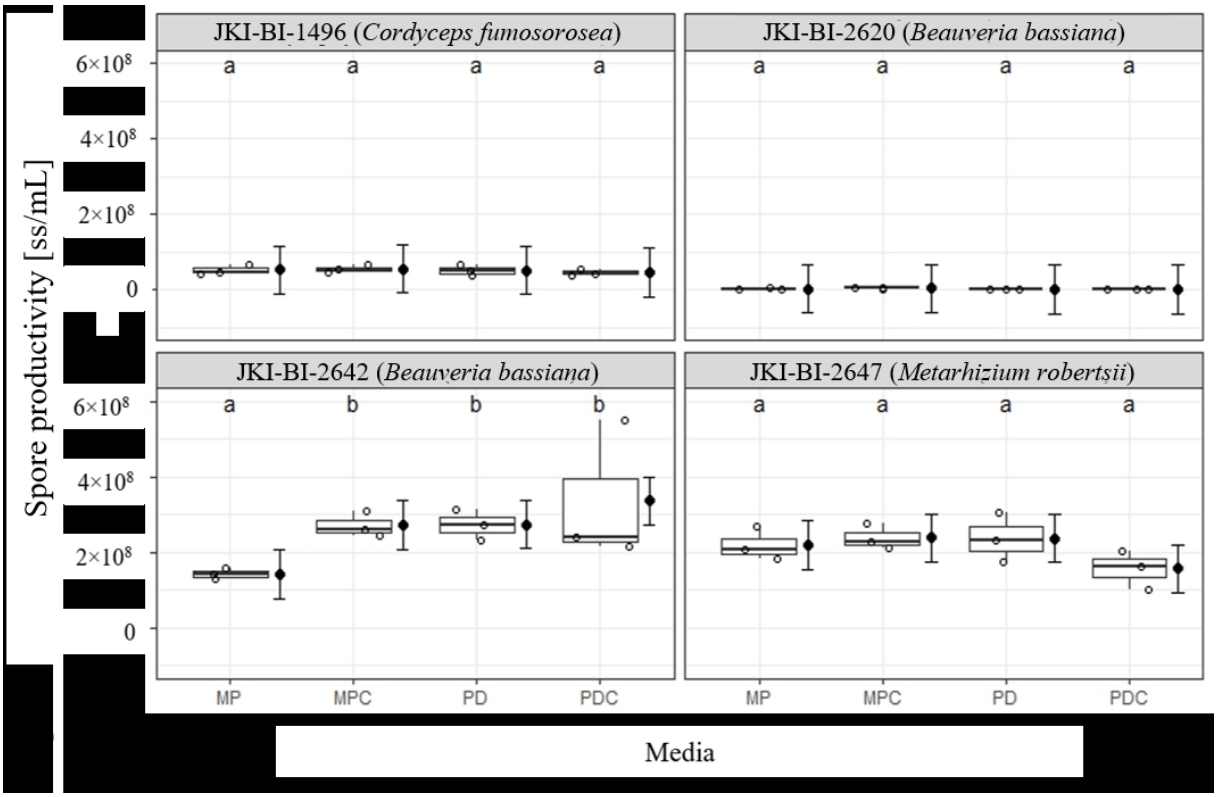

Figure 5. Effect of four different media on spore productivity [ss/mL] of four entomopathogenic fungi strains. Jittered boxplots consist of the median and the 25 % and 75 % quantile. Black dots and error bars represent adjusted mean with 95 % confidence limits per medium. Means followed by the same letters are not significantly different (Tukey HSD test, $\alpha = 0.05$, n = 3), (MP = Malt peptone, MPC = Malt peptone + 1 % chitin, PD = Potato dextrose, PDC = Potato dextrose + 1 % chitin).

When 1 % of chitin was added to the PD medium, the spore count of JKI-BI-2642 (*B. bassiana*; 3.4×10^8 ss/mL), JKI-BI-2647 (*M. robertsii*; 1.6×10^8 ss/mL) and JKI-BI-1496 (*C. fumosorosea*; 4.1×10^7 ss/mL) were higher, whereas JKI-BI-2620 (*B. bassiana*; 1.8×10^5 ss/mL) was indifferent. Using MP media, *M. robertsii* increased to its highest spore count/mL (2.2×10^8 ss/mL), while *C. fumosorosea* showed a lower spore count (5.2×10^7 ss/mL).

When 1 % chitin was added to MP medium, JKI-BI-2647 and JKI-BI-2642 showed a higher spore number, while the other two strains JKI-BI-1496 and JKI-BI-2620 showed a lower spore number. However, for most strains this effect was not significant, with the exception of JKI-BI-2642, where adding 1 % chitin in MP medium, resulted in a significantly higher spore number (1.4×10^8 from 2.7×10^8 ss/mL, $df = 30$, $p = 0.03$).

Determination of dry biomass

There was no significant effect of the type of media for the different strains on their dry biomass (ANOVA; $F = 1.1$, $df = 9$, $p = 0.42$). However, adding 1 % chitin showed an effect for some strains. All strains, except JKI-BI-1496 (*C. fumosorosea*), showed a significant increase of dry biomass (Figure 6).

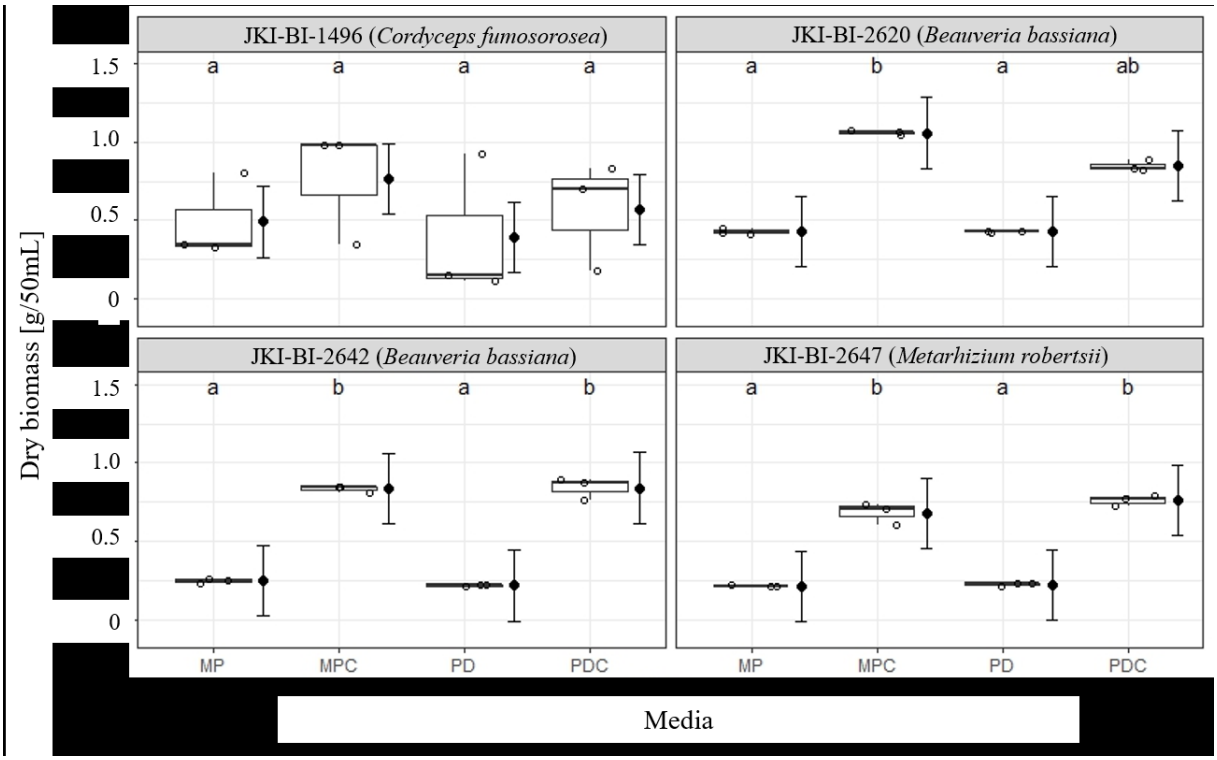

Figure 6. Effect of four different media on dry biomass [g/50mL] of four entomopathogenic fungi strains. Jittered boxplots consist of the median and the 25 % and 75 % quantile. Black dots and error bars represent adjusted mean with 95 % confidence limits per medium. Means followed by the same letters are not significantly different (Tukey HSD test, $\alpha = 0.05$, n = 3), (MP = Malt peptone, MPC = Malt peptone + 1 % chitin, PD = Potato dextrose, PDC = Potato dextrose + 1 % chitin).

Adding chitin to MP medium for JKI-BI-2620, effected the increase in biomass significantly (0.4 g/50mL to 1.1 g/50mL, $df = 30$, $p = 0.002$). While, adding 1 % chitin to PD did not effect the biomass of JKI-BI-2620 (0.4 g/50mL to 0.9 g/50mL, $df = 30$, $p = 0.05$) significantly. In

the JKI-BI-2642 (*B. bassiana*) adding 1 % chitin showed a significant increase of biomass in both media (MP medium, 0.3 g/50mL to 0.8 g/50mL, $df = 30$, $p = 0.004$; PD medium, 2.2 g/50mL to 0.8 g/50mL, $df = 30$, $p = 0.002$). A similar result was observed in JKI-BI-1647 (*M. robertsii*) when adding chitin (MP medium, 0.2 g/50mL to 0.7 g/50mL, $df = 30$, $p = 0.02$, PD medium, 0.2 g/50mL to 0.8 g/50mL, $df = 30$, $p = 0.009$).

Measuring the optical density of fungal culture

The optical density was different between EPF strains (ANOVA; $F = 2.0$, $df = 9$, $p = 0.08$), with an effect of the type of medium (ANOVA; $F = 6.3$, $df = 3$, $p = 0.002$). If the initial optical density was compared in different media (Figure 7), it can be seen that adding 1 % of chitin to the MP and PD media had an effect on all the strains. When adding 1 % chitin to the PD medium only JKI-BI-1496 (*C. fumosorosea*) showed a significant higher optical density after 72 hours of incubation (Figure 8).

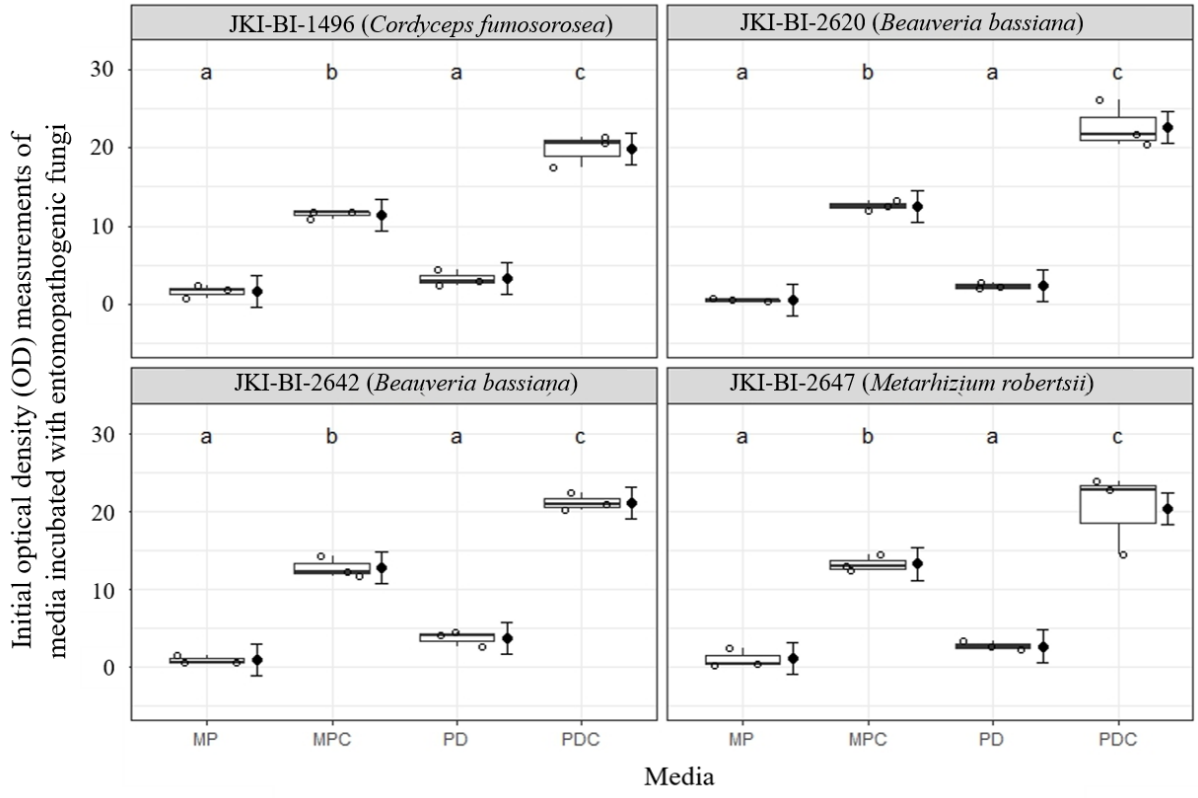

Figure 7. Effect of four different media on intial optical density [OD] of media incubated with four entomopathogenic fungi strains in the first hour. Jittered boxplots consist of the median and the 25 % and 75 % quantile. Black dots and error bars represent adjusted mean with 95 % confidence limits per medium. Means followed by the same letters are not significantly different (Tukey HSD test, $\alpha = 0.05$, n = 3), (MP = Malt peptone, MPC = Malt peptone + 1 % chitin, PD = Potato dextrose, PDC = Potato dextrose + 1 % chitin).

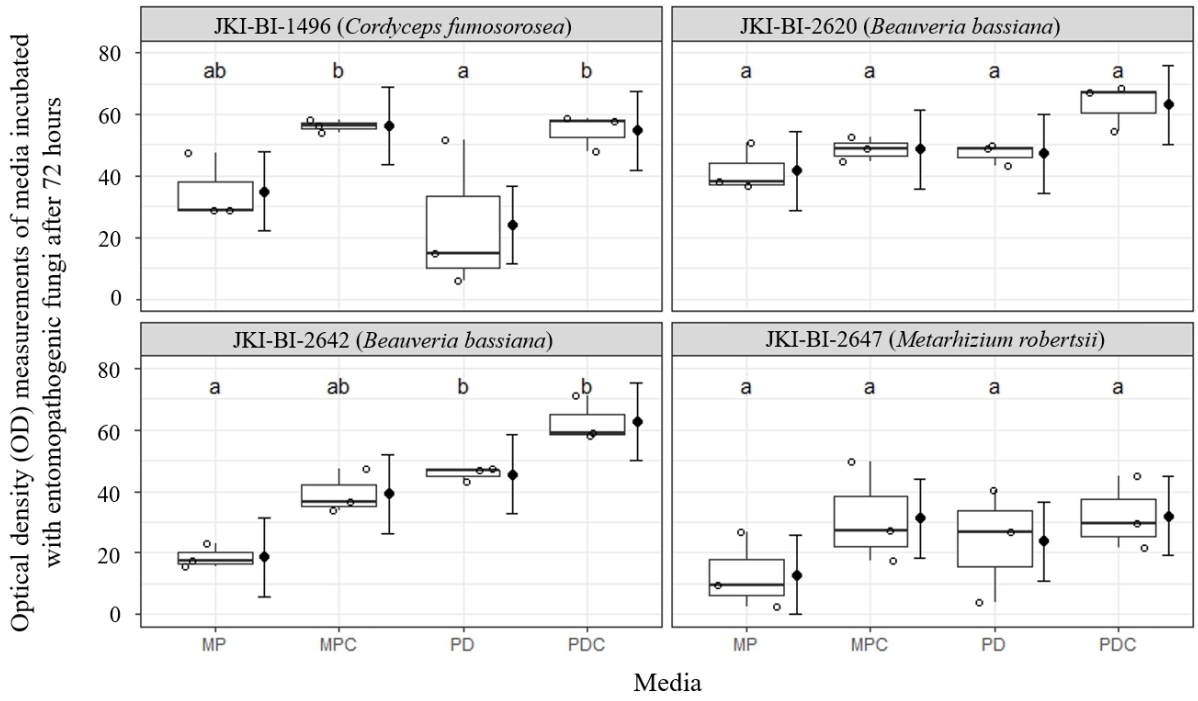

Figure 8. Effect of four different media on optical density [OD] of media incubated with four entomopathogenic fungi strain after 72 hours. Jittered boxplots consist of the median and the 25 % and 75 % quantile. Black dots and error bars represent adjusted mean with 95 % confidence limits per medium. Means followed by the same letters are not significantly different (Tukey HSD test, $\alpha = 0.05$, n = 3), (MP = Malt peptone, MPC = Malt peptone + 1 % chitin, PD = Potato dextrose, PDC = Potato dextrose + 1 % chitin)

Discussion

The primary aim of this study was to isolate EPF from an apple orchard in Germany and evaluate their potential to control *C. pomonella.* EPF are known to be adapted to local ecosystems and climates (Abaajeh and Nchu 2015; Lestari et al. 2016). The orchard's nutrient-rich soil on rocky ridges likely supports diverse microorganisms, including EPF, potentially offering effective control against local fruit pests like *C. pomonella*. The unique microclimate of the rocky ridges could further enhance the survival and effectiveness of these microorganisms.

In this study all strains, including control strain, from three different genera were distinguished based on their morphological features (Abaajeh 2014). All *Beauveria* strains were whitish in colour with a fluffy appearance, whereas *Metarhizium* strains were greenish in colour with a powdery texture (Islam et al. 2021). *Cordyceps* strains were characterized by a whitish, creamy or yellowish aerial mycelium (Zimmermann 2008). The identification of

EPF based on molecular techniques is a prerequisite for distinguishing species more accurately. Internal transcribed spacer ITS1-5.8S-ITS4 region of ribosomal DNA (rDNA-ITS) is the most widely used for detection and identification of EPF (Islam et al. 2014; Gebremariam et al. 2021). The sequences of the ITS1-5.8S-ITS4 rDNA region of all unknown EPF isolates showed 95 – 100 % sequence identity with the NCBI/Genebank data and they matched with the morphological identification as well. Molecular identification at species level was essential to my research. *Beauveria* strains were pinpointed using primers B22U and B822L (Ramos et al. 2017; Kasambala Donga et al. 2021; Soth 2022), while the 5´ region of the elongation factor-1-alpha was sequenced for *Metarhizium* (Bischoff et al. 2009; Fisher et al. 2011). Phylogenetic trees with bootstrap values over 50 provided strong support for current findings (Huang et al. 2009; Apollos et al. 2017; Russo and Selvatti 2018), and the monophyletic groups indicated a common ancestor (Reddy 2011), affirming the morphological and molecular consistency of the EPF species identified.

The precise molecular and morphological identification of EPF not only strengthens the understanding of their genetic and evolutionary backgrounds, but also may correlates with their effectiveness in controlling *C. pomonella.* The virulence study on *C. pomonella* demonstrated that *Beauveria* and *Metarhizium* are more virulent than *Cordyceps* strain confirming their potential as bioinsecticides (Abaajeh and Nchu 2015; Islam et al. 2021). *B. bassiana's* broad-spectrum activity, conducive to various formulations, underscores its versatility as a non-selective pesticide (Islam et al. 2021). *Cordyceps'* specificity, as noted by Qu et al. (2022), aligns with its targeted pest control capabilities.

The effectiveness of EPF, demonstrated by their virulence against *C. pomonella*, can be partly attributed to their enzymatice mechanisms, such as chitinase production. Chitin serves as a versatile substrate with notable antimicrobial, anti-inflammatory, and antioxidative properties, integral to fungal cell walls and invertebrate exoskeletons (Park and Kim 2010; Murthy and Bleakley 2012). Chitinase, key to hydrolyzing chitin, works alongside proteases to break down insect cuticles, aiding in cellular wall degradation and defense against other fungi (Mondal et al. 2016). This study employed a modified assay using Lugol's Iodine (Ai Xia et al. 2011), achieving rapid chitinase activity detection as evidenced by clear zones within 5 minutes, a significant reduction from the traditional hour-long staining methods (Duncan et al. 2008).

EPF that can adapt to local temperature variations is crucial for their effectiveness in the field. Temperature extremes can either deactivate fungal spores through dehydration at high temperatures or inhibit growth at low temperatures (Inglis et al. 2001). Research by Abaajeh (2014) suggests that while EPF generally thrive across a range of temperatures, they grow best between 25 °C and 35 °C. Strains adapted to local climates demonstrate enhanced performance, underlining the importance of region-specific strain selection. In current study, JKI-BI-1496 (*C. fumosorosea*) emerged as particularly robust, showing optimal radial growth at 20 °C and 25 °C, whereas JKI-BI-2647 (*M. robertsii*) performed best at 30 °C. This highlights the potential of *C. fumosorosea* as a versatile candidate for biological control in varying temperature conditions. Da Couceiro et al. (2021) also observed similar patterns with *M. robertsii*, where the mycelia of this fungus grew faster at 33 °C. It has been suggested that *Metarhizium* strains can be used primarily as insect pathogens in tropical and sub-tropical regions (Yeo et al. 2003). According to Seib et al. (2023), *Metarhizium* strains, incubated at 25 - 30 °C, showed strong mycelial growth with rapid germination and fast virulence, although the fungi had a lower mortality against *G. mellonella*. Temperature tolerance varies among *C. fumosorosea* and *B. bassiana* isolates, influenced by their geographic origins. European strains of *C. fumosorosea* grow best at 20 - 25 °C, while isolates from warmer regions like the southern United States and western Asia have optimal growth at slightly higher temperatures of 25 - 28 °C. Indian *C. fumosorosea* strains show the greatest heat tolerance, thriving even at 32 - 35 °C (Zimmermann 2007b). *B. bassiana* exhibits a broader optimal range of 23 - 28 °C, suggesting its adaptability across diverse climates (Zimmermann 2007b). These findings, consistent with the superior potential of *Cordyceps* strains in warmer agricultural soils (Islam et al. 2021), underline the need for context-specific selection of biocontrol agents to ensure their effectiveness (Yeo et al. 2003).

Considering the environmental fluctuations, sunlight can be an important factor for the effectiveness of EPF. For instance, Iwanicki et al. (2021) reported that *Metarhizium* are light sensitive. Fernandes et al. (2015) and Couceiro et al. (2021) reported that 2 hours of sunlight simulation are sufficient to reduce germination and virulence. Similar experiments were also conducted by Fargues et al. (1996), who compared different EPF under several sunlight simulations. They found that the degree of light tolerance is strain/species specific. In this study, after 3 hours of simulated sunlight, JKI-BI-1496 (*C. fumosorosea*) showed higher

germination compared to the other three strains. JKI-BI-2647 (*M. robertsii*) showed the lowest germination among all the strains.

The necessity of understanding environmental factors in the application of EPF, paralleling the influence of nutrient availability on fungal growth. Nutrients play a key role in promoting fungal growth as they provide the building blocks, energy source and co-factors for biochemical reactions. Mar et al. (2012) demonstrated that a nutrient-rich medium influences mycelial growth, whereas a nutrient poor medium stimulates sporulation. This study aimed to optimize nutrient compositions for cultivating EPF in liquid fermentation, focusing on maximizing yield and efficiency for large-scale production. Key nutrients, including carbon, nitrogen, and essential minerals, were adjusted to suit specific fungal species and strains, enhancing growth and propagule quality. This optimization is crucial for improving the effectiveness of EPF as biological control agents (BCAs) (Jaronski 2014).

Moreover, in EPF, fast conidia germination, radial growth and high sporulation rates can determine virulence, potentially can act as catalysts for fungal virulence (Gebremariam et al. 2021). In this study, spore production, dry biomass, and optical density were examined. Between two *B. bassiana* strains. JKI-BI-2642 showed higher spore production, indicating efficient carbon and nitrogen usage, essential for sporulation (Cojocaru and Lumînare 2021). Adding 1 % chitin to MP medium significantly boosted spore numbers for JKI-BI-2642, aligning with prior findings that chitin can enhance submerged spore production (Cojocaru and Lumînare 2021). However, the same chitin addition led to reduced spore numbers in PD medium and had no effect on JKI-BI-2620, illustrating the variable influence of nutrient adjustments on fungal growth (Feng et al. 1994).

Optical density in EPF studies measures light absorption, indirectly indicating fungal biomass and growth. However, it is not directly proportional to biomass. Therefore, dry biomass was also evaluated alongside optical density for a more accurate assessment. This study identified varying growth responses in two *B. bassiana* strains (JKI-BI-2620 and JKI-BI-2642), both showing increased biomass and optical density, though JKI-BI-2620 had minimal spore production. The addition of chitin influenced these parameters across these two strains produced in different media, potentially due to peptone serving as a nitrogen source (Senthamizhlselvan et al. 2010). While JKI-BI-2620 exhibited superior mycelial growth, the chitin-induced enhancement in optical density was not sustained after 72 hours, likely affected

by incubation shaking. In contrast, JKI-BI-1496 (*C. fumosorosea*) showed different spore production patterns, unaffected by alterations in the carbon-to-nitrogen ratio or chitin addition. This highlights the distinct nutrient dependencies for growth and spore production in these fungal strains.

On the other hand, the dry biomass of JKI-BI-2647 (*M. robertsii*) showed similar results for the other three strains. The addition of 1 % chitin increased the dry biomass this strain. However, optical density of *M. robertsii* in different media showed a different picture after 72 hours of incubation. *M. robertsii* cultured in PD medium with or without 1 % chitin showed no difference in optical density, which was somewhat surprising, while the same fungus produced in MP medium with and without chitin gave the usual result. Iwanicki et al. (2021) reported that spores of *Metarhizium* are sensitive when submerged. This could be the reason of the results of current study.

It was found that different strains react differently to different environmental parameters. In terms of light tolerance such information could be important when it comes to shelf life of a particular strain (Iwanicki et al. 2021). In addition, fungal stability and conidial yield may vary. Stable strains last for several generations, whereas unstable strains usually decline after a few subcultures (Ansari and Butt 2011).

In conclusion, this study was initiated with collecting soil samples from one apple orchard, which had a well-documented history with *C. pomonella*. Additionally, on the sampling sides no EPF were ever applied before and field trials are foreseen to control white grubs with *Beauveria brongniartii*. In the orchard various EPF were isolated with potential to control *C. pomonella*. Four out of these 13 strains showed high chitinase activity, which may positively influence the effectiveness of EPF. Furthermore, the EPF differed in temperature optima and sunlight sensitivity, which may influence the incidence in the field. Based on the producability some of the strains are interesting candidates for further development. It suggests that a deeper understanding of the potential of these EPF as effective biocontrol strategy may be gained by enhancing their virulence and by subjecting them to field trials against *C. pomonella*.

Chapter II

Chitin-amended media: A solution to improve effectiveness of *Cordyceps fumosorosea* as a biological control agent (BCA) against *Cydia pomonella*

Abstract

The microbial control of insect pests such as the codling moth *Cydia pomonella* is a major challenge in agriculture. This study investigates the potential of chitin-amended medium to improve the effectiveness of entomopathogenic fungus JKI-BI-1496 (*Cordyceps fumosorosea*) for control of *C. pomonella*. Chitin, a key component of insect exoskeletons, colloidal chitin (CC) or chitosan were added in two concentrations to the liquid growth media. In first bioassay with larvae of *C. pomonella* the virulence of the submerged spores produced in these different media was compared. Spores produced from medium containing 1 % CC exhibited the highest virulence, resulting in a mortality of 66.0 %. Additionally, the influence of the chitin-amended media on submerged spore and biomass production and on the speed of germination were analysed. Then, it investigated the impact of the most promising media containing 1 % CC or 1 % chitin on lethal concentration, sunlight sensitivity and on the formulation process. To achieve a mortality of 75 % a concentration of 1.6×10^7 submerged spores/mL (ss/mL) was required for the media containing 1 % CC, compared to media containing no CC or chitin, which required a more than four times higher concentration of 7.1×10^7 ss/mL. However, the addition of 1 % CC had a negative effect on the fungus' resilience during prolonged sunlight simulation. On the other hand, the media containing 1 % CC had a positive effect on maintaining the viability within the formulation process via freeze-drying. These findings offer valuable insights into strategies for enhancing the effectiveness of *C. fumosorosea* including the down-stream process.

Introduction

Entomopathogenic fungi (EPF) can be described as multi-taskers. On the one hand, they are an excellent alternative to chemical insecticides and, on the other, they are environmentally friendly. EPF are widely distributed in our ecosystems and in the fungal kingdom. Many of these EPF are currently having a major impact on the global biopesticide market. In a large number of EPF, the genera *Beauveria*, *Cordyceps* and *Metarhizium* contribute as active ingredients to commercially available formulated products (Montesinos-Matías et al. 2021). In Europe, the number of organic pesticides are lower compared to the global market. The European Commission is currently considering strengthening the risk assessment of pesticides, facilitating the authorisation of organic pesticides and improving the approval process by Member States. There are EU-based pesticide regulations that provide for several steps to enter the market. This includes evaluation of the active substance and approval at EU level (European Commission, accessed 25 March 2022). According to the EU, seven strains of *Beauveria bassiana*, one of *Cordyceps. fumosorosea* and one of *Metarhizium anisopliae* are approved as active ingredients for (Stephan et al. 2021; European Commission, Accessed on 25 March 2022). The development of a potential biopesticide involves a number of steps. For example, selecting the best fungal strain to control the target insect based on virulence, enzymatic activity profile, influence of nutrients, tolerance to environmental stressors and stability. *C. fumosorosea* was considered a potential candidate for formulated EPF against *Cydia pomonella*, as it has already been approved as an active ingredient for pesticides in the EU.

When developing the formulation of a potential EPF, the culture medium has a major influence on the growth characteristics and virulence towards the target insect. Therefore, several studies have focused on modifying the nutrient composition of culture media to improve the efficacy of EPF, including spore number, germination, optical density, lethality, etc. (Jackson 1997; Safavi et al. 2007). These parameters have been shown to be indicators of EPF effectiveness. In this context, it has been described that fungi with a higher production capacity of viable conidia and a higher invasiveness of the mycelium have a better performance because the infection of the target insects is faster (Montesinos-Matías et al. 2021). Therefore, the correct choice of culture media has a major impact not only on effectiveness but also on large-scale production. In addition, it has been shown that nutritional

and environmental conditions in liquid media can have significant effects on spore yield, stability and biocontrol efficacy of entomopathogenic fungi (Vega 2003; Jackson 1997). Furthermore, the formulation of a liquid culture medium enriched with increased nitrogen content is critical for the production of desiccation-tolerant blastospores of *C. fumosorosea*. These blastospore preparations have significant potential as bioinsecticidal propagules compared to conidia because they germinate rapidly on agar and on the cuticle of insects such as the silver leaf whitefly (Jackson 1997). Safavi et al. (2007) tested different concentrations of carbon and nitrogen ratios with 1 % chitin in liquid media. The present study focuses on the suitability of culture media containing different amounts of chitin and its derivatives for the cultivation of *C. fumosorosea* against *C. pomonella*.

After the French chemist Henri Braconnot first isolated and characterised it from fungi in 1811, chitin $(C_8H_{13}O_5N)n$ was identified as the second most abundant natural biopolymer on Earth.(Lavall et al. 2007; Ngo et al. 2008; Abdou et al. 2008; Park and Kim 2010; Abo Elsoud & El Kady 2019). It is a natural nitrogenous polysaccharide composed of β-1, 4 N-acetyl-D-glucosamine units. It is widely distributed in nature and can be found in insect exoskeletons, crustacean shells and fungal cell walls (Nithya et al. 2015). Chitin, its deacetylated form, chitosan, and the deminarilised form, colloidal chitin (CC), have attracted considerable interest in terms of their proposed new applications. Chitosan is a partially deacetylated form of chitin, and consists of polymers of b-1, 4-glucosamine subunits, with a molecular weight of up to 400 kDa. It is environmentally safe and non-toxic to higher organisms (Palma-Guerrero et al. 2008). In insects, chitin and chitosan serve as structural barriers that can only be overcome by the action of extracellular chitinases, allowing the fungus to enter and spread into the haemocoel. This suggests that chitinases may play a critical role in both insect cuticle degradation and host invasion (Ali et al. 2010a). Incorporation of chitin and its derivatives into the culture medium significantly enhances chitinase production by EPF and increases EPF efficiency (Nithya et al. 2015). In the last decade, interest in chitinases has increased due to their diverse applications, one of the most important being their use in the biocontrol of plant pathogens (Mondal et al. 2016). Cuticle-degrading enzymes include proteases, lipases, and chitinases, of which chitinases contribute significantly to the degradation of protective sheaths of pests (Deshpande 1999).

It was very critical to select the potential EPF to determine the best production of submerged spores with high virulence against *C. pomonella*, when chitin and its derivatives were added in media. After standardized virulence test and agar-chitin plate assay, potential entomopathogenic fungus JKI-BI-1496, *C. fumosorosea* was found. In virulence test, JKI-BI-1496 showed more than 85 % mortality. In agar-chitin plate assay, it showed the enzymatic activity more than 1. After these two tests, it can be said that JKI-BI-1496 has virulence effect on *Cydia pomonella*, as well as a chitinase effect (Chapter I). Therefore, further experiments with nitrogenous polysaccharides, i.e., chitin to formulate potential media and to evaluate the effectiveness against *C. pomonella* were intended.

The main idea of this chapter is that the addition of chitin and its derivatives in the production of *C. fumosorosea* is beneficial in two ways. Firstly, it could play a positive role in the growth and degradation of the fungal cell wall. Secondly, it could have an effect on the effectiveness of the EPF in degrading the insect cuticle. Chitin, which is a major component of both these structures, is also a critical factor in virulence (Charnley 2003; Wu et al. 2010). The involvement of chitinases in insect cuticle degradation could be a valuable tool for more efficient insect control, particularly in the case of pests such as *C. pomonella*. The first objective of this study is to select the potential medium containing chitin and its derivatives for JKI-BI 1496 (*C. fumosorosea*) which can produce submerged spores with high virulence against *C. pomonella*.

Nevertheless, it is important to address concerns about the stability of such a formulation under field conditions, which warrants further investigation in the future. The efficacy of EPF in the field is affected by various environmental stress factors (Mascarin et al. 2018; Irsad et al. 2023). The ability of EPF to survive stress factors prior to infection, during cuticular penetration, and in vivo is a prerequisite for successful biological control agents (BCAs). There are numerous reports on the effects of various environmental factors on *Beauveria* and *Metarhizium* (Aw and Hue 2017; Rangel and Roberts 2018; Wang et al. 2021; Liu et al. 2023; Quesada-Moraga et al. 2024). Furthermore, in previous chapter (Chapter I), it was observed that *C. fumosorosea* showed higher radial growth at 25 ºC and higher germination after 3 hours of sunlight simulation compared to other three EPF. However, little is known about the behaviour of *C. fumosorosea* under changing environmental stress factors. Although many questions remain unanswered about the effect of nutrition on blastospore stability under

environmental stress and the effectiveness of *C. fumosorosea* blastospores under field conditions, many strains of *C. fumosorosea* can rapidly produce substantial amounts of desiccation-tolerant blastospores in liquid culture when provided with media containing appropriate levels of carbon and nitrogen (Jackson et al. 1997; Jackson 1997; Xie et al. 2016). In the current study, adding chitin and its derivatives to the culture media increased the carbon content of the media. Therefore, the second objective was to determine the influence of chitin and its derivatives on the effectiveness of JKI-BI 1496 (*C. fumosorosea*) in relation to simulated sunlight for different durations (1 to 4 hours).

Desiccation-tolerant submerged spores also affected viability in long-term storage (Feng et al. 1994; Jackson et al. 2004; Jaronski 2023). Freeze-drying is a commonly used technique for long-term storage (Tan et al. 1995). However, freeze-dried submerged spores are sometimes associated with difficulties in preserving live blastospores. For example, the ability to withstand desiccation during the drying process and maintain stability during storage (Jackson et al. 1997; Jackson et al. 2004). The method or formulation used to dry *C. fumosorosea* blastospores has been shown to significantly affect the desiccation tolerance and storage stability of these propagules (Jackson et al. 1997; Jackson 1997; Jackson et al. 2006). In addition, nutrients in the growth medium, especially carbon and nitrogen, also influence the stability of the dried spores (Cliquet and Jackson 1999; Cliquet and Jackson 2005; Dietsch et al. 2021). Therefore, the third objective of this research was to determine the effects of chitin-enriched media on long-term storage viability by freeze-drying.

Materials and Methods

Fungal strain *Cordyceps fumosorosea*

For present work, JKI-BI-1496 (*Cordyceps fumosorosea*) was used. This strain was isolated from *Cydia pomonella* in 1971 by Müller-Kögler in Darmstadt, Germany (Inventory data, JKI). The hosts of *C. fumosorosea* include amongst others the whitefly (*Bemisia tabaci*), codling moth (*C. pomonella*), plum moth (*Cydia funebrana*) and peach moth (*Cydia molesta*). JKI-BI-1496 was taken from the institute's own strain collection, which is stored at - 80 °C and was cultivated on malt-peptone-agar (MPA) containing 3 % (w/v) malt extract (Merck,

Darmstadt, Germany), 0.5 % (w/v) peptone from soybean (Merck, Darmstadt, Germany) and 1.8 % (w/v) agar-agar (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). For all further experiments, the fungus was cultivated in liquid medium (see below).

Primary selection of suitable media through submerged spores and biomass measurement

To prepare the starter culture, previously prepared 100-mL Erlenmeyer flasks containing 50 mL autoclaved malt-peptone broth containing 30 g/L malt extract (Merck, Darmstadt, Germany) and 9 g/L peptone (Sigma Aldrich, Buchs, Switzerland) were used. Flasks were inoculated with a loop of a sterile inoculation needle conidia of *C. fumosorosea* grown for around 14 days on MPA at 25 °C. The inoculated Erlenmeyer flasks were incubated for 72 hours at 150 rpm and 25 °C on a horizontal shaker (Novotron, 50mm deflection, Infors, Bottmingen, Switzerland). The submerged spore number was measured by Thoma counting chamber (Improved Neubauer, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). New starter cultures were inoculated for each experiment (spore productivity, dry biomass measurement, online biomass monitoring by backscattered light detection, detection of germination speed and bioassay), and all media used in the subsequent experiments were inoculated with fungal material from these starter cultures with 1×10^6 submerged spores per mL (ss/mL).

For observing the influence of the media composition, three different liquid media has been used. The media used were (i) malt peptone, (ii) potato dextrose (Merck, Darmstadt, Germany), containing 26.5 g/L potato dextrose, and (iii) modified Samsi8 (S8) (Samšináková 1966), containing 25 g/L glucose (Merck, Darmstadt, Germany), 20 g/L corn steep solid (Sigma Aldrich, Buchs, Switzerland) and 5 g/L sodium chloride (Merck, Darmstadt, Germany).

50 mL of liquid medium were inoculated with 1×10^6 ss/mL from the starter culture. After cultivation for 72 hours at 25 °C and 150 rpm on a horizontal shaker, the number of spores and dry biomass were detected. Furthermore, it was determined how the fungal isolate behaves during growth in the liquid medium. The experiment repeated three times.

For dry biomass measurement, whole 50 mL spore suspension was poured into a filter paper (diameter 150 mm), and a vacuum pump was used for filtration. Then this filter paper was placed into a bowl (the weight of the bowl was measured beforehand) and weighted the wet mass. Then this bowl with wet filter paper was put at 60 °C for 72 hours, dry biomass was weighted. This experiment was conducted independently with three repetitions.

Influence of chitin derivatives on *C. fumosorosea*

After the primary selection of basic media, submerged spore count, biomass monitoring by backscattred light detection and germination were evaluated with chitin and its derivatives with different concentrations with S8 medium (0.5 % (w/v) and 1 % (w/v) of chitin (Merck, Darmstadt, Germany), chitosan (Merck, Darmstadt, Germany) and colloidal chitin (CC) (modified from chitin)). Preparation of CC was described in Chapter I.

First bioassay

First bioassay was designed to examine the effects of chitin derivatives on *C. fumosorosea* spores against codling moth (*C. pomonella*). Fifth instar larvae (L_5) of *C. pomonella* were used for the virulence test. L_1 larvae were obtained from the Julius Kühn Institute (JKI) and reared with until reaching the L_5 stage. The rearing procedure was explained in chapter I. Submerged spores of *C. fumosorosea* were produced through liquid fermentation for 96 hours at 25 °C and 150 rpm, followed by filtration and centrifugation. The spore pellet was washed once and resuspended in 0.5 % Tween 80. The spore concentration was adjusted to 1×10^6 ss/mL. The bioassay included an untreated control and a control with 0.5 % Tween 80. Each treatment involved dipping 20 larvae into the respective spore suspension. After a gentle rotation, larvae were placed on autoclaved gauze in plastic boxes with cardboard hiding places and incubated in the dark at 25 °C for 21 days. Feeding was unnecessary for L_5 larvae. After 3 weeks, the experiment was evaluated based on the mortality of moths. Virulence of *C. fumosorosea* was measured based on the percentage of mortality of L_5 *C. pomonella* larvae. The experiment was independently repeated seven times.

Growth

For counting submerged spores, the same protocol was used as for submerged spores production and counting for primary media selection except the incubation time was 96 hours at 25 °C and 150 rpm. This experiment was conducted by three repetitions.

The biomass monitoring by backscattered light detection of the fungal culture during liquid fermentation was measured using the Cell Growth Quantifier (CGQ) (Aquila biolabs GmbH, Baesweiler, Germany), a measuring device that enables non-invasive, real-time monitoring of organism growth in culture vessels. For this purpose, the flasks were placed on a CGQ sensor plate during the incubation, which repetitively measures the biomass density through the wall of the shake flask. The sensor emits light in the form of light pulses into the culture medium and measures the intensity of the light that is scattered back from the biomass (backscatter measurement). The higher the concentration of microorganisms in the medium, the higher the intensity of the reflected light. The device took a measurement every 5 seconds including rpm and temperature. The biomass monitoring data was recorded every minute for 96 hours. This experiment had been conducted with seven repetitions.

Speed of germination

To check the speed of germination of the submerged spores produced in the different media, 100 µl of spore suspension were pipetted onto MPA-plate and spread with a sterile Drigalski spatula. The plates were incubated at 25 °C and the germination was determined from every hour over 8 hours. For this purpose, a piece of agar measuring approx. 2 cm × 2 cm was cut out of the plate with a sterile scalpel and placed on a slide. A drop of lactophenol blue (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was placed on the agar piece to stain the spores and germ tubes and to stop growth. A cover slip was then placed on top, which caused the dye to spread evenly over the agar piece. Spores were counted as germinated when the length of the germination tube was as long as the width of the spore. This experiment was done with three repetition and three replications.

Impact of the amendment of 1 % chitin and 1 % CC on *Cordyceps fumosorosea*

Second bioassay: Lethal concentration

Additionally, the lethal concentration for 75 % mortality of *C. pomonella* was determined through statistical analysis using a generalized linear model. Spore concentrations of 1×10^6, 1×10^7, and 1×10^8 ss/mL were evaluated with three repetitions for determining the lethal concentration (LC_{75}) of submerged spores. Even by applying the lowest concentration, the probability of mortality of insects was relatively high. Therefore, LC_{75} was used here for analysis instead of LC_{50} or LC_{90}.

Influence of sunlight simulation

To assess the chitin amendment on the impact of sunlight on the produced submerged spores, simulated sunlight exposure was conducted for 1, 2, 3, and 4 hours at 350 W/m^2. For preparing the spores, after filtering over four layers of cotton gauze, submerged spores were kept for 3 minutes in the sonicator (Bandeln Sonorex RK 52, Berlin, Germany). 10 mL spore suspension at a concentration of 1×10^6 ss/mL was prepared, and 1 mL was pipetted in one compartment of the 24-well control plate (cell culture multiwell plate 24 wells, PS, Greiner Bio-One GmbH, Frickenhausen, Germany). This plate was irradiated without lid in a SUNTEST XXL + FD sunlight simulator (Atlas Material Testing Technology, Illinois, USA). The lid was removed to avoid light refraction and bundling effects due to evaporation and subsequent condensation. In the sample chamber of the sunlight simulator, the highest possible humidity (95 %) was ensured to prevent the samples from drying out. Other 24-well control plate was wrapped with lid aluminum foil to expose the spores to the same temperature conditions but protect them from solar radiation. All the samples were irradiated with 300 - 800 nm. The black standard (BST) served as the temperature sensor and the fan was set to 2000 rpm. The samples were thus exposed to a total of 3780 kJ/m^2 for 4 hours. After each hour of exposure, samples were taken and transferred to the 24-well plate covered with aluminum foil. At the end of the exposure three droplets (10 µL) of each sample were pipetted on a MPA-plate. Samples from MPA-plates were taken after 16 hours of incubation at 25 ºC. Germination was evaluated at each time point as described above. The germination of control plates were considered 100 %, serving as a reference for comparison. The experiment was repeated three times with three replications each.

Influence of freeze-drying

Freeze-drying of submerged spores aimed to extend product shelf life while maintaining microbial viability and ensuring ease of use. 20 % (w/v) lactose solution was used as cryo-protectant, which was autoclaved after boiling. The cooled solution was mixed 1:2 with a submerged spore suspension (1×10^6 ss/mL). 3 mL of this mixture were pipetted into 15-mL vials (Ismatec, Wertheim, Germany) and the rubber stoppers were then loosely placed on the test vials and these were placed in the freeze-dryer (Virtis, Advantage El automated version, Gardiner, New York, USA). Due to the loose fitting, the lids were not closed and the moisture could escape under vacuum during the freeze-drying. The freeze-drying was done through a program SynWiz started and checked. The freeze-drying process included three main phases, named freezing, additional freezing and drying phase. In freezing phase, the temperature was decreased to + 5 °C over 20 minutes, followed by a decrease to - 20 °C over 5.3 hours and a further decrease to - 40 °C over 11.4 hours. The additional freezing phase involved maintaining the temperature at - 40 °C for 15 minutes before lowering it to - 80 °C. Finally, in the drying phase, the temperature was held at - 20 °C for 3 days. After 3 days of freeze-drying was stopped and the vials were sealed immediately sealed at 0.2 mbar in the freeze-drying chamber of the freeze-dryer. After freeze-drying, germination capacity of the spores was determined. Freeze-dried material was resuspended with 2.7 mL of 0.5 % Tween 80, and 100 µl per plate were pipetted for germination testing. The viability of non-freeze-dried and freeze-dried submerged spores was measured after incubation for 16, 24, and 48 hours of incubation at 25 °C. This experiment was independently repeated three times with nine replications.

Statistical Analysis

Statistical analyses were conducted using R Studio software (Version 1.4.1106) (RStudio Team, 2020). The data are presented as the mean and standard deviation (± SD) or mean and standard error (± SE), depending on which model was used for calculation. The underlying assumptions of the ANOVA were met. To ensure normal distribution and variance homogeneity, the residuals of the respective model were visually inspected. The QQ-Plot (sample quantile-theoretical quantile) was used to assess normal distribution, and the

Residuals-Prediction-Plot was used to check for variance homogeneity. The best-fitted model was selected based on the Akaike criteria. The Information Criterion (AIC) (Burnham and Anderson, 2002) was used after backward elimination of the full model. Unless otherwise stated, differences between factor levels were examined using the post hoc Tukey HSD test ($\alpha = 0.05$) with the R package emmeans (Russell, 2023) in the context of an ANOVA. For submerged spore count, dry biomass and bioassay, following formula for linear model (LM) has been used:

$$\text{LM (y} \sim \text{media + repetition)}$$

For estimating germination test, biomass monitoring, sunlight simulation tolerance and freeze-drying, following LM has been executed:

$$\text{LM (y} \sim \text{media + hour + media: hour + repetition + replication)}$$

For estimating lethal concentration for 75 % mortality of *C. pomonella* was done by fitting a GLM with binomial family for each concentration of each medium tested followed by predictions using the MASS package in R (Ripley, 2023).

For Kaplan-Meier analysis, survival curves over time were calculated with the survival R package (Therneau, 2023) with survfit function and compared with survival curves using the log-rank test (survdiff function) to determine global effects.

Results

Primary selection of suitable media through submerged spores and biomass measurement

Three liquid media were compared to select a suitable medium for production of submerged spores of *C. fumosorosea*. For both, submerged spores (ANOVA; $F = 10.1$, $df = 4$, $p = 0.03$) and dry biomass (ANOVA; $F = 1203.5$, $df = 2$, $p < 0.001$) significant differences among media were determined after 72 hours cultivation. In S8 medium, *C. fumosorosea* produced the significant highest number of submerged spores with 2.0×10^8 ss/mL and highest amount of dry weight (Table 1) in pairwise comparison. Therefore, S8 medium was chosen for further experimentation.

Table 1. Submerged spores and biomass production of *C. fumosorosea* in three different liquid standard media.

Media	Concentration of submerged spores [ss/mL]	Dry biomass [g/50mL]
S8	$2.1 \times 10^8 \pm 8.2 \times 10^7$ (b)*	1.1 ± 0.02 (C)
Malt peptone	$5.2 \times 10^7 \pm 1.3 \times 10^7$ (a)	0.3 ± 0.01 (B)
Potato dextrose	$5.1 \times 10^7 \pm 1.5 \times 10^7$ (a)	0.2 ± 0.04 (A)

*: Mean ± SD with the same letters after executing linear model are not significantly different (Tukey HSD test, $\alpha = 0.05$, n = 3)

Influence of different chitin derivatives on *C. fumosorosea*

After the primary selection of media, S8 medium was amended with chitin and its derivatives with different concentrations (0.5 and 1 % of chitin, chitosan or CC).

First Bioassay

Bioassay with submerged spores produced in different media demonstrated a direct effect of the media composition on the spore virulence, which was measured as percentage of mortality of L_5 *C. pomonella* larvae (ANOVA; $F = 6.6$, $df = 8$, $p < 0.001$). The effect of submerged spores produced in S8 + 1 % chitin ($df = 48$, $p = 0.002$ (untreated); $p = 0.001$ (tween)) and S8 + 1 % CC ($df = 48$, $p = 0.0001$ (untreated); $p = 0.0001$ (tween)) showed significantly higher mortality of the larvae compared to the untreated and tween control (Figure 1). Only the submered spores produced in medium S8 + 1 % CC showed significantly higher virulence (mortality = 66.4 %, $df = 48$, $p = 0.004$) in comparison to spores produced in S8 medium. However, there was no significant differences observed, in case of mortality among the spores produced in the media, containing chititn and its derivatives.

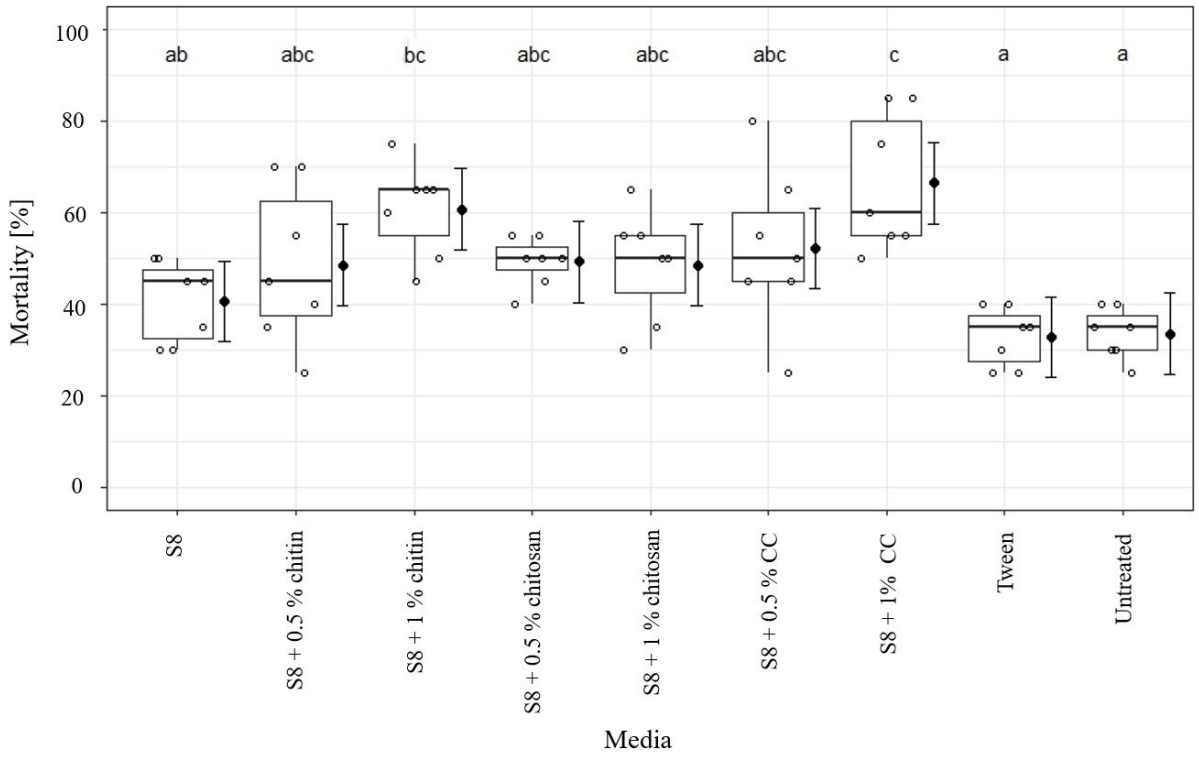

Figure 1. Pairwise comparison of the mortality of *C. pomonella* L_5 larvae caused by *C. fumosorosea* grown with different chitin derivatives. Jittered boxplots consist of the median and the 25 % and 75 % quantile. Black dots and error bars represent adjusted mean with 95 % confidence limits per medium. Means with the same letters are not significantly different (Tukey HSD test; $\alpha = 0.05$, n = 7).

Growth

After 96 hours of cultivation, significant effects of chitin derivatives on the production of submerged spores were observed (ANOVA; $F = 3.5$, $df = 6$, $p < 0.01$). The highest yield of submerged spores was obtained with S8 + 0.5 % chitosan (2.8×10^8 ss/mL) and was significantly different from S8 ($df = 36$, $p = 0.02$) and S8 + 0.5 % chitin ($df = 36$, $p < 0.01$) (Figure 2). For all other media, the number of produced submerged spores did not differ.

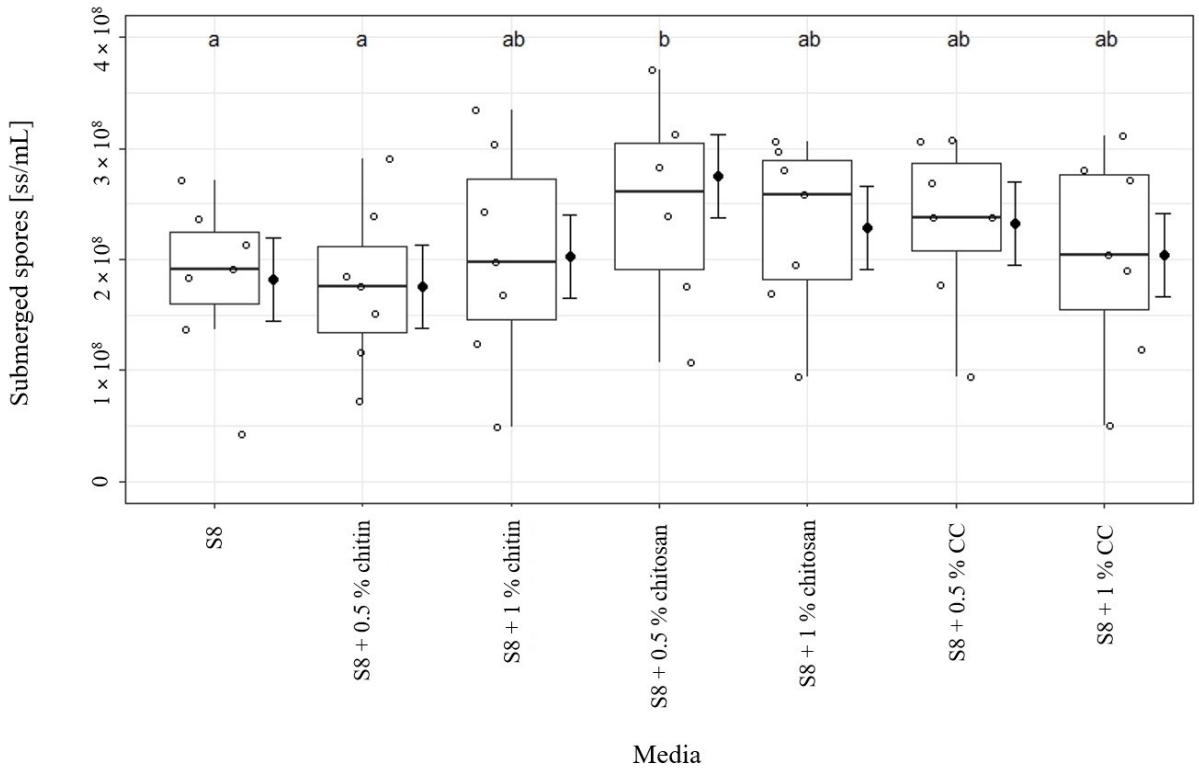

Figure. 2. Pairwise comparison of the effect of seven different media on *C. fumosorosea* on spore number. Submerged spores in [ss/mL]. Jittered boxplots consist of the median and the 25 % and 75 % quantile. Black dots and error bars represent adjusted mean with 95 % confidence limits per medium. Means with the same letters are not significantly different (Tukey HSD test; $\alpha = 0.05$, n = 7).

Biomass monitoring by backscattered light detection of seven media were measured over 96 hours (Table 2). Statistical significance was found for the effect of growing biomass in different media interacting with time (ANOVA; $F = 6.5$, $df = 24$, $p < 0.001$). Notably, after 48 hours, S8 and S8 + 0.5 % CC had slightly higher biomass compared S8 + 0.5 % chitosan, S8 + 1 % chitosan and S8 + 1 % CC ($df = 203$, $p < 0.01$). The whole scenario changed after 72 hours of incubation, when S8 + 0.5 % chitin, S8 + 1 % chitin S8 + 0.5 % CC and S8 + 1 % CC produced higher biomass compared to S8 + 0.5 % chitosan and S8 + 1 % chitosan by backscatter intensity values.

Table 2. Effect of chitin derivatives on biomass by backscatter intensity [a.u.] after incubation for 24, 48 and 96 hours.

Media	24 hours	48 hours	72 hours	96 hours
S8	19.9 ± 33.9 (a)*	1149.1 ± 475.0 (b)	3260.9 ± 427.0 (e)	3207.4 ± 1445.0 (d)
S8 + 0.5 % chitin	34.6 ± 46.4 (a)	1006.4 ± 454.0 (ab)	2926.7 ± 535.0 (de)	3359.6 ± 548.0 (d)
S8 + 1 % chitin	17.0 ± 29.0 (a)	817.7 ± 344.0 (ab)	2653.7 ± 385.0 (cd)	3039.4 ± 354.0 (d)
S8 + 0.5 % chitosan	10.0 ± 17.2 (a)	463.3 ± 171.0 (a)	1661.7 ± 199.0 (ab)	1852.0 ± 146.0 (ab)
S8 + 1 % chitosan	14.4 ± 31.0 (a)	512.3 ± 223.0 (a)	1406.4 ± 332.0 (a)	1440.1 ± 298.0 (a)
S8 + 0.5 % CC	6.0 ± 15.9 (a)	1200.7 ± 1091.0 (b)	2481.6 ± 273.0 (cd)	2927.4 ± 248.0 (cd)
S8 + 1 % CC	20.4 ± 39.6 (a)	540.0 ± 378.0 (a)	2153.6 ± 335.0 (bc)	2384.9 ± 332.0 (bc)

*: Mean ± SD with the same letters within one incubation time after executing linear model are not significantly different (Tukey HSD test, $\alpha = 0.05$, n = 7) (a.u. = Arbitrary unit).

Speed of germination

To analyze the speed of germination, germination was observed every hour over 8 hours. The speed of germination of submerged spores produced in media with chitosan was slower compared to other media. When all the media were compared regarding the germination time (Table 3), no significant differences were noted within the first 2 hours. Starting from 3 hours incubation there were first signs that the media may influence the speed of germination. After 8 hours incubation it was confirmed that spores from S8 + 0.5 % chitosan media significantly reduced the germination ($df = 444$, $p < 0.0001$) and spores grown in S8 + 1 % CC germinated significantly faster ($df = 444$, $p < 0.0001$) compared to spores produced in S8 medium.

Table 3. Effect of chitin and its derivatives on germination [%] of submerged spores of *C. fumosorosea* after 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours and 8 hours of incubation.

Media	1 hour	2 hours	3 hours	4 hours	5th hours	6th hours	7th hours	8th hours
S8	0.3 ± 0.7 (a)*	2.6 ± 2.0 (a)	5.3 ± 3.9 (ab)	6.8 ± 3.2 (ab)	13.7 ± 1.7 (b)	28.6 ± 6.6 (b)	37.3 ± 4.7 (cd)	52.4 ± 6.2 (c)
S8 + 0.5 % chitin	1.1 ± 1.4 (a)	0.9 ± 1.5 (a)	4.1 ± 3.3 (ab)	6.0 ± 2.0 (ab)	10.0 ± 1.2 (ab)	20.6 ± 5.6 (a)	34.6 ± 7.3 (bc)	56.3 ± 12.3 (cd)
S8 + 1 % chitin	1.0 ± 1.1 (a)	1.0 ± 1.1 (a)	2.6 ± 2.5 (a)	5.1 ± 1.6 (a)	15.3 ± 2.5 (bc)	21.6 ± 3.3 (a)	31.8 ± 11.1 (bc)	46.0 ± 5.4 (b)
S8 + 0.5 % chitosan	0.0 ± 0.0 (a)	0.0 ± 0.0 (a)	1.2 ± 1.1 (a)	4.3 ± 2.0 (a)	7.2 ± 0.9 (a)	16.3 ± 1.7 (a)	23.7 ± 6.5 (a)	36.3 ± 2.3 (a)
S8 + 1 % chitosan	0.5 ± 1.2 (a)	2.1 ± 1.9 (a)	3.9 ± 2.9 (ab)	4.76 ± 1.9 (a)	13.3 ± 4.2 (b)	20.0 ± 5.5 (a)	30.1 ± 5.7 (b)	46.1 ± 4.6 (b)
S8 + 0.5 % CC	2.7 ± 2.3 (a)	3.5 ± 3.0 (a)	6.1 ± 4.9 (ab)	7.6 ± 3.7 (ab)	11.3 ± 2.6 (ab)	19.6 ± 4.9 (a)	41.1 ± 4.9 (d)	59.9 ± 4.9 (d)
S8 + 1 % CC	1.2 ± 1.5 (a)	3.8 ± 2.23 (a)	9.3 ± 5.1 (b)	11.4 ± 5.7 (b)	19.9 ± 7.1 (c)	36.8 ± 12.2 (c)	53.1 ± 7.3 (e)	75.7 ± 5.9 (e)

*: Mean ± SD with the same letters after executing linear model are not significantly different (Tukey HSD test, $\alpha = 0.05$, n = 3×3)

Impact of the amendment of 1 % chitin and 1 % CC on *C. fumosorosea*

Following analysis of the virulence, growth, biomass monitoring and speed of germination of submerged spores of *C. fumosorosea*, the potential media S8 + 1 % chitin and S8 + 1 % CC were selected for further experiments and were compared together with the S8 medium.

Second bioassay: Lethal concentration

Exposure to different concentrations of submerged spores (1×10^6, 1×10^7 and 1×10^8 ss/mL) of all three media had significant effect on the mortality of larvae (binomial test; $df = 24$, $p < 0.01$). When the effect of media were pairwise compared (Tukey test; $\alpha = 0.05$) (Figure. 3), it can be observed that at 1×10^6 ss/mL, spores produced in S8 medium, showed significantly lower mortality (43 %) than S8 + 1 % chitin (66.7 %, $df = 24$, $p = 0.02$) and S8 + 1 % CC (68.3 %, $df = 24$, $p = 0.01$).

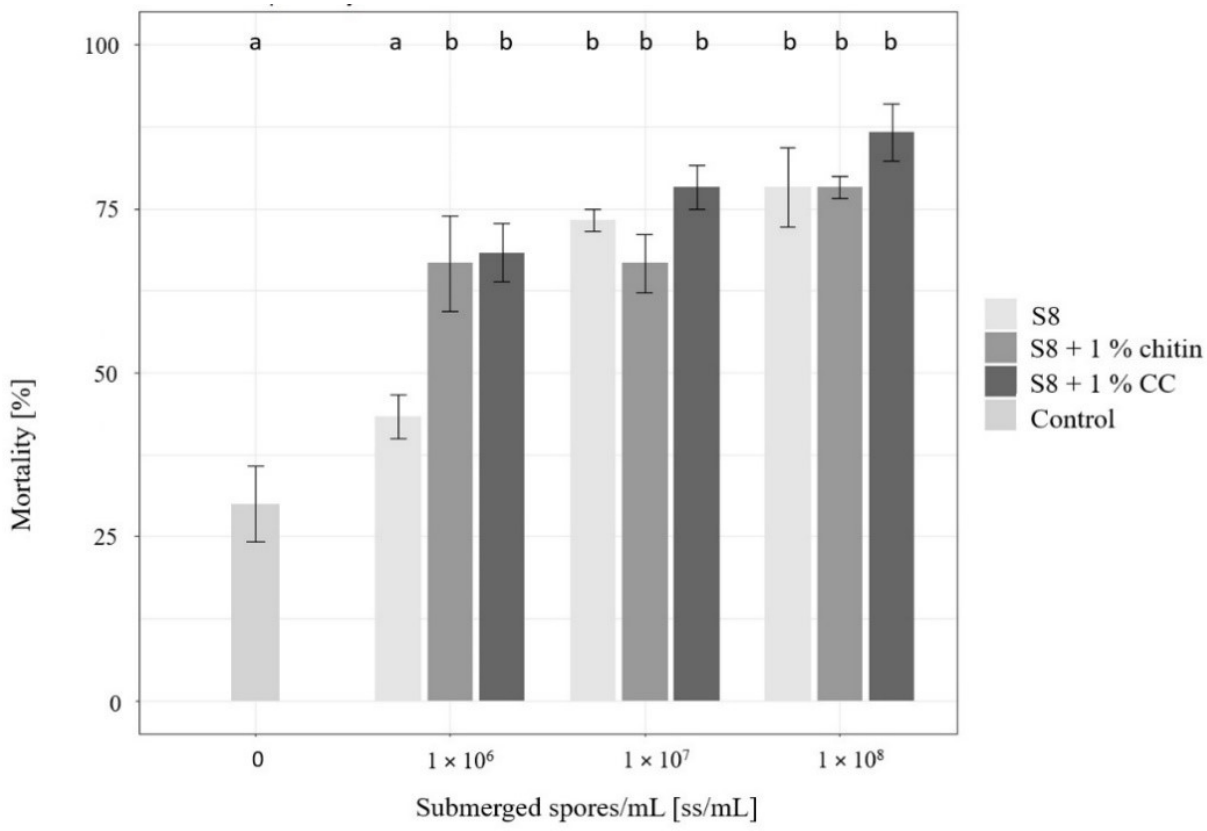

Figure 3. Effect of S8, S8 + 1 % chitin and S8 + 1 % CC media on mortality of *C. pomonella* larvae at spore concentration of 1×10^6, 1×10^7 and 1×10^8 ss/mL. Each bar represents the average mortality, with error bars indicating the standard error, calculated as the standard deviation of mortality divided by the square root of the number of repetitions within each media. Means with the same letters are not significantly different (Tukey HSD test; $\alpha = 0.05$, n = 3).

To achieve 75 % mortality of *C. pomonella* larvae, the required lethal spore concentration were 7.1×10^7 ss/mL, 7.01×10^7 ss/mL and 1.6×10^7 ss/mL for S8 medium, S8 + 1 % chitin, and S8 + 1 % CC, respectively. Moreover, S8 + 1 % CC media required a spore concentration more than four times lower than S8 (binomial test; $p = 0.03$) to achieve the same level of larval mortality.

Influence of sunlight simulation

A Kaplan-Meier survival curve was determined to analyze the effect of sunlight on submerged spores grown in three potential media (Figure 4). There was noteworthy reduction of germination probability in sunlight simulated hours with significant differences among three media (log-rank test; $p < 0.0001$).

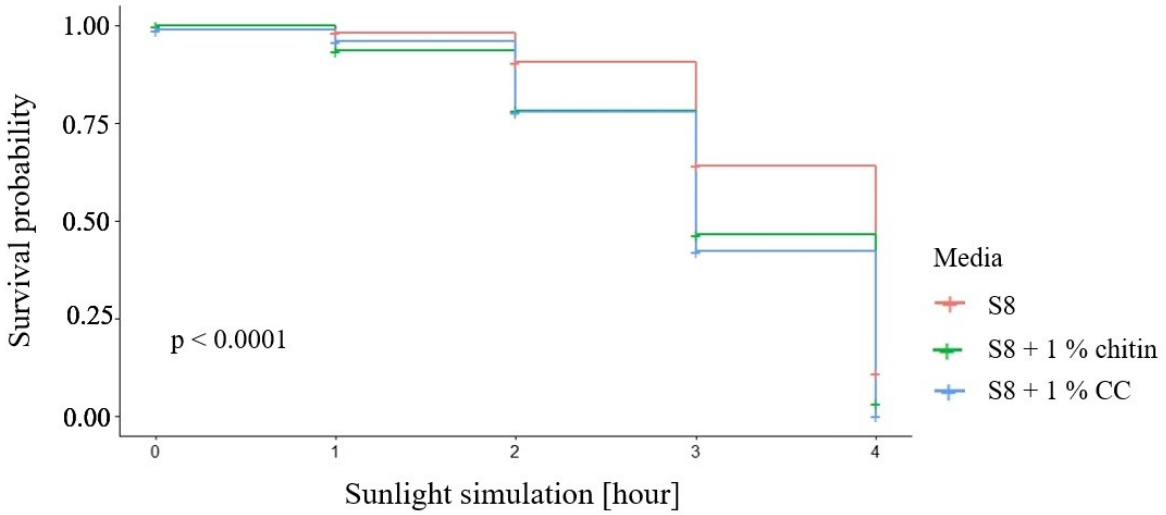

Figure 4. Overall survival probability (Kaplan-Meier analysis) of the spores produced in the media with S8, S8 + 1 % chitin and S8 + 1 % CC after simulated sunlight exposure for continuous 4 hours. The statistics of shown p-values (log-rank test; $\alpha = 0.05$, n = 3 × 3) indicates differences among the germination of submerged spores produced in three media within the respective sunlight simulation time.

When submerged spores were exposed to simulated sunlight, differences have been observed in germination among the three media (Figure 5).

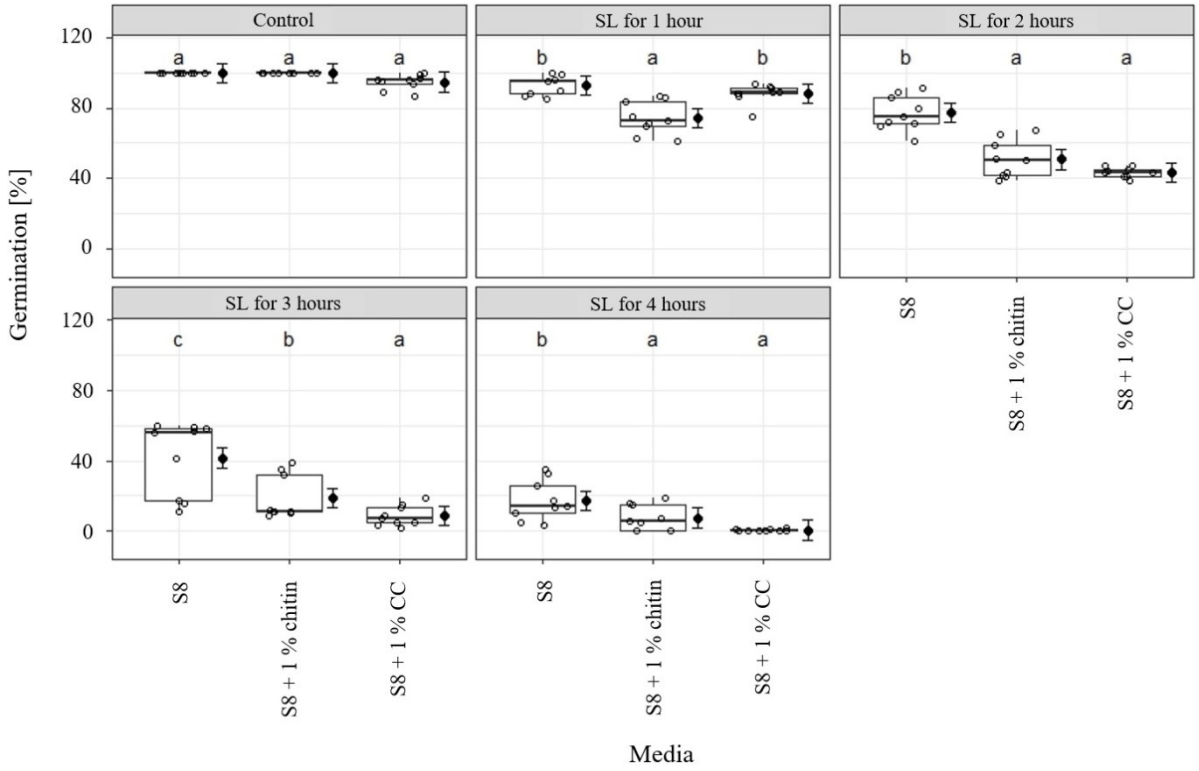

Figure 5. Effect of S8, S8 + 1 % chitin and S8 + 1 % CC on germination [%] of simulated sunlight exposed (for 0, 1, 2, 3 and 4 hours) submerged spores of *C. fumosorosea* after 16 hours of incubation. Jittered boxplots consist of the median and the 25 % and 75 % quantile. Black dots and error bars represent adjusted mean with 95 % confidence limits per medium. Means followed by the same letters are not significantly different (Tukey HSD test; $\alpha = 0.05$, n = 3 × 3) (SL= Sunlight simulation).

Pairwise comparison showed that without sunlight exposure, spores produced in all three media rendered over 90 % germination (S8 = 100 %, S8 + 1 % chitin = 100 %, S8 + 1 % CC = 94.8 %) without any significant difference. After 1 hour of sunlight simulation, spores produced in S8 + 1 % chitin (74.4 %) media germinated slower than spores produced in S8 (92.8 %, $df = 116$, $p < 0.0001$) and S8 + 1 % CC (88.1 %, $df = 116$, $p = 0.003$). As sunlight simulation hours increased, after 2 hours, spores produced in S8 + 1 % chitin (50.8 %, $df = 116$, $p < 0.0001$) and S8 + 1 % CC (43.3 %, $df = 116$, $p < 0.0001$) spores showed significantly lower germination compared to spores from S8 (77.2 %). After continuous sunlight simulation for 3 hours, spores from S8 + 1 % CC media showed a rapid reduction (8.7 %) in germination, whereas spores from S8 + 1 % chitin (18.9 %, $df = 116$, $p = 0.03$) and S8 (41.7 %, $df = 116$, $p < 0.0001$) media showed slightly higher germination, with all results being

significantly different from each other. After 4 hours of sunlight simulation, spores produced in S8 + 1 % CC (0.4 %, $df = 116$, $p = 0.0001$) and S8 + 1 % chitin media (7.6 %, $df = 116$, $p = 0.04$) showed lower germination, with no significant difference between them, while spores produced in S8 medium (17.3 %) had an increased germination and showed significance in comparison with both of the other media. Spores produced in S8 medium had their germination reduced by 50 % after 2.8 hours of simulated sunlight. In S8 + 1 % chitin medium, this reduction occurred faster, at 2.02 hours. Spores produced in S8 + 1 % CC medium initially showed over 90 % germination for the first hour, but dropped sharply to 50 % at 1.8 hours.

Influence of freeze-drying on formulation process

Freeze-drying of submerged spores produced in three potential media showed differences in their germination capacity. Non-freeze-dried spores began germinating after 16 hours of incubation (Figure 6 (A)). After ANOVA analysis for non-freeze-dried spores, significant effects of media ($F = 923.04$, $df = 2$, $p < 0.001$) and media-hours interaction ($F = 299.7$, $df = 4$, $p < 0.001$) were observed on spore germination. After 16 hours, the germination of spores from S8 + 1 % CC media (91.9 %) was significantly higher than from S8 + 1 % chitin (77.5 %, $df = 224$, $p < 0.001$) or S8 medium (66.3 %, $df = 224$, $p < 0.001$). At 24 hours and 48 hours of incubation, there were no significant differences among the spore germination of the three media.

On other hand, freeze-dried spores showed varied germination over time (Figure 6 (B)), with significant media-hour interaction (ANOVA; $F = 3.4$, $df = 4$, $p < 0.05$). After 16 hours, submerged spores grown in all media showed low germination (S8 = 3.1 %, S8 + 1 % chitin = 4.2 %, S8 + 1 % CC = 5.0 %), which were insignificantly different from each other. Spores produced in S8 + 1 % CC displayed higher germination after 24 hours (32.6 %), surpassing germination of spores produced in S8 (20.0 %, $df = 224$, $p < 0.0001$) and S8 + 1 % chitin (24.4 %, $df = 224$, $p < 0.004$). After 48 hours, spores produced in S8 + 1 % CC (69.6 %) medium showed significantly greater response in germination than spores produced in S8 (56.5 %, $df = 224$, $p < 0.0001$) and S8 + 1 % chitin (60.4 %, $df = 224$, $p < 0.001$). As the germination of submerged spores grown in three media were over 50 % after 48 hours of incubation, Spores cultured in S8 medium required 44.4 hours of incubation for germinating of 50 % spores. On other hand, 42.2 hours incubation was needed to have 50 % of

germination of spores from S8 + 1 % chitin medium. Among the media, least amount of incubation time (37.5 hours) was required to accomplish 50 % of spore germination, when the spores were produced in S8 + 1 % CC medium.

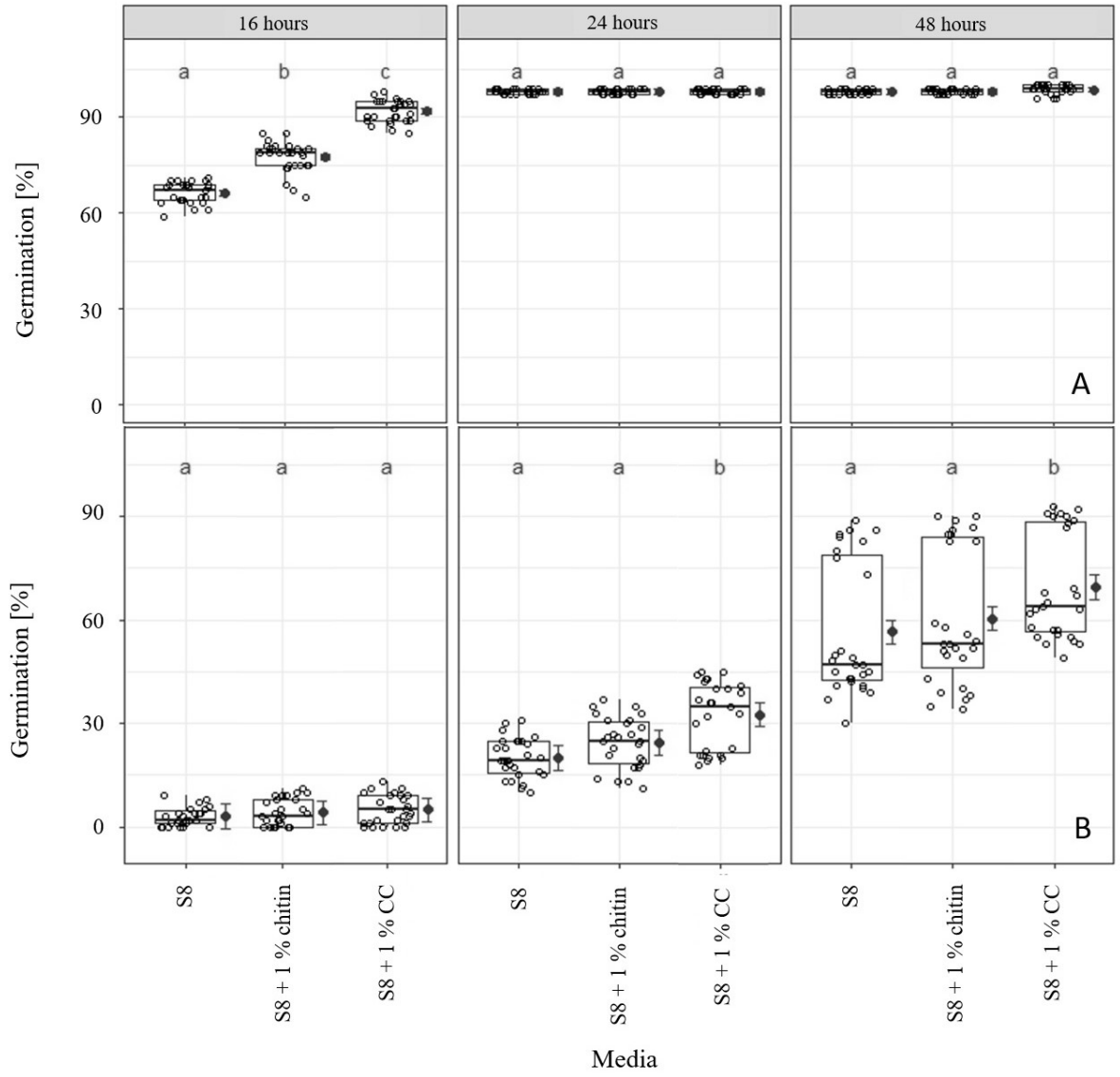

Figure 6. Effect of S8, S8 + 1 % chitin and S8 + 1 % CC on germination [%] of (A) non-freeze-dried spores and (B) freeze-dried spores of *C. fumosorosea* after 16, 24 and 48 hours of incubation. Jittered boxplots consist of the median and the 25 % and 75 % quantile. Black dots and error bars represent adjusted mean with 95 % confidence limits per medium. Means with the same letters are not significantly different (Tukey HSD test; $\alpha = 0.05$, n = 3 × 9).

Discussion

This study primarily aimed to optimize an effective basic medium, containing chitin and its derivatives for improving the effectiveness of submerged spores of *C. fumosorosea*. Malt peptone and potato dextrose media were found insufficient, leading to the inclusion of modified Samsi 8 (S8), described by Samsinakova (1966), which demonstrated superior growth (Stephan et al. 2016). In this study, best growth of *C. fumosorosea* was achieved in a low-cost S8 medium with glucose, corn steep flour, and NaCl, which was also supported by previous works from Stephan et al. (2016) and Mascarin (2015).

After the initial medium selection, the effect of chitin-amendments of chitin to *C. fumosorosea* were investigated. Two concentrations of chitin derivatives were added to the S8 medium, and the incubation period was extended from 72 to 96 hours because Ali et al. (2010a) observed that chitinase production by *C. fumosorosea* stabilized between 96 and 120 hours with anincrease in chitinase levels after 48 hours when the fungus was cultivated in a liquid medium with 1 % chitin or colloidal chitin (CC) as the sole carbon source. Chitin and CC induce chitinases in EPF, potentially contributing to control insect (Safavi et al. 2007; Mondal et al. 2016). Given that *C. fumosorosea* exhibits chitinase activity, the addition of chitin or CC was expected to trigger this enzyme production and consequently to enhance virulence against *C. pomonella* (Ali et al. 2010a). Previous reports indicated that chitinases activity in *C. fumosorosea* was affected by CC as a carbon source (Ali et al., 2010b). In the current study, addition of 1 % CC to the culture medium resulted in the highest virulence against *C. pomonella*, while chitosan did not show significant differences among other media. For virulence, EPF spores might react to their previous growing environment; certain genes or metabolic pathways could be upregulated and may exhibit adaptive responses (e.g., during host invasion) by adjusting their gene expression profiles (Sahai and Manocha 1993; Yang et al. 2020; Kamboj et al. 2022; Zhang et al. 2023; Thakur et al. 2023). Chitosan's potential to alter hyphal growth and reduce toxin production might explain this outcome (Reddy et al. 1998; European Patent Office 2009). Another study corroborates my findings by demonstrating that the EPF *Lecanicillium lecanii* exhibits enhanced pathogenicity against *Aphis craccivora* when cultivated in media containing chitin and CC (Nithya et al. 2015).

Regarding the production of submerged spores of *C. fumosorosea*, spores produced in S8 + 0.5 % chitosan medium exhibited significantly higher spore yield compared to other media formulations. This finding aligns with a previous study, where corn cased medium supplemented with chitosan resulted in increased spore production (European Patent Office 2009). Results of the current study also exhibit a similar tendency as those reported by Nithya et al. (2015), showing that *L. lecanii* produced more spores in medium with 0.5 % or 1 % chitosan compared to pure chitin or chitin extra pure.

Biomass production was online measured using backscattering light in this study (Luong et al. 2011; Griffiths et al. 2011; Nguyen and Rittmann 2018). S8 and S8 + 0.5 % chitin, S8 + 1 % chitin, S8 + 0.5 % CC and S8 + 1 % CC resulted in higher biomass of *C. fumosorosea* after 96 hours compared to biomass cultured in S8 + 0.5 % chitosan and S8 + 1 % chitosan media, consistent with findings by Ali et al. (2010a, 2010b). Gryndler et al. (2003) also noted the potential of chitin to stimulate microbial biomass and optical density, remaining relatively stable over time.

Spore germination on the host cuticle marks the inception of the EPF growth (Islam et al. 2021). The speed of germination is a critical factor influencing rapid infection and depends on the nutritional components of the production medium (Boucias and Pendland 1983; Seib et al. 2023). Similar findings for *C. fumosorosea* were reported by Jackson (1997), indicating the impact of nutrition on spore germination. Despite chitosan's antimicrobial activities, it often inhibits spore germination and affects growth (Reddy et al. 1998; Saniewska 2001; Park et al. 2002; Rabea et al. 2003; Palma-Guerrero et al. 2008; European Patent Office 2009), supporting the present study where chitosan addition was not beneficial for germination despite its ability to increase the number of submerged spores per mL. Similar results were found for *Metarhizium anisopliae* (Montesinos-Matías et al. 2021). Contrarily, in the current study, spore germination was enhanced, when only 0.5 % CC or 1 % CC were added to the S8 medium. Rapid chitinase increase during spore formation has been linked to significant differences in germination (Charnley 2003), supporting the observed improvements in germination of spores produced in media with chitin and CC.

Numerous studies have established a direct link between germination and virulence (Park et al. 2002; Charnley 2003; Safavi et al. 2007; Palma-Guerrero et al. 2008; European Patent Office 2009; Abaajeh and Nchu 2015; Islam et al. 2021). In this study, the addition of chitin

or CC to S8 medium influenced biomass production, germination, and subsequently, the virulence of *C. fumosorosea* against *C. pomonella*. As chitinases are the enzymes that specifically hydrolyze chitin and during germination process, chitinase activity increases. Moreover, chitinases are important for fungal spore germination as they help with cell wall remodeling, hyphal development, and breaking down exogenous chitin for nutrition (Gooday et al. 1992; Gomaa 2021; Thakur et al. 2023). Their enzymatic reaction involves breaking down chitin into smaller components such as N-acetylglucosamine monomers, chitobioses, or chitooligosaccharides (Yang and Zhang 2019; Gomaa 2021; Kumar et al. 2022). As a result, when higher germination occurs and enhance virulence, they form germ tube that can penetrate the host cuticle and enter the insect body. Infectious spores proliferate, producing toxins that cause insect death (Jackson 1997; Islam et al. 2021).

Chitinases, working alongside proteases, play a crucial role in insect cuticle degradation during the infection initiation of EPF, influencing various stages such as germination, hyphal growth, morphogenesis, nutrition, and defense against insects (Mondal et al., 2016). In first bioassay, submerged spores produced in S8 + 1 % CC showed the best mortality. In second bioassay, the LC_{75} (lethal concentration) results showed that spores produced in S8 + 1 % CC required a lower concentration for 75 % mortality against *C. pomonella*. Furthermore, the CC particles are smaller due to the dispersion of chitin particles, resulting in a larger surface area compared to bulk chitin. The reason leads to increased availability of the active ingredient (Murthy and Bleakley 2012; Kaya et al. 2015; Kidibule et al. 2018). Limited water solubility, often a challenge with chitin, is addressed by synthesizing CC through chemical modification, enabling a more uniform distribution (Vinothkumar and Paterson 2023). Chitinases have been shown to mediate the degradation of the insect cuticle, creating openings that facilitate pathogen entry into susceptible insect tissues (Bahar et al. 2012; Dukare et al. 2021; Zeng et al. 2022). CC, used as a carbon source in submerged fermentation, has demonstrated effectiveness against cotton bollworm (*Helicoverpa armigera*), causing significant mortality, particularly up to the pupa stage (Binod et al. 2007). Ali et al. (2010a) evaluated *C. fumosorosea* fungal culture filtrates containing chitinases against the pest insect *Plutella xylostella*, showing adverse effects on larval growth and metamorphosis. They observed low survival times with CC compared to the control, with 1.5 % CC proving to be the most effective concentration (Ali et al. 2010b). In current study, higher larval mortality and lower concentration was also achieved with the spores cultured in 1 % CC amended medium.

EPF must meet specific criteria to be considered as BCA, including efficient conidia production, high virulence, and resilience against abiotic stressors like elevated temperatures and sunlight. After confirming the virulence effects of *C. fumosorosea* spores, those were cultured in S8 + 1 % CC and S8 + 1 % chitin media, underwent an environmental stress test using a sunlight simulator. Surprisingly, the amendment of 1 % CC to the cultivation medium negatively affected the germination of *C. fumosorosea* spores after 4 hours of sunlight radiation at 350 W/m^2. While studies have reported that 4 hours of UV-B radiation exposure can significantly reduce conidial viability and germination of *Metarhizium* (Braga et al. 2015; Rojas et al. 2023), the observed reduction in germination of spores produced in S8 + 1 % CC suggests an intriguing and potentially protective response by the fungi. Chitin, a major component of insect exoskeletons, could be perceived by EPF as a signal of potential host presence. In response, EPF may downregulate germination, possibly as a strategy to await the actual presence of an insect host (Nahar et al. 2003). Additionally, higher carbon concentrations in the culture media may promote fungal growth, but when exposed to solar radiation, energy and resources may be redirected towards repairing radiation-induced damage, potentially negatively impacting UV tolerance (Torres et al. 2019). Optimal carbon concentration for EPF growth and UV tolerance is crucial, as insufficient carbon can limit growth, while excess carbon may divert resources from protective mechanisms. Achieving the right balance in culture media composition becomes vital for optimizing UV tolerance (Lübeck and Lübeck 2022).

Stability is crucial for the long-term preservation of EPF, and freeze-drying is a widely used technique for this purpose. The current study reveals that adding 1 % CC enhances the stability of *C. fumosorosea* spores, making freeze-drying an effective method for long-term storage. To improve desiccation tolerance, 20 % lactose was included in the media composition, a strategy supported by previous research (Cliquet and Jackson 1997; Jackson et al. 2006; Stephan et al. 2016; Dietsch et al. 2021). The addition of lactose serves as a readily available carbon source, promoting higher germination. EPF efficiently utilize sugars for growth, and the fungus benefits from an easily utilizable carbon source for regrowth (Jackson et al. 1997; Jackson 1997; Cliquet and Jackson 1997; Cliquet and Jackson 2005). The presence of carbon sources during spore prodution helps preserving the metabolic activity of *C. fumosorosea*, increasing its likelihood of remaining viable and functional after rehydration, an essential aspect for its intended applications (He et al. 2021). Furthermore, the addition of

carbon sources, especially 1 % CC, may induce the production of stress-response proteins, antioxidants, and other protective molecules in *C. fumosorosea* before freeze-drying. This upregulation supports the fungus in coping with freeze-drying-induced damage, ensuring the viability and functionality of the fungus after rehydration (Roy and Gupta 2004). Culturing *C. fumosorosea* in a medium with abundant carbon sources, such as 1 % CC, promotes robust growth and reproduction, enhancing the fungus's ability to withstand stressors like freeze-drying (Khan et al. 2023).

In conclusion, it can be said that incorporating chitin and its derivatives into culture media significantly influences the effectiveness of submerged spores of *C. fumosorosea*. Chitin-amended media had a complex impact on the whole downstream process of growth, effectiveness and environmental stability. Notably, the inclusion of 1 % colloidal chitin (CC) in the growth significantly enhanced the fungal virulence against *C. pomonella* larvae. However, trade-offs were observed: while 1 % CC improved viability (including freeze-drying) during the formulation process, it negatively affected the fungus' resilience under prolonged simulated sunlight.

Chapter III

Formulation of spray-dried *Cordyceps fumosorosea* submerged spores containing water-soluble sunlight protectants

Abstract

This research was conducted to develop a spray-drying process by integrating water-soluble sunlight protectants into the formulation process of submerged spores of JKI-BI-1496 (*Cordyceps fumosorosea*), aiming to enhance spore stability under simulated sunlight. After proving that submerged spores of *C. fumosorosea* survive the spray-drying process, nine water-soluble sunlight protectants (Humic acid Na, humic acid K, lignin, black tea, cocoa, coffee, skimmed milk, green tea, and calcofluor white) were evaluated for their potential incorporation into the spray-drying process. Absorbance profiles of the protectants were examined across a spectrum ranging from 280 - 800 nm. Following evaluation, all protectants underwent spray-drying with key parameters set at 65 °C inlet temperature, 48 °C outlet temperature, and maximal final product temperature of 35 °C. Skimmed milk powder was chosen as the protective agent during spray-drying, forming a final suspension containing 5 % (w/v) skimmed milk, 5×10^6 ss/mL submerged spores, and 5 % (w/v) sunlight protectants. Germination of spray-dried spores were assessed after exposure to simulated solar radiation for 1 - 4 hours. Spores formulated with black tea exhibited the highest germination after 4 hours, notably 48.3 %, compared to minimal viability (< 18 %) with other protectants. Calcofluor white displayed stability over 12 weeks storage at 6 °C with 44.9 % germination. These findings highlight the potential of water-soluble sunlight protectants, particularly black tea and calcofluor white, to enhance the environmental stability of JKI-BI-1496 spores, offering a promising strategy for their survival and growth in outdoor agricultural settings.

Introduction

Radiant energy emitted by the sun, known as solar radiation, is essential for sustaining life on earth, encompasses ultraviolet (UV), visible, and infrared bands, each with distinct impacts on the environment and its inhabitants (Gurjar et al. 2017). While UV radiation comprises a minor portion of total solar radiation, it significantly affects various biological processes, including gene expression, metabolism, and plant-insect interactions (Kotilainen et al. 2020). In terms of the light that reaches the earth's surface, 49.4 % is attributed to infrared radiation (> 700 nm), while visible light (400 - 700 nm) contributes 42.3 %, whereas ultraviolet radiation (UV-A (320 - 400 nm), UV-B (280 - 320 nm) and UV-C (100 - 280 nm)) makes up slightly more than 8 % of the total solar radiation (Zerefos et al. 2000; Gurjar et al. 2017; Burt 2018; Buban et al. 2019; Kotilainen et al. 2020; Rahoma et al. 2021).

Moreover, sunlight has long been recognized as a significant factor in the fungal life cycle, affecting processes like sexual and asexual reproduction, growth, virulence, and secondary metabolism (Fuller et al. 2015; Fuller et al. 2016; Dias et al. 2020; Vrabl et al. 2023). Furthermore, solar radiation, particularly in the form of UV-B, presents a substantial risk to fungal propagules, exerting a significant influence on their capacity to endure and efficiently engage in insect control within the natural environment (Inglis et al. 1995; Inglis et al. 2001; Fernandes et al. 2015). Several researches have been conducted to find out the negative impact of solar radiation to entomopathogenic fungi (EPF) (Cabanillas and Jones 2009; Fernandes et al. 2015; Gurjar et al. 2017; Sharma et al. 2023). For examle, the impact of simulated sunlight on the survival of conidia from four entomopathogenic hyphomycetes species was investigated (Fargues et al. 1996). Conidia from *Beauveria bassiana*, *Metarhizium anisopliae*, *Metarhizium flavoviride* and *Cordyceps fumosorosea* isolates were exposed to artificial sunlight (295 - 1100 nm at UV-B irradiance of 0.3 W/m^2) for 0, 1, 2, 4 and 8 hours. Survival rates decreased with prolonged exposure, particularly after 2 hours. Overall, *M. flavoviride* isolates showed the highest resistance to irradiation, followed by *B. bassiana* and *M. anisopliae*, while *C. fumosorosea* conidia were the most susceptible. Intraspecies variation indicated the importance of selecting strains with irradiation tolerance for developing microbial control agents in environments where increased persistence is desirable (Fargues et al. 1996). Previous research highlighted that UV-B light exposure for 2 hours (at irradiances of 0.9 or 1.2 W/m^2) led to a substantial decrease in conidial efficiency. In

certain instances, there was more than a 50 % reduction in germination, particularly observed in *Metarhizium* (Braga et al. 2001b; Fernandes et al. 2007). In recent work of Da Couceiro et al. (2021), a notable impact on germination was observed only after 6 hours of exposure (total dose of 0.6 W/m^2) for nearly all 12 isolates of *Metarhizium* from Brazil. Subsequently, five of these isolates were rendered completely inactive following 8 hours of exposure (total dose of 0.6 W/m^2). In addition to conidia, blastospores produced in liquid culture can also serve in biocontrol applications. However, their tolerance to stressors like heat and UV-B radiation can be lower than that of aerial conidia, with the degree of sensitivity varying across different fungal species (Bernardo et al. 2020). Therefore, formulation of EPF by e.g. physical manipulation has been investigated as a tool to protect fungi to some extent from solar radiation (Fernandes et al. 2015). In Chapter II, it was observed that media ammended with chitin derivatives was benificial for infuencing virulence and storage capacity of *C. fumosorosea*, but had negative effect on germiantion under continuous simulated sunlight. Therefore, it was obvious to protect the submerged spores of *C. fumosorosea* against solar radiation through bioprocess engineering (Drying).

Proper handling during drying and formulation is crucial for developing microbial biopesticides, especially for blastospores (Bernardo et al. 2020). Pioneering the spray-drying technique, Stephan and Zimmermann (1998) dehydrated submerged spores (spores, which are formed in submerged culture) of *M. anisopliae* and *M. flavoviride*, achieving high blastospore viability (> 80 %) immediately after drying. On other hand, progress has been made with *C. fumosorosea* and *B. bassiana*, demonstrating high blastospore survival after freezing, spray- or air drying (Cliquet and Jackson 1997; Jackson et al. 1997; Sandoval-Coronado et al. 2001; Mascarin et al. 2015; Correa 2020; Iwanicki et al. 2021). Laboratory manipulations offer opportunities to enhance sunlight tolerance within the formulation process of for field applications. This type of manipulation can be done through spray-drying or ionic gelation methods. In spray-drying, tiny droplets are formed by spraying a mix of the material and active compounds into hot air, making them dry up and turn into particles (Felizatti et al. 2021). Therefore, the first objective of this research was to establish a suitable spray-drying process to formulate submerged spores of *C. fumosorosea*.

Furthermore, to formulate spray-dried spore with sunlight protectant, it is crucial to choose the appropriate coating material. Oil-based formulations, particularly those with mineral or

vegetable oils, enhance conidia tolerance to UV radiation and improve germination compared to water-based formulations (Leland and Behle 2004; Jordan et al. 2021). Chemical sunscreens added to both, water- and oil-based formulations, further protect conidia against UV radiation (Inglis et al. 1995; Iwanicki et al. 2021). UV radiation protectants are more effective in water-based formulations due to the thicker protective layer created by water droplets during evaporation (Leland and Behle 2004). Promising substances like calcofluor white and natural compounds have shown UV protection in controlled environments, but field persistence reports are limited (Kaiser et al. 2019; Kaiser et al. 2020). In my study, I focused on water-soluble sunlight protectants, considering their effects on germination of fungus. The limited selection of co-formulants for effective sunlight protection poses a challenge in open field applications of entomopathogenic fungi (Kaiser et al. 2020). Hence, the second objective was to identify water-soluble protectants based on their light absorbance capacity using a photometer in the laboratory.

The main aim was to protect submerged spores of *C. fumosorosea* against sunlight by adding sunlight protectants to the spray-drying process. Therefore, the third objective of this work was to evaluate the effects of time of exposure to simulated sunlight on spore germination of *C. fumosorosea* in formulation with potential sunlight protectants, and to determine whether these formulations are viable for enhancing the sunlight stress tolerance of spores.

Certain studies have focused on the shelf life of spray-dried submerged spores, revealing cell viabilities of up to 90 % for *B. bassiana* blastospores when stored in a modified atmosphere for 9 months at 26 °C (Jackson et al. 2006; Mascarin et al. 2015). Nevertheless, the comparative evaluation of shelf life and bioactivity in spray-dried formulations of *C. fumosorosea* submerged spores remains limited within existing literature. Dry microgranular formulations (spray-drying), can be characterized by advantageous traits such as extended shelf life and ease of handling, demonstrate suitability for water-based spray applications in agricultural settings (Leland and Behle 2004; Fernandes et al. 2015; Mascarin et al. 2015; Kaiser et al. 2019; Iwanicki et al. 2021). Hence, the forth objective of the current study was the estimation of effect of sunlight protectants on storability of spray-dried submerged spores.

Material and Methods

Production of spray-dried submerged spores of *C. fumosorosea*

For the present work, JKI-BI-1496 (*C. fumosorosea*) was used, which was isolated from *Cydia pomonella* in 1971 by Müller-Kögler in Darmstadt, Germany (Inventory data, JKI). Production of submerged spores of *C. fumosorosea* though liquid fermentation in S8 medium has been described in Chapter II. Produced submerged spores were filtered through three layers of gauze and were centrifuged for 5 minutes at 3000 *g*. The resulting pellet was then re-suspended in deionized water. A suspension was prepared, containing 2×10^7 ss/mL submerged spores, using autoclaved water. A 20 % (w/v) solution of skimmed milk was made with boiled autoclaved water. After that, spore suspension and solution of skimmed milk were combined in a 1:1 ratio, resulting in a 50 mL suspention containing 10 % (w/v) skimmed milk and 1×10^7 ss/mL submerged spores. Subsequently, all spray-drying parameters of the spray-dryer (Mini spray dryer S-300, BÜCHI Labortechnik AG, Flawil, Switzerland) were adjusted (Table 1). After spray-drying the powder containing submerged spores was taken out from the jar carefully and kept in sterile 25-mL Eppendorf conical tubes (Carl Roth GmbH + Co. KG, Karlsruhe, Germany).

Table 1. Spray dryer parameters with range

Parameter	Range
Dry Gas	30 m^3/hour
Inlet Temperature	65 °C
Spray Gas	900 L/hour
Pump	1.4 mL/minute
Outlet Temperature	48 °C
Product Temperature	35 °C
Unclogging	10 bpm
Filter pressure	- 49 mbar

Feasibility test

After spray-drying, the feasibility of spray-drying was tested by comparing the viability of not spray-dried and spray-dried spores. For this purpose, not spray-dried and spray-dried submerged spores were mixed thoroughly in 10 mL of autoclaved water. Three 10 µL droplets from both sample were pipetted onto a MPA-plate. All plates were then incubated at 20 °C for 16 hours, after which the samples were stained with Lactophenol blue (Carl Roth GmbH, Karlsruhe, Germany) and the germination was determined by counting 100 spores. A spore was considered germinated when the length of the germ tube equaled or exceeded the width of the spore. The experiment was repeated three times with three replications.

Selection of water-soluble sunlight protectants

The selection of water-soluble additives (Table 2) with potential sunlight protective effects was grounded in prior research (Kaiser et al. 2020). For preparation of liquid stock solution, 20 % (w/v) of humic acid Na, humic acid K, lignin and skimmed milk were mixed with autoclaved distilled water. Calcofluor white solution was also prepared with the same manner. 20 % (w/v) of black tea, coffee, cocoa and green tea was taken and brewed with sterile distilled water at 70 °C for 15 minutes. After that samples were diluted again twice at 1:10 ratio for wavelength spectrum by spectrometer.

Table 2. List of the water-soluble sunlight protectants

Sunlight protectants	Active substances	Manufacturer
Humic acid Na	Assay of humic acid ~ 45 - 75 %, Loss on drying ~ 25 %	Carl Roth GmbH + Co. KG, Karlsruhe, Germany.
Humic acid K	Potassium – Humates = 80 - 85 %, Total humic Acid = 68 - 73 %, Fulvic Acid = 5 - 6 %, Potassium (K_2O) = 10 - 12 %, Dry matter = 83 - 85 %, Organic substances = 68 - 73 %, pH value = 9.5 - 10.5 %, Bulk density = 0.55 - 0.65 Kg/L	Humintech GmbH, Grevenbroich, Germany.
Lignin, alkali	In powder form, transition temp sintering point 188 °C, density = 1.3 g/mL at 25 °C, surface tension = 43 mN/m (1 % aqueous), 5 % moisture, 13.4 wt. % loss on heating at 316 °C	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany.
Skimmed milk	Calorific value 1519 kJ/358 kcal, Fat (thereof saturated fatty acids 1.0 g), 0.6 g Carbohydrates (thereof sugar 51.7 g), Protein 35.5 g, Salt 1.4 g	J. M. Gabler-Saliter Milchwerk GmbH and Co. KG, Obergünzburg, Germany.
Black tea	Harbal tea, produced by, organic farming from the best growing areas of India, weight = 35g, 20 infusion bags per pack.	Alnatura GmbH, Darmstadt, Germany.
Coffee	Roasted Coffee, finish: ground, coffee type: filter coffee, coffee characteristic: organic, content: 500g, produced in long-term and fair partnerships with farmers in the country of origin, Brazil, Arabica and Robusta beans in organic quality	Alnatura GmbH, Darmstadt, Germany.
Cocoa	95 % Cocoa powder, 5 % Kaliumcarbonat (E501), Energy content/ calorific value 1620/387 kJ/ kcal, Fat 21 g = thereof saturated fatty acids 13 g, Carbohydrates 12 g (thereof sugar 0.5 g) Protein 0.05 g, Dietary fiber 22 g, Salt 0.04 g (thereof starch 31 g). From Africa, middle and south America.	Alnatura GmbH, Darmstadt, Germany.
Calcofluor white	Calcofluor white M2R, 1 g/L, Evans blue, 0.5 g/L, λmax = 423 - 443 nm, form: liquid	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany.
Green tea	Comes from organic farming	Alnatura GmbH, Darmstadt, Germany.

Absorption measurement

To measure the absorption over the sunlight spectrum of nine potential sunlight protectants, a FLUOstar® Omega spectrometer (BMG LABTECH, Ortenberg, Germany) was used, which is a filter-based multi-mode microplate reader. 100 µL sample was added to each well of a 96-well plate (GREINER 96 U-BOTTOM, Greiner Bio-One GmbH, Frickenhausen, Germany). To capture the data, ten flashes for each well in the microplate were performed, ensuring thorough data acquisition. Samples were analysed over a wavelength range from 280 - 800 nm with a wavelength step width of 2 nm. The settling time was set to 0.1 seconds, allowing

the instrument to stabilize before taking measurements. This analysis was repeated three times with three replications each. After capturing the absorbance data, analysis was done by MARS software. The MARS Data Analysis Software serves as an integral tool for the comprehensive analysis of data acquired from the FLUOstar Omega microplate reader.

Production of spray-dried submerged spores coated with sunlight protectants

To produce spray-dried submerged spores coated with sunlight protectants, 50 mL suspension of submerged spores (containing 10 % skimmed milk and 1×10^7 ss/mL submerged spores) has been prepared, as described before. In parallel, a 10 % solution of selected sunlight protectants was prepared using autoclaved water. Afterwards, spore suspension and solution of sunlight protectants were also mixed in a 1:1 ratio (25 mL of 5 % skimmed milk with 5×10^6 ss/mL submerged spores + 25 mL of 5 % sunlight protectants). Then, resulting 50 mL supension was turned into powder form by spray-drying following the same protocol as described earlier. Production of spray-dried submerged spores coated with sunlight protectants was repeated three times independently. The spray-dried spores were kept for maximal 24 hours at 6 °C before testing at simulated sunlight.

Sunlight simulation through Atlas XXL Sunlight simulator

After production of submerged spores coated with sunlight protectants, all samples of spray-dried spores were exposed to simulated sunlight in a SUNTEST XXL + FD sunlight simulator (Atlas Material Testing Technology, Illinois, USA) for 1, 2, 3 and 4 hours. Therefore, the spray-dried spores were mixed thoroughly in 10 mL of autoclaved water. Subsequently, 1 mL of this mixture was added to each well of a 24-well plate (cell culture multiwell plate 24 well, PS, Greiner Bio-One GmbH, Frickenhausen, Germany) for sunlight simulation exposition. Another multi-well plate, designated as a control, was covered with aluminum foil and was also exposed to sunlight. In between every hour of sunlight exposure, the samples were transferred (in additional 5 minutes) to the foil-covered 24-well plate, and optional markings were made in the wells to enable replenishing with autoclaved water if needed to prevent drying.

Table 3. Sunlight simulator parameter with range

Shut down criteria	Running time
Filter system	Day light
Irradiation control	300 - 800 nm
Sub-cylinders	Not necessary

Phase	Duration [minute]	Irradiation [W/m^2]	Chamber Temp [°C]	Black standard [°C]	Rel. Moist. [%]
1	60	350	20	35	95
2	5	-	-	-	-
3	60	350	20	35	95
4	5	-	-	-	-
5	60	350	20	35	95
6	5	-	-	-	-
7	60	350	20	35	95
8	5	-	-	-	-

Viability test

Following the exposure, three 10 µL droplets from each sample were pipetted onto a quarter of MPA-plates. All plates were then incubated at 20 °C for 16 hours. To check the viability of spray-dried spores coated with sunlight protectants, germination test was conducted in the same manner as described before. The experiment was repeated three times with three replications.

Storability test

Storability test was done in two steps. The first step was to select the right temperature to analyze a potential effect of sunlight protectants on the storability of submerged spores. The second step was to store the formulated spores for different duration at previously selected temperature.

Storage temperature

Freshly produced spray-dried spores were kept at 20 °C and 6 °C for 1 week to check the influence of storage temperature on the viability of spores. Spray-dried spores were stored in a

sterile 15-mL Eppendorf conical test tubes (Carl Roth GmbH + Co. KG., Karlsruhe, Germany). The experiment was repeated three times.

Storage weeks

For long-term storage test, spray-dried spores were kept for 1, 2, 4, 8 and 12 weeks at 6 °C. After that, germination test was done by the same manner after the respective weeks. Sample of spray-dried spores were taken from the same conical test tube for the following weeks of storage. The experiment was conducted with three repetitions and three replications.

Statistical Analysis

For screening of water-soluble sunlight protectants, the analysis of the experimental data using software R Studio (Version 1.4.1106) (RStudio Team 2020) revealed a clear correlation between wavelength and optical density. For the rest of the statistical analyses, data are presented as the arithmetic mean (mean) and standard deviation (± SD) or adjusted mean (adjusted mean) and standard error (± SE), depending on the model used for calculation. To meet the ANOVA's underlying assumptions of normal distribution and variance homogeneity, residuals of the respective model were visually inspected. Normal distribution was checked with the QQ-Plot (sample quantile-theoretical quantile) and variance homogeneity with the Residuals-Prediction-Plot. Selection of the best-fitted model was based on the Akaike Information Criterion (AIC) (Burnham and Anderson 2002) after backward elimination of the full model. Unless otherwise noted, the post hoc Tukey HSD test ($\alpha = 0.05$) was performed with the R package emmeans (Russell V. Lenth 2023) to examine differences between the respective factor levels in the context on an ANOVA. For estimating germination test for feasibility, viability and storability test, following LM has been executed:

$$\text{LM (y} \sim \text{media + hour + media: hour + repetition + replication)}$$

For Kaplan-Meier analysis, survival curves over time were created with the survival R package (Therneau 2023) with survfit function and compared with survival curves using the log-rank test (survdiff function) to determine global effects.

Results

Feasibility test

First, a feasibility test was performed to proof whether freshly produced submerged spores from liquid culture can survive the spray-drying process. It was found that spray-drying has a direct influence on the viability (ANOVA; $F = 8.5$, $df = 1$, $p < 0.01$). After pairwise comparison (Figure 1) germination of freshly produced spores (99.2 %) was significantly higher ($df = 8$, $p < 0.01$) than of spray-dried spores (95.6 %). Though spray-dried spores showed significantly lower germination than freshly produced spores, for both the germination was higher than 95 %. Therefore, it can be said that spray-drying is feasible and this formulation technique can be used for further experiments.

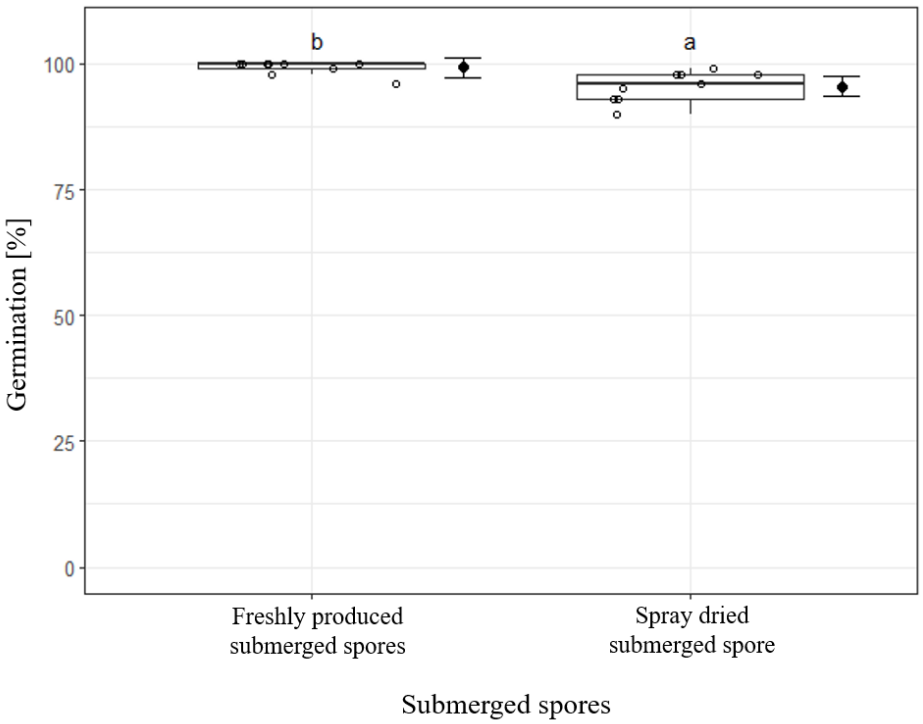

Figure 1. Germination [%] of submerged spores of *C. fumosorosea* before and after spray-drying. The germination was analysed after 16 hours of incubation on MPA at 20 ºC. Jittered boxplots consist of the median and the 25 % and 75 % quantile. Black dots and error bars represent adjusted mean with 95 % confidence limits. Means with the same letters are not significantly different (Tukey HSD test, $\alpha = 0.05$, n = 3 × 3).

Screening of water-soluble sunlight protectants

While screening of water-soluble sunlight protectants, it was observed that all the samples (Humic acid Na, humic acid K, lignin, green tea, cocoa, calcofluor white, black tea, coffee, skimmed milk) showed absorbance > 0 OD_{600} over the whole wavelength range (280 - 800 nm), but with different curve characteristics (Figure 2).

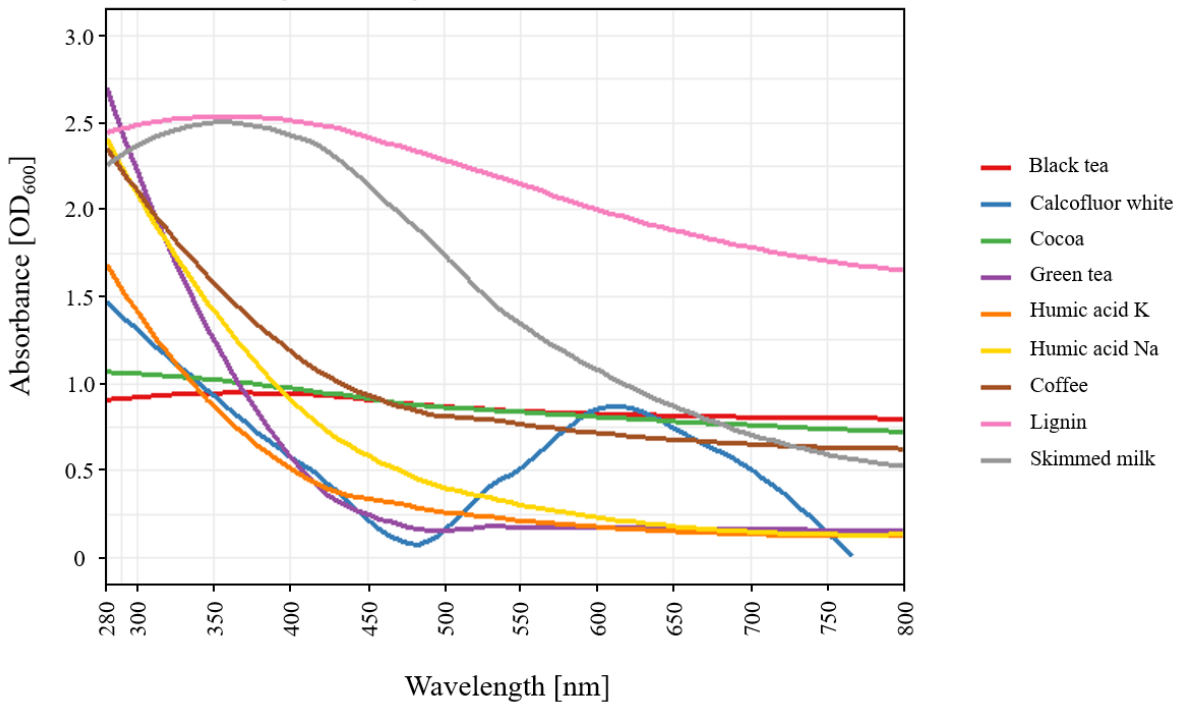

Figure 2. Absorbance [OD_{600}] versus different wavelengths [nm] of water-soluble sunlight protectants from 280 - 800 nm. n = 3.

Viability test

Submerged spores coated with six different sunlight protectants were tested for viability test through germination test under 1, 2, 3 and 4 hours of simulated sunlight. Here, humic acid Na, humic acid K and lignin were taken out from the study, as they had too sticky properties and by using the spray-drying adjustments described above nearly all of the dried product accumulated on the inner surface of the spray cylinder and on the cyclone. With submerged spore formulations coated with different sunlight protectants along with control (spores coated with 5 % skimmed milk), viability tests were done by determining the germination. At this point, cocoa was excluded for further analysis, because MPA-plates became hazy and unclear so that a microscopical evaluation became impossible. Firstly, Kaplan-Meier survival curves

(Figure 3) suggested that the germination over simulated sunlight time, varied significantly among different formulations. The log-rank test results revealed significant variations in germination distributions. Specifically, the germination probability distribution for submerged spores coated with black tea was significantly higher than control ($p < 0.0001$), skimmed milk ($p < 0.001$), coffee ($p < 0.0001$), calcofluor white ($p < 0.001$) and green tea ($p < 0.0001$).

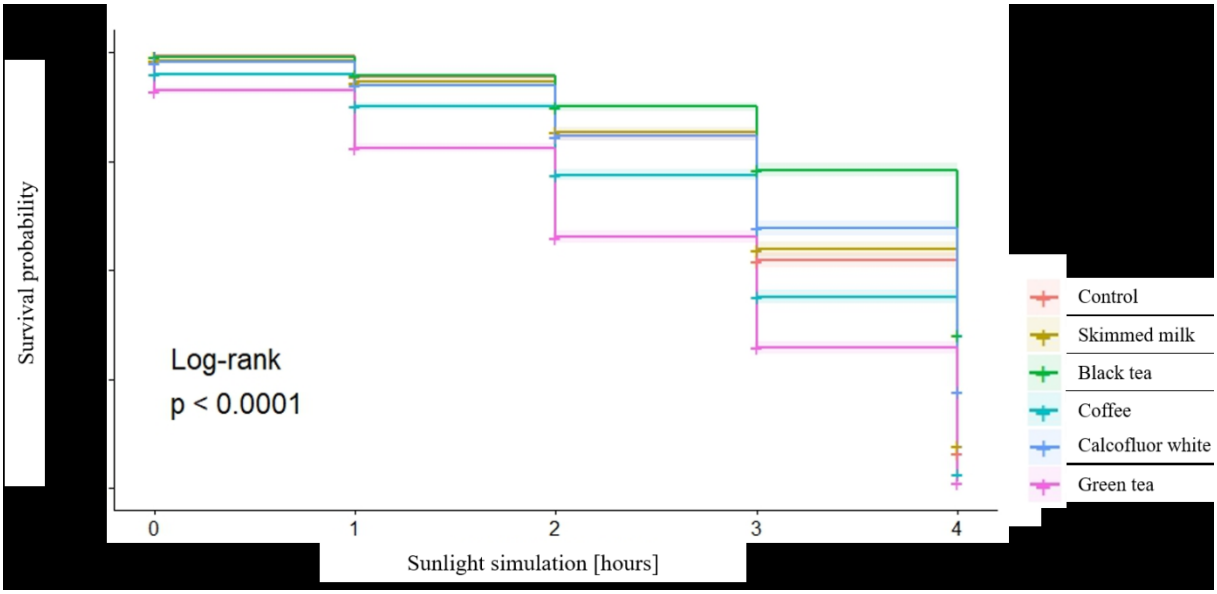

Figure 3. Overall survival probability (Kaplan-Meier analysis) of the spray-dried submerged spores of *C. fumosorosea* coated with different sunlight protectants (control, skim milk, black tea, coffee, calcofluor white and green tea) over 4 hours simulated sunlight exposure. The p-value (log-rank test, $\alpha = 0.05$, n = 3 × 3) indicates differences in the germination of submerged spores formulated with sunlight protectants within the respective sunlight simulation time.

After Kaplan-Meier probability analysis, a comprehensive analysis was conducted to analyze the impact of different hours of sunlight simulation on germination. This analysis showed that spores coated with different formulations significantly affected the germination of spores exposed to simulated sunlight (ANOVA; $F = 37.4$, $df = 5$, $p < 0.001$). This suggests that the formulations had distinct impacts on spore germination. Additionally, the duration of exposure time to simulated sunlight also significantly influenced germination, with clear differences noted between different durations ($F = 180.7$, $df = 4$, $p < 0.001$). In the absence of simulated sunlight, pairwise comparisons showed no significant difference between the germination of spores coated with black tea (94.8 %, $df = 325$, $p = 1.00$) and calcofluor white (88.8 %, $df = 325$, $p = 0.43$) compared to the control (Figure 4). However, spores coated with coffee (75.2 %, $df = 325$, $p < 0.0001$) and green tea (56.2 %, $df = 325$, $p < 0.0001$) exhibited significantly lower germination. Under one hour of simulated sunlight, similar pattern has

been observed. After 2 hours of sunlight exposure, germination of control started differing significantly from spores coated with black tea (75.6 %, $df = 325$, $p < 0.0001$). After 3 hours of sunlight simulation, spores coated with both black tea (66.8 %, $df = 325$, $p < 0.0001$) and calcofluor white (47.8 %, $df = 325$, $p < 0.0001$) maintained higher germination compared to the control. After 4 hours of continuous sunlight simulation, spores coated with black tea exhibited the most robust germination (48.3 %) and were significantly more effective compared to the spores coated with skimmed milk (17.8 %, $df = 325$, $p < 0.0001$), coffee (7.75 %, $df = 325$, $p < 0.0001$), calcofluor white (37.1 %, $df = 325$, $p = 0.02$), green tea (4.67 %, $df = 325$, $p < 0.0001$) and the control (15.8 %, $df = 325$, $p < 0.0001$). It has been observed that the survival under simulated sunlight of spores coated with black tea was higher resulting in a longer period of 3.9 hours of simulated sunlight for achieving 50 % germination, compared to spores coated with calcofluor white (2.9 hours), skimmed milk (2.4 hours) and control (2.3 hours).

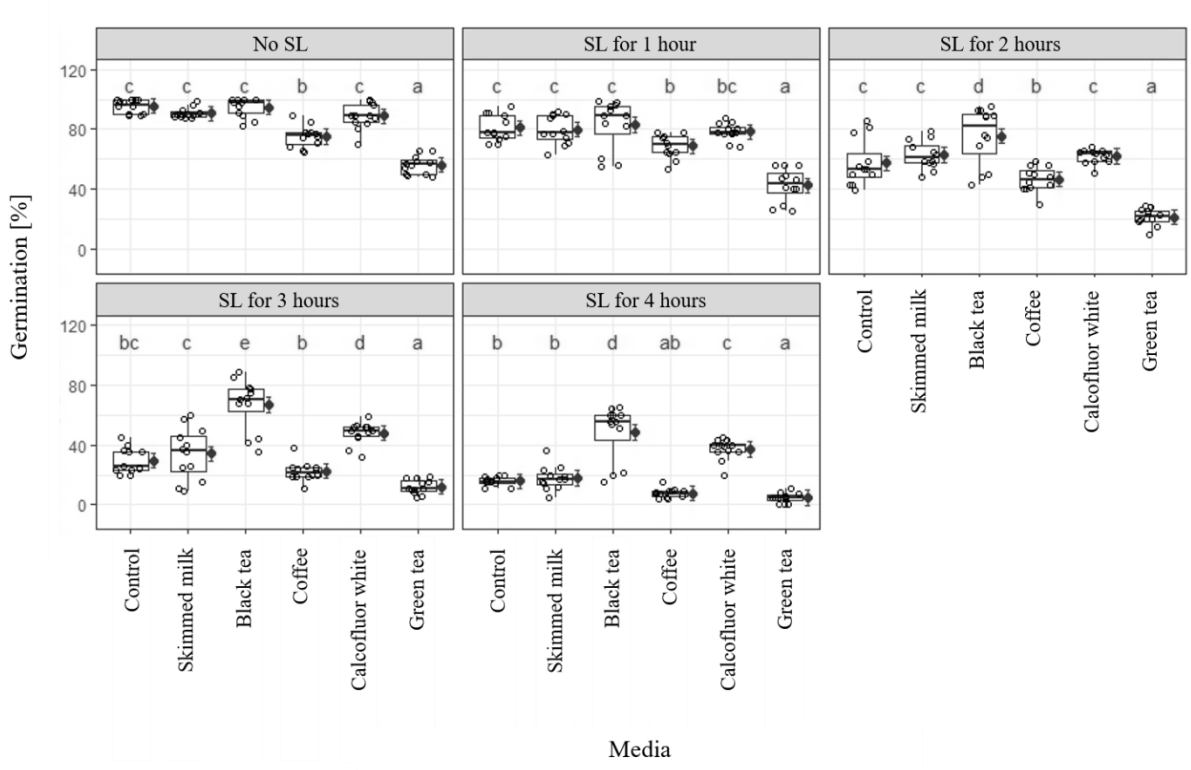

Figure 4. Germination [%] of spray-dried submerged spores of *C. fumosorosea* coated with different sunlight protectants (control, skimmed milk, black tea, coffee, calcofluor white and green tea) under simulated sunlight (for 0, 1, 2, 3 and 4 hours). The germination was analysed after 16 hours of incubation on MPA at 20 ºC. Jittered boxplots consist of the median and the 25 % and 75 % quantile. Black dots and error bars represent adjusted mean with 95 % confidence limits. Means with the same letters are not significantly different (Tukey HSD test, $\alpha = 0.05$, n= 3 × 3) (SL = Sunlight simulation).

Storability test

Storage temperature

After storing the spray-dried submerged spores at 6 °C and 20 °C for 1 week, the result reveals that temperature significantly influenced germination (ANOVA; $F = 237.5$, $df = 1$, $p < 0.001$). When pairwise Tukey test was done (Figure 5), it was seen that at 20 °C, none of the different formulated spray-dried spores germinated more than 10.3 %. On other hand, at 6 °C, spores formulated with different sunlight protectants germinated between over 50 % and 90 % after 1 week of storage. Therefore, for further storage experiment, 6 °C was taken as standard temperature.

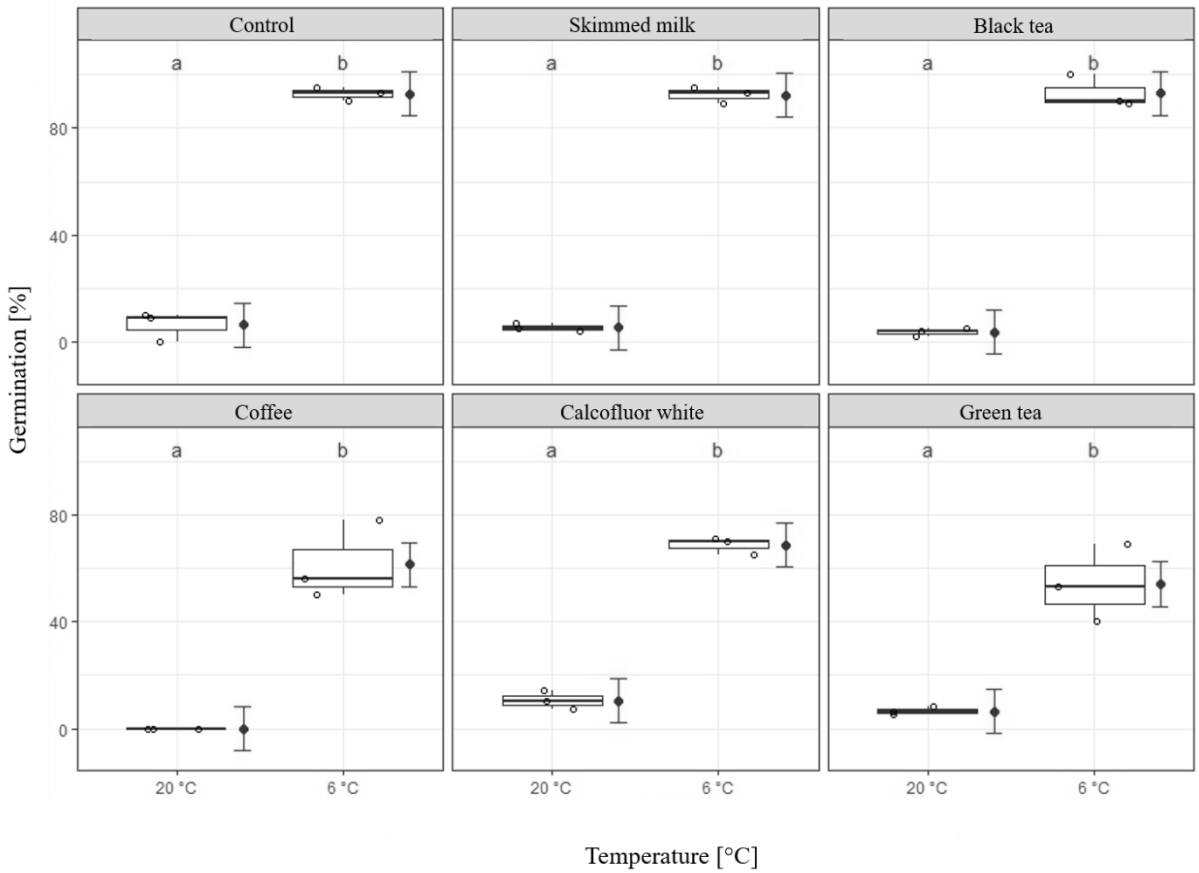

Figure 5. Influence of two different storage temperatures on germination [%] of submerged spores of *C. fumosorosea* coated with five potential sunlight protectants. Duration of the storage was 1 week. The germination was analysed after 16 hours of incubation on MPA at 20 ºC. Jittered boxplots consist of the median and the 25 % and 75 % quantile. Black dots and error bars represent adjusted mean with 95 % confidence limits. Means with the same letters are not significantly different. (Tukey HSD test, $\alpha = 0.05$, n = 3).

Storage over time

The resulting Kaplan-Meier survival curves (Figure 6) depicted distinct patterns for each formulation, indicating varying germination over time. The log-rank test revealed a highly significant difference among the formulation groups ($p < 0.0001$).

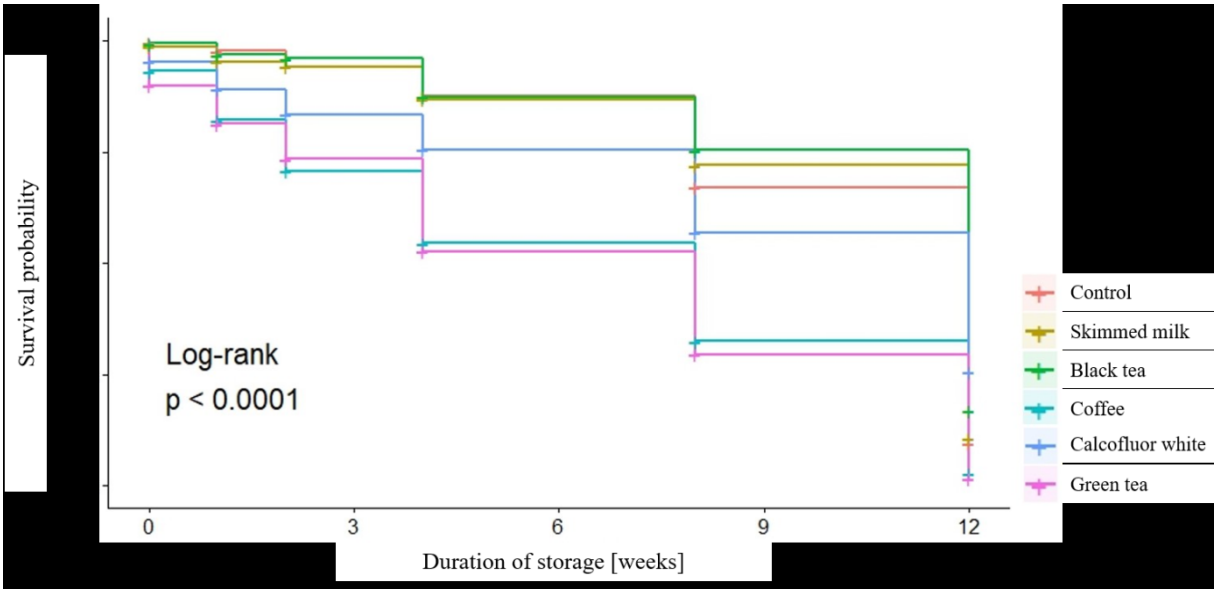

Figure 6. Overall survival probability (Kaplan-Meier analysis) of the spray-dried submerged spores *C. fumosorosea* coated with sunlight protectants (control, skim milk, black tea, coffee, calcofluor white and green tea) after storage of 1, 2, 4, 8 and 12 weeks. The statistic of shown p-values (log-rank test, $\alpha = 0.05$, n = 3 × 3) and indicates differences in the germination of submerged spores coated with different sunlight protectants for respective storage time.

For germination for different weeks of storage reveal statistically significant effects of multiple factors. Specifically, the formulation, type of sunlight protectant, significantly influenced germination outcomes (ANOVA; $F = 40.5$, $df = 5$, $p < 0.001$). Additionally, the time of storage representing a substantial impact on germination as well ($F = 108.7$, $df = 5$, $p < 0.001$).

Notably, pairwise comparisons (Figure 7) showed that skimmed milk (96.7 %) and black tea (96.1 %) coated spores maintained germination comparable to the control (97.2 %) over the initial storage period. However, spores coated with calcofluor white (71.8 %), coffee (57.1 %), and green tea (58.4 %) exhibited significantly lower germination than the control ($df = 284$, $p < 0.0001$). As storage time increased, spores coated with skimmed milk and black tea displayed progressively higher germination, closely followed by the control, particularly in the first week. This pattern persisted into the second week. By the eighth week, the

germination of black tea coated spores (72.4 %, *df* = 284, *p* = 0.0002) and skimmed milk coated spores (65.8 %, *df* = 284, *p* = 0.052) continued to show marginal to significant differences compared to the control, while those coated with coffee (19.0 %, *df* = 284, *p* < 0.0001) and green tea (12.1 %, *df* = 284, *p* < 0.0001) significantly decreased. Interestingly, by the twelfth week, spores coated with calcofluor white (44.9 %) emerged as the preferred protectant, showing significantly higher germination compared to all other formulations except black tea (*df* = 284, *p* < 0.0001).

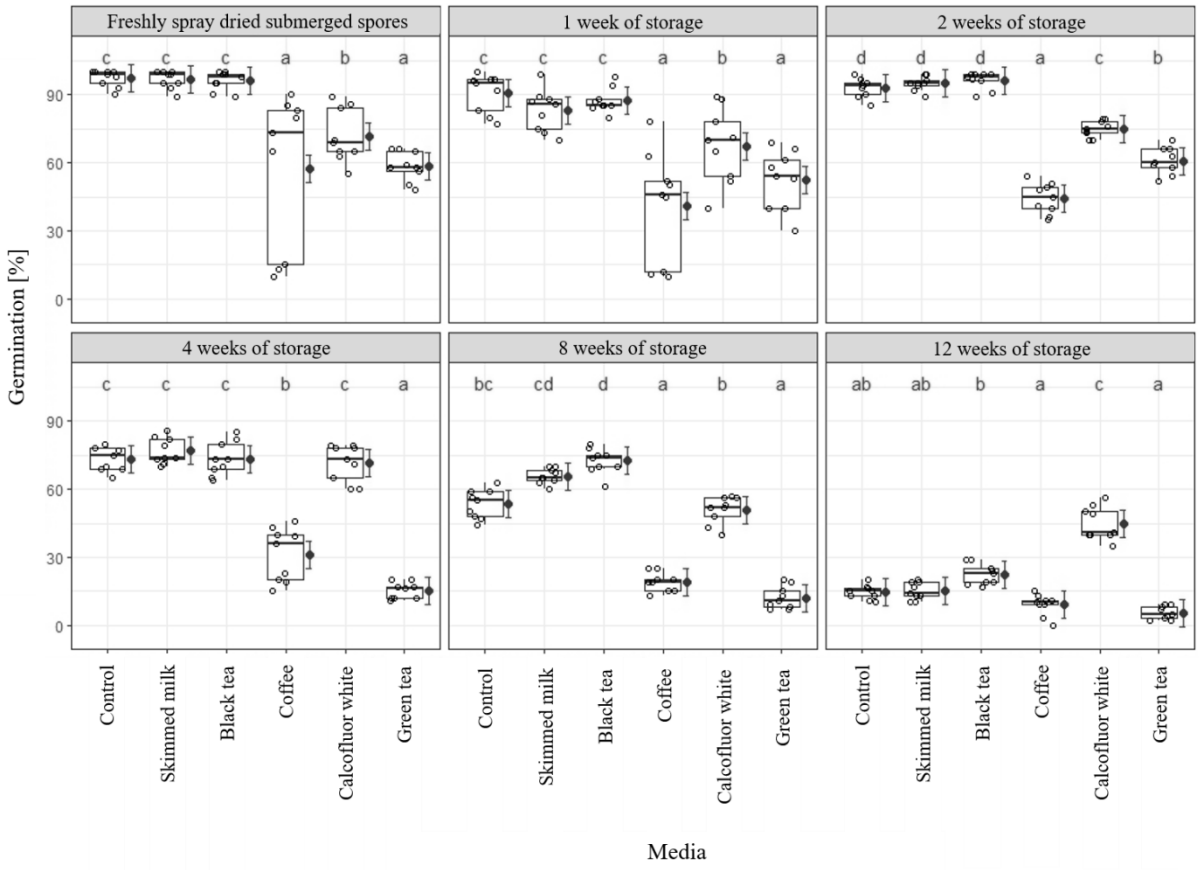

Figure 7. Germination [%] of spray-dried submerged spores of *C. fumosorosea* coated with different sunlight protectants for storage after freshly produced spores, 1 week, 2 weeks, 4 weeks, 8 weeks, and 12 weeks of storage. The germination was analysed after 16 hours of incubation on MPA at 20 ºC. Jittered boxplots consist of the median and the 25 % and 75 % quantile. Black dots and error bars represent adjusted mean with 95 % confidence limits. Means with the same letters are not significantly different (Tukey HSD test, $\alpha = 0.05$, n = 3 × 3).

The study assessed the time required to reach a 50 % reduction in germination under various treatments. Spores coated with skimmed milk and black tea showed more prolonged viability, with a reduction at 8.3 weeks and 9.1 weeks, respectively. Conversely, coffee-coated spores demonstrated a rapid decline, achieving a 50 % reduction in just 0.4 weeks. Calcofluor white

coated spores had the most extended effect, delaying the reduction to 9.9 weeks. Green tea coated spores reached the 50 % mark at 1.4 weeks.

Discussion

The main focus of this study was to develop an efficient spray-drying process for *C. fumosorosea* that ensures viability and long-term storage while incorporating sunlight protectants. One crucial part was to optimize the spray-drying technique for submerged spores of *C. fumosorosea* and evaluating the effectiveness of spray-dried spores. Earlier studies on *C. fumosorosea* have identified specific nutritional and environmental conditions that facilitate the rapid production of submerged spores with high desiccation tolerance (Jackson et al. 1997; Jackson et al. 2003; Jackson et al. 2006). The viability and survival of submerged spores produced in liquid culture during drying and storage are crucial prerequisites for their effectiveness as a microbial biocontrol agent (Mascarin et al. 2015; Mascarin et al. 2016). Formulating submerged spores in a dry state presents limited options, primarily solid formulations (powders, granules, beads, etc.) or oil-based formulations without water. The lipophobicity of blastospores presents a challenge to the development of oil-based formulations, although this is possible with dried blastospores (Stephan 1998). Therefore, solid formulations are considered the easiest and most cost-effective method for formulating dried blastospores (Horaczek and Viernstein 2004; Fernandes et al. 2015; Mascarin et al. 2016). For practical applications, an effective drying method must yield a uniform product with minimal damage to cell integrity and viability. It should also be easily scalable without significant additional processing costs (Horaczek and Viernstein 2004). As in this study, submerged spores were in focus, spray-drying was chosen over other drying techniques. Spray-drying is efficient for processing heat-sensitive materials due to short drying times and low temperatures (Stephan and Zimmermann 1998). It can also serve as a cost-effective encapsulation method, producing microcapsules in a simple, continuous operation, making it six times cheaper than freeze-drying in terms of water removal cost (Horaczek and Viernstein 2004). On other hand, spray-drying offers the advantage of combining particle formation and rapid drying in a single step, distinguishing it from air drying (Mascarin et al. 2016). This process fully fit to this study, as the main purpose was to protect or to coat the spores. However, in various studies, it was observed that submerged spores are susceptible to drying. Particularly with spray-drying, they undergo rapid water loss, causing damage to microbial

cells (Stephan and Zimmermann 1998; Fu and Chen 2011; Mascarin et al. 2016). Therefore, choosing appropriate protectants to be added to the liquid submerged spore suspension is crucial for effective formulation (Iwanicki et al. 2021). Survival of blastospores after drying is influenced by drying conditions, formulations, and blastospore preparation. Early studies in a biological containment hood showed variability in desiccation tolerance and storage stability (Cliquet and Jackson 1997; Stephan and Zimmermann 1998; Jackson et al. 2003; Leland and Behle 2004; Jackson et al. 2006; Fu and Chen 2011; Fernandes et al. 2015; Mascarin et al. 2016). For example, skimmed milk and polyvinylpyrrolidone matrix was examined for its protective function, leveraging skimmed milk's known membrane-stabilizing, emulsifying, and sunlight protectant properties during spray-drying (Stephan and Zimmermann 1998; Leland and Behle 2004; Horaczek and Viernstein 2004; Bianca Correa 2020; Iwanicki et al. 2021). In the current work, 5 % skimmed milk was used as protective agent for spray-dried submerged spores of JKI-BI-1496 and resulting over 90 % germination of spores. This supports other research as well (Stephan and Zimmermann 1998; Leland and Behle 2004; Holka and Kowalska 2023).

Following the feasibility testing of spray-dried submerged spores, the next step involved screening for suitable water-soluble sunlight protectants. There is always a question that arises that why water-based sunlight protector should be taken into account over oil-based formulation. Plenty of research had been conducted using oil based sunlight protectants (Inglis et al. 1995; Leland and Behle 2004; Leland et al. 2004; Jackson et al. 2006; Fernandes et al. 2015; Holka and Kowalska 2023). But no studies had been done using water-soluble sunlight protectants solely with *C. fumosorosea*. Water-soluble protectants also possess some benefits. The water-soluble sunlight protectant serves the dual purpose of safeguarding the bioactive agent from sunlight by absorbing, blocking, or reflecting radiation (Leland et al. 2004). In this study, all nine sunlight protectants had been used and they had been found to show absorbance OD in the range of > 0. That means, all the material had the minimum transmission rate, in other word, sunlight protection power (Anonymous 2023a). Moreover, all these samples had been used in several researches as a sunlight protector (Leland and Behle 2004; Leland et al. 2004; Braga et al. 2006; Arthurs et al. 2008; Shapiro et al. 2008; El-Salamouny et al. 2009; Kaiser et al. 2019; Wong et al. 2019; Kaiser et al. 2020; Dias et al. 2020; Iwanicki et al. 2021; Rojas et al. 2023; Nasir et al. 2023; Bayramoğlu 2023; Holka and Kowalska 2023).

When it comes to protect the submerged spores from sunlight, one potential approach to address the environmental challenge is to incorporate sunlight light protectants into fungal formulations (Fargues et al. 1996). There have been several researches conducted with formulated fungal spores under simulated solar radiation in laboratory settings (Inglis et al. 1995; Fargues et al. 1996; Fernandes et al. 2015; Acheampong et al. 2020; Kaiser et al. 2020; Dietsch et al. 2021), although the level of protection achieved was limited. In this study, without sunlight protectant, submerged spores started to decrease the germination after 2 hours of sunlight simulation. Similar result was also found previous studies conducted with *Metarhizium* without any protective additives (Braga et al. 2001; Bayramoğlu 2023). On other hand, among all the formulation, spores coated with black tea showed the best (over 50 % germination) result, even after 4 hours of continuous solar simulation. The finding was consistent with previous research showing that caffeinated tea or caffeine is more effective in inhibiting UVB (Shapiro et al. 2008; El-Salamouny et al. 2009). According to Rechner et al. (2002), black tea contains the predominant phenolic compounds (four theaflavins, epicatechin gallate, theogallin, quercetin-3-rutinoside, and 4-caffeoyl quinic acid). Thearubigins constitute an estimated 75 - 82 % of the total phenolics (Rechner et al. 2002). Additionally, black tea contains caffeine, chlorogenic acid, and gallic acid. This suggests that caffeine plays a crucial role in UV protection (Shapiro et al. 2008; El-Salamouny et al. 2009; Kaiser et al. 2019; Kaiser et al. 2020; Iwanicki et al. 2021). Another research was conducted by Bayramoğlu (2023), where it was mentioned that black tea extracts are rich in polyphenol, which act as antioxidants against radicals generated by solar radiation. This kind of actions also minimize oxidative damage and thereby promoting the integrity of the spores, which is crucial for successful germination. In current study, spray-dried submerged spores coated with black tea did not have negative influence in germination of JKI-BI-1496 (*C. fumosorosea*). While, that was the case with coffee and green tea. Coffee and green tea coated submerged spores were very vulnerable, when it comes to germination. Though, green tea and coffee had solar radiation absorbance capacity (El-Salamouny et al. 2009). There have been several researches conducted using black tea, green tea and coffee, using as UV protector for *Beauveria* and *Metarhizium* (El-Salamouny et al. 2009; Kaiser et al. 2019; Kaiser et al. 2020; Bayramoğlu 2023), but hardly any work has been done with *C. fumosorosea* spores. Kaiser et al. (2018) found that black tea, when combined with *B. bassiana*, substantially increased the exposure of the fungus to UV-B, as indicated by the colony-forming units (CFU). In contrast, green tea showed not such an effect (Kaiser et al. 2019; Kaiser et al. 2020). Another study conducted by

Ibrahim et al. (2019) demonstrated that extracts from both black tea and green tea have the potential to act as UV protectants for baculovirus. Notably, black tea exhibited a more pronounced protective effect used for baculovirus (Ibrahim et al. 2019; Anggraini et al. 2022; Bayramoğlu 2023). All these align with the findings of the current study, indicating that black offers increased solar radiation protection for submerged spores of JKI-BI-1496 (*C. fumosorosea*). Green tea was used in this study was not caffeinated. This might be a cause to why spores with green tea was not germinating as spores coated with black tea.

After confirming the solar radiation protection, the influence of the sunlight protectants on the storability was analysed. In current study, formulated submerged spores were very sensitive to storage temperatures at 20 °C (lower than 10.3 % germination) compared to 6 °C (over 50 % germination). This result aligns with several studies suggesting that lower temperatures are beneficial for the long-term preservation of spray-dried spores. For example, a study conducted by Stephan and Zimmermann (2001), where the storability of spray-dried spore of Metarhizium-isolates at 5 °C and 30 °C was assessed. The spray-dried spores showed no reduction in germination at 5 °C, while declined faster at 30 °C. After four years, the viability of these isolates remained at over 70 %, when stored at 5 °C. Another study on the spray-drying encapsulation of *Beauveria bassiana* conidia demonstrated that 80 % viability was maintained for six months when stored at 4 °C (Liu and Liu 2009; Felizatti et al. 2021). Similar result was also found by Mascarin et al. (2016), when *B. bassiana* spores stored at 4 °C for 9 months with high viability rate. Consistent with current findings, studies have shown that blastospores of *C. fumosorosea* and *B. bassiana* exhibit good survival and stability under refrigerated storage (Jackson et al. 2006; Mascarin et al. 2015; Mascarin et al. 2016). In a previous study (Iwanicki et al. 2021), it was suggested that storing formulations at 4 °C under modified atmosphere, significantly increased the half-life 2.1 times of the blastospores of *Metarhizium.*

After selecting the storage temperature of formulated submerged spores, storage duration experiments had been conducted. Until 8 weeks of storage, spores coated with black tea was showing the best result. However, situation suddenly changed after 12 weeks of storage. Spores coated with clacofluor white showed over 50 % germination with longer storage period among other formulation. There have been several works conducted using calcofluor white as UV protectant or adjuvants for EPF (Shapiro 1992; Inglis et al. 1995; Reddy et al.

2008; Reddy et al. 2009; Fernandes et al. 2015). For example, Reddy et al. (2008) found that calcofluor white, at concentrations of 1 to 10 g/L, provided protection against UV radiation without inhibiting *B. bassiana* growth. Similarly, Fernandes et al. (2015) discovered that oxybenzone, added at 0.5 % (w/v) to water suspension, significantly increased conidial tolerance to UV in comparison to controls for four out of six isolates studied. In current study, calcofluor white coated submerged spores also showed significant higher germination under simulated sunlight than other water-soluble sunlight protectants, except black tea coated spores. Added to that, in 2009, Reddy et al. invented the tablet form of *B. bassiana* conidia, where calcofluor white was used as UV protectant. This formulation was taken also for the shelf life assessment. It was observed that at lower temperature (4 - 8 °C) helped to store tablet conidia for 18 months (Reddy et al. 2009). However, concrete researches on the evaluation of the shelf life of submerged *C. fumosorosea* spores coated with calcofluor white may not be well documented in the literature till now. Nevertheless, studies on the use of calcofluor white in fungi staining techniques and its applications in microbiology, histology and others have been extensively documented (Lynchand Gibson 1987; Nickle and Shapiro 1992; Harrington and Hageage 2003; Dalynn Biologicals 2014).

Researchers interested in evaluating the shelf life of fungal spores or assessing the stability of biocontrol agents may have conducted related studies using different methodologies, storage conditions and fluorescent dyes or stains. Therefore, it is possible that relevant research exists, but may not have explicitly focused on *C. fumosorosea* spores coated with calcofluor white. To understand why calcofluor white has a longer shelf life than other samples, underlying fundamental aspects were considered. Calcofluor white is basically composed of nitrogenous elements, which binds to the cellulose and chitin (Ruiz-Herrera et al. 2003; Roncero and Durán 2003; Harringtonand Hageage 2003; Plásek and Hosková 2010; Rasconi et al. 2010; Xueling et al. 2021). Therefore, this can act as a preservative by inhibiting fungal growth and reproduction. When fungal spores are coated with calcofluor white, it creates an environment less conducive to fungal growth, thus extending the shelf life of the submerged spores of JKI-BI-1496 even after 12 weeks. Moreover, calcofluor white is itself a relatively stable compound, meaning it doesn't easily degrade or undergo chemical reactions that could render it ineffective (Harrington and Hageage 2003, Xueling et al. 2021). This stability contributes to its longer shelf life. In current study, using this sunlight protectant was beneficial for required purpose.

In conclusion, it can be said that spray-drying has been shown to be an effective preservation method, achieving a 90 % germination of submerged spores of JKI-BI-1496 (*C. fumosorosea*). The addition of black tea, a natural source of polyphenols and antioxidants, further reinforces this approach as a shield against solar radiation. Black tea has properties that can protect against sunlight-induced degradation, which can help to extend the longevity of treated materials. Additionally, the inclusion of calcofluor white, which is known for its preservative qualities and moderate sunlight protection capabilities, can help to further extend the shelf life of treated substances while maintaining their effectiveness over prolonged periods. This approach provides a comprehensive solution for ensuring material integrity and performance under varying environmental conditions, highlighting the reliability of the preservation process and its practical applicability in a variety of academic and industrial settings.

General discussion

An effective method for the biocontrol of pest insects by entomopathogenic fungi (EPF) requires a methodical and strategic approach. Identifying and selecting potential EPF for biocontrol involves several critical steps. The first step in the development of an effective EPF is the identification of a highly virulent strain of the fungus against its target pest insect. This is then followed by the development of suitable production and formulation techniques for the EPF. Once these two steps have been completed, the final stage of the process is to assess the long-term storage capability of the formulation. The final step is to confirm that the EPF formulation is effective and suitable for use in real agricultural settings, in accordance with the guidelines established by Montesinos (2003). Throughout this thesis, priority has been given to selecting a fungus with the potential to act as a biocontrol against *Cydia pomonella*, through a series of experiments and meeting several criteria (Figure 1). Afterwards, the selected potential fungus was formulated in such a way to be useful in Integrated Pest Management (IPM) for controlling *Cydia pomonella* (Figure 2).

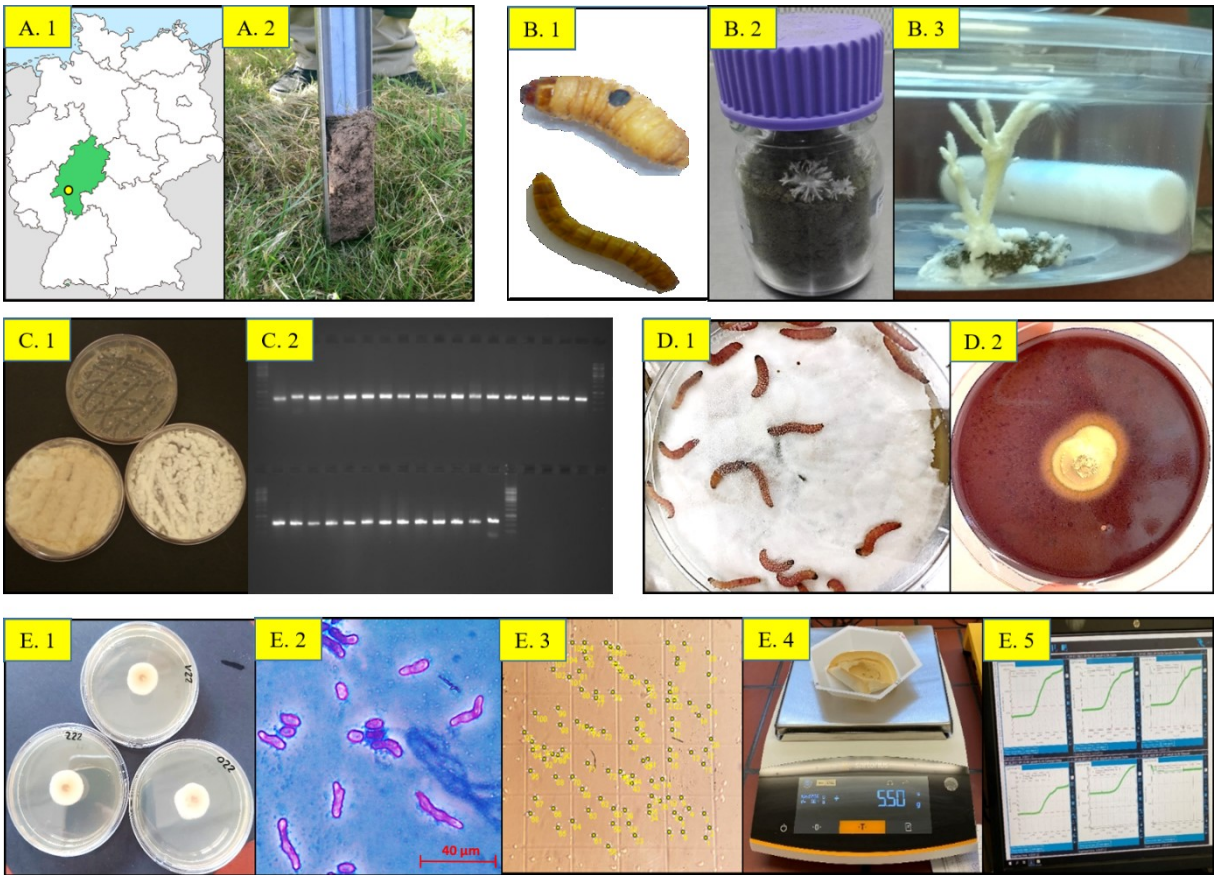

Figure 1. Process of screening for entomopathogenic fungi. Soil sample collection: (A.1) Location (Hessen) of soil sample collection, (A.2) soil sample collection with bore hole (Photo: Paluch, M., JKI); Isolation of EPF: (B.1, B.2, B.3) Insect (*Galleria* (Top) and *Tenebrio* (Bottom)) bait method for EPF isolation (Photo: Ehrich, C., JKI); Characterization of isolated samples: (C.1) Morphological characterization, (C.2) Molecular identification; Screening of the strains: (D.1) Virulence test, (D.2) Chitinase activity test; Compatibility test with environmental and nutritional growth parameter: (E.1) Radial growth after incubation at different temperatures, (E.2) Germination test after simulated sunlight for 3 hours, (E.3) Submerged spore count, (E.4) Dry biomass weight, (E.5) Optical density measurement.

Figure 2. The fungus JKI-BI-1496 (*C. fumosorosea*), from harvesting to the production of a formulation. (A.1) 14 day old *C. fumosorosea* in MPA-plate, (A.2) Liquid fermentation of *C. fumosorosea*, (A.3) Submerged spores after liquid fermentation after 72 hours at 25 ºC at 150 rpm; Final product: (B.1) Freeze-dried submerged spores, (B.2) Spray-dried spores with coated with black tea, (B.3) Spray-dried spores with calcofluor white.

Selecting an entomopathogenic fungus for controlling the pest insect *Cydia pomonella*

Selecting a potential candidate for a suitable biocontrol agent requires an understanding of the complex interplay of multiple factors that collectively affect the efficacy of the entomopathogen (Jaronski 2010). Environmental and nutritional aspects, including virulence and chitinase activity were evaluated (Chapter I), *C. fumosorosea* was selected as a candidate for controlling *C. pomonella* and further analyses were conducted (Chapter II).

Chapter I of this dissertation focuses on the critical process of selecting potential entomopathogenic fungi (EPF) from soil. Developing biological control agents (BCAs) requires finding a fungal strain that meets specific criteria for a marketable product. This involves evaluating potential fungal candidates for their ecological characteristics, ability to be produced at scale, environmental safety, and, most importantly, their virulence against

target pests. Virulence is widely regarded as a critical factor as it directly impacts the fungus's ability to control or eliminate pests, making it the primary determinant of the product's effectiveness in pest management (Jones and Burges 1998; Maina et al. 2018; Paluch 2021). This comprehensive approach ensures that the selected fungal isolate is both effective and sustainable for use in agricultural settings. In Chapter I, 32 strains were isolated from soil samples and one strain was included from strain collection of JKI. Before evaluating the effectiveness of these fungi, characterization (morphological and molecular) was essential, as it helps to identify effective strains, optimize culture conditions, assess the environmental impact and develop formulations that enhance their use as biopesticides in IPM (Gebremariam et al. 2021; Bich et al. 2021; Rejula et al. 2021; Bali et al. 2022). In current study, 33 EPF belonging to the genera *Beauveria*, *Metarhizium* and *Cordyceps* have been identified. All these three genera have been proven being effective to control *C. pomonella* (Lacey and Unruh 2005; Zimmermann et al. 2013; Abaajeh and Nchu 2015; Gürlek et al. 2018; Iwanicki et al. 2019; Stone and Bidochka 2020; An et al. 2021; Ahmed et al. 2024). Consequently, it was expected that after assessing virulence, environmental conditions, and nutrient variations in the culture medium, an effective strain would be identified for development as a BCA.

After morphological and molecular characterization, mortality tests were conducted to assess their effectiveness against *C. pomonella* larvae as a first virulence parameter. The results showed varying levels of virulence among the strains, with *Beauveria* and *Metarhizium,* exhibiting higher virulence compared to *Cordyceps*. The study found that *Beauveria* and *Metarhizium* species are effective insect pathogens, which is consistent with a study by Ahmad et al. (2024). In their study *B. bassiana* treatment resulted in higher mortality among *C. pomonella* larvae and pupae, with fewer adults emerging, compared to *C. fumosorosea*, which had lower mortality but higher adult emergence (Ahmad et al. 2024). This suggests that *B. bassiana* is more effective in suppressing *C. pomonella* populations due to its higher virulence against the pest. In 2015, Abaajeh and Nchu conducted a research concerning indigenous fungal isolates, collected from South African soils. Two fungal isolates of *Metarhizium robertsii* showed virulence against *C. pomonella* (Abaajeh and Nchu 2015). On other hand, according to Zimmermann et al. (2013), *B. bassiana* and *C. farinosa* may act as natural regulators of *Cydia pomonella* populations in orchard ecosystems. In particular *B.*

bassiana shows a high prevalence in hibernating *C. pomonella* populations, indicating its significance as a natural mortality factor (Zimmermann et al. 2013).

The reason for the differences in effectiveness between different fungal isolates may be related not only to their distinct virulence (Nielsen et al. 2005), but also to their remarkable enzymatic and molecular characteristics (Maistrou et al. 2020). Research on entomopathogenic fungi such as *B. bassiana* and *M. anisopliae* shows that host death is primarily caused by the production of mycotoxins, such as beauvericin and destruxin (Moino Jr. et al. 2002; Wang and Xu 2012; Zhang et al. 2017; Weng et al. 2019). While some bioactive molecules of *C. fumosorosea* have been identified, it remains unclear whether its mode of action against pests, is similar to that of toxin-secreting fungi (Weng et al. 2019). The effectiveness *C. fumosorosea*, is thought to be related to its ability to penetrate, colonise, and produce conidia within the host, leading to faster insect mortality (Lei et al. 2021; Deka et al. 2021). Despite the broad host range of *C. fumosorosea* and the current gaps in understanding of its toxins and metabolites (Sharma et al. 2023), the strain has demonstrated high effectiveness. According to Bugti et al. (2018), *C. fumosorosea* (strain Ifu13a) achieved mortality of 81 to 100 % against various pests, including the tea jassid (*Jacobiasca formosana*), cotton aphid (*Aphis gossypii*), tobacco whitefly (*Bemisia tabaci*), and pear lace bug (*Stephanitis nashi*), at a concentration of 10^8 conidia/mL. This indicates the potential of *C. fumosorosea* as a versatile and effective tool in integrated pest management strategies.

According to Wang et al. (2023), the higher the activity of the cuticle-degradation enzyme, the higher the virulence of the fungus against the target pest. *B. bassiana*, a fungus that preys on insects, uses chitinase (Fan et al. 2007; Fang et al. 2009) to breach the insect's chitin-based epidermis. This enzyme breaks down the chitin, allowing the fungal mycelia to penetrate the cuticle and gain access to the insect's body fluids. Toxicity to insects was found to increase significantly when *B. bassiana* chitinase was overexpressed along with proteases compared to when the proteases were not included. Similarly, the chitinase from *C. fumosorosea* acts as a virulence factor by targeting and degrading the insect cuticle components, facilitating pathogen entry into the host and the assimilation of carbohydrates released from the host tissue (Fang et al. 2009). In *M. anisopliae* it has been shown by Niassy et al. (2013) that it upregulates the chitinase gene to degrade chitin as part of its infection process. Activation of this gene is critical for fungal penetration of the host epidermis and the initial phase of

infection in insects (Jeong et al. 2023). One hypothesis of this thesis was that adding chitin derivatives to the media composition will increase the chitinase activity of EPF (Chapters I and II), which will later positively affect spore growth, biomass, germination and virulence. Therefore, a chitinase activity test was conducted as a parameter of virulence.

From 33 strains, four strains were selected for their potential to combat insect pests, based on their virulence parameters (virulence test and chitinase activity). However, these virulence parameters were measured under laboratory conditions and it is not clear how they will perform under field conditions. Therefore, additional environmental and nutritional aspects of the strains are very important to consider when selecting the best candidates (Montesinos 2003; Mascarin et al. 2010; Köhl et al. 2011). The strains showed differential sensitivity to temperature and sunlight exposure, highlighting the need for environmental considerations in the implementation of EPF-based biopesticides (Jaronski 2010; Jackson et al. 2010; Deka et al. 2021; Islam et al. 2021; Quesada-Moraga et al. 2024). Temperature tolerance was found to be a critical factor, with JKI-BI-1496 (*C. fumosorosea*) demonstrating optimal growth at 20 - 25 °C, while *M. robertsii* showed strong mycelial growth at 25 - 30 °C, indicating suitability for tropical and subtropical regions. Also at 25 °C, *C. fumosorosea* showed the higher adaptability than the other three fungi. Several studies have shown the temperature tolerance of different EPF (Wu et al. 2020; Seib et al. 2023; Quesada-Moraga et al. 2024). In the present study, it was notable that strains isolated from the same field exhibited variations in their temperature optima. Collectively, these differences suggest that the strains span a broader temperature spectrum, potentially enhancing their ecological resilience. In the case of sunlight simulation, *C. fumosorosea* showed higher germination even after 3 hours of simulated sunlight, compared to other three strains. In particular, *M. robertsii* showed the lowest tolerance to simulated sunlight, which was also supported by several other studies, where *Metarhizium* were studied for their response to UV-A and UV-B (Braga et al. 2001; Fernández-Bravo et al. 2016; Fernández-Bravo et al. 2017; Quesada-Moraga et al. 2024).

Furthermore, my study showed strain specific effects of different media compositions on growth parameters (Chapter I and Chapter II). Nutrient availability significantly affected fungal growth and sporulation with the consequence that media composition is playing a critical role for optimizing spore production and biomass yield (Mascarin et al. 2010; Quesada-Moraga et al. 2024). It was hypothesized that altering the nutrients would have a

direct effect on spore growth, germination, biomass or virulence. For example, adding chitin derivatives for the production of *C. fumosorosea*, significantly improved all parameters. Adding 1 % colloidal chitin (CC) to the basic media composition, increased the germination, biomass and overall stimulated the virulence (Mascarin et al. 2010; Mascarin 2015; Yang and Zhang 2019; Gomaa 2021; Lei et al. 2021; Moharram et al. 2021; Jeong et al. 2023).

C. fumosorosea was the only one of the four selected fungi that successfully fulfilled all criteria (virulence, chitinase activity, temperature optima, tolerance of simulated sunlight and growth in different culture media). Furthermore, this fungus shows a wider host range compared to the other.

Liquid fermentation versus solid-state fermentation

The key factor in developing EPF as BCA is the ability for mass production and efficient application. Among the various fermentation methods, much emphasis has been on refining solid-state or liquid fermentation to improve biomass production and ensure high survival rates after drying and throughout storage (Jackson 1997). The choice between using solid-state fermentation and liquid fermentation depends on the desired form of the fungal strain for field application. Currently, aerial conidia, the asexual spores, are the most commonly used active component of BCA worldwide (Faria and Wraight 2007), for dispersal, survival, and infecting hosts. The production of these infective propagules is typically achieved through solid-substrate cultivation. The scale-up process involves growing the fungus on moist cereal grains for several weeks under controlled conditions (Mascarin et al. 2010; Mascarin 2015; Jaronski 2023). This is due to challenges faced by the latter, including high substrate costs, labor-intensive processes, non-uniform and poorly controlled production conditions leading to contamination, and low energy efficiency with production often exceeding 10 days (Mascarin 2015). However, liquid fermentation has been found to be a more efficient method for producing EPF compared to solid-state fermentation (Jackson 1997; Jackson et al. 1997; Jackson et al. 2004; Mascarin 2015; Jaronski 2023).

Liquid fermentation is suitable for producing submerged spores, which can germinate rapidly and potentially offer an advantage in terms of the speed of pest control. However, it should be noted that spores produced through this method are less environmentally stable than conidia (Jackson et al. 1997; Jackson et al. 2004; Jackson et al. 2010; Jackson and Jaronski 2012;

Mascarin 2015; Jaronski 2023). In the current study, the downstream process involved liquid fermentation of *C. fumosorosea*. This was followed by stabilization and an increase in shelf life through a drying process (Chapter II, Chapter III). The liquid culture media was enriched with carbon and nitrogenous substrates, which influenced the growth, germination, and virulence of the submerged spores. The media consisted of low-cost materials, which supports research conducted by Jackson (1997). Previous studies on *C. fumosorosea* have demonstrated that nutrient-rich media are mostly supported in submerged liquid cultures (Jackson 1997; Jackson et al. 1997). In liquid fermentation, the transition from mycelium to blastospore in filamentous fungi such as *C. fumosorosea* is significantly influenced by oxygen supply. Aerobic conditions are required for this growth change (Mascarin 2015). Additionally, the enhanced desiccation tolerance of *C. fumosorosea* and its blastospores has been primarily linked to the initial nitrogen concentration in the culture medium and the residual glucose concentration in the spent medium (Jackson et al. 1997; Cliquet and Jackson 2005; Mascarin 2015). Liquid fermentation was chosen for the production of submerged spores of *C. fumosorosea* due to its advantages.

Downstream processing: Fermentation to formulation

In this study, I first addressed the nutritional aspects of cultivating fungal spores, particularly blastospores, before discussing the drying methods employed. Spores were grown in media enriched with colloidal chitin to enhance their nitrogen content, which is crucial for their growth and stability. This approach mirrors findings by Mascarin et al. (2015), who noted that cottonseed flour was an effective nitrogen source for *C. fumosorosea* blastospore production, enhancing both desiccation tolerance and storage stability, although it may not be economically viable for large-scale production (Jaronski 2023). Following fermentation, two drying techniques were applied: freeze-drying and spray-drying, detailed in Chapters II and III, respectively. Freeze-drying was specifically chosen for spores cultivated in chitin derivative-rich media, with lactose utilized as a protective agent to minimize damage during the drying process. This method is particularly beneficial for heat-sensitive materials, as it avoids high temperatures, thereby preserving the spores' viability. The protective role of lactose and the suitability of freeze-drying for these applications are supported by previous studies indicating extended shelf life and easy rehydration of the spores (Jackson et al. 1997; Morgan et al. 2006; Mascarin et al. 2015; Stephan et al. 2016). Conversely, spores dried

without protective agents were found to be unstable under simulated sunlight exposure. Therefore, as an alternative option to stabilize the spores for outer field, spray-drying was chosen as the formulation process (Chapter III). Spray-drying is a fast process that allows for efficient production and produces fine, uniform particles, which can enhance product quality. Spray-dried spores are often stable and have a longer shelf life (Stephan and Zimmermann 1998; Horaczek and Viernstein 2004; Mascarin 2015; Mascarin et al. 2016; Jaronski 2023). Stephan and Zimmermann (1998) discovered that by suspending blastospores of *B. bassiana*, *M. anisopliae*, *M. flavoviride*, and *C. fumosorosea* in a solution of 20 % skimmed milk powder and 2.5 % sugar-beet syrup before spray drying, a 90 % viability rate was achieved. In contrast, unprotected spores were germinable. The addition of other additives such as yeast extract and bentonite clay proved to be ineffective. The study also identified critical temperatures for minimizing blastospore damage during spray-drying, which were 64 °C (inlet) and 48 °C (outlet) (Stephan and Zimmermann 1998). Chen and Feng (2002) found that low vacuum and heat are optimal for maintaining the viability of *C. fumosorosea* conidia, while high vacuum and temperature processes are harmful. This underscores the significance of controlling desiccation and temperature during fungal conidia processing, especially for delicate forms such as submerged spores (Chen and Feng 2002; Jaronski 2023). In the current study (Chapter III), skimmed milk was used as a protective agent for spray-drying. This protection prevented damage to the spores. Additionally, the use of water-soluble sunlight protectants was found to enhance the stability of the spores during spray-drying, simulated sunlight exposure, and extended shelf life. This could be due to the double protection from the high heat during the drying process. This drying process produces fine, uniform, free-flowing spore powders that enhance dispersibility and efficacy, simplifying handling during formulation and application (Stephan and Zimmermann 1998; Horaczek and Viernstein 2004; Mascarin 2015; Mascarin et al. 2016; Jaronski 2023).

Another drying process is fluid-bed drying, which is cost-effective and easily scalable for large-scale production with minimal loss of viability, despite its non-uniform particle distribution (Horaczek and Viernstein 2004; Stephan et al. 2021; Jaronski 2023; Seib et al. 2023). Horaczek and Viernstein (2004) conducted a comparison of freeze-drying, spray-drying, and fluid-bed drying for *B. brongniartii* and *M. anisopliae* conidia. Several observations were made, including the fact that *M. anisopliae* exhibited greater heat resistance. Spray-drying resulted in significant mortality (~ 65 %) for both fungi, while

freeze-drying effectively preserved *B. brongniartii* but not *M. anisopliae*. The preservation of *M. anisopliae* was improved by skim milk and polyvinylpyrrolidone, and fluid-bed drying at 60 °C was fatal for both. Another study has been conducted by Stephan et al. (2021) on the granule-based formulation of *C. fumosorosea*, *B. bassiana*, and *M. brunneum* using fluid-bed drying. They found that *C. fumosorosea* and *B. bassiana* achieved nearly 100 % colonization at 0.03 % dry weight, while *M. brunneum* reached only 50 % at 0.7 % after formulation. Moisture significantly affected conidiation in sterile soil. To enhance granule colonization of *M. brunneum*, nutrient pre-coating was used, which benefited both biomass and submerged spores. Malt extract was found to be particularly effective, indicating that aerial conidia can also be coated (Stephan et al. 2021).

Spray and fluid-bed drying can produce large quantities of product quickly, but they subject fungi to strong thermal stress, which not all strains can withstand. Freeze-drying, although gentler, is energy-intensive and not suitable for all fungi. These methods can create sprayable formulations, with spray and fluid-bed drying also coated particles and granules can be produced. Developing wettable powders or granules via these two methods makes it easy to handle during application (Jaronski 2010; Mascarin 2015; Islam et al. 2021; Méndez-González et al. 2022; Sharma et al. 2023). In summary, each method has its trade-offs, and the choice depends on factors such as product characteristics, cost considerations, and intended applications and is as well strain dependent. Researchers and manufacturers carefully evaluate these pros and cons to select the most suitable drying technique. In the current study, fluid-bed drying was not conducted during the formulation process. This type of drying could be helpful for comparison studies of different drying method for *C. fumosorosea* spores in future.

Importance of testing the formulated microorganism under simulated conditions before real field-testing

Despite the advancements in science and technology, there is still a crucial need for research to expand the range of species used in pest management and improve their field performance. Furthermore, it is essential to increase user awareness about proper application to encourage widespread adoption and realize their full market potential (Santos et al. 2022). Throughout Chapters I, II, and III of this study, environmental stability was a key consideration in ensuring that the formulations are suitable for effective real-world field applications. This strategy prioritizes ecological safety and practicality, aiming to create solutions that align with

sustainable agricultural practices. In Chapter I, experiments were conducted to determine the tolerance of selected potential EPF to both temperature and simulated sunlight. Chapter II describes the production of submerged spores in modified media. The viability of the spores under simulated sunlight was then examined, but the results were not satisfactory. Chapter III details the further formulation conducted to make the spores viable under simulated sunlight. The ultimate goal is to produce viable spores for application in the agricultural field. Understanding the physical and environmental factors that affect the transfer of fungal propagules to the insect cuticle is crucial for optimizing the use of this biocontrol strategy (Jackson et al. 2010). In this study, although field trials were not conducted, extensive experiments were carried out in a simulated environment. These experiments are expected to provide valuable insights and results that will be useful for practical field applications. It is important to note that outcomes from real field tests can sometimes differ significantly from in vitro results. For example, in 2021, Paluch conducted a study on using *M. brunneum* for potato cultivation showed differing results between lab and field applications. Although laboratory tests have shown the effectiveness of biocontrol agents (BCAs) against wireworms and BCA formulations have been developed, their effectiveness in the field has been inconsistent. This is partly due to lower than expected soil temperatures. It is challenging to translate laboratory success to field conditions, and the factors that affect the field performance of BCAs are not yet fully understood. Therefore, it is equally important to conduct pilot trials in the field before applying and upscaling the product. My thesis has begun an analysis of temperature and sunlight tolerance as important indicators for environmental stability. This analysis will be essential for optimizing further experiments involving other variables such as humidity and rainfall.

Application timing

Integrating entomopathogenic fungi (EPF) into IPM strategies in apple orchards, where fungicides are frequently used, involves careful planning to maximize effectiveness and manage resistance. By rotating fungicides, creating biological buffers, and incorporating EPF into Integrated Pest Management (IPM), orchard managers can enhance pest control, reduce chemical use, and promote sustainable practices, leading to healthier crops and improved yields. The primary objective of this research is to control *C. pomonella*. Therefore, it is essential to have information about the application time and location. The occurrence and

activity of EPF are affected by various factors, including soil type, air temperature, humidity, treatment timing, host availability, and agricultural practices (Lacey and Unruh 2005). For instance, the effectiveness of the desired biocontrol strategy was compromised by insufficient fungal proliferation of *Metarhizium* in the potato ridge after applying AgriMet formulations. This was primarily due to low soil temperatures (< 17 °C) during spring application (Paluch 2021). The challenge of achieving successful pest management is complicated by the timing of application. There is no direct information on the specific application timing of *C. fumosorosea* for controlling *C. pomonella* in orchards. Instead, several studies primarily focus on the antimicrobial activities of *C. fumosorosea*, including detailed analyses of its main metabolites and their effects (Weng et al. 2019; Wei et al. 2022; Sharma et al. 2023). While this information provides insight into the potential benefits and applications of *C. fumosorosea*, it does not directly address its use for pest control in orchards. However, *B. bassiana* has been successfully used as a BCA agent for controlling *C. pomonella* for many years. By applying the information from *B. bassiana*, the application time of *C. fumosorosea* could be optimized. For example, Zimmermann et al. (2013) conducted extensive research on this topic using *B. bassiana.* They found that applying this fungus to branches and stems in late summer or autumn, targeting the last larval or diapausing instars of *C. pomonella*, along with soil application under trees to reach overwintering larvae. In another report, Balaško et al. (2020) also mentioned *B. bassiana* as an effective EPF for controlling *C. pomonella*. The optimal time to attack with EPF is when the insect is in its cocoon or overwintering larvae stage (Balaško et al. 2020). However, it is important to note that the timing of application may be critical based on general biological control practices and the life cycle of *C. pomonella*. To ensure maximum effectiveness, the application should coincide with the early larval stages of the pest, which are more susceptible to infection by the fungus. This period is typically shortly after egg hatch, before the larvae bore into fruit, making them more difficult to target with biological control agents. The time for submitting an application may vary depending on the geographic location, occurring in late summer, autumn, or late spring to early summer (Lacey and Unruh 2005; Zimmermann et al. 2013). The challenge is to identify successful synergies between entomopathogens (both fungi and nematodes), natural predators, and parasitoids, alongside the use of both traditional and novel insecticide formulations, semiochemicals and habitat adjustments, to establish a profitable and environmentally sustainable pest management system for orchards (Lacey and Unruh 2005). In 2005, Lacey and Unruh observed that biocontrol strategies such as the suppression of codling moth populations using

granuloviruses, the use of entomopathogenic nematodes (EPNs) to target cocooned larvae and thereby reduce overwintering populations, and the introduction of parasitoids specific to the codling moth are all biological strategies that work well with mating disruption techniques. Therefore, the period when L_5 larvae exit damaged fruit to pupate in the tree's stem or bark presents a critical window for intervention with fungal entomopathogens such as *C. fumosorosea*. Targeting larvae during this transition could be an optimal strategy for pest control and could minimize the population after the first generation, as they are more exposed and potentially more susceptible to fungal infection before they reach the protective pupal stage. Timely treatments could enhance the effectiveness of biological control measures against *C. pomonella,* addressing this gap.

Conclusion and Outlook

In recent decades, plant protection strategies focused on ensuring sufficient food production for a growing population (Alexandratos and Bruinsma 2012). While chemical pesticides have significantly increased crop yields (Stetter and Lieb 2000), they have also led to pesticide resistance and harmful residues (Nicolopoulou-Stamati et al. 2016). Recently, there has been a shift towards evaluating the environmental risks of pesticide use, driven by an ecologically aware society that values biodiversity (Carvalho 2017). The negative impacts of agricultural intensification on insect abundance and biodiversity (Pettis et al. 2013, Vanbergen & Initiative 2013, Newbold et al. 2015, Seibold et al. 2019) prompted Germany towards expanding organic food and farming to 30 % by 2030 (Anonymous 2023b, BMEL, accessed on 24.05.2024). Globally, organic cultivation increased from 15 million hectares in 2000 to 96.4 million hectares in 2022 (FiBL 2024). Moreover, despite stringent risk assessments for pesticide registration (Villaverde et al. 2014, Gehen et al. 2019), biological control practices remain underutilized in agriculture (Kumar & Singh 2015). This is partly due to farmers' perception that this practice is less effective than chemical alternatives. However, the long-term benefits of biological control methods for biodiversity conservation may justify their lower effectiveness, though these benefits are not yet quantitatively measured. Monetizing both the positive and negative externalities could better reflect the long-term socioeconomic benefits of pesticides.

In this context, the findings of this dissertation contribute to the development of environmentally friendly and sustainable pest management strategies in agriculture. By optimizing culture media with chitin and its derivatives, the study delivers crucial insights into boosting the effectiveness and stability of entomopathogenic fungi for biocontrol purposes. This research represents a significant advancement in the development of integrated pest management strategies that reduce reliance on chemical pesticides and promote environmentally conscious farming practices.

Entomopathogenic fungi (EPF) such as *Beauveria* and *Metarhizium* have been used for a longer period of time to control *C. pomonella*. However, little work has been done with *C. fumosorosea*, as recent publications indicate (Zimmermann et al. 2013; Ahmad et al. 2024). Therefore, this thesis aimed to fill this gap by exploring the advancements and limitations of

formulating desiccated submerged spores for long-term shelf life. It also encourages the development of novel biocontrol products due to its interesting biological activity and uncomplicated production of liquid culture (Stephan et al. 2021). Furthermore, *C. fumosorosea* exhibits a high degree of environmental adaptability (Wu et al. 2020), produces a greater quantity of spores (Stephan et al. 2021), and contains secondary metabolites (Weng et al. 2019; Lei et al. 2021), that facilitate a faster mode of action. This dissertation demonstrates that *C. fumosorosea* has the potential to be an effective biological control agent (BCA) against *C. pomonella*, in case it is formulated correctly.

Although there are still unresolved questions regarding the impact of environmental factors on submerged spores stability and field effectiveness, current findings suggest that the rapid germination of liquid fermentation-produced submerged spores may enhance their potential to control *C. pomonella*. This is especially true in field situations where moisture availability is a limiting factor for biocontrol success (Jackson et al. 1997). This thesis prioritises sunlight simulation among other environmental factors. To protect the submerged spores, only water-soluble sunlight protectants have been experimented with for *C. fumosorosea*. Although there are other options available, such as oil-based protectants or a mixture of the two, they have not been tested. Choosing a specific tube size for spray-drying formulation has limited the use of common protectants such as humic acid and lignin for sunlight protection. This study highlights the effects of sunlight simulation on the viability and storage of protectant-coated spores, identifying a crucial research gap regarding stability under different temperature, humidity, and rainfall conditions. There is potential for additional research to investigate the impact of environmental factors on spore behavior, using the foundational data presented in the dissertation as a starting point.

This study highlights the expensive nature of drying processes such as freeze-drying and spray-drying. Furthermore, it stresses the significance of conducting field trials for the BCA product to guarantee its effectiveness and viability in real-world agricultural settings. This indicates a path forward for comprehensive evaluation and development.

Overall, this study emphasises the significance of carefully selecting highly virulent fungal strains, optimising mass production through liquid fermentation, and creating effective formulations with suitable additives. The liquid fermentation method developed in this research enables rapid production of desiccation-resistant, shelf-stable submerged spores,

representing a significant advancement in the mass production of *C. fumosorosea*, which could also be applied to other dimorphic entomopathogenic fungi. The findings contribute to the broader adoption of liquid fermentation techniques for producing cost-effective, shelf-stable fungal BCAs. This aids the biopesticide industry in managing a variety of insect pests in conventional agriculture. Sustainable agriculture is increasingly relying on alternatives to conventional chemical insecticides. This is to seek solutions that are eco-friendly and minimize human exposure to pesticides. BCAs, when integrated with other components of IPM, can effectively manage pests like *C. pomonella* and other orchard nuisances. Despite the promise of biological control methods, there is still a significant amount of both basic and applied research to be conducted. It is important to re-evaluate the use of inundative releases, considering that these biological organisms function differently from chemical treatments. Chemical pesticides are often used reactively, once a pest outbreak has occurred and economic thresholds have been exceeded. However, this approach may not be suitable for EPF as they act more slowly and may require frequent applications (Jaronski 2010). Integrating BCAs within Integrated Pest Management (IPM) strategies can enhance the sustainability of pest control. Combining EPF with other control measures, such as predators and microbial agents, offers a comprehensive and sustainable solution, demonstrating their potential in effective pest management practices. Additionally, merging fungal agents, such as *B. bassiana* with other microbial controls, such as *B. thuringiensis tenebrionis*, can mitigate the limitations inherent in each control method alone. This creates a more robust defense against pests, such as the Colorado potato beetle (Wraight and Ramos 2005). Fungal entomopathogens can be powerful components in the toolkit of farmers when used thoughtfully and in combination with other strategies. (Jaronski 2010), enhancing pest management and supporting the move towards more sustainable agricultural practices.

Incorporating a combination of biological control methods, including the combination of two or three EPF, is a forward-thinking strategy within IPM. This hand-in-hand approach reduces the risk of pest resistance development by not relying on a single method. Combining various biological strategies ensures a more robust and resilient pest management system, aligning with the principles of sustainability and environmental stewardship that are central to the future of IPM.

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Appendix

Table 1: DNA sequences of the ITS region of the isolated strains

Strain number	DNA sequences of the ITS region
JKI-BI-2619	GCGGAGGGATCATTACCGAGTTTTCAACTCCCTAACCCTTCTGTGAACCTACCTATCGTTGCTTCGGCGGACTCGCCCCAGCCCGGACGCGGACTGGACCAGC GGCCCGCCGGGGACCTCAAACTCTTGTATTCCAGCATCTTCTGAATACGCCGCAAGGCAAAACAAATGAATCAAAACTTTCAACAACGGATCTCTTGGCTCT GGCATCGATGAAGAACGCAGCGAAACGCGATAAGTAATGTGAATTGCAGAATCCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTG GCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACCTCCCCTTGGGGAGGTCGGCGTTGGGGACCGGCAGCACACCGCCGGCCCTGAAATGGAGTGGCG GCCCGTCCGCGGCGACCTCTGCGCAGTAATACAGCTCGCACCGGAACCCCGACGCGGCCACGCCGTAAAACACCCAACTTCTGAACGT
JKI-BI-2620	GGATCATTACCGAGTTTTCAACTCCCTAACCCTTCTGTGAACCTACCTATCGTTGCTTCGGCGGACTCGCCCCAGCCCGGACGCGGACTGGACCAGCGGCCCG CCGGGGACCTCAAACTCTTGTATTCCAGCATCTTCTGAATACGCCGCAAGGCAAAACAAATGAATCAAAACTTTCAACAACGGATCTCTTGGCTCTGGCATC GATGAAGAACGCAGCGAAACGCGATAAGTAATGTGAATTGCAGAATCCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGG CATGCCTGTTCGAGCGTCATTTCAACCCTCGACCTCCCCTTGGGGAGGTCGGCGTTGGGGACCGGCAGCACACCGCCGGCCCTGAAATGGAGTGGCGGCCCG TCCGCGGCGACCTCTGCGCAGTAATACAGCTCGCACCGGAACCCCGACGCGGCCACGCCGTAAAACACCCAACTTC
JKI-BI-2632	GGGATCATTACCGAGTTTTCAACTCCCTAACCCTTCTGTGAACCTACCTATCGTTGCTTCGGCGGACTCGCCCCAGCCCGGACGCGGACTGGACCAGCGGCCC GCCGGGGACCTCAAACTCTTGTATTCCAGCATCTTCTGAATACGCCGCAAGGCAAAACAAATGAATCAAAACTTTCAACAACGGATCTCTTGGCTCTGGCAT CGATGAAGAACGCAGCGAAACGCGATAAGTAATGTGAATTGCAGAATCCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGG GCATGCCTGTTCGAGCGTCATTTCAACCCTCGACCTCCCCTTGGGGAGGTCGGCGTTGGGGACCGGCAGCACACCGCCGGCCCTGAAATGGAGTGGCGGCCC GTCCGCGGCGACCTCTGCGCAGTAATACAGCTCGCACCGGAACCCCGACGCGGCCACGCCGTAAAACACCCAACTTCTGAACGTT
JKI-BI-2642	GGGATCATTACCGAGTTTTCAACTCCCTAACCCTTCTGTGAACCTACCTATCGTTGCTTCGGCGGACTCGCCCCAGCCCGGACGCGGACTGGACCAGCGGCCC GCCGGGGACCTCAAACTCTTGTATTCCAGCATCTTCTGAATACGCCGCAAGGCAAAACAAATGAATCAAAACTTTCAACAACGGATCTCTTGGCTCTGGCAT CGATGAAGAACGCAGCGAAACGCGATAAGTAATGTGAATTGCAGAATCCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGG GCATGCCTGTTCGAGCGTCATTTCAACCCTCGACCTCCCCTTGGGGAGGTCGGCGTTGGGGACCGGCAGCACACCGCCGGCCCTGAAATGGAGTGGCGGCCC GTCCGCGGCGACCTCTGCGCAGTAATACAGCTCGCACCGGGACCCCGACGCGGCCACGCCGTAAAACACCCAACTTCTGAACG
JKI-BI-2637	GCGGAGGGATCATTACCAGAGTTTTACAACTCCCAACCCTTCTGTGAACCTACCCATAGTTGCTTCGGCGGACCCGCCCCAGCGTCCGGACGGCCCAGCGCC GGCCCGCGACCTGGACCCAGGCGGCCGCCGGGGACCACGCAACCCTGTATCTGTCAGCCTCTCTGAATCCGCCGCAAGGCAACACAAACGAATCAAAACTTT CAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCA CATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACGTCCCCCGGGACGTCGGCCTTGGGGACCGGCAGCACCCCGCC GGCCCTGAAATGGAGTGGCGGCCCGTCCGCGGCGACCTCTGCGCAGTACAAGCACTCGCACCGGGAACCCGACGCGGCCCGCCGTGAAACCCCCAACCTCT GAACGTTGACCTC

JKI-BI-2638	GGATCATTACCAGAGTTTTACAACTCCCAACCCTTCTGTGAACCTACCCATAGTTGCTTCGGCGGACCCGCCCCAGCGTCCGGACGGCCCAGCGCCGGCCCGC GACCTGGACCCAGGCGGCCGCCGGGGACCACGCAACCCTGTATCTGTCAGCCTCTCTGAATCCGCCGCAAGGCAACACAAACGAATCAAAACTTTCAACAAC GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCG CCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACGTCCCCCGGGACGTCGGCCTTGGGGACCGGCAGCACCCCGCCGGCCCTG AAATGGAGTGGCGGCCCGTCCGCGGCGACCTCTGCGCAGTACAAGCACTCGCACCGGGAACCCGACGCGGCCCGCCGTGAAACCCCCAACCTCT
JKI-BI-2623	CATTACCAGAGTTTTCACAACTCCCAACCCTCCTGTGAACCTACCCATCGTTGCTTCGGCGGACTCGCCCCAGCGTCCGGACGGCCTCGCGCCGGCCCGCGAC CTGGACCCAGGCGGCCGCCGGAGACCACGCAACCCTGCATCCATCAGTCTCTCTGAATCCGCCGCAAGGCAACACAAACGAATCAAAACTTTCAACAACGG ATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCC CGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACGTCCCCCGGGACGTCGGCCTTGGGGACCGGCAGCACCCCGCCGGCCCTGAA ATGGAGTGGCGGCCCGTCCGCGGCGACCTCTGCGAAGTACTACAGCTCGCACCGGAAACCCGACGCGGCCCCGCCGTGAAACCCCCAACTCTGAACGT
JKI-BI-1496	TCATTACCAGAGTTTTACAACTCCCAACCCTTCTGTGAACCTACCCATAGTTGCTTCGGCGGACCCGCCCCAGCGTCCGGACGGCCCAGCGCCGGCCCGCGAC CTGGACCCAGGCGGCCGCCGGGGACCACGCAACCCTGTATCTGTCAGCCTCTCTGAATCCGCCGCAAGGCAACACAAACGAATCAAAACTTTCAACAACGG ATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCC CGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACGTCCCCCGGGACGTCGGCCTTGGGGACCGGCAGCACCCCGCCGGCCCTGAA ATGGAGTGGCGGCCCGTCCGCGGCGACCTCTGCGCAGTACAAGCACTCGCACCGGGAACCCGACGCGGCCCGCCGTGAAACCCCCAACCTCTGAACGTTGA CCT
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Appendix

JKI-BI-2639	AGCGGAGGGATCATTACCGAGTTATCCAACTCCCAACCCCTGTGAATTATACCTTTAATTGTTGCTTCGGCGGGACTTCGCGCCCGCCGGGGACCCAAACCTT CTGAATTTTTTAATAAGTATCTTCTGAGTGGTTAAAAAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGTCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTAC GCCCCTCAAGTCCCCTGTGGACTTGGTGTTGGGGATCGGCGAGGCTGGTTTTCCAGCGCAGCCGTCCCTCAAATCAATTGGCGGTCTCGCCGTGGCCCTCCTC TGCGCAGTAGTAAAACACTCGCAACAGGAGCCCGGCGCGGTCCACTGCCGTAAAACCCCCCAACTTTTTATAGTTGACCTCGAATC
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JKI-BI-2621

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JKI-BI-2653

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JKI-BI-2622

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JKI-BI-2648

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JKI-BI-2652

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JKI-BI-2651

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Appendix

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Appendix

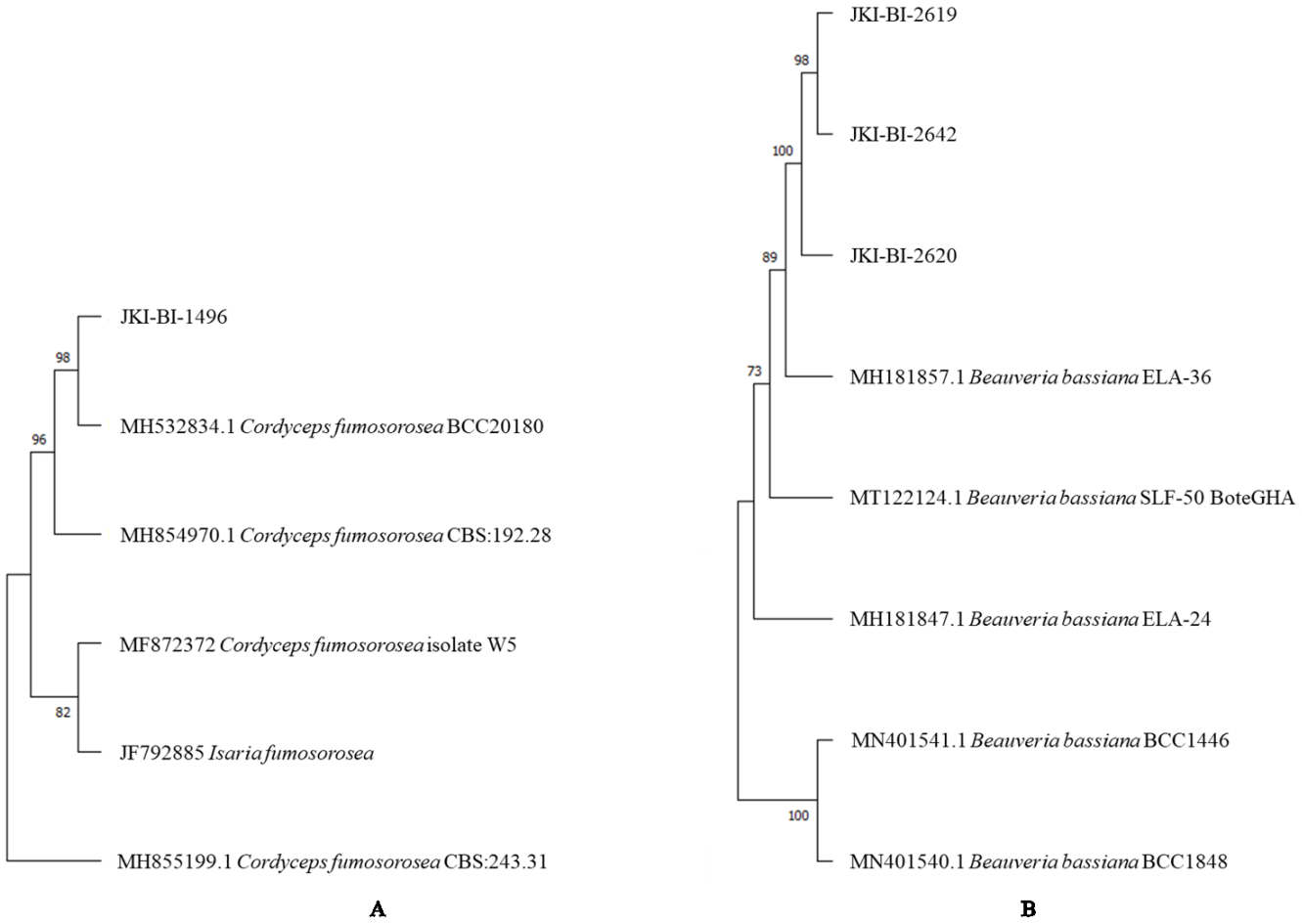

Figure 1. A. Phylogenetic tree of *Cordyceps fumosorosea*, B. Phylogenetic tree of *Beauveria bassiana* (continuing to next page)

Appendix

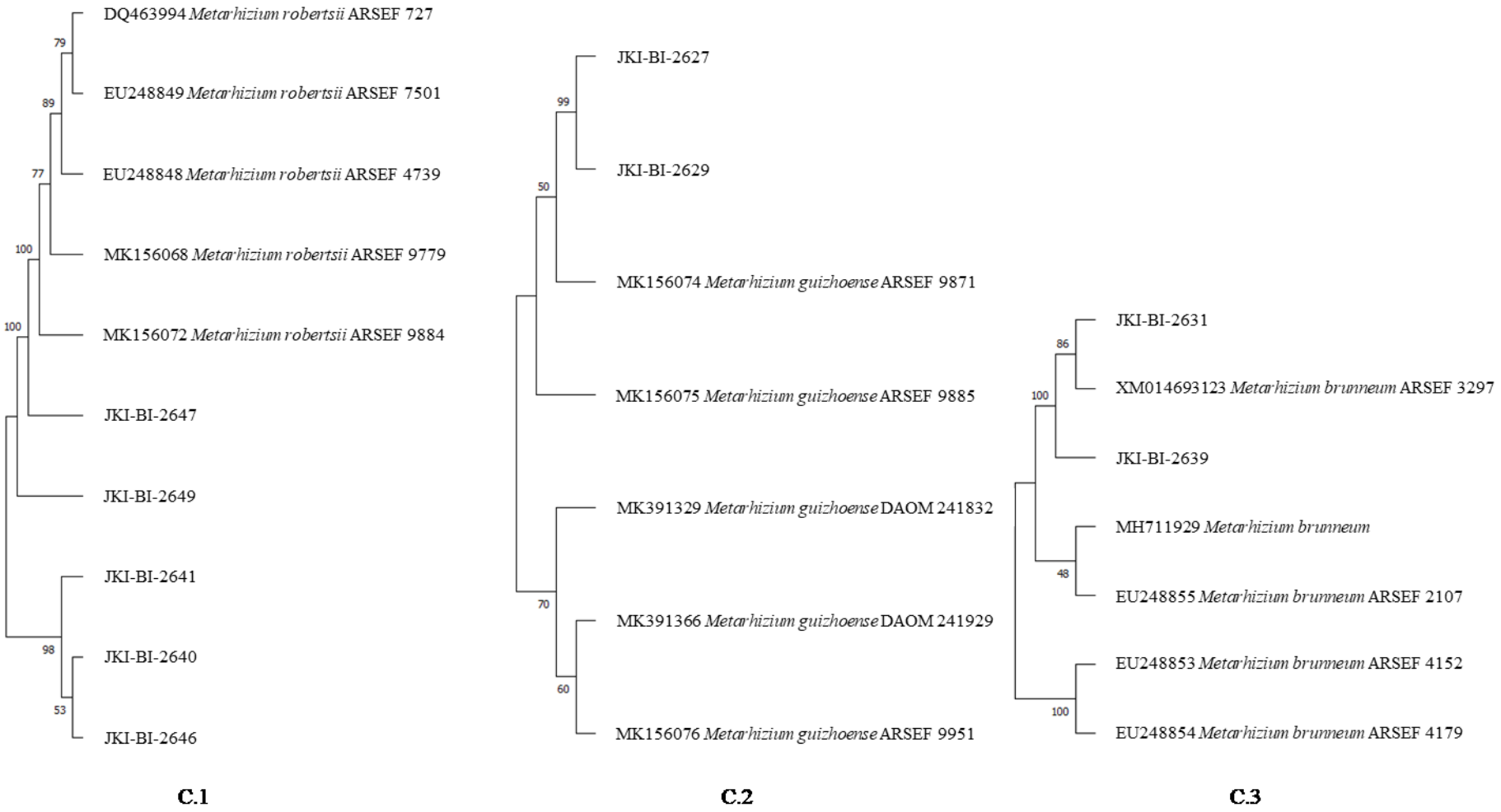

Figure 1. C.1 Phylogenetic tree of *Metarhizium robertsii*, C.2 Phylogenetic tree of *Metarhizium guizhoense*, C.3 Phylogenetic tree of *Metarhizium brunneum*.

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Ferner erkläre ich, dass ich bei der Verfassung der Dissertation die "Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Technischen Universität Darmstadt" und die "Leitlinien zum Umgang mit digitalen Forschungsdaten an der TU Darmstadt" in den jeweils aktuellen Versionen beachtet habe. Die Arbeit wurde bisher bei keiner anderen Hochschule zu Prüfungszwecken eingereicht.

Darmstadt, den 18.07.2024
…….
Ort, Datum

……
Unterschrift

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- Isolation and Characterization of entomopathogenic fungi as biocontrol agent for codling moth: insights into their variation in growing parameters.
- Investigation of the influence of chitin and its derivatives on efficacy of entomopathogenic fungi in terms of virulence against *Cydia pomonella*, environmental stress factors and long term storability through bioassay, germination test, UV tolerance and lypolization.
- Screening a number of water-soluble protectants through their UV absorbance by using a photometer.
- Development of spray dried submerged spores of Cordyceps fumosorosea coated by water-soluble UV protectants.
- Evaluation of the effects of time of exposure to simulated solar radiation on spore germination of *Cordyceps fumosorosea* in formulation with potential UV protectants.

11/2016- 09/2018 **Spacenus GmbH**
Marienburgstraße 27, 64297 Darmstadt, Germany.

Agronomist

- Research on nutrient deficiency, symptoms and field nutrition budgeting, developing crop disease modelling.
- Maintenance of database of diseased crops.

12/2015-08/2016 **Max Planck Institute for Biology of Aging**
Valenzano lab, Evolutionary and experimental biology of aging, Joseph-Stelzmann-Str 9b, Cologne, Germany.

Intern

- Molecular biological method (PCR) in S1 area and maintenance of African turquoise killifish.

- Maintenance of water quality of fish living aquarium.
- Data analysis and management of water quality and lifespan of killifish including insitu hybridization.
- Updating database in the online platform.

Education

01/2020-Onwards Ph.D student at Julius Kühn Institute, Federal Research Centre for Cultivated Plants, Institute for Biological Control, Schwabenheimer Str. 101, D-69221 Dossenheim, Germany.
First supervisor: Prof. Dr. Andreas Jürgens (Technical University Darmstadt, Biology Department)
Second supervisor: Prof. Dr. Johannes A. Jehle (Julius Kühn Institute, Federal Research Centre for Cultivated Plants, Institute for Biological Control)

01/2010 – 08/2011 Master of Science in Biotechnology and Genetic Engineering (Grade point: 1.2)
Biotechnology and Genetic Engineering Department, University of Development Alternatives, Dhaka, Bangladesh.
Master Thesis: Antinociceptive Activity Studies in Mice with Methanol Extracts of Mashroom "Ganoderma lucidum (Ganodermataceae)" (Grade point 1.0).

02/2004- 12/2008 Bachelor of Science in Biotechnology and Genetic Engineering (Grade point 1.5)
Biotechnology and Genetic Engineering Discipline, Life Science School, Khulna University, Khulna, Bangladesh.
Bachelor Thesis: Plant tissue culture of Strawberry (Grade Point 1.0).

Publications

- Antara N-H, Stephan D (2023), Entwicklung von sprühgetrockneten Submerssporen von Cordyceps fumosorosea, beschichtet mit wasserlöslichen UV-Schutzmitteln. 63. Deutsche Pflanzenschutztagung: Pflanzenschutz morgen - Transformation durch Wissenschaft; 26. bis 29. September 2023, - Kurzfassungen der Vorträge und Poster. https://www.openagrar.de/receive/openagrar_mods_00088713
- Antara N-H, Hoibian S, Stephan D (2021), Characterization of entomopathogenic fungi as biocontrol agent for codling moth: insights into their variation in growing parameters. 13th Young Scientists Meeting 2021 213:7. https://www.openagrar.de/receive/openagrar_mods_00074651
- Antara NH, Hoibian S, Stephan D (2021) Chitin amended media: A solution for improved entomopathogenic fungi against codling moth. 2021 International Congress on Invertebrate Pathology and Microbial Control & 53rd Annual Meeting of the Society for Invertebrate Pathology; 28th June - 2nd July 2021, Virtual Meeting 127. https://www.openagrar.de/receive/openagrar_mods_00075598
- Antara N-H, Hoibian S, Stephan D (2021) Optimierung nachhaltiger entomopathogener Pilze durch wirksame Formulierung zur Überwindung von Umweltstress. 62. Deutsche Pflanzenschutztagung: Gesunde Pflanzen in Verantwortung für unsere Welt ; 21. - 23. September 2021, -Kurzfassungen der Vorträge und Poster- 467:380. https://www.openagrar.de/receive/openagrar_mods_00074345
- [1]Fatema Toz Zohra, [1]Nusrat Harun Antara, [1]Shahnaz Rahman, [1]Aynal Haq Rana, [1]Farjana Akther Noor, [1]Himel Nahreen Khaleque, [1]Sherezad Sanam, [1]Farhana Islam, [1]Alok Kumar Paul, [2] Nirod Chandra Sarker, [1] Mohammed Rahmatullah (2011), Antinociceptive Activity Studies in Mice with Methanol Extracts of Two Mushroom Species: Lentinula edodes (Marasmiaceae) and Ganoderma lucidum (Ganodermataceae), Advances in Natural and Applied Sciences, 5(2): 127-130, ISSN 1995-0772. http://www.aensiweb.com/old/anas/2011/127-130.pdf

Conferences

- 63. Deutsche Pflanzenschutztagung – from 26th to 29th September 2023, Georg-August-Universität Göttingen.
- SIP 2022, 1 to 4 August 2022, Nelson Mandela Bay, South Africa.
- 2021 International Congress on Invertebrate Pathology and Microbial Control & 53rd Annual Meeting of the Society for Invertebrate Pathology, June 28, 2021 - July 02, 2021, Mexico and France.
- 23. Jahrestagung des AK Biologischer Pflanzenschutz -online- 18.-19.03.2021.
- 62. Deutsche Pflanzenschutztagung – digital from 21st to 23rd September 2021, Georg-August-Universität Göttingen.
- Young Scientists Meeting 2021, Julius Kühn Institute, Quedlinburg.

Training and Workshop

- 'Data science for experimental life sciences with R (Part 1 and 2)', organized by Federal Ministry of Food and Agriculture (BMLE).
- 'German Science System', Online course (October 04-29, 2021), organized by Plant academy 2030, sponsored by Federal Ministry of Education and Research.
- 'Mentoring Program Kick-off and Sustainable Career Workshop', Online course (August 03-07, 2020), organized by Plant academy 2030, sponsored by Federal Ministry of Education and Research.
- 'International Zebrafish and Medaka Course (IZMC)' by European Zebrafish Resource Center (EZRC), Karlsruhe Institute of Technology (KIT).
- Joint Student Project, Egypt, (www.elgouna2014.wordpress.com/) and Spain.
- Intercultural Communication: Problem-Solving Skills for the International Workplace. Part 1, 2 and 3.

Membership

- Society of Invertebrate Pathology (SIP).
- Deutscher Akademischer Austausch Dienst (DAAD) alumni.

Personal skills

Laboratory skills PCR, Agarose electrophoresis, DNA isolation and purification, Solid and liquid fungi fermentation, Spray drying, Lypolyzation, Microscopy, UV spectroscopy, Light absorption through Flurorescence photo-meter, GMP, GLP.

Language Skills Bengali: Native
English: Excellent in Reading, Writing and Speaking
German: Good in Reading, Writing and Speaking

IT Skills
- Data Management (R studio, Excel, Access and SPSS, Sigmaplot, SAS, SAP)
- Office Packages (MS Word, MS Excel, MS PowerPoint, MS Project, MS Access, Outlook)
- Grafik Design (Adobe Photoshop, Adobe Illustrator)
- Citavi
- ArcGIS,QGIS
- Project Management (Trello,Slack)