Development of attract and kill formulations for biological psyllid pest control

Dissertation

to obtain the doctoral degree “Dr. rer. nat.”

at the Faculty of Agricultural Sciences,

Georg-August-University Göttingen,

Germany

Submitted by

Linda Claire Muskat

born in Rüdesheim am Rhein, Germany

Göttingen, July 2022

1. Name of supervisor: Prof. Dr. Michael Rostás

2. Name of first co-supervisor: Prof. Dr. Anant Patel

3. Name of second co-supervisor: Prof. Dr. Stefan Vidal

Date of oral examination: 28.10.2022

Contents

Summary

Psyllid pests are distributed all over the world causing damage in various crop plants by serving as vector insects for phytoplasmosis diseases. One of these psyllids distributed in Europe is *Cacopsylla picta* (Hemiptera: Psyllidae), which is the only known vector of *Candidatus* Phytoplasma mali, the causing agent of apple proliferation. There are no direct measures to combat phytoplasmosis bacteria, thus, the vector itself needs to be controlled. As chemical insecticides have been increasingly restricted in recent years due to their high risk to non-target organisms, new strategies for psyllid control are needed. Innovative attract-and-kill strategies using semiochemicals and beneficial microorganisms offer insect-specific and environmentally friendly control options.

One of the potential allomones released by apple trees attractive towards *Cacopsylla picta* is β-caryophyllene. Due to the instability against UV and oxidation and their high volatility, protection and release control of semiochemicals by formulation is needed. Entomopathogenic fungi are considered as promising alternative to common synthetic insecticides. In 2016, the new entomophthoralean fungus *Pandora* sp. nov. inedit. (ARSEF 13372) was isolated from a *Cacopsylla* sp. collected in a Danish pear orchard. Species of the Entomophthorales are known for their strict host specificity, their fast speed-to-kill and for their ability to cause epizootics. To enable commercial applicability of *Pandora* sp. nov., the non-economic mass production and inconsistent efficiency after field application needs to be overcome.

The overall aim of this work was the development of innovative formulations that can be applied in attract-and-kill-strategies for the biological control of *Cacopsylla picta* in apple orchards. Special emphasize was laid on the development of a slow release formulation for the attractant β-caryophyllene and the establishment of a fermentation and formulation process suitable for mass production and propagation of the entomopathogenic fungus *Pandora* sp. nov. inedit. (ARSEF 13372) in psyllid pest control.

Chapter 1 provides important background information and the state-of-the-art of the research topics addressed in this thesis. In addition, this chapter contains a review providing an overview of recent formulations developed for insect behavior manipulation by semiochemicals, focusing on formulation aspects and the potential for slow and controlled release. This chapter also covers the state-of-the-art on entomophthoralean fungi as biocontrol agents and on attract-and-kill strategies.

In **Chapter 2**, a novel formulation based on an ethylcellulose-candelilla wax-oleogel was developed for the slow release of β-caryophyllene as attract formulation for *C. picta*. The developed and patented formulation combines unique properties that makes it highly suitable for semiochemical formulation and release, such as a reduced processing temperature, improved oil-binding capacity and mechanical stability, self-adhesive properties on leaves and release modifiability.

Chapter 3 deals with the potential of the attract formulation developed in **Chapter 2** for a controlled release. β-caryophyllene release was found to be affected by melting of candelilla wax as a thermo-responsive oleogelator. Thus, the novel ethylcellulose-candelilla wax-oleogel formulation bears the potential for a temperature-triggered release of β-caryophyllene. With the formulation presented in **Chapter 2 and 3**, a novel matrix-type semiochemical formulation based on nontoxic, biobased and

biodegradable materials is provided that will contribute to the establishment of oleogels as delivery systems for semiochemicals in general.

In **Chapter 4,** the biomass production of the new entomophthoralean psyllid-pathogenic fungus *Pandora* sp. nov. inedit (ARSEF 13372) in a liquid culture was explored. *Pandora* sp. nov. was found to grow best in media containing skimmed milk, but even faster in a mixture of skimmed milk, yeast extract and a low-cost protein hydrolysate from animal by-products. Increased media osmolality through the addition of sodium chloride promoted growth as finely dispersed mycelium, which is suitable for subsequent encapsulation in hydrogel beads. After the medium had been transferred to a stirred tank bioreactor with a working volume of 8 L, a maximum biomass dry weight of 21 $g \cdot L^{-1}$ was reached after 48 hours. These promising results pave the way for large-scale fermentation processes of the new *Pandora* species.

In **Chapter 5,** *Pandora* sp. nov. inedit. (ARSEF 13372) was converted into an easily applicable form by encapsulation in Ca-alginate beads. *Pandora* sp. nov. grew from the beads and discharged conidia over 12 days. Supplementing the beads with skimmed milk as nutrient source increased conidial numbers by 2.95-fold. Beads containing skimmed milk as nutritional additive and 10% *Pandora* sp. nov. biomass led to the highest mortalities of 48.3% and 75.0% in the two target psyllid species *Cacopsylla picta* and *Cacopsylla pyri*, respectively. In a second bioassay, the survival time of *Cacopsylla pyri* was significantly reduced when exposed to beads containing 20% *Pandora* sp. nov with a median survival time of 5–6 days past inoculation and a cumulative mortality of 89%. The promising results of this study will ease the way for large-scale field application of the new *Pandora* species in biological psyllid pest control.

Chapter 6 addresses the high humidity requirements necessary for successful sporulation of entomophthoralean fungi. *Pandora* sp. nov. was found to sporulate only when the water activity was higher than 0.99. Co-application with a paste-type formulation based on biopolymers with high water sorption capacities developed and patented in this work allowed encapsulated *Pandora* sp. nov. to sporulate under very dry humidity conditions of 30 – 40% RH in the laboratory for at least 6 days and even under dry conditions in a semi-field trial during summertime.

In **Chapter 7**, a computer-assisted image analysis method was developed for the rapid, simple and objective quantification of actively discharged conidia of *Pandora* sp. nov. The key to faster quantification is the calculation of the linear relationship between the gray value and the automatically counted number of conidia, which needs to be performed only once in the beginning of the analysis. Afterwards, the gray value is used as single parameter for quantification. The method can be performed with conventional laboratory equipment by using bright-field microscopes, standard scanners and the open-source software ImageJ and, thus, can serve as a manual for other researchers for quantification of fungal spores.

In **Chapter 8** the findings of this work are summarized and discussed, the impact is pointed out and prospects for future research and developments are given.

Chapter 1

Introduction

General Introduction

One of the main challenges of our century is to provide food for a steadily growing global human population from crops grown on steadily shrinking arable land (Lambin & Meyfroidt, 2011; FAO, 2019). Already in the year 2030 one expects 8.5 billion people and 9.7 billions in 2050 (United Nations 2019). Climate change and increasing temperatures favour insect pest proliferation due to favourable climatic conditions and new invasive species are exploring new areas and crop plants (Schneider et al., 2022). More than 67,000 different crop pest species are responsible for an annual yield reduction of 40% (Oerke et al., 1994). The damage by insect pests is usually caused by herbivory in the form of feeding on the plant, plant sap, fruits or roots. Herbivorous insects can further serve as vectors of microbiological plant pathogens (Eigenbrode et al., 2018).

One of the most disastrous microbial plant pathogen responsible for economic damage in various crop plants all over the world are phytoplasmas (Bertaccini, 2007; Lee et al., 2000). Phytoplasmas are commonly vectored by phloem-feeding insects, such as psyllids (Weintraub & Beanland, 2006; Hogenhout et al., 2008). One of the phytoplasma diseases is apple proliferation, one of the most important diseases in apple trees (Seemüller & Schneider, 2004). The vector insect of the causing agent of apple proliferation, *Candidatus* Phytoplasma mali, is the summer apple psyllid *Cacopsylla picta* (Jarausch et al., 2007; 2019). As there is no direct measure to combat phytoplasmas, the vector itself needs to be controlled.

In recent decades, chemical insecticides have been used in huge amounts to reduce crop losses due to insect pests, also for the control of psyllids. The massive use of chemical insecticides has introduced serious problems: resistance in many target insects, leading to reduced efficacy of the insecticide, secondary pest outbreaks and high pesticide residues in food (Thakore et al., 2006; Glare et al., 2012; Chandler et al., 2011; Blackburn et al., 2016). Additionally, chemical pesticides have fallen into infamy because of their toxic effects on non-target organisms and humans. Therefore, a fundamental paradigm change is currently emerging. There is an increasing demand for new, more sustainable and environmental-friendly pest control options. Even the European Parliament recommends “when pesticides are used, appropriate risk management measures should be established and low risk pesticides as well as biological control measures should be considered in the first place” (European Parliament 2009). Biological control measures also include so-called ´biopesticides´, living microorganisms or natural products to suppress pest populations (Chandler et al., 2011). Living microorganisms, often termed ´biological control agent´ or shorter ´biocontrol agents´ (Eilenberg et al., 2001) can include viruses, fungi, bacteria, protozoa and nematodes (Lacey et al., 2015). Also insect predators or parasitoids can be used as biocontrol agents (Waage & Hassel, 1982). Natural products are commonly extracts or metabolites of plants or microorganisms and further include semiochemicals (Chandler et al., 2011; Villaverde et al., 2016). The advantages of biopesticides over synthetic chemical pesticides are their higher target specificity, less toxicity to non-target organisms, less likelihood of insect

resistances and that they commonly originate from renewable resources (Seiber et al., 2006; Thakore et al., 2014; Villaverde et al., 2016). The global biopesticide market is expected to exceed USD 10 billion by 2025 with 15-18% compounded annual growth rate. Biopesticides will reach 10% share of the global crop protection market in 2025 (DunhamTrimmer®, 2022), which is still very low in relation to the total global pesticide market. This is attributed to non-economic mass-production methods of biopesticides, limited stability and inconsistent efficacy under field conditions (Glare et al., 2012).

New insights in the scent preferences and chemical ecology of psyllids and other pest insects offer new control options (Mayer et al., 2008a; 2008b). Innovative attract-and-kill or push-pull-kill strategies aim to improve the efficacy of biopesticides by combination of different active agents (Cook et al., 2006; Gregg et al., 2018). To improve the killing effect of a bioinsecticide, such as an entomopathogenic microorganism or insecticidal plant extract, it is combined with an attractant, in order to attract the target insect to the kill compound and thus, increase the contact possibility (Gregg et al., 2018). The attract-and-kill concept can be combined with repellent stimuli that push the target insect away from the crop plant in a push-pull-kill strategy (Cook et al., 2006).

Different dispenser types for the release of semiochemicals, like pheromones, as ´attract´ compounds have been developed during the last decades, which have already successfully been applied in mating disruption strategies (Smart et al., 2014). For the formulation of microbial biopesticides, that can be applied as the ´kill´ compound, various bioencapsulation materials and techniques have been developed and tested (Burges, 1998; de Faria & Wraight, 2007; Vemmer & Patel, 2013). A suitable formulation transfers the active ingredients into an easily applicable form, stabilize them and improve their performance and efficacy after field application (Burges, 1998). The choice of a suitable formulation material is the key to control the release and let the microbial biocontrol agent efficiently perform after field application.

Thus, there is a need to develop and improve mass production methods for microbial biocontrol agents and semiochemical formulations in order to realize attract-and-kill strategies for integrated and biological psyllid pest control.

Aims

The overall aim of this work was the development of formulations that can be applied in attract-and-kill-strategies for the biological control of *Cacopsylla picta* in apple orchards (see **Figure 1.1**). Special emphasize was laid on the development of a slow release formulation for the attractant β-caryophyllene and the establishment of a fermentation and formulation process suitable for mass production and propagation of the entomopathogenic fungus *Pandora* sp. nov. inedit. (ARSEF 13372) in psyllid pest control.

The research presented in this work was split into seven chapters (**Chapter 2 – 7**), dealing with different topics and aims. **Chapter 2 and 3** deal with the development of the attract formulation releasing β-caryophyllene. **Chapter 4 – 7** deal with the fermentation and formulation of the kill compound *Pandora* sp. nov. inedit. (ARSEF 13372).

Chapter 2 aimed to develop an oleogel-based formulation for the slow release of β-caryophyllene as attractant for *C. picta.* The specific objectives were (1) to lower the temperature at which the volatile compound is added to the oleogel to improve entrapment efficiency, (2) to slow the release of β-caryophyllene from the oleogel, (3) to improve the compatibility of the formulation for plant leaves by reduced oil leakage and improved mechanical stability, (4) to evaluate the self-adhesive properties of the formulation on plant leaves and (5) to validate the insect behavior manipulating effect of the formulation.

Chapter 3 aimed to proof whether the formulation developed in **Chapter 2** bears the potential for a controlled release of β-caryophyllene. The starting assumption was that melting and crystallization of candelilla wax as a thermo-responsive oleogelator will enable a temperature-triggered release.

Chapter 4 aimed to identify an optimal fermentation medium suitable for mass production of *Pandora* sp. nov. The specific objectives were to (1) screen for a suitable complex nitrogen source for fast growth and high biomass production, (2) evaluate growth kinetics in the resulting media, (3) reduce pellet formation in order to promote growth as finely dispersed mycelia and (4) prove the scalability of the fermentation in a stirred tank bioreactor.

Chapter 5 aimed for the transfer of *Pandora* sp. nov. inedit. (ARSEF 13372) into easily applicable calcium alginate beads. The specific objectives of this study were (1) to investigate the maintenance of sporulation duration and intensity of *Pandora* sp. nov. after encapsulation in calcium alginate beads, (2) to improve and prolong the sporulation by addition of nutritional formulation additives and (3) to evaluate the pathogenicity and virulence of the *Pandora*. sp. nov. formulations against the target species, pear psyllid *C. pyri* and summer apple psyllid *C. picta* under laboratory conditions.

Chapter 6 aimed to development and evaluate a formulation that compensate for the specific humidity requirements of *Pandora* sp. nov. (ARSEF13372) and supports sporulation under insufficient humidity conditions. The specific objectives of this study were (1) to identify the water activity values necessary for growth and sporulation of *Pandora* sp. nov., (2) to prove a formulation that maintains a sufficiently high water activity under non-saturated humidity conditions to allow sporulation of *Pandora* sp. nov., (3) to validate the potential of the formulation for improved sporulation under semi-field conditions and (4) evaluate the distance of conidial distribution in a semi-field trial.

Chapter 7 aimed to develop a fast, simple, reproducible and objective computer-assisted image analysis method for the quantification of actively discharged conidia of encapsulated *Pandora* sp. nov. with potential for analysis of large-scale sample quantities.

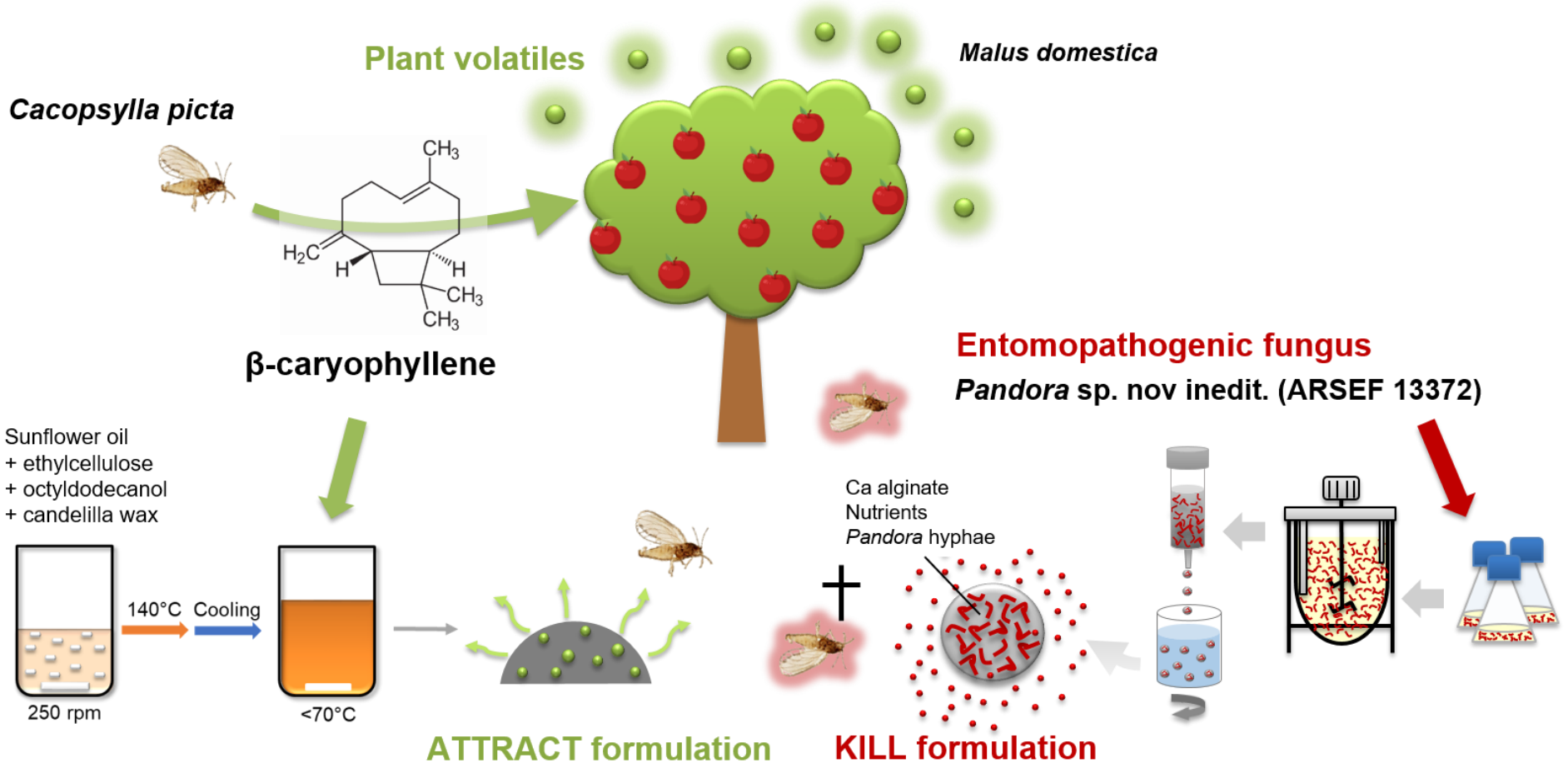


Figure 1.1. Graphical abstract of this PhD thesis with the title „Development of attract and kill formulations for biological psyllid pest control".

State-of-the-art

Crop plant apple

Apple *Malus domestica* (Rosales:Rosaceae) is the most important pome fruit of temperate regions worldwide. Up to 87.2 million t are harvested annually, 20 million t of them in Europe and 1 million t in Germany. Sales in Germany amounted to more than € 200 million in 2018 (Behr 2019). The cultivation area for apples in Germany is around 33,600 hectares, which is around 70% of the tree fruit growing area. Organically produced apples were cultivated on an area of 6,092 hectares in 2017, which corresponds to a proportion of 18 percent (Bundesinformationszentrum Landwirtschaft BZL).

Apple proliferation and *Candidatus* Phytoplasma mali

One of the most important diseases in apple trees is apple proliferation, causing annual yield losses in European apple production. Typical symptoms of apple proliferation (**Figure 1.2**) are witches´ brooms and poor tasting fruits with small size and weight which are untradeable (Bovey 1961, Seemüller et al., 2011, Hadidi 2011, Bertaccini 2014). An outbreak of apple proliferation in 2001 caused € 25 million yield losses in Germany and € 100 million in Italy (Strauss 2009). Besides the morphological effects on the plant, phytoplasma infections have several effects on physiology of an infected plant, such as altered phloem composition (Görg et al., 2021a) and volatile emission (Mayer et al., 2008a; b; Gallinger et al., 2021). The causing agent of apple proliferation is the phytoplasma *Candidatus* Phytoplasma mali.

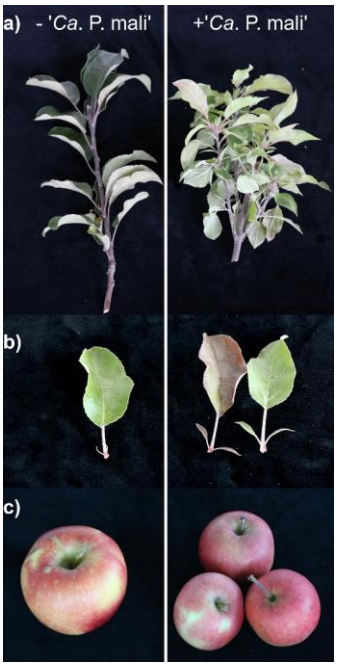


Figure 1.2. Symptoms of apple proliferation disease (+ 'Ca. P. mali', right) in comparison to non-infected apple *M. domestica* cv. Mairac (- '*Ca*. P. mali', left): a) witches' broom, b) enlarged stipules and c) smaller fruit. (Picture: Felix Briem & Kerstin Zikeli)

Phytoplasmas

Phytoplasmas are obligate phytopathogenic bacteria which are characterized by lacking of cell wall, a small size (200-800 nm), reduced genome size and missing of some key metabolic pathways (IRPCM 2004; Bai et al., 2006; Lee et al., 2000). Phytoplasmas are obligat parasitic and can only survive in the host plant´s phloem or in their insect vector´s cells and fluids (Weintraub & Beanland, 2006; Bai, 2006; Bertaccini et al., 2007; IRPCM 2004; Huang et al., 2020). Phytoplasmas cannot be cultivated in-vitro to date (Bendix & Lewis, 2018; Lee, 2000; Huang et al., 2020). Commonly, phytoplasmas are transmitted by phloem-feeding insect vectors and are responsible for plant diseases in hundreds of plant species, including many economically important crops, fruit trees and ornamental plants (Hogenhout et al. 2008; Oshima et al. 2013). Besides leafhoppers and planthoppers, psyllids are the most important vector insects for phytoplasmas (Huang et al., 2020).

Psyllids

Psyllids or jumping plant lice belong to the order Hemiptera. They are classified in 8 families and more than 3500 species are described which are distributed nearly all over the world (Hodkinson 2009;

Burckhardt and Ouvrard, 2012), from which 400 occur in Europe (Jarausch et al., 2019). *Cacopsylla* species belonging to the Psyllidae family are restricted to Europe and infest Rosaceae species. Nymph and adult *Cacopsylla* are phloem-feeding phytophagous insects with piercing-sucking mouthparts. They are often mono- or oligophagous. They include important crop pests in plants cultivated for fruit production, e.g. *Cacopsylla picta*, *C. pyri*, *C. pyricola*, *C. pruni* causing economic damage in apple, pear and stone fruit cultivation (Seemüller & Schneider, 2004). Direct damage is caused by feeding from the sugar rich phloem sap and by excretion of honeydew, which favours mold growth. The most important damage is caused by the transmission of the phytoplasmas (Weintraub & Beanland, 2006; Hogenhout et al., 2008; Jarausch et al., 2019).

Cacopsylla picta

One of these phytoplasma-vectoring psyllids is the summer apple psyllid *Cacopsylla picta* (Hemiptera:Psyllidae; **Figure 1.3**), the vector of *Candidatus* Phytoplasma mali (Jarausch et al., 2007; 2019). *C. picta* belongs to the *Cacopsylla* species and has an egg stage and five unwinged instars. It is univoltine and migrates between divergent plant species during its lifecycle: *C. picta* reproduces on *Malus* spp. (Rosaceae). Newly emerged adults leave after some days or weeks to conifers in mountainous regions for overwintering, they are therefore called ´emigrants´. In early spring, the same individuals remigrate to *Malus* spp. for mating and oviposition, then they are called ´remigrants´ (Mayer et al., 2011). *C. picta* is currently the only known vector of *Candidatus* Phytoplasma mali, the causal agent of apple proliferation in Germany (Frisinghelli et al., 2000; Jarausch et al, 2019; Mayer et al., 2009). The mean naturally occurring phytoplasma infection rate of *C. picta* populations in Southern Germany is around 10% (Jarausch et al., 2019).

Figure 1.3. *Cacopsylla picta* female. Picture: Mayer and Gross, JKI Dossenheim.

Crop protection

Phytoplasma infected plants cannot be cured. Preventive and phytosanitary measures are limited to planting of phytoplasma-free plants and removal of infected plants from the orchard (Weintraub & Wilson, 2010; Barthel et al., 2020). Another option is the control of the vector insects. Management of psyllids, including *C. picta*, is reliant on insecticides to limit initial infection and reinfection of trees (Grafton-Cardwell, 2013; Jarausch et al., 2019). Synthetic and biological insecticides based on pyrethrum, neonicotinoids, azadirachtin and diazomet have been proven for psyllid control in Germany (BVL, 2021), but they are harmful for non-target insects, including beneficial insects. Moreover, as part of the current trend towards a more sustainable agriculture (European Parliament, 2019), the demand for alternative environmentally friendly pest management options is increasing.

Attract-and-Kill and Push-Pull-Kill strategies as alternative control options

Novel insect pest control concepts exploit new insights into the chemical ecology of insect pests, their host plants and their natural enemies (Pickett et al., 1997; Khan et al, 2008). Innovative approaches such as attract-and-kill strategies, also called lure-and-kill or attracticide (Gregg et al., 2017) or push-pull-kill strategies, also called stimulo-deterrent strategies (Cook et al., 2006), combine behavior

modifying stimuli in order to manipulate the distribution and abundance of pest insects with compounds that kill the pest insect apart from the crop plant (**Figure 1.4**). Attract-and-kill strategies typically combine a semiochemical attractant with a toxicant within a slow-release formulation or device that enables the reduction of active ingredients applied (Gregg et al. 2018).

Semiochemicals have the most versatility and potential for use in attract-and-kill or push-pull-kill strategies as ´attract´ (=pull) or `push` (=repel) compound to manipulate the insect's behavior (Agelopoulos et al., 1999). Semiochemicals offer the promise of selective insect manipulation and pheromones have long been applied successfully in mating disruption (Smart et al., 2014). Also other stimuli, such as visual, acoustic or gustatory stimuli, can be elements of such strategies. Consequently, there is a wide range of possible combinations (Eigenbrode et al., 2016). The kill compound can be a synthetical insecticide, but a natural and more selective kill compound is preferable. This can be plant extracts, so called ´biologicals´, insect growth regulators or living biocontrol agents, such as entomopathogenic fungi, applied at the place to where the pest insects are attracted. When the pest insect is attracted to a trap crop or semiochemical release system, which simultaneously serves as a habitat or attractive cue for parasitoids or predators (Landis et al., 2000), the strategy can be combined with conservational pest control management (Cook et al., 2007). Also mass trapping of the attracted pest insect is an option (El-Sayed et al., 2006).

When the attract-and-kill tactic is combined with a ´repel´ (=push) compound, it becomes a push-pull-kill strategy. This can be implemented by planting of repellent plants in the field, to push the insect out to alternative host-plants outside (pull). The aim of push-pull strategies is to divert pest insect populations from the crop plant to another less valued trap plant, by making the crop plant difficult to locate, unattractive or unsuitable for survival of the offspring. Push-pull concepts have long been proven by traditional small farms in Afrika and realized by usage of locally available plants as intercrops with a pest repellent plant, which drives away or deters pest insects from the target food crop in combination with attractant trap plants (ICIPE, 2022).

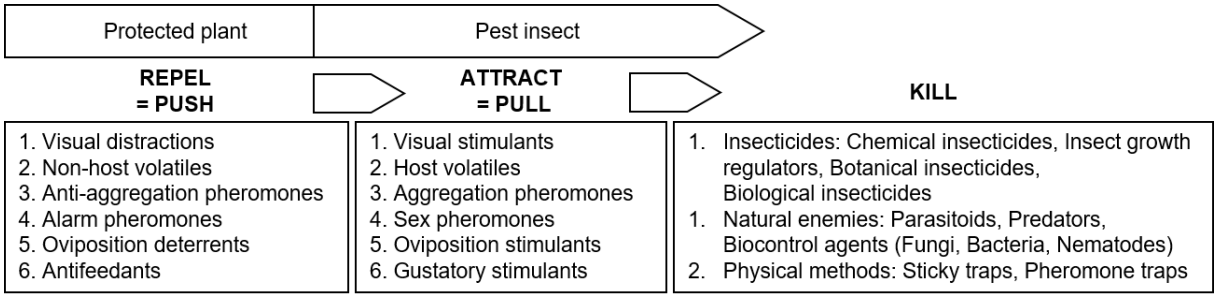


Figure 1.4. Elements of attract-and-kill and push-pull-kill strategies. Modified from Zhang et al., 2013.

Advantages of attract-and-kill or push-pull-kill strategies are the reduction of risk for contact between the kill compound and the crop plant, beneficials and non-target organisms (Griggs et al., 2017), thereby, a higher specificity for the target pest insect and targets both sexes, when the right kairomones are selected as attract compound. A challenge is the behavior manipulation in a complex environment (El-Sayed et al., 2009) and finding the right mix and dosage of the attractants to be perceptible to the target insect and triggering the desired behavior (Bruce & Pickett, 2011).

Current research in the field of psyllid pest control mainly focusses on citrus psyllid *Diaphorina citri*, vectoring *Candidatus* Liberibacter citri, the causing agent of Huanglongbing disease (Citrus greening),

another economically important psyllid with a similar pathosystem like *C. picta.* Recently, Martini et al. (2020) tested the attract-and-kill approach in a citrus orchard in Florida for population reduction in *D. citri*. A semiochemical blend was used as an attractant; yellow sticky traps or the insecticide spinosad achieved killing and successfully reduced population. Chow et al. (2018; 2019) used visual cues as attractant with either β-cyfluthrin or the entomopathogenic fungus *Isaria fumosorosea* as killing compound. No reduction of infestation was achieved with the fungus as killing agent, but was with the insecticide. George et al. (2020) presented a multimodal attract-and-kill system, using a combination of different attractive cues, namely color, semiochemical attractant, phagostimulant and ultraviolet (UV) reflectant in combination with the insecticide β-cyfluthrin and found a higher mortality in *D. citri* compared to yellow sticky traps. These studies reinforce the assumption that attract-and-kill is a promising tool for integrated or biological pest management of psyllids.

In order to make the active agents work in the field, specific formulations individually developed for each compound are needed, which should also take into account the current trend towards biobased and biodegradable materials.

Specific environmentally friendly formulations to be applied in an attract-and-kill strategy for *C. picta* and other psyllids distributed in European fruit orchards have not yet been developed.

ATTRACT

In the next paragraph, recent progress on the development of slow and controlled release semiochemical formulations is reviewed.

Innovations in semiochemical formulations

Linda C. Muskat and Anant V. Patel

Bielefeld University of Applied Sciences, Faculty of Engineering and Mathematics, WG Fermentation and Formulation of Biologicals and Chemicals

Published in: Entomologia Generalis (ISSN 0171-8177), 2021, Vol. 42(2), 231–249

DOI: 10.1127/entomologia/2021/1230

Abstract

The potential of semiochemicals for the targeted behavior manipulation of insects has been known for a long time. Their low impact on non-target organisms makes them interesting candidates for use in insect control for various applications such as agricultural and forestry pest control, stored-product protection and protection against hematophagous insects. Due to their high volatility and chemical instability against UV light and oxidation, their use often remains limited. Tailor-made formulations can protect semiochemicals from environmental factors and can improve release performance and duration triggering a desired reaction in the target insect at the place of application. This review provides an overview of recent formulation types developed for insect behavior manipulation by semiochemicals, with emphasize on formulation aspects and formulation potential for slow and controlled release. The focus was set on inventions and studies aiming to elucidate material and formulation properties that affect the semiochemical release and, with specific attention, enable targeted release manipulation.

Keywords: Encapsulation; Slow release; Controlled release; Triggered release; Pheromone dispenser; Semiochemical release systems; Microencapsulation; Nanoencapsulation; Push-Pull strategy; Attract-and-Kill; Pheromones; Allomones; Agricultural pest control; Forestry pest control; Mosquitoe repellent formulation; Stored-product pest insects; Essential oil formulation.

Introduction

Semiochemicals play a key role in the interaction of insects with their environment. Considering their purpose, semiochemicals can be divided into two main subgroups: pheromones and allelochemicals. Pheromones act intraspecifically between individuals of one species as sex pheromones, aggregation pheromones, alarm pheromones, stimulo-deterrent pheromones, trail pheromones or host marking pheromones. Allelochemicals are responsible for interspecific chemical communication with other species and can be classified into allomones, beneficial for the emitter, kairomones, beneficial for the receptor and synomones, beneficial for emitter and receptor (Heuskin et al., 2011; Suckling et al., 2000). During the past years, several of these substances have been identified and their potential for insect behavior manipulation has been evaluated.

The major applications for semiochemicals as insect behavior manipulators are insect pest control in agriculture and forestry, stored-product protection and the protection of humans and animals from hematophagous insects (**Figure 1.5**).

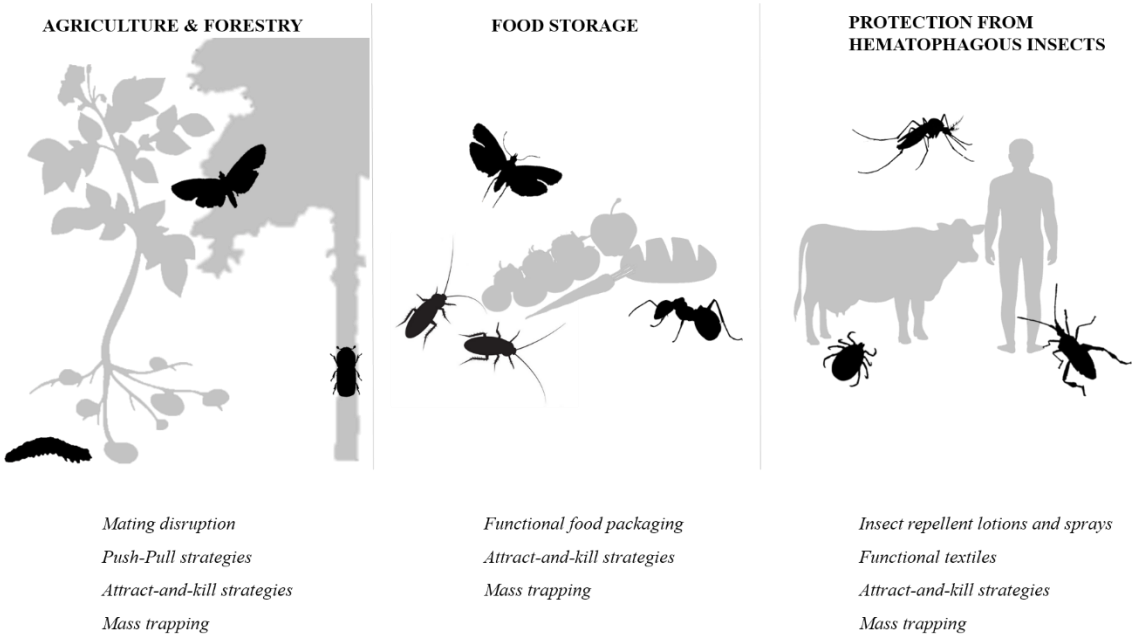


Figure 1.5. Applications of semiochemicals for insect behavior manipulation

For agriculture and forestry, insects are of great relevance in various ways. On the one hand, there are the beneficial insects, which include important pollinator insects, but also predators or parasitoids of herbivores. On the other hand, insect pests are responsible for economic and ecological damage in cultivated crops and trees. The damage is usually caused by feeding on the plant, fruits or roots or through herbivory by bark-, wood- and phloem-borers, foliage-feeders and sap feeders (Liebhold et al., 2013). Herbivorous insects can further serve as vectors of microbiological plant pathogens (Paine et al., 1997). Post-harvest pest insects cause serious qualitative and quantitative losses in raw and processed products, directly by feeding or indirectly by contamination of stored food with microorganisms, and for some people the insect-derived proteins in food can cause allergic reactions (Arlian, 2002; Marsin et al., 2020). Hematophagous insects serve as vectors for dangerous diseases (Lehane & Lehane, 2005). In areas with high pressure from these vector-borne diseases transmitted by insects, protection measures and vector control strategies are indispensable.

The application of semiochemicals offer environmental friendly control options and have come into focus in recent years as the use of synthetic insecticides becomes increasingly restricted due to the negative effects on non-target organisms (Cardé & Minks, 1997; Howse et al., 1997; Hummel & Miller, 1984). The potential of species-specific pheromones is well known and has been established for decades in mating disruption strategies, monitoring and mass trapping (Cardé & Elkinton, 1984; Lakatos & Tuba, 2011; Smart et al., 2014). Based on the increasing knowledge of pheromone chemistry, so called parapheromones, that mimic the effect of naturally occurring pheromones, were developed. These synthesized pheromone analogues have the potential to overcome some of the drawbacks of pheromone use in practice, such as instability and high costs (Renou & Guerrero, 2000; Sellanes & González, 2014; Ujváry et al., 1993). Other alternative strategies for the control of insect pests that rely on the insect behavior manipulating effect of semiochemicals are attract-, repell-, push-pull or attract-and-kill strategies, sometimes called “lure-and-kill” or simply as “attracticide” (Trematerra, 2012). As part of these strategies, attractive and repellent stimuli are applied individually or in combination, e.g. to

attract natural enemies of the insect pest or to repel the insect pest and lure it to an alternative target to be killed by insecticides (Gregg et al., 2018; Kovanci et al., 2011; Xu et al., 2018).

In many of these cases, the use of semiochemicals for insect control purposes is limited. Due to the high volatility and chemical instability of semiochemicals against UV light and oxidation (Heuskin et al., 2011; Lopez et al., 2012) and further for their large-scale application, there is always a need for formulation improvement. An optimal semiochemical formulation releases the active ingredient at an effective rate for a prolonged period (Stelinski et al., 2005). Moreover, by formulation the toxicity for humans, animals and plants can be reduced. Furthermore, formulations mitigate excessive loss of the active compound during storage and high levels of the compound in the environment or at the place of application by reduction of evaporation. The choice of a suitable formulation material is the key to modulate the release rate in a desired way. Another essential factor for the development of a tailor-made formulation is the knowledge about the physico-chemical properties of the active agent (**Table 1.1**) and the conditions at the place of application and release.

Table 1.1. Properties of semiochemicals and profile of requirements for semiochemical formulations

Properties of semiochemicals	Profile of requirements for semiochemical formulations
Highly volatile Reactive in presence of oxygen or UV light Hydrophobic/oily	Slow release Controlled release High encapsulation efficiency and loading Low cost/economic viability Easy, non-toxic and scalable preparation procedure Storability and long shelf life Easy-to-apply Low toxicity during application Biodegradability but sufficient persistence during application duration Compatibility with matrix/lotion/membrane/packaging material

In the case of insect semiochemicals, a prerequisite for success is to know about the concentration perceptible by the insect to achieve the desired behavior manipulating effect. Pheromones are effective in very low concentrations due to the presence of specialized receptors to provoke a desired effect on the insect (Deisig et al., 2014; Fleischer & Krieger, 2018; Renou, 2014). On the contrary, allelochemicals are often needed in much higher concentrations to be perceptible to the target insect and their effect can change depending on the concentration. Many publications in this field focus on the potential of plant-derived essential oils as allelochemical sources (Marsin et al., 2020; Müller & Buchbauer, 2011). Moreover, in the case of allelochemicals, mixtures of more than one component proofed as more effective than single components (Bruce & Pickett, 2011; Tóth et al., 2009). Hence, complex essential oils or plant extracts have come into focus during the last years.

When developing formulations, the most important aspect is the release behavior of a semiochemical formulation, including release rate and release duration. The release kinetics can be expressed as zero

or first order kinetics. The preferred release profile follows zero-order kinetics, characterized by a constant release rate independent from the semiochemical concentration. More common, however, is a first-order release rate, which, depending on the concentration, has initially high release rates that decrease with decreasing concentrations (saturation curve) (Akelah, 2013), resulting in a high residual load of the semiochemicals in the release system, well known from pheromone dispensers. Other important formulation aspects are listed in **Table 1.1**.

In spite of the rapidly growing knowledge on semiochemicals and their role in the environment (Ando, 2004; Fleischer & Krieger, 2018; Leal, 2013; Schulz, 2005), an overview on advances in formulation materials and methods with potential for slow or controlled volatile semiochemical release has been missing in the literature so far. In this review, recent formulation technologies in the field of semiochemical release are described with emphasize on the formulation materials, methods and properties affecting the semiochemical release.

Formulation methods

In the following section, innovative semiochemical formulation materials and methods will be presented. They were classified by their design into passive dispensers, active aerosol dispensers, bio-polymer based microcapsules (consisting of a core-shell structure), matrix types (monolithic and monophasic structures) including biopolymer beads, organic nanoparticles (zein nanoparticles and solid-lipid nanoparticles), nanogels, emulsions (SPLAT® and nanoemulsions), nanofibers, cyclodextrin inclusion complexes, porous materials (silicates, metal-organic frameworks and silica gels) and chemical entrappment (photoremovable protection groups). The formulations are illustrated in **Figure 1.6**.

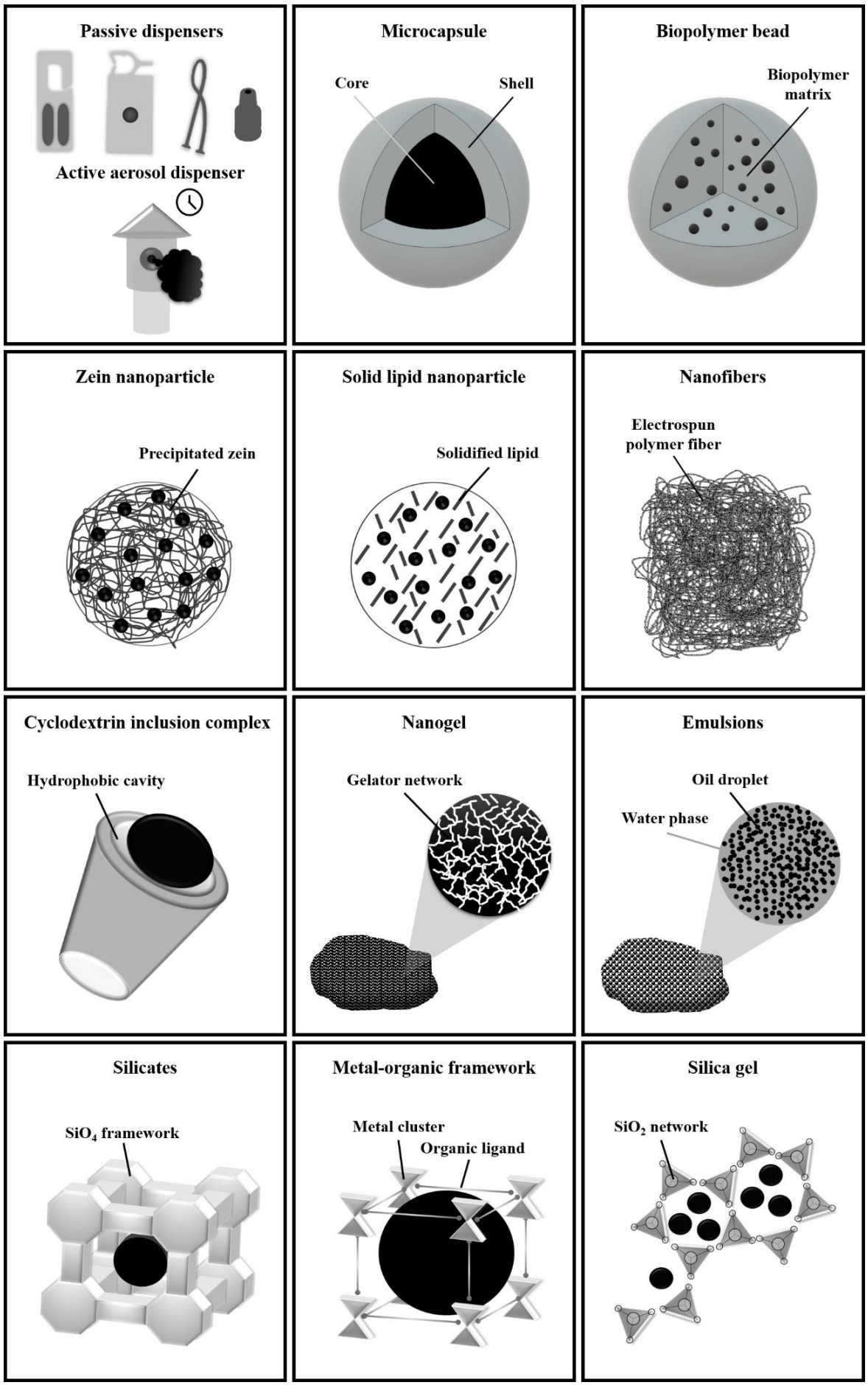


Figure 1.6. Semiochemical formulations. Black = semiochemical.

Dispensers

Passive dispensers

Different passive dispenser types for the propagation of semiochemicals have been developed in the past decades for insect pest control in agriculture and forestry, food storage and other applications like textile protection. The most classic dispenser types are made from natural or synthetic polymers, impregnated or filled with the semiochemical (Ando, 2004; Butler & McDonough, 1981).

Due to their simple preparation and suitability for several compounds, different depot dispensers or rubber septa are commonly used for insect behavioral studies and also for mating disruption in agriculture and forestry. These dispenser types became popular in practice because of their preferably long-term (up to 3-4 month) release behavior (Heuskin et al., 2011). Many of them were innovated by industry and are available in the world markets.

Release is mainly affected by the permeability of the dispenser wall material and can further be modified the thickness, the combination of different material-layer or additional coatings or membranes.

A popular example is a commercialized depot dispenser made of a thermoformed thermoplastic polymer blend made of polylactid and polyester filled with (Z)-9-dodecen-1-yl acetate and (E,Z)-7,9-dodecadienyl acetate (Auffermann & Heck, 2015) with the trade name RAK® 1+2 annually applied in European vineyard for simultaneous mating disruption of *Lobesia botrana* and *Eupoecilia ambiguella* (BASF).

The PE bag dispensers marketed by Suterra® as CheckMate® work on a similar principle and are available for mating disruption in various agricultural insect pests. They are filled with the sex pheromone and release it continuously throughout the season from a huge depot (https://www.suterra.com/products_pest).

One of the best-selling mating disruption dispensers is a plastic tube developed and patented by Shin-Etsu Chemical Co., Ltd. and traded with the name Isomate®. This dispenser is made of an inner barrier wall made of a polyethylene or a copolymeric resin of vinyl acetate and polyethylene, formed as a long tubular body, which is filled with the pheromone and sealed at both ends. The tube is partly coated by a polymeric material (e.g. polyvinyl chloride (PVC), polyvinylidene fluoride (PVDF), polyacrylonitrile (PAN), polysulfone (PSU), polycarbonate (PC), polyamide (PA), polyurethane (PU), polyvinyl alcohol (PVA) and so on), which is impermeable or hardly permeable by the pheromone, thus, the pheromone can only permeate through the first barrier wall and is sustainably released from the outer surface and release can be slowed down by the type and amount of the additional coating polymer (Ogawa et al., 1989).

Another carrier material used for a long time as semiochemical release dispenser is rubber. Rubber septa are commonly based on natural rubber, composed of polyisoprene. Also synthetic rubbers, such as nitrile butadiene rubber (NBR), styrene butadiene rubber (SBR), styrol butadiene styrol (SBS), styrol isopren styrol (SIS) or silicone rubbers are available (Baxter, 2009). The semiochemical is impregnated onto the rubber septum by organic solvent support, followed by solvent evaporation. The release from the rubbers differ dependent from the rubber type, but commonly follows first order kinetics (McDonough, 1991).

However, these passive dispensers are usually non-biodegradable and very persistent in the field and require manual application techniques. Besides the dispenser design and material and pheromone

volatility, temperature and air-flow are the main factors, affecting pheromone emission rates. Thus, another drawback of these dispensers is the loss of expensive pheromones, regardless of the time of day or the flight activity of the target insects, as the dispensers also release when the target insect is absent.

Active aerosol dispensers

In view of increasing digitalization in agriculture, established passive dispenser types now have to compete with commercialized active dispensers. While passive dispensers have the drawback of continuously releasing pheromones even when the target insect is inactive, active battery-powered aerosol dispensers can be programmed to release pheromones from a large depot at selected time intervals when the target pest is active and thereby, are suitable for long-term controlled release of sufficient pheromone amounts (Baldessari et al., 2013; Benelli et al., 2019; Burks & Thomson, 2020). Several aerosol devices at the experimental or commercial development level have already been reviewed by Benelli et al. (2019). As there is a lack of knowledge on active aerosol dispensers performance in the field, current research aim (1) to explore their efficacy compared to established passive dispenser types (2) to find the right release dosage and (3) to minimize the density of dispensers under maintenance of their efficiency (Burks & Thomson, 2019; Shorey et al., 1996; Vacas et al., 2016). Burks and Thomson (2020) demonstrated that the active aerosol dispensers are highly suitable to insect behavioral studies, as aerosol dispensers can release the semiochemical, in the study the sex pheromone for navel orangeworm for mating disruption, in a controlled manner, unaffected by the formulation properties and the effect of abiotic factors, in particular temperature and wind-speed. Opposed to classical passive dispensers, from which the release increases with temperature, active aerosol devices enable release under cold conditions which makes them a promising release device for mating disruption in night-active insects, such as noctuid moths.

Despite their improved performance compared to passive dispenser types, the novel active aerosol dispensers still suffer from manual application, the requirement for equipment, their non-degradability and the need for an additional power source.

MICROCAPSULES

Microcapsules, composed of a core-shell-system in which the semiochemical is trapped within the core surrounded by a polymeric wall has been developed as technical applicable alternative to dispenser systems described in section 2.1.

Biopolymer-based microcapsules

The classical microencapsulation method for pheromones is so-called complex coacervation technique, which bases on the electrostatic interaction of oppositely charged polymers in aqueous solution (Scher, 1977; Vemmer & Patel, 2013).

The principle release mechanisms of volatiles from microcapsules is one single burst-release by rupture from internal or external pressure, rupture from dissolution by hydrolysis or by diffusion through the wall. The diffusion-mediated release rate can be controlled by particle size distribution, wall thickness, density and degree of cross-linking or by combining different microcapsules of different release profiles (Scher, 1977).

In order to replace toxic cross-linkers for the preparation of microcapsules by complex coacervation, alternatives were tested. A promising alternative might be genipin, which is naturally occurring in *Genipa americana* and has the property to cross-link proteins, including gelatine and chitosan (Muzzarelli, 2009; Ramos-de-la-Pena et al., 2016).

The cross-linker genipin was tested for the encapsulation of mosquito repellent *Zanthoxylum limonella* oil in microcapsules prepared from chitosan and gelatin as wall materials (Maji & Hussain, 2009). Results show that the release rate of the encapsulated oil can be modified by the cross-linker concentration. Increased concentration increased the density of the shell and thereby reduce the oil release rate. Moreover, by increasing the chitosan concentration, thermal stability of the beads was improved. It has to be mentioned that genipin is a relatively expensive compound that has to be extracted from plant material.

Chitosan which is a derivative of chitin, the second most abundant polysaccharide on earth, can be purchased as cheap by-product from marine food industry and is a well-established microencapsulation wall material. Core-shell capsules using chitosan as wall material also form by the use of NaOH as hardening agent in a simple coacervation around the oily core. This method was demonstrated to be suitable for the encapsulation of citronella oil as mosquito repellent by Hsieh et al. (2006) and of limonene for insect repellent textile impregnation by Souza et al. (2014). Both found that the release can be modified by the concentration of wall material and hardening agent. Higher NaOH concentrations lead to larger particle size, increased wall thickness and a slower release rate (Souza et al., 2014).

In conclusion, core-shell microcapsules allow semiochemical release control and might be suitable for the slow release of pheromones needed at low concentrations or for short-lasting applications (2-8 h), e.g. of mosquito repellents. Due to their usually small (<200 µm) (Scher, 1977) and modifiable size, microcapsules are favorable for easy incorporation into insect repellent carrier formulations, e.g. lotions and sprays or into textile impregnation formulations.

Yeast cell wall microcapsules

Yeast is one of the most important microorganisms in biotechnology. They are used in a variety of processes, thus, yeast biomass waste is generated as a biotechnological by-product and is therefore a cheap raw material. In the 1990s, the potential of yeast cell walls as a protective shell for bioactive compounds was explored (Bishop et al., 1998). The morphology of this formulation type is like that from microcapsules by its core-shell composition with the active agent expected in the core. Since yeast cell wall microcapsules have been reported to protect from degradation by oxygen or UV radiation and from fast volatilization of the active ingredient (Madene et al., 2006; Shi et al., 2008) they are highly suitable for volatile semiochemicals.

The preparation procedure is easy. The yeast cell walls have to be moistened with water to become permeable and enable the diffusion of the active ingredient into the interior of the yeast cell. A subsequent drying process seals the cell walls and the substance is encapsulated inside the cell. Lima et al. (2019) used this technique for the encapsulation of carvacrol as repellent against the tick *Rhipicephalus* (Boophilus) *microplus* (Canestrini) within *Saccharomyces cerevisiae* cell walls. They applied the freeze-dried yeast cell microcapsules in a laboratory experiment and found improved repellency, compared to unformulated carvacrol, for at least 6 hours.

Photosensitive Microcapsules

An innovative approach to microcapsule-based semiochemical encapsulation is the integration of photosensitive adjuvants within the microcapsule shell providing the triggered release of the semiochemical localized in the core under light exposure. Titanium dioxide (TiO_2) nanoparticles are well known as suitable photosensitive adjuvants (Prieto et al., 2015). Due to its photocatalytic properties hydroxyl radicals are formed, when exposed to UV light. Ribeiro et al. (2016) used this material by functionalization of polyurethane microcapsules with TiO2 nanoparticles and demonstrated that the release rate of encapsulated citronella oil increased when the microcapsules were treated with UV light. However, polyurethane is not biologically degradable and TiO_2 is currently under debate in the REACH due to the suspicion of being carcinogenic (ECHA, 2020).

MATRIX-TYPE FORMULATIONS

In matrix-type formulations, the semiochemical is embedded within the solidified matrix and the release is driven by diffusion of the semiochemical through the formulation material.

Biopolymer beads

The use of biobased and biodegradable materials is one of the current global trends also in formulation technology. Due to their biocompatibility and biodegradability, the potential of alginate beads has been proven in several studies for cell encapsulation for other agricultural applications (Nussinovitch, 2010; Vemmer & Patel, 2013). Alginate is a polysaccharide derived from marine brown algae (Phaeophyceae) and is composed of α-L-guluronic acid und β-D-mannuronic acid. The preparation of alginate beads is simple and the matrix material is available at different qualities and costs and allows the encapsulation under mild conditions. The principle of alginate bead formation is based on ionic gelation. By dripping of an aqueous sodium alginate solution into a solution containing divalent ions, the alginate chains are cross-linked in a three-dimensional network. The supramolecular gel structure is explained by an “egg-box” model (Grant et al., 1973).

Since alginate hydrogels are hydrophilic in nature, pre-emulsification of the hydrophobic volatiles within an oil-in-alginate emulsion is necessary to enable successful distribution of the semiochemicals within the bead (Martins et al., 2017). Once encapsulated, alginate beads have been proven to protect the encapsulated volatile from rapid degradation by oxidation due to low oxygen permeability (Heuskin et al., 2010).

Encapsulation efficiency, loading and release rate of semiochemicals from alginate-based hydrogels beads is directly affected by the gel network tightness, i.e. the degree of cross-linking. Cross-linking can be modified by the type and concentration of alginate and cross-linker ions, ionic strength, cross-linker ion to COO^- ratio and maturation time and also by the content of the encapsulated volatile, as demonstrated by Heuskin et al. (2012) who encapsulated E-β-farnesene and E-β-caryophyllene in a gelled oil-in-water emulsion to attract the parasitoid *Aphidius ervi* for the biological control of *Acyrtosiphon pisum*.

When the semiochemical itself should serve as the oil-phase embedded within the aqueous alginate, stabilization by an additional emulsifier is needed. Yosha et al. (2008) demonstrated, that gelatin is a suitable stabilizer for the encapsulation of the model parapheromone dodecyl acetate within alginate beads, cross-linked with $CaCl_2$. With increasing gelatin concentration, the emulsion stability was

improved. Furthermore, by varying the alginate and gelatin concentrations, bead porosity changed and affected the dodecyl acetate release from the beads. As alginate or gelatin concentration increased, the release rate decreased.

The formulation of semiochemicals within alginate beads was adapted by others for the release of E-β-farnesene for realization of a push-pull strategy by Xu et al. (2018) and for methyl salicylate by Wang et al. (2019) for the control of aphids in field trials. Xu et al. (2018) found better attraction to natural enemies of aphids by E-β-farnesene formulated within oil than in alginate beads, whereas Wang et al. (2019) observed significantly higher numbers of aphid predator *Metasyrphus corollae* in wheat fields treated with methyl salicylate loaded alginate beads.

However, Lopez et al. (2012) found that conventional alginate beads did not provide slower release of linalool by encapsulation. Therefore, they improved encapsulation efficiency, loading, and release control of linalool by the addition of starch and especially glycerol. Moreover, they found better performance by the inverse gelation technique, which resulted in an alginate-based microcapsule formulation, as they were described in section 2.2, realized by dripping the cross-linker solution into alginate for gelation from inside to outside, in combination with glycerol and starch addition.

Besides their advantages of ease and non-toxicity of preparation at low temperatures and low energy input and their biodegradability, there are some disadvantages negatively affecting the usability for controlled semiochemical release. Hydrogel capsules commonly suffer from a low initial loading due to high polymer and water content, low encapsulation efficiencies (<90%) and additionally, the release can be negatively affected by humidity due to swelling of the hydrogel (Heuskin et al., 2012; Lopez et al., 2012; Mao et al., 2020). Daems et al. (2016) found that the release of E-β-caryophyllene from solidified oil-in-alginate beads, dried under pressure (2 bar), did not release E-β-caryophyllene at humidity conditions >85% r.h. due to increased water content of the beads, which makes them unsuitable for insect control under high humidity conditions. A promising study of Valladares et al. (2016) indicate that swelling of alginate beads might be reduced by an additional chitosan coating. They found more rigid and stable pores by increased pH of the gelling solution, which supported a higher release rate for a longer period of 6-methyl-5-hepten-2-ol as sex pheromone encapsulated within chitosan-coated alginate beads for the behavior manipulation of *Megaplatypus mutates*.

For use as effective delivery system for semiochemicals in agriculture and forestry, further research is required aiming a size reduction to enable technical application by conventional spraying instruments (Yosha et al., 2008) as the biopolymer beads developed in the reviewed studies are commonly of a large size up to several mm.

Zein nanoparticles

Zein is the main storage protein in maize. It consists of lipophilic amino acid residues that give it a hydrophobic character. As a protein, zein exhibits a special solubility behavior depending on the solvent and the pH value and can be classified by its solubility and sequence length into four fractions (α-, β-, γ-, and δ-zein) with α-zein as the most abundant and commercially available (Bourbon et al., 2019; Fathi et al., 2019). Several methods, like nanoprecipitation, liquid-liquid dispersion, phase separation and electrospraying, have been described for the preparation of different zein-based formulations with a focus on nanoscale carrier systems. Details on the methodologies were described by Pascoli et al.

(2018). A typical manufacturing process, known as solvent-emulsification-evaporation, produces zein nanoparticles by dissolving zein in an ethanolic solution (70 - 85% in water), containing the active ingredient, followed by solvent evaporation. Thereby, the ethanol evaporates while stirring with high speed in a water-surfactant mixture in which the formed zein-active ingredient-droplet is insoluble. In the resulting solid nanoparticles, the semiochemical is embedded within the zein matrix (Kasaai, 2018).

The hydrophobic character of zein supports a high solubility and thus good distribution of the hydrophobic compounds within the matrix and high loading capacity. Another major benefit is the protection from photodegradation as demonstrated by Oliveira et al. (2018), who formulated geraniol and R-citronellal as mite repellents within zein nanoparticles. The encapsulation within zein nanoparticles resulted in a 17-fold decrease in degradation of geraniol by UV radiation. This result was in line with several other reports on formulation of oils in zein nanoparticles as antimicrobial or antioxidant formulations (Oliveira et al., 2018; Pascoli et al., 2018). Due to their excellent protection of some encapsulated compounds against photodegradation, zein nanoparticles seem to be very well suited for applications under intensive light exposure.

Solid lipid nanoparticles

Solid lipid nanoparticles (SLNs) present an alternative drug delivery system to liposomes, micelles and nanoemulsions by differing from other lipid based systems by a solidified matrix in which the active agent is incorporated. SLNs are commonly of spherical shape and a mean particle size about 40 to 1000 nm (Pardeike et al., 2009; San Martin-Gonzalez, 2015). Several preparation methods are available: high pressure homogenization, microemulsification, solvent emulsification-evaporation, ultrasonification/high speed homogenization, super critical fluid, spray chilling and double emulsion method (W/O/W) (Tavares et al., 2018).

SLNs are prevalently prepared by high pressure homogenization which includes melting of the lipid carrier, dispersion of the active compound within the melt followed by emulsification at temperatures above the melting point of the lipid under high pressure in an aqueous phase containing emulsifiers to enable the formation of small lipid droplets, followed by rapid cooling to initiate crystallization of the lipid (Mehnert & Mäder, 2012; Müller et al., 2002).

Due to their hydrophobic character and solid matrix, SLNs provide a good barrier against environmental factors such as water and light. The release is less affected by moisture compared to e.g. hydrogels as described above.

The main application in semiochemicals release from SLNs is the application on the skin to form a repellent reservoir against mosquitoes. Biocompatible lipids as matrix material reduce dermal irritation potential and skin permeation compared to ethanolic sprays or unformulated compounds (Müller et al., 2002; Pardeike et al., 2009).

One of the most important parameters during development of SLNs is the compatibility of the lipid carriers with the active compound. İşcan et al. (2005) found that some lipids were unsuitable for the encapsulation of N,N-diethyl-meta-toluamide (DEET), the most common insect repellent against medical important pest insects (Syed & Leal, 2008; Tavares et al., 2018). Thus, the nature of the lipid matrix is very important for high semiochemical loading and sufficient encapsulation efficiency. The compatibility or solubility can be examined by the crystalline status of the SLN formulation and

determined by differencial scanning calorimetry (DSC). Stearic acid –Tween 80-based SLNs performed best for the encapsulation of DEET and were adapted by other groups, e.g. Puglia et al. (2009). To investigate the in-vitro performance of the developed SLNs for topical application, they focused on the benefit of the formulation on reduced skin permeation of the formulated repellents.

Nanogels

An efficient measure to reduce the semiochemicals volatility is the direct gelling of the oily compound with low molecular mass gelators (LMMGs). Up-to-date, there has been only one publication on this formulation type in the field of semiochemical release (Bhagat et al., 2013). The described method is quite simple and does not require any environmentally harmful and toxic chemicals: the LMMG all-trans tri(p-phenylenevinylene) bis-aldoxime was dispersed in methyl eugenol as an attractant for *Bactrocera dorsalis* and heated above the melting point of the LMMG (65°C). By self-assembly through weak intermolecular noncovalent interactions such as hydrogen bonding, π–π -stacking and van der Waals forces of the gelator, methyl eugenol was gelled. At 50°C, unformulated methyl eugenol evaporates within 3 weeks, formulated methyl eugenol within 30 weeks, when 0.2 ml of the gel was placed in a glass vial (Ø=5 mm) open at the top. At lower temperatures the release was much slower.

Since the gel is hydrophobic, similar to solid lipid nanoparticles and zein, release is less affected by ambient moisture, making the formulation also useful for pest control in crop protection in rainy climates or during rainy seasons.

Emulsions

Emulsions are biphasic systems of two non-miscible liquids, typically composed of water, oil and emulsifier(s).

SPLAT®

One of the first slow release formulations to compete with the classic passive dispenser types was an emulsion-based technology developed by Delwiche et al. (1998) and Atterholt et al. (1999), patented (Delwiche et al., 1999) and later commercialized with the trade name SPLAT® (Specialized Pheromone and Lure Application Technology) for use in agriculture and forestry.

In order of emulsion preparation, the paraffin wax was molten above its melting point to incorporate the pheromone, an emulsifier and other additives, followed by the addition of hot water under rapid stirring and emulsification with a high-speed mixer. The resulting emulsion proved to be practical for spray application. After application, the water will evaporate from the emulsion and a solidified, rain-proofed formulation remains on the bark or foliage of the treated plant (Delwiche et al., 1998).

The pheromone release from this formulation was found to be mainly affected by the solubility of the pheromone within the formulation matrix. The addition of vitamin E and soy oil improved solubility of the pheromone within the paraffin wax matrix and thereby decreased the release-rate. Furthermore, higher pheromone content and increased surface area increased the release-rate, ranging from 0.4 to 2 mg/day and release lasts for at least 100 days at 27°C (Atterholt et al., 1999).

Several studies and field trials have been conducted using modified SPLAT® for various components and insects, as it is commercially available for different applications, mainly in agriculture and forestry, such as mating disruption, attract-and-kill strategies, repellency of pest insects or attraction of pollinators (https://www.iscatechnologies.com/collections/lures).

Nanoemulsions

A more recent emulsion-based formulation for semiochemicals are so called nanoemulsions. Nanoemulsions differ from classic emulsions by their smaller droplet size, ranging from 10 to 200 nm and their stability (Ray et al., 2015).

Nanoemulsions can be prepared by high or low energy methods: high-pressure homogenization and ultrasonification are high energy methods, phase inversion temperature (PIT) and emulsion inversion point (EIP) method are low energy methods (Gupta et al., 2016). Compared to larger scale emulsions, nanoemulsions are transparent or translucent due to a smaller droplet size and exhibit a larger surface area and thereby a higher loading capacity. Nanoemulsions are kinetically and long-term physically stable (with no apparent flocculation or coalescence) but not thermodynamically stable. Due to their remarkable low viscosity they can be sprayed easily. As metastable formulations, nanoemulsions can be further dispersed in aqueous solutions without phase separation (Balaji et al., 2015; Bouchemal et al., 2004; Ray et al., 2015).

The release of semiochemicals from nanoemulsions can be modified by changing the droplet size, viscosity and homogeneity, flow behavior, contents of water, surfactant and co-solvent concentrations and ratios. By increasing the surfactant concentration and emulsification speed or pressure, the droplet size can be reduced, homogeneity increased, the stability improved and thereby release can be slowed down (Sakulku et al., 2009).

Pascual-Villalobos et al. (2017) found that the smaller the droplet size within the nanoemulsion, the higher the activity of the encapsulated essential oils against aphids. They also found increased nanoemulsion stability by glycerol addition of and/or lecithin as an additional surfactant. The results were confirmed by Narawi et al. (2020), who formulated nutmeg essential oil as mosquito repellent in nanoemulsions. Their findings demonstrate that the oil mobility within the emulsion affected the release rate. By reducing the water content and increasing the glycerol content, the emulsion viscosity and stability increased. As the formulation was designed as a mosquito repellent formulation, repellent studies were carried out for a duration of only 8 hours.

Nanofibers

Nanofibers, characterized by their diameter on nanometer scale, can be produced with an electrospinning set-up, which consists of a high-voltage source, an electrically conductive blunted needle, a syringe pump and a collector. The spinning solution, composed of a polymer, dissolved in an organic solvent and the active ingredient, is filled into the syringe, connected to the spinneret and subjected to an electric field. Due to high voltage (10 – 30 kV), the surface of the fluid spinning solution droplet gets electrostatically charged at the tip of the needle. This leads to an elongation of the liquid drop towards a conical object, the so-called Taylor cone. The formation of electrospun nanofibers relies on the electrical field between the positively charged spinneret to which the needle is connected and the negatively charged collector (Osanloo et al., 2019).

In the resulting nanofiber, the active ingredient is embedded in a fiber-shaped matrix made of synthetic or natural polymers. The release of semiochemicals from electrospun nanofibers is mainly affected by the matrix forming polymer, the content of the active and fiber morphology. Fiber formation, diameter and morphology can be controlled by the spinning solution properties such as viscosity, solvent volatility,

surface tension, conductivity and dielectric constant (Muñoz et al., 2019) but also by voltage, flow rate and distance between tip and collector (Osanloo et al., 2019).

The first report on electrospun nanofibers for the release of semiochemicals was published by Hellmann et al. (2011). In their in-vitro study, grape berry moth synthetic sex pheromone (Z)-9-dodecenyl acetate was formulated within electrospun cellulose acetate (CA) and polyamid 6 (PA6) nanofibers. They found linear release kinetics (zero-order) from fibers and the fibers released the pheromone for a duration of more than 100 days. One of their main finding is the slower release from fibers made of CA than from PA6. Direct spinning of the nanofibers in the vineyard for mating disruption of *Lobesia botrana* by an electrospinning apparatus attached to tractor was discussed as future application technique.

The potential of CA as a carrier for long-lasting semiochemical release was confirmed later by Iliou et al. (2019) who formulated citronella oil as mosquito repellent against *Aedes albopictus* in cellulose acetate and polyvinylpyrrolidone and found a release duration for more than 4 weeks from the CA nanofibers. This finding was explained by the importance of the solubility, which cause a higher amount of the semiochemicals trapped in CA than in PA6 (Hellmann et al., 2011; Iliou et al., 2019; Rempel et al., 2019).

Another feature of electrospun nanofibers to manipulate the release behavior is the ability to produce multilayer systems by simply spinning nanofiber mats of a desired thickness and layers made of one or more polymers. Iliou et al. (2019) developed a triple layer nanofiber mat, composed of CA and polyvinylpyrrolidone individually or in combination and found prolonged release duration compared to the single layer mats up to four weeks.

Muñoz et al. (2019) were able to produce core-enriched fibers by use of a coaxial nozzle. Core-enriched fibers performed better by a longer release and efficiency of citrodiol as repellent against *Aedes aegyptii* compared to monolithic fibers prepared by a common single nozzle. Moreover, they found the ratio and total content of the repellent affected the release.

Ryan et al. (2020) also produced monolithic and coaxial fibers for the formulation of picaridin within nylon-6,6 as a textile relevant polymer to be applied for tick and mosquito repellent textile advices.

Rempel et al. (2019) gives an alternative technique for the formation of nanofibers by solution blow spinning technique with conventional airbrush equipment. They were able to produce fibers of 95–426 nm diameter made of ethylen vinyl-acetate by extrusion of the solution through a small diameter nozzle (0.3 mm) under high pressure (5 bar) to encapsulate synthetic sex pheromones from the oriental fruit moth, *Grapholita molesta* and citrus leafminer *Phyllocnistis citrella*.

In general, electrospun nanofibers exhibit interesting properties like long-term zero-order release, broad diameter distribution, high stiffness and strength due to strong chain and crystal orientations induced by the spinning process, thus enhancing the fiber stability during field application. Moreover, due to the fibrous shape, this formulation type was found being well suited to be combined with textiles for insect repellent advices. Especially for use in the environment, e.g. in agriculture, care should be taken to ensure that the polymer used is made of biodegradable materials.

Cyclodextrin inclusion complexes

Cyclodextrins (CDs) are cyclic oligosaccharides derived from enzymatically treated starch, consisting of α-1,4-glycosidic linked α-D-glucopyranose subunits. They have a toroidal shape and due to hydroxyl groups present in the interior, they are sufficiently apolar to reversibly bind hydrophobic molecules, depending on their size and shape, within their cavity by weak bonding. Due to their hydrophilic exterior, CDs became prominent in pharmaceuticals and food technology for improved bioavailability of poorly water soluble molecules in aqueous environments (Del Valle, 2004; Ullmann et al., 1985; Wadhwa et al., 2017). Due to their special binding properties, GC-columns containing CDs for selective detection of chiral insect pheromones are available.

Three cyclodextrins differing in the number of α-D-glucopyranose subunits are technical available: α -, β- and γ -CDs of six, seven and eight subunits, respectively. The different length causes a variation of the cavity diameter as follows: α = 4.7, β = 6.0, γ = 7.5 Å, with β as most commonly used because of its low costs (Ullmann et al., 1985).

The most frequently used methods for the preparation of inclusion complexes are the so-called kneading method and the co-precipitation method, commonly followed by freeze-drying and spray-drying (Marques, 2010). CDs are known and used to improve light, oxidative and reactive stability of the enclosed molecule, increase water solubility of water-insoluble substances, enable the pulverization of volatile liquids and most importantly reduce volatilization (Wadhwa et al., 2017). A precondition for inclusion is the geometry and size of the molecule that has to be compatible with the cavity diameter (Yang, 2005).

A typical current application is grafting of semiochemical-loaded CDs on textiles through covalent bonds via the hydroxyl groups of the cellulose, in order to improve washing resistance of mosquito or tick repellents (Hebeish et al., 2008; Inceboz et al., 2015; Khanna & Chakraborty, 2018; Romi et al., 2005).

Yannakopoulou et al. (2002) elucidated the configuration and deposition of the CD in interaction with (Z)-tetradec-7-en-1-al. They found two binding types of guest molecules in the crystalline structure of the complex: one inside the β-CD cavity and another trapped and held loosely outside the cavity. They concluded that CDs are efficient for slowing down the release. One of their most relevant observations was that the pheromone was well stabilized and released more slowly when encapsulated within the cavity compared to the exterior.

One of the main factors affecting encapsulation efficiency and thereby the release from CDs is the semiochemical itself (Campos et al., 2018; Khanna & Chakraborty, 2018). Some works observed only partial complexation of the molecule within CD resulting in a very fast release (Khanna & Chakraborty, 2018; Yannakopoulou et al., 2002). Other factors are preparation parameters affecting encapsulation efficiency, such as CD:semiochemical ratio, embedding time, embedding temperature and CD:ddH2O ratio (Huang et al., 2020).

Arad-Yellin et al. (2001) strengthened the interaction of the host molecule 1,7-dioxaspiro-5,5-undecane by UV irradiation of the solution containing the pheromone and β-cyclodextrin percinnamate (β-CD-PC). UV radiation induced the formation of intramolecular cyclobutane bridges that trap the pheromone within the CD cavity and release the pheromone by bond-breaking chemical reactions.

POROUS MATERIALS

Micro- and nanoporous materials are characterized by high interior surface area. They can be classified by their pore size into microporous: <2 nm; mesoporous: 2-50 nm and macroporous:>50 nm (Everett, 1972). Depending on the selected porous material, the release of bioactive molecules can be controlled by modification of the pore size, shape and chemical properties to manipulate the interaction of the molecule with the material. For slow or controlled release formulation of semiochemicals silicates (zeolites, sepiolites), metal-organic frameworks and silica gels were evaluated as carriers (Paseta et al., 2016).

Silicates

Zeolites are naturally occurring aluminosilicates building a network of channels and cavities. For each framework type identified in natural or synthetical zeolites, a three-letter code (e.g. MFI, ZSM) was assigned by the International Zeolite Association (IZA, 2020). Due to their high adsorption capacity, zeolites have been used as carriers for several bioactive molecules and fragrances, are commonly used as molecular sieves, catalysts or as ion exchange materials (Li et al., 2017). Volatile semiochemicals or other non-polar molecules are immobilized by adsorption (De Smedt et al., 2015). The adsorption mechanism mainly bases on medium-range or van-der-Waals forces, but depending on the molecule and the zeolite, hydrogen bonds or other heteropolar interactions can be involved (Muñoz-Pallares et al., 2001). Depending on the molecule size and conformation, the molecular diffusion of the active ingredient can be controlled by selection of the zeolite with the desired pore diameter, which can vary in the range of 0.7-1.2 nm, by the SiO2/Al2O3 molar ratio or by the presence of acidic or other functional groups (De Smedt et al., 2015).

When investigating release behavior from different zeolite types, Muñoz-Pallares et al. (2001) found that zeolite pore dimensions and the presence or absence of acidic sites have the greatest effect on the release rate of n-decanol. Release duration decreased with decreasing pore diameter from a half-life of 2 days (pore diameter: 7.6 x 5.4 Å) to 136 days (pore diameter: 5.3 x 5.6 Å) and by increasing acidity. Further factors affecting the release were polarity and polarizability of the framework, modifiable by Si/Al ratio variation. Lower polarity resulted in a slower release due to stronger adsorption of the pheromone to the zeolite.

Ramos et al. (2017) formulated rhynchophorol in the two different MFI zeolite types ZSM-5 and silicalite-1. Thereby, adequate zeolite dimensions to host the guest molecule were considered. Unfortunately, rhynchophorol was completely degraded in ZSM-5, rendering this material unsuitable to use as matrix for the release of rhynchophorol or similar molecules. As explanation, they found strong chemical interactions between rhynchophorol and acid sites of ZSM-5 zeolites promoting the degradation of this pheromone. As a supporting result, in silicalite-1, which has no acidic sites in a sufficient amount, rhynchophorol was not degraded. However, Viana et al. (2018) also formulated rhynchophorol and observed interaction of the pheromone with zeolite L and sodium magadiite. In these materials rhynchophorol remained stable and did not degrade.

To achieve more controlled release options, zeolites can be chemically modified. Seo et al. (2016) were able to improve pheromone adsorption capacity of zeolite A by modification with cetyltrimethylammonium ions (CTA+) by ion-exchange. Due to the low Si/Al ratio it has higher cation-exchange capacity than other zeolites and they demonstrated, that the modified zeolite A prolonged the

release of aggregation pheromones of *Riptorturus pedestris*. Only half of the pheromone was released after 10 weeks in a field trial.

Another mesoporous material that can be used for pheromone release is sepiolite, a magnesium silicate. Tabletized sepiolite formulations were developed and patented by Corma et al. (2000). The inventors demonstrated that the release of the model semiochemical trimedlure can be modified by the compacting pressure, the associated cation and the adsorption layer. This formulation material was used by Vacas et al. (2009) in field trials for the long-term release of sex pheromones for mating disruption in California red scale, *Aonidiella aurantii*.

As silicates, such as zeolites and sepiolites are available at low cost, the formulation by tableting and adsorption is cheap and easy and it has the potential for long-term release duration, these materials seems to be promising alternatives for the common passive dispensers made from synthetic plastics.

Metal-organic frameworks (MOFs)

Compared to naturally occurring zeolites, metal-organic frameworks (MOFs) are synthetically created from building blocks of metal ions or clusters with organic ligands to form 1D, 2D and 3D crystal lattices. MOFs are characterized by having large pore volumes and high surface areas and due to their great synthesis control, MOFs exhibit structural flexibility, wider range of porosity and surface area and modifiability by chemical functionalization (Furukawa et al., 2013).

The incorporation and slow release of bioactive molecules from MOFs, first reported by Horcajada et al. (2012) can be modified by the framework architecture.

There are two principle methodologies for the formulation of bioactive molecules in MOFs, the one-step and the multi-step encapsulation procedure. During the one-step procedure, the active ingredient is added into the synthesis solution of the MOF. In the multi-step procedure, first the MOF is synthesized and then subsequently activated to enable the adsorption of molecules (Paseta et al., 2015).

The release of volatile molecules adsorbed on pore surfaces is driven by slow diffusion through open pores or decomposition of the MOF. One of the most important prerequisites is the amount of adsorbed compounds into the MOF. In view of the semiochemical uptake, the space-filling model using van-der-Waals radii of the atoms is an important preview option on successful uptake (Hamzah et al., 2020). Due to the high chemical flexibility during preparation, functional groups can be added to modify the trapping and release of semiochemicals. Hamzah et al. (2020) demonstrated, that the addition of alkyl groups or the presence of an amino group to provide a hydrophobic or hydrophilic pore environment improved 3-octanone uptake and retention. The presence of large octyl groups take up too much pore space to allow 3-octanone inclusion.

An interesting feature of many MOFs such as MIL-53(Al) and MIL-88A(Al) (Paseta et al., 2016) is their decomposition in the presence of water, also supporting good biodegradability of these MOFs. Paseta et al. (2016) make use of this property, because they aimed a triggered release of insect repellents from pool treatment tablets at the place of application. At the same time, the fast degradation in presence of water is a disadvantage as semiochemicals will be released immediately compromising sufficient persistence. To overcome this problem, moisture-stabilized MOFs were designed. The moisture-stabilized zirconium (IV) MOF used by Hamzah et al. (2020) enabled the release of 3-octanone over a period of 100 days.

Silica gels

Silica or so called sol-gel materials are amorphous inorganic materials of metal alkoxides. The most frequently used alcoxidic pre-cursors are silanes. In the presence of water and under acidic conditions, the pre-cursors form silanol (Si-OH) groups by hydrolysis. A three-dimensional network is formed by the subsequent condensation reaction between the partially hydrolyzed silanol pre-cursors. Guest molecules added to the sol are enclosed within the resulting silica gel. After full gelation, sol-gel materials exhibit glass-like properties (Zada et al., 2009).

Silica matrices are chemically inert (Kandimalla et al., 2006; Zada et al., 2009) which supports low reactivity and interaction with semiochemicals and improves shelf life. Moreover, sol–gel materials can be prepared and applied in any desired shape and thickness and thereby modify release rates.

To adjust the release of active compounds from silica gels, pore size and density can be modified by the choice of the pre-cursors, pre-cursor concentration, and degree of pre-cursor hydrolysis, which affects the degree of cross-linking during the polymerization process in order to provide an optimal release rate from the silica gel (Zada et al., 2009).

Zada et al. (2009) investigated the effect of preparation parameters, namely water/tetramethyl orthosilicate pre-cursor (TMOS) ratios, pH, solvents in which the semiochemical and the surfactants are dissolved, and surface tension of surfactants on the stability of the silica matrix and the release rate of (Z)-7-dodecenyl acetate. The formulation stability was hardly affected by water/TMOS ratio, since at some ratios the silica tends to crack under room temperature causing excessive semiochemical leakage. They found that this cracking is related to the pH value and can be prevented by the addition of an excess of an ammonium base or by addition of a surfactant.

Moreover, precursor side chains of the comonomers of sol-gels affect the release of semiochemicals. To give an example, Zada et al. (2009) revealed that the phenyl tail of the phenyltrimethoxysilane (PTMOS) used as comonomer, may enhance gel hydrophobicity. They also found that increased comonomer content decreased cross-linking and thereby enlarged the cavities and microporous structure, resulting in pheromone loss (Bian et al., 2014).

Semiochemical release rates can be modified by increased pheromone/pre-cursor ratios and the gel concentration. Bian et al. (2014) found, as the loading volume increased, the half-life of trans-2-hexen-1-al release increased in a linear relationship. Moreover, the persistence of the encapsulated volatile depends on its physico-chemical properties, as can be seen from the observation that alcohols with 6-10 carbons had the longest half-life, while esters with 6-12 carbons had the shortest. In general, the persistence of small molecules is shorter than that of large molecules, rendering sol-gels more suitable for long-chain insect sex pheromones than for small semiochemicals (Bian et al., 2014).

The polarity of the gel may be too high for the incorporation and the diffusion mediated release of lipophilic compounds such as pheromones. Another disadvantage is the need for convective or hypercritical drying, neither suitable for semiochemical formulation due to high losses or degradation of the semiochemical. Another limitation is the low modifiability of pore size uniformity which is essential to facilitate the reproducibility and control of release rates. Bian et al. (2014) reduced the pore size by using a co-monomer with a short alkyl group and a low comonomer to TMOS ratio to slow down the release.

Photoremovable protection groups

Pro-fragrances have long been known in perfume formulation technology. The volatile compound is chemically trapped by a group that can be easily eliminated from the molecule by an external trigger. Despite the potential of this technique and the physico-chemical similarity of volatile semiochemicals to perfumes and fragrances, it has remained nearly unexplored for the controlled release of semiochemicals.

Atta et al. (2013) proved the potential of the four different photoremovable protecting groups 7-hydroxy-4-hydroxymethylcoumarin (=coumarin), 1-pyrenemethanol (=pyrene), 9-anthracenemethanol (=anthracene) and 2-(hydroxymethyl)anthraquinone (=anthraquinone) for the controlled release of (Z)-11-hexadecen-1-ol, a sex pheromone of *Chilo infuscatellus* for mating disruption. They reported that the pheromone was only released under UV light (≥350 nm) or direct sunlight irradiation and release was thereby controllable by an external trigger.

Table 1.2. Formulations and materials for semiochemicals, insect species and applications

Formulation	Material	Semiochemical	Insect species	Application	References
Passive dispenser					
CheckMate®	PE bags	Sex pheromones of the target insect species	*Amyelois transitella, Aonidiella aurantii, Planococcus ficus, Cydia pomonella, Grapholita molesta*	Mating disruption	(https://www.suterra.com/products_pest)
RAK® 1 + 2; RAK® 3	Plastic vials	(Z)-9-dodecen-1-yl acetate and (E,Z)-7,9-dodecadienyl acetate; Codlemone ((*E,E*)-8,10-dodecadien-1-ol and tetradecyl acetate)	*Lobesia botrana* and *Eupoecilia ambiguella; Cydia pomonella*	Mating disruption	Auffermann and Heck (2015)
Isomate®	Capillary made of polymeric materials	Sex pheromones of the target insect species	*Cydia pomonella, Grapholita funebrana, Synanthedon myopaeformis, Grapholita lobarzewskii*	Mating disruption	Ogawa *et al.* (1989)
ISCAlure®	Rubber septa	Pheromones of the target insect species	Diverse North American agricultural pest insects	Trapping	(https://www.iscatechnologies.com/collections/lures)
Active aerosol dispenser					
Aerosol dispenser	Aerosol dispenser	(*E,E*)-8,10-Dodecadien-1-ol	*Cydia pomonella*	Mating disruption	Baldessari *et al.* (2013)
Aerosol dispenser	Aerosol dispenser	(*Z,Z*)-11,13-hexadecadienal	*Amyelois transitella*	Mating disruption	Burks and Thomson (2020)
Neburel® - Aerosol dispenser	Aerosol dispenser	(*Z*)-11,16-hexadecenal	*Chilo suppressalis*	Mating disruption	Vacas *et al.* (2016)
Aerosol dispenser	Aerosol dispenser	(*E*)-11-14:OAC and (*Z*)-11-14:OAC, (*Z,E*)-9,12-14:OAc and (*Z*)-9-14:OH	*Spodoptera exigua, Platynota stultana*	Mating disruption	Shorey *et al.* (1996)

Table 1.2 (continued)

Formulation	Material	Semiochemical	Insect species	Application	References
Microcapsules					
Simple coacervation	Chitosan - NaOH	Citronella oil	Mosquitoes	Mosquito repellent	Hsieh *et al.* (2006)
Complex coacervation	Chitosan-Gelatine - Genipin	*Zanthoxylum limonella* oil	Mosquitoes	Mosquito repellent	Maji and Hussain (2009)
Simple coacervation	Chitosan - NaOH	Limonene	-	Insect repellent - Textile impregnation	Souza *et al.* (2014)
Yeast cell wall-microcapsule	Yeast cell walls	Carvacrol	*Rhipicephalus (Boophilus) microplus*	Tick (larvae) repellent	Lima *et al.* (2019)
Photosensitive microcapsules – Interfacial polymerization	PU + Titanium dioxide nanoparticles	Citronella oil	*Anopheles stephensi*	Mosquito repellent	Ribeiro *et al.* (2016)
Alginate beads					
Ionic gelation	Alginate + oil + alpha-tocopherol (CaCl2)	*E*-β-farnesene, *E*-β-caryophyllene	*Aphidius ervi*	Parasitoid attractant	Heuskin *et al.* (2012)
Ionic gelation	Alginate; Alginate-Chitosan; Alginate + glycerol + starch	Linalool	-	-	Lopez *et al.* (2012)
Ionic gelation + interfacial precipitation	Alginate + Chitosan coated	6-methyl-5-hepten-2-ol (sulcatol)	*Megaplatypus mutatus*	Attract formulation	Valladares *et al.* (2016)
Ionic gelation	Alginate + oil + alpha-tocopherol ($CaCl_2$)	*E*-β-caryophyllene	*Aphidius ervi*	Parasitoid attractant	Daems *et al.* (2016)
Ionic gelation	Alginate + oil ($CaCl_2$)	*E*-β-farnesene, Methyl salicylate	Aphids	Push-Pull strategy	Xu *et al.* (2018)
Ionic gelation	Alginate + oil ($CaCl_2$)	Methyl salicylate	*Sitobion avenae, Metasyrphus corollae*	Push-Pull strategy	Wang *et al.* (2019)
Ionic gelation	Alginate + gelatin ($CaCl_2$)	Dodecyl acetate	-	-	Yosha *et al.* (2008)

Table 1.2 (continued)

Formulation	Material	Semiochemical	Insect species	Application	References
Nanoparticles					
Protein nanoparticle	Zein	Geraniol and *R*-citronellal	*Tetranychus urticae*	Mite repellent	Oliveira *et al.* (2018)
Solid lipid nanoparticles					
SLN high-pressure homogenization	Stearic acid - Tween80	DEET	-	-	İşcan *et al.* (2005)
SLN precipitation technique	Hydrogenated vegetable oil and lecithin	DEET	*Aedes aegypti, Anopheles stephensi*	Mosquito repellent	Domb *et al.* (1995)
SLN simple coacervation	Spermaceti®, Polawax®	DEET	-	-	Kasting *et al.* (2008)
SLN ultrasonification	Stearic acid	Diethyltoluamide, Ethylhexyl *p*-methoxycinnamate	-	-	Puglia *et al.* (2009)
Nanogels					
Thermal gelation by LMMG	LMMG	Methyl eugenol	*Bactrocera dorsalis*	Attract fruit pests; Attract and Trap	Bhagat *et al.* (2013)

Table 1.2 (continued)

Formulation	Material	Semiochemical	Insect species	Application	References
Emulsions					
SPLAT®					
SPLAT® OFM/CYDIA/Lobesia/GM-O/LBAM HD-O/EC-O/PBW/TUTA/HELIO/FAW	Aqueous paraffin emulsion	Sex pheromone of the target insect	*Grapholita molesta/Cydia pomonella/Lobesia botrana/Lymantria dispar/Epiphyas postvitanna,/Ectomyelois ceratoniae/Pectinophora gossypiella/Tuta absoluta/Helicoverpa spp./Spodoptera frugiperda*	Mating disruption	(Delwiche *et al.*, 1999; https://www.iscatechnologies.com/collections/lures)
SPLAT® SWD/MAT SPINME/MAT CL/MAT TML; HOOK® RPW/SPW/RB/TUTA; NOCTOVI®; ANAMED®	Aqueous paraffin emulsion	Sex pheromone, parapheromone, aggregation pheromone (or other attractants) of the target insect	*Drosophila suzukii*/Tephritidae/*Bactrocera oleae/Ceratitis capitate; Rhynchophorus ferrugineus/Rhynchophorus palmarum/Dynastinae spp./Tuta absoluta;* Noctuidae; Tephritidae	Attract and Kill	(Delwiche *et al.*, 1999; https://www.iscatechnologies.com/collections/lures)
SPLAT® VERB	Aqueous paraffin emulsion	Anti-aggregation pheromone	*Dendroctonus ponderosa*	Repell mountain pine beetle	(Delwiche *et al.*, 1999; https://www.iscatechnologies.com/collections/lures)
APIS Bloom™	Aqueous paraffin emulsion	Nasonov pheromone	*Apis mellifera*	Attract pollinator bees	(Delwiche *et al.*, 1999; https://www.iscatechnologies.com/collections/lures)

Table 1.2 (continued)

Formulation	Material	Semiochemical	Insect species	Application	References
Nanoemulsions					
Nanoemulsion - Ultrasound	Tween 80, Soy lecithin, Glycerol, Water	Various essential oils	*Rhopalosiphum padi*	Aphid repellent for leaf application	Pascual-Villalobos *et al.* (2017)
Nanoemulsion	Poloxamer 407	Ethyl butylacetylaminopropionate (IR3535)	-	Insect repellent for dermal application	Pinto *et al.* (2017)
Nanoemulsion - Spontaneous emulsification	Montanov®82 (+glycerol)	Citronella oil	*Aedes aegypti*	Mosquito repellent	Sakulku *et al.* (2009)
Nanoemulsion - Phase inversion method	Tween 80	*Pilocarpus spicatus* essential oil, (Limonene)	*Rhipicephalus microplus*	Cattle tick repellent	Nogueira *et al.* (2020)
Nanoemulsion	Montanov®82 (+glycerol) nanoemulsion	Nutmeg oil	*Aedes aegypti*	Mosquito repellent	Narawi *et al.* (2020)
Nanoemulsion	Ammonia, ethanol, Tween 80	Amyl acetate	*Drosophila melanogaster*	Fruit fly attractant	Krittika *et al.* (2019)
Nanoemulsion – Phase inversion temperature (PIT) method	PEG	*N,N*-diethyl phenylacetamide (DEPA)	*Culex tritaeniorhynchus*	Insect repellent	Balaji *et al.* (2015)

Table 1.2 (continued)

Formulation	Material	Semiochemical	Insect species	Application	References
Nanofibres					
Electrospinning	Cellulose acetate and Polyamid 6 nanofibres	Grape berry moth *Lobesia botrana* synthetic sex pheromone	*Lobesia botrana*	Mating disruption	Hellmann *et al.* (2011)
Electrospinning	Polycaprolactone, PEG-polycaprolactone, Ethyl cellulose and Polyvinyl acetate-PVP.	Trimedlure	*Ceratitis capitata*	Attract formulation	Bisotto-de-Oliveira *et al.* (2014)
Electrospinning	Cellulose acetate (CA), Polyvinyl acetate (PVAc), Polycaprolactone (PCL), Polyvinyl pyrrolidone (PVP), Styrene-butadienestyrene (SBS) copolymer, and blends thereof	*Grapholita molesta* synthetic sex pheromone	*Grapholita molesta*	Mating disruption	Bisotto-De-Oliveira *et al.* (2015)
Electrospinning	Polycaprolactone (PCL):polyethylene glycol (PEG) (1:1)	(*E*)-8,(*Z*)-8-dodecenyl acetate, (*Z*)-8-dodecanol	*Grapholita molesta*	Attract-and-kill strategy (in combination with cypermethrin as kill component)	Czarnobai De Jorge *et al.* (2017)
Electrospinning	Polycaprolactone, Cellulose acetate and Polyhydroxybutyrate	1,7-dioxaspiro-5.5-undecane, (*Z*)-7-tetradecenal	*Bactrocera oleae, Prays oleae*	Mass trapping (Attract formulation)	Kikionis *et al.* (2017)
Electrospinning	PLA	DEET	*Aedes aegypti*	Mosquito repellent, Textile integration	Annandarajah *et al.* (2019)
Electrospinning	Cellulose acetate, Polyvinylpyrrolidone	Citronella oil	*Aedes albopictus*	Mosquito repellent	Iliou *et al.* (2019)

Table 1.2 (continued)

Formulation	Material	Semiochemical	Insect species	Application	References
Blow spinning	Ethylene–vinyl acetate (EVA)	(*Z,Z*)-7,11-hexadecadienal and (*Z,Z,E*)-7,11,13-hexadecadienal, (*E*)-8-dodecenyl acetate, (*Z*)-8-dodecenyl acetate, *Z*-8-dodecenol	*Grapholita molesta, Phyllocnistis citrella*	Mating disruption	Rempel *et al.* (2019)
Electrospinning	Ethylcellulose (Ethocel) nanofibre	Citriodiol	*Aedes aegypti*	Mosquito repellent; Mats	Muñoz *et al.* (2019)
Electrospinning	Nylon-6,6 nanofibers	Picaridin	-	Mosquito and tick repellent	Ryan *et al.* (2020)
Cyclodextrin inclusion complexes					
Inclusion complex	β-Cyclodextrin	*(Z)*-7-tetradecenal, (*E)*-11-hexadecenal, (*E)*-11-hexadecenyl acetate	*Prays oleae, Palpita unionalis*	Mating disruption (in delta sticky traps or polyethylene vials)	Mazomenos *et al.* (2002)
Inclusion complex	β-Cyclodextrin	(*Z*)-tetradec-7-en-1-al	*Prays oleae*	slow release of sex pheromone of olive pest	Yannakopoulou *et al.* (2002)
Inclusion complex	β-Cyclodextrin	DEET	*Aedes aegypti and Anopheles stephensi*	Mosquito repellent (Textile impregnation)	Romi *et al.* (2005)
Inclusion complex	Monochlorotriazinyl-β-Cyclodextrin (MCT-β-CD)	Limonene	Mosquitoes	Mosquito repellent (Textile impregnation)	Hebeish *et al.* (2008)
Inclusion complex	β-Cyclodextrin (BHT stabilized)	(*Z*)-7-tetradecenal (*Z*)-7-14:Ald)	*Prays oleae*	Mating disruption (Polypropylene tube dispenser)	Hegazi *et al.* (2009)
Inclusion complex - Kneading method	β-Cyclodextrin	Citronella oil, Citronellal, Citronellol	*Aedes aegypti*	Mosquito repellent; Lotion for human skin application	Songkro *et al.* (2012)

Table 1.2 (continued)

Formulation	Material	Semiochemical	Insect species	Application	References
Inclusion complex - Kneading method	β-Cyclodextrin	Eucalyptol	*Hyalomma marginatum*	Tick repellent for cotton fabric impregnation	Inceboz *et al.* (2015)
Inclusion complex	β-Cyclodextrin	*Melaleuca alternifolia* oil	*Rhipicephalus australis*	Cattle tick repellent	Yim *et al.* (2016)
Inclusion complex	β-Cyclodextrin	Citronella oil	Mosquitoes	Mosquito repellent (Textile impregnation)	Lis *et al.* (2018)
Inclusion complex -	β-Cyclodextrin	Essential oils of cedarwood, clove, eucalyptus, peppermint, lavender and jasmine	*Anopheles stephensi*	Mosquito repellent (Textile impregnation)	Khanna and Chakraborty (2018)
Inclusion complex -	γ-Cyclodextrin	*Elsholtzia ciliata* oil, *Ilex chinensis* oil	*Blattella germanica*	Cockroach repellent	Huang *et al.* (2020)
Inclusion complex + UV irradiation	β-Cyclodextrin percinnamate	1,7-dioxaspiro-5,5-undecane	-	-	Arad-Yellin *et al.* (2001)
Inclusion complex - kneading method	β-Cyclodextrin	Carvacrol and linalool	*Tetranychus urticae*	Mite repellent	Campos *et al.* (2018)
Inclusion complex -	β-Cyclodextrin	Linalool	-	-	Lopez *et al.* (2012)

Table 1.2 (continued)

Formulation	Material	Semiochemical	Insect species	Application	References
Zeolite					
Adsorption	Zeolites differing in their Si/Al ratio, polarity, compensating cation, Brönsted acidity and pore size	*n*-decanol, Trimedlure (tert-butyl 4(5)chloro-2-methylcyclohexane carboxylate)	*Agrotis segetum, Cydia pomonella, Ceratitis capitata*	-	Muñoz-Pallares *et al.* (2001)
Adsorption	SMZ-A (cetyltrimethylammonium ion (=surfactant)-modified zeolite A)	(*E*)-2-hexenyl-(*Z*)-3-hexenoate (*E,Z*), (*E*)-2-hexenyl-(*E*)-2 hexenoate (*E,E*), Myristyl isobutyrate	*Riptortus pedestris*	Aggregation pheromone trap	Seo *et al.* (2016)
Adsorption	ZSM-5	Rhynchophorol (2-methyl-5(*E*)-heptene-4-ol)	*Rhynchophorus palmarum*	Trapping	Ramos *et al.* (2017)
Adsorption	Composite membranes (PBAT:Zeolite Y)	Rhynchophorol (2-methyl-5(*E*)-heptene-4-ol)	*Rhynchophorus palmarum*	-	Correia *et al.* (2018)
Adsorption	Zeolite L, Na-magadiite	Rhynchophorol (2-methyl-5(*E*)-heptene-4-ol) or 6-methyl-2-hepten-4-ol	*Rhynchophorus palmarum*	-	Viana *et al.* (2018)
Sepiolite					
Adsorption	Sepiolite tablets	-	-	-	Corma *et al.* (2000)
Adsorption	Sepiolite tablets	Sex pheromone of *Aonidiella aurantii*	*Aonidiella aurantii*	Mating disruption	Vacas *et al.* (2009)

Table 1.2 (continued)

Formulation	Material	Semiochemical	Insect species	Application	References
Metal-organic frameworks					
MOF	Zinc(II) and zirconium(IV) metal–organic frameworks ([Zn4O(bdc)3] (IRMOF-1),19,22 [Zn4O(bdc-NH2)3](IRMOF-3),19,23 [Zn4O(bdc-NHPr)3] (IRMOF-NHPr), [Zn4O(bdc-NHBu)3] (IRMOF-NHBu) and [Zn4O(bdc-NHOc)3] (IRMOF-NHOc))	3-octanone, 4-methyl-3-heptanone (ant alarm pheromones)	*Atta* and *Acromyrmex* species *(Atta sexdens)*	Leaf-cutting ant control; Attract-and-Kill strategy; Trapping	Hamzah *et al.* (2020)
MOF	Copper-benzene-1,3,5-tricarboxylic acid (Cu-BTC)	DEET	Mosquitoes	Insect/Mosquito repellent; Textile impregnation	Emam and Abdelhameed (2017)
MOF	MIL-53(Al), MIL-88A(Al))	Geranic acid, citronellic acid, geraniol and IR3535	-	Insect repellent; Trichloroisocyanuric acid tablet for water treatment in swimming pools	Paseta *et al.* (2016)

Table 1.2 (continued)

Formulation	Material	Semiochemical	Insect species	Application	References
Silica gels					
Silica gel	TMOS/PTMOS	Peach twig borer pheromone, (8*E*,10*E*)-dodecadienol sex pheromone	*Cydia pomonella*	Mating disruption, hung in in funnel traps (IPS)	Zada *et al.* (2009)
Silica gel	TMOS/MTMOS, TMOS/ETMOS, TMOS/PTMOS	Trans-2-hexen1-al, cis-3-hexen-1-ol, 3,7-dimethylocta-1,6-dien3-ol	Black citrus aphid	Aphid attractant for trapping	Bian *et al.* (2014)
Silica gel	IBERSIL A-400	Geranic acid, citronellic acid, geraniol and IR3535	-	Insect repellent; Trichloroisocyanuric acid tablet for water treatment in swimming pools	Paseta *et al.* (2016)
Photoremovable protection groups	Coumarin, pyrene, anthracene (=photoremovable protecting groups)	(*Z*)-11-hexadecen-1-ol	*Chilo infuscatellus*	Mating disruption	Atta *et al.* (2013)

Comparison of formulations

In order to make the right choice when selecting the formulation method taking into account the individual characteristics, it is advisable to subject it to a comparison of the advantages and disadvantages listed in **Table 1.3**. It must also be noted that not every formulation method is suitable for the intended application.

Table 1.3. Advantages and disadvantages of different formulation types

FORMULATION	ADVANTAGES	DISADVANTAGES
Passive dispensers	• High loading capacity • Universal applicability	• Manual application • Non-degradability
Active aerosol dispenser	• Programmable release • High loading capacity • Release duration for the whole season • Universal applicability	• Manual application • Aerosol dispenser equipment required • Non-degradability
Biopolymer-based microcapsules	• Suitable for spray application • Incorporation into delivery systems • Flexible material selection	• Low loading (per unit) • Short-term release duration
Yeast cell wall-microcapsules	• Available from cheap industrial by-product waste	• Low loading (per unit) • Low release variation • Drying equipment required
Photosensitive MCs	• UV triggered controlled release	• Expertise on synthesis required • Toxicity of TiO_2
Alginate-based formulations	• Biocompatibility and biodegradability • Cheap and easy preparation procedure • Mild preparation conditions	• Swelling and shrinkage • Release suppressed under high humidity • Low loading capacity
Zein nanoparticles	• Excellent protection from photodegradation • Biodegradability	• High energy preparation procedure • High speed homogenization equipment required • Use of solvents
Solid lipid nanoparticles	• Release less affected by moisture	• High energy preparation procedure • High speed homogenization equipment required • Short-term release duration
Nanogels	• Very high loading capacity • Release less affected by moisture • Easy preparation procedure	• Heat treatment during preparation
Paraffin emulsions	• High loading capacity	• Mineral oil based
Nanoemulsions	• Large surface area • High loading capacity • Dispersion in aqueous solutions without phase separation	• Burst release • Low physical stability (Ostwald ripening and coalescence)
Nanofibers	• Long-term zero-order release • Flexible material selection • High mechanical strength	• Electrospinning equipment and knowledge required • Use of solvents • High voltage
Cyclodextrins	• Biodegradability	• Expensive • Low loading capacity • Low release control • Use of solvents
Silicates (Zeolites, Sepiolites)	• Clay as degradation product • Cheap and easy preparation procedure	• Low release control
Metal-organic frameworks	• Modifiable architecture • High release control • Degradability	• Expertise on synthesis required
Silica gels	• Degradability	• Use of solvents
Photoremovable protection groups	• UV or light-triggered controlled release	• Expertise on synthesis required • Limited molecule spectrum

Future perspectives and conclusion

Most of the reviewed technologies, although partially successful in other fields, are at an early stage of development. Notable exceptions are well-established commercialized passive dispenser types, which are broadly used in agriculture, active aerosol dispensers, microcapsules and the paraffin-based emulsion SPLAT®. Novel developments always compete with these established well-working release systems and some of the studies showed that they are less appropriate in terms of costs and performance. If commercialization for practical large-scale application is the overall aim, scalability of the methods has to be considered. Safety and registration issues of nanoformulations, but also for not yet registered biobased or clay materials, is one of the most important obstacles. Consequently, some of them will be placed ad acta or remain stand-alone studies.

As most of the studies focused on model compounds or single semiochemicals of relevance for their target insect, the potential of the developments has to be proofed for their specific applicability. More materials and methods still warrant investigation. Many (bio)polymer options remain unexplored despite their potential to be an excellent base for semiochemical formulations. In view of formulation technologies for perfumes, flavors and fungicides, there are a lot of unexplored options to develop tailor-made formulations also for the slow or controlled release of semiochemicals. It can also be noted that many studies deal with slow release formulations, while a few studies address controlled release options in spite of fruitful material research in other disciplines such as pharmacy.

Some of the reviewed studies are fundamental studies of scientific interest on the general release potential without proposing applications. Future work is needed to demonstrate the potential of the new technologies in application studies. Moreover, some key aspects in formulation sciences as encapsulation efficiency and loading were often not reported. In the special case of methods requiring heating or high energy input during formulation preparation, loss of semiochemicals due to evaporation or degradation, which is also a problem for all technologies including a drying step, should be considered. Hence, the release duration reported as one of the most important properties is often only a few hours instead of several weeks, which is a major drawback of nanoparticles. This release behavior will be suitable in short-duration applications, such as mosquito repellent formulations. But this release duration is definitely too short for mating disruption or attract-and-kill strategies in agricultural insect pest control, since classical dispenser types are the benchmark, which perform for several weeks. In view of these considerations, it has to be emphasized that there is no “silver bullet” formulation for all insect species and applications that ensure a zero-order kinetic. The release behavior from most of the formulations developed up-to-date is mainly affected by temperature, resulting in waste of expensive substances even when the target insect is inactive. Thus, there is a need for further development of tailor-made formulations that release the semiochemical only when the target insect is active.

To conclude, more research efforts into new materials, methods and technologies are required to fill the gap between identification of new semiochemicals in chemical ecology and their successful application.

Author contributions

LCM wrote the manuscript; AVP edited the manuscript.

References

Akelah A (2013) Polymers in the controlled release of agrochemicals. In Akelah A (Eds.), *Functionalized Polymeric Materials in Agriculture and the Food Industry* (pp. 133-192). Springer, Boston, MA.

Ando T (2004) *The Chemistry Of Pheromones And Other Semiochemicals I*. Springer Science & Business Media.

Annandarajah C, Norris EJ, Funk R, Xiang CH, Grewell D, Coats JR, Mishek D & Maloy B (2019) Biobased plastics with insect-repellent functionality. *Polymer Engineering and Science*, 59, 460-467. https://doi.org/10.1002/pen.25083.

Arad-Yellin R, Tsoucaris G & Green BS (2001) Reversible encapsulation of guests in modified cyclodextrins: studies with the beta-cyclodextrin percinnamate 1,7-dioxaspiro 5,5 undecane system. *Tetrahedron Letters*, 42, 1335-1337. https://doi.org/10.1016/s0040-4039(00)02170-5.

Arlian LG (2002) Arthropod allergens and human health. *Annual Review of Entomology*, 47, 395-433.

Atta S, Ikbal M, Boda N, Gauri SS & Singh NDP (2013) Photoremovable protecting groups as controlled-release device for sex pheromone. *Photochemical & Photobiological Sciences*, 12, 393-403. https://doi.org/10.1039/c2pp25118c.

Atterholt CA, Delwiche MJ, Rice RE & Krochta JM (1999) Controlled release of insect sex pheromones from paraffin wax and emulsions. *Journal of Controlled Release*, 57, 233-247. https://doi.org/10.1016/s0168-3659(98)00119-9.

Auffermann & Heck (2015) Pheromone dispenser made of plastic material, WO2015128428A1.

Balaji APB, Mishra P, Kumar RSS, Mukherjee A & Chandrasekaran N (2015) Nanoformulation of poly(ethylene glycol) polymerized organic insect repellent by PIT emulsification method and its application for Japanese encephalitis vector control. *Colloids and Surfaces B-Biointerfaces*, 128, 370-378. https://doi.org/10.1016/j.colsurfb.2015.02.034.

Baldessari M, Ioriatti C & Angeli G (2013) Evaluation of Puffer® CM, a release device of pheromone to control codling moth on apple in Italy. *IOBC-WPRS Bulletin*, 91, 199-204.

BASF https://www.agrar.basf.de/de/Produkte/Produktdetails/RAK%C2%AE-1-2-M.html.

Baxter IH (2007) A Trap or Dispenser. WO2007/110601A1.

Benelli G, Lucchi A, Thomson D & Ioriatti C (2019) Sex pheromone aerosol devices for mating disruption: Challenges for a brighter future. *Insects*, 10, 308.

Bhagat D, Samanta SK & Bhattacharya S (2013) Efficient management of fruit pests by pheromone nanogels. *Scientific Reports*, 3, 8. https://doi.org/10.1038/srep01294.

Bian L, Sun XL, Cai XM & Chen ZM (2014) Slow release of plant volatiles using sol-gel dispensers. *Journal of Economic Entomology,* 107, 2023-2029. https://doi.org/10.1603/ec14054.

Bishop JRP, Nelson G & Lamb J (1998) Microencapsulation in yeast cells. *Journal of Microencapsulation*, 15, 761-773. https://doi.org/10.3109/02652049809008259.

Bisotto-de-Oliveira R, De Jorge B, Roggia I, Sant'Ana J & Pereira C (2014) Nanofibers as a vehicle for the synthetic attactant TRIMEDLURE to be used for *Ceratitis capitata* Wied:(Diptera, Tethritidae) capture. *Journal of Research Updates in Polymer Science*, 3, 40-47.

Bisotto-De-Oliveira R, Morais RM, Roggia I, Silva SJ, Sant'Ana J & Pereira CN (2015) Polymers nanofibers as vehicles for the release of the synthetic sex pheromone of *Grapholita molesta* (Lepidoptera, Tortricidae). *Revista Colombiana De Entomologia,* 41, 262-269.

Bouchemal K, Briançon S, Perrier E & Fessi H (2004) Nano-emulsion formulation using spontaneous emulsification: solvent, oil and surfactant optimisation. *International Journal of Pharmaceutics,* 280, 241-251.

Bourbon AI, Pereira RN, Pastrana LM, Vicente AA & Cerqueira MA (2019) Protein-based nanostructures for food applications. *Gels*, 5, 17. https://doi.org/10.3390/gels5010009.

Bruce TJ & Pickett JA (2011) Perception of plant volatile blends by herbivorous insects–finding the right mix. *Phytochemistry*, 72, 1605-1611.

Burks CS & Thomson DR (2019) Optimizing efficiency of aerosol mating disruption for navel orangeworm (Lepidoptera: Pyralidae). *Journal of Economic Entomology*, 112, 763-771.

Burks CS & Thomson DR (2020) Factors affecting disruption of navel orangeworm (Lepidoptera: Pyralidae) using aerosol dispensers. *Journal of Economic Entomology*, 113, 1290-1298. https://doi.org/10.1093/jee/toaa010.

Butler LI & McDonough LM (1981) Insect sex-pheromones - evaporation rates of alcohols and acetates from natural-rubber septa. *Journal of Chemical Ecology*, 7, 627-633. https://doi.org/10.1007/bf00987710.

Campos EVR, Proenca PLF, Oliveira JL, Pereira AES, Ribeiro LND, Fernandes FO, Goncalves KC, Polanczyk RA, Pasquoto-Stigliani T, Lima R, Melville CC, Della Vechia JF, Andrade DJ & Fraceto LF (2018) Carvacrol and linalool co-loaded in beta-cyclodextrin-grafted chitosan nanoparticles as sustainable biopesticide aiming pest control. *Scientific Reports,* 8, 14. https://doi.org/10.1038/s41598-018-26043-x.

Cardé R & Minks A (1997) *Insect Pheromone Research: New Directions.* Springer Science & Business Media.

Cardé RT & Elkinton J (1984) Field trapping with attractants: methods and interpretation. In Hummel, HE, Miller TA (Eds.), *Techniques in Pheromone Research* (pp. 111-129). Springer, New York, NY. https://doi.org/10.1007/978-1-4612-5220-7_4.

Corma A, Muñoz-Pallares J & Primo-Yufera E (2000) Emitter of semiochemical substances supported on a sepiolite, preparation process and applications. WO2000002448A1.

Correia PRC, Ramos IG, Viana AC, Mascarenhas AJS, Sant'ana AEG, Goulart HF & Druzian JI (2018) Development of composite membrane PBAT: Zeolite Y for application as rhynchophorol release system. *Journal of Applied Polymer Science,* 135, 12. https://doi.org/10.1002/app.45757.

Czarnobai De Jorge B, Bisotto-de-Oliveira R, Pereira CN & Sant'Ana J (2017) Novel nanoscale pheromone dispenser for more accurate evaluation of *Grapholita molesta* (Lepidoptera: Tortricidae) attract-and-kill strategies in the laboratory. *Pest Management Science,* 73, 1921-1926.

Daems F, Bera F, Lorge S, Fischer C, Brostaux Y, Francis F, Lognay G & Heuskin S (2016) Impact of climatic factors on the release of E-beta-caryophyllene from alginate beads. *Biotechnologie Agronomie Societe Et Environnement,* 20, 130-142.

De Smedt C, Someus E & Spanoghe P (2015) Potential and actual uses of zeolites in crop protection. *Pest Management Science,* 71, 1355-1367. https://doi.org/10.1002/ps.3999.

Deisig N, Dupuy F, Anton S & Renou M (2014) Responses to pheromones in a complex odor world: sensory processing and behavior. *Insects,* 5, 399-422.

Del Valle EM (2004) Cyclodextrins and their uses: a review. *Process Biochemistry*, 39, 1033-1046.

Delwiche M, Atterholt C & Rice R (1998) Spray application of paraffin emulsions containing insect pheromones for mating disruption. *Transactions of the ASAE*, 41, 475.

Delwiche M, Krochta JM, Rice RE & Atterholt C (1999) Aqueous emulsion comprising biodegradable carrier for insect pheromones and methods for controlled release thereof. US6001346A.

Domb A, Marlinsky A, Maniar M & Teomim L (1995) Insect repellent formulations of N, N-diethyl-m-toluamide (deet) in a liposphere system: efficacy and skin uptake. *Journal of the American Mosquito Control Association*,11, 29-34.

ECHA (2020) https://echa.europa.eu/de/substance-information/-/substanceinfo/100.033.327: European Chemicals Agency.

Emam HE & Abdelhameed RM (2017) In-situ modification of natural fabrics by Cu-BTC MOF for effective release of insect repellent (N,N-diethyl-3-methylbenzamide). *Journal of Porous Materials*, 24, 1175-1185. https://doi.org/10.1007/s10934-016-0357-y.

Everett D (1972) Manual of symbols and terminology for physicochemical quantities and units, appendix II: Definitions, terminology and symbols in colloid and surface chemistry. *Pure and Applied Chemistry,* 31, 577-638.

Fathi M, Vinceković M, Jurić S, Viskić M, Režek Jambrak A & Donsì F (2019) Food-grade colloidal systems for the delivery of essential oils. *Food Reviews International*, 37(1), 1-45.

Fleischer J & Krieger J (2018) Insect pheromone receptors - key elements in sensing intraspecific chemical signals. *Frontiers in Cellular Neuroscience*, 12, 14. https://doi.org/10.3389/fncel.2018.00425.

Furukawa H, Cordova KE, O'Keeffe M & Yaghi OM (2013) The chemistry and applications of metal-organic frameworks. *Science,* 341(6149), 1230444

Grant GT, Morris ER, Rees DA, Smith PJ & Thom D (1973) Biological interactions between polysaccharides and divalent cations: the egg-box model. *FEBS Letters,* 32, 195-198.

Gregg PC, Del Socorro AP & Landolt PJ (2018) Advances in attract-and-kill for agricultural pests: beyond pheromones. *Annual Review of Entomology*, 63, 453-470.

Gupta A, Eral HB, Hatton TA & Doyle PS (2016) Nanoemulsions: formation, properties and applications. *Soft Matter*, 12, 2826-2841. https://doi.org/10.1039/c5sm02958a.

Hamzah HA, Rixson D, Paul-Taylor J, Doan HV, Dadswell C, Roffe GW, Sridhar A, Hobday CL, Wedd C, Duren T, Hughes WOH, Spencer J & Burrows AD (2020) Inclusion and release of ant alarm pheromones from metal-organic frameworks. *Dalton Transactions*, 49, 10334-10338. https://doi.org/10.1039/d0dt02047h.

Hebeish A, Fouda MMG, Hamdy IA, El-Sawy SM & Abdel-Mohdy FA (2008) Preparation of durable insect repellent cotton fabric: Limonene as insecticide. *Carbohydrate Polymers,* 74, 268-273. https://doi.org/10.1016/j.carbpol.2008.02.013.

Hegazi E, Konstantopoulou M, Herz A, Mazomenos B, Khafagi W, Agamy E, Zaitun A, Abd El-Aziz G, Showiel S & Abdel-Rahman S (2009) Is mating disruption effective in controlling the olive moth, *Prays oleae*? *Crop Protection*, 28, 181-189.

Hellmann C, Greiner A & Wendorff JH (2011) Design of pheromone releasing nanofibers for plant protection. *Polymers for Advanced Technologies*, 22, 407-413.

Heuskin S, Lorge S, Godin B, Leroy P, Frère I, Verheggen FJ, Haubruge E, Wathelet JP, Mestdagh M & Hance T (2012) Optimisation of a semiochemical slow-release alginate formulation attractive towards *Aphidius ervi* Haliday parasitoids. *Pest Management Science*, 68, 127-136.

Heuskin S, Rozet E, Lorge S, Farmakidis J, Hubert P, Verheggen F, Haubruge E, Wathelet JP & Lognay G (2010) Validation of a fast gas chromatographic method for the study of semiochemical slow release formulations. *Journal of Pharmaceutical and Biomedical Analysis*, 53, 962-972. https://doi.org/10.1016/j.jpba.2010.07.014.

Heuskin S, Verheggen FJ, Haubruge E, Wathelet JP & Lognay G (2011) The use of semiochemical slow-release devices in integrated pest management strategies. *Biotechnologie Agronomie Societe Et Environnement,* 15, 459-470.

Horcajada P, Gref R, Baati T, Allan PK, Maurin G, Couvreur P, Ferey G, Morris RE & Serre C (2012) Metal-organic frameworks in biomedicine. *Chemical Reviews*, 112, 1232-1268. https://doi.org/10.1021/cr200256v.

Howse P, Stevens I & Jones O (1997) *Insect pheromones and their use in pest management.* Springer Science & Business Media.

Hsieh W-C, Chang C-P & Gao Y-L (2006) Controlled release properties of chitosan encapsulated volatile citronella oil microcapsules by thermal treatments. *Colloids and Surfaces B: Biointerfaces*, 53, 209-214.

https://www.iscatechnologies.com/collections/lures.

https://www.suterra.com/products_pest, Vol. 2021: Suterra.

Huang K, Zhang D, Ren J-J, Dong R & Wu H (2020) Screening of the repellent activity of 12 essential oils against adult german cockroach (Dictyoptera: Blattellidae): Preparation of a sustained release repellent agent of binary oil-γ-cd and its repellency in a small container. *Journal of Economic Entomology,* 113(5), 2171-2178.

Hummel HE & Miller TA (1984) *Techniques in pheromone research.* Springer Series in Experimental Entomology, Springer, USA.

Iliou K, Kikionis S, Petrakis PV, Ioannou E & Roussis V (2019) Citronella oil-loaded electrospun micro/nanofibrous matrices as sustained repellency systems for the Asian tiger mosquito *Aedes albopictus*. *Pest Management Science*, 75, 2142-2147.

Inceboz T, Erkan G, Turkoglu GC, Sariisik AM, Bakirci S, Uner S & Uner A (2015) In-vivo and in-vitro tick repellent properties of cotton fabric. *Textile Research Journal*, 85, 2071-2082. https://doi.org/10.1177/0040517515578326.

İşcan Y, Wissing S & Müller R (2005) Solid lipid nanoparticles (slntm) for topical drug delivery: Incorporation of the lipophilic drugs n, n-diethyl-m-toluamide and vitamin K. *Die Pharmazie-An International Journal of Pharmaceutical Sciences,* 60, 905-909.

IZA (2020) http://www.iza-online.org/.

Kandimalla VB, Tripathi VS & Ju H (2006) Immobilization of biomolecules in sol–gels: biological and analytical applications. *Critical Reviews in Analytical Chemistry*, 36, 73-106.

Kasaai MR (2018) Zein and zein -based nano-materials for food and nutrition applications: A review. *Trends in Food Science & Technology,* 79, 184-197. https://doi.org/10.1016/j.tifs.2018.07.015.

Kasting GB, Bhatt VD & Speaker TJ (2008) Microencapsulation decreases the skin absorption of N, N-diethyl-m-toluamide (DEET). *Toxicology in Vitro*, 22, 548-552.

Khanna S & Chakraborty JN (2018) Mosquito repellent activity of cotton functionalized with inclusion complexes of beta-cyclodextrin citrate and essential oils. *Fashion and Textiles*, 5, 18. https://doi.org/10.1186/s40691-017-0125-x.

Kikionis S, Ioannou E, Konstantopoulou M & Roussis V (2017) Electrospun micro/nanofibers as controlled release systems for pheromones of *Bactrocera oleae* and *Prays oleae*. *Journal of Chemical Ecology*, 43, 254-262. https://doi.org/10.1007/s10886-017-0831-2.

Kovanci OB, Knight A & Larsen T (2011) Combined sprays of sex pheromone and insecticides to attract-and-kill codling moth. *IOBC/WPRS Bulletin,* 72, 83-88.

Krittika S, Indhumathi P, Hari BNV, Devi DR & Yadav P (2019) Evidence of nanoemulsion as an effective control measure for fruit flies *Drosophila melanogaster*. *Scientific Reports,* 9, 11. https://doi.org/10.1038/s41598-019-47045-3.

Lakatos F & Tuba K (2011) The use of pheromones in the Hungarian forest management. *IOBC/WPRS Bulletin*, 72, 15-21.

Leal WS (2013) Odorant reception in insects: roles of receptors, binding proteins, and degrading enzymes. *Annual Review of Entomology,* 58, 373-391.

Lehane MJ & Lehane M (2005) *The biology of blood-sucking insects*. Cambridge University Press. https://doi.org/10.1017/CBO9780511610493.

Li Y, Li L & Yu J (2017) Applications of zeolites in sustainable chemistry. *Chem,* 3(6), 928-949.

Liebhold AM, McCullough DG, Blackburn LM, Frankel SJ, Von Holle B & Aukema JE (2013) A highly aggregated geographical distribution of forest pest invasions in the USA. *Diversity and Distributions,* 19, 1208-1216.

Lima AD, Landulfo GA & Costa LM (2019) Repellent effects of encapsulated carvacrol on the *Rhipicephalus* (Boophilus) *microplus* (Acari: Ixodidae). *Journal of Medical Entomology*, 56, 881-885. https://doi.org/10.1093/jme/tjy240.

Lis MJ, Carmona OG, Carmona CG & Bezerra FM (2018) Inclusion complexes of citronella oil with cyclodextrin for controlled release in biofunctional textiles. *Polymers*, 10, 14. https://doi.org/10.3390/polym10121324.

Lopez M, Maudhuit A, Pascual-Villalobos M & Poncelet D (2012) Development of formulations to improve the controlled-release of linalool to be applied as an insecticide. *Journal of Agricultural and Food Chemistry*, 60, 1187-1192.

Madene A, Jacquot M, Scher J & Desobry S (2006) Flavour encapsulation and controlled release - a review. *International Journal of Food Science & Technology,* 41, 1-21. https://doi.org/10.1111/j.1365-2621.2005.00980.x.

Maji TK & Hussain MR (2009) Microencapsulation of *Zanthoxylum limonella* oil (ZLO) in genipin crosslinked chitosan–gelatin complex for mosquito repellent application. *Journal of Applied Polymer Science,* 111, 779-785.

Mao L, Lu Y, Cui M, Miao S & Gao Y (2020) Design of gel structures in water and oil phases for improved delivery of bioactive food ingredients. *Critical Reviews in Food Science & Nutrition*, 60, 1651-1666.

Marques HMC (2010) A review on cyclodextrin encapsulation of essential oils and volatiles. *Flavour and Fragrance Journal,* 25, 313-326.

Marsin AM, Muhamad, II, Anis SNS, Lazim NAM, Ching LW & Dolhaji NH (2020) Essential oils as insect repellent agents in food packaging: a review. *European Food Research and Technology*, 246, 1519-1532. https://doi.org/10.1007/s00217-020-03511-1.

Martins E, Poncelet D, Rodrigues RC & Renard D (2017) Oil encapsulation techniques using alginate as encapsulating agent: applications and drawbacks. *Journal of Microencapsulation,* 34, 754-771. https://doi.org/10.1080/02652048.2017.1403495.

Mazomenos BE, Pantazi-Mazomenou A & Stefanou D (2002) Attract and kill of the olive fruit fly *Bactrocera oleae* in Greece as a part of an integrated control system. *IOBC/WPRS Bulletin,* 25, 137-146.

McDonough LM (1991) Controlled release of insect sex-pheromones from a natural-rubber substrate. *ACS Symposium Series,* 449, 106-124.

Mehnert W & Mäder K (2012) Solid lipid nanoparticles: production, characterization and applications. *Advanced Drug Delivery Reviews*, 64, 83-101.

Müller M & Buchbauer G (2011) Essential oil components as pheromones. A review. *Flavour and Fragrance Journal,* 26(6), 357-377.

Müller RH, Radtke M & Wissing SA (2002) Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. *Advanced Drug Delivery Reviews*, 54, S131-S155.

Muñoz-Pallares J, Corma A, Primo J & Primo-Yufera E (2001) Zeolites as pheromone dispensers. *Journal of Agricultural and Food Chemistry*, 49, 4801-4807. https://doi.org/10.1021/jf010223o.

Muñoz V, Buffa F, Molinari F, Hermida LG, García JJ & Abraham GA (2019) Electrospun ethylcellulose-based nanofibrous mats with insect-repellent activity. *Materials Letters*, 253, 289-292.

Muzzarelli RAA (2009) Genipin-crosslinked chitosan hydrogels as biomedical and pharmaceutical aids. *Carbohydrate Polymers,* 77, 1-9. https://doi.org/10.1016/j.carbpol.2009.01.016.

Narawi MM, Chiu HI, Yong YK, Zain NNM, Ramachandran MR, Tham CL, Samsurrijal SF & Lim V (2020) Biocompatible nutmeg oil-loaded nanoemulsion as phyto-repellent. *Frontiers in Pharmacology*, 11, 15. https://doi.org/10.3389/fphar.2020.00214.

Nogueira JAP, Figueiredo A, Duarte JL, de Almeida FB, Santos MG, Nascimento LM, Fernandes CP, Mourao SC, Toscano JHB, Rocha LM & Chagas ACS (2020) Repellency effect of *Pilocarpus spicatus* A. St.-Hil essential oil and nanoemulsion against *Rhipicephalus microplus* larvae. *Experimental Parasitology*, 215, 5. https://doi.org/10.1016/j.exppara.2020.107919.

Nussinovitch A (2010) Beads and special applications of polymers for agricultural uses. In Nussinovitch A (Eds.), *Polymer macro-and micro-gel beads: Fundamentals and applications.* (pp. 231-253), Springer.

Ogawa K, Yamamoto A, Aiba N & Nagura S (1989) Sustained-release dispenser of sex pheromone of insects. US8834910B2.

Oliveira JLd, Campos EnV, Pereira AE, Pasquoto T, Lima R, Grillo R, Andrade DJd, Santos FAd & Fraceto LF (2018) Zein nanoparticles as eco-friendly carrier systems for botanical repellents aiming sustainable agriculture. *Journal of Agricultural and Food Chemistry*, 66, 1330-1340.

Osanloo M, Arish J & Sereshti H (2019) Developed methods for the preparation of electrospun nanofibers containing plant-derived oil or essential oil: a systematic review. *Polymer Bulletin*, 77(11), 6085-6104.

Paine TD, Raffa KF & Harrington TC (1997) Interactions among scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology,* 42, 179-206. https://doi.org/10.1146/annurev.ento.42.1.179.

Pardeike J, Hommoss A & Müller RH (2009) Lipid nanoparticles (SLN, NLC) in cosmetic and pharmaceutical dermal products. *International Journal of Pharmaceutics*, 366, 170-184.

Pascoli M, de Lima R & Fraceto LF (2018) Zein nanoparticles and strategies to improve colloidal stability: A mini-review. *Frontiers in Chemistry*, 6, 6. https://doi.org/10.3389/fchem.2018.00006.

Pascual-Villalobos MJ, Canto-Tejero M, Vallejo R, Guirao P, Rodriguez-Rojo S & Cocero MJ (2017) Use of nanoemulsions of plant essential oils as aphid repellents. *Industrial Crops and Products*, 110, 45-57. https://doi.org/10.1016/j.indcrop.2017.05.019.

Paseta L, Potier G, Abbott S & Coronas J (2015) Using Hansen solubility parameters to study the encapsulation of caffeine in MOFs. *Organic & Biomolecular Chemistry*, 13, 1724-1731.

Paseta L, Simon-Gaudo E, Gracia-Gorria F & Coronas J (2016) Encapsulation of essential oils in porous silica and MOFs for trichloroisocyanuric acid tablets used for water treatment in swimming pools. *Chemical Engineering Journal*, 292, 28-34. https://doi.org/10.1016/j.cej.2016.02.001.

Pinto IC, Cerqueira-Coutinho CS, Santos EP, Carmo FA & Ricci-Junior E (2017) Development and characterization of repellent formulations based on nanostructured hydrogels. *Drug Development and Industrial Pharmacy*, 43, 67-73.

Giamberini M, Prieto SF & Tylkowski B (2015) *Microencapsulation: innovative applications.* De Gruyter.

Puglia C, Bonina F, Castelli F, Micieli D & Sarpietro MG (2009) Evaluation of percutaneous absorption of the repellent diethyltoluamide and the sunscreen ethylhexyl p-methoxycinnamate-loaded solid lipid nanoparticles: an in-vitro study. *Journal of Pharmacy and Pharmacology*, 61, 1013-1019.

Ramos-de-la-Pena AM, Renard C, Montanez J, Reyes-Vega MD & Contreras-Esquivel JC (2016) A review through recovery, purification and identification of genipin. *Phytochemistry Reviews*, 15, 37-49. https://doi.org/10.1007/s11101-014-9383-z.

Ramos IG, Viana AC, dos Santos EL, Mascarenhas AJS, Sant'Ana AEG, Goulart HF, Druzian JI & Andrade HMC (2017) Synthesis, characterization and evaluation of MFI zeolites as matrixes for rhynchophorol prolonged release. *Microporous and Mesoporous Materials*, 242, 99-108. https://doi.org/10.1016/j.micromeso.2016.12.034.

Ray M, Gupta R & Rousseau D (2015) Properties and applications of multilayer and nanoscale emulsions. In Sabliov C, Chen H & Yada R (Eds.), *Nanotechnology and Functional Foods*: *Effective Delivery of Bioactive Ingredients* (p. 175). John Wiley & Sons.

Rempel SP, Engler LG, Soares MRF, Catafesta J, Moura S & Bianchi O (2019) Nano/microfibers of EVA copolymer obtained by solution blow spinning: Processing, solution properties, and

pheromone release application. *Journal of Applied Polymer Science*, 136, 8. https://doi.org/10.1002/app.47647.

Renou M (2014) Pheromones and general odor perception in insects. *Neurobiology of Chemical Communication*, 1, 23-56.

Ribeiro AD, Marques J, Forte M, Correia FC, Parpot P, Oliveira C, Pereira AI, Andrade L, Azenha C, Mendes A, Alves GM, Sousa CA & Tavares CJ (2016) Microencapsulation of citronella oil for solar-activated controlled release as an insect repellent. *Applied Materials Today*, 5, 90-97. https://doi.org/10.1016/j.apmt.2016.09.003.

Romi R, Nostro PL, Bocci E, Ridi F & Baglioni P (2005) Bioengineering of a cellulosic fabric for insecticide delivery via grafted cyclodextrin. *Biotechnology Progress*, 21, 1724-1730.

Ryan JJ, Casalini R, Orlicki JA & Lundin JG (2020) Controlled release of the insect repellent picaridin from electrospun nylon-6,6 nanofibers. *Polymers for Advanced Technologies*, 31(12), 3039-3047. https://doi.org/10.1002/pat.5028.

Sakulku U, Nuchuchua O, Uawongyart N, Puttipipatkhachorn S, Soottitantawat A & Ruktanonchai U (2009) Characterization and mosquito repellent activity of citronella oil nanoemulsion. *International Journal of Pharmaceutics*, 372, 105-111.

San Martin-Gonzalez MF (2015) Solid lipid nanoparticles and applications. In Sabliov C, Chen H & Yada R (Eds.), *Nanotechnology and Functional Foods: Effective Delivery of Bioactive Ingredients* (p.214), John Wiley & Sons.

Scher H (1977) Microencapsulated pesticides. In Scher H (Eds.) *Controlled Release Pesticides* (pp. 126-144). ACS Symposium Series, Vol. 53, Washington, DC, USA. https://doi.org/10.1021/bk-1977-0053.ch012

Schulz S (2005) *The chemistry of pheromones and other semiochemicals II.* Springer Science & Business Media.

Seo SM, Lee JM, Lee HY, An J, Choi SJ & Lim WT (2016) Synthesis of nanoporous materials to dispense pheromone for trapping agricultural pests. *Journal of Porous Materials*, 23, 557-562.

Shi G, Rao L, Yu H, Xiang H, Yang H & Ji R (2008) Stabilization and encapsulation of photosensitive resveratrol within yeast cell. *International Journal of Pharmaceutics*, 349, 83-93. https://doi.org/10.1016/j.ijpharm.2007.07.044.

Shorey HH, Sisk CB & Gerber RG (1996) Widely separated pheromone release sites for disruption of sex pheromone communication in two species of Lepidoptera. *Environmental Entomology*, 25, 446-451.

Smart LE, Aradottir GI & Bruce TJA (2014) *Role of Semiochemicals in Integrated Pest Management*. In Abrol DP (Eds.) *Integrated Pest Management: Current Concepts and Ecological Perspective* (pp. 93–109). Elsevier Academic Press Inc, San Diego.

Songkro S, Hayook N, Jaisawang J, Maneenuan D, Chuchome T & Kaewnopparat N (2012) Investigation of inclusion complexes of citronella oil, citronellal and citronellol with β-cyclodextrin for mosquito repellent. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, 72, 339-355.

Souza JM, Caldas AL, Tohidi SD, Molina J, Souto AP, Fangueiro R & Zille A (2014) Properties and controlled release of chitosan microencapsulated limonene oil. *Revista Brasileira De*

Farmacognosia-Brazilian Journal of Pharmacognosy, 24, 691-698. https://doi.org/10.1016/j.bjp.2014.11.007.

Stelinski LL, Gut LJ, Mallinger RE, Epstein D, Reed TP & Miller JR (2005) Small plot trials documenting effective mating disruption of oriental fruit moth by using high densities of wax-drop pheromone dispensers. *Journal of Economic Entomology*, 98, 1267-1274. https://doi.org/10.1603/0022-0493-98.4.1267.

Suckling D, Karg G, Rechcigl JE & Rechcigl NA (2000) Pheromones and other semiochemicals. In Rechcigl JE & Rechcigl NA (Eds.), *Biological and Biotechnological Control of Insect Pests* (pp. 63-99). CRC Press, Boca Raton, FL.

Tavares M, da Silva MRM, de Siqueira LBdO, Rodrigues RAS, Bodjolle-d'Almeida L, Dos Santos EP & Ricci-Júnior E (2018) Trends in insect repellent formulations: A review. *International Journal of Pharmaceutics*, 539, 190-209.

Tóth M, Szentkirályi F, Vuts J, Letardi A, Tabilio MR, Jaastad G & Knudsen GK (2009) Optimization of a phenylacetaldehyde-based attractant for common green lacewings (*Chrysoperla carnea*). *Journal of Chemical Ecology*, 35, 449-458.

Trematerra P (2012) Advances in the use of pheromones for stored-product protection. *Journal of Pest Science*, 85, 285-299.

Ullmann F, Gerhartz W, Yamamoto YS, Campbell FT, Pfefferkorn R & Rounsaville JF (1985) *Ullmann's Encyclopedia of Industrial Chemistry*.

Vacas S, Alfaro C, Navarro-Llopis V & Primo J (2009) The first account of the mating disruption technique for the control of California red scale, *Aonidiella aurantii* Maskell (Homoptera: Diaspididae) using new biodegradable dispensers. *Bulletin of Entomological Research*, 99, 415.

Vacas S, Navarro I, Primo J & Navarro-Llopis V (2016) Mating disruption to control the striped rice stem borer: pheromone blend, dispensing technology and number of releasing points. *Journal of Asia-Pacific Entomology*, 19, 253-259.

Valladares GA, Audino PG & Strumia MC (2016) Preparation and evaluation of alginate/chitosan microspheres containing pheromones for pest control of *Megaplatypus mutatus* Chapuis (Platypodinae: Platypodidae). *Polymer International,* 65, 216-223. https://doi.org/10.1002/pi.5049.

Vemmer M & Patel AV (2013) Review of encapsulation methods suitable for microbial biological control agents. *Biological Control*, 67, 380-389.

Viana AC, Ramos IG, dos Santos EL, Mascarenhas AJS, Lima MD, Sant'Ana AEG & Druzian JI (2018) Validation of analytical method for rhynchophorol quantification and stability in inorganic matrix for the controlled release of this pheromone. *Chemistry Central Journal*, 12, 9. https://doi.org/10.1186/s13065-018-0426-1.

Wadhwa G, Kumar S, Chhabra L, Mahant S & Rao R (2017) Essential oil-cyclodextrin complexes: an updated review. J*ournal of Inclusion Phenomena and Macrocyclic Chemistry*, 89, 39-58. https://doi.org/10.1007/s10847-017-0744-2.

Wang K, Liu J, Zhan Y & Liu Y (2019) A new slow-release formulation of methyl salicylate optimizes the alternative control of *Sitobion avenae* (Fabricius) (Hemiptera: Aphididae) in wheat fields. *Pest Management Science*, 75, 676-682.

Xu Q, Hatt S, Lopes T, Zhang Y, Bodson B, Chen J & Francis F (2018) A push–pull strategy to control aphids combines intercropping with semiochemical releases. *Journal of Pest Science*, 91, 93-103.

Yang Y (2005) Study on the inclusion compounds of eugenol with α-, β-, γ-and heptakis (2, 6-di-O-methyl)-β-cyclodextrins. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, 53, 27-33.

Yannakopoulou K, Ripmeester JA & Mavridis IM (2002) Release of the *Prays oleae* pheromone as a consequence of supramolecular structure: study of the beta-cyclodextrin-(Z)-tetradec-7-en-1-al complex by X-ray crystallography and NMR spectroscopy in the solid state and in solution. *Journal of the Chemical Society-Perkin Transactions*, 2, 1639-1644. https://doi.org/10.1039/b111632k.

Yim WT, Bhandari B, Jackson L & James P (2016) Repellent effects of *Melaleuca alternifolia* (tea tree) oil against cattle tick larvae (*Rhipicephalus australis*) when formulated as emulsions and in beta-cyclodextrin inclusion complexes. *Veterinary Parasitology*, 225, 99-103. https://doi.org/10.1016/j.vetpar.2016.06.007.

Yosha I, Shani A & Magdassi S (2008) Slow release of pheromones to the atmosphere from gelatin−alginate beads. *Journal of Agricultural and Food Chemistry*, 56, 8045-8049.

Zada A, Falach L & Byers JA (2009) Development of sol–gel formulations for slow release of pheromones. *Chemoecology*, 19, 37-45.

Oleogels

A gel type that gained increasing attention during the last decade as formulation matrix for reactive, lipophilic substances in cosmetic, pharmaceutical and food formulation technology are oleogels (O´Sullivan et al., 2016; Gom, 2001; Singh et al., 2013; Esposito et al., 2018; Park & Maleky, 2020; Mao et al., 2020). Oleogels are semi-solid systems, in which a liquid oil phase is entrapped by a structuring agent, the oleogelator, forming a three-dimensional network (Vintiliou & Leroux, 2008; Mao et al., 2020). They are a subclass of so called organogels, a broader class of colloidal systems immobilizing any organic solvent (Patel, 2015). In the literature also the terms molecular gel (Jadhav et al., 2011) or nanogel (Bhagat et al., 2013) can be read.

Dependent from the oleogelator, relatively large amounts of oil can be gelled by a low amount of the oleogelator (Patel, 2015). Different gelator molecules have been identified and evaluated in the last years (Co & Marangoni, 2018). Oleogelators can be classified based on their molecular weight into low-molecular-weight organogelators (LMOGs), such as 12-hydroxystearic acid, and high-molecular-weight oleogelators, mainly polymeric gelators, such as ethylcellulose (Flöter et al., 2021). LMOGs can be crystalline particles or self-assembled structures of low-molecular-weight compounds. Hybrid oleogels, gelled by different synergistically acting oleogelators, offer a variety of combinations and enable the modulation of gel properties (Pakseresht, & Mazaheri Tehrani, 2022).

When using polymeric oloegelators, different gel formation options are possible, which are illustrated in **Figure 1.7**. In the direct methods, the gelator is dispersed in the oil and gelation occurs after heating and cooling of the mixture. Other principles are emulsion based methods or a more labour-intensive method, the solvent exchange, in which after first preparing a hydrogel, the hydrophilic solvent is replaced by an oil phase (Martins et al., 2018).

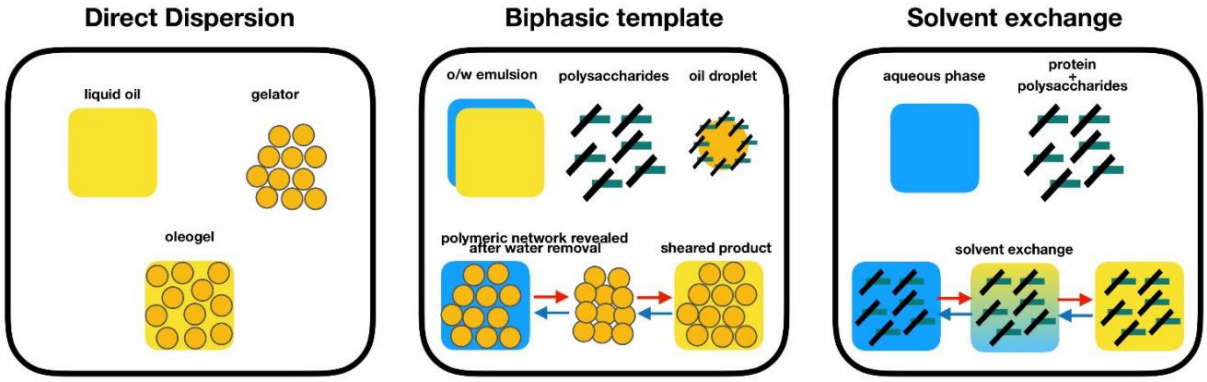


Figure 1.7. Principles of oleogelation using biopolymers (Martins et al., 2018).

One of the easiest methods is direct dispersion of ethylcellulose (EC) in oil. EC is a linear polysaccharide derived from regrowable cellulose. EC is prepared by replacement of the hydroxyl end groups by ethyl end groups. Therefore, cellulose is dissoluted in an alkali solution to break down the cellulose supramolecular structure. Afterwards, ethyl chloride gas is added, which interacts with the alkalized cellulose (Atalla and Isogai, 1998). The resulting degree of substitution (DS) is responsible for the EC properties: at low DS <1.5, EC is water soluble, at DS of 2.4 – 2.5 the EC is soluble in organic solvents (Koch, 1937).

The gelling properties of EC have first been described in 1991 by Aiache et al. (1991). Gel formation is induced by heating the dispersion of EC and oil above the glass transition temperature of the EC (~140°C). At this temperature, the polymer chains become unfolded. Under subsequent cooling, the unfolded polymer chains form a backbone of a cross-linked gel network. The gel is stabilized by self-assembly through hydrogen bonding among the polymer chains along with some hydrophobic interactions between the ester groups of the oil and the side chains of the ethyl cellulose. The oil is physically entrapped in the EC network (Davidovich-Pinhas et al., 2015).

Oleogels for entrapment of lipophilic compounds and volatiles

The attention that oleogels have gained as formulation matrices for lipophilic compunds can be attributed to the oleogel´s desirable properties for such substances: a high loading capacity (Vintiloiu & Leroux, 2008; Martin et al., 2017), a low risk for microbial contamination, due to the lack of an aquatic medium required for growth of most microbial contaminants (Vintiliou & Leroux, 2008) and extended shelf life of reactive compounds by improving oxidative stability (Shi et al., 2014; O´Sullivan et al., 2016).

Few attempts have been made aiming for a slowed or controlled release of volatile substances from oleogels (Yang et al., 2018; Yılmaz et al., 2015; Yılmaz & Demirci, 2021; Valls et al., 2020; Pang et al., 2021). For the release of semiochemicals for agricultural applications or at least volatiles than can act as semiochemicals, but were formulated for non-agricultural applications, only a few studies have been conducted: the model volatiles 2-heptanone and lauryl acetate were directly gelled with the LMOG mannitol dioctanoate by Jadhav et al. (2011); ethyl eugenol as fruit pest attractant was directly gelled by Bhagat et al. (2013) with a synthetized LMOG and a very long release duration was found of up to several weeks; linalool, citral and *Mentha arvensis* essential oil as fungicides for food preservation and hoof treatment were directly gelled with ethylcellulose by Yogev & Mizrahi (2020), but they were only interested in a short-term release duration of up to some hours.

To the best of my knowledge, there is no study about the development of an ethylcellulose oleogel for the slow and controlled release of semiochemicals for application in insect pest behavior manipulating strategies.

β-caryophyllene as semiochemical

During its migratory flights and host finding behavior *C. picta* orientates by volatile cues released by its host plant *Malus* spp. Moreover, it was found, that phytoplasma-infections alter the volatile emission and host-finding behavior of infected plants and vector insects (Gallinger, 2019; Mayer, 2008a; b). Mayer et al. (2008a; b) have found that young *C. picta* emigrants are more attracted by the scent of infected trees than by the scent of healthy trees. This means that the apple proliferation phytoplasma alters the scent of apple trees in such a way that the psyllids are attracted to the infected trees in early summer and acquire the phytoplasma before emigrating to their overwintering host plants. During the overwintering, the phytoplasmas multiply in the insect and invade the salivary glands. When returning in spring, the eggs are preferentially laid on uninfected plants. In the process, the plants are pierced with the proboscis

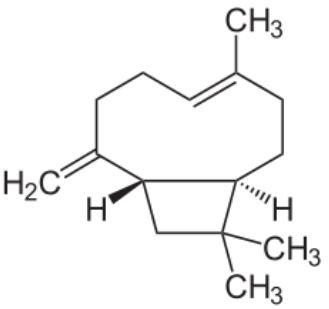


Figure 1.8. Chemical structure of β-caryophyllene. Structural formula was obtained as open source from Wikipedia.org.

before and during egg laying, thus transferring the phytoplasmas from the saliva. As a result, the phytoplasma spread in the apple orchard increases.

As one of the key substances released by the infected apple trees and involved in the strong attraction of *C. picta* the sesquiterpene β-caryophyllene (**Figure 1.8**) was identified (Mayer et al., 2008a).

Such semiochemicals are suitable candidates for application in attract-and-kill or push-pull-kill strategies. As the use of semiochemicals is restricted due to their high volatility and chemical instability, formulation is needed.

KILL

Entomophthorales as biocontrol agents

Between 700 and 1000 species of fungi are recognized as pathogens of insects (Goettel et al., 2010; Vega et al., 2012). They are found all over the world and play an important role as natural regulators of insect populations (Keller, 2006). Entomopathogenic fungi have been considered as promising environmentally friendly alternative to synthetic chemical insecticides (Glare et al., 2012; Lacey et al., 2015). Unlike other entomopathogenic microorganisms, the infection of the insect by an entomopathogenic fungus takes place percutaneously, not perorally (Jaronski, 2014). Thus, when entomopathogenic fungi are applied as biocontrol agents, they are not actively taken up by the target insect, but an external contact with the infection units is sufficient to induce an infection.

More than 170 entomopathogenic fungal strains have been commercialized as biocontrol agents (Jaronski, 2014). The majority of these products contain species of the hypocrealean fungi *Beauveria* and *Metarhizium* and have proven to be effective bioinsecticides for the control of a variety of agriculturally and horticulturally relevant insect pests (De Faria & Wraight, 2007; Lacey, 2007; Jaronski, 2014). They are mostly less specialized with characteristically broad host ranges, occur mainly in the soil and in other protected places and can easily be cultivated. They are hemibiotrophic, as they switch from a biotrophic phase in the hemocoel of the living insect to a saprophytic phase when colonizing the insect´s body after death. The insect death is usually achieved by organ damage, enzymatic activity, exhaustion of nutrients and toxic metabolites (Hajek & St. Leger, 1994; Roy et al., 2006; Goettel et al., 2001; Strasser et al., 2000).

Another group of entomopathogenic fungi, the Entomophthorales, bear some highly advantageous characteristics that make them suitable candidates as biocontrol agents. Compared to hypocrealean fungi, species of the Entomophthorales are primarily occurring above-ground, are highly specialized and very virulent species (Keller et al., 2006), are known for their strict host specificity (Eilenberg & Michelsen 1999; Jensen et al. 2001; Keller 2007), their fast speed-to-kill (Pell et al., 2001) and for their ability to cause epizootics (Jaques & Patterson 1962; Vega et al., 2012; Eilenberg et al., 2019). The high specificity towards particular host species reduces the risk towards non-target organisms (Latgé & Papierok, 1988).

A main characteristic of the Entomophthorales is the active discharge of conidia, the asexual spores that are the infection units of these fungi. Many insects infected by Entomophthorales are known to be induced by the fungus to move to high plant parts or exposed sites shortly before death. This

phenomenon is called ´summit disease´ and was described for many entomophthoralean species (Roy et al., 2006; Elya et al., 2018). Summiting occurs mainly in the late afternoon or evening hours. This has two advantages for the fungus: The death of the insect occurs in the evening hours. The dew allows the fungus to take up moisture quickly and sufficiently for sporulation. Due to the exposed position, the actively discharged spores spread over a large area, they are also exposed to the wind and can be further dispersed (Keller, 2008). After landing, the conidia stick, by aid of mucoid substances and a partly detached cell wall, to surfaces like the host insect cuticle (Keller, 1991; Olsen et al., 2019). When landing on non-suitable surfaces, ´primary conidia´ are able to form ´secondary conidia´ or higher orders. When landing on the host insect cuticle, the germing tubes penetrate, by enzymatic activity and mechanical force, the insect integument to enter the insect´s hemolymph. Within the hemolymph, the entomophthorales multiply as cell-wall less protoplasts (Butt et al., 1981), to overcome the insect immune system by avoidance of detection of fungal cell-wall components or as hyphal bodies (Beauveais & Latgé, 1988). Under unfavorable conditions the zygo- or azygospores may become thick-walled resting spores to overcome e.g. the absence of the host insect during winter time (Scorsetti et al., 2012). After metabolization of essential not yet identified nutrients from the insect hemolymph and body, the fungus starts to grow out of the insect, form conidiophores on the insect surface and rhizoids on the lower part of the insect body to fix it for optimal deposition of the sporulating cadaver and the infection cycle starts all over again.

Fermentation and mass production of Entomophthorales

A requirement for the establishment of a fungus as biocontrol agent in agriculture is the mass-production of fungal biomass, which is essential to provide inoculant in a sufficient quantity for large scale field application (Shah & Pell, 2003; Jaronski, 2014). It is a general assumption, that insect pest control by entomopathogenic fungus is successful, when a sufficient amount of inoculum is applied (Jackson et al., 2010).

Different mass-production methods have been established and optimized during the last decades to maximize viable and virulent biomass at lowest possible costs. Solid state fermentation is the most utilized method for the production of aerial conidia of many hypocrealean entomopathogenic fungi, which naturally produce those infection units (Jaronski & Mascarin, 2017).

For entomophthoralean fungi, whose conidia are difficult to collect, submerged fermentation processes for the production of hyphal bodies or mycelia is superior. Furthermore, submerged fermentation processes allow for short fermentations times, high yields, control of the biomass morphology and can easily be scaled up for mass production (Jackson, 1997; Ravensberg, 2011; Jaronski, 2014).

Fungi from the Entomophthorales have often been reported to be difficult to grow in vitro because of their special nutritional requirements (Latgé, 1981; Eilenberg et al., 1992; Wilding & Latteur, 1987; Papierok & Hajek, 1997; Pell et al., 2001; Hajek et al., 2012; Jaronski, 2014). The most commonly used media for isolation and laboratory scale growth in vitro contain complex nutrient sources like egg yolk (Latgé, 1981; Keller, 1997; Freimoser et al., 2001) supplemented with fetal bovine serum or vitamins and amino acid. Thus, the difficulty to grow an entomophthoralean fungus on technical scale in vitro at low costs remains one of the main challenges on the way to utilization as biocontrol agent.

A more detailed introduction, history and state-of-the-art of the mass production of the Entomophthorales is given in **Chapter 4**.

Formulation of entomopathogenic fungi

Since the switch from hyphal body to mycelia growth is required for sporulation (Freimoser et al., 2003), a transfer of the fungal material produced in submerged culture to some kind of a solid stage is necessary.

Previous research has evaluated different types of propagules for the application of entomophthoralean fungi in insect pest control. The majority of field trials used in-vitro infected insect cadavers as propagules for the introduction of an entomophthoralean fungus into insect pest populations in the field or greenhouse (Wilding et al., 1990; Dara & Semptner, 2005; Dinu et al., 2016). This method lacks from its low potential for scale-up and commercialization. McCabe & Soper (1985) patented their 'dry-marcescent process' for the preparation of dried mycelial mats from which the fungus will sporulate after full rehydration.

Different formulations for various microbial biocontrol agents have been established and commercialized: wettable powders, granules, bates, water dispersable granules, contact powder, suspension concentrate, oil miscible concentrate, ultra-low volume suspensions, oil dispersions (de Faria & Wraight, 2007) for drenching, spraying, baiting or as seed coating. Most commercialized fungal-based insecticide formulations are emulsifiable suspensions or wettable powders. Although unformulated spores with a small separate quantity of wetting agent are sold (Jaronski, 2014; Jaronski & Mascarin, 2017).

A biomaterial that has gained much attention in encapsulation of living cells is calcium alginate (Nussinovitch, 2010; Vemmer & Patel, 2013). Alginate refers to the salts of alginic acids, first described in 1881 in a patent by Stanford (1881). Alginic acids can be found in various marine brown algae (Phaeophyceae) acting as structuring agents and cell wall stabilizers (Helgerud et al., 2010). It forms flexible gels with calcium, magnesium, strontium and barium ions (Rehm, 2009; Thu et al., 1996). Globally production of alginate is 38,000 t per year (Helgerud et al., 2010). Commonly, alginate is traded and used in form of sodium alginate, the water-soluble sodium salt of alginic acid. Alginates are high-molecular (molecular weight 10-600 kDa) polysaccharides based on α-L-guluronic acid and β-D-mannuronic acid, 1,4-glycosidally connected to linear chains. The homopolymeric regions composed of α-L-guluronic acid are referred to as G-blocks, areas composed of β-D-mannuronic acid as M-blocks, heteropolymeric areas as GM-blocks. The G-blocks are mainly involved in the gelation process, but also the GM-blocks. Dependent from the content/proportion of G- and M-blocks and concentration of cross-linker ions stronger or weaker gels are formed.

A commonly used cross-linker is a calcium chloride. Gelation occurs when sodium alginate, associated with monovalent Na^+ cations, gets in contact with divalent Ca^{2+} cations. The Na^+ ions are replaced by the divalent Ca^{2+} ions. The Ca^{2+} ions interact with the anionic carboxylgroups of opposite G-blocks of two different polymer chains. By the formation of cavities in which the Ca^{2+} ions are localized, the arrangement looks like eggs in an egg-box, the model of alginate gelation is often called ´egg-box model´ (**Figure 1.9**).

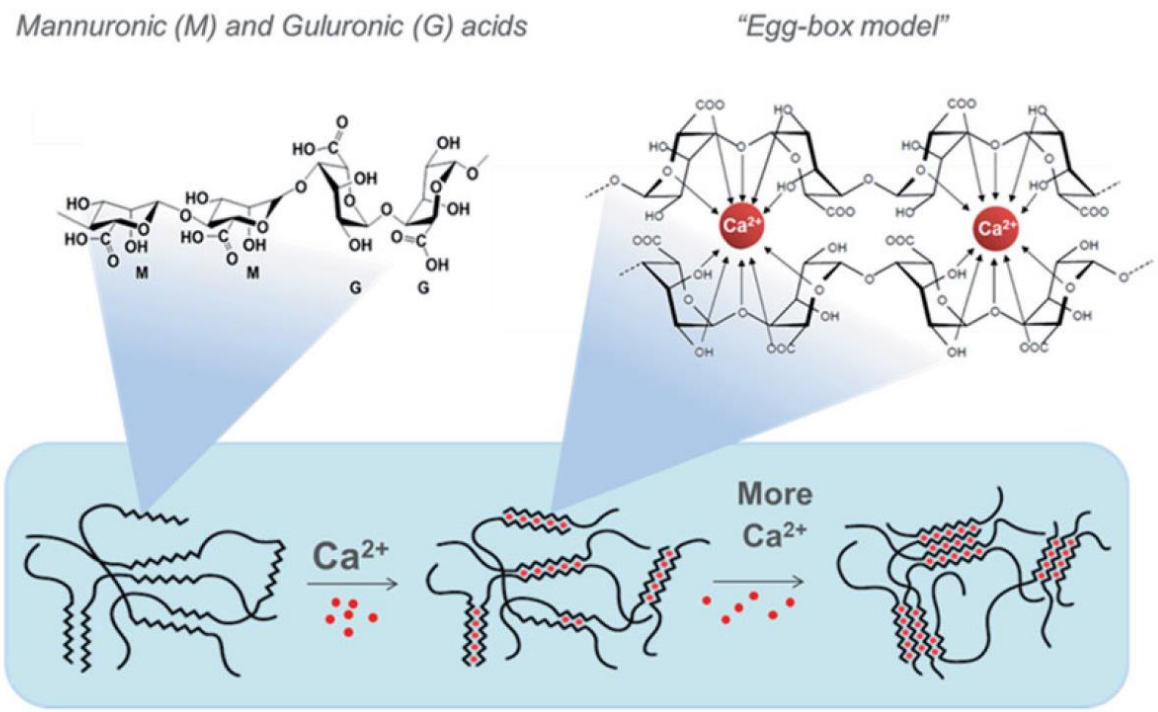


Figure 1.9. The "Egg-box model": Principle of alginate gelation. Martins et al. (2017).

There are various advantages making Ca-alginate a highly suitable encapsulation matrix for living fungal biocontrol agents and for their application in agriculture: gelation is possible at ambient temperatures, spherical beads can be produced from alginate by the use of simple techniques, water is used as reaction medium, it has been proven for living cells, it is not toxic to humans and the environment, it is vegan and it is a biobased material from renewable resources and is biodegradable, which is desired for applications in biological plant protection.

Formulation additives

Another advantage of Ca-alginate encapsulation is the ease to co-formulate the entomopathogenic fungus with beneficial formulation additives (Burges, 1998). Classical additives belong to the group of filling agents, which increase drying survival of entomopathogenic fungi (Przyklenk et al., 2017). Nutrients can improve growth and sporulation duration and intensity after field application by serving as a ´microfermenter´ formulation and, thus, saving valuable biomass in the production process (Pereira and Roberts, 1991; McLoughlin, 1994; Burges, 1998; Przyklenk et al., 2017). Also protective agents, such as UV-protectants, drying protectants or superabsorbents for improved rehydration (Zhou et al., 2009) or attractants for improved contact of the insect with the entomopathogenic fungus (Vemmer et al., 2016) are possible additives. Recent developments by Hermann et al. (2021) aimed for an enfastened mode of infection by improved water absorption due to the addition of additives that increased the capillarity of the EPF formulation matrix. The efficiency of EPF formulations can further be imporved by additives, that support the infection and colonization of the target organism or its enzymatic activity: Krell et al. (2018) demonstrated enhanced endophytism of an entomopathogenic *Metarhizium brunneum* in tomato plants by addition of cellulase in order to ease the entry of the fungus into the plant via the roots. Another class of additives are virulence-enhancing additives. Numerous publications highlight the importance of chitinolytic enzyme activity as a key feature of

entomopathogenic fungi for successful host insect cuticle penetration and the presence of chitin within the insect cuticle or within artificial substrates or formulations may improve virulence of entomopathogenic fungi on their host insects and, thus, chitin may serve as a virulence-enhancing formulation additive (Charnley & St. Leger, 1991; Charnley, 2003; Gerding-Gonzalez et al., 2007; Małagocka et al., 2015; Shah et al., 1999).

A more detailed state-of-the-art of the formulation of the Entomophthorales is given in **Chapter 5.**

Pandora sp. nov. inedit. (ARSEF 13372)

In 2016, a new entomophthoralean species of the genus *Pandora* (Entomophthorales:Entomophthoraceae) was isolated from infected *Cacopsylla* spp. collected in a Danish pear orchard (Jensen 2017; Jensen et al., 2018). The fungus is not yet named, but based on morphological characteristics of the conidia and DNA sequences of the ITS-region the fungus proved to be a new, not yet named species (Jensen, 2017; Görg et al., 2021b; Figure 1.10). *Pandora* species can be classified in the *Erynia* – *Pandora* – *Furia* genera clade (Gryganskyi et al., 2013). These fungi are obligate entomopathogens. The pathogenicity of *Pandora* sp. nov. has already been demonstrated for several psyllid species under laboratory conditions by exposure of the insects to sporulating mycelia grown on solid media (Jensen 2017; Jensen et al., 2018; Herren, 2018; Görg et al., 2021b).

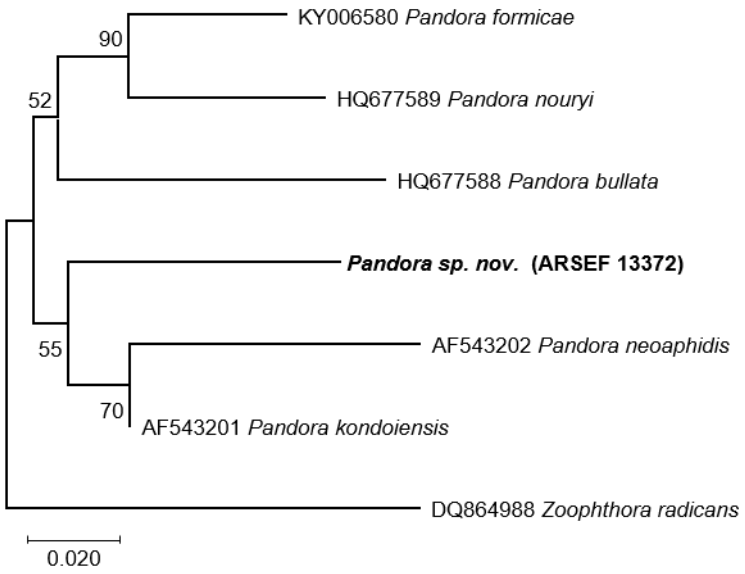


Figure 1.10. Neighbor-joining tree of the evolutionary history of *Pandora* sp. nov. (ARSEF 13372) based on the ITS sequence. Görg et al. (2021b).

Despite the potential of the Entomophthorales for insect pest control, until today, no Entomophthorales-based insecticide was established at all and in particular for psyllid pest control above ground in fruit trees.

References

Agelopoulos N, Birkett, MA, Hick AJ, Hooper AM, Pickett JA, Pow EM, Smart LE, Smiley DWM, Wadhams LJ & Woodcock CM (1999) Exploiting semiochemicals in insect control. Pesticide Science 55(3): 225-235.

Aiache JM, Gauthier P, Aiache S (1992) New gelification method for vegetable oils I: Cosmetic application. International Journal of Cosmetic Science 14(5): 228–234

Atalla RH, Isogai A (1998) Recent developments in spectroscopic and chemical characterization of cellulose. In Dumitriu S (ed.) Polysaccharides: structural diversity and functional versatility, 2nd Edition, Marcel Dekker, New York: 123–157.

Bai X, Zhang J, Ewing A, Miller SA, Jancso Radek A, Shevchenko DV, Tsukerman K, Walunas T, Lapidus A, Campbell JW & Hogenhout SA (2006) Living with genome instability: the adaptation of phytoplasmas to diverse environments of their insect and plant hosts. Journal of Bacteriology 188(10): 3682-3696.

Barthel D, Bianchedi P, Campisano A, Covelli LT, Dallago G, Ioriatti C, Jarausch W, Letschka T, Mittelberger C, Moser M, Öttl S, Österreicher J, Schweigkofler W, Tedeschi R, Unterthurner M, Janik K (2020) Plant hosts of apple proliferation phytoplasma. In Janik K, Barthel D, Oppedisano T, Anfora G (ed.) Apple proliferation: A joint review. Fondazione Edmund Mach: 9–32.

Beauvais A & Latgé JP (1988) A simple medium for growing entomophthoralean protoplasts. Journal of Invertebrate Pathology 51(2): 175-178.

Behr H-C (2019) AMI Markt Bilanz Obst 2019. Agrarmarkt Informations-Gesellschaft mbH, Bonn.

Bendix C & Lewis JD (2018) The enemy within: phloem-limited pathogens. Molecular Plant Pathology 19(1): 238-254.

Bertaccini A (2007) Phytoplasmas: diversity, taxonomy, and epidemiology. Frontiers in Bioscience-Landmark 12(2): 673-689.

Bhagat D, Samanta S & Bhattacharya S (2013) Efficient management of fruit pests by pheromone nanogels. Scientific Reports 3: 1294.

Blackburn D, Shapiro-Ilan DI, Adams BJ (2016) Biological control and nutrition: Food for thought. Biological Control 97: 131-138.

Bovey R (1961) Apple proliferation disease. Stations fédérales d'essais agricoles, Lausanne.

Bruce TJ & Pickett JA (2011) Perception of plant volatile blends by herbivorous insects – finding the right mix. Phytochemistry 72(13): 1605-1611.

Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL) (2022) Online-Datenbank Pflanzenschutzmittel: Verzeichnis zugelassener Pflanzenschutzmittel. https://www.bvl.bund.de/DE/Arbeitsbereiche/04_Pflanzenschutzmittel/01_Aufgaben/02_ZulassungPSM/01_ZugelPSM/psm_ZugelPSM_node.html. Accessed 2022/06/30

Bundesinformationszentrum Landwirtschaft (BZL; 2022). https://www.landwirtschaft.de/landwirtschaftliche-produkte/wie-werden-unsere-lebensmittel-erzeugt/pflanzlicheprodukte/aepfel.#:~:text=Die%20Anbaufl%C3%A4che%20von%20%C3%84pfeln%20liegt,70%20Prozent%20der%20gesamten%20Baumobstanbaufl%C3%A4che. Accessed: 06/25/2022

Burckhardt D & Ouvrard D (2012) A revised classification of the jumping plant-lice (Hemiptera: Psylloidea). Zootaxa 3509(1): 1-34.

Burges HD (1998) Formulation of microbial biopesticides: beneficial microorganisms, nematodes and seed treatments. Kluwer, Dordrecht.

Butt TM, Beckett A & Wilding N (1981) Protoplasts in the in vivo life cycle of *Erynia neoaphidis*. Microbiology 127(2): 417-421.

Chandler D, Bailey AS, Tatchell GM, Davidson G, Greaves J, Grant WP (2011) The development, regulation and use of biopesticides for integrated pest management. Philosophical Transactions of the Royal Society of London B: Biological Sciences 366(1573): 1987-1998.

Charnley AK & St. Leger RJ (1991) The role of cuticle-degrading enzymes in fungal pathogenesis in insects. In Cole GT, Hoch HC (ed.) The fungal spore and disease initiation in plants and animals. Springer, Boston, MA: 267-286.

Charnley AK (2003) Fungal pathogens of insects: Cuticle degrading enzymes and toxins. Advances in Botanical Research 40: 241-321.

Charnley AK & Collins SA (2007) Entomopathogenic fungi and their role in pest control. Environmental and Microbial Relationships 4: 159.

Chow A, Czokajlo D, Patt JM & Sétamou M (2019) Development and field validation of a beta-cyfluthrin-based 'attract-and-kill' device for suppression of Asian citrus psyllid (Hemiptera: Liviidae) on residential citrus. Journal of Economic Entomology 112(6): 2824-2832.

Chow A, Dunlap CA, Jackson MA, Avery PB, Patt JM & Sétamou M (2018) Field efficacy of autodissemination and foliar sprays of an entomopathogenic fungus, *Isaria fumosorosea* (Hypocreales: Cordycipitaceae), for control of Asian citrus psyllid, *Diaphorina citri* (Hemiptera: Liviidae), on residential citrus. Journal of Economic Entomology 111(5): 2089-2100.

Co ED & Marangoni AG (2018) Oleogels: an introduction. In Marangoni AG, Garti N (ed.) Edible oleogels, Vol. 2, AOCS Press, Urbana, IL, USA: 1-29.

Cook SM, Khan ZR & Pickett JA (2006) The use of push-pull strategies in integrated pest management. Annual Review of Entomology 52: 375-400.

Dara SK, Semtner PJ (2005) Introducing *Pandora neoaphidis* (Zygomycetes: Entomophthorales) into populations of *Myzus persicae* (Homoptera: Aphididae) on flue-cured tobacco. Journal of Agricultural and Urban Entomology 22: 173-180.

Davidovich-Pinhas M, Barbut S, Marangoni AG (2015) The gelation of oil using ethyl cellulose. Carbohydrate Polymers 117: 869–878.

Davidovich-Pinhas M (2016) Oleogels: a promising tool for delivery of hydrophobic bioactive molecules. Therapeutic Delivery 7(1): 1-3.

de Faria MR, & Wraight SP (2007) Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. Biological Control 43(3): 237-256.

de Lima IJ, Carneiro Leão MP, da Silva Santos AC, da Costa AF & Tiago PV (2021) Production of conidia by entomopathogenic isolates of *Fusarium caatingaense* on different vegetable substrates. Biocontrol Science & Technology 31(2): 206-218.

Dinu M, Bloemhard C, van Holstein-Saj R, Messelink G (2016) Exploring opportunities to induce epizootics in greenhouse aphid populations. Acta Horticulturae 1164: 371-376.

DunhamTrimmer® Global Biocontrol Market Overview Update 2022 (2022) https://dunhamtrimmer.com/products/biocontrol-global-market-report/. Accessed: 06/24/2022

Eigenbrode SD, Birch ANE, Lindzey S, Meadow R & Snyder WE (2016) A mechanistic framework to improve understanding and applications of push-pull systems in pest management. Journal of Applied Ecology 53(1): 202-212.

Eigenbrode SD, Bosque-Pérez NA & Davis TS (2018) Insect-borne plant pathogens and their vectors: ecology, evolution, and complex interactions. Annual Review of Entomology 63: 169-191.

Eilenberg J, Hajek A, Lomer C (2001) Suggestions for unifying the terminology in biological control. BioControl 46(4): 387-400.

Eilenberg J, Bresciani J & Latgé JP (1986) Ultrastructural studies of primary spore formation and discharge in the genus *Entomophthora*. Journal of Invertebrate Pathology 48(3): 318-324.

Eilenberg J, Michelsen V (1999) Natural host range and prevalence of the genus *Strongwellsea* (Zygomycota: Entomophthorales) in Denmark. Journal of Invertebrate Pathology 73(2): 189–198.

Eilenberg J, Saussure S, Fekih IB, Jensen AB & Klingen I (2019) Factors driving susceptibility and resistance in aphids that share specialist fungal pathogens. Current Opinion in Insect Science 33: 91-98.

Eilenberg J, Wilding N & Bresciani J (1992) Isolation *in vitro* of *Strongwellsea castrans* (Fungi: Entomophthorales) a pathogen of adult cabbage root flies, *Delia radicum* (Dipt.: Anthomyiidae). Entomophaga 37(1): 65-77.

El-Sayed AM, Suckling DM, Byers JA, Jang EB, Wearing CH (2009) Potential of "lure and kill" in long-term pest management and eradication of invasive species. Journal of Economic Entomology 102(3): 815-835.

Elya C, Lok TC, Spencer QE, McCausland H, Martinez CC & Eisen M (2018) Robust manipulation of the behavior of *Drosophila melanogaster* by a fungal pathogen in the laboratory. eLife 7: e34414.

Esposito CL, Kirilov P & Roullin VG (2018) Organogels, promising drug delivery systems: An update of state-of-the-art and recent applications. Journal of Controlled Release 271: 1-20.

European Parliament (2009) Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009 establishing a framework for Community action to achieve the sustainable use of pesticides. Official Journal of the European Union 309: 71-86.

Flöter E, Wettlaufer T, Conty V, Scharfe M (2021) Oleogels - Their applicability and methods of characterization. Molecules 26(6): 1673.

Food and Agriculture Organization of the United Nations (FAO; 2019) https://www.fao.org/state-of-food-agriculture/2019/en/. Accessed: 06/25/2022

Freimoser FM, Grundschober A, Tuor U & Aebi M (2003) Regulation of hyphal growth and sporulation of the insect pathogenic fungus *Entomophthora thripidum* in vitro. FEMS Microbiology Letters 222(2): 281-287.

Freimoser FM, Jensen AB, Tuor U, Aebi M & Eilenberg J (2001) Isolation and in vitro cultivation of the aphid pathogenic fungus *Entomophthora planchoniana*. Canadian Journal of Microbiology 47(12): 1082-1087.

Frisinghelli C, Delaiti L, Grando MS, Forti D, Vindimian ME (2000) *Cacopsylla costalis* (Flor 1861), as a vector of apple proliferation in Trentino. Journal of Phytopathology 148(7-8): 425–431.

Gallinger J, Zikeli K, Zimmermann MR, Görg LM, Mithöfer A, Reichelt M (2021) Specialized 16SrX phytoplasmas induce diverse morphological and physiological changes in their respective fruit crops. PLoS Pathogens 17(3): e1009459.

Gallinger J, Jarausch B, Jarausch W, Gross J (2019) Host plant preferences and detection of host plant volatiles of the migrating psyllid species *Cacopsylla pruni*, the vector of European Stone Fruit Yellows. Journal of Pest Science 93(1): 461-475.

George J, Lapointe SL, Markle LT, Patt JM, Allan SA, Setamou M, Rivera MJ, Qureshi JA, Stelinski LL (2020) A multimodal attract-and-kill device for the Asian citrus psyllid *Diaphorina citri* (Hemiptera: Liviidae). Insects 11(12): 870.

Gerding-Gonzalez M, France A, Sepulveda ME and Campos J (2007) Use of chitin to improve a *Beauveria bassiana* alginate-pellet formulation. Biocontrol Science & Technology 17(1): 105–110.

Glare T, Caradus J, Gelernter W, Jackson T, Keyhani N, Köhl J, Marrone P, Morin L, Stewart A (2012) Have biopesticides come of age? Trends in Biotechnology 30(5): 250-258.

Goettel MS, Eilenberg J & Glare T (2010) Entomopathogenic fungi and their role in regulation of insect populations. In Gilbert LI & Gill SS (ed.) Insect Control: Biological and Synthetic Agents, Academic Press, Elsevier: 387–438.

Goettel MS, Hajek AE, Siegel JP & Evans HC (2001) Safety of fungal biocontrol agents. In: Butt TM, Jackson C, Magan N (ed) Fungi as biocontrol agents: progress problems and potential, CABI Publishing, Wallingford, Oxon, UK: 347-375.

Gom T (2001) Oleogels and their applications. In Osada Y, Kajiwara K, Fushimi T, Irasa O, Hirokawa Y, Matsunaga T, Shimomura T, Wang L, Ishida H (ed.) Gels Handbook. Academic Press: 368-380.

Görg LM, Gallinger J & Gross J (2021a) The phytopathogen '*Candidatus* Phytoplasma mali' alters apple tree phloem composition and affects oviposition behavior of its vector *Cacopsylla picta*. Chemoecology 31(1): 31-45.

Görg LM, Eilenberg J, Jensen A, Jensen A & Gross J (2021b) Pathogenicity against hemipteran vector insects of a novel insect pathogenic fungus from Entomophthorales (*Pandora* sp. nov. inedit.) with potential for biological control. Journal of Invertebrate Pathology 183: 107621.

Grafton-Cardwell EE, Stelinski LL & Stansly PA (2013) Biology and management of Asian citrus psyllid, vector of the huanglongbing pathogens. Annual Review of Entomology 58(1): 413-432.

Gregg PC, Del Socorro AP & Landolt PJ (2018) Advances in attract-and-kill for agricultural pests: Beyond pheromones. Annual Review of Entomology 63: 453-470.

Gryganskyi AP, Humber RA, Smith ME, Hodge K, Huang B, Voigt K & Vilgalys R (2013) Phylogenetic lineages in Entomophthoromycota. In Persoonia: Molecular Phylogeny and Evolution of Fungi 30(1): 94-105.

Hadidi A, Barba M, Candresse T & Jelkmann W (2011) Virus and virus-like diseases of pome and stone fruits. APS Press/American Phytopathological Society.

Hajek AE, & St. Leger RJ (1994) Interactions between fungal pathogens and insect hosts. Annual Review of Entomology 39(1): 293-322.

Hajek AE, Papierok B & Eilenberg J (2012) Methods for study of the Entomophthorales. In Lacey LA (ed.) Methods in Insect Pathology, Academic Press Amsterdam, 2nd Edition: 285-316.

Hall RA & Papierok B (1982) Fungi as biological control agents of arthropods of agricultural and medical importance. Parasitology 84(4): 205-240.

Helgerud T, Gaserød O, Fjæreide T, Andersen PO, Larsen CK (2010) Alginates. In Imeson A (ed.) Food stabilisers, thickeners and gelling agents, John Wiley & Sons, Inc, Oxford: 50-72.

Hermann KM, Grünberger A & Patel AV (2021) Formalin-casein enhances water absorbency of calcium alginate beads and activity of encapsulated *Metarhizium brunneum* and *Saccharomyces cerevisiae*. World Journal of Microbiology and Biotechnology 37(9): 1-16.

Herren P (2018) Conidial discharge of *Pandora* sp., a potential biocontrol agent against *Cacopsylla* spp. in apple and pear orchards, MSc thesis, Department of Plant and Environmental Sciences, University of Copenhagen, Denmark.

Hodkinson ID (2009) Life cycle variation and adaptation in jumping plant lice (Insecta: Hemiptera: Psylloidea): a global synthesis. Journal of Natural History 43(1-2): 65–179.

Hogenhout SA, Oshima K, Ammar ED, Kakizawa S, Kingdom HN & Namba S (2008) Phytoplasmas: Bacteria that manipulate plants and insects. Molecular Plant Pathology 9(4): 403-423.

Huang W, Reyes-Caldas P, Mann M, Seifbarghi S, Kahn A, Almeida RPP, Béven L, Heck M, Hogenhout SA & Coaker G (2020) Bacterial vector-borne plant diseases: unanswered questions and future directions. Molecular Plant 13(10): 1379–1393.

International Centre of Insect Physiology and Ecology (ICIPE) (2022) http://www.icipe.org/research/plant-health/push-pull-ipm-technology. Accessed 2022/06/30

IRPCM Phytoplasma/Spiroplasma Working Team–Phytoplasma Taxonomy Group (2004) '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. International Journal of Systematic and Evolutionary Microbiology 54: 1243–1255.

Jackson M (1997) Optimizing nutritional conditions for the liquid culture production of effective fungal biological control agents. Journal of Industrial Microbiology and Biotechnology 19(3): 180-187.

Jackson MA, Dunlap CA & Jaronski ST (2010) Ecological considerations in producing and formulating fungal entomopathogens for use in insect biocontrol. BioControl 55(1): 129-145.

Jadhav SR, Chiou BS, Wood DF, DeGrande-Hoffman G, Glenn GM & John G (2011) Molecular gels-based controlled release devices for pheromones. Soft Matter 7(3): 864-867.

Jaques RP & Patterson NA (1962) Control of the apple sucker, *Psylla mali* Schmidb., by the fungus *Entomophthora sphaerosperma* (Fresenius). The Canadian Entomologist 94(8): 818-825.

Jarausch B, Schwind N, Jarausch W, Krczal G, Dickler E, Seemüller E (2003) First report of *Cacopsylla picta* as a vector of apple proliferation phytoplasma in Germany. Plant Disease 87(1): 101.

Jarausch B, Tedeschi R, Sauvion N, Gross J, Jarausch W (2019) Psyllid Vectors. In Bertaccini A, Weintraub PG, Rao GP, Mori N (ed.) Phytoplasmas: Plant Pathogenic Bacteria II, Vol 92. Springer Singapore, Singapore: 53–78.

Jarausch B, Fuchs A, Schwind N, Krczal G & Jarausch W (2007) *Cacopsylla picta* as most important vector for '*Candidatus* Phytoplasma mali' in Germany and neighbouring regions. Bulletin of Insectology 60(2): 189-190.

Jaronski S (2010) Ecological factors in the inundative use of fungal entomopathogens. BioControl 55(1): 159-185.

Jaronski ST (2014) Mass production of entomopathogenic fungi: State of the art. In Morales-Ramos JA, Guadalupe Rojas M & Shapiro-Ilan DI (ed.) Mass production of beneficial organisms. Elsevier Inc., Amsterdam: 357-413.

Jaronski ST & Mascarin GM (2017) Mass production of fungal entomopathogens. In Lacey LA (ed.) Microbial control of insect and mite pests: From theory to practice, Academic press: 141-155.

Jensen AH (2017) A new insect pathogenic fungus from Entomophthorales with potential for psyllid control, MSc thesis, Department of Plant and Environmental Sciences, University of Copenhagen, Denmark.

Jensen AH, Gross J, Jensen AB, Gallinger J & Eilenberg J (2018) A new insect pathogenic fungus from Entomophthorales with potential for psyllid control. Mitteilungen der Deutschen Gesellschaft für allgemeine und angewandte Entomologie 21: 283-286.

Keller S (1991) Arthropod-pathogenic Entomophthorales of Switzerland. II. *Erynia*, *Eryniopsis*, *Neozygites*, *Zoophthora* and *Tarichium*, Sydowia 43: 39-122.

Keller S (1997) The genus *Neozygites* (Zygomycetes, Entomophthorales) with special reference to species found in tropical regions. Sydowia 49(2): 118-146.

Keller S (2007) Systematics, taxonomy and identification. In Keller S (ed.) Arthropod-pathogenic Entomophthorales: Biology, ecology, identification. Office for Official Publications of the European Communities: 110-132.

Khan ZR, James DG, Midega CA & Pickett JA (2008) Chemical ecology and conservation biological control. Biological control 45(2): 210-224.

Koch W (1937). Properties and uses of ethylcellulose. Industrial & Engineering Chemistry 29(6): 687-690.

Krell V, Jakobs-Schönwandt D, Vidal S & Patel AV (2018) Cellulase enhances endophytism of encapsulated *Metarhizium brunneum* in potato plants. Fungal Biology 122(5): 373-378.

Lacey L, Grzywacz D, Shapiro-Ilan D, Frutos R, Brownbridge M, Goettel M (2015) Insect pathogens as biological control agents: Back to the future. Journal of Invertebrate Pathology 132: 1-41.

Lambin EF & Meyfroidt P (2011) Global land use change, economic globalization, and the looming land scarcity. Proceedings of the National Academy of Sciences 108(9): 3465-3472.

Landis DA, Wratten SD & Gurr GM (2000) Habitat management to conserve natural enemies of arthropod pests in agriculture. Annual Review of Entomology 45(1): 175-201.

Latgé JP, Silvie P, Papierok B, Remaudière G, Dedryver C & Rabasse J (1983) Advantages and disadvantages of *Conidiobolus obscurus* and of *Erynia neoaphidis* in the biological control of aphids. Aphid Antagonists, AA Balkema, Rotterdam: 20-32.

Latgé JP & Papierok B (1988) Aphid pathogens. In Minks AK, Harrewijn P (ed.) Aphids. Their Biology, Natural Enemies and Control, Vol. 2B, Elsevier, Amsterdam: 323–335.

Lee IM, Davis RE & Gundersen-Rindal DE (2000) Phytoplasma: phytopathogenic mollicutes. Annual Review of Microbiology 54(1): 221–255.

Małagocka J, Grell MN, Lange L, Eilenberg J & Jensen AB (2015) Transcriptome of an entomophthoralean fungus (*Pandora formicae*) shows molecular machinery adjusted for successful host exploitation and transmission. Journal of Invertebrate Pathology 128: 47-56.

Mao L, Lu Y, Cui M, Miao S & Gao Y (2020) Design of gel structures in water and oil phases for improved delivery of bioactive food ingredients. Critical Reviews in Food Science & Nutrition 60(10): 1651-1666.

Martin B, Brouillet F, Franceschi S & Perez E (2017) Evaluation of organogel nanoparticles as drug delivery system for lipophilic compounds. AAPS Pharmscitech 18(4): 1261-1269.

Martini X, Hoyte A, Mafra-Neto A, Aksenov AA, Davis CE & Stelinski LL (2020) Progress toward an attract-and-kill device for Asian citrus psyllid (Hemiptera: Liviidae) using volatile signatures of citrus infected with Huanglongbing as the attractant. Journal of Insect Science 20(6): 25.

Martins AJ, Pastrana LM, Vicente AA & Cerqueira MA (2018) Food grade polymers for the gelation of edible oils envisioning food applications. In Gutiérrez TJ (ed.) Polymers for food applications, Springer: 591-608.

Martins E, Poncelet D, Rodrigues RC & Renard D (2017) Oil encapsulation techniques using alginate as encapsulating agent: Applications and drawbacks. Journal of Microencapsulation 34(8): 754-771.

Mayer CJ, Jarausch B, Jarausch W, Jelkmann W, Vilcinskas A & Gross J (2009) *Cacopsylla melanoneura* has no relevance as vector of apple proliferation in Germany. Phytopathology 99(6): 729-738.

Mayer CJ, Vilcinskas A & Gross J (2011) Chemically mediated multitrophic interactions in a plant–insect vector-phytoplasma system compared with a partially nonvector species. Agricultural and Forest Entomology 13(1): 25-35.

Mayer CJ, Vilcinskas A & Gross J (2008a) Pathogen-induced release of plant allomone manipulates vector insect behaviour. Journal of Chemical Ecology 34(12): 1518-1522.

Mayer CJ, Vilcinskas A & Gross J (2008) Phytopathogen lures its insect vector by altering host plant odor. Journal of Chemical Ecology 34(8): 1045-1049.

McCabe D & Soper RS (1985) U.S. Patent No. 4,530,834. Washington, DC: U.S. Patent and Trademark Office.

McLoughlin AJ (1994) Controlled release of immobilized cells as a strategy to regulate ecological competence of inocula. In Scheper T (ed.) Biotechnics/Wastewater. Advances in Biochemical Engineering/Biotechnology, Vol. 51, Springer, Berlin, Heidelberg: 1-45.

Nussinovitch A (2010) Polymer macro- and micro-gel beads: fundamentals and applications. Springer Science & Business Media.

Oerke EC, Dehne HW, Schoenbeck F & Weber A (1994) Crop production and crop protection: estimated losses in major food and cash crops. Elsevier Science Publishers, Amsterdam, The Netherlands.

Olsen NL, Herren P, Markussen B, Jensen AB & Eilenberg J (2019) Statistical modelling of conidial discharge of entomophthoralean fungi using a newly discovered *Pandora* species. PloS one 14: e0215914.

Oshima K, Maejima K, Namba S (2013) Genomic and evolutionary aspects of phytoplasmas. Frontiers in Microbiology 4: 230.

O'Sullivan CM, Barbut S & Marangoni AG (2016) Edible oleogels for the oral delivery of lipid soluble molecules: Composition and structural design considerations. Trends in Food Science & Technology 57: 59-73.

Pakseresht S & Mazaheri Tehrani M (2022) Advances in multi-component supramolecular oleogels - A review. Food Reviews International 38(4): 760-782.

Pang M, Cao L, Kang S, Jiang S & Cao L (2021) Controlled release of flavor substances from sesame-oil-based oleogels prepared using biological waxes or monoglycerides. Foods 10(8): 1828.

Papierok B & Hajek AE (1997) Fungi: entomophthorales. In Lacey LA (ed.) Manual of techniques in insect pathology. Academic Press, San Diego, CA: 187-212.

Park C & Maleky F (2020) A critical review of the last 10 years of oleogels in food. Frontiers in Sustainable Food Systems 4: 139.

Patel AR (2015) Alternative routes to oil structuring. Springer International Publishing, Cham, Switzerland.

Pell JK, Eilenberg J, Hajek AE & Steinkraus D (2001) Biology, ecology and pest management potential of Entomophthorales. In Butt TM, Jackson C and Magan N (ed.) Fungi as biocontrol agents: progress, problems and potential, CABI: 71-154.

Pereira RM & Roberts DW (1991) Alginate and cornstarch mycelial formulations of entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*. Journal of Economic Entomology 84(6): 1657-1661.

Pickett JA, Wadhams LJ & Woodcock CM (1997) Developing sustainable pest control from chemical ecology. Agriculture, Ecosystems & Environment 64(2): 149-156.

Przyklenk M, Vemmer M, Hanitzsch M & Patel A (2017) A bioencapsulation and drying method increases shelf life and efficacy of *Metarhizium brunneum* conidia. Journal of Microencapsulation 34(5): 498-512.

Ravensberg WJ (2011) A roadmap to the successful development and commercialization of microbial pest control products for control of arthropods. Vol. 10, Springer Science & Business Media.

Rehm BHA (2009) Alginates. Biology and Applications, Springer, Berlin, Heidelberg, Germany.

Roy HE, Steinkraus DC, Eilenberg J, Hajek AE & Pell JK (2006) Bizarre interactions and endgames: Entomopathogenic fungi and their arthropod hosts. Annual Review of Entomology 51: 331-357.

Schneider L, Rebetez M & Rasmann S (2022) The effect of climate change on invasive crop pests across biomes. Current Opinion in Insect Science: 100895.

Scorsetti AC, Jensen AB, Lastra CL & Humber RA (2012) First report of *Pandora neoaphidis* resting spore formation in vivo in aphid hosts. Fungal Biology 116(2): 196-203.

Seemüller E & Schneider B (2004) '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri' and '*Candidatus* Phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. International Journal of Systematic and Evolutionary Microbiology 54(4): 1217-1226.

Seemüller E, Carraro L, Jarausch W & Schneider B (2011) Apple proliferation phytoplasma. Virus and virus-like diseases of pome and stones fruits. The American Phytopathological Society, St. Paul, Minnesota, USA: 67-76.

Seiber JN, Coats J, Duke SO, Gross AD (2014) Biopesticides: State of the art and future opportunities. Journal of Agricultural and Food Chemistry 62(48): 11613-11619.

Shah PA, Aebi M & Tuor U (1999) Production factors involved in the formulation of *Erynia neoaphidis* as alginate granules. Biocontrol Science & Technology 9(1): 19-28.

Shah P & Pell JK (2003) Entomopathogenic fungi as biological control agents. Applied Microbiology and Biotechnology 61(5): 413-423.

Shi R, Zhang Q, Vriesekoop F, Yuan Q & Liang H (2014) Preparation of organogel with tea polyphenols complex for enhancing the antioxidation properties of edible oil. Journal of Agricultural and Food Chemistry 62(33): 8379-8384.

Singh VK, Pal K, Pradhan DK & Pramanik K (2013) Castor oil and sorbitan monopalmitate based organogel as a probable matrix for controlled drug delivery. Journal of Applied Polymer Science 130(3): 1503-1515.

Smart LE, Aradottir GI & Bruce TJA (2014) Role of Semiochemicals in Integrated Pest Management. Elsevier Academic Press Inc, San Diego, CA.

Stanford ECC (1881) Improvements in the manufacture of useful products from seaweeds. British Patent 142.

Strasser H, Vey A & Butt TM (2000) Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium*, *Tolypocladium* and *Beauveria* species. Biocontrol Science & Technology 10(6): 717-735.

Strauss E (2009) Phytoplasma research begins to bloom. Science 325: 388–390.

Thakore Y (2006) The biopesticide market for global agricultural use. Industrial Biotechnology 2(3): 194-208.

Thu B, Smidsrød O, Skjåk-Bræk G (1996) Alginate gels - some structure-function correlations relevant to their use as immobilization matrix for cells. In Wijfels RH, Buitelaar RM, Bucke C, Tramper J (ed.) Immobilized Cells: Basics and Applications, Vol. 11. Elsevier Science: 19-30.

United Nations (2019) World Population Prospects: The 2019 Revision, Volume I: Comprehensive Tables (ST/ESA/SER.A/426) Population Division of the Department of Economic and Social Affairs of the United Nations Secretariat, New York, USA.

Valls A, Castillo A, Porcar R, Hietala S, Altava B, García-Verdugo E & Luis SV (2020) Urea-based low-molecular-weight pseudopeptidic organogelators for the encapsulation and slow release of (R)-limonene. Journal of Agricultural and Food Chemistry 68(26): 7051-7061.

Vega FE, Goettel MS, Blackwell M, Chandler D, Jackson MA, Keller S, Koike M, Maniania NK, Monzón A, Ownley BH, Pell JK, Rangel DEN & Roy HE (2009) Fungal entomopathogens: New insights on their ecology. Fungal Ecology 2(4): 149-159.

Vega FE, Meyling NV, Luangsa-Ard JJ & Blackwell M (2012) Fungal entomopathogens. Insect Pathology 2. Academic Press, San Diego, CA: 171-220.

Vemmer M, Schumann M, Beitzen-Heineke W, French BW, Vidal S & Patel AV (2016) Development of a CO_2-releasing coformulation based on starch, *Saccharomyces cerevisiae* and *Beauveria bassiana* attractive towards western corn rootworm larvae. Pest Management Science 72(11): 2136-2145.

Vemmer M & Patel AV (2013) Review of encapsulation methods suitable for microbial biological control agents. Biological Control 67(3): 380-389.

Villaverde JJ, Sandín-España P, Sevilla-Morán B, López-Goti C & Alonso-Prados JL (2016) Biopesticides from natural products: Current development, legislative framework, and future trends. BioResources 11(2): 5618-5640.

Vintiloiu A & Leroux JC (2008) Organogels and their use in drug delivery—a review. Journal of Controlled Release 125(3): 179-192.

Waage JK & Hassell MP (1982) Parasitoids as biological control agents–a fundamental approach. Parasitology 84(4): 241-268.

Weintraub PG & Beanland L (2006) Insect vectors of phytoplasmas. Annual Review of Entomology 51: 91-111.

Weintraub PG, Wilson MR & Jones P (2010) Control of phytoplasma diseases and vectors. In Weintraub PG, Wilson MR & Jones P (ed.) Phytoplasmas: genomes, plant hosts and vectors, CABI: 233-249.

Wilding N & Latteur G (1987) The Entomophthorales - problems relative to their mass production and their utilisation. Mededelingen van de Faculteit landbouwwetenschappen. Rijksuniversiteit Gent 52(2): 159-164.

Wilding N, Mardell S., Brobyn PJ, Wratten SD & Lomas J (1990) The effect of introducing the aphid-pathogenic fungus *Erynia neoaphidis* into populations of cereal aphids. Annals of Applied Biology 117(3): 683-691.

Yang DX, Chen XW & Yang XQ (2018) Phytosterol-based oleogels self-assembled with monoglyceride for controlled volatile release. Journal of the Science of Food and Agriculture 98(2): 582-589.

Yilmaz E & Demirci Ş (2021) Preparation and evaluation of virgin olive oil oleogels including thyme and cumin spices with sunflower wax. Gels 7(3): 95.

Yılmaz E, Öğütcü M & Yüceer YK (2015) Physical properties, volatiles compositions and sensory descriptions of the aromatized hazelnut oil-wax organogels. Journal of Food Science 80(9): 2035-2044.

Yogev S & Mizrahi B (2020) Organogels as a delivery system for volatile oils. ACS Applied Polymer Materials 2(5): 2070-2076.

Zhang Z, Sun X, Luo Z, Gao Y & Chen Z (2013) The manipulation mechanism of “push–pull” habitat management strategy and advances in its application. Acta Ecologica Sinica 33(2): 94-101.

Zhou X & Feng MG (2009) Sporulation, storage and infectivity of obligate aphid pathogen *Pandora* nouryi grown on novel granules of broomcorn millet and polymer gel. Journal of Applied Microbiology 107(6): 1847-1856.

Chapter 2

Development of a self-adhesive oleogel formulation designed for the slow release of semiochemicals

Linda C. Muskat[1,2,3], Lin Jiang[2], Johannes Brikmann[3], Michael Rostás[2], Anant V. Patel[1,3]

[1]Bielefeld University of Applied Sciences, Faculty of Engineering and Mathematics, WG Fermentation and Formulation of Biologicals and Chemicals

[2]Georg-August-University Goettingen, Department of Crop Sciences, Agricultural Entomology

[3]Bielefeld University of Applied Sciences, Faculty of Engineering and Mathematics, Bielefeld Institute of Applied Materials Research

Published in: Macromolecular Materials and Engineering, 2022, 307, 2200276 (ISSN: 1439-2054)

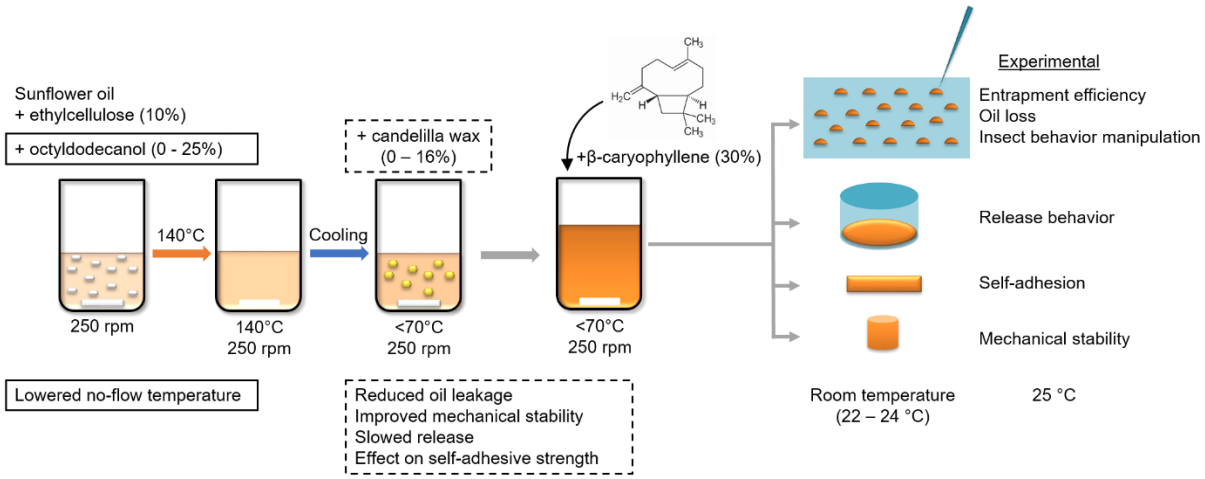
DOI: 10.1002/mame.202200276

Abstract

Oleogels have been widely studied as delivery matrices for lipophilic compounds. Despite their potential, oleogels are underexplored for the formulation and release of semiochemicals for insect behavior manipulation strategies. We present an ethylcellulose-candelilla wax-oleogel as suitable carrier for the slow release of volatile semiochemicals, employing the sesquiterpene β-caryophyllene as a model substance. A current drawback of ethylcellulose oleogels is the high temperature needed during the oleogel preparation procedure, leading to excessive volatilization of the active compounds. Reducing the gelling temperature from 104.8 to 66.9 °C and, thus, the volatile addition temperature by addition of the softener octyldodecanol results in 95.28% entrapment efficiency. Experimental results demonstrate release modifiability by tailoring the matrix crystalline state due to candelilla wax addition. Furthermore, a method is established for analyzing the self-adhesive properties of the gel on plant leaves. In addition, the gel's self-adhesive properties employed on apple leaves are synergistically affected by oil leakage and gel stiffness. Olfactometer experiments showed that the formulation was able to enhance the attractiveness of aphid-infested plants for parasitic wasps. The present study provides a novel matrix-type semiochemical formulation based on non-toxic, biobased and biodegradable materials and will contribute to the establishment of oleogels as delivery systems for semiochemicals.

Keywords: Organogel, Ethylcellulose, Self-assembly, Volatile diffusion, Biomaterial composite, No-flow temperature, Insect behavior manipulation

Graphical abstract



Introduction

Plant volatiles play a key role in the interaction of insect pests, their host plants and their natural enemies. The use of these volatiles as semiochemicals in insect pest control strategies permits a specific effect on the target insect and reduces the risk for non-target organisms (Howse et al., 1997).

The sesquiterpene β-caryophyllene is a semiochemical of broad application potential for behavior manipulation of different agricultural important organisms. β-caryophyllene was identified as an attractant for the summer apple psyllid *Cacopsylla picta* (Mayer et al., 2008) and as a potential repellent for the citrus psyllid *Diaphorina citri* (Alquézar et al., 2017), both being important phytoplasma vector insects. It was also identified as an attractant for the parasitic wasps *Aphidius ervi* (Heuskin et al., 2012) and *A. colemani* (Vitiello et al., 2021) and was found to be released by root-damaged maize plants as an attractant for the predatory nematode *Heterorhabditis megidis* (Rasmann et al., 2005).

Due to the high volatility and chemical instability of semiochemicals against UV light and oxidation (Heuskin et al., 2011) the use of semiochemicals for insect control purposes is often limited. For their large-scale application for insect pest control, protection of semiochemicals by formulation is needed (Plimmer, 1981; Sucklng & Karg, 2000). Therefore, different dispenser and formulation types have been developed in the last decades (Muskat & Patel., 2022).

An ideal semiochemical formulation protects the active ingredient during storage, is inexpensive and non-toxic to produce, improves technical application in the field and, most importantly, increases shelf life and fine-tunes release into the environment (Plimmer, 1981; Bieri-Leonhardt, 1982; Muskat & Patel., 2022). The choice of a suitable formulation material is the key to modulating the release rate in a desired way (Muskat & Patel., 2022). Due to their biocompatibility and biodegradability, the potential of alginate hydrogel beads has been proven in several studies for a variety of active agents Nussinovitch, 2010; Vemmer & Patel., 2013), and have also been tested as carriers for the slow-release of β-caryophyllene (Heuskin et al., 2012). A drawback of hydrogel capsules for the formulation of hydrophobic substances such as β-caryophyllene is that they commonly suffer from a low initial loading and that the release is strongly affected by humidity caused by swelling of the hydrogel (Mao et al., 2020; Daems et al., 2016).

A gel type that is gaining more and more interest for the formulation of reactive, lipophilic substances in cosmetics, pharmaceuticals and food technology is the so-called organogel (Gom, 2001; Davidovich-Pinhas, 2015; Esposito, 2018; Park & Maleky, 2020). Organogels are defined as semi-solid systems with hydrophobic nature stabilized by the three-dimensional network of an organogelator formed in an organic solvent. If oil is the organic solvent, the gels are also termed oleogels (Mao et al., 2020). Other synonyms can be found in the literature, too, e.g., molecular gels (Jadhav et al., 2011) or nanogels (Bhagat et al., 2013). They are practically organogels, as a lipophilic solvent is gelled by a gelator molecule and gelation is sufficient to gel the lipophilic liquid even at low concentrations (Davidovich-Pinhas, 2018). A growing number of oleogelator molecules that are able to gel oil have been identified during the last years (Co & Marangoni, 2018). Based on their molecular weight, oleogelators can be classified into low-molecular-weight organogelators LMOGs, such as 12-hydroxystearic acid, and polymeric gelators, such as ethylcellulose (Co & Marangoni, 2018). The combination of different synergistically acting gelators to create hybrid oleogels enable the modulation of gel properties in a desired way for special application (Davidovich-Pinhas, 2018; Buerkle & Rowan, 2012; Gravelle et al., 2017; Gravelle et al., 2018; da Silva et al., 2019).

Oleogels as formulation matrices for lipophilic compounds offer high loading capacity (Vintiloiu & Leroux, 2008; Martin et al., 2017), they are of low risk for microbial contamination and extend shelf life of reactive compounds by improving oxidative stability (Shi et al, 2014; O'Sullivan et al., 2016) Numerous studies have dealt with the structure-functional relationship of the delivery rate of active lipophilic compounds from oleogels (Mao et al., 2020; O'Sullivan et al., 2016; Yang et al., 2018). However, only a few studies have focused on the formulation and release of volatile substances from organogels (Yang et al., 2018; Yilmaz et al., 2015; Yılmaz & Demirci, 2021; Valls et al., 2020; Pang et al., 2021).

Despite their potential, oleogels are underexplored for the release of semiochemicals in agricultural applications. Recent attempts have demonstrated suitability of organogels as semiochemical carriers: Jadhav et al. (2011) directly gelled the model pheromones 2-heptanone and lauryl acetate with the sugar alcohol-based amphiphile mannitol dioctanoate at a very low concentration of the gelator molecule. Bhagat et al. (2013) directly gelled methyl eugenol as fruit pest attractant with a synthesized low-molecular mass gelator and found a very long release duration of up to several weeks. Yogev and Mizrahi (2020) evaluated the potential of ethylcellulose for the direct gelation of the volatile oils linalool, citral and *Mentha arvensis* essential oil as fungicide formulation for food preservation and hoof treatment, but were only interested in a short-term release of up to some hours. However, none of these studies investigated whether the release can be further modified by adding additives and how they affect gel properties. An optimal semiochemical formulation for use in insect manipulating strategies should achieve high loading capacity and entrapment efficiency, be stable under storage, shipping and technical application, be easy to apply at the application site and, most importantly, releases the semiochemical for a prolonged time period and in a consistent manner.

Therefore, the aim of the present study was the in-depth development of an oleogel-based formulation for the slow release of semiochemicals in agricultural pest control applications. β-caryophyllene was employed as a model substance.

The specific objectives of this study were

(1) to lower the temperature at which the volatile compound is added to the oleogel to improve entrapment efficiency,

(2) to slow the release of β-caryophyllene from the oleogel,

(3) to improve the compatibility of the formulation for plant leaves by reduced oil leakage and improved mechanical stability,

(4) to evaluate the self-adhesive properties of the formulation on plant leaves

(5) to validate the insect behavior manipulating effect of the formulation.

Results and Discussion

Ethylcellulose was selected as oleogelator because the oleogel preparation procedure is simple and ethylcellulose is generally considered as safe for food and pharmaceutical applications (Gravelle et al., 2018; O'Sullivan et al., 2016; ECHA, 2022) and thus will not have any negative impact on the environment or when consumed by animals. Furthermore, its odorless and physiologically inert character (Rekhi & Jambhekar, 1995) makes it a suitable polymer for the release of semiochemicals. Moreover, ethylcellulose has recently been demonstrated to be a promising structuring agent for volatile oils (Yogev & Mizrahi, 2020). Sunflower oil as the major oil phase of the gel is considered non-toxic to humans and animals and is registered for pesticide applications even in organic agriculture (Mäder et al., 2019). With regard to future large-scale field application of the formulation, a technical grade β-caryophyllene of at least 80% purity was used in this study for financial reasons. Details on the preparation procedure and **Table 2.2** containing all formulation compositions tested in this study can be found in **4. Experimental Section**.

Octyldodecanol reduces no-flow temperature of the sol for improved β-caryophyllene entrapment efficiency and softens the gel

A drawback of oleogelation is the need for heating during preparation, which can result in oxidation of the carrier oil (Mao et al., 2020) and in the oxidation and excessive volatilization of the active ingredient. The onset volatilization temperature of unformulated β-caryophyllene was 73.3 °C (±0.3). For the formation of ethylcellulose oleogels, a heating step above the glass transition temperature of the ethylcellulose is necessary (Davidovich-Pinhas et al., 2014) The glass transition temperature of the ethylcellulose used in this study was approximately 129–133 °C (Dow, 2005). Gelation occurs during cooling below the sol-gel transition temperature (Kavanagh & Ross-Murphy, 1998), also called gelling point or no-flow temperature. Addition and proper mixing of β-caryophyllene into the sol is only sufficient above the no-flow temperature. In order to avoid β-caryophyllene heating up to the glass transition temperature of ethylcellulose and above its volatilization temperature, the ethylcellulose-sunflower oil-oleogel was prepared first in order to facilitate complete dissolution of the ethylcellulose before addition of β-caryophyllene in the last preparation step. The onset temperature of the β-caryophyllene volatilization from the formulation (W0+bCAR-OD) was 97.0 °C (±1.5). Thus, β-caryophyllene seems to be protected from excessive volatilization by the sol, as unformulated β-caryophyllene onset temperature was much lower (73.3 °C ±0.3). This is consistent with other studies that found increased

volatilization temperature of active ingredients by their formulation (Rezaei et al., 2019; Jelic, 2021; Sun et al., 2021). The no-flow temperature of the ethylcellulose-sunflower oil-oleogel (OD0) proved to be 104.8 °C (±1.1), which was higher than the β-caryophyllene volatilization temperature. The entrapment efficiency of 77.05% (±1.8) of β-caryophyllene in this gel (W0+bCAR-OD) was expectedly low.

Hence, the first aim of this study was to reduce the no-flow temperature of the sol to protect β-caryophyllene from excessive heat during entrapment. Plasticizers can improve processability of polymers with high glass transition temperatures like ethylcellulose (Rekhi & Jambhekar, 1995; Sears & Touchette, 1985; Thakkar et al., 2020; Maru et al., 2011). In the present study, octyldodecanol was selected as a softener due to its unique solution properties for ethylcellulose described by Melzer et al. (2003).

The effect of different octyldodecanol concentrations (0, 5, 10, 15, 20 and 25%) on the no-flow temperature and the viscosity of the melt at the no-flow temperature is shown in **Figure 2.1**.

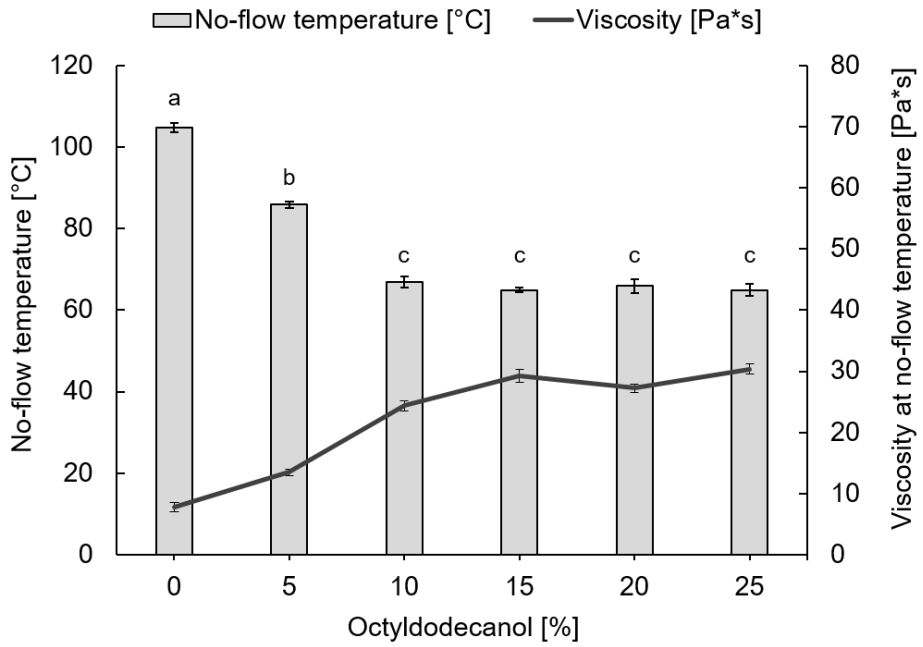


Figure 2.1. Effect of octyldodecanol on no-flow temperature and viscosity of the ethylcellulose-sunflower oil-oleogels before β-caryophyllene addition. Different letters above bars indicate significant differences based on one-way ANOVA and Tukey post-hoc test ($p<0.001$). Mean ±SD, n = 5.

The addition of octyldodecanol had a significant effect on the no-flow temperature ($F_{5,20} = 407.008$; $p<0.001$; one-way ANOVA and *Tukey post-hoc* test) of the sol. Without the addition of octyldodecanol, the no-flow temperature was 104.8 °C. By addition of 5%, the no-flow temperature was significantly reduced to 85.8 °C (±0.8), at 10%, the no-flow temperature further decreased significantly to 66.9 °C (±1.4). Higher concentrations (>10%) had no further decreasing effect. A saturation effect was ruled out, as the no-flow temperature of a gel composed of ethylcellulose and octyldodecanol (OD60; **Table 2.2**) did not occur in the measurable range down to 50 °C (data not shown).

As the no-flow temperature was lowered to 66.9 °C by octyldodecanol addition, we expected a low β-caryophyllene loss during the preparation process and thus a higher entrapment efficiency. The mean temperature at which the formulated β-caryophyllene was completely volatilized (endset at the DTG

curve) from the W0+bCAR variant was 168.0°C (±3.3; n = 30). The entrapment efficiency in the gel with the preferred octyldodecanol concentration of 10% (W0+bCAR) was 95.28% (± 2.14).

Compared to other semiochemical formulation techniques, the entrapment efficiency of the present method is comparatively high. Lopez et al. (2012) tested different encapsulation techniques for the formulation and slow release of linalool and found the highest entrapment efficiency of 89% for an alginate microcapsule. Unfortunately, for the few studies on volatile encapsulation in oleogels, entrapment efficiency is often not reported. Milanovic et al. (2010) reported an entrapment efficiency of 86.5% for the microencapsulation of ethyl vanillin in carnauba wax thermally solidified by an emulsion-based technique. Martin et al. (2017) found a high encapsulation efficiency for indomethacin and ketoconazole formulated in castor oil-oleogel-nanoparticles, but these are non-volatile drugs and the method only needs a short heat treatment, as low-molecular-weight organogelators (LMOGs), such as 12-hydroxystearic acid, were used as oleogelators.

The viscosity at the no-flow temperature is an important parameter for processability in future technical-scale production processes (Thakkar et al., 2020). As the no-flow temperature decreased, the viscosity of the sol at the no-flow temperature increased from 7.7 (0%), 13.8 (5%), 24.3 (10%) to 29.2 (15%) Pa*s. At 20% octyldodecanol (27.3 Pa*s) and 25% (30.4 Pa*s), no significant increase in viscosity was observable.

In terms of applicability in crop protection, mechanical stability of the formulation plays a major role, especially during storage, shipping and technical application. We hypothesized that the softening effect of octyldodecanol is not only observable for the sol at the no-flow temperature, but also on the stiffness of the gelled formulation.

The stiffness of the gel measured in form of the secant modulus differed for various octyldodecanol concentrations, Welch's $F_{4,\ 9.709} = 419.794$ ($p < 0.001$). Octyldodecanol strongly softened the ethylcellulose-sunflower oil-oleogel (OD0–OD20; **Figure 2.2**). Games-Howell post-hoc analysis revealed a significant difference ($p < 0.001$) between the stiffness of all octyldodecanol concentrations. Mean stiffness decreased from 0.32 MPa (0% octyldodecanol) to 0.17 (5%), 0.08 (10%), 0.06 (15%) and 0.01 (20%).

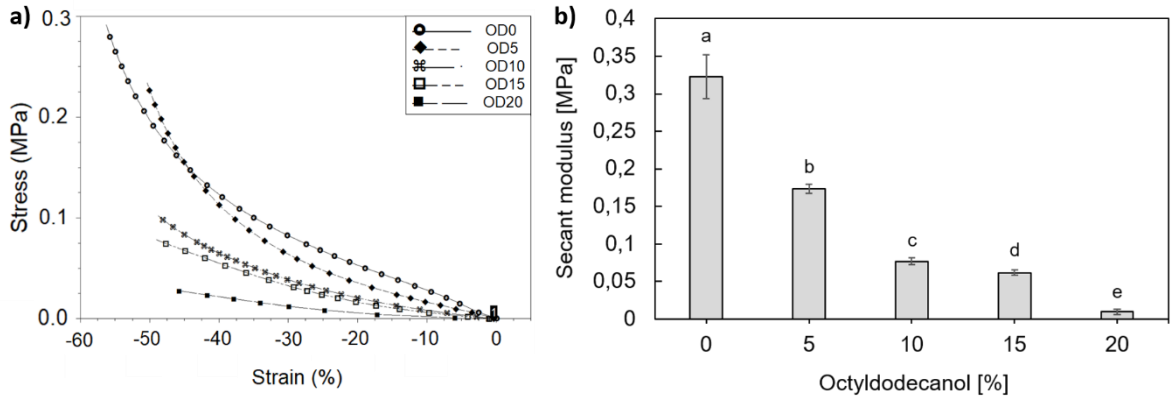


Figure 2.2. Effect of octyldodecanol on ethylcellulose-sunflower oil-oleogel (OD0–OD20) stiffness without β-caryophyllene addition. **a)** Stress-strain diagrams of representative samples. **b)** Secant modulus dependent from the octyldodecanol content determined between 0 and 10% compression. Different letters above bars indicate significant differences based on Welch's test and Games-Howell post-hoc test ($p<0.001$). Mean ± SD, n = 5.

The softening effect of octyldodecanol can be explained by polymer–solvent interactions according to the observation by Bisschops (1955) that chain junctions are hindered by a good solvent and promoted by a poor solvent, which has been commonly accepted for several other polymeric systems (Laredo et al., 2011) and also for ethylcellulose oleogels (Gravelle et al., 2016). Considering the ethylcellulose-sunflower oil-octyldodecanol gel, the addition of octyldodecanol improves solubility of ethylcellulose in the solvent, resulting in a hindrance of ethylcellulose self-assembly via hydrogen bonding between the ethylcellulose polymer chains. This hindrance caused a lowered gelation temperature and a softer gel.

Nevertheless, as octyldodecanol has a softening effect on the resulting gel and is a costly additive, its concentration should be as low as possible. Therefore, as 10% was sufficient to significantly lower the no-flow temperature the following experiments were carried out with the gel containing 10% octyldodecanol.

Ethylcellulose-sunflower oil-octyldodecanol oleogels slow down release of β-caryophyllene

The most important aspect of a semiochemical formulation is the release rate and release duration. For agricultural application, a long and consistent release is required (Yoon et al., 2017). Novel formulations have to compete with dispensers of uniform zero-order kinetics and a long release persistence of up to 6 months for successful use in mating disruption or push-pull strategies (Muskat & Patel, 2022).

One of the aims of this study was to slow down the release of β-caryophyllene to prolong its persistence in the field after application. It is generally accepted that (1) a high solubility of a molecule in the matrix material and (2) the structuring of a liquid by a gelator molecule or polymer chain acting as a barrier for the diffusion of a molecule through the matrix slows the release (Scher, 1977; Kaplan et al., 2019).

A β-caryophyllene loading of 30% was chosen, as at this concentration, a sufficient dissolution of ethylcellulose in the sunflower oil-octyldodecanol mixture was given. A repeated measures ANOVA determined that the release of β-caryophyllene was significantly affected by formulation ($F_{3, 14}$ = 11.115, $p < 0.001$, partial $\eta^2 = 0.704$). Bonferroni-adjusted post-hoc analysis revealed significantly ($p < 0.005$) slower release of β-caryophyllene by formulation in crude sunflower oil (SO+bCAR) or ethylcellulose-sunflower oil-oleogels (W0+bCAR-OD and W0+bCAR) compared to unformulated β-caryophyllene. While unformulated β-caryophyllene was completely volatilized after 26 days, 66% (W0+bCAR), 63% (W0+bCAR-OD) and 50% (SO+bCAR) of the initial β-caryophyllene amount remained in the formulations at the same time point (**Figure 2.3**). Since the unformulated β-caryophyllene was completely volatilized after 26 days and the effects of the formulation composition on the release were already apparent at this point, all release experiments were terminated after 27 days.

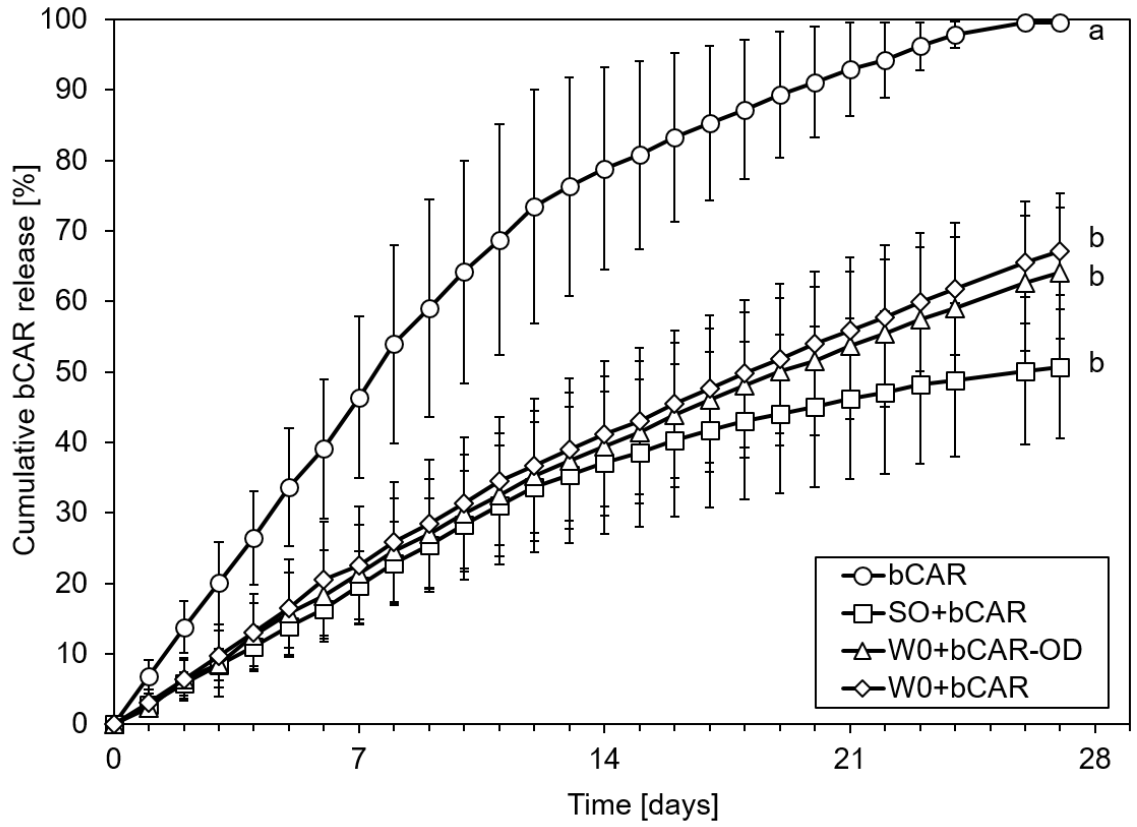


Figure 2.3. Release behavior of unformulated β-caryophyllene (bCAR; ○), β-caryophyllene in sunflower oil (SO+bCAR; □), β-caryophyllene formulated in ethylcellulose-sunflower oil-oleogel without octyldodecanol (W0+bCAR-OD; Δ) and with (10%) octyldodecanol (W0+bCAR; ◊). Different letters indicate significant differences based on a RM-ANOVA and Bonferroni post-hoc test ($p<0.005$). Mean ± SD, n = 5.

Partial replacement of the sunflower oil with the softener octyldodecanol (10%; w/w) had no significant effect on the β-caryophyllene release from the ethylcellulose oleogel over time. Thus, octyldodecanol seems not to influence the solubility of β-caryophyllene in the matrix.

Interestingly, release from crude sunflower oil was as slow as from the ethylcellulose structured oil. This result was unexpected, as the structuring of solutes is generally accepted as a measure to slow down the diffusion of a molecule by entrapment within any material or gel (Scher, 1977; Kaplan et al., 2019), including oleogels (Kavanagh & Ross-Murphy, 1998; Nguyen et al., 2019).

However, similar observations have been made by others (Jadhav et al., 2011; Pang et al., 2021; Vallet et al., 1991). The inefficiency of some oleogelators in slowing the release was explained by the presence of hydrophilic domains formed by the intermolecular hydrogen bonding responsible for the self-assembly of the gelling agents (den Adel et al., 2010), which reduces the hydrophobicity of the oil (Pang et al., 2021; Chen et al., 2016). This gelling mechanism is well described for the mixtures of β-sitosterol and γ-oryzanol (Bot et al., 2008; Duffy et al., 2009; Liu et al., 2021) and ethylcellulose alike (Gravelle et al., 2018; Dey et al., 2011)

With this result, we demonstrate that sunflower oil can also serve as a sprayable carrier for slowing the release of β-caryophyllene. Nevertheless, such a formulation is only suitable when small amounts are needed, as the oil spreads as a thin film. The advantage of the gel is that it can be prepared in different sizes and shapes and thereby release can be prolonged by increasing the formulation size. Moreover,

gelation enables a higher rainfastness compared to pure oil and will reduce drift and waste of the formulated β-caryophyllene during application, making it more suitable for spot applications.

Candelilla wax improves oil-binding capacity and mechanical stability

An initial problem of the ethylcellulose oleogel formulation (W0) was excessive oil leakage, which is a disadvantage for subsequent foliar application due to a potential negative effect on the leaf. Furthermore, as shown in **Figure 2.3**, β-caryophyllene release from the ethylcellulose oleogel was as fast as from unstructured oil.

Candelilla wax, a natural wax derived from *Euphorbia* spp., had previously been described as an oleogelator of high oil-binding capacity (Blake et al., 2014) and was therefore incorporated into the ethylcellulose oleogel as an additional oleogelator.

The addition of candelilla wax significantly reduced oil-loss from ethylcellulose-oleogels without β-caryophyllene ($F_{5,33}$ = 499.754; p<0.001; one-way ANOVA and Tukey post hoc test) and with β-caryophyllene (30%) ($F_{5,27}$ = 1077,376; p<0.001; one-way ANOVA and Tukey post hoc test; **Figure 2.4**).

The addition of the second oleogelator candelilla wax at a concentration between 4 and 8% suppressed any oil-leakage from the gel and will thereby reduce or inhibit spreading of the oil over the leaf surface.

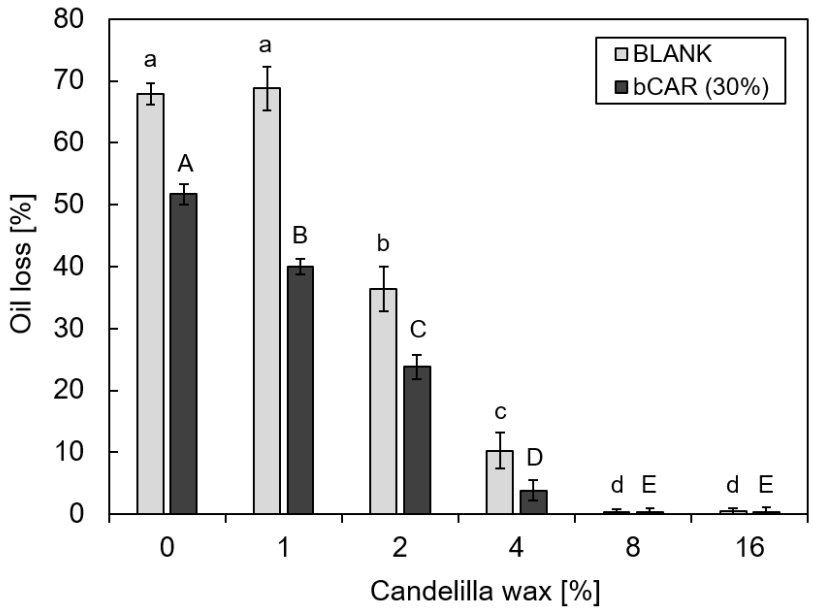


Figure 2.4. Effect of candelilla wax on oil loss from blank ethylcellulose-sunflower oil-oleogels (BLANK; light grey; lower case letters) and ethylcellulose-sunflower oil-oleogels with 30% β-caryophyllene (bCAR (30%); dark grey; capital letters). Different letters above bars indicate significant differences based on one-way ANOVA and Tukey post hoc test (p<0.001). Mean ± SD, n = 5.

As expected, entrapment efficiency was not affected by variation of the wax concentrations ($F_{5,24}$ = 2.173; p = 0.091; one-way ANOVA and *Tukey post-hoc test*). Mean entrapment efficiency for all wax concentrations (W0+bCAR – W16+bCAR) was 95.35% ±1.16 (n = 30).

Besides reduced oil-leakage, the addition of candelilla wax was assumed to have a positive effect on the mechanical stability of the gel (Lim et al., 2017) and mechanical stability will positively correlate with the wax concentration (Rocha et al., 2013; Martins et al., 2016).

The addition of candelilla wax had a significant effect on the mechanical stability of the gel without β-caryophyllene ($F_{5,24}$ = 968.189; p<0.001; one-way ANOVA and Tukey post-hoc test). The stiffness of the candelilla wax-free gel (W0_blank) was relatively low (0.16 MPa). The addition of 1% candelilla wax (W1_blank) had no significant effect on the stiffness (p=0.790). From a wax concentration ≥2%, a linear increase correlating with candelilla wax concentration was observable. The mechanical stability increased significantly from 0.49 MPa for 2% (W2_blank) to 0.88 (W4_blank), 1.32 (W8_blank) and 2.65 MPa for 16% (W16_blank) candelilla wax (**Figure 2.5**).

Candelilla wax also had a significant effect on the mechanical stability of the gel with a 30% β-caryophyllene loading ($F_{5,24}$ = 227.151; p<0.001; one-way ANOVA and Tukey post-hoc test). 1% candelilla wax (W1+bCAR) had no significant effect on the stiffness (p=0.992). When 2% candelilla wax (W2+bCAR) was added, the stiffness increased slightly but significantly to 0.24 MPa. 4% candelilla wax (W4+bCAR) had no significant increasing effect compared to 2% (p = 0.102). When 8% candelilla wax (W8+bCAR) was added, stiffness significantly increased to 0.71 MPa, a concentration of 16% candelilla wax (W16+bCAR) with 0.72 MPa had no further significant stabilizing effect on the gel (p = 1.000). There was no significant difference between both candelilla wax free gels, but a higher stabilizing effect in β-caryophyllene free gels. Thus, β-caryophyllene acts as a softener on the ethylcellulose-candelilla wax-oleogel, assumed to be attributed to the solvent properties of β-caryophyllene for the candelilla wax.

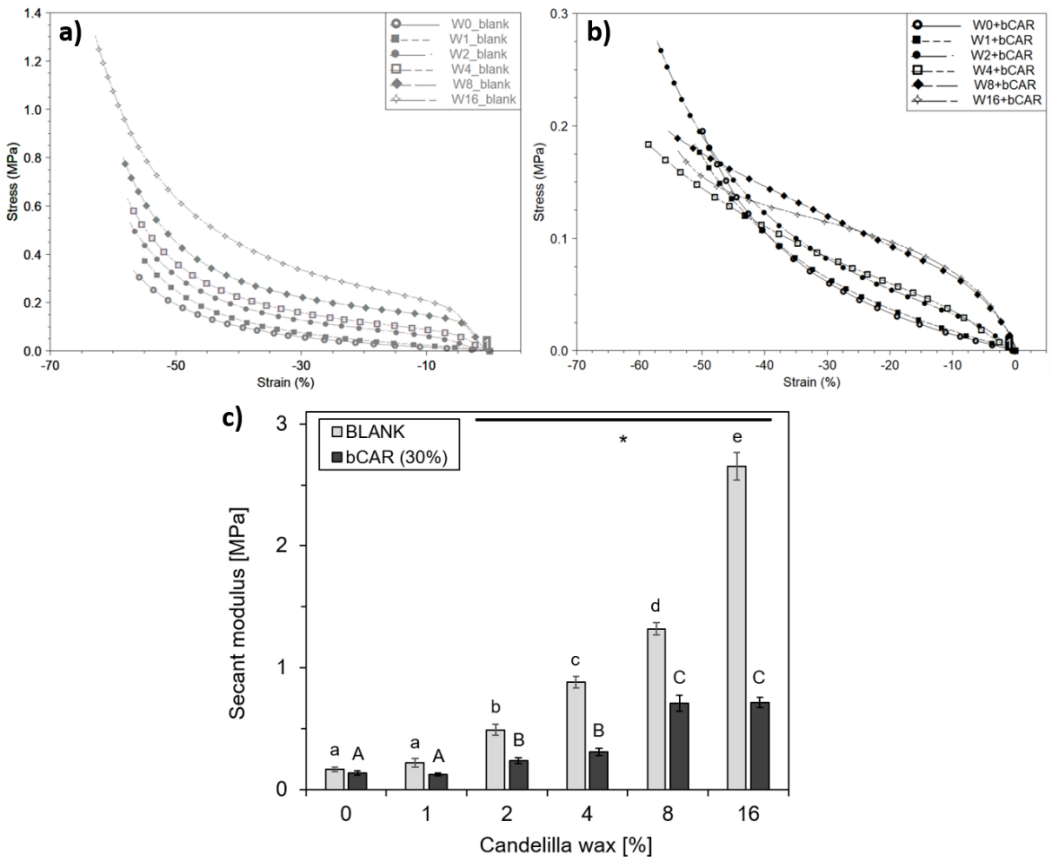


Figure 2.5. Effect of candelilla wax on the stiffness of the ethylcellulose-sunflower oil-octyldodecanol (10%) oleogel without (BLANK; light grey; lowercase letters) and with β-caryophyllene (bCAR (30%); dark grey; capital letters). **a)** Stress-strain diagram of representative samples without β-caryophyllene. **b)** Stress-strain diagram of representative samples with β-caryophyllene (30%). **c)** Secant modulus dependent from the candelilla wax content of samples determined between 0 and 10% compression. Different letters above bars indicate significant differences between different wax concentrations, asterisk indicates significant differences between gels with and without β-caryophyllene but containing the same wax concentration based on one-way ANOVA and Tukey post-hoc test (p<0.05). Mean ± SD, n = 5.

Candelilla wax slows β-caryophyllene release

As the addition of the second oleogelator candelilla wax reduced oil leakage, we assumed that the candelilla wax also slows the release of the entrapped β-caryophyllene. Our initial hypothesis was that the candelilla wax crystals serve as a barrier for β-caryophyllene diffusion.

This hypothesis was accepted for candelilla wax concentrations between 1 and 4%, where a repeated measures ANOVA determined that the release of β-caryophyllene was significantly affected by the candelilla wax content $F_{5, 18} = 19.799$, $p < 0.001$, partial $\eta^2 = 0.846$ and a Bonferroni-adjusted post-hoc analysis revealed significantly slower release from the formulations containing 1% (W1+bCAR), 2% (W2+bCAR) and 4% (W4+bCAR) candelilla wax compared to the wax-free oleogel (W0+bCAR) ($p < 0.01$; **Figure 2.6**).

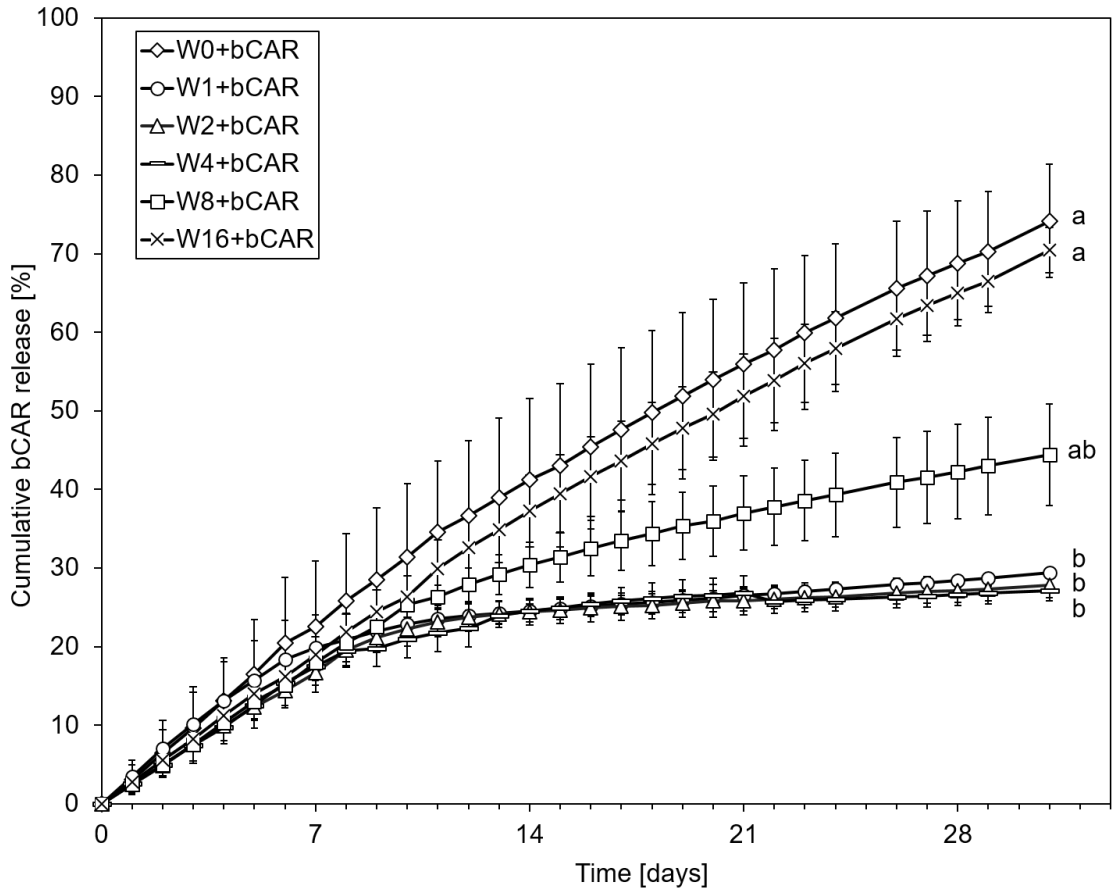


Figure 2.6. Effect of candelilla wax concentration on β-caryophyllene release from ethylcellulose-sunflower oil-oleogels. Wax concentrations tested: 0% (W0+bCAR; ◊), 1% (W1+bCAR; ○), 2% (W2+bCAR; Δ), 4% (W4+bCAR; ─), 8% (W8+bCAR; □), 16% (W16+bCAR; X). Different letters indicate significant differences based on a RM-ANOVA and Bonferroni post-hoc test ($p<0.001$). Mean ± SD, n = 5.

Surprisingly, with increasing wax content higher than 8% (W8+bCAR), the release increased compared to lower wax concentrations between 1 and 4%. From the gel containing 16% wax (W16+bCAR), release was as fast as from W0+bCAR. W8+bCAR did not significantly differ from both groups, thus, release increased from a wax content between 4 and 8%. This result was unexpected, as it is generally accepted that the higher the crystallinity the slower the diffusion of molecules through a material (Yoon et al., 2017; Scher, 1977; Frank et al., 2005), including oil diffusion through crystalline matrice (Nelis et al., 2021).

For natural waxes, in particular, a volatile release retardation was found by others and explained by the formation of closed spaces within the formulation matrix that reduce the mobility of molecules within the crystalline network and by reduced solubility of the encapsulated molecule (Pang et al., 2021; Yoon et al., 2017; Slodowicz et al., 2017). Atterholt et al. (1999) found soy oil as a volatility suppressant of pheromone release from paraffin wax based emulsion. They suggest an increased solubility of the pheromone in the paraffin matrix by the soy oil, causing a decrease in the release rate.

In none of these studies did the wax contents vary, the wax concentrations were at low levels (<8%) and it does not explain the lower release from oleogels of low wax contents <8%.

The explanation for the faster release of β-caryophyllene from gels of higher wax content can be found in the microstructure of the gels: Looking at the microscopic images in **Figure 2.7**, the majority of the gels containing 1–4% candelilla wax appears more amorphous with only some single wax crystals present. Crystals dispersed throughout an oil phase were declared to increase the tortuosity factor of a system (Dey et al., 2011; Nelis et al., 2021; Valoppi et al., 2020) and thereby slow diffusion of the liquid phase through the matrix. In gels with higher candelilla wax content, it looks like the majority of the gel is present in a crystalline state, which leads to the conclusion that at these (>8% candelilla wax) concentrations, the wax co-crystallizes with the oil and β-caryophyllene exists between the crystallized areas.

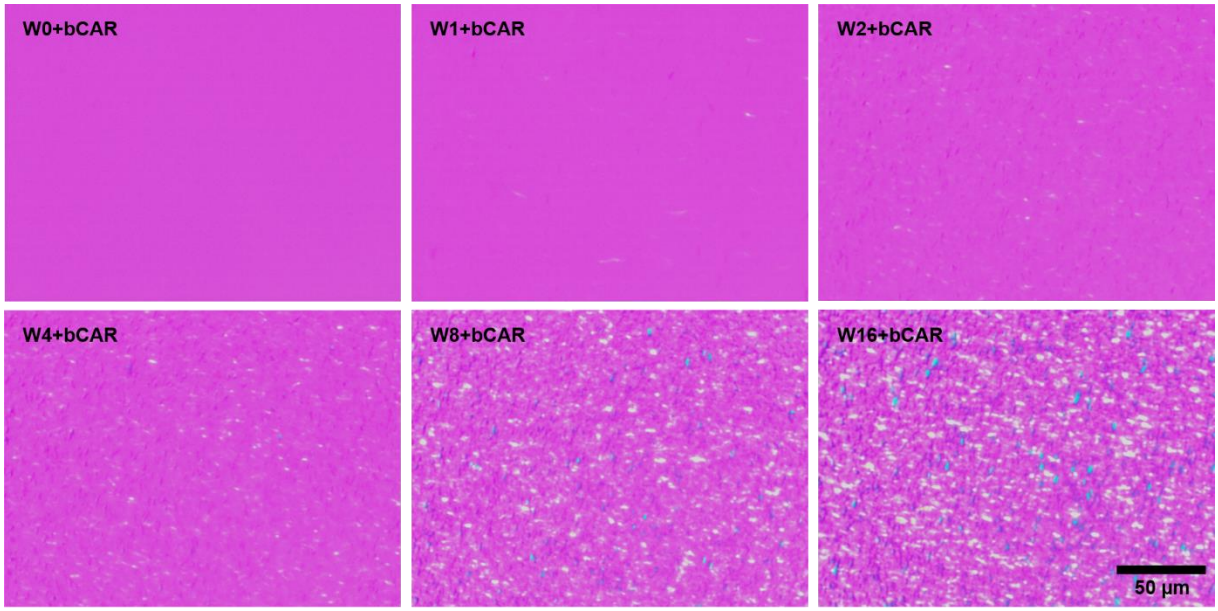


Figure 2.7. Polarized light microscopic images of ethylcellulose-sunflower oil-β-caryophyllene oleogels containing different concentrations of candelilla wax: No wax (W0+bCAR), 1% (W1+bCAR), 2% (W2+bCAR), 4% (W4+bCAR), 8% (W8+bCAR), 16% (W16+bCAR). White and blue areas represent crystals; violet areas are amorphous/non-crystalline phases. Magnification 20x.

These results indicate that the barrier effect of the wax crystals acts at low concentration (<8%) and the refasten release at higher candelilla wax concentrations can be explained by a “channel effect”. This effect is caused by reduced solubility of β-caryophyllene in the matrix, which is in a more solid and aggregated state with the non-crystalline β-caryophyllene present in the spaces and being thereby unformulated due to a co-crystallization and aggregation of the sunflower oil with the wax.

This explanation is supported by the study of Yadav et al. (Yadav et al., 2017) who observed increased release rates of the model drug ciprofloxacin hydrochloride from a stearic acid:stearyl alcohol rice bran

oil oleogel with increasing stearic acid contents. They explained their results with a hindrance of the diffusion of the drug within the oleogel by a smaller mesh size in the oleogels at lower stearic acid content and improved synergistic interactions among the stearyl alcohol and stearic acid molecules at higher contents, which did not allow the drug molecules to accommodate within the gelator network structure.

Ongoing experiments will elucidate the nature and strength of the interaction between β-caryophyllene and the wax crystals and provide proof of the “channel effect”.

Another simpler explanation is based on Hansen solubility parameters (Hansen, 2007): The best solubility of β-caryophyllene is expected in the gels containing 1% (W1+bCAR)–8% (W8+bCAR) candelilla wax due to the matrix polarity by presence of different candelilla wax contents.

The modelling of the β-caryophyllene release with the Korsmeyer-Peppas (Korsmeyer et al., 1983) and Peppas-Sahlin (1989) diffusion models reveals non-linear release kinetics (**Table 2.1**). This indicates an anomalous release of β-caryophyllene as characterized by Crank (1979), which can be described very well with the Peppas-Sahlin model with acceptable coefficients of determination. It should be noticed that the variants W1+bCAR, W2+bCAR and W4+bCAR are not well represented by this model, since these variants almost stop their release after 14 days. From W0+bCAR and W16+bCAR it can be predicted that 100% of the formulated β-caryophyllene will be released within 47 days based on a calculation with the Peppas-Sahlin model. In comparison, from W8+bCAR β-caryophyllene will be completely released within 183 days.

Table 2.1 Coefficients of the Korsmeyer-Peppas and Peppas-Sahlin diffusion models. k = kinetic constant; n,m = release exponents; k1 = Fickian diffusion constant; k2 = relaxation transport constant; R2 = coefficient of determination

	Korsmeyer-Peppas Model $\frac{M_t}{M_\infty} = kt^n$			**Peppas-Sahlin Model** $\frac{M_t}{M_\infty} = k_1 t^m + k_2 t^{2m}$			
Formulation	k	n	R^2	k_1	k_2	m	R^2
bCAR	16,385	0,560	0,959	-166,688	168,273	0,106	0,979
SO+bCAR	6,838	0,639	0,964	-78,898	76,998	0,123	0,981
W0+bCAR-OD	4,805	0,788	0,995	-15,880	16,963	0,274	0,998
W0+bCAR	4,988	0,791	0,998	-12,653	14,571	0,293	0,999
W1+bCAR	8,718	0,369	0,938	-151,876	156,753	0,038	0,966
W2+bCAR	7,155	0,423	0,911	-138,214	140,650	0,044	0,944
W4+bCAR	7,194	0,420	0,910	-149,432	152,099	0,041	0,944
W8+bCAR	5,464	0,625	0,982	-46,868	46,953	0,137	0,994
W16+bCAR	3,720	0,862	0,998	-7,143	8,788	0,346	0,999

It has to be noted that the release from W1+bCAR, W2+bCAR and W4+bCAR is very slow from day eleven. This can be explained by a concentration or equilibrium effect between β-caryophyllene and wax crystalline state. This release behavior will result in a high residual load of β-caryophyllene in the formulation and there is a risk of insufficient concentrations for target organism behavior manipulation. Thus, when huge amounts are needed, slow release is not major to a higher, but more constant release rate of the semiochemical as realized by W0+bCAR (no wax) or W16+bCAR (16% candelilla wax).

Candelilla wax influences adhesive properties on apple leaves

Retention and rainfastness of non-dispenser semiochemical formulations is important for prolonged release in the place of application. Several studies on microencapsulated pheromone formulations are reliant on additional adhesive adjuvants or surfactants to improve the retention of the formulation on leaves (Taylor & Matthews, 1986; Knight et al., 2004).

The developed oleogel formulation has self-adhesive properties on apple leaves, which allow for application without additional surfactants and will give it a high rainfastness. To the best of our knowledge, there is no study on the adhesive properties of an oleogel on plant leaves. Therefore, a dynamic-mechanical shear experiment method from conventional sticker technology was adapted for the investigation of the adhesive properties of the oleogel on apple leaves.

Adhesive properties of the gel on apple leaves were significantly dependent on the candelilla wax content ($F_{5,24}$ = 45.262; p<0.001; one-way ANOVA and *Tukey post hoc test*; **Figure 2.8b**). The shear stress under which the gels slip from the leaf samples was between 483.3 (8%) and 1133.4 (2%) Pa. The gels without candelilla wax (0%) withstood a shear stress of 735.8 Pa. With increasing candelilla wax content, the shear stress at break increased to 1133.4 Pa for the gel with 2% candelilla wax. When the wax content was further increased, the ability to withstand shear stress decreased significantly to 916.3 Pa (4%). The gels with the highest candelilla wax contents had the lowest adhesive abilities with 483.3 (8%) and 521.6 Pa (16%).

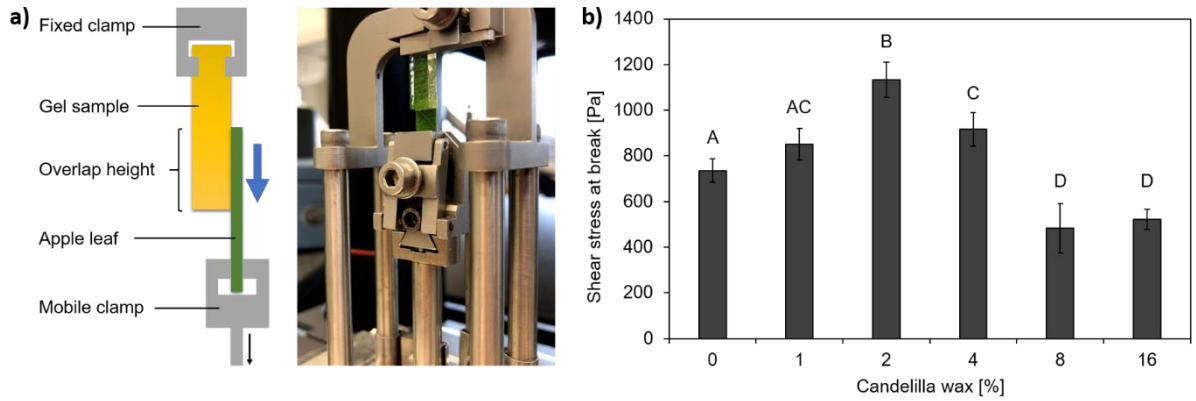


Figure 2.8. **a)** Experimental set-up of the self-adhesive properties analysis of the gel on apple leaves by a tensile shear test. **b)** Influence of candelilla wax on self-adhesive strength of the formulation (ethylcellulose-sunflower oil-octyldodecanol-oleogel+bCAR (30%)) on apple leaves. Different letters above bars indicate significant differences based on one-way ANOVA and Tukey post hoc test (p<0.001). Mean ± SD, n = 5.

Comparing the influence of the wax on both the oil binding properties (**Figure 2.4**) and the stiffness of the gel (**Figure 2.5**), it can be assumed that, on the one hand, stiffer gels adhere more poorly than elastic ones due to a lower direct contact area and, on the other hand, the oil leaking out of the formulation apparently serves as an adhesion agent. Nevertheless, if the quantities leaking out become

too high, the oil acts as a lubricant, so that the formulation slips off the leaf at low wax contents due to the high oil loss. The formulation W2+bCAR (wax content 2%), which is still elastic enough, with at the same time medium oil leakage, therefore exhibits the best adhesion properties. How the gels behave on the leaves under field conditions needs further investigation.

Aphidius colemani response to formulation

Aphid parasitoids in search for host insects are attracted to the odors that plants emit in response to aphid feeding (da Silva et al., 2016; Aartsma et al., 2017). Olfactometer experiments with the aphid parasitoid *A. colemani* were performed to test whether a single β-caryophyllene emitting oleogel capsule (W2+bCAR) would enhance the attractiveness of odors emanating from an aphid infested plant. β-caryophyllene emission of one capsule was 4.3 µg h^{-1} (±0.48; n=5). As mentioned above, for financial reasons a technical grade β-caryophyllene of at least 80% purity was used in this study. The main component besides β-caryophyllene emitted by the capsules was α-humulene with 0.165 µg h^{-1} (±0.04; n=5). The results showed that naïve parasitoids which had not learned to associate β-caryophyllene with successful parasitization of aphids, did not find the enhanced odor blend more attractive. Aphid infested plants with odor capsule (bCAR) were not significantly preferred over aphid infested plants without capsules (CONTROL) ($Z= 0.97$, $p > 0.33$; Wilcoxon matched pairs test; **Figure 2.9a**). However, β-caryophyllene became a conditioned stimulus when *A. colemani* females were allowed to associate the volatile compound with a positive experience (aphid parasitization). Hence, in the second experiment significantly more parasitoids were attracted by the odour blend of sugar beet plants treated with aphids and capsule (bCAR) compared to plants treated with aphids only (CONTROL) ($Z=2.17$, $P=0.030$; Wilcoxon matched pairs test; **Figure 2.9b**). Overall, naïve parasitoids (n=95) were more motivated to respond to volatiles in our experiments than experienced insects (n=46). This could be due to the fact that experienced parasitoids had parasitized a few aphids immediately before the test and needed time to recover before continuing with further host searching.

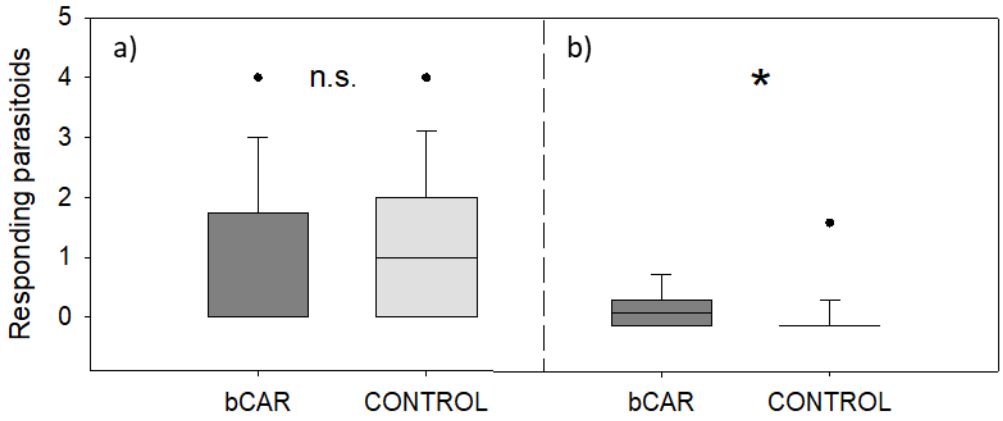


Figure 2.9. Attraction of the parasitoid *Aphidius colemani* to odours from a single β-caryophyllene emitting capsule placed on a sugar beet plant with 5 aphids. **a)** Response of naïve parasitoids (*without* previous oviposition experience in the presence of capsule odour prior to olfactometer experiment). **b)** Response of experienced parasitoids (*with* previous oviposition experience in the presence of capsule odour prior to olfactometer experiment). bCAR = single β-caryophyllene capsule placed on a sugar beet plant with 5 aphids, CONTROL = sugar beet plant with 5 aphids. Boxes show median numbers of responding parasitoids per released group of 6 (line), 25th and 75th percentiles. Whiskers are 5th and 95th percentiles. Dots represent outliers. Wilcoxon matched pairs test, n.s. = not significant, *$p<0.05$). n = 32 (8 replicate days, 4 releases per day)

Conclusion

Oleogel based formulation engineering is in its early days, and for several applications, such as semiochemical release, only few attempts have been made. The present study demonstrates that ethylcellulose-candelilla wax-oleogels are suitable matrices for the slow release of semiochemicals, in particular β-caryophyllene. The developed formulations exhibit a combination of unique properties that make it highly suitable for semiochemical formulation and release, such as a reduced processing temperature, high loading capacities due to the high solubility of hydrophobic volatiles in the oil based matrix, improved oil-binding capacity and mechanical stability, self-adhesive properties on leaves and release modifiability. Thus, the developed gel and method can be used as a prototype to establish universally usable slow-release semiochemical delivery systems. The formulation's shape stability is given by ethylcellulose, but release modifiability by a second oleogelator, in this case candelilla wax. Hybrid oleogelator systems offer unlimited opportunities and will enable release retardation and control of a variety of semiochemicals and volatile compounds. For the intended application scenario of using the formulation for behavioral manipulation of insects in push-pull, attract-and-reward or attract-and-kill strategies, the formulation variant containing 2% candelilla wax (W2+bCAR) proved to be the most suitable, as it has the best self-adhesive properties on leaves in addition to a strongly slowed and thus long-term β-caryophyllene release. However, the release of β-caryophyllene from this variant was slowed to such an extent that after eleven days only very low amounts of β-caryophyllene were released. When further dispersed in the environment, the concentration might be too low to be perceptible to any target insect. For applications where a high concentration and more uniform release of β-caryophyllene is of utmost importance, the formulation variant containing a higher candelilla wax content of 16 % (W16+bCAR) would be a more suitable choice, which further exhibits high mechanical stability and suppressed oil loss. The mechanism of release modification needs further investigation to elucidate the multiple interaction of the ethylcellulose network – candelilla wax – β-caryophyllene and oil component, which is responsible for the release behavior. Ongoing experiments deal with the climatic impact on the release and material behavior in order to make predictions on release rate and duration under application conditions.

Experimental Section

Chemicals

Sunflower oil (SO) was purchased from a local supermarket, ethylcellulose (EC; ETHOCELTM Standard 100 Industrial Ethylcellulose; batch code: D184IA9031; Dow Chemical Company, Midland, MI, USA) with ethoxyl content 48.7% and a viscosity of 98.0 mPa*s was obtained from ChemPoint (Bergen op Zoom, Netherlands), candelilla wax (W; 2039; batch code: 10-1185) from Kahlwax GmbH & Co. KG (Trittau, Germany), 2-octyldodecan-1-ol (OD; Eutanol® G; batch code: 18112004) from Caelo (Caesar & Loretz GmbH, Hilden, Germany) and β-caryophyllene (bCAR; ≥80%, FCC, FG; batch code: MKCG3589) from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Preparation procedure

Sunflower oil was optionally mixed with octyldodecanol in a beaker and ethylcellulose was added. The mixture was heated above the ethylcellulose glass transition temperature (140 °C) under continuous

stirring at 250 rpm on a magnetic hotplate stirrer (IKA® C-MAG HS 7, IKA®-Werke GmbH & CO. KG, Staufen, Germany) set to 180 °C. After full dissolution of the ethylcellulose (~1 hour), the mixture was cooled to a temperature above the no-flow temperature and candelilla wax was added. After full melting and solving of the candelilla wax, β-caryophyllene was added and the sol was set to room temperature for thermal gelation. Samples were stored in closed containers in a fridge at 5 °C or used immediately. All formulation compositions tested in this study are listed in **Table 2.2**.

Table 2.2. List and composition of all formulation matrices without β-caryophyllene and formulations containing β-caryophyllene tested in this study. SO = sunflower oil, EC = ethylcellulose, OD = octyldodecanol, W = candelilla wax, bCAR = β-caryophyllene. All data are given in weight% (w/w).

Name	SO	EC	OD	W	bCAR
bCAR	0	0	0	0	100
SO+bCAR	70	0	0	0	30
OD0	85.71	14.29	0	0	0
OD5	78.57	14.29	7.14	0	0
OD10	71.42	14.29	14.29	0	0
OD15	64.29	14.29	21.42	0	0
OD20	57.14	14.29	28.57	0	0
OD25	50	14.29	35.71	0	0
OD60	0	14.29	85.71	0	0
W0_blank	80	10	10	0	0
W1_blank	79	10	10	1	0
W2_blank	78	10	10	2	0
W4_blank	76	10	10	4	0
W8_blank	72	10	10	8	0
W16_blank	64	10	10	16	0
W0+bCAR-OD	60	10	0	0	30
W0+bCAR	50	10	10	0	30
W1+bCAR	49	10	10	1	30
W2+bCAR	48	10	10	2	30
W4+bCAR	46	10	10	4	30
W8+bCAR	42	10	10	8	30
W16+bCAR	34	10	10	16	30

Analysis

Rheological properties of the sol

To determine the no-flow temperature and the effects of octyldodecanol addition on the gel processability, the viscosity of the melt was continuously analyzed under constant cooling (3 K min^{-1}) from the initial preparation temperature of 140 °C to 50 °C with a high-pressure capillary rheometer (HPCR; RHEOGRAPH 20, GÖTTFERT Werkstoff-Prüfmaschinen GmbH, Buchen, Germany). Therefore, the gels composed of sunflower oil, ethylcellulose and different octyldodecanol contents were maintained at 140 °C and filled into the pre-heated (140 °C) barrel of the high-pressure capillary rheometer. The constant shear rate was calculated following Pérez et al. (2006) for a 250 rpm stirring speed, which corresponds to an average shear rate of 244.08 s^{-1} (Eq. 1). The piston speed of 0.212 mm sec^{-1} was calculated following the HPCR equipment instructions (Eq. 2) for the diameter of the barrel (Ø=12 mm) and the capillary (Ø=1 mm; length = 20 mm) and the previously calculated average shear rate.

$$\dot{\gamma} = 33.1\, N^{1.4} \quad (1)$$

Where $\dot{\gamma}$ is the average shear rate (in s^{-1}) and N is the agitation speed (in s^{-1}). The average shear rate was then inserted in the following equation:

$$v = \frac{\dot{\gamma}\, R^3}{D^2} \quad (2)$$

Where v is the piston speed of the rheometer (in mm s^{-1}), $\dot{\gamma}$ is the average shear rate (in s^{-1}), R is the radius of the capillary (in mm) and D is the diameter of the barrel (in mm).

The no-flow temperature was set to the point at which the viscosity increases:

$$\eta = \frac{\tau}{\dot{\gamma}} \quad (3)$$

Where η is viscosity (in Pa*s), τ is the shear stress (in Pa) and $\dot{\gamma}$ is the average shear rate (in s^{-1}).

$$\tau = \frac{p\, R}{2\, L} \quad (4)$$

Where τ is the shear stress (in Pa), p is the continuously measured pressure (in Pa), R is the radius of the capillary (in mm) and L is the length of the capillary (in mm).

Mechanical properties of the oleogel

Mechanical stability of the oleogels was evaluated by dynamic-mechanical analysis (DMA Q800, TA Instruments, New Castle, Delaware, USA) in a compression test. Therefore, oleogel samples were prepared by pouring the melt in cylindrical cavities (Ø = 5 mm; height = 4 mm) of a self-made silicone form. After gelation at room temperature, the samples were analyzed starting from a preload of 0.0001 N and a ramp of 2 N min^{-1} to 18 N. The measurements were carried out at a constant temperature of 25 °C and at 60% r.h. The stiffness of the gels was determined by a secant modulus at a compression between 0 and 10%, as the gel was more elastic up to this compression before undergoing a constant flow phase.

Thermal behavior and entrapment efficiency

The onset volatilization temperature of β-caryophyllene and the sufficiently formulated amount of β-caryophyllene in the oleogels (entrapment efficiency, EE) was determined by thermogravimetric analysis

(Hi-Res TGA 2950 Thermogravimetric Analyzer, TA Instruments, New Castle, Delaware, USA). For the analysis of the entrapment efficiency, oleogel samples were prepared were prepared in lenticular droplet shape (10–15 mg) by dropping the sol on a silicone mat (Kaiser®, WMF Group GmbH, Geislingen/Steige, Germany) by means of a pipette.

Individual gel samples (10–15 mg) or β-caryophyllene (9–11 mg) were placed in a 100-µl-platinum pan. The run was performed with a quasi-isotherm high resolution method: equilibration at 30 °C, Ramp 50 °C min^{-1}, res 5 °C to 600 °C, Hi-res sensitivity 1.0 and a nitrogen flow of 60 ml min^{-1}. The entrapment efficiency was calculated by the difference of weight loss (%) between a blank sample without β-caryophyllene and a sample prepared with a loading of 30% at the endset temperature in the differential thermogravimetric (DTG) curve when the whole β-caryophyllene was volatilized from the formulation (temp($bCAR_{CV}$)) as follows:

$$EE\ [\%] = \frac{Weight\ loss\ at\ temp(bCAR_{CV})\ [\%]}{Loading\ [\%]} * 100\ \% \quad (5)$$

Release kinetics

In order to characterize the release of β-caryophyllene, samples were prepared as described above according to the compositions listed in **Table 2.2** by pouring the hot oleogel melts into crystallization dishes (Crystallization dishes ROTILABO® without spout, 20 ml, Ø = 40 mm, Carl Roth GmbH, Karlsruhe) and allowing them for gelation at room temperature. In the case of the oleogel and the oil samples, 3.5 g was poured per dish. For the bCAR samples, 1.16 g β-caryophyllene (ρ = 0.9) per dish was used to correspond to the 30% β-caryophyllene loading of the gel and the oil samples. The samples filled in the crystallization dishes were then placed in a climatic chamber (KBF P 240 E5.2, Binder GmbH, Tuttlingen, Germany) in a random order and incubated at constant 25 °C, 60% room humidity and an air flow of 1 m/sec. The release of β-caryophyllene was determined gravimetrically by weight loss, which was measured on an analytical balance (AG204 DeltaRange®, Mettler Toledo GmbH, Gießen, Germany) every 24 hours.

$$Cumulative\ bCAR\ release\ [\%] = 100\ [\%] - \frac{Weight\ loss\ bCAR\ (t_x)[\%]}{Loading\ (t_0)\ [\%]} * 100\ [\%] \quad (6)$$

Where Weight loss bCAR (t_x) is related to the initial β-caryophyllene amount (in %).

Each experimental run was performed with five replicates per sample composition.

Diffusion modeling

The release of β-caryophyllene was modelled using the mean values from the release experiments with the Korsmeyer-Peppas (Korsmeyer et al., 1983; Eq. (7)) and Peppas-Sahlin (1989) model (Eq. (8)).

$$\frac{M_t}{M_\infty} = kt^n \quad (7)$$

$$\frac{M_t}{M_\infty} = k_1 t^m + k_2 t^{2m} \quad (8)$$

The coefficients of the models were determined with the non-linear regression procedure of SPSS Statistics V28.0 (SPSS, Chicago, IL), which uses an iterative estimation algorithm.

Oil loss determination

Oil loss from oleogels was determined following the procedure of Dibildox-Alvarado et al. (2004) with some modifications. Therefore, oleogel samples were prepared in lenticular droplet shape (10–15 mg). One droplet was placed in the middle of a pre-weighted filter paper (Ø = 55 mm, particle retention 12–15 µm, VWR International, Leuven, France). Blank filter papers without a sample were used as a control. The filter papers carrying the samples were individually placed on small petri dishes in order to prevent oil loss from the filter paper during the experiment. The filter papers were placed in a climatic chamber (INCU-Line, VWR International, Leuven, France) and incubated at constant temperature of 25 °C and 30–40% r.h. for 24 h. Afterwards, the oleogel samples were discarded and the filter papers were weighed again. The oil loss was calculated as follows:

$$Oil\ loss\ [\%] = \frac{w_{filter+oil\,(t24)} - w_{filter\,(t0)}}{w_{sample\,(t0)}} * 100\ \% \quad (9)$$

Where w is weight (in g) and t is time (in h).

Polarized light microscopy

For microscopic images, a drop of the sol was placed on a pre-heated glass microscope slide. The sol was overlaid with a pre-heated glass cover slip and pressed to ensure a thin sample suitable for microscopy. The samples were cooled to room temperature and then stored in a closed container at 5 °C in the fridge for at least 24 h. Before and during microscopy, the samples were set to room temperature. Microscopic images were taken with a Standard WL microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with a polarization filter and a λ-plate analyzer at 20-fold magnification.

Adhesive properties

In order to characterize the adhesive properties of the oleogels on apple leaves, the oleogel samples were prepared by pouring the hot melt on a silicone mat with a block thickness of 4 mm, which were then cut to an exact width of 12.65 mm with the punch device of the shear set (TA Instruments, New Castle, Delaware, USA) and a length of 25 mm with a scalpel. With the same instruments, slices from freshly collected apple leaves (*Malus domestica* var. 'Cox Orange') were freshly cut immediately before the run. The oleogel sample was fixed in the fixed clamp, the apple leave slice was fixed in the mobile clamp (**Figure 2.8a**). The analysis by a tensile shear test by dynamic-mechanical analysis (DMA Q800, TA Instruments, New Castle, Delaware, USA) was performed with the following parameters: preload 0.001 N; ramp of 0.01 N min^{-1} to 1 N at constant 25 °C and at 60% r.h. The adhesive strength was calculated by the shear stress at break as follows:

$$Shear\ stress\ at\ break\ [Pa] = \frac{Force\ at\ break\ [N]}{Overlap\ area\ [m^2]} \quad (10)$$

Aphidius colemani *response to formulation*

The olfactory attraction of the aphid parasitoid *Aphidius colemani* to a single β-caryophyllene emitting oleogel capsule was assessed in a six-arm-olfactometer (Turlings et al., 2004). Two series of experiments were conducted, testing naïve and experienced parasitoid wasps. Mated 2–5 d old females of *A. colemani* were used for all experiments. Naïve parasitoids had no prior exposure to aphids, plants or oleogel capsule odor. To give *A. colemani* experience, 3-4 female parasitoids were allowed to

parasitize 12 aphids while being exposed to the odor of an oleogel capsule as described in Costa et al. (2010). Experience events were performed twice for each parasitoid: the day before and 0.5 h prior olfactometer experiments commenced.

One day before the test, sugar beet seedlings were infested with five aphid nymphs (instar 3-4) of the species *Aphis fabae*. On the following day, two plants with aphids were placed individually into the cup of an odor source vessel of the olfactometer. A single oleogel capsule was placed on a leaf of an aphid infested plant, the other aphid infested plant served as control and remained without capsule. The two treatments were placed vis-à-vis, the other four vessels remained empty. The flow rate was 1.0 L min^{-1} for incoming air and 0.5 L min^{-1} for air going out to the behavioral arena and the volatile traps, respectively. The air stream was stabilized for 10 min before the behavioral assays started. Parasitoid wasps were released in groups of 6 into the olfactometer. After 30 min, their choices were recorded and the group was replaced by a new one. Four groups of wasps were tested on the same day. The positions of the treatments were changed each day, but were always vis-à-vis. Eight replicate days were carried out with a new pair of plants and new parasitoids each day.

The emission rate of β-caryophyllene in the olfactometer was measured according to Winter and Rostás (2010). For 1 h, the headspace was collected with a volatile trap containing 30 mg Porapak Q as adsorbens. Trapped volatiles were eluted with 1250 µl dichloromethane. An aliquot of 250 µl was transferred to a GC vial and tetralin (200 ng) was added as an internal standard. A sample of 2 µl was injected into the GC-MS (Agilent 5977B) equipped with a HP-5ms (30 m × 0.25 mm inner diameter, 0.25 µm film thickness).

Statistical analysis

Statistical analysis was carried out using the software SPSS Statistics V25.0 (SPSS, Chicago, IL). All data are given as mean values ± standard deviations (SD). The level of significance was set to $p < 0.05$. All treatments were performed with at least five samples, unless otherwise stated.

All data were checked for normality by a *Shapiro-Wilk* test at $\alpha = 0.05$ and homogeneity of variance by a *Levene* test. Data on entrapment efficiency, oil-loss, effect of octyldodecanol on the gel stiffness and release behavior were arcsin transformed prior to analysis. The data on no-flow temperature, entrapment efficiency, effect of candelilla wax and β-caryophyllene on gel stiffness, oil-loss and the adhesive properties of the gel on apple leaves were analyzed by one-way ANOVA followed by a *Tukey post-hoc* test. Data on the effect of octyldodecanol on the gel stiffness were analyzed by Welch's test for nonhomogeneity, followed by a Games-Howell correction. Data on release of β-caryophyllene were proofed for outliers and extreme values, which were excluded from further analysis. A repeated measures analysis (RM-ANOVA) was conducted, followed by a Bonferroni post hoc test to examine the effect of the formulation on the release rate. Data on parasitoid olfactory responses did not follow normal distribution and were therefore analyzed by Wilcoxon matched pairs test.

Acknowledgements

This study was supported by means of the German Federal Ministry of Food and Agriculture (Bundesministerium für Ernährung und Landwirtschaft, BMEL) as part of the Picta-Kill project [No. 2814900415]. Open access publication was funded by the Deutsche Forschungsgemeinschaft (DFG,

German Research Foundation) – 490988677 – and Bielefeld University of Applied Sciences. We thank Ana-Katrina Büttner for language editing.

Author Contributions

LCM designed and conducted the experiments on the formulation development and characterization, analyzed and visualized the data, conducted statistical analyses and wrote the manuscript. LJ conducted insect olfaction experiments and GC-MS analyses. JB calculated the diffusion model. MR conceptualized olfaction experiments and analyzed data. AVP secured funding and edited the manuscript.

References

Aartsma, Y., Bianchi, F. J., van der Werf, W., Poelman, E. H., & Dicke, M. (2017). Herbivore-induced plant volatiles and tritrophic interactions across spatial scales. New Phytologist, 216(4), 1054-1063.

Alquézar, B., Volpe, H. X. L., Magnani, R. F., de Miranda, M. P., Santos, M. A., Wulff, N. A., Simões Bento, R. M., Postali Parra, J. R., Bouwmeester, H. & Peña, L. (2017). β-caryophyllene emitted from a transgenic *Arabidopsis* or chemical dispenser repels *Diaphorina citri*, vector of *Candidatus* Liberibacters. Scientific Reports, 7(1), 1-9.

Atterholt, C. A., Delwiche, M. J., Rice, R. E., & Krochta, J. M. (1999). Controlled release of insect sex pheromones from paraffin wax and emulsions. Journal of Controlled Release, 57(3), 233-247.

Bhagat, D., Samanta, S. & Bhattacharya, S (2013). Efficient management of fruit pests by pheromone nanogels. Scientific Reports, 3, 1294.

Bieri-Leonhardt, B. A. (2019). Release rates from formulations and quality control methods. In Kydonieus, A. F. (ed.) Insect Suppression with Controlled Release Pheromone Systems: Volume I. CRC Press: 245.

Bisschops, J. (1955). Gelation of concentrated polyacrylonitrile solutions. II. Journal of Polymer Science, 17(83), 89-98.

Blake, A. I., Co, E. D., & Marangoni, A. G. (2014). Structure and physical properties of plant wax crystal networks and their relationship to oil binding capacity. Journal of the American Oil Chemists' Society, 91(6), 885-903.

Bot, A., den Adel, R., & Roijers, E. C. (2008). Fibrils of γ-oryzanol+ β-sitosterol in edible oil organogels. Journal of the American Oil Chemists' Society, 85(12), 1127-1134.

Buerkle, L. E., & Rowan, S. J. (2012). Supramolecular gels formed from multi-component low molecular weight species. Chemical Society Reviews, 41(18), 6089-6102.

Chen, X. W., Guo, J., Wang, J. M., Yin, S. W., & Yang, X. Q. (2016). Controlled volatile release of structured emulsions based on phytosterols crystallization. Food Hydrocolloids, 56, 170-179.

Co, E. D., & Marangoni, A. G. (2018). Oleogels: an introduction. In Marangoni, A. G. & Garti, N. (ed.) Edible oleogels: structure and health implications. AOCS Press, Urbana, IL, USA: 1-29.

Costa, A., Ricard, I., Davison, A. C., & Turlings, T. C. (2010). Effects of rewarding and unrewarding experiences on the response to host-induced plant odors of the generalist parasitoid *Cotesia marginiventris* (Hymenoptera: Braconidae). Journal of Insect Behavior, 23(4), 303-318.

Crank, J. (1979). The Mathematics of Diffusion. Oxford University Press, London, UK.

da Silva, S. E., França, J. F., & Pareja, M. (2016). Olfactory response of four aphidophagous insects to aphid-and caterpillar-induced plant volatiles. Arthropod-Plant Interactions, 10(4), 331-340.

da Silva, T. L. T., Arellano, D. B., & Martini, S. (2019). Interactions between candelilla wax and saturated triacylglycerols in oleogels. Food Research International, 121, 900-909.

Daems, F., Béra, F., Lorge, S., Fischer, C., Brostaux, Y., Francis, F., Lognay, G. & Heuskin, S. (2016). Impact of climatic factors on the release of E-β-caryophyllene from alginate beads. Biotechnologie, Agronomie, Société et Environnement, 20(2): 130-142.

Davidovich-Pinhas, M. (2016). Oleogels: a promising tool for delivery of hydrophobic bioactive molecules. Therapeutic Delivery, 7(1), 1-3.

Davidovich-Pinhas, M. (2018). Oleogels. In Pal, K. & Banerjee, I. (ed.) Polymeric Gels: Characterization, properties and biomedical applications. Woodhead Publishing Series in Biomaterials, 231-249.

Davidovich-Pinhas, M., Barbut, S., & Marangoni, A. G. (2014). Physical structure and thermal behavior of ethylcellulose. Cellulose, 21(5), 3243-3255.

den Adel, R., Heussen, P. C., & Bot, A. (2010, October). Effect of water on self-assembled tubules in β-sitosterol+ γ-oryzanol-based organogels. Journal of Physics: Conference Series 247(1): 012025.

Dey, T., Kim, D. A., & Marangoni, A. G. (2011). Ethylcellulose oleogels. In Marangoni, A. G. & Garti, N. (ed.) Edible oleogels: structure and health implications. AOCS Press, Urbana, IL, USA: 295-311.

Dibildox-Alvarado, E., Rodrigues, J. N., Gioielli, L. A., Toro-Vazquez, J. F., & Marangoni, A. G. (2004). Effects of crystalline microstructure on oil migration in a semisolid fat matrix. Crystal Growth & Design, 4(4), 731-736.

Duffy, N., Blonk, H. C., Beindorff, C. M., Cazade, M., Bot, A., & Duchateau, G. S. (2009). Organogel-based emulsion systems, micro-structural features and impact on in vitro digestion. Journal of the American Oil Chemists' Society, 86(8), 733-741.

ECHA, https://echa.europa.eu/substance-information/-/substanceinfo/100.126.240; accessed: 01/2022.

Esposito, C. L., Kirilov, P., & Roullin, V. G. (2018). Organogels, promising drug delivery systems: An update of state-of-the-art and recent applications. Journal of Controlled Release, 271, 1-20.

Frank, A., Rath, S. K., & Venkatraman, S. S. (2005). Controlled release from bioerodible polymers: Effect of drug type and polymer composition. Journal of Controlled Release, 102(2), 333-344.

Gom, T. (2001). Oleogels and their applications. In Osada Y, Kajiwara K, Fushimi T, Irasa O, Hirokawa Y, Matsunaga T, Shimomura T, Wang L, Ishida H (ed.) Gels Handbook. Academic Press: 368-380

Gravelle, A. J., Davidovich-Pinhas, M., Barbut, S., & Marangoni, A. G. (2017). Influencing the crystallization behavior of binary mixtures of stearyl alcohol and stearic acid (SOSA) using ethylcellulose. Food Research International, 91, 1-10.

Gravelle, A. J., Davidovich-Pinhas, M., Zetzl, A. K., Barbut, S., & Marangoni, A. G. (2016). Influence of solvent quality on the mechanical strength of ethylcellulose oleogels. Carbohydrate Polymers, 135, 169-179.

Gravelle, A. J., Marangoni, A. G., & Davidovich-Pinhas, M. (2018). Ethylcellulose oleogels. In Marangoni AG, Garti N (ed.) Edible oleogels, Vol. 2, AOCS Press, Urbana, IL, USA: 331-362.

Hansen, C. M. (2007). Hansen solubility parameters: A user's handbook, CRC Press, Boca Raton, Florida, USA.

Heuskin, S., Lorge, S., Godin, B., Leroy, P., Frère, I., Verheggen, F.J., Haubruge, E., Wathelet, J.-P., Mestdagh, M., Hance, T. & Lognay, G. (2012). Optimisation of a semiochemical slow-release alginate formulation attractive towards *Aphidius ervi* Haliday parasitoids. Pest Management Science, 68(1), 127-136.

Heuskin, S., Verheggen, F. J., Haubruge, E., Wathelet, J. P., & Lognay, G. (2011). The use of semiochemical slow-release devices in integrated pest management strategies. BASE 15(3), 459-470.

Howse, P., Stevens, J. M., & Jones, O. T. (2013). Insect Pheromones and their Use in Pest Management. Springer Science & Business Media.

Jadhav, S. R., Chiou, B. S., Wood, D. F., DeGrande-Hoffman, G., Glenn, G. M., & John, G. (2011). Molecular gels-based controlled release devices for pheromones. Soft Matter, 7(3), 864-867.

Jelić, D. (2021). Thermal stability of amorphous solid dispersions. Molecules, 26(1), 238.

Kaplan, S., Colak, M., Hosgoren, H., & Pirinccioglu, N. (2019). Design of L-lysine-based organogelators and their applications in drug release processes. ACS Omega, 4(7), 12342-12356.

Kavanagh, G. M., & Ross-Murphy, S. B. (1998). Rheological characterisation of polymer gels. Progress in Polymer Science, 23(3), 533-562.

Knight, A. L., Larsen, T. E., & Ketner, K. C. (2004). Rainfastness of a microencapsulated sex pheromone formulation for codling moth (Lepidoptera: Tortricidae). Journal of Economic Entomology, 97(6), 1987-1992.

Korsmeyer, R. W., Gurny, R., Doelker, E., Buri, P., & Peppas, N. A. (1983). Mechanisms of solute release from porous hydrophilic polymers. International Journal of Pharmaceutics, 15(1), 25-35.

Laredo, T., Barbut, S., & Marangoni, A. G. (2011). Molecular interactions of polymer oleogelation. Soft Matter, 7(6), 2734-2743.

Lim, J., Hwang, H. S., & Lee, S. (2017). Oil-structuring characterization of natural waxes in canola oil oleogels: Rheological, thermal, and oxidative properties. Applied Biological Chemistry, 60(1), 17-22.

Liu, R., Xu, Y., Chang, M., Tang, L., Lu, M., Liu, R., Jin, Q. & Wang, X. (2021). Antioxidant interaction of α-tocopherol, γ-oryzanol and phytosterol in rice bran oil. Food Chemistry, 343, 128431.

Lopez, M. D., Maudhuit, A., Pascual-Villalobos, M. J., & Poncelet, D. (2012). Development of formulations to improve the controlled-release of linalool to be applied as an insecticide. Journal of Agricultural and Food Chemistry, 60(5), 1187-1192.

Mao, L., Lu, Y., Cui, M., Miao, S., & Gao, Y. (2020). Design of gel structures in water and oil phases for improved delivery of bioactive food ingredients. Critical Reviews in Food Science & Nutrition, 60(10), 1651-1666.

Martin, B., Brouillet, F., Franceschi, S., & Perez, E. (2017). Evaluation of organogel nanoparticles as drug delivery system for lipophilic compounds. AAPS Pharmscitech, 18(4), 1261-1269.

Martins, A. J., Cerqueira, M. A., Fasolin, L. H., Cunha, R. L., & Vicente, A. A. (2016). Beeswax organogels: Influence of gelator concentration and oil type in the gelation process. Food Research International, 84, 170-179.

Maru, S. M., de Matas, M., Kelly, A., & Paradkar, A. (2011). Characterization of thermal and rheological properties of zidovidine, lamivudine and plasticizer blends with ethyl cellulose to assess their suitability for hot melt extrusion. European Journal of Pharmaceutical Sciences, 44(4), 471-478.

Mayer, C. J., Vilcinskas, A., & Gross, J. (2008). Pathogen-induced release of plant allomone manipulates vector insect behavior. Journal of Chemical Ecology, 34(12), 1518-1522.

Melzer, E., Kreuter, J., & Daniels, R. (2003). Ethylcellulose: A new type of emulsion stabilizer. European Journal of Pharmaceutics and Biopharmaceutics, 56(1), 23-27.

Milanovic, J., Manojlovic, V., Levic, S., Rajic, N., Nedovic, V., & Bugarski, B. (2010). Microencapsulation of flavors in carnauba wax. Sensors, 10(1), 901-912.

Muskat, L.C. & Patel, A.V. (2022). Innovations in semiochemical formulations. Entomologia Generalis, 42(2): 231–249. https://doi.org/10.1127/entomologia/2021/1230

Nelis, V., De Neve, L., Danthine, S., Dewettinck, K., Martins, J. C., & Van der Meeren, P. (2021). Oil diffusion in fat crystal matrices: Characterization by NMR relaxometry and diffusometry. European Journal of Lipid Science & Technology, 123(4), 2000237.

Nguyen, D. V., Li, F., Li, H., Wong, B. S., Low, C. Y., Liu, X. Y., & Kang, L. (2015). Drug permeation through skin is inversely correlated with carrier gel rigidity. Molecular Pharmaceutics, 12(2), 444-452.

Nussinovitch, A. (2010). Polymer macro-and micro-gel beads: fundamentals and applications. Springer Science & Business Media.

O'Sullivan, C. M., Barbut, S., & Marangoni, A. G. (2016). Edible oleogels for the oral delivery of lipid soluble molecules: Composition and structural design considerations. Trends in Food Science & Technology, 57, 59-73.

Pang, M., Cao, L., Kang, S., Jiang, S., & Cao, L. (2021). Controlled release of flavor substances from sesame-oil-based oleogels prepared using biological waxes or monoglycerides. Foods, 10(8), 1828.

Park, C., & Maleky, F. (2020). A critical review of the last 10 years of oleogels in food. Frontiers in Sustainable Food Systems, 4, 139.

Peppas, N. A., & Sahlin, J. J. (1989). A simple equation for the description of solute release. III. Coupling of diffusion and relaxation. International journal of pharmaceutics, 57(2), 169-172.

Pérez, J. S., Porcel, E. R., López, J. C., Sevilla, J. F., & Chisti, Y. (2006). Shear rate in stirred tank and bubble column bioreactors. Chemical Engineering Journal, 124(1-3), 1-5.

Plimmer, J. R. (1981). Formulation and regulation: constraints on the development of semiochemicals for insect pest management. In Mitchell, E. R. (ed.) Management of Insect Pests with Semiochemicals, Springer, Boston, MA, 403-420.

Mäder, R., Leopold, J., Gatzert, X., Milan, M., Gräber, H., Hermanowski, S., Zapf C. (2019). Betriebsmittelliste 2019 für den ökologischen Landbau in Deutschland, FiBL, Frick, Switzerland 2019.

Rasmann, S., Köllner, T. G., Degenhardt, J., Hiltpold, I., Toepfer, S., Kuhlmann, U., Gershenzon, J. & Turlings, T. C. (2005). Recruitment of entomopathogenic nematodes by insect-damaged maize roots. Nature, 434(7034), 732-737.

Rekhi, G. S., & Jambhekar, S. S. (1995). Ethylcellulose-a polymer review. Drug Development and Industrial Pharmacy, 21(1), 61-77.

Rezaei, A., Fathi, M., & Jafari, S. M. (2019). Nanoencapsulation of hydrophobic and low-soluble food bioactive compounds within different nanocarriers. Food Hydrocolloids, 88, 146-162.

Rocha, J. C. B., Lopes, J. D., Mascarenhas, M. C. N., Arellano, D. B., Guerreiro, L. M. R., & da Cunha, R. L. (2013). Thermal and rheological properties of organogels formed by sugarcane or candelilla wax in soybean oil. Food Research International, 50(1), 318-323.

Scher, H. B. (1977). Microencapsulated pesticides. Scher HB (1977) Microencapsulated pesticides. In Scher H (ed.) Controlled Release Pesticides. ACS Publications, Washington, DC, USA: 126-144.

Sears, J. K., Touchette, N. W., & Darby, J. R. (1985). Plasticizers. In Tess, R. W., Poehlein G.W. (ed.) Applied Polymer Science, Vol. 2, ACS Symposium Series, American Chemical Society, Washington, DC, USA, 611-641.

Shi, R., Zhang, Q., Vriesekoop, F., Yuan, Q., & Liang, H. (2014). Preparation of organogel with tea polyphenols complex for enhancing the antioxidation properties of edible oil. Journal of Agricultural and Food Chemistry, 62(33), 8379-8384.

Slodowicz, M. P., Ceriani-Nakamurakare, E., Carmarán, C., & González-Audino, P. A. (2017). Physicochemical properties and field evaluation of monolithic wax formulations for the controlled release of a forest pest pheromone. Journal of the Brazilian Chemical Society, 28, 348-359.

Suckling, D. M., & Karg, G. (1999). Pheromones and other semiochemicals. In Rechcigl, J. E. & Rechcigl, N. A. (ed.), Biological and Biotechnological Control of Insect Pests. CRC Press, Boca Raton, FL, 63-99.

Sun, Y., Fan, S., Liang, R., Ni, X., Du, Y., Wang, J., & Yang, C. (2021). Design and characterization of starch/solid lipids hybrid microcapsules and their thermal stability with menthol. Food Hydrocolloids, 116, 106631.

Taylor, N., & Matthews, G. A. (1986). Effect of different adjuvants on the rainfastness of bendiocarb applied to Brussels sprout plants. Crop Protection, 5(4), 250-253.

Thakkar, R., Thakkar, R., Pillai, A., Ashour, E. A., & Repka, M. A. (2020). Systematic screening of pharmaceutical polymers for hot melt extrusion processing: A comprehensive review. International Journal of Pharmaceutics, 576, 118989.

Dow (2005) Ethylcellulose polymers technical handbook. The Dow Chemical Company.

Turlings, T. C., Davison, A. C., & TamÒ, C. (2004). A six-arm olfactometer permitting simultaneous observation of insect attraction and odour trapping. Physiological Entomology, 29(1), 45-55.

Vallet, A., Cassier, P., & Lensky, Y. (1991). Ontogeny of the fine structure of the mandibular glands of the honeybee (*Apis mellifera* L.) workers and the pheromonal activity of 2-heptanone. Journal of Insect Physiology, 37(11), 789-804.

Valls, A., Castillo, A., Porcar, R., Hietala, S., Altava, B., García-Verdugo, E., & Luis, S. V. (2020). Urea-based low-molecular-weight pseudopeptidic Organogelators for the encapsulation and slow release of (R)-limonene. Journal of Agricultural and Food Chemistry, 68(26), 7051-7061.

Valoppi, F., Salmi, A., Ratilainen, M., Barba, L., Puranen, T., Tommiska, O., Helander, P., Heikkilä, J. & Haeggström, E. (2020). Controlling oleogel crystallization using ultrasonic standing waves. Scientific Reports, 10(1), 1-13.

Vemmer, M., & Patel, A. V. (2013). Review of encapsulation methods suitable for microbial biological control agents. Biological Control, 67(3), 380-389.

Vintiloiu, A., & Leroux, J. C. (2008). Organogels and their use in drug delivery - A review. Journal of Controlled Release, 125(3), 179-192.

Vitiello, A., Molisso, D., Digilio, M. C., Giorgini, M., Corrado, G., Bruce, T. J., D'Agostino, N. & Rao, R. (2021). Zucchini plants alter gene expression and emission of (E)-β-Caryophyllene following *Aphis gossypii* infestation. Frontiers in Plant Science, 11, 592603.

Winter, T. R., & Rostás, M. (2010). Nitrogen deficiency affects bottom-up cascade without disrupting indirect plant defense. Journal of Chemical Ecology, 36(6), 642-651.

Yadav, I., Kasiviswanathan, U., Soni, C., Paul, S. R., Nayak, S. K., Sagiri, S. S., Anis, A. & Pal, K. (2017). Stearic acid modified stearyl alcohol oleogel: Analysis of the thermal, mechanical and drug release properties. Journal of Surfactants and Detergents, 20(4), 851-861.

Yang, D. X., Chen, X. W., & Yang, X. Q. (2018). Phytosterol-based oleogels self-assembled with monoglyceride for controlled volatile release. Journal of the Science of Food and Agriculture, 98(2), 582-589.

Yilmaz, E., & Demirci, Ş. (2021). Preparation and evaluation of virgin olive oil oleogels including thyme and cumin spices with sunflower wax. Gels, 7(3), 95.

Yılmaz, E., Öğütcü, M., & Yüceer, Y. K. (2015). Physical properties, volatiles compositions and sensory descriptions of the aromatized hazelnut oil-wax organogels. Journal of Food Science, 80(9), 2035-2044.

Yogev, S., & Mizrahi, B. (2020). Organogels as a delivery system for volatile oils. ACS Applied Polymer Materials, 2(5), 2070-2076.

Yoon, J. Y., Yang, C. Y., & Kim, J. D. (2017). Sustainable delivery of a sex pheromone with an ester wax to disrupt *Grapholita molesta* mating. Macromolecular Research, 25(4), 374-380.

Chapter 3

Temperature-triggered release of β-caryophyllene from an ethylcellulose-candelilla wax oleogel

Abstract

Insect flight activity of a majority of agricultural important insects increases with increasing temperature. Stimuli-responsive semiochemical delivery systems can match the release with the insect´s flight activity. Oleogels as thermo-responsive formulation matrices bear the potential for such a release behavior. In this study, the potential of an ethylcellulose–candelilla wax hybrid oleogel for the controlled release of β-caryophyllene as model semiochemical was investigated. The peak melting temperature of the candelilla wax in the two gels investigated in the present study was found at 29.1 and 38.9 °C. The release from the two EC-CLW gels with different candelilla wax contents significantly increase when the temperature was increased above the peak melting temperature of the candelilla wax. The candelilla wax free gel showed no switch but a linear release increase with increasing temperature. A fine-tuned temperature-triggered release could be obtained by altering the wax concentration or replacement by other thermo-responsive oleogelators with a different melting and crystallization temperature.

Graphical abstract

Introduction

The release behavior from most semiochemical formulations or dispensers developed to date is a passive release, resulting in waste of expensive substances even when the target insect is inactive (Muskat & Patel., 2021). Thus, there is a need for further developments of formulations that release the semiochemical matching with the target insect´s behavior.

Insect flight activity of a majority of agricultural important insects is temperature dependent and increases with increasing temperature up to a temperature optimum individual for each insect and then decrease at further temperature increase (Taylor, 1963; Goller & Esch, 1990; Hall, 2009). Hence, temperature is a reasonable stimulus when developing controlled release formulations for application in push-pull or attract-and-kill strategies for the behavior manipulation of these insects. Aiming at a controlled release by temperature as stimulus is a special challenge, as temperature is the major determinant of the semiochemical release rate, even from passive dispensers (Nielsen et al., 2019). Hence, a formulation with a temperature-triggered release needs to be prepared from a thermo-responsive material. The development of stimuli-responsive gels is one of the top subjects of current soft materials science (Ishi & Shinkai, 2005; Esser-Kahn et al., 2009). Thermo-responsive drug release is well established in pharmaceutical formulation technology, where a thermo-triggered release at body temperature is preferred (Fitzpatrick et al., 2012; Bikram & West, 2008). Few attempts have been made for the temperature-triggered release of semiochemicals. Xiao et al. (2021) developed a microcapsule with adjustable controlled-release characteristics by the change in temperature based on phase transition of the n-hexadecane core of the capsules. However, microcapsules commonly suffer from a very low deposit of the active ingredient and are unsuitable for a long-term release of semiochemicals (Muskat & Patel, 2022). An interesting matrix type formulation with a high loading capacity for lipophilic compounds are so called organogels or oleogels. Numerous studies deal with the structure-functional relationship of the delivery rate of active lipophilic compounds from oleogels (Lupi et al., 2013; O´Sullivan et al., 2016; Yang et al., 2018; Mao et al., 2019). In fact, most organogels are thermo-responsive (Patel et al., 2013) and thereby enable the controlled release of incorporated active substances by phase transition through heating (Sagiri et al., 2014; Fasolin et al. 2018), as shown for the model drug indomethacin by Tokuyama & Kato (2010). Only a few studies have focused on the formulation and release of volatiles from organogels and moreover the potential of organogels for the controlled and stimuli-triggered release of volatile substances such as semiochemicals is not yet explored.

Recently, Muskat et al. (2022) developed an oleogel formulation for the slow release of the model semiochemical β-caryophyllene. The formulation contains ethylcellulose and candelilla wax as oleogelators and it was shown that the release is mainly affected by the candelilla wax (CLW) content under static temperature conditions at 25 °C.

In this study it was hypothesized that this formulation bears the potential for a temperature-triggered release of β-caryophyllene as candelilla wax is a thermo-responsive oleogelator with melting and crystallization points at moderate temperatures when incorporated as oleogelator in an organic phase (Toro-Vazquez et al., 2007). It is further expected that the oil-leakage from the gel will increase with temperature due to melting of the wax, as the wax was found to be the major stabilizer of the formulation.

Results and Discussion

The combination of different synergistically acting oleogelators to create hybrid oleogels enable the modulation of gel properties in a desired way for special applications (Davidovich-Pinhas et al., 2018; Buerkle & Rowan, 2012; Gravelle et al., 2017; Gravelle et al., 2018; da Silva et al., 2019).

In a previous study (Muskat et al., 2022), the gel investigated in the present study have been proven as highly suitable for the formulation and release of semiochemicals, in particular the model semiochemical β-caryophyllene. In view of later field application in insect behavior manipulating strategies, the effect of temperature on release and material properties plays a crucial role. The starting hypothesis of the present study was, that the melting of the thermo-responsive oleogelator candelilla wax will enable release control.

Melting behavior of the CLW in the EC-CLW-bCAR oleogel

The second oleogelator candelilla wax serves as a thermo-responsive additive at the same time. In **Figure 3.1** the differential scanning calorimetry results of the melting behavior of the second oleogelator candelilla wax is shown. As expected, the melting temperature and enthalpy increases with increasing candelilla wax content. This is in well accordance with previous studies dealing with wax as oleogelators (Toro-Vazquez et al., 2007; Blake et al., 2014; Martins et al., 2016). For lowest CLW content of 1%, the peak melting temperature was 23.9 °C. With increasing CLW concentration the peak melting temperature increased to 29.1 (W2), 38.9 (W4), 40.8 (W8) and 44.0 °C (W16). Moreover, it was observed, that the CLW as oleogelator has a wide melting range, indicated in **Figure 3.1** by onset melting temperature (Tm Onset) and offset melting temperature (Tm Offset) of each wax content. This can be attributed to the composition of CLW: CLW has a high content of hydrocarbons and a medium amount of esters and some minor compounds (Blake et al., 2014). Furthermore, it was observed that the increase in the melting point was not linear or exponential correlating to the CLW content. This can be attributed to a change in solubility of the gel components, as a solubility optimum or saturation seems to be reached around 4% CLW. This assumption is supported by the data on melting enthalpy (ΔH). Melting enthalpy was very low at low CLW concentrations (1% and 2% CLW), followed by a shift at 4% and then did not further increase at 8%, but was much higher at 16%.

The melting temperatures or areas are suggested to be the temperature at which a change in release will be observable. A switch should not be present in the W0 gel without the second thermo-responsive oleogelator wax.

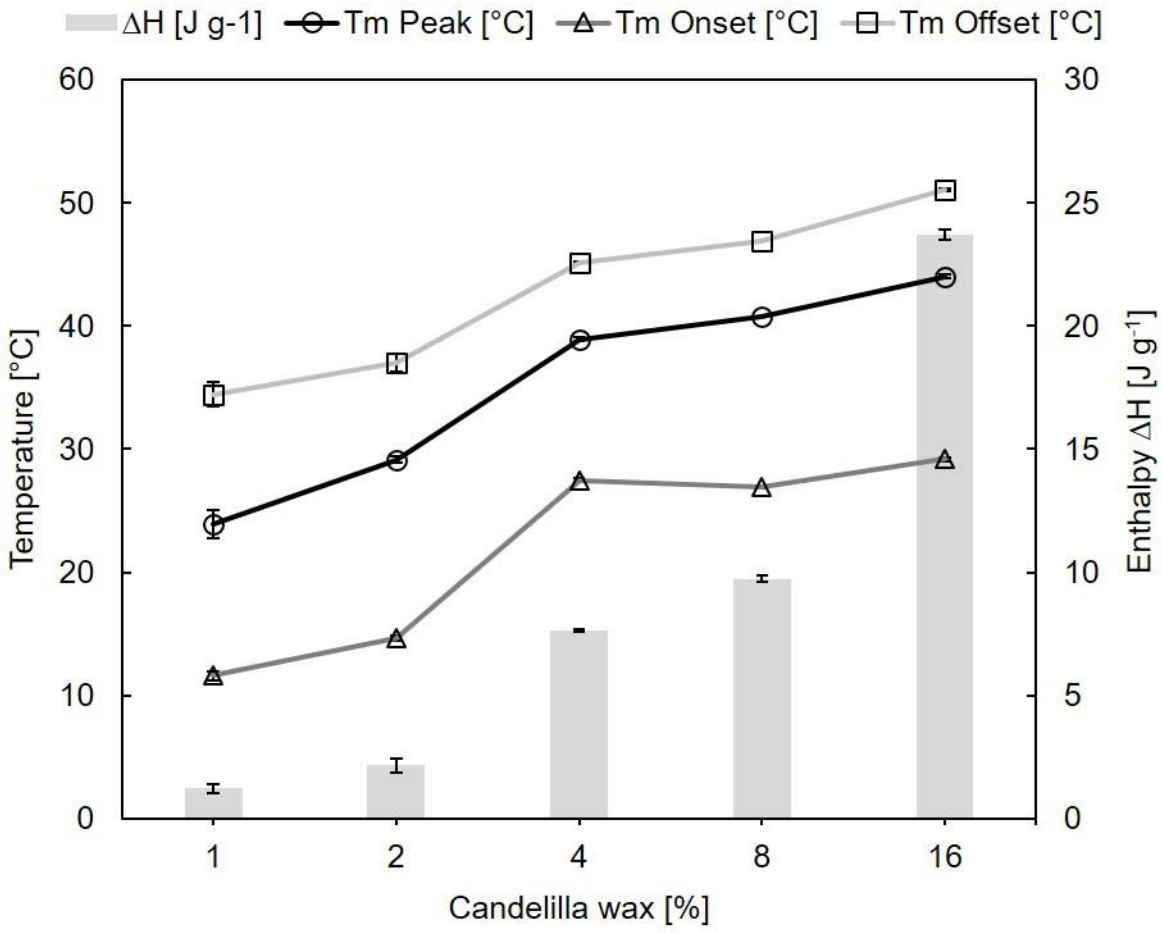


Figure 3.1 Peak melting temperature (Tm Peak), Onset melting temperature (Tm Onset), Offset melting temperature (Tm Offset) and melting enthalpy (ΔH) of the EC-CLW-oleogel+bCAR dependent from the candelilla wax content. Mean ±SD, n = 3.

Effect of temperature on release

As shown in **Figure 3.2** and determined by a repeated measures ANOVA with a Greenhouse-Geisser correction temperature was found having a significant effect on the release from all formulation variations ($F_{3,30}$ = 233.501; p<0.001). In all variations an increase in release is observable. The release increase from W0+bCAR is likely linear when the temperature increased from 15 to 25, 35 and 45 °C, which was expected, as there is no temperature-induced material modification expected besides softening of the EC-oleogel. The relationship of increased release rates with increased temperatures has been found in several other studies (Kraan and Ebbers 1990; Bradley et al. 1995; Stipanovic et al. 2004; Zhu et al. 2015; Daems et al., 2016).

The candelilla wax content also has a significant impact on the β-caryophyllene release ($F_{2,10}$ = 40.444; p<0.001). In both formulation compositions (W2+bCAR and W4+bCAR) release was slower when temperature was ≤35 °C compared to the wax free formulation. As hypothesized, the increase in release matches with the melting of the wax (see **Figure 3.1**): Peak melting temperature for W2+bCAR is at 29.1 °C, onset at 14.6 °C, offset at 37.0 °C, for W4+bCAR peak melting temperature is 38.9 °C, onset at 27.5 °C, offset at 45 °C. The switch in increased release up to an amount as high as W0 (without a thermo-responsive, meltable additive) was for W2+bCAR between 25 to 35 °C and for W4+bCAR from 35 to 45 °C, which matches with the mean melting area. The melting of the wax crystals of W4+bCAR is also visible in the **Graphical abstract**.

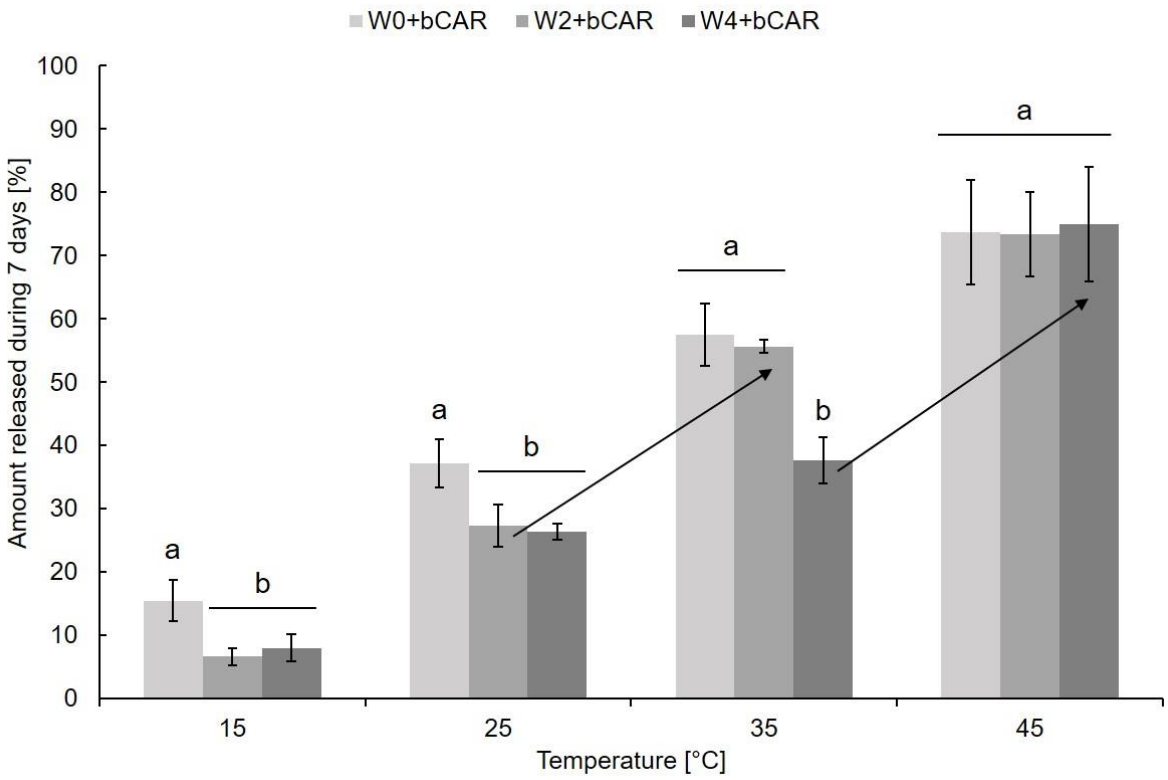


Figure 3.2 Release after 7 days of incubation under different temperature conditions from the gel containing 0% (W0+bCAR), 2% (W2+bCAR) and 4% (W4+bCAR) candelilla wax. Different letters above bars indicate significant differences between the released amounts of bCAR from the formulations containing different wax contents within one temperature regime based on a RM-ANOVA and pairwise comparation followed by a Bonferroni post-hoc test at $p<0.05$. Mean ± SD; n = 5. Arrows highlight the release increase of W2+bCAR and W4+bCAR correlating with the peak melting temperature.

Oil loss

It is well known, that CLW is a wax with a high oil-binding capacity and it was found, that with increasing CLW content the oil-loss can be reduced under a constant temperature of 25 °C (Muskat et al., 2022). Data on the effect of temperature on oil-loss from wax oleogels are rare.

Here we hypothesized, that melting of the oil will lead to gel properties similar to the gel without wax. Therefore, it is important to set the oil-loss data under different temperatures in correlation with the melting temperature.

A repeated measures ANOVA with a Greenhouse-Geisser correction determined that temperature had a significant effect on oil-loss ($F_{4,80}=343.576$; $p<0.001$), candelilla wax content had a significant effect on oil-loss ($F_{3,20}=104.659$; $p<0.001$) and the combination of temperature with the wax content had a significant effect on oil-loss ($F_{12,80}=12.110$; $p<0.001$). Bonferroni-adjusted post-hoc analysis revealed significant differences within the groups, as indicated in **Figure 3.3**.

As expected, the gel without wax (W0+bCAR) showed a temperature independent oil-loss. Thus, this is in well accordance with the fact, that the wax is the main compound of the gel hindering the oil leaking from the EC network. Only small amounts of CLW (2%; W2+bCAR) were able to significantly reduce oil loss from the gel, in as far as the temperature was lower than the melting temperature of the wax incorporated in the gel, as it is obvious in the gel W2+bCAR, where the oil-loss increased to a similar manner like the gel without wax (W0+bCAR) when the samples were incubated above the melting

temperature (~27 °C; **Figure 3.1**). Interestingly, when the wax content was further increased to ≥4%, the oil-loss below 45 °C and thus, a temperature at which the wax is molten, did not reach the high oil-loss like W0+bCAR.

This can be explained by the interaction of the oil-phase and the molten wax with the EC network. Frolova et al. (2022) found that the content of total polar materials in oils is associated with a decrease in the oil-binding capacity of oleogels. Giacintucci et al. (2018) found as the minor compounds from olive oil were removed, gel strength decreased, mainly due to the decrease in oil polarity and concomitant decreased EC solubility in the oil. Thus, an increase of oil polarity by melting of the wax and thereby an increase of the amount of molten wax compounds in the oil phase which is at the same time the solvent of EC, changes the interaction of the oil phase with the EC network.

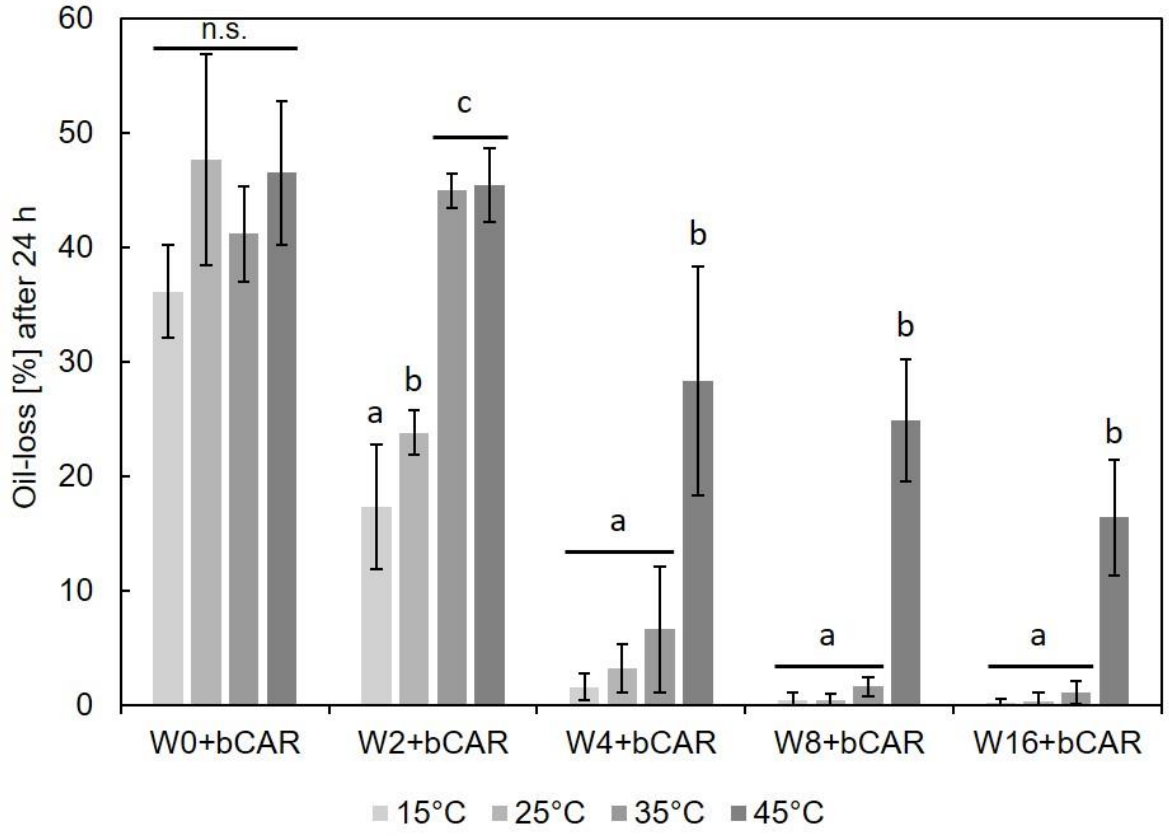


Figure 3.3 Oil loss from the oleogel containing 0, 2, 4, 8 and 16% candelilla wax after 24 h incubation under different temperature conditions. Different letters above bars indicate significant differences within one formulation composition based on RM-ANOVA and Bonferroni post-hoc test at $p<0.05$. Mean ±SD; n=5.

Analysis of the crystallization of the wax showed that the melting of the CLW is reversible and the wax recrystallizes under cooling of the gel, as shown in **Figure 3.4**. This indicates that the release will be slowed down when the gel is cooled below the crystallization temperature of the wax.

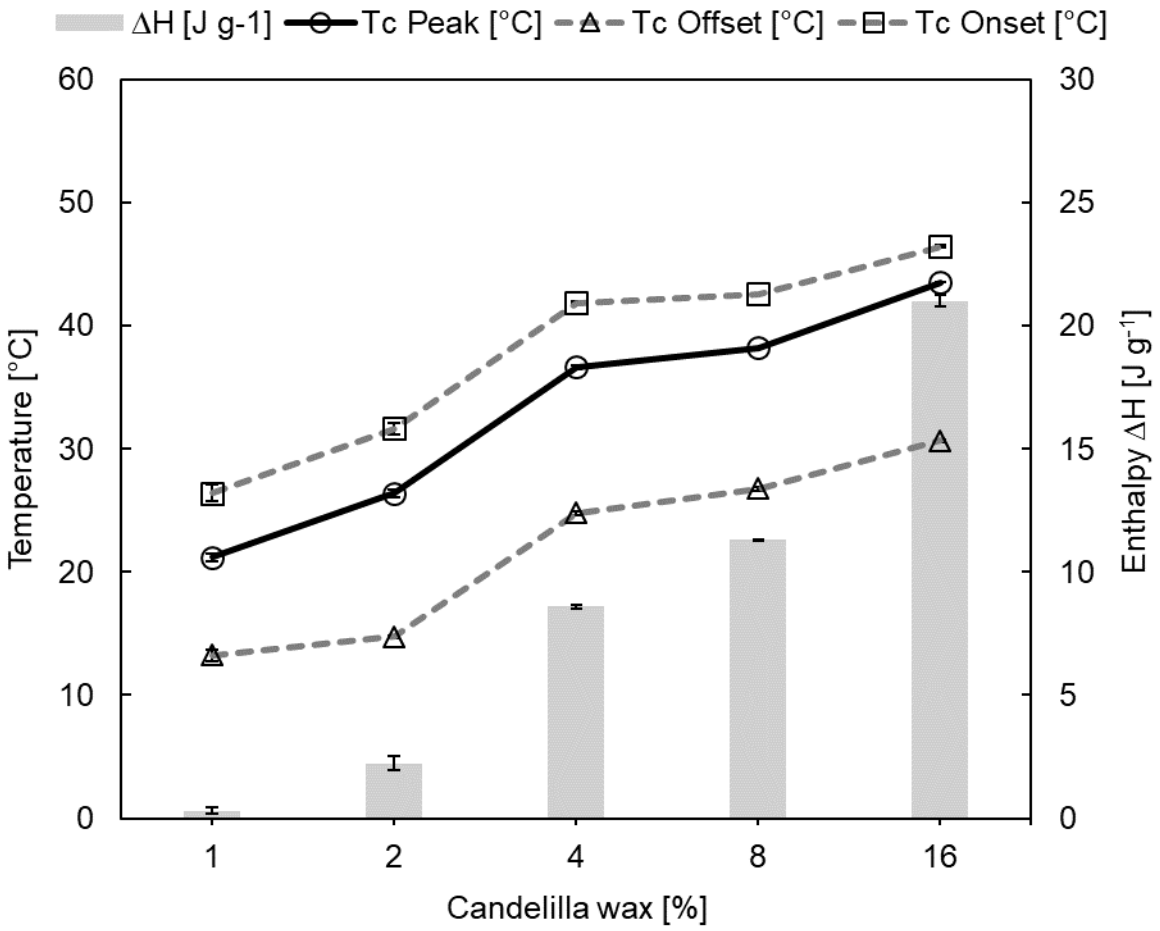


Figure 3.4 Peak crystalization temperature (Tc Peak), Onset crystalization temperature (Tc Onset), Offset crystalization temperature (Tc Offset) and crystalization enthalpy (ΔH) of the EC-CLW-oleogel+bCAR dependent from the candelilla wax content. Mean ±SD, n = 3.

Conclusion

This study presents the first report of thermo-tiggered release of a semiochemical from an ethylcellulose-candelilla wax oleogel. It was found, that the release increase matches with the melting of the second oleogelator candelilla wax. As shown in a previous study (**Chapter 2**), the “outer” gel containing only ethylcellulose as single oleogelator is thermally stable up to 66.9 °C (sol-gel temperature; Muskat et al., 2022). Hence, at temperatures present in Middle European fruit orchards, the formulation proposes shape stability under simultaneously temperature induced material modifications due to melting of the second oleogelator candelilla wax. Such a form stability in combination with a temperature triggered release represents a novel highly innovative delivery system for semiochmicals. The melting range of the thermo-responsive additive, e.g. by replacement with another wax with a narrower melting and crystallization range, is proposed to fine-tune the release to match it with a target insect´s behavior. The On/Off release behavior will prolong release by protection of the deposit during cold temperatures. In order to improve the predictability of the temperature induced release, future work should investigate modelling with the free volume – diffusion model described by Rafailovich & Sokolov (2011) as there is a shrinking and expansion of the gel under alternating temperature due to melting and crystallization of the wax.

Experimental Section

Chemicals

Sunflower oil (SO) was purchased from a local supermarket, ethylcellulose (EC; ETHOCELTM Standard 100 Industrial Ethylcellulose; batch code: D184IA9031; Dow Chemical Company, Midland, MI, USA) with ethoxyl content 48.7% and a viscosity of 98.0 mPa*s was obtained from ChemPoint (Bergen op Zoom, Netherlands), candelilla wax (W; 2039; batch code: 10-1185) from Kahlwax GmbH & Co. KG (Trittau, Germany), 2-octyldodecan-1-ol (OD; Eutanol® G; batch code: 18112004) from Caelo (Caesar & Loretz GmbH, Hilden, Germany) and β-caryophyllene (bCAR; ≥80%, FCC, FG; batch code: MKCG3589) from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Preparation procedure

Gels were prepared according to the method described in Muskat et al. (2022). Sunflower oil was mixed with octyldodecanol in a beaker and ethylcellulose was added. The mixture was heated above the ethylcellulose glass transition temperature (140 °C) under continuous stirring at 250 rpm on a magnetic hotplate stirrer (IKA® C-MAG HS 7, IKA®-Werke GmbH & CO. KG, Staufen, Germany) set to 180 °C. After full dissolution of the ethylcellulose (~1 hour), the mixture was cooled to a temperature above the no-flow temperature (70 °C) and candelilla wax was added. After full melting and solving of the candelilla wax, β-caryophyllene was added and the sol was set to room temperature for thermal gelation. Samples were stored in closed containers in a fridge at 5 °C or used immediately. All formulation compositions tested in this study are listed in **Chapter 2** in **Table 2.2.**

Analysis

Thermal properties

The melting temperatures (T_m), crystalization temperatures (T_C) and melting and crystallization enthalpy (ΔH) of the gels were determined by using a DSC 3 STARe System differential scanning calorimeter (Mettler-Toledo GmbH, Gießen, Deutschland). Oleogel samples were prepared were prepared in lenticular droplet shape (8-12 mg), were weighted and placed in 40 µL aluminium crucibles (Mettler-Toledo, Gießen, Germany), which were hermetically sealed. Measurements were carried out with the TOPEM® method with a pulse of ±1 K. Initial temperature was set to 5 °C. With a rate of 2 K/min the temperature was raised to 60 °C and held for 1 min. In the case of the samples containing 16% candelilla wax (W16+bCAR), the temperature maximum was 90 °C. Afterwards, the sample was cooled with a rate of -2 K/min to 5 °C and held for 1 min. The heating-cooling cycle was run twice. Nitrogen flow was set to 60 ml/min. Data were analyzed using the STARe software (Mettler-Toledo) and mean values of three replicates were given as results.

β-caryophyllene release

In order to characterize the release of β-caryophyllene, samples were prepared by pouring the oleogel melt into crystallization dishes (Crystallization dishes ROTILABO® without spout, 20 ml, Ø = 40 mm, Carl Roth GmbH, Karlsruhe). For the oleogel and the oil samples 3.5 g were poured per dish. Equal to the 30% loading, 1.16 g β-caryophyllene (ρ = 0.9) was filled per dish. Every experiment run was performed with five samples per oleogel composition and control. The samples were then placed in a climatic chamber (KBF P 240 E5.2, Binder GmbH, Tuttlingen, Germany) and incubated at the following temperatures: 15, 25, 35 and 45 °C at 60% relative humidity. The release of β-caryophyllene was

determined gravimetrically by weight loss, which was measured on an analytical balance (AG204 DeltaRange®, Mettler Toledo GmbH, Gießen, Germany) every 24 hours.

$$Cumulative\ bCAR\ release\ [\%] = 100\ [\%] - \frac{Weight\ loss\ bCAR\ (t_x)[\%]}{Loading\ (t_0)\ [\%]} * 100\ [\%] \quad (6)$$

Results are given in % released after 7 days of incubation in relation to the initial loading.

Oil loss determination

Oil loss from oleogels was determined following the procedure of Dibildox-Alvarado et al. with some modifications. Therefore, oleogel samples were prepared in lenticular droplet form (10–15 mg). One droplet was placed in the middle of a pre-weighted filter paper (Ø = 55 mm, particle retention 12–15 µm, VWR International, Leuven, France). Blank filter papers without a sample were used as a control. The filter papers carrying the samples were individually placed on small petri dishes in order to prevent oil loss from the filter paper during the experiment. The filter papers were placed in climatic chambers (INCU-Line, VWR International, Leuven, France) and incubated at the following temperatures: 15, 25, 35 and 45 °C at 30–40% relative humidity for 24 h. Afterwards, the oleogel samples were discarded and the filter papers were weighed again. The oil loss was calculated as follows:

$$Oil\ loss\ [\%] = \frac{w_{filter+oil\ (t24)} - w_{filter\ (t0)}}{w_{sample\ (t0)}} * 100\ \% \quad (7)$$

Where w is weight (in g) and t is time (in h).

Statistical analysis

Statistical analysis was carried out using the software SPSS Statistics V25.0 (SPSS, Chicago, IL). All data are given as mean values ± standard deviations (SD). The level of significance was set to $p < 0.05$. All treatments were performed with at least five samples, unless otherwise stated. All data were checked for normality by a *Shapiro-Wilk* test at $\alpha = 0.05$ and homogeneity of variance by a *Levene* test. Data on oil-loss and β-caryophyllene release were arcsin transformed prior to analysis. Data on oil-loss were analyzed by one-way ANOVA followed by a *Tukey post-hoc* test. Data on release of β-caryophyllene were proofed for outliers and extreme values, which were excluded from further analysis. A repeated measures analysis (RM-ANOVA) was conducted, followed by a Bonferroni post hoc test and Greenhouse-Geisser correction to examine the effect of the temperature in combination with candelilla wax content on the release rate.

References

Blake, A.I., Co, E., Marangoni, A.G. (2014) Structure and physical properties of plant wax crystal networks and their relationship to oil binding capacity. Journal of the American Oil Chemists' Society 91, 885e903.

Bradley SJ, Suckling DM, McNaughton KG, Wearing CH, Karg G. 1995. A temperature-dependent model for predicting release rates of pheromone from a polyethylene tubing dispenser. Journal of Chemical Ecology, 21(6), 745–760.

Buerkle, L. E., & Rowan, S. J. (2012). Supramolecular gels formed from multi-component low molecular weight species. Chemical Society Reviews, 41(18), 6089-6102.

Chen, X. W., Li, X. X., Ma, C. G., Wan, Z. L., & Sun, S. D. (2021). Structuring of edible liquid oil into smart thermo-triggered soft matters for controlled bioactive delivery. Journal of Agricultural and Food Chemistry, 70(1), 309-318.

da Silva, T. L. T., Arellano, D. B., & Martini, S. (2019). Interactions between candelilla wax and saturated triacylglycerols in oleogels. Food Research International, 121, 900-909.

Daems F, Béra F, Lorge S, Fischer C, Brostaux Y, Francis F, Lognay G, Heuskin S. (2016). Impact of climatic factors on the release of E-β-caryophyllene from alginate beads. Biotechnologie, Agronomie, Société et Environnement, 20(2), 130–142.

Davidovich-Pinhas, M. (2018). Oleogels. In Pal, K. & Banerjee, I. (ed.) Polymeric Gels: Characterization, properties and biomedical applications. Woodhead Publishing Series in Biomaterials, 231-249.

Esser-Kahn, A. P., Odom, S. A., Sottos, N. R., White, S. R., & Moore, J. S. (2011). Triggered release from polymer capsules. Macromolecules, 44(14), 5539-5553.

Frolova, Y., Sarkisyan, V., Sobolev, R., Makarenko, M., Semin, M., & Kochetkova, A. (2022). The influence of edible oils' composition on the properties of beeswax-based oleogels. Gels, 8(1), 48.

Giacintucci, V., Di Mattia, C. D., Sacchetti, G., Flamminii, F., Gravelle, A. J., Baylis, B., Dutcher, J. R., Marangoni, A. G. & Pittia, P. (2018). Ethylcellulose oleogels with extra virgin olive oil: The role of oil minor components on microstructure and mechanical strength. Food Hydrocolloids, 84, 508-514.

Goller, F. & Esch, H. (1990). Comparative study of chill-coma temperatures and muscle potentials in insect flight muscles. Journal of Experimental Biology, 150(1), 221-231.

Gravelle, A. J., Davidovich-Pinhas, M., Barbut, S., & Marangoni, A. G. (2017). Influencing the crystallization behavior of binary mixtures of stearyl alcohol and stearic acid (SOSA) using ethylcellulose. Food Research International, 91, 1-10.

Gravelle, A. J., Marangoni, A. G., & Davidovich-Pinhas, M. (2018). Ethylcellulose oleogels. In Marangoni A. G., Garti N. (ed.) Edible oleogels, Vol. 2, AOCS Press, Urbana, IL, USA: 331-362.

Hall, D. G. (2009). An assessment of yellow sticky card traps as indicators of the abundance of adult *Diaphorina citri* (Hemiptera: Psyllidae) in citrus. Journal of Economic Entomology, 102, 446–452.

Kraan C. & Ebbers A. (1990). Release rates of tetradecen-1-ol acetates from polymeric formulations in relation to temperature and air velocity. Journal of Chemical Ecology. 16(4), 1041–1058.

Li, X., Wen, Y., Zhang, Y., & Ge, Z. (2022). Pheromone enclosed in halloysite with n-octadecane releases rhythmically under simulated diurnal temperature. Applied Clay Science, 217, 106386.

Lupi, F. R., Gabriele, D., Baldino, N., Mijovic, P., Parisi, O. I., & Puoci, F. (2013). Olive oil/policosanol organogels for nutraceutical and drug delivery purposes. Food & Function, 4(10), 1512-1520.

Malavosklish, B. & West, J. L. (2008). Thermo-responsive systems for controlled drug delivery. Expert Opinion on Drug Delivery, 5(10), 1077-1091.

Mao, L., Lu, Y., Cui, M., Miao, S., & Gao, Y. (2019). Design of gel structures in water and oil phases for improved delivery of bioactive food ingredients. Critical Reviews in Food Science & Nutrition, 60(10), 1651-1666.

Martins, A.J., Cerqueira, M.A., Fasolin, L.H., Cunha, R.L., Vicente, A.A. (2016). Beeswax organogels: Influence of gelator concentration and oil type in the gelation process. Food Research International, 84, 170-179.

Muskat, L.C. & Patel, A.V. (2022). Innovations in semiochemical formulations. Entomologia Generalis, 42(2), 231–249.

Muskat, L.C., Jiang L., Rostás M., Patel A.V. (2022). Development of a self-sticking oleogel formulation for the slow release of semiochemicals. Macromolecular Materials & Engineering, 307(10), 2200276.

Nielsen, M. C., Sansom, C. E., Larsen, L., Worner, S. P., Rostás, M., Chapman, R. B., Butler, R. C., de Kogel, W. J., Davidson, M. M., Perry, N. B. & Teulon, D. A. (2019). Volatile compounds as insect lures: factors affecting release from passive dispenser systems. New Zealand Journal of Crop and Horticultural Science, 47(3), 208-223.

O'Sullivan, C. M., Barbut, S. & Marangoni, A. G. (2016). Edible oleogels for the oral delivery of lipid soluble molecules: composition and structural design considerations. Trends in Food Science & Technology, 57, 59-73.

Patel, A. R., Schatteman, D., De Vos, W. H., & Dewettinck, K. (2013). Shellac as a natural material to structure a liquid oil-based thermo reversible soft matter system. RSC Advances, 3(16), 5324-5327.

Rafailovich, M. H., & Sokolov, J. (2011). Networks: Diffusion. Encyclopedia of Materials: Science and Technology, 6111-6113.

Sagiri, S. S., Behera, B., Rafanan, R. R., Bhattacharya, C., Pal, K., Banerjee, I., & Rousseau, D. (2014). Organogels as matrices for controlled drug delivery: A review on the current state. Soft Materials, 12(1), 47-72.

Scott D Fitzpatrick, Lindsay E Fitzpatrick, Ajit Thakur, Mohammad A Jafar Mazumder & Heather Sheardown (2012) Temperature-sensitive polymers for drug delivery, Expert Review of Medical Devices, 9, 4, 339-351.

Stipanovic AJ, Hennessy PJ, Webster FX, Takahashi Y. (2004). Microparticle dispensers for the controlled release of insect pheromones. Journal of Agricultural and Food Chemistry, 52(8): 2301–2308.

Taylor, L. R. (1963). Analysis of the effect of temperature on insects in flight. The Journal of Animal Ecology, 99-117.

Tokuyama, H., & Kato, Y. (2010). Preparation of thermosensitive polymeric organogels and their drug release behaviors. European Polymer Journal, 46(2), 277-282.

Toro-Vazquez, J.F., Morales-Rueda, J.A., Dibildox-Alvarado, E., Charo-Alonso, M., Alonzo-Macias, M., Gonzalez-Chavez, M.M. (2007). Thermal and textural properties of organogels developed by candelilla wax in safflower oil. Journal of the American Oil Chemists' Society, 84, 989-1000.

van Esch, J. H., & Feringa, B. L. (2000). New functional materials based on self-assembling organogels: From serendipity towards design. Angewandte Chemie International Edition, 39(13), 2263-2266.

Xiao, D., Liang, W., Xie, Z., Cheng, J., Du, Y., & Zhao, J. (2021). A temperature-responsive release cellulose-based microcapsule loaded with chlorpyrifos for sustainable pest control. Journal of Hazardous Materials, 403, 123654.

Yang, D. X., Chen, X. W., & Yang, X. Q. (2018). Phytosterol-based oleogels self-assembled with monoglyceride for controlled volatile release. Journal of the Science of Food and Agriculture, 98(2), 582-589.

Zhu H, Thistle HW, Ranger CM, Zhou H, Strom BL (2015). Measurement of semiochemical release rates with a dedicated environmental control system. Biosystems Engineering, 129, 277–287.

Chapter 4

Fermentation of the psyllid-pathogenic fungus *Pandora* sp. nov. inedit. (Entomophthorales: Entomophthoraceae)

Linda C. Muskat[1,2]; Michael Przyklenk[3]; Pascal Humbert[1]; Jørgen Eilenberg[4]; Anant V. Patel[1]

[1]Bielefeld University of Applied Sciences, Faculty of Engineering and Mathematics, WG Fermentation and Formulation of Biologicals and Chemicals, Bielefeld, Germany; [2]Georg-August-University Goettingen, Agricultural Entomology, Department of Crop Sciences, Goettingen, Germany; [3]BIOCARE Gesellschaft für biologische Schutzmittel mbH, Einbeck, Germany; [4]University of Copenhagen, Department of Plant and Environmental Sciences, Copenhagen, Denmark

Published in: Biocontrol Science and Technology, 2022, Vol 32 (5), 564-585.

DOI: 10.1080/09583157.2022.2035680

Abstract

Pandora sp. nov. inedit. (ARSEF13372) is a recently isolated entomophthoralean fungus with high potential for psyllid pest control. The use of entomopathogenic fungi as biocontrol agents in large scale field application requires biomass production in a sufficient quantity and quality. This work is the first attempt to explore the biomass production of the novel entomophthoralean *Pandora* species in a liquid medium. This study aims at establishing a submerged fermentation process with potential for mass production of the fungus. Three different complex nutrient sources were evaluated for maximizing biomass in a liquid shaking culture. *Pandora* sp. nov. grew best in media containing skimmed milk, but even faster in a mixture of skimmed milk, yeast extract and a low-cost protein hydrolysate from animal by-products. Subsequently, the produced biomass can be encapsulated in biopolymer beads or granules in order to transfer the fungus into an easily applicable form. Therefore, growth as finely dispersed mycelium was promoted by increased media osmolality through the addition of sodium chloride. After the medium had been transferred to a stirred tank bioreactor with a working volume of 8 L, a maximum biomass dry weight of 21 $g \cdot L^{-1}$ was reached after 48 hours. These promising results can pave the way for large scale fermentation and formulation processes of this novel *Pandora* species for biological psyllid pest control.

Keywords: Entomophthorales, Biological control, Entomopathogenic fungi, Fermentation, Fungal morphology, Stirred tank bioreactor

Introduction

Entomopathogenic fungi are considered a promising alternative to common synthetic insecticides (Glare et al., 2012; Lacey et al., 2015). Compared with hypocrealean fungi, such as species from the genera *Metarhizium* or *Beauveria*, species of the Entomophthorales are known for their high host specificity, their fast speed-to-kill (Pell et al., 2001) and for their ability to cause epizootics (Vega et al., 2012; Eilenberg et al., 2019). In 2016, a new entomophthoralean species of the genus *Pandora* was isolated from infected psyllids collected in a Danish pear orchard (Jensen 2017; Jensen et al., 2018). Psyllids are distributed all over the world and cause damage in various crop plants by serving as vector insects for phytoplasmosis diseases. Phytoplasma infected plants cannot be cured and defense strategies are limited to the use of chemical insecticides of high negative impact on non-target organisms (Jarausch & Jarausch, 2009). The pathogenicity of *Pandora* sp. nov. has already been demonstrated for several psyllid species under laboratory conditions (Jensen 2017; Jensen et al., 2018; Herren, 2018; Görg et al., 2021b; Muskat et al., 2021b).

For inundative control strategies, the mass-production of fungal biomass is essential for providing inoculant in a sufficient quantity for large scale field application (Shah & Pell, 2003; Jaronski, 2014). Despite their many aforementioned advantages, the fungi from Entomophthorales have often been reported to be difficult to grow *in vitro* (Latgé, 1981; Eilenberg et al., 1992; Papierok & Hajek, 1997; Pell et al., 2001; Hajek et al., 2012; Jaronski, 2014). By their individual requirements for *in vitro* growth, the different entomophthoralean genera can be categorized into four groups (Latgé, 1981; Keller, 1997; Pell et al., 2001): The first group can be easily grown in classical mycological media, such as *Conidiobolus* spp. (Latgé et al., 1983; 1985). The second group is slightly more difficult; therefore, fungi belonging to this group need some more supplements, but have been cultivated in submerged culture, including *Batkoa*, *Erynia/Pandora* and *Zoophthora* spp. (Latgé et al., 1983; Li et al, 1993; Wraight et al., 2003; Leite et al., 2005). Members of the third group only grow in complex media, e.g. some *Entomophthora* and *Entomophaga* spp. (Wolf, 1951; Jönsson, 1968; Latgé et al., 1977; Latgé & Sanglier, 1985; Beauvais & Latgé, 1988; Nolan, 1988; 1993b; Freimoser et al., 2001). Fungi like *Strongwellsea* and *Neozygites* belong to the fourth group, which so far have only been cultivated in costly tissue culture media (Grundschober et al., 1998; Eilenberg et al., 1992; Leite et al., 2000; Delalibera et al., 2003). Some Entomophthorales cannot be grown *in vitro* at all (Hajek & St. Leger, 1994; Leite et al., 2000).

Thus, the difficulty to grow an entomophthoralean fungus on a technical scale *in vitro* in low cost media remains one of the main challenges on the way to utilization as a biocontrol agent. Three stages of an entomophthoralean fungus can be considered for large scale production: 1) conidia, 2) resting spores and 3) hyphal bodies (Latgé et al., 1983). Conidia of Entomophthorales are the infective units themselves, but they have a low shelf life and are sensitive of UV and desiccation (Brobyn et al., 1985). Furthermore, the active conidial discharge and the mucous layer typical for the Entomophthorales (Eilenberg et al., 1986; Hajek et al., 2012; Olsen et al., 2019) causes difficulties in conidia collection (Muskat et al., 2021a) and down-stream processing (Soper, 1985). Resting spores are resistant to environmental stresses (Hajek & Roberts, 1991) and have the potential for long-term storage (Hajek et al., 2001, 2008). At the same time, their dormancy is a disadvantage for technical scale application because it is difficult to reactivate them to form conidia synchronously (Latgé et al., 1983). Hyphal bodies

of Entomophthorales occur naturally in infected hosts and can be grown in submerged fermentation (Hajek et al., 2012). Submerged fermentation allows for short process times and can easily be scaled up for mass-production (Jackson, 1997; Jaronski, 2014). The aphid pathogenic species *Pandora neoaphidis* has been reported to grow in the form of hyphal bodies in classical mycological media supplemented with milk and egg yolk (Latgé et al., 1978) or in a medium composed of yeast extract, glucose and milk (Li et al., 1993). *P. neoaphidis* was subjected to several studies aimed at using this fungus as a biocontrol agent, e.g. by Shah et al. (1998, 1999), who adapted Li's simple medium for subsequent encapsulation of the produced biomass in hydrogel beads. They showed that the hyphae grown in the liquid shaking culture measured 220 to 620 µm in length and 7 to 19 µm in diameter with a 74 to 83% cytoplasmic content, but they did not further modify the hyphal morphology or increase biomass production by adjustment of the cultivation parameters or medium improvement. Leite et al. (2005) tested different nitrogen sources for the production of *Batkoa* sp., *Furia* sp. and *Neozygites floridana* in liquid culture. The combination of different nitrogen sources improved the production of hyphal bodies of the studied entomophthoralean species, but the most suitable combination for maximizing biomass is actually species and even isolate dependent (Papierok & Hajek, 1997; Pell et al., 2001; Leite et al., 2005). It has been previously reported that protein hydrolysates as complex nitrogen sources support the growth of various Entomophthorales (Latgé, 1975b; 1977; 1981; Latgé & Remaudière, 1975; Freimoser et al., 2000; Leite et al., 2003; Hajek et al., 2012). Furthermore, fermentation costs can be lowered by selecting complex nutrient substrates that do not require other supplements, such as salts, vitamins or amino acids. The reduction of fermentation costs is of actual interest in fermentation studies of a diversity of fungi, as demonstrated for *Fusarium catingaense* (de Lima et al., 2021), *Metarhizium robertsii* (Iwanicki et al, 2020) or *Beauveria bassiana* (Mascarin et al., 2018).

In order to transfer the viable biomass of an entomophthoralean fungus into an easily applicable and stable form, a suitable morphology of the fungal material is needed. Filamentous organisms tend to form pellet-like structures under submerged culture conditions (Cox et al., 1989). Conglomerated mycelium is unsuitable for the formulation within hydrogel beads, as described by Shah et al. (1989) who filtered the grown biomass before encapsulation in order to separate pellets from finely dispersed hyphae. To prevent the formation of pellets during fermentation, there are various options for modifying the fermentation parameters or the media composition, such as increased osmolality and viscosity of the media (Wucherpfennig et al., 2011; Krell et al., 2019) or variation of the stirring speed (Patel et al., 2011). Notable studies aiming at morphological modifications of an Entomophthorales grown under mass fermentation conditions in liquid culture were conducted by Nolan (1990; 1991). He found increased hyphal body production from protoplasts of *Entomophaga aulicae* when stirred tank bioreactors were configured with neutral or positively charged disks. As yet, however, there have not been any studies on targeted production of finely dispersed mycelium of an Entomophthorales in submerged culture suitable for later formulation.

The aim of this study was to identify an optimal fermentation medium suitable for mass production of *Pandora* sp. nov., paving the way for largescale field application of the novel fungus in biological control strategies. The specific objectives were to (1) screen for a suitable complex nitrogen source for fast growth and high biomass production, (2) evaluate growth kinetics in the resulting media, (3) reduce

pellet formation in order to promote growth as finely dispersed mycelia and (4) prove the scalability of the fermentation in a stirred tank bioreactor.

Materials and Methods

Chemicals

All chemicals used in this study were acquired from Carl Roth GmbH (Karlsruhe, Germany) or VWR International GmbH (Darmstadt, Germany) and concentrations are given as (w/w), unless otherwise stated.

Fungal isolate

The *Pandora* isolate used in this study originated from an infected *Cacopsylla* sp. collected in Danish pear orchards by Jensen (2017) and was named KVL 1644. The isolate is deposited in the USDA Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (Ithaca, NY) as ARSEF 13372. The fungus is under description (inedit.) and will hereinafter be referred to as *Pandora* sp. nov.

Cultivation of *Pandora* sp. nov.

To ensure that there were no contaminations, all experiments were conducted under sterile conditions. All solutions, media, flasks and tools that came in contact with the culture were washed in ultrapure water to ensure a salt free state and were autoclaved for 6 min at 121 °C and 2 bar. The media components were dissolved in ultrapure water prior to autoclaving and cooled to room temperature prior to inoculation.

Solid state cultivation

Pandora sp. nov. was grown on a solid medium adapted from Hajek et al. (2012) composed of 4.0% glucose, 2.0% casein and 2.0% agar (Saboraud Dextrose Agar (SDA) supplemented with 20% of a mixture of 60% egg yolk and 40% fresh skimmed milk (SDAME)) on Petri dishes (diameter 90 mm) sealed with Parafilm® and incubated at its optimal growth temperature of 18 °C in the dark. To maintain the culture, mycelial plugs (0.5 cm^2) were cut and transferred to fresh media when the mycelium reached the edge of the plate. In order to prevent a loss of virulence, the fungus was frequently transferred through the host insect *C. pyri* and the solid culture used for experiments was transferred onto fresh artificial media for a maximum of two times.

Submerged cultivation in shaking flasks

Submerged cultures of *Pandora* sp. nov. were grown in 100 ml of medium in 250 ml shaking flasks with four baffles at 18 °C and 170 rpm and an amplitude of 20 mm (IKA KS 4000 ic control, Staufen, Germany). In order to transfer the fungus from the solid media into liquid culture, three pieces of mycelia (0.5 cm^2) grown on SDAME agar plates were cut with a scalpel and transferred to a pre-culture medium composed of fresh skimmed milk (100 $g \cdot L^{-1}$). The initial pH value of the pre-culture medium was 6.9. The hyphal material grown in the pre-culture for 72 h was collected by centrifugation (4700 g; 15 min; 18 °C) and washed twice in glucose solution (26.6 $g \cdot L^{-1}$) to equal the original volume under centrifugation for removal of the pre-culture medium. After the final centrifugation step, the washed and pelletized hyphal bodies were resuspended in glucose solution (26.6 $g \cdot L^{-1}$) to equal the original volume. The main culture was inoculated with 10% (v/v) of the suspension. As the hyphal bodies were of different size and shape and the cells agglomerate, it was not possible to adjust the inoculant in hyphal

bodies/mL. The biomass dry weight used for inoculation of the main culture was 0.028 g · 10 ml^{-1} ±0.005 (n=25).

In order to find a suitable medium for optimal growth of *Pandora* sp. nov. in submerged culture, two different experiments were carried out, which are described in detail below. The different media compositions tested are listed in **Table 4.1**.

Table 4.1 Media compositions used in this study. FSM = fresh skimmed milk (1.5% fat), AN = ANiMOX, SM = skimmed milk powder, YE = yeast extract, NaCl = sodium chloride. The highlighted medium (grey background) was used in the scale-up experiment in a stirred tank bioreactor. All contents are given in [g · L-1].

	Medium	FSM	Glucose	AN	SM	YE	NaCl
	Pre-culture	100	0	0	0	0	0
Experiment 1 - N screening	**AN**	0	26.60	10	0	0	0
	SM	0	26.60	0	10	0	0
	YE	0	26.60	0	0	10	0
	AN+SM	0	26.60	5	5	0	0
	AN+YE	0	26.60	5	0	5	0
	YE+SM	0	26.60	0	5	5	0
	AN+SM+YE	0	26.60	3.3	3.3	3.3	0
Experiment 2 - Pellet reduction	**NaCl 0**	0	26.60	3.3	3.3	3.3	0
	NaCl 0.5	0	26.60	3.3	3.3	3.3	5
	NaCl 1	0	26.60	3.3	3.3	3.3	10
	NaCl 2	0	26.60	3.3	3.3	3.3	20
	NaCl 4	0	26.60	3.3	3.3	3.3	40

Screening for suitable complex nitrogen sources

In order to find a suitable medium for the *in vitro* cultivation of *Pandora* sp. nov., the effect of different complex nitrogen (N) sources on biomass production in shaking culture was investigated. The N sources tested in this study were yeast extract (YE; Carl Roth GmbH, Karlsruhe, Germany), skimmed milk prepared from powder (SM; HEIRLER CENOVIS GMBH, Radolfzell, Germany) and a low-cost protein hydrolysate from animal by-products (AN; ANiPept, ANiMOX GmbH, Berlin, Germany, batch No. 1176). The N sources were added at a fixed total amount of 10 g · L^{-1}, individually or in combinations: when added individually, 10 g · L^{-1} of the single N source, in binary combination, 5 g · L^{-1} of each N source and in the AN+SM+YE mixture 3.3 g · L^{-1} of each N source. All N sources were dissolved in ultrapure water and autoclaved separately to prevent protein precipitation. Glucose was used as main carbon source at a fixed amount of 26.6 g · L^{-1}, adapted from Leite et al. (2005). The initial pH value of all main culture media was 6.3 and not controlled during the fermentation. The total organic carbon (TOC) content was determined with the LCK 381 cuvette test and the total nitrogen (N) content with the LATON® LCK 338 cuvette test according to the manufacturer instructions (HACH LANGE GMBH, Düsseldorf, Germany)

in one sample of each medium prior to inoculation. The TOC and N content and the C/N ratio of the media tested in the N screening experiments are listed in **Table 4.2**.

Table 4.2 Total nitrogen (N) content, total organic carbon (TOC) content and C/N ratio of the media tested in the N screening experiment.

	N (mg/L)	TOC (mg/L)	C/N
Pre-culture	627	5339	8.52
AN	1089	14298	13.13
SM	618	14114	22.84
YE	1264	15188	12.02
AN+SM	868	14753	17.00
AN+YE	1145	13175	11.51
SM+YE	932	14746	15.83
AN+SM+YE	1080	13406	12.41

The main culture was grown for a further 120 h under the same conditions as the pre-culture. The experiments were carried out with five shaking flasks per treatment.

Analysis of biomass formation, glucose consumption, specific growth rate, yield coefficient and mean hyphal length

Every 24 h, one 10 ml sample was taken from each shaking flask. Fungal material of *Pandora* sp. nov. was separated from the broth culture media by vacuum filtration onto pre-dried and pre-weighted filter papers (qualitative filter paper 401, 12–15 µm pore size, VWR, Germany). Unless otherwise stated, all analyses were conducted with five replicates.

For determination of biomass formation, the samples on the filter papers were rinsed separately with water under vacuum filtration in order to remove media residuals and filters were subsequently dried for 48 h at 60 °C. After cooling the filters to room temperature, the biomass dry weight was determined gravimetrically and calculated as g biomass dry weight per L^{-1}.

Glucose concentration in the culture media was analyzed by HPLC. Glucose solutions of known concentration were used as references.

The specific growth rate was determined following the equation (Takors, 2014):

$$\mu = \frac{1}{X}\frac{dX}{dt} \quad \text{(Eq. 1)}$$

where µ is the specific growth rate (in 1/h), X is biomass dry weight (in g · L^{-1}) and t is time (in h).

The yield coefficient based on glucose consumption was calculated as follows:

$$Yield\ coefficient = \frac{biomass\ dry\ weight\ (t_{120}) - biomass\ dry\ weight\ (t_0)}{glucose\ (t_0) - glucose\ (t_{120})} \quad \text{(Eq. 2)}$$

where biomass dry weight (in g · L^{-1}) was determined as described above and t is time (in h).

The mean hyphal length was determined from pictures taken under the light microscope with the length measurement tool of the ImageJ open-source software (version Fiji 1.52; Wayne Rasband; National Institutes of Health, Maryland, USA).

Effect of sodium chloride on pellet formation

In order to reduce pellet formation, media osmolality was increased by adding different sodium chloride concentrations (0, 0.5, 1, 2, 4%) to the culture medium composed of glucose (26.6 $g \cdot L^{-1}$), ANiPept (3.3 $g \cdot L^{-1}$), skimmed milk powder (3.3 $g \cdot L^{-1}$) and yeast extract (3.3 $g \cdot L^{-1}$). The experiment was carried out with five shaking flasks per treatment. Every 24 hours, one 10 ml sample was taken from each flask. The sample was then divided: 5 ml were used for biomass dry weight determination as described in 2.6.1, the other 5 ml were used for the analysis of pellet formation as described below.

Determination of medium osmolality

Osmolality [osmol/kg] of the culture media was determined measuring the melting point by Differential Scanning Calorimetry (DSC3+, Mettler-Toledo, Gießen, Germany), using sodium chloride solutions of known osmolality as references.

Analysis of pellet formation

The quantification of pellets formed by *Pandora* sp. nov. in shaking cultures was measured by software-supported image analysis. In order to prepare reproducible macroscopic images of the fungal morphology grown in shaking cultures, one 5 ml sample per flask and time point was transferred separately into a Petri dish (Ø = 35 mm). The dishes were subsequently placed on a cold cathode lamp light panel (5000 K ± 5 %; Slimlite 2420, Kaiser, Germany) in the dark hood of a gel documentation system (UVsolo TS, Analytik Jena AG, Jena, Germany) and images were taken with the integrated camera system (monochrome camera, 5.0-megapixel resolution, 8–48 mm f/1.2 zoom lens).

Images were processed and analyzed with the ImageJ open-source software. By manual adjustment of the color threshold brightness, the pellet area was measured in relation to the magnification and pixel size and given in percent of the whole sample area as parameter for pellet formation. The image processing workflow is illustrated in **Figure 4.1**.

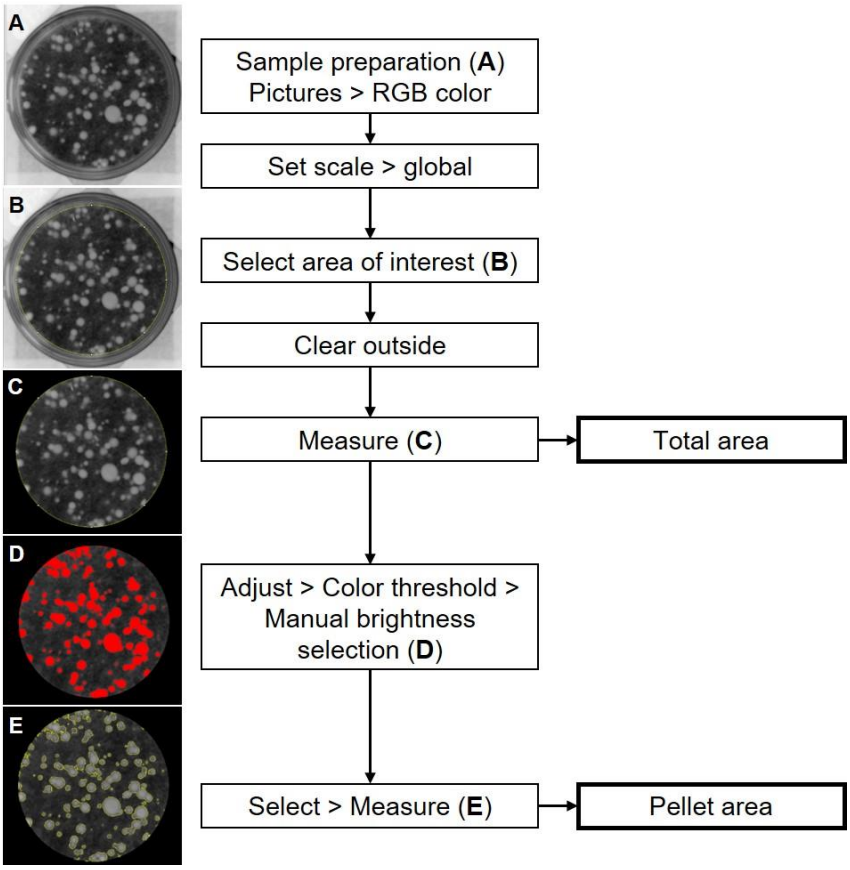


Figure 4.1 Workflow of the software supported image analysis for pellet area determination. 5 ml samples were taken every 24 h from the liquid shaking culture of *Pandora* sp. nov. and placed in Petri dishes (Ø = 35 mm). The dishes were placed on a cold cathode lamp light panel in the dark hood of a gel documentation system and images were taken with the integrated camera system (monochrome camera, 5.0-megapixel resolution, 8 - 48 mm f/1.2 zoom lens). Images were processed and analyzed with the open source software ImageJ. By manual adjustment of the color threshold brightness, the pellet area was measured in relation to the magnification and pixel size and given in percent of the whole sample area as parameter for pellet formation. White and grey = Fungal material. Black = No fungal material.

Submerged cultivation in stirred tank bioreactors

In order to prove the scalability of the developed medium and procedure, upscaling of the submerged cultivation was carried out in a 10 L stirred-tank bioreactor (Biostat B, Type 884032/6, Braun, Germany) equipped with a three 6 wing/blade disc stirrer (Ø = 75 mm) and with a working volume of 8 L. The main culture medium consisted of 26.6 g · L^{-1} glucose as main carbon source, supplemented with YE, SM and AN as nitrogen source (10 g · L^{-1}) and sodium chloride (10 g · L^{-1}) as osmolality modifier. All main culture media components were dissolved in deionized water and filled into the bioreactor before autoclaving at 121 °C and 2 bar for 20 min (Varioklav®, Type 400, H+P Labortechnik AG, Germany), except for the skimmed milk (SM). In order to prevent protein precipitation, SM was dissolved and autoclaved separately at 121 °C and 2 bar for 6 min and added before the fermentation process. The total culture volume was 8 L. *Pandora* sp. nov. was transferred from solid into liquid culture and grown in the pre-culture as described above. The bioreactor was inoculated with 800 mL (10% (v/v)) of the pre-culture under sterile conditions. In the bioreactor, *Pandora* sp. nov. was grown for 96 h at 18 °C. During the fermentation process, the pO_2 content was controlled by the stirrer speed within a range of 50–350 rpm, at a gas rate of 0.8 vvm, to ensure that the pO_2 concentration stayed above 60% over the whole period of time. The initial pH value was 7.5 and not controlled during the fermentation.

Two 50 ml samples were taken every 24 h to determine the biomass dry weight as described above. pH, pO_2 and rpm were automatically recorded every 24 hours. The fermentation was repeated three times.

Statistical analysis

Statistical analysis was carried out using the SPSS Statistics V25.0 software (SPSS, Chicago, IL). All data are given as mean values ± standard deviations (SD).

Data on pellet area were arcsine transformed prior to analysis. All data were checked for normality and homogeneity of variance using the *Shapiro–Wilk* and *Levene* tests. Means were examined for significant differences according to one-way analysis of variance (ANOVA), followed by a *Tukey post hoc range test* and *Bonferroni* correction. If the criteria for variance homogeny and normal distribution were not met, data were calculated by a nonparametric *Kruskal-Wallis* test followed by *Dunn-Bonferroni* for multiple comparisons with one treatment. The level of significance was set to $p < 0.05$, unless otherwise stated.

Results

Effect of different nitrogen sources on biomass production and specific growth rate in shaking culture

The composition of the culture medium affected the biomass production by *Pandora* sp. nov. in submerged culture significantly ($F_{6,28}$ = 11.943; p<0.001; one-way ANOVA). Growth was markedly improved when *Pandora* sp. nov. was cultured in media containing skimmed milk (SM; **Figure 4.2**). There was no significant difference between all tested media containing SM (p=0.104; *Tukey post-hoc range test*) with a maximum of 5.038 g biomass dry weight per L and a yield coefficient of 0.19 based on glucose after 120 h of cultivation.

The specific growth rate during the first two days of cultivation significantly differed between the different media compositions ($F_{6,28}$ = 5.761; p<0.001; one-way ANOVA). When SM was combined with yeast extract (YE), the specific growth rate increased from 0.0134 (±0.0029) to 0.0193 (±0.0035), but growth was not significantly faster (p = 0.391; *Tukey post-hoc range test*). In the medium containing SM in combination with YE and animal based protein hydrolysate (AN) the specific growth rate further increased to 0.0250 (±0.0054) and was significantly higher compared with the SM medium (p = 0.012; **Figure 4.2**). Further experiments on fermentation were conducted with the medium containing glucose (26.6 g L^{-1}), AN (3.3 g L^{-1}), SM (3.3 g L^{-1}) and YE (3.3 g L^{-1}).

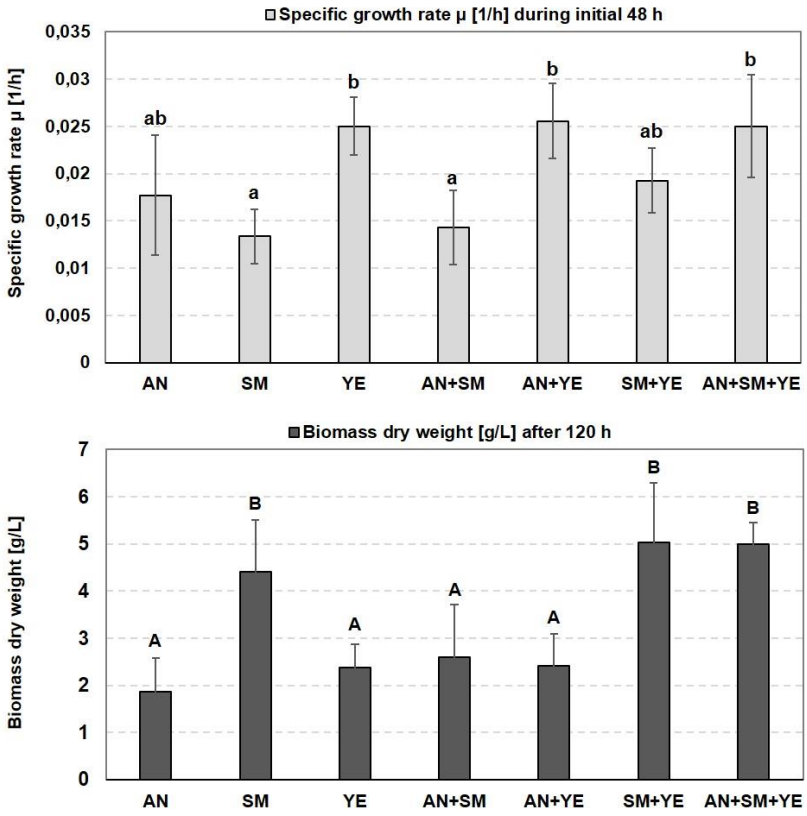


Figure 4.2 Biomass production of *Pandora* sp. nov. in media containing different complex nitrogen sources in shaking culture after 120 h and specific growth rate during the first 48 h. All media contain glucose (26.6 g . L-1). The nitrogen source was added at a fixed amount of 10 g . L-1 individually or in combinations. AN = protein hydrolysate from animal by-products; SM = skimmed-milk; YE = yeast extract. Different letters above bars indicate significant differences between biomass dry weight (capital letters) or between specific growth rate (lowercase letters) based on one-way ANOVA and Tukey post-hoc range test at p < 0.05 (means ± SD, n = 5).

Growth kinetics in liquid shaking culture

Fungal growth increased within the first 48 h and correlated with glucose consumption (**Figure 4.3**). Subsequently, fungal growth appeared to stagnate, but at the same time glucose consumption increased noticeably and the formation of pellets occurred. Following the growth plateau, a second growth phase was observable after 72 hours and the formation of pellets increased rapidly, until more than 50% of the biomass was present in form of pellets after 120 h. After 120 h, >90% of the initial glucose was consumed and the cultivation was discontinued. The pH value did not change noticeably during the entire cultivation and remained between 5.9 and 6.3. Lactose concentration was determined by HPLC, but was not consumed by *Pandora* sp. nov. (data not shown).

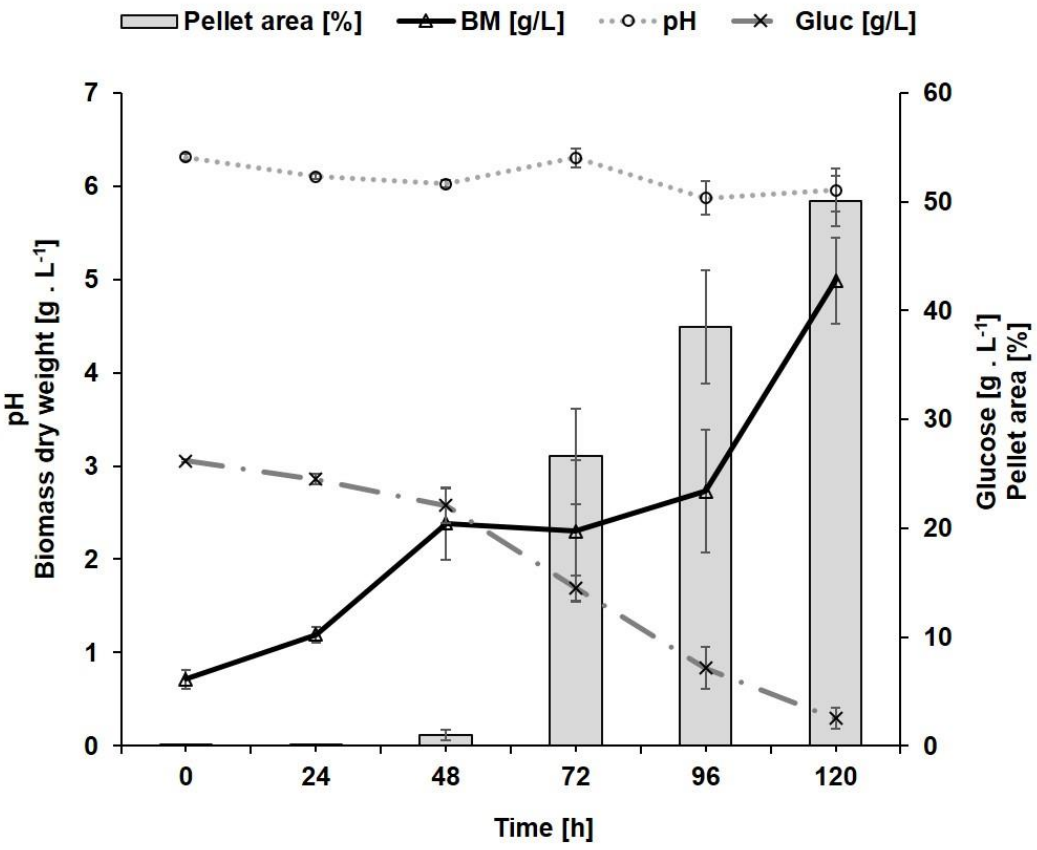


Figure 4.3 Growth kinetic of *Pandora* sp. nov. (ARSEF 13372) in liquid shaking culture at 18 °C and 170 rpm for 120 h in a medium containing glucose (26.6 g · L^{-1}), AN (3.3 g · L^{-1}), SM (3.3 g · L^{-1}) and YE (3.3 g · L^{-1}). Means ± SD; n = 5.

Effect of media osmolality on production of finely dispersed mycelium

By supplementing the liquid medium composed of glucose (26.6 g L^{-1}), AN (3.3 g L^{-1}), SM (3.3 g L^{-1}) and YE (3.3 g L^{-1}) with different amounts of NaCl, osmolality increased from 120.67 osmol/kg (0%) to 340.23 osmol/kg (0.5%), 461.40 osmol/kg (1%), 892.27 osmol/kg (2%) and 1280.75 osmol/kg (4%), as listed in **Table 4.3.**

Table 4.3 Modification of media osmolality by NaCl addition, measured by the melting temperature.

NaCl content [%]	Melting point [°C]	Osmolality [osmol/kg]
0	-2.453	120.666
0.5	-3.554	340.233
1	-4.161	461.399
2	-6.321	892.269
4	-8.268	1280.751

Pellet formation was significantly affected by the addition of NaCl ($F_{4,20}$ = 233.3; p<0.001; one-way ANOVA), as illustrated in **Figure 4.4** and **4.5.** In media containing no NaCl or a low concentration of 0.5% NaCl, intense pellet formation occurred after 72 hours (data not shown) and increased until more than 50% of the grown biomass showed pellet morphology after 120 h of cultivation (**Figure 4.3**). In media containing NaCl concentrations >1%, pellet formation was significantly reduced (p<0.001; *Tukey post hoc range test*; **Figure 4.5**).

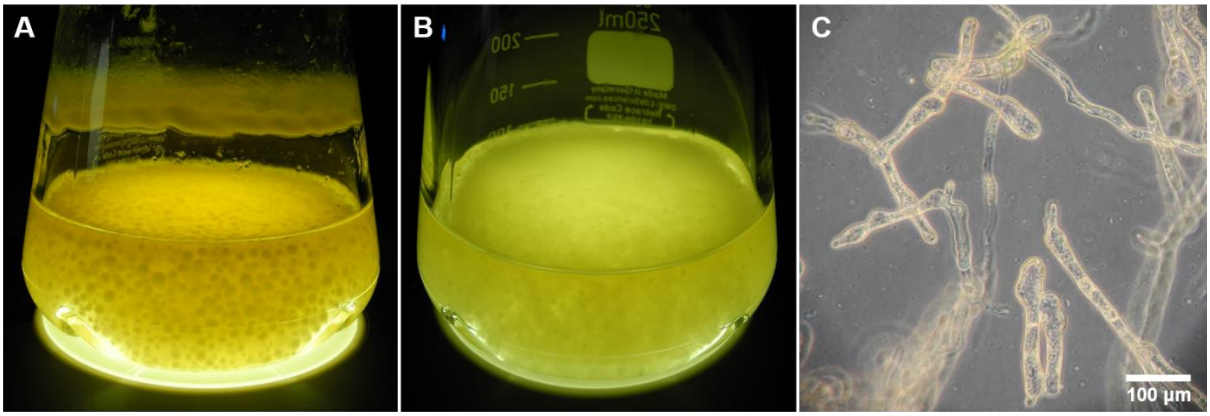


Figure 4.4 *Pandora* sp. nov. grown in shaking culture after 72 h. A = Medium without NaCl addition. B = Medium supplemented with 1% NaCl and an osmolality of 461.4 osmol/kg. C = Hyphal bodies of *Pandora* sp. nov. grown in the main culture after 48 h. Media based on glucose (26.6 g · L^{-1}), AN (3.3 g · L^{-1}), SM (3.3 g · L^{-1}) and YE (3.3 g · L^{-1}). The shaking-flasks were placed on a light panel for taking the pictures.

Moreover, the addition of NaCl significantly affected biomass production ($F_{2,20}$ = 32.483; p<0.001; one-way ANOVA). Fungal growth was nearly suppressed by a NaCl concentration of 4% (p>0.001; *Tukey post hoc test*), resulting in a black picture after image processing, as no hyphal material was observable in the sample. NaCl concentrations between 0 and 2% did not significantly affect the biomass production.

The mean hyphal length in the medium containing glucose (26.6 g · L^{-1}), AN (3.3 g · L^{-1}), SM (3.3 g · L^{-1}), YE (3.3 g · L^{-1}) and NaCl (10 g · L^{-1}) was 558.253 µm (± 163.042; n=28) after 48 h of cultivation.

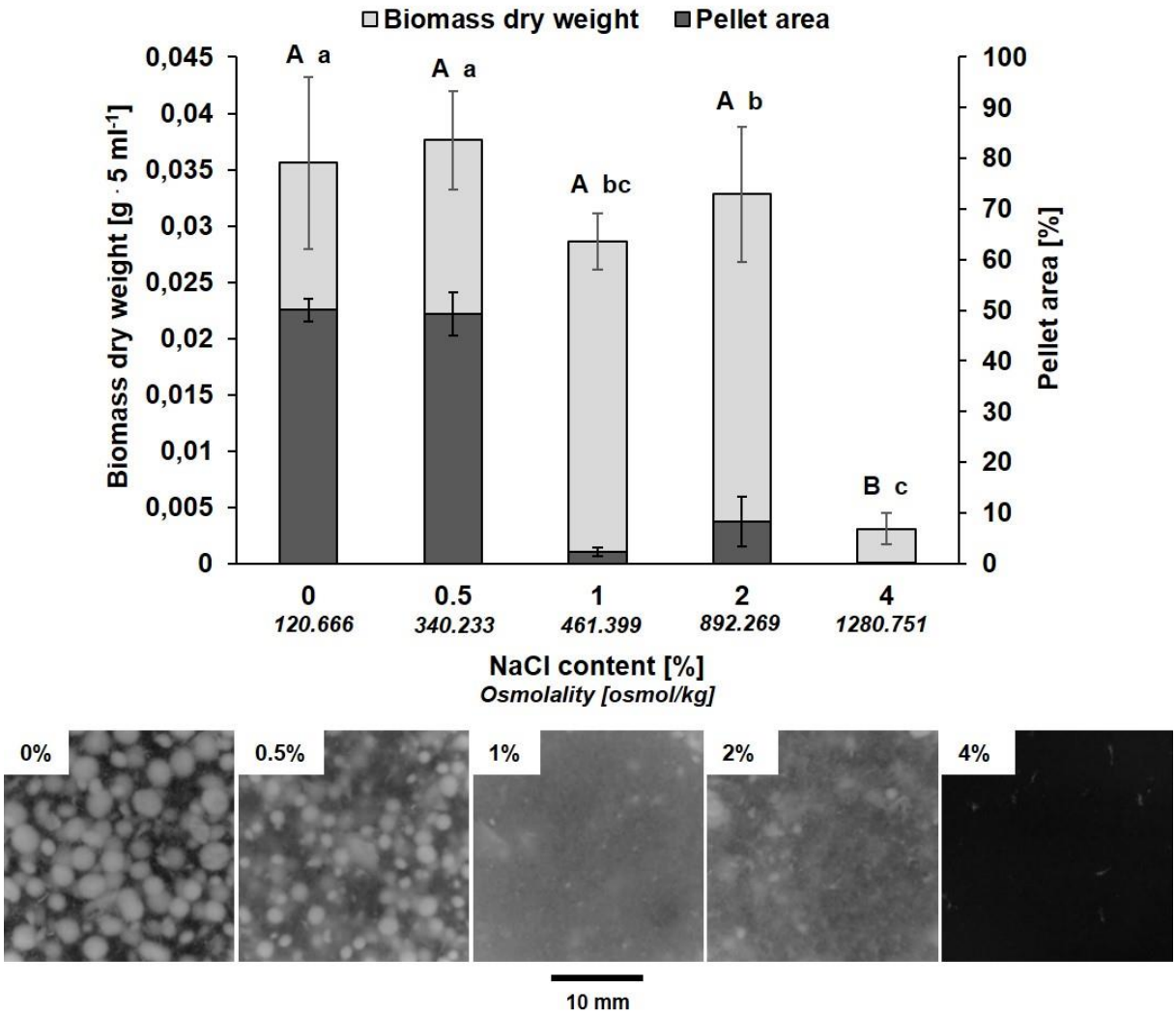


Figure 4.5 Biomass production (light-grey) and pellet formation (dark-grey) by *Pandora* sp. nov. grown in shaking cultures of different osmolalities. All media based on glucose (26.6 g . L-1), AN (3.3 g . L-1), SM (3.3 g . L-1) and YE (3.3 g . L-1). Media osmolality [osmol/kg] was modified by addition of sodium chloride as follows: 120.67 (0%), 340.23 (0.5%), 461.40 (1%), 892.27 (2%), 1280.75 (4%). During incubation for 120 h at 18 °C and 170 rpm, every 24 h, one 5 ml sample was taken from each flask and transferred into Petri dishes for documentation. The samples were placed on a cold cathode lamp light panel in the dark hood of a gel documentation system and pictures (shown below the plot) were taken with the integrated camera system. Pictures were processed and analyzed with the open source software ImageJ. By manual adjustment of the color threshold brightness, the pellet area was measured in relation to the magnification and pixel size and given in percent of the whole sample area as parameter for pellet formation. Different letters above bars indicate significant differences between biomass dry weight (capital letters) or pellet area (lowercase letters) based on one-way ANOVA and Tukey post hoc range test at $p < 0.05$ (means ± SD, n = 5).

Scale-up

The submerged fermentation process for *Pandora.* sp. nov. was transferred from shaking flask to a stirred bioreactor. The highest biomass production in submerged fermentation in a 10 L stirred tank reactor was reached after 48 h of cultivation with a maximum of 21 g biomass dry weight per L (**Figure 4.6**). After that point, the biomass concentration started to decrease during the cultivation process until the fermentation was stopped after 96 h. With increasing biomass, the stirrer speed increased from 50 to 350 rpm in order to compensate for the reduced O_2 transfer rate due to the high viscosity in the later stages of the fermentation.

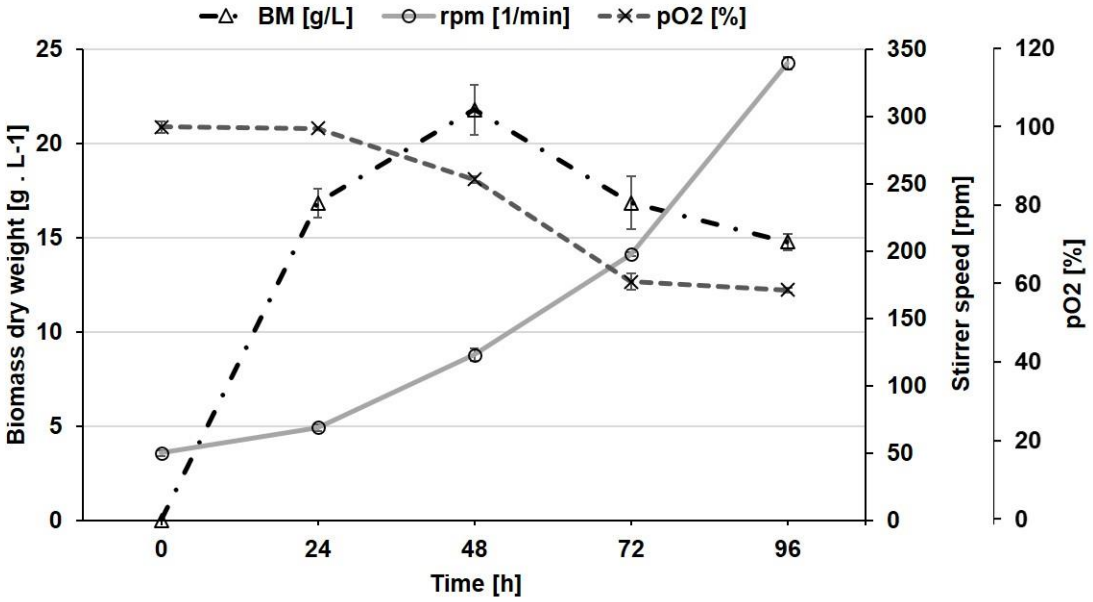


Figure 4.6 Growth kinetics of *Pandora* sp. nov. in submerged fermentation in a stirred tank bioreactor in a medium containing glucose (26.6 g . L-1), AniPept (3.3 g . L-1), skimmed milk (3.3 g . L-1), yeast extract (3.3 g . L-1) and NaCl (10 g . L-1). The fermentation was carried out with following parameters: temperature 18°C, dissolved oxygen level (pO2) 60%, initial pH-value 6.0 (not controlled) and a gas flow of 0.8 vvm. The stirrer speed (rpm) was controlled by the pO2 value within a range of 50 – 350 rpm that the pO2 concentration stayed above 60% over time. The total culture volume was 8 L. Means ± SD; n = 3.

Discussion

Effect of different nitrogen sources on biomass production and specific growth rate in shaking culture

In our search for suitable media components for the liquid cultivation of *Pandora* sp. nov., we were mainly guided by the works of other researchers who studied culture media development for phylogenetically related Entomophthorales, namely *Pandora neoaphidis* (Latgé et al., 1983; Shah et al., 1999) and *Furia* sp. (Leite et al., 2005).

Our results demonstrate that *Pandora* sp. nov. can be grown in common fungal media components and the highest biomass production was observable in the media containing skimmed milk (SM). Skimmed milk is a nutrient source commonly used for the cultivation of entomophthoralean fungi (Papierok & Hajek 1997; Leite et al., 2005; Hajek et al., 2012), but it is not yet clear why it works so well. Possible factors are (1) that skimmed milk provides proteins utilizable by *Pandora* sp. nov., as previous studies imply, in which high protease activity was observed in other Entomophthorales when they grew in skimmed milk containing media (Jönsson, 1968; Urbańczyk et al., 1992), (2) the high amino acid content or an amino acid profile similar to the host insect's hemolymph, as amino acids are suitable nitrogen sources for growth of the Entomophthorales (Wolf, 1951; Latgé et al., 1975b; Nolan, 1990; Leite et al., 2005) or (3) that skimmed milk has a comparatively low total nitrogen content (**Table 4.2**), making it less toxic than the other tested complex N sources, as higher nitrogen concentrations possibly lead to excessive release of cell toxic ammonia and result in growth inhibition (Griffin 1993; Leite et al. 2005). (4) We further speculate that the fungus is adapted to the milk based nutrient source, as in the present study the pre-culture contained skimmed milk as sole nutrient source.

The specific growth rate, in combination with a high biomass production, was even better when SM was combined with YE and animal based protein hydrolysate (AN). This is consistent with Hajek et al. (2012) who pointed out that most Entomophthorales can be grown in media supplemented with SM and YE. Moreover, this is in line with the results of Latgé et al. (1983) who found out that *P. neoaphidis* can be grown in classical mycological media supplemented with milk and egg yolk and also in a medium composed of yeast extract, glucose and milk with potential for scale-up in the bioreactor (Li et al., 1993; Pell et al., 2001).

For the *Pandora* sp. nov. used in this study, YE seems to be less important for biomass maximization than SM. Since this protein hydrolysate is comparable with common beef extract, our results are in line with Leite et al. (2005). They found out that *Furia* sp., a fungus belonging to the *Erynia* – *Pandora* – *Furia* genera clade (Gryganskyi et al., 2013) and thereby being phylogenetically near to *Pandora* sp. nov., grew well in a combination of SM, YE and beef extract, but also in the SM+YE combination, as well.

Glucose was selected as main carbon source, as several studies indicated that the Entomophthorales prefer monosaccharides and are unable to metabolize disaccharides, such as sucrose (Latgé, 1975a; Leite et al., 2003). Thus, it was not unexpected when we found that the disaccharide lactose from the skimmed milk was not metabolized by *Pandora* sp. nov. either (data not shown).

A yield coefficient of 0.19 is similar to the results of Russell and Paterson (1982) who determined a yield of 0.10–0.28 for *Erynia radicans (= Zoophthora radicans*) grown in a medium composed of glucose and yeast extract. Compared with other EPF, these yield coefficients can be considered low, as for example finely dispersed mycelium of *Metarhizium brunneum* BIPESCO5 was cultivated with a yield coefficient of 0.5 (Krell, unpublished), *Beauveria bassiana* ATP-02, DSM 24665 with 0.36 (Lohse et al., 2015) and the nematophagous fungus *Hirsutella rhossiliensis* BBA with 0.44 (Patel et al., 2011). To improve the competitiveness of the Entomophthorales with other entomopathogenic fungi, future studies on media optimization are needed to address yield enhancement. Furthermore, at present, it is not possible to estimate the biomass needed or the cost per hectare for controlling psyllids.

Growth kinetics

Describing the kinetic stages, it was observed that after an initial growth phase of two days with low glucose consumption, fungal growth appeared to stagnate on day three. Afterward, glucose consumption increased noticeably, accompanied by formation of pellets (**Figure 4.3**). These observations indicate that during the early stages, other nutrients from the complex nitrogen sources are metabolized for biomass formation. After the first growth phase, the fungus requires energy that is not invested in biomass formation, but in other metabolic processes related to the transition from hyphal bodies to mycelial growth morphology (Papierok & Hajek, 1997; Freimoser et al., 2003). This phenomenon has long been recognized in conventional fermentation studies with filamentous fungi (Cox et al., 1998; Gibbs et al., 2000) and was also shown to occur within the Entomophthorales: Nolan (1993a) described a shift in glucose consumption by *Entomophaga aulicae* grown in stirred tank bioreactors in different culture media during the first days, when protoplasts turned into hyphal bodies. Freimoser et al. (2000; 2003) found walled mycelium formation from *Entomophthora thripidum* protoplasts after exhaustion of complex nitrogen sources. More recently, Iwanicki et al. (2020) gave evidence about gene expression distinctions between blastospores that are comparable to hyphal bodies in Entomophthorales and mycelial growth stages of *Metarhizium anisopliae*. Genes up-regulated during blastospore stages were involved in oxidative stress, amino acid metabolism, respiration processes, transmembrane transport and production of secondary metabolites. In the later mycelial growth phase, up-regulated genes were associated with increased growth, metabolism and cell wall re-organization.

Effect of osmolality on the production of finely dispersed mycelium

One common problem when culturing filamentous microorganisms in submerged culture that also occurred in this study is the formation of pellet-like structures. Pellets suffer from reduced viable biomass (Clark, 1962), limitation of oxygen and nutrient diffusion (Prosser & Tough, 1991) and being unsuitable for encapsulation in small sized beads (Patel et al., 2011; Krell et al., 2018) due to their size of up to several mm (Cox et al., 1998). Shah et al. (1998) solved this problem by sieving the mycelia of *P. neoaphidis* grown in shaking-culture before encapsulation at the cost of high biomass loss.

The preferred biomass morphology for ensuing formulation are finely dispersed mycelium or hyphal bodies that can be produced by modification of fermentation parameters as demonstrated for other filamentous fungi (Patel et al., 2011; Jaronski, 2014; Krell et al., 2018; Hallmann et al., 2019). In the present study, pellet formation was reduced by adding salt in order to increase media osmolality in a

cheap and simple way. Other cultivation studies on filamentous fungi also succeeded in manipulating the hyphal morphology, e.g. of *Aspergillus niger* by salt addition (Wucherpfennig et al., 2011) or in promotion of finely dispersed mycelium morphology of *M. brunneum* by a combination of increased medium osmolality and viscosity (Krell et al., 2018). In this way, the entire biomass can be used for encapsulation in hydrogel beads without sieving and loss of viable biomass.

Moreover, it has to be noted that the osmolality of standard Grace's insect culture media used for growth of entomophthoralean cells is around 340–580 osmol/kg (Beauvais and Latgé, 1988; Lopez Lastra et al., 2001; Freimoser et al., 2003). Consequently, the medium containing sodium chloride between 0.5 – 1% simulates optimal growth conditions in terms of osmolality.

Scale-up and media costs

The last aim of this study was to prove the scalability of the medium and method for production of *Pandora* sp. nov. biomass by transferring the cultivation from shaking culture flasks into a stirred tank bioreactor. After the transfer, four times more biomass was produced in the stirred tank bioreactor compared with the shaking culture in flasks. This can be explained by improved pO_2 and nutrient supply.

After 48 h, the fungal cells became increasingly attached to the vessel wall and stirrer in the area of the medium-air interface and formed a growing mycelial ring. This mycelium was not collected for biomass dry weight determination, but was probably viable contributing to oxygen consumption. The ensuing decrease of pO_2 explains the increasing rpm, although the biomass from sampling appears to be decreasing at this time (**Figure 4.6**). It cannot be ruled out that a stirrer speed of 350 rpm at the end of the fermentation damages fungal cells. Damage of fungal cells by high stirring speed is mainly caused by fluid-mechanical stress from turbulence flow or bursting gas bubbles or direct shear stress by the stirring devices (Van Suijdam & Metz, 1981; König, 1981; Märkl & Bronnenmeier, 1985; Chisti, 2009). Cells with weakened outer cell layers such as protoplasts or hyphal bodies (Butt et al., 1981) are sensitive to shear, as they are not experienced in physiological stress. For the stirring device used in the present study with a 75 mm diameter stirrer at a speed of 350 rpm, the impeller tip speed is 1.37 m/s. For the fungi whose damaging threshold is known, it varies between 0.4 (*Trichoderma harzianum* (Chisti, 2009)) and 2.5 (*Penicillium chrysogenum* (Makagiansar et al., 1993)), but it also depends on the stirrer type and geometry as they generate different shear fields (Hardy et al., 2017). For *Pandora* or any entomophthoralean species, the damaging threshold value of the stirrer speed is unknown. Nevertheless, biomass needs to be harvested earlier in the fermentation process when highly viable cells are needed for further formulation and application in biocontrol strategies.

The costs for the media combinations used in this study were calculated (**Table 4.4**) in order to find the most cost efficient medium for cultivation of *Pandora* sp. nov. with potential for mass-production. The combination of SM with YE or with YE and AN leads to both the highest biomass production and highest specific growth rate (**Figure 4.2**). Since YE, even the technical YE used in this study, is an expensive media component, media costs were reduced by partially replacing YE with SM and AN, a low-cost protein hydrolysate from animal by-products ($X^2 = 23.93$; df = 7; $p < 0.1$; *Kruskall-Wallis*; **Table 4.4**).

Table 4.4 Media costs. All media, except the pre-culture medium, contain a fixed glucose amount of 26.6 g . L-1 and a fixed amount of a complex nitrogen source of 10 g . L-1 individual or in combination. AN = protein hydrolysate from animal by-products, SM = skimmed milk powder, YE = yeast extract, NaCl = sodium chloride. Medium used for further experiments is highlighted. Different letters indicate significant differences of the media costs according to a Kruskall-Wallis test followed by Dunn-Bonferroni at p<0.1.

Medium	€/L medium	€/kg biomass dry weight	Sig.
Pre-culture	0.1	36.7 ± 7.2	-
AN	0.2025	151.6 ± 115.8	a
SM	0.3933	94.8 ± 23.6	a
YE	0.9775	427.1 ± 80.2	b
AN+SM	0.2979	134.7 ± 47.8	ab
AN+YE	0.5900	276.0 ± 117.8	ab
SM+YE	0.6854	144.0 ± 32.6	ab
AN+SM+YE	0.5210	105.3 ± 9.8	a
AN+SM+YE + NaCl (1%)	**0.5638**	**99.3 ± 8.4**	**a**

Conclusion

One of the main reasons for the failure to establish an entomophthoralean fungus as a biocontrol agent for practical use has so far been the difficulty of growing these fungi *in vitro* on a large scale and thereafter formulating them. In this study, we described the production of finely dispersed mycelium of the new *Pandora* species on a technical scale in a submerged fermentation procedure. Furthermore, as the fungus used in this study was recently isolated, this is the first report on *in vitro* cultivation of *Pandora* sp. nov, inedit. (ARSEF 13372) on a technical scale in liquid culture. The first essential step of mass production on the way to large scale field application of the promising novel fungus has been taken. The results provide a valuable basis for urgently needed novel control approaches for psyllids as agricultural pest insects. Further studies focus on the formulation of *Pandora* sp. nov. in order to improve the sporulation capacity and infection potential (Muskat et al., 2021b) and enable practical use in field application.

Author contributions

Linda C. Muskat: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing – original draft; Writing – review and editing; Project administration.

Michael Przyklenk: Methodology; Investigation; Writing – original draft.

Pascal Humbert: Conceptualization; Writing – review and editing; Project administration.

Jørgen Eilenberg: Conceptualization; writing – review and editing.

Anant V. Patel: Conceptualization; Funding acquisition; Methodology; Supervision; Writing – review and editing.

References

Beauvais, A. & Latgé, J. P. (1988). A simple medium for growing entomophthoralean protoplasts. Journal of Invertebrate Pathology 51: 175-178. https://doi.org/10.1016/0022-2011(88)90079-1.

Brobyn, P. J., Wilding, N. & Clark, S. J. (1985). The persistence of infectivity of conidia of the aphid pathogen *Erynia neophidis* on leaves in the field. Annals of Applied Biology 107: 365-376. https://doi.org/10.1111/j.1744-7348.1985.tb03153.x.

Butt, T. M., Beckett, A., & Wilding, N. (1981). Protoplasts in the in vivo life cycle of *Erynia neoaphidis*. Microbiology 127(2): 417-421. https://doi.org/10.1099/00221287-127-2-417.

Chisti, Y. (2009). Shear sensitivity. Encyclopedia of industrial biotechnology: Bioprocess, Bioseparation and Cell Technology. John Wiley & Sons: 1-40.

Clark, D. S. (1962). Submerged citric acid fermentation of ferrocyanide-treated beet molasses: morphology of pellets of *Aspergillus niger*. Canadian Journal of Microbiology 8: 133-136. https://doi.org/10.1139/m62-017.

Cox, P. W., Paul, G. C. & Thomas, C. R. (1998). Image analysis of the morphology of filamentous microorganisms. Microbiology 144: 817-827. https://doi.org/10.1099/00221287-144-4-817.

Cui, Y. Q., Okkerse, W. J., Van der Lans, R. G. J. M., & Luyben, K. C. A. (1998). Modeling and measurements of fungal growth and morphology in submerged fermentations. Biotechnology and Bioengineering, 60(2): 216-229.

Delalibera Jr, I., Hajek, A. E., & Humber, R. A. (2003). Use of cell culture media for cultivation of the mite pathogenic fungi *Neozygites tanajoae* and *Neozygites floridana*. Journal of Invertebrate Pathology, 84(2): 119-127. https://doi.org/10.1016/j.jip.2003.09.005.

de Lima, I. J., Carneiro Leão, M. P., da Silva Santos, A. C., da Costa, A. F. & Tiago, P. V. (2021). Production of conidia by entomopathogenic isolates of *Fusarium caatingaense* on different vegetable substrates. Biocontrol Science & Technology 31: 206-218. https://doi.org/10.1080/09583157.2020.1843599.

Eilenberg, J., Bresciani, J. & Latgé, J. P. (1986). Ultrastructural studies of primary spore formation and discharge in the genus *Entomophthora*. Journal of Invertebrate Pathology 48: 318-324. https://doi.org/10.1016/0022-2011(86)90060-1.

Eilenberg, J., Saussure, S., Fekih, I. B., Jensen, A. B. & Klingen, I. (2019). Factors driving susceptibility and resistance in aphids that share specialist fungal pathogens. Current Opinion in Insect Science 33: 91-98. https://doi.org/10.1016/j.cois.2019.05.002.

Eilenberg, J., Wilding, N. & Bresciani, J. (1992). Isolation in vitro of *Strongwellsea castrans* (Fungi: Entomophthorales) a pathogen of adult cabbage root flies, *Delia radicum* (Dipt.: Anthomyiidae). Entomophaga 37: 65-77. https://doi.org/10.1007/BF02372975.

Freimoser, F. M., Grundschober, A., Aebi, M. & Tuor, U. (2000). In vitro cultivation of the entomopathogenic fungus *Entomophthora thripidum*: isolation, growth requirements and sporulation. Mycologia 92: 208-215. https://doi.org/10.1080/00275514.2000.12061146.

Freimoser, F. M., Jensen, A. B., Tuor, U., Aebi, M. & Eilenberg, J. (2001). Isolation and in vitro cultivation of the aphid pathogenic fungus *Entomophthora planchoniana*. Canadian Journal of Microbiology 47: 1082-1087. https://doi.org/10.1139/w01-115.

Freimoser, F. M., Grundschober, A., Tuor, U. & Aebi, M. (2003). Regulation of hyphal growth and sporulation of the insect pathogenic fungus *Entomophthora thripidum* in vitro. FEMS Microbiology Letters 222: 281-287. https://doi.org/10.1016/S0378-1097(03)00315-X.

Gibbs, P., Seviour, R. & Schmid, F. (2000). Growth of filamentous fungi in submerged culture: Problems and possible solutions. Critical Reviews in Biotechnology 20: 17-48. https://doi.org/10.1080/07388550091144177.

Glare, T., Caradus, J., Gelernter, W., Jackson, T., Keyhani, N., Köhl, J., Marrone, P., Morin, L. & Stewart, A. (2012). Have biopesticides come of age? Trends in Biotechnology 30: 250-258. https://doi.org/10.1016/j.tibtech.2012.01.003.

Görg, L.M., Eilenberg, J., Jensen, A., Jensen, A. & Gross, J. (2021). Pathogenicity against hemipteran vector insects of a novel insect pathogenic fungus from Entomophthorales (*Pandora* sp. nov. inedit.) with potential for biological control. Journal of Invertebrate Pathology 183: 107621. https://doi.org/10.1016/j.jip.2021.107621.

Griffin, D. H. (1993) Fungal Physiology. Wiley, New York.

Grundschober, A., Tuor, U., & Aebi, M. (1998). In vitro cultivation and sporulation of *Neozygites parvispora* (Zygomycetes: Entomophthorales). Systematic and Applied Microbiology 21(3), 461-469. https://doi.org/10.1016/S0723-2020(98)80057-X.

Gryganskyi, A. P., Humber, R. A., Smith, M. E., Hodge, K., Huang, B., Voigt, K. & Vilgalys, R. (2013). Phylogenetic lineages in Entomophthoromycota. Persoonia: Molecular Phylogeny and Evolution of Fungi 30: 94-105. https://doi.org/10.3767/003158513X666330.

Hajek, A. E. & Roberts, D. W. (1991). Pathogen reservoirs as a biological control resource: Introduction of *Entomophaga maimaiga* to north american gypsy moth, *Lymantria dispar*, populations. Biological Control 1: 29-34. https://doi.org/10.1016/1049-9644(91)90098-k.

Hajek, A. E., & St. Leger, R. J. (1994). Interactions between fungal pathogens and insect hosts. Annual Review of Entomology 39(1): 293-322.

Hajek, A. E., Wheeler, M. M., Eastburn, C. C. & Bauer, L. S. (2001). Storage of resting spores of the gypsy moth fungal pathogen, *Entomophaga maimaiga*. Biocontrol Science & Technology 11: 637-647 https://doi.org/10.1080/09583150120076184.

Hajek, A. E., Burke, A. E., Nielsen, C., Hannam, J. J. & Bauer, L. S. (2008). Nondormancy in *Entomophaga maimaiga* azygospores: effects of isolate and cold exposure. Mycologia 100: 833-842. https://doi.org/10.3852/07-160.

Hajek, A. E., Papierok, B. & Eilenberg, J. (2012). Methods for study of the Entomophthorales. In Methods in Insect Pathology, Academic Press Amsterdam, 2nd Edition: 285-316. https://doi.org/10.1016/B978-0-12-386899-2.00009-9.

Hallmann, J., Gutberlet, V., Jakobs-Schönwandt, D., Vorlop, K. D., Müller, J. & Patel, A. V. (2019). Effect of additives on the efficacy of microencapsulated *Hirsutella rhossiliensis* controlling *Heterodera schachtii* on sugar beets. Biological Control 128: 40-47. https://doi.org/10.1016/j.biocontrol.2018.09.005.

Hardy, N., Augier, F., Nienow, A. W., Béal, C., & Chaabane, F. B. (2017). Scale-up agitation criteria for *Trichoderma reesei* fermentation. Chemical Engineering Science 172: 158-168. https://doi.org/10.1016/j.ces.2017.06.034.

Herren, P. (2018). Conidial discharge of *Pandora* sp., a potential biocontrol agent against *Cacopsylla* spp. in apple and pear orchards. [Unpublished master's thesis]. University of Copenhagen, Department of Plant and Environmental Sciences.

Iwanicki, N. S. A., Delalibera Júnior, I., Eilenberg, J. & De Fine Licht, H. H. (2020). Comparative RNAseq analysis of the insect-pathogenic fungus *Metarhizium anisopliae* reveals specific transcriptome signatures of filamentous and yeast-like development. G3: Genes, Genomes, Genetics 10: 2141-2157. https://doi.org/10.1534/g3.120.401040.

Jackson, M. (1997). Optimizing nutritional conditions for the liquid culture production of effective fungal biological control agents. Journal of Industrial Microbiology and Biotechnology 19: 180-187. https://doi.org/10.1038/sj.jim.2900426.

Jarausch, B. & Jarausch, W. (2009). Psyllid vectors and their control. In Phytoplasmas: genomes, plant hosts and vectors. CABI: 250-271.

Jaronski, S. T. (2014). Mass production of entomopathogenic fungi: state of the art. Mass production of beneficial organisms. Elsevier Inc., Amsterdam: 357-413. https://doi.org/10.1016/B978-0-12-391453-8.00011-X.

Jensen, A. H. (2017). A new insect pathogenic fungus from Entomophthorales with potential for psyllid control. [Unpublished master's thesis]. University of Copenhagen, Department of Plant and Environmental Sciences.

Jensen, A. H., Gross, J., Jensen, A. B., Gallinger, J. & Eilenberg, J. (2018). A new insect pathogenic fungus from Entomophthorales with potential for psyllid control. Mitteilungen der Deutschen Gesellschaft für allgemeine und angewandte Entomologie 21: 283-286.

Jönsson, A. G. (1968). Protease production by species of *Entomophthora*. Applied Microbiology 16: 450-457. https://doi.org/10.1128/AM.16.3.450-457.1968.

Keller, S. (1997). The genus *Neozygites* (Zygomycetes, Entomophthorales) with special reference to species found in tropical regions. Sydowia 49(2): 118-146.

König, B., Seewald, C., & Schügerl, K. (1981). Process engineering investigations of penicillin production. European Journal of Applied Microbiology and Biotechnology 12(4): 205-211. https://doi.org/10.1007/BF00499488.

Krell, V., Jakobs-Schönwandt, D., Vidal, S. & Patel, A. V. (2018). Cellulase enhances endophytism of encapsulated *Metarhizium brunneum* in potato plants. Fungal Biology 122: 373-378. https://doi.org/10.1016/j.funbio.2018.03.002.

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M. & Goettel, M. S. (2015). Insect pathogens as biological control agents: Back to the future. Journal of Invertebrate Pathology 132: 1-41. https://doi.org/10.1016/j.jip.2015.07.009.

Latgé, J. P. (1975a). Croissance et sporulation de 6 espèces d'entomophthorales 1. Influence de la nutrition carbonée. Entomophaga 20: 201-207. https://doi.org/10.1007/BF02371660.

Latgé, J. P. (1975b). Croissance et sporulation de 6 espèces d'Entomophthorales II. Influence de diverses sources d'azote. Mycopathologia 57: 53-57. https://doi.org/10.1007/BF00431180.

Latgé, J. P., Soper, R. S., & Madore, C. D. (1977). Media suitable for industrial production of *Entomophthora virulenta* zygospores. Biotechnology and Bioengineering, 19(9): 1269-1284. https://doi.org/10.1002/bit.260190903.

Latgé, J. P. (1981). Comparaison des exigences nutritionnelles des Entomophthorales. Annales de Microbiologie 132(3): 299-306.

Latgé, J. P. & Remaudière, G. (1975). Croissance et sporulation de six espèces d'Entomophthorales. III. Influence des concentrations de carbone et d'azote et du rapport C/N. Revue de Mycologie 39(4): 239-250.

Latgé, J. P., Remaudière, G. & Diaquin, M. (1978). Un nouveau milieu pour la croissance et la sporulation d'Entomophthorales pathogenes d'aphides: [A new culture medium for growth and sporulation of Entomophthorales species, pathogens of aphids]. Annales de Microbiologie 129B: 463-476.

Latgé, J. P., Silvie, P., Papierok, B., Remaudière, G., Dedryver, C. & Rabasse, J. (1983). Advantages and disadvantages of *Conidiobolus obscurus* and of *Erynia neoaphidis* in the biological control of aphids. Aphid Antagonists, AA Balkema, Rotterdam: 20-32.

Latgé, J. P., & Sanglier, J. J. (1985). Optimisation de la croissance et de la sporulation de *Conidiobolus obscurus* en milieu defini. Canadian Journal of Botany, 63(1): 68-85. https://doi.org/10.1139/b85-011.

Leite, L. G., Smith, L., Moraes, G. J., & Roberts, D. W. (2000). In vitro production of hyphal bodies of the mite pathogenic fungus *Neozygites floridana*. Mycologia, 92(2): 201-207. https://doi.org/10.1080/00275514.2000.12061145.

Leite, L. G., Alves, S. B., Batista Filho, A. & Roberts, D. W. (2003). Effect of salts, vitamins, sugars and nitrogen sources on the growth of three genera of Entomophthorales: *Batkoa*, *Furia* and *Neozygites*. Mycological Research 107: 872-878. https://doi.org/10.1017/S0953756203007974.

Leite, L. G., Alves, S. B., Batista Filho, A. & Roberts, D. W. (2005). Simple, inexpensive media for mass production of three entomophthoralean fungi. Mycological Research 109: 326-334. https://doi.org/10.1017/s0953756205002406.

Li, Z., Butt, T. M., Beckett, A. & Wilding, N. (1993). The structure of dry mycelia of the entomophthoralean fungi *Zoophthora radicans* and *Erynia neoaphidis* following different preparatory treatments. Mycological Research 97: 1315-1323. https://doi.org/10.1016/S0953-7562(09)80163-9.

Lohse, R., Jakobs-Schönwandt, D., Vidal, S. & Patel, A. V. (2015). Evaluation of new fermentation and formulation strategies for a high endophytic establishment of *Beauveria bassiana* in oilseed rape plants. Biological Control 88: 26-36. https://doi.org/10.1016/j.biocontrol.2015.05.002.

Lopez Lastra, C. C., Gibson, D. M. & Hajek, A. E. (2001). Survival and differential development of *Entomophaga maimaiga* and *Entomophaga aulicae* (Zygomycetes : Entomophthorales) in *Lymantria dispar* hemolymph. Journal of Invertebrate Pathology 78: 201-209. https://doi.org/10.1006/jipa.2001.5069.

Makagiansar, H. Y., Shamlou, P. A., Thomas, C. R., & Lilly, M. D. (1993). The influence of mechanical forces on the morphology and penicillin production of *Penicillium chrysogenum*. Bioprocess Engineering, 9(2): 83-90. https://doi.org/10.1007/BF00369035.

Märkl, H.; Bronnenmeier, R. (1985). Mechanical stress and microbial production: Fundamentals of Biochemical Engineering, Vol. 2: 370-392. Brauer, H. (Ed.), Germany; VCH Verlagsgesellschaft.

Mascarin, G. M., Kobori, N. N., Jackson, M. A., Dunlap, C. A., & Delalibera Jr, Í. (2018). Nitrogen sources affect productivity, desiccation tolerance and storage stability of *Beauveria bassiana* blastospores. Journal of Applied Microbiology, 124(3): 810-820. https://doi.org/10.1111/jam.13694.

Muskat, L. C., Kerkhoff, Y., Humbert, P., Nattkemper, T. W., Eilenberg, J., & Patel, A. V. (2021a). Image analysis-based quantification of fungal sporulation by automatic conidia counting and gray value correlation. MethodsX, 8, 101218. https://doi.org/10.1016/j.mex.2021.101218

Muskat, L. C., Görg, L. M., Humbert, P., Gross, J., Eilenberg, J., & Patel, A. V. (2021b). Encapsulation of the psyllid-pathogenic fungus *Pandora* sp. nov. inedit. and experimental infection of target insects. Pest Management Science. https://doi.org/10.1002/ps.6710

Nolan, R. A. (1986). Effects of an altered developmental pattern on amino acid uptake for a protoplast isolate of the fungus *Entomophaga aulicae* under mass-fermentation conditions. Canadian Journal of Microbiology, 32(11), 855-860. https://doi.org/10.1139/m86-157.

Nolan, R. A. (1988). A simplified, defined medium for growth of *Entomophaga aulicae* protoplasts. Canadian Journal of Microbiology, 34(1), 45-51. https://doi.org/10.1139/m88-008.

Nolan, R. A. (1990). Enhanced hyphal body production by *Entomophaga aulicae* protoplasts in the presence of a neutral and a positively charged surface under mass fermentation conditions. Canadian Journal of Botany-Revue Canadienne De Botanique 68: 2708-2713. https://doi.org/10.1139/b90-343.

Nolan, R. A. (1991). Influence of a negatively charged surface (teflon disk) on *Entomophaga aulicae* protoplast morphogenesis under mass fermentation conditions. Canadian Journal of Botany-Revue Canadienne De Botanique 69: 2578-2581. https://doi.org/10.1139/b91-321.

Nolan, R. A. (1993a). Physiological studies with the fungus *Entomophaga aulicae* during morphogenesis in 3 different media under fermentation conditions. Canadian Journal of Microbiology 39: 701-708. https://doi.org/10.1139/m93-100.

Nolan, R. A. (1993b). An inexpensive medium for mass fermentation production of *Entomophaga aulicae* hyphal bodies competent to form conidia. Canadian Journal of Microbiology, 39(6): 588-593.

Olsen, N. L., Herren, P., Markussen, B., Jensen, A. B. & Eilenberg, J. (2019). Statistical modelling of conidial discharge of entomophthoralean fungi using a newly discovered *Pandora* species. PloS One 14: e0215914. https://doi.org/10.1371/journal.pone.0215914.

Papierok, B. & Hajek, A. E. (1997). Fungi: Entomophthorales. In Manual of techniques in insect pathology. Academic Press, San Diego, CA: 187-212. https://doi.org/10.1016/B978-012432555-5/50012-9.

Patel, A. V., Jakobs-Schönwandt, D., Rose, T. & Vorlop, K. D. (2011). Fermentation and microencapsulation of the nematophagous fungus *Hirsutella rhossiliensis* in a novel type of hollow beads. Applied Microbiology and Biotechnology 89: 1751-1760. https://doi.org/10.1007/s00253-010-3046-9.

Pell, J., Eilenberg, J., Hajek, A. & Steinkraus, D. (2001). Biology, ecology and pest management potential of Entomophthorales. In Fungi as biocontrol agents: progress, problems and potential, CABI: 71-154. https://doi.org/10.1079/9780851993560.0071

Prosser, J. I. & Tough, A. (1991). Growth mechanisms and growth kinetics of filamentous microorganisms. Critical Reviews in Biotechnology 10: 253-274. https://doi.org/10.3109/07388559109038211.

Russell, R., & Paterson, M. (1982). Effects of variations in nitrogen and carbon sources on the physiology of growth of *Erynia radicans*, a potential mycoinsecticide. Biotechnology Letters 4: 115-120. https://doi.org/10.1007/BF01091346.

Shah, P. & Pell, J. (2003). Entomopathogenic fungi as biological control agents. Applied Microbiology and Biotechnology 61: 413-423. https://doi.org/10.1007/s00253-003-1240-8.

Shah, P. A., Aebi, M. & Tuor, U. (1998). Method to immobilize the aphid-pathogenic fungus *Erynia neoaphidis* in an alginate matrix for biocontrol. Applied and Environmental Microbiology 64: 4260-4263. https://doi.org/10.1128/AEM.64.11.4260-4263.1998.

Shah, P. A., Aebi, M. & Tuor, U. (1999). Production factors involved in the formulation of *Erynia neoaphidis* as alginate granules. Biocontrol Science & Technology 9: 19-28. https://doi.org/10.1080/09583159929875.

Soper, R. S. (1985). *Erynia radicans* as a mycoinsecticide for spruce budworm control. Proceedings Symposium: Microbial control of spruce budworms and gypsy moths; 1984 April 10-12; Windsor Locks, CT. Gen. Tech. Rep. NE-100. Broomall, PA: US Department of Agriculture, Forest Service, Northeastern Forest Experiment Station. 69-76.

Takors, R. (2014). Kommentierte Formelsammlung Bioverfahrenstechnik. Springer Spektrum.

Urbańczyk, M. J., Zabża, A., Bałazy, S., & Peczyńska-Czoch, W. (1992). Laboratory culture media and enzyme activity of some entomopathogenic fungi of *Zoophthora* (Zygomycetes: Entomophthoraceae). Journal of Invertebrate Pathology 59: 250-257. https://doi.org/10.1016/0022-2011(92)90129-R.

Van Suijdam, J. C., & Metz, B. (1981). Influence of engineering variables upon the morphology of filamentous molds. Biotechnology and Bioengineering 23(1): 111-148. https://doi.org/10.1002/bit.260230109.

Vega, F. E., Meyling, N. V., Luangsaard, J. J. & Blackwell, M. (2012). Fungal entomopathogens. Insect Pathology. Academic Press, San Diego, CA: 171-220.

Wolf, F. T. (1951). The cultivation of two species of *Entomophthora* on synthetic media. Bulletin of the Torrey Botanical Club 78: 211–220. https://doi.org/10.2307/2481976.

Wraight, S. P., Galaini-Wraight S., Carruthers, R. I., & Roberts, D. W. (2003). *Zoophthora radicans* (Zygomycetes: Entomophthorales) conidia production from naturally infected *Empoasca kraemeri* and dry-formulated mycelium under laboratory and field conditions. Biological Control 28(1): 60-77. https://doi.org/10.1016/S1049-9644(03)00035-5.

Wucherpfennig, T., Hestler, T. & Krull, R. (2011). Morphology engineering - osmolality and its effect on *Aspergillus niger* morphology and productivity. Microbial Cell Factories 10: 58. https://doi.org/10.1186/1475-2859-10-58.

Chapter 5

Encapsulation of the psyllid-pathogenic fungus *Pandora* sp. nov. inedit. and experimental infection of target insects

Linda C. Muskat[1,2], Louisa M. Görg[3], Pascal Humbert[1], Jürgen Gross[3], Jørgen Eilenberg[4], Anant V. Patel[1]

[1]Bielefeld University of Applied Sciences, Faculty of Engineering and Mathematics, WG Fermentation and Formulation of Biologicals and Chemicals; [2]Georg-August-University Goettingen, Agricultural Entomology, Department of Crop Sciences; [3]Institute for Plant Protection in Fruit Crops and Viticulture, Julius Kühn-Institut, Federal Research Institute for Cultivated Plants; [4]University of Copenhagen, Department of Plant and Environmental Sciences

Published in: Pest Management Sciences, 2022, Vol. 78, 991-999

DOI: 10.1002/ps.6710

Abstract

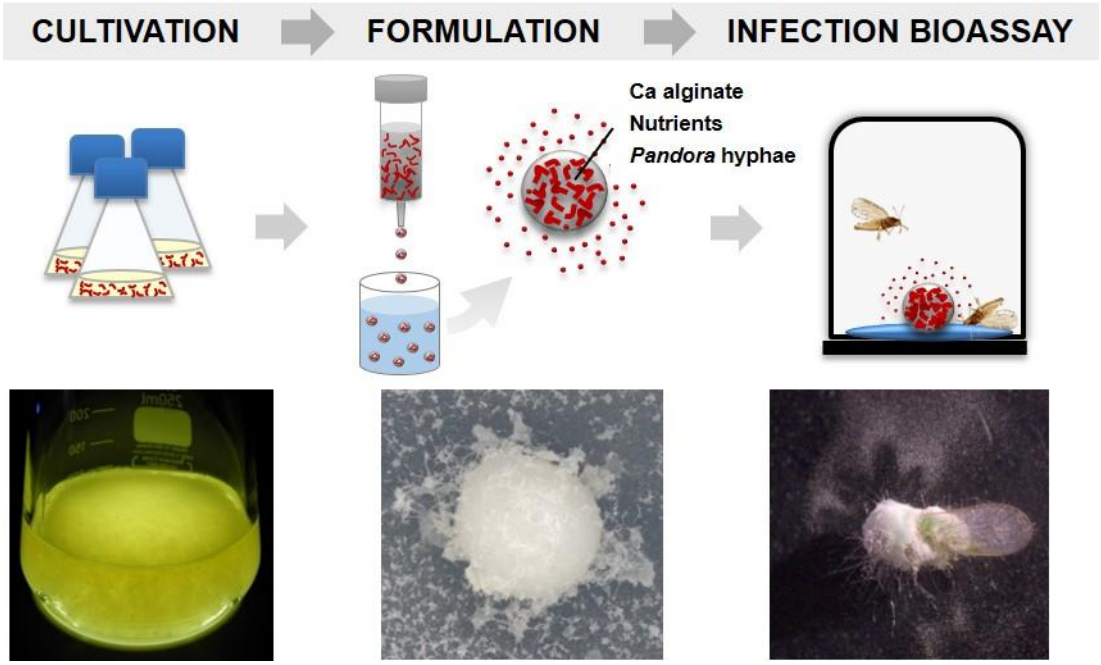
BACKGROUND: *Pandora* sp. nov. inedit. (Entomophthorales: Entomophthoraceae) (ARSEF 13372) is a recently isolated entomophthoralean fungus with potential for psyllid pest control. This study aimed to develop a formulation based on biocompatible hydrogel beads in order to transfer the fungus into an easily applicable form and to test the effects on psyllids.

RESULTS: After encapsulation in calcium alginate beads, *Pandora* sp. nov. grew from the beads and discharged conidia over 12 days under optimal humidity conditions at 18 °C. Conidial number was increased 2.95-fold by the addition of skimmed milk as nutritional formulation additive to the beads. The virulence of the encapsulated fungus was assessed with the two target psyllid species; the summer apple psyllid, *Cacopsylla picta* and the pear psyllid, *Cacopsylla pyri*. Beads containing skimmed milk as nutritional additive led to the highest mortalities (48.3% on *C. picta* and 75.0 % on *C. pyri*). In a second bioassay, survival time of *C. pyri* exposed to beads containing different concentration (10, 20 or 40%) of *Pandora* sp. nov. was tested. The survival time of *C. pyri* was significantly reduced when exposed to beads containing 10% or 20% *Pandora* sp. nov. The median survival time was reached after 5-6 days past inoculation and the cumulative mortality for *C. pyri* treated with *Pandora* sp. nov. beads showed up to 89% mortality.

CONCLUSION: The promising results of this study will ease the way for large-scale field application of a novel *Pandora* species in biological psyllid pest control.

Keywords: Entomophthorales, Encapsulation, Biological control, Entomopathogenic fungi, Virulence, Psyllid control

Graphical abstract



Introduction

Jumping plant lice (Hemiptera: Psyllidae) are phloem feeding insects damaging fruit trees by serving as vectors of phytoplasmas, inducing diverse morphological and physiological changes in their plant hosts (Weintraub & Beanland, 2006; Jarausch et al., 2019; Gallinger et al., 2021). The pear psyllid *C. pyri* vectors the phytoplasma '*Candidatus* Phytoplasma pyri' in pear tree *Pyrus communis* L., the migrating summer apple psyllid *Cacopsylla picta* (Foerster 1848) vectors '*Candidatus* Phytoplasma mali', the causing agent of apple proliferation disease in *Malus domestica* Borkh. (Carraro et al., 1997; Frisinghelli et al., 2000; Seemüller & Schneider, 2004). In European fruit production, high economic losses are caused by reduced fruit yield and quality and dying plants following phytoplasma infection (Bovey, 1961; Civolani et al., 2010; Seemüller et al., 2011). In Italy, an outbreak of apple proliferation disease caused up to € 100 million losses (Strauss, 2009). So far, phytoplasma infected plants cannot be cured and only preventive management strategies such as vector control e.g. with chemical insecticides aiming to minimize the spread of phytoplasma diseases are available (Jarausch & Jarausch, 2009).

In 2016, an entomophthoralean species of the genus *Pandora* was isolated from infected psyllids collected in a Danish pear orchard (Jensen et al., 2018). The species is currently under description as a new species, since it differs from the known *Pandora* species infecting Hemiptera (Görg et al., 2021). Under laboratory conditions, the pathogenicity of *Pandora* sp. nov. mycelial mats grown *in-vitro* on solid media was already demonstrated for several *Cacopsylla* pests such as *C. pyri*, *C. pyricola, C. picta*, *C. mali* and *C. pruni* but also Triozidae (Jensen et al., 2018; Görg et al., 2021; Jensen, 2017; Herren, 2018). Fungi from the Entomophthorales are known for their host specificity, their fast speed-to-kill and for their ability to cause natural epizootics among insects from different orders (Pell et al, 2001; Eilenberg & Philipsen, 1988; Nielsen et al., 2001; Filotas et al., 2003; Vega et al., 2009). Despite their potential, no biological control agents based on an Entomophthorales have been commercialized so far, due to difficulties of growing and mass-producing them *in-vitro* and inconsistent results in field trials (Pell et al.,

2001; Barta & Cagáň, 2006; Jaronski, 2014; Shap & Pell, 2003; Wilding & Latteur, 1987; Wraight et al., 2001). Moreover, research regarding pathogenicity and virulence and thus the suitability of entomophthoralean species for pest control strategies of psyllids in orchards is scarce (Gross et al., 2021).

The conversion of *Pandora* sp. nov. into a biocontrol agent for psyllid pest control requires a formulation that secure the virulence of the fungus when applied against the target insects. The formulation of living biocontrol agents within a carrier material may improve it´s applicability, shelf life, growth and sporulation duration and intensity after field application. The use of biocompatible and biodegradable calcium alginate was demonstrated as a promising carrier for the encapsulation of some members of the Entomophthorales (Chen et al., 2014; Shah et al., 1998; 1999; Zhou et al., 2016). Moreover, the formulation within alginate beads enables the co-formulation of entomopathogenic fungi with beneficial formulation additives. Additives may be nutrients, that enable fungal multiplication after field application, host insect attractants, filling agents for reduced compression during drying, UV and drying protectants or virulence-enhancing components, such as host insect cuticle components that can act as enzyme inducers (e.g. proteins or chitin) (Przyklenk et al., 2017; Burges, 1998; Gerding-Gonzalez et al., 2007; Krell et al., 2018; Hallmann et al., 2019; Vemmer et al., 2016). In order to enable a future utilization of *Pandora* sp. nov. for psyllid pest control we aimed to develop a formulation based on calcium alginate beads.

The specific objectives of this study were (1) to investigate the maintenance of sporulation duration and intensity of *Pandora* sp. nov. after encapsulation in calcium alginate beads, (2) to improve and prolong the sporulation by addition of nutritional formulation additives and (3) to evaluate the pathogenicity and virulence of various *Pandora.* sp. nov. formulations against the target species, pear psyllid *C. pyri* and summer apple psyllid *C. picta,* under laboratory conditions.

Materials and Methods

Chemicals

All chemicals used in this study were acquired by Carl Roth GmbH (Karlsruhe, Germany) or VWR and concentrations are given as (w/w), unless otherwise stated.

Plants

Plants were kept in an insect proof screen house under ambient conditions. Healthy potted pear trees (*Pyrus communis* L.) cultivar ‘Williams Christ’ grown on cv. 'Kirchensaller Mostbirne' rootstocks and potted apple trees (*Malus domestica* Borkh.) cultivar ‘Golden Delicious' grown on 'M9' rootstocks were used to rear insects and as donor of leaves for infection bioassays.

Insects

All insects were collected by the beating tray sampling method (Müther & Vogt, 2005). Adult *C. pyri* specimens (F0) were collected in spring 2018 and 2019 from *Pyrus* sp. at the conventionally managed experimental field at the Julius Kühn - Institut Dossenheim, Germany (49°27'02.1"N 8°38'23.0"E). Overwintered adults (remigrants, F0) of the migrating apple psyllid species *C. picta* were collected in March/April 2019 at an extensively managed *Malus* sp. orchard in Dossenheim, Germany (49°26'45.5"N 8°38'59.0"E.). The summer apple psyllids *C. picta* could only be maintained for one generation

(emigrants, F1). Rearing conditions for both psyllid species were as follows: 20 °C day and 15 °C night temperatures under long day conditions (L16:D8) and 55% relative humidity in a climatic chamber. The psyllids were placed on their respective host plant in 47.5 x 47.5 x 93 cm BugDorm rearing cages (NHBS, UK). The insect specimens used in bioassays were randomly selected from rearing cages containing one single generation.

Fungal isolate

The *Pandora* isolate used in this study originated from an infected *Cacopsylla* sp. collected in a Danish pear orchard (55°50'24.3"N 12°33'46.5"E).[14] The strain is deposited in the USDA Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (Ithaca, NY) as ARSEF 13372. The fungus is under description (inedit.) and will here be referred to as *Pandora* sp. nov.

Solid state cultivation

Pandora sp. nov. was grown on solid medium composed of 4.0% glucose, 2.0% casein and 2.0% agar (Saboraud Dextrose Agar (SDA) supplemented with 20% of a mixture of 60% egg yolk and 40% fresh skimmed milk (SDAME) on Petri dishes (diameter 90 mm) sealed with parafilm® and incubated at 18 °C in the dark (Hajek et al., 2012). To prevent the loss of virulence, the fungus was frequently transferred through the host insect *C. pyri* and the solid culture used for experiments was transferred not more than two times onto fresh artificial media. As *C. picta* can only be maintained for one generation (see. 2.3) and is more difficult to collect and rear compared to *C. pyri*, and thus, not available throughout the whole year, the fungus was only transferred through *C. pyri.*

Submerged cultivation in shake flasks

Submerged cultures of *Pandora* sp. nov. were grown in 100 ml liquid medium in 250 ml shake flasks with four baffles at 18 °C and 170 rpm and an amplitude of 20 mm (IKA KS 4000 ic control, Staufen, Germany). In order to transfer the fungus from the solid media into liquid culture, three pieces of mycelia (0.5 cm^2) were cut with a scalpel and transferred to the pre-culture, composed of fresh skimmed milk (100 $g \cdot L^{-1}$) in ultra-pure water (MilliQ) and incubated for 48 h. The hyphal bodies grown in the pre-culture were collected by centrifugation (4700 g; 15 min; 18 °C) and washed twice in glucose solution (26.6 $g \cdot L^{-1}$) to equal the original volume under repeated centrifugation. Then, the washed and pelletized hyphal bodies were resuspended in glucose solution (26.6 $g \cdot L^{-1}$) to equal the original volume. The main culture was inoculated by 10% (v/v) of the suspension. The main culture medium was composed of glucose (26.6 $g \cdot L^{-1}$), sodium chloride (NaCl; 10 $g \cdot L^{-1}$), yeast extract (3.33 $g \cdot L^{-1}$), skimmed milk powder (HEIRLER CENOVIS GMBH, Radolfzell, Germany; 3.33 $g \cdot L^{-1}$) and a low-cost protein hydrolysate from animal by-products (ANiPept, ANiMOX GmbH, Berlin, Germany, batch No. 1176; 3.33 $g \cdot L^{-1}$). All media components were dissolved in ultrapure water and separately autoclaved for 6 min at 121 °C and 2 bar. The main culture was grown for further 48 h at the same conditions like the pre-culture.

Preparation of *Pandora* sp. nov. for encapsulation

In order to collect finely dispersed hyphae of *Pandora* sp. nov. grown in the main-culture, the cultures from five shaking flasks were unified and centrifuged at 4700 g at 18 °C for 15 min and the hyphal material was washed twice in NaCl (0.9%) to equal the original volume under repeated centrifugation

(4700 g; 15 min; 18 °C). After the final centrifugation step, the washing solution was discarded and the pelletized hyphal material was used for encapsulation.

Preparation of beads

All bead compositions used in this study are listed in **Table 5.1**. The experiments were carried out under sterile conditions and all solutions and components were autoclaved for 6 min at 121 °C and 2 bar, unless otherwise stated. Prior to encapsulation, sodium alginate (Manugel GMB, FMC Corporation, PA, USA, batch No. G7708901) was dissolved in ultrapure water to a final concentration of 3.0% and autoclaved for 6 min at 121 °C. The encapsulation suspension was prepared by mixing 10% (w/w) heat-sterilized native corn starch (Maisita, Agrana Beteiligungs-AG, Vienna, Austria; 10%) into the sodium alginate. Depending on the treatment, pre-dissolved and autoclaved skimmed milk powder was added as nutritional formulation additive at a final concentration of 4% (w/w). To evaluate, if chitin from crab shell can serve as a virulence-enhancing factor, the chitin (Roth) was ground by using a ball mill (MM400 ball mill, Retsch GmbH, Haan, Germany) at 30 Hz for 5 min to a particle size <300 µm. This was dispersed and autoclaved in ultrapure water. Skimmed milk was partly replaced by chitin (Sm2Chi2) at a final concentration of 2% skimmed milk and 2% chitin (w/w). Finally, hyphal material of *Pandora* sp. nov. (see 2.5.1) was added to final concentrations of 10% (Pandora10), 20% (Pandora20) or 40% (Pandora40) (w/w). The suspension was gently stirred for 5 min. For bead formation, the solution was dripped into a stirred (250 rpm) calcium chloride solution (0.1 M) by using a syringe with a cannula (diameter 2.1 × 0.8 mm, Sterican, B. Braun AG, Melsungen, Germany). Beads were kept in the solution under stirring for 20 min and subsequently washed with ultrapure water for 1 min.

Table 5.1 Composition (in % w/w) of calcium alginate bead formulations used in this study. Chi = chitin. Sm = skimmed milk from powder.

	Starch	***Pandora* sp. nov.**	**Chitin**	**Skimmed milk**
Blank	10	0	0	4
Alginate	0	10	0	0
Starch	10	10	0	0
Chi4	10	10	4	0
Sm4	10	10	0	4
Sm2Chi2	10	10	2	2
Pandora10 (=Sm4)	10	10	0	4
Pandora20	10	20	0	4
Pandora40	10	40	0	4

Quantification of fungal sporulation from beads

In order to quantify conidial discharge (see **Figure 5.1a**), moist beads, randomly selected from the batch, were individually placed on water agar (2%) in Petri dishes (Ø = 70 mm) and incubated at 18 °C in the dark in a closed container to maintain high humidity conditions >96% RH. To collect the discharged conidia, the petri dishes containing the beads were inverted and placed above a smaller petri dish (Ø = 35 mm) filled with 3 ml of 0.5% sodium dodecyl sulfate (SDS grained pure, AppliChem GmbH, Darmstadt, Germany) in order to eliminate the effect of aggregation of conidia or formation of secondary conidia or germ-tubes (Hajek et al., 2012). Conidia numbers were determined with a Fuchs-Rosenthal counting chamber from five beads per treatment and 12 samples in a fixed time-interval of 24 h. If counting was performed to observe sporulation duration and intensity, the SDS solution was replaced by a fresh one every 24 h.

Infection bioassay

The laboratory infection bioassays (see **Figure 5.1b**), following the recommendations of Hajek et al. (2012), were performed in small UV sterilized polypropylene (PP) cups (Ø: 69 mm, height: 68 mm; bikapack GmbH, Feldkirch, Austria) as inoculation units as described by Görg et al. (2021). Details on preparation of the PP cups are given in Görg et al. (2019). One bead per cup was placed directly on the agar in the middle of the inoculation unit and the cups were sealed with their lids. The beads were incubated for 3 days at 18 °C, relative humidity conditions >96% and darkness in a climatic chamber (Rumed® Type 3201, Rubarth Apparate GmbH, Laatzen, Germany). Afterwards, the parameters were set to 20 °C and short-day conditions (L:D 10:14 h) to better meet the insects rearing conditions, relative humidity was maintained >96%. Three psyllids were then introduced in each inoculation unit and exposed to conidia discharged by the *Pandora* sp. nov beads for 24 h. Insects in inoculation units without beads were treated similarly and used as control group (Control). After the 24 h exposure time, the insects were transferred to new bioassay units free of beads. A leaf from the insects' respective host plant was put into the agar and served as nutrient source. In the middle of each trial period, all insects were transferred into completely new bioassay units with fresh leaves. Insect mortality was monitored daily over a period of 10 days. Dead insects were observed for signs of fungal growth 24 h after death by using a stereomicroscope (Stemi 508, Carl Zeiss AG, Oberkochen, Germany). Jumping plant lice individuals which escaped or died due to handling mishaps were marked as censored (See 2.11 Statistical Analysis).

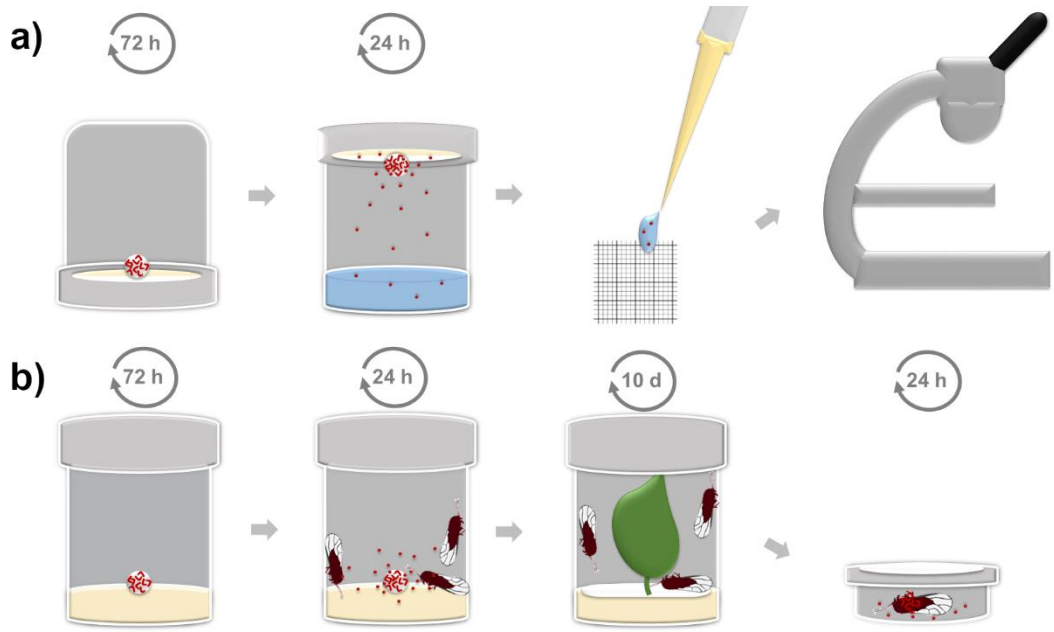


Figure 5.1 Experimental setup of quantification of discharged conidia (a) and infection bioassays with *Pandora* sp. nov. beads (b).

In the first bioassay, survival of two psyllid species, the pear psyllid *C. pyri* (F2, summer forms) and the summer apple psyllid *C. picta* (F1, emigrants) were exposed to one of the following treatments; *Pandora* sp. nov. formulations (Sm4 or Sm2Chi2), blank bead (Blank) or control treatment without beads (Control). Assays were conducted at the same time using the same production batch of formulation. For each treatment, 16 cups each containing one bead of the respective formulation was prepared and the survival of 48 insects per treatment was monitored for each species ($N_{C.\ pyri}$ = 192, $N_{C.\ picta}$ = 192). In the second infection bioassay, *C. pyri* was exposed to different concentrations of *Pandora* sp. nov. in bead formulations Pandora10 (10% *Pandora* sp. nov.), Pandora20 (20% *Pandora* sp. nov.), Pandora40 (40% *Pandora* sp. nov.), blank beads (Blank) and Control (without beads). For each treatment, 10 cups each containing one bead of the respective formulation was prepared and 3 *C. pyri* (F3, summer forms) were added to each cup. Survival of *C. pyri*, observation of *Pandora* sp. nov. outgrowth from killed psyllids and quantification of conidia discharged from the beads placed in the PP cups was conducted as described above (2.9).

Statistical analysis

Statistical analysis of sporulation behavior of encapsulated *Pandora* sp. nov. from beads and the effect of variations of the bead composition was carried out using the software SPSS Statistics V25.0 (SPSS, Chicago, IL). All data are given as mean values ± standard deviations (SD). Data for conidial discharge from beads were checked for normality and homogeneity of variance using *Shapiro–Wilk* and *Levene* test. Mean numbers of discharged conidia were tested for significant differences by one-way ANOVA followed by a *Tukey post hoc range test* and *Bonferroni* correction. If the criteria for variance homogeny and normal distribution was not met, data were calculated by nonparametric *Kruskal-Wallis* test followed by *Dunn-Bonferroni* for multiple comparisons with one treatment or a *Welch* correction for nonhomogeneity followed by a *Games-Howell* test. The effect of different formulation additives (treatment) on number of conidia discharged from beads across time was compared with a repeated measures ANOVA, with time and treatment as independent variables. The sphaericity of the matrix assumption was assessed with the *Mauchly sphericity test*. If the outcome of the test was significant,

the *Greenhouse–Geisser* adjustment was used to correct for violations of sphericity. The level of significance was set to $p < 0.05$.

The statistical analyses of infection bioassays were run in R (Version°1.2.5033; RStudio Team 2019) and figures were generated using "ggplot2" (Wickham et al., 2019) and "survminer" (Kassambara et al., 2017) packages for time-to-event curves. Analysis of time-to-event data (event: death) for censored data according to *Kaplan-Meier method* (Jager et al., 2008) was performed using the *survfit* function of the "survival" package (Therneau & Lumley, 2009). The median survival time and the cumulative mortality [%] ± standard error (SE) as 100% minus the cumulative survival [%] at the end of the experiment (day 10) were calculated after Jager (2008). A violation of the proportional hazard assumption occurred visibly in the crossing of survival curves in each infection bioassay. Hence, in order to assess treatment effects, *parametric models* with different parametric distributions (exponential, weibull, loglogistic) were fitted using the *survreg* function of the "survival" package (Therneau & Lumley, 2009) and compared according to Akaike information criterions (AIC). The parametric models were simplified by removal of non-significant interactions and terms ($p > 0.05$) using the *step* function and the best model identified. Multiple pairwise comparison between effects of psyllid species and treatments were performed with estimated marginal means and 95 % confidence intervals with the function *emmeans* from "emmeans" package (Lenth et al., 2018) and p-value adjustment by the method of *Tukey* (Supplementary table 1 and 2). Significance levels were set to $p < 0.05$. The conidia production of the beads (conidia number ± SE) under infection bioassay conditions were compared by *Kruskal and Wallis one-way analysis of variance by ranks* and *Dunn's-test* for multiple comparisons with one treatment (Sm4 = Pandora10) with p-value adjustment after the *Bonferroni* method using the "PMCMRplus" package (Pohlert, 2020).

Results

Effect of nutrients on duration and intensity of sporulation

Pandora sp. nov. was able to grow and sporulate after encapsulation within calcium alginate beads, as illustrated in **Figure 5.2**.

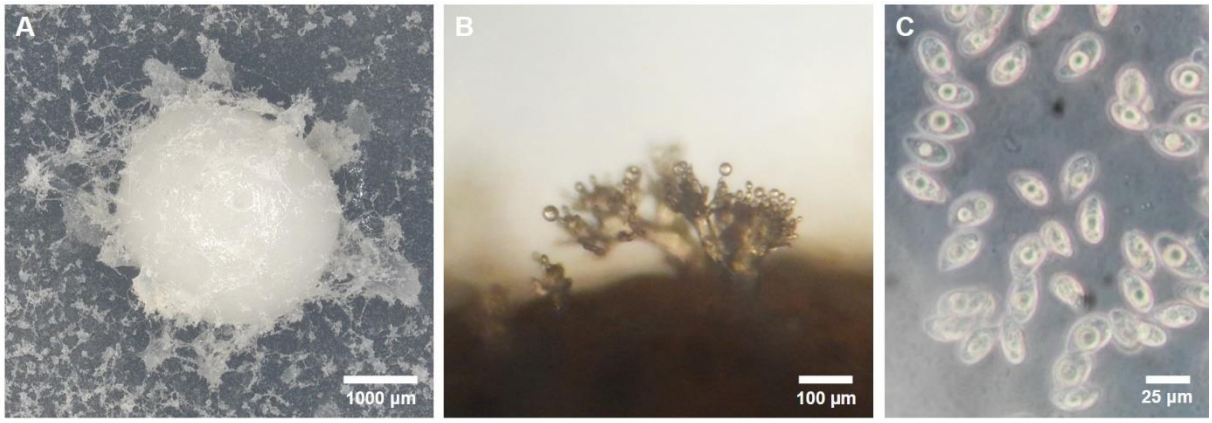


Figure 5.2 *Pandora* sp. nov. growing from calcium alginate beads after 6 days of incubation on water agar plates (A). Conidiophores are observable on the bead surface within the first 24 h (B). Actively discharged conidia from encapsulated *Pandora* sp. nov. (C).

The number of conidia discharged from the beads was significantly affected by the formulation additives ($F_{2,177}$ = 986.751; p < 0.001), the time ($F_{6.946,\ 1229.449}$ = 757.302; p < 0.001) and the interaction of formulation additives and time ($F_{13.892,\ 1229.449}$ = 184,022; p < 0.001). Within the first 24 h, only a few conidia were discharged from all beads (**Figure 5.3**). Sporulation from starch beads increased distinctly at day 2 of incubation and reached a plateau with a maximum of 6.59 x 10^5 (± 1.94 x 10^5) conidia per bead and at day 3 followed by a constant regression in conidia numbers. Sporulation from Sm4 beads increased rapidly up to 2.1 x 10^6 (± 3.73 x 10^5) conidia per bead and reached a single peak sporulation event at day 4 after bead preparation. After 12 days, only a few conidia were discharged from all bead variations compared to conidia numbers during peak sporulation within the first 6 days after bead preparation.

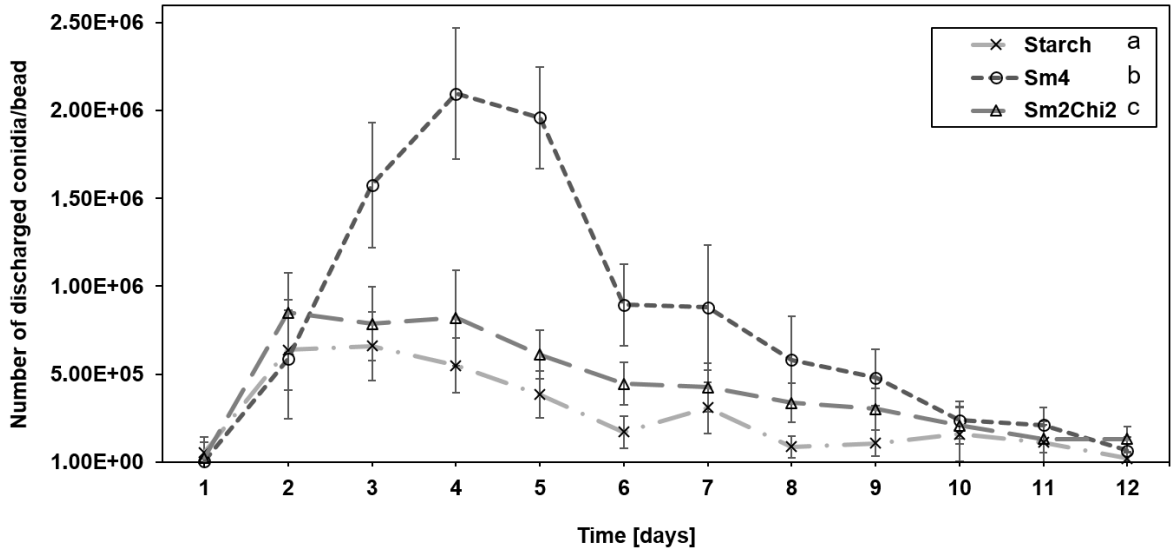


Figure 5.3 Duration and intensity of conidial discharge by *Pandora* sp. nov. from different beads within the first 12 days after bead preparation. Different letters in the legend indicate significant differences according to RM-ANOVA with Games-Howell post hoc test at p < 0.05. Means ± SD, n = 5.

By the addition of skimmed milk (4%; w/w), the total number of conidia discharged from the beads (9.57 x 10^6) was significantly increased by 2.95-fold compared to starch beads (3.25 x 10^6) (X^2 = 12.500; df = 2; p < 0.001; *Bonferroni*).

From the beads containing chitin (2%; w/w) and skimmed milk (2%; w/w) there was only a slight but not significant increase of total conidia numbers (5.30 x 10^6) by 1.63-fold compared to beads without nutritional additives (p = 0.231; *Bonferroni*), but much less than beads containing skimmed milk (p < 0.001).

Effect of *Pandora* sp. nov. concentration in beads on sporulation

The *Pandora* sp. nov. concentration within the beads significantly affected the number of discharged conidia (X^2 = 77.640; df = 2; p < 0.05; *Kruskall-Wallis*; **Figure 5.4**). Significantly more conidia were discharged from beads containing 20% *Pandora* sp. nov. to beads with 10% or 40% ($F_{2,177}$ = 56,745; p < 0.0001; *Games-Howell*). There was a significant difference between beads with a *Pandora* sp. nov. concentration of 10% and 40% (p = 0.037; *Games-Howell*).

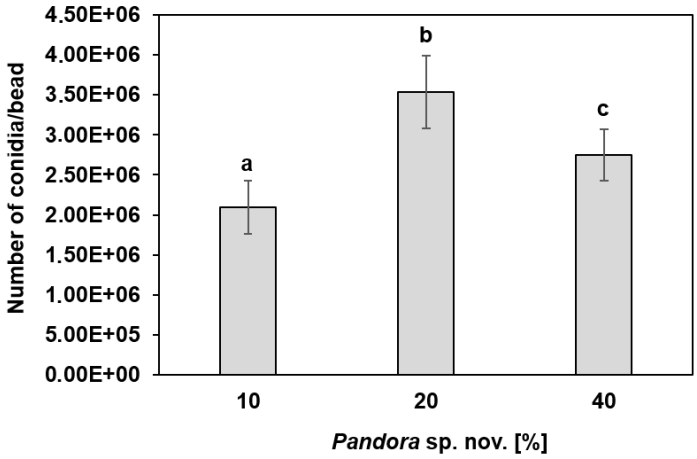


Figure 5.4 Effect of *Pandora*. sp. nov. concentration in beads on conidial discharge during day 4 after bead preparation. Different letters above bars indicate significant differences based on Kruskal-Wallis and Games-Howell post-hoc range test at $p < 0.05$. Means ± SD, n = 5.

Infection bioassay for evaluation of pathogenicity of formulated *Pandora* sp. nov. on *Cacopsylla picta* and *Cacopsylla pyri*

Sm2Chi2 beads produced significantly more conidia ($3x10^4 \pm 7x10^3$ conidia) than Sm4 beads ($1x10^4 \pm 1x10^3$) during the 24 h exposure time (X^2 = 7.43, df = 1, p = 0.006; *Kruskal-Wallis*). Beads without *Pandora* sp. nov. did not produce any conidia.

Psyllids were exposed to the beads during peak sporulation at day four (see **Figure 5.3** – duration Sm4). Treatment (X^2 = 43.30, df = 3, p < 0.0001; *survreg*) and insect species (X^2 = 14.24, df = 1, p = 0.0002; *survreg*) had highly significant effects for the survival of *C. pyri* and *C. picta* (Supplementary table 1). For both species, treatments with *Pandora* sp. nov. beads (Sm4, Sm2Chi2) significantly reduced the survival time compared to beads without fungus (Blank) or no beads (Control) (**Figure 5.5**). For the Sm4 formulation, the pear psyllid *C. pyri* had a significantly decreased mean survival time (6.5 ± 0.7 d) compared to *C. picta* (9.5 ± 0 d, **Figure 5.5**).

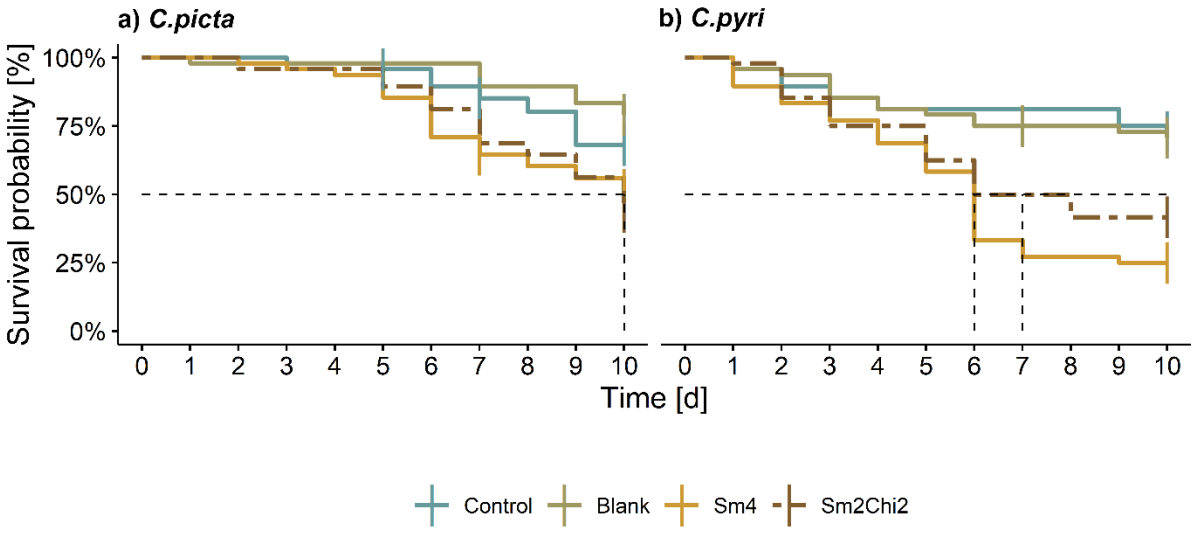


Figure 5.5 Survival [%] for psyllids a) *C. picta* (n = 48/treatment and b) *C. pyri* (n = 48/treatment) 10 days after exposure to beads containing *Pandora* sp. nov. visualized by Kaplan–Meier curves. The dashed black lines indicate the median survival time. Individuals which escaped or died due to handling mishaps were marked as censored events, indicated by tick marks on the Kaplan-Meier curve (Jager et al., 2008).

Infection bioassay for evaluation of dose-response effects against *C. pyri*

The proportion of *Pandora* sp. nov. in beads had a significant effect on mean numbers of conidia produced (X^2 = 33.04, df = 4, p < 0.0001; *Kruskal-Wallis*). Pandora20 beads produced $4x10^4 \pm 2x10^3$ conidia. This was significantly more (z = 3.49, p = 0.001; *Dunn-Bonferroni*) than for Pandora40 beads that produced $3x10^3 \pm 5x10^2$ conidia). Both Pandora20 and Pandora40 beads produced significantly more conidia than Pandora10 beads (z = 2.39, p = 0.03; *Dunn-Bonferroni*), that produced $1x10^4 \pm 2x10^3$ conidia. Beads without *Pandora* sp. nov. material did not produce any conidia.

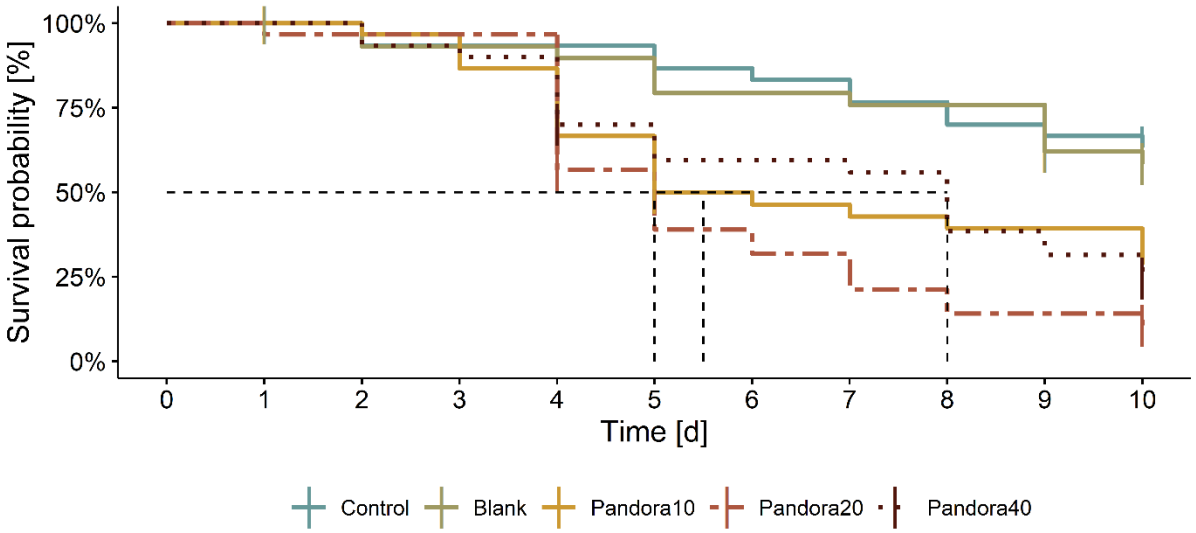


Figure 5.6 Survival [%] for pear psyllid *C. pyri* (n = 30/treatment) 10 days past inoculation visualized by Kaplan–Meier curves. The dashed black lines indicate the median survival time. Individuals which escaped or died due to handling mishaps were marked as censored events, indicated by tick marks on the Kaplan-Meier curve (Jager et al., 2008).

Treatment with distinct bead formulations with varying *Pandora* sp. nov. concentrations had a highly significant effect on the mean survival time of *C. pyri* (X^2 = 31.25, df = 4, p < 0.0001; *survreg*; Supplementary table 2). The survival time of *C. pyri* treated with Pandora20 and Pandora10 beads was significantly reduced in comparison to *C. pyri* treated with Control or Blank beads (**Figure 5.6**). The cumulative mortality for pear psyllids *C. pyri* treated with *Pandora* sp. nov. beads showed up to 89% (± 6) mortality. For insects exposed to beads containing *Pandora* sp. nov., the median survival time was reached between 5-6 days or 8 days after inoculation with Pandora20 and Pandora10 or Pandora40 beads, respectively (**Figure 5.6**). Since the cumulative mortality did not reach 50% for *C. pyri* without fungal exposure (Control, Blank) the median survival time could not be determined for these treatments. Even though a natural cumulative mortality up to about 42% (± 9) was observed within 10 days, *C. pyri* cadavers without fungal exposure (Control, Blank) did not show post-mortem signs of *Pandora* sp. nov. infection.

Discussion

Several studies on conidial discharge in Entomophthorales have reported a rapid increase of conidia numbers discharged from infected cadavers and fresh or dried mycelial mat preparations as well, with a peak occurring within the first 2 days after death of the host insect followed by a fast decrease and

cessation of sporulation within only 3 days (Aoki 1981; Kalsbeek et al., 2001; Wraight et al., 2003; Li et al., 2006; Olsen et al., 2019). After encapsulation of *Pandora* sp. nov., a sporulation peak occurred at day 4 after bead preparation and conidial discharge was observable for at least 12 days. This is much longer, compared to the report on the same fungus by Olsen et al. (2019), who observed a single peak sporulation event <50 h and a maximal sporulation duration of 125 h using mycelia mats grown on solid culture media, transferred to wet filter-paper and incubated under similar temperature and humidity conditions. Moreover, in the present study the total number of discharged conidia increased by the addition of skimmed milk as nutritional additive compared to beads without nutritional additives. This is in accordance with the observation of Shah et al. (1999), who increased conidial discharge by *P. neoaphidis* encapsulated in alginate beads by addition of milk based nutrients. There are several other reports on the positive effects of formulation on sporulation capacity and infection potential of other entomophthoralean fungi, especially in comparison to the sporulation capacity of infected cadavers (Hua & Feng, 2003; Zhou & Feng, 2010; Zhou et al., 2014). Compared to these studies, the total number of 9.57×10^6 conidia discharged by formulated *Pandora* sp. nov. per bead is even higher. Thus, the formulation within calcium alginate beads had at least two beneficial effects: (1) Prolonged sporulation duration and (2) Increased conidia production due to nutrient addition. After peak sporulation, conidia numbers decreased rapidly but since it was reported that 50 conidia of *P. neoaphidis* per m^3 air are sufficient to cause an epizootic in the field (Coremans, 1976; Latgé et al., 1983) the beads may still remain infective after peak sporulation. Our results indicate that formulation possess a promising option to overcome the well-known problem of the Entomophthorales of very short sporulation capacities observable from cadavers.

In the infection bioassays, summer apple psyllid *Cacopsylla picta* and pear psyllid *C. pyri* were successfully infected and killed by *Pandora* sp. nov. formulations. Moreover, a fast speed-to-kill was observed with a median survival time of only 5 to 6 days for pear psyllid *C. pyri* exposed to formulated *Pandora* sp. nov. In comparison, the screening of seventeen Hypocreales species or strains on Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) revealed a median survival time of approx. 6 to 10 days whereas for eight fungal species or strains the mortality rate did not even reach 50% within 10 days (Ausique et al., 2017). In the same experiment, we hypothesized that chitin may serve as a virulence-enhancing formulation additive for improved infection efficiency of *Pandora* sp. nov. as reported for other entomopathogenic fungi (Gerding-Gonzalez et al., 2007; Charnley, 2003; Fang et al., 2005). Previous experiments indicate that *Pandora* sp. nov. can metabolize chitin, as significantly more conidia were discharged from beads containing chitin compared to beads without this additive ($p < 0.001$; *Tukey post hoc test*; **Supplementary figure 5.1**). Even though beads containing chitin in combination with skimmed milk produced higher amounts of conidia, there was no effect on mean survival time compared to the formulation without chitin for both tested psyllid species.

In the second experiment, 20% appears to be an optimal *Pandora* sp. nov. concentration to obtain high conidia numbers. In comparison, formulations containing 40% *Pandora* sp. nov., however, produced significantly less conidia. This may be explained by (1) a faster metabolization of the nutrients due to more viable fungal cells or (2) self-metabolization of the fungus due to a lack of nutrients or (3) release of toxic components by the fungus e.g. ammonium or (4) a temporal shift of the peak sporulation and warrants further investigations. Moreover, it was found that a formulation containing 10% of *Pandora* sp.

nov. was sufficiently effective in killing *C. pyri* and *C. picta*., as neither 20% nor 40% *Pandora* sp. nov. concentrations were able to significantly reduce the mean survival times any further. Taken together with the results obtained in earlier studies on *Pandora* sp. nov.'s pathogenicity for four additional *Cacopsylla* sp. using mycelia mats (Jensen et al., 2018; Görg et al., 2021; Jensen, 2017; Herren, 2018), the findings indicate the high potential of our formulation (Sm4) for pest control strategies of several psyllid pest species of fruit crops. Noteworthy, in infection bioassays with pear psyllid *C. pyri* and summer apple psyllid *C. picta*, we found that there were significant differences in susceptibility to *Pandora* sp. nov. infections between the two psyllid species. The significantly reduced mean survival time as well as the higher cumulative mortality of pear psyllids *C. pyri* suggests a higher susceptibility of *C. pyri* in comparison to *C. picta*. This finding is especially interesting as the fungus was originally isolated from a *Cacopsylla* spp. on pear trees. However, the difference in virulence towards *C. pyri* and *C. picta* could also be due to the effect of frequently transferring *Pandora* sp. nov. through *C. pyri* as culture maintenance practice and not through *C. picta*. Nevertheless, the results of our bioassay are in accordance to another study, which reported that aphid species were more susceptible to infection by entomophthoralean fungi when the fungal inoculum originated from cadavers of the same species (conspecific) opposed to other aphid species (heterospecific) (Ben Fekih et al., 2019). Nonetheless, this finding may also be caused by the high variation in conidia produced of respective formulations in both studies. Given the high variance in conidia produced of beads within the same formulations here observed under infection bioassay conditions, we propose that this issue may be the starting point for further standardization and improvement of the formulation. Additional improvements should be aimed at objective conidia quantification. In the present study conidia were collected in SDS solution to prevent germination and eliminate the stickiness, however this method is labour-intense and subjective. As conidial discharge is one key parameter to evaluate the infection potential of an Entomophthorales formulation, automatized conidia quantification methods were recently developed by our group to further improve the comparability of conidia quantification (Muskat et al., 2021).

Conclusion

The results of this study provide a valuable basis for an urgently needed novel environmentally friendly and economically justifiable control approach for psyllids as agricultural pest insects. Nonetheless, experiments were carried out under laboratory conditions with fresh beads. It is well known that sporulation of fungi from Entomophthorales is strongly affected by temperature and humidity conditions. Ongoing experiments deal with drying and shelf life of the formulation as well as improved water retention to enable sporulation under non-saturated humidity conditions present in European fruit orchards. Further studies are needed to prove the potential of the developed formulation under field conditions in order to develop a commercial product.

Acknowledgements

This study was conducted as part of the PICTA-KILL project which aims to develop innovative attract-and-kill formulations for biological psyllid pest control, supported by funds of the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support program numbers

2814900415 and 2814900515. We thank Sabine Wetzel and Thimo Braun (JKI, Dossenheim, Germany) for excellent assistance in the lab and Felix Hergenhahn (JKI, Dossenheim, Germany) for grafting and cultivation of the plants.

Author contributions

Conceptualization: LCM, LMG, PH, JG, JE and AVP. Data curation: LCM and LMG. Formal analysis: LCM and LMG. Investigation: LCM and LMG. Methodology: LCM, LMG, JG and AVP. Validation: LCM and LMG. Statistical analysis: LCM and LMG. Visualization: LCM and LMG. Writing - original draft: LCM and LMG. Writing - review and editing: LCM, LMG, PH, JG, JE and AVP. Supervision: JG and AVP. Funding acquisition: JG and AVP. Project administration: LCM and PH.

References

Aoki J (1981) Pattern of conidial discharge of an *Entomophthora* species ("grylli" type) (Entomophthorales: Entomophthoracea) from infected cadavers of *Mamestra brassicae* L. (Lepidoptera:Noctuidae). Appl Entomol Zool 16:216-224. https://doi.org/10.1303/aez.16.216

Ausique JJS, D'Alessandro CP, Conceschi MR, Mascarin GM and Delalibera Jr I (2017) Efficacy of entomopathogenic fungi against adult *Diaphorina citri* from laboratory to field applications. J Pest Sci 90:947-960. https://doi.org/10.1007/s10340-017-0846-z. 10.1007/s10340-017-0846-z

Barta M, Cagáň L (2006) Aphid-pathogenic Entomophthorales (their taxonomy, biology and ecology). Biologia 61:543-616. https://doi.org/10.2478/s11756-007-0100-x

Ben Fekih I, Jensen AB, Boukhris-Bouhachem S, Pozsgai G, Rezgui S, Rensing C, Eilenberg J (2019) Virulence of two entomophthoralean fungi, *Pandora neoaphidis* and *Entomophthora planchoniana*, to their conspecific (*Sitobion avenae*) and heterospecific (*Rhopalosiphum padi*) aphid hosts. Insects 10:54. https://doi.org/10.3390/insects10020054

Bovey R (1961) Apple proliferation disease. Stations fédérales d'essais agricoles, Lausanne.

Burges HD (1998) Formulation of mycoinsecticides. In Formulation of microbial biopesticides, Springer, Dordrecht:131-185

Carraro L, Loi N, Ermacora P, Gregoris A, Osler R (1997) Transmission of pear decline by using naturally infected *Cacopsylla pyri* L. In XVII International Symposium Virus and Virus-Like Diseases of Temperate Fruit Crops 472:665-668. https://doi.org/10.17660/ActaHortic.1998.472.89

Charnley AK (2003) Fungal pathogens of insects: cuticle degrading enzymes and toxins. Adv Bot Res 40:241-321. https://doi.org/10.1016/S0065-2296(05)40006-3

Chen C, Ye S, Wang D, Hatting JL, Yu X (2014) Alginate embedding and subsequent sporulation of in vitro-produced *Conidiobolus thromboides* hyphae using a pressurised air-extrusion method. Biol Control 69:52-58. https://doi.org/10.1016/j.biocontrol.2013.10.016

Civolani S (2010) The past and present of pear protection against the pear psylla, *Cacopsylla pyri* L. In Insecticides - pest engineering: 385–408

Coremans P (1976) Note preliminaire sur l'isolement d'entomophthora en belgique. Bull Soc Fr Mycol Méd 5(2):153-156

Eilenberg J, Philipsen H (1988) The occurrence of Entomophthorales on the carrot fly (*Psila rosae* F.) in the field during two successive seasons. Entomophaga 33:135-144. https://doi.org/10.1007/BF02372648

Fang W, Leng B, Xiao Y, Jin K, Ma J, Fan Y, Feng J, Yang X, Zhang Y, Pei Y (2005) Cloning of *Beauveria bassiana* chitinase gene Bbchit1 and its application to improve fungal strain virulence. Appl Environ Microbiol 71:363-370. https://doi.org/10.1128/AEM.71.1.363-370.2005

Filotas MJ, Hajek AE, Humber RA (2003) Prevalence and biology of *Furia gastropachae* (Zygomycetes : Entomophthorales) in populations of forest tent caterpillar (Lepidoptera : Lasiocampidae). Can Entomol 135:359-378

Frisinghelli C, Delaiti L, Grando MS, Forti D, Vindimian ME (2000) *Cacopsylla costalis* (Flor 1861), as a vector of apple proliferation in Trentino. J Phytopathol 148:425-431.

Gallinger J, Zikeli K, Zimmermann MR, Görg LM, Mithöfer A, Reichelt M, Seemüller E, Gross J, Furch ACU (2021) Specialized 16SrX phytoplasmas induce diverse morphological and physiological changes in their respective fruit crops. PLoS Pathog 17(3):e1009459.

Gerding-Gonzalez M, France A, Sepulveda ME, Campos J (2007) Use of chitin to improve a *Beauveria bassiana* alginate-pellet formulation. Biocontrol Sci Technol 17:105-110.

Görg LM, Jensen AH, Eilenberg J, Gross J (2019) Developing a new biological control strategy for *Cacopsylla* spp. with the novel entomopathogenic fungus *Pandora* sp. In Joint Meeting of the IOBC-WPRS Working Groups “Pheromones and other semiochemicals in integrated production” and “Integrated Protection of Fruit Crops” 146:88-90

Görg LM, Eilenberg J, Jensen AB, Jensen AH, Gross J (2021) Pathogenicity against hemipteran vector insects of a novel insect pathogenic fungus from Entomophthorales (*Pandora* sp. nov. inedit.) with potential for biological control. J Invertebr Pathol, in press.

Gross J, Gallinger J, Görg LM (2021) Interactions between phloem-restricted bacterial plant pathogens, their vector insects, host plants, and natural enemies mediated by primary and secondary plant metabolites. Entomol Gen, in press.

Hajek AE, Papierok B, Eilenberg J (2012) Methods for study of the Entomophthorales. In Manual of techniques in invertebrate pathology, Academic Press Amsterdam, 2nd Edition:285-316. https://doi.org/10.1016/B978-0-12-386899-2.00009-9

Hallmann J, Gutberlet V, Jakobs-Schönwandt D, Vorlop KD, Müller J, Patel AV (2019) Effect of additives on the efficacy of microencapsulated *Hirsutella rhossiliensis* controlling *Heterodera schachtii* on sugar beets. Biol Control 128:40-47. https://doi.org/10.1016/j.biocontrol.2018.09.005

Herren P (2018) Conidial discharge of *Pandora* sp., a potential biocontrol agent against *Cacopsylla* spp. in apple and pear orchards. M.Sc. thesis. Department of Plant and Environmental Sciences. University of Copenhagen.

Hua L, Feng MG (2003) New use of broomcorn millets for production of granular cultures of aphid-pathogenic fungus *Pandora neoaphidis* for high sporulation potential and infectivity to *Myzus persicae*. FEMS Microbiol Lett 227:311-317. https://doi.org/10.1016/S0378-1097(03)00711-0

Jager KJ, Van Dijk PC, Zoccali C, Dekker FW (2008) The analysis of survival data: the Kaplan–Meier method, Kidney international 74(5):560-565. https://doi.org/10.1038/ki.2008.217

Jarausch B, Jarausch W (2009) Psyllid vectors and their control. In Phytoplasmas: genomes, plant hosts and vectors: 250-271.

Jarausch B, Tedeschi R, Sauvion N, Gross J, Jarausch W (2019) Psyllid vectors. In Phytoplasmas: plant pathogenic bacteria, Volume 2, Transmission and management of phytoplasma associated diseases:53-78.

Jaronski ST (2013) Mass production of entomopathogenic fungi: state of the art. In Morales-Ramos JA, Rojas MG, Shapiro-Ilan DJ (ed), Mass Production of Beneficial Organisms. Elsevier Inc., Amsterdam: 357–415.

Jensen AH (2017) A new insect pathogenic fungus from Entomophthorales with potential for psyllid control. MSc thesis. Department of Plant and Environmental Sciences, University of Copenhagen.

Jensen AH, Gross J, Jensen AB, Gallinger J, Eilenberg J (2018) A new insect pathogenic fungus from Entomophthorales with potential for psyllid control. Mitt Dtsch Ges Allg Angew Entomol 21:283-286

Kalsbeek V, Pell JK, Steenberg T (2001) Sporulation by *Entomophthora schizophorae* (Zygomycetes : Entomophthorales) from housefly cadavers and the persistence of primary conidia at constant temperatures and relative humidities. J Invertebr Pathol 77:149-157. https://doi.org/10.1006/jipa.2001.5012

Kassambara A, Kosinski M, Biecek P, Fabian S (2017) survminer: Drawing Survival Curves using 'ggplot2'. R package version 0.3, 1.

Krell V, Jakobs-Schoenwandt D, Vidal S, Patel AV (2018) Cellulase enhances endophytism of encapsulated *Metarhizium brunneum* in potato plants. Fungal Biol 122:373-378. https://doi.org/10.1016/j.funbio.2018.03.002

Latgé J, Silvie P, Papierok B, Remaudière G, Dedryver C, Rabasse J (1983) Advantages and disadvantages of *Conidiobolus obscurus* and of *Erynia neoaphidis* in the biological control of aphids. Aphid Antagonists, AA Balkema, Rotterdam:20-32

Lenth R, Singmann H, Love J, Buerkner P, Herve M (2018) Emmeans: Estimated marginal means, aka least-squares means. R package version 1(1):3.

Li W, Xu WA, Sheng CF, Wang HT, Xuan WJ (2006) Factors affecting sporulation and germination of *Pandora nouryi* (Entomophthorales: Entomophthoraecae), a pathogen of *Myzus persicae* (Homoptera : Aphididae). Biocontrol Sci Technol 16:647-652. https://doi.org/10.1080/09583150500532840

Muskat LC, Kerkhoff Y, Humbert P, Nattkemper TW, Eilenberg J, Patel AV (2021) Image analysis-based quantification of fungal sporulation by automatic conidia counting and gray value correlation. MethodsX 8:101218. https://doi.org/10.1016/j.mex.2021.101218

Müther J, Vogt H (2003) Sampling methods in orchard trials: a comparison between beating and inventory sampling. IOBC/WPRS Bulletin 26:67–72

Nielsen C, Eilenberg J, Harding S, Oddsdottir E, Haldorsson G (2001) Geographical distribution and host range of Entomophthorales infecting the green spruce aphid *Elatobium abietinum* Walker in Iceland. J Invertebr Pathol 78:72-80. https://doi.org/10.1006/jipa.2001.5045

Olsen NL, Herren P, Markussen B, Jensen AB, Eilenberg J (2019) Statistical modelling of conidial discharge of entomophthoralean fungi using a newly discovered *Pandora* species. PloS One 14(5):e0215914. https://doi.org/10.1371/journal.pone.0215914

Pell JK, Eilenberg J, Hajek AE, Steinkraus DC (2001) Biology, ecology and pest management potential of Entomophthorales. In Butt TM, Jackson C and Magan N (ed.) Fungi as biocontrol agents: progress, problems and potential, CABI: 71-154.

Pohlert T (2020) PMCMRplus: Calculate Pairwise Multiple Comparisons of Mean Rank Sums Extended. R package version 1.4. 4

Przyklenk M, Vemmer M, Hanitzsch M, Patel A (2017) A bioencapsulation and drying method increases shelf life and efficacy of *Metarhizium brunneum* conidia. J Microencapsul 34:498-512. https://doi.org/10.1080/02652048.2017.1354941

Seemüller E, Schneider B (2004) '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri' and '*Candidatus* Phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. J Syst Evol Microbiol 54:1217–1226. https://doi.org/10.1099/ijs.0.02823-0

Seemüller E, Carraro L, Jarausch W, Schneider B (2011) Apple proliferation phytoplasma. In Virus and virus-like diseases of pome and stones fruits. The American Phytopathological Society, St. Paul, Minnesota, USA:67-76. https://doi.org/10.1094/9780890545010.014

Shah PA, Aebi M, Tuor U (1998) Method to immobilize the aphid-pathogenic fungus *Erynia neoaphidis* in an alginate matrix for biocontrol. Appl Environ Microbiol 64(11):4260-4263. https://doi.org/10.1128/AEM.64.11.4260-4263.1998

Shah PA, Aebi M, Tuor U (1999) Production factors involved in the formulation of *Erynia neoaphidis* as alginate granules. Biocontrol Sci Technol 9:19-28. https://doi.org/10.1080/09583159929875

Shah P, Pell J (2003) Entomopathogenic fungi as biological control agents. Appl Microbiol Biotechnol 61:413-423. https://doi.org/10.1007/s00253-003-1240-8

Strauss E (2009) Phytoplasma research begins to bloom. Science 325:388-390. https://doi.org/10.1126/science.325_388

Therneau T, Lumley T (2009) Survival: Survival analysis, including penalised likelihood. R package version, 2(6). R Found. Stat. Comput.

Vega FE, Goettel MS, Blackwell M, Chandler D, Jackson MA, Keller S, Koike M, Maniania NK, Monzon A, Ownley BH, Pell JK, Rangel DEN and Roy HE (2009) Fungal entomopathogens: new insights on their ecology. Fungal Ecol 2:149-159. https://doi.org/10.1016/j.funeco.2009.05.001

Vemmer M, Schumann M, Beitzen-Heineke W, French BW, Vidal S, Patel AV (2016) Development of a CO2 -releasing coformulation based on starch, *Saccharomyces cerevisiae* and *Beauveria bassiana* attractive towards western corn rootworm larvae. Pest Manag Sci 72:2136-2145. https://doi.org/10.1002/ps.4245

Weintraub PG, Beanland L (2006) Insect vectors of phytoplasmas. Annu Rev Entomol 51:91-111. https://doi.org/10.1146/annurev.ento.51.110104.151039

Wickham H, Chang W, Henry L, Pedersen TL, Takahashi K, Wilke C, Woo K, Yutani H (2019) ggplot2: Elegant Graphics for Data Analysis. RStudio. New York.

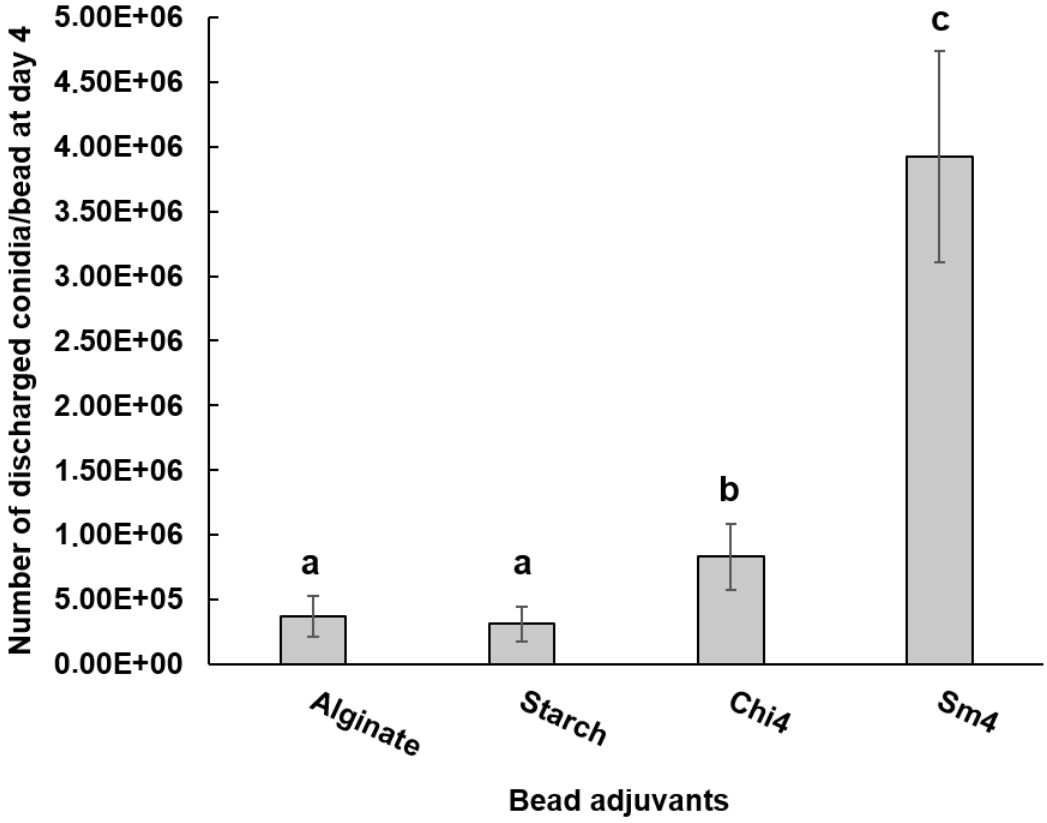
Wilding N, Latteur G (1987) The Entomophthorales - problems relative to their mass production and their utilisation. Meded. Fak. Landbouwwet. R. U. Gent 52:159-164

Wraight S, Jackson M, De Kock S (2001) Production, stabilization and formulation of fungal biocontrol agents. In Butt TM, Jackson C and Magan N (ed) Fungi as biocontrol agents: progress, problems and potential, CABI:253-287

Wraight SP, Galaini-Wraight S, Carruthers RI, Roberts DW (2003) *Zoophthora radicans* (Zygomycetes: Entomophthorales) conidia production from naturally infected *Empoasca kraemeri* and dry-formulated mycelium under laboratory and field conditions. Biol Control 28:60-77.

Zhou X, Su X, Liu H (2016) A floatable formulation and laboratory bioassay of *Pandora delphacis* (Entomophthoromycota: Entomophthorales) for the control of rice pest *Nilaparvata lugens* Stal (Hemiptera: Delphacidae). Pest Manag Sci 72:150-154.

Supplementary



Supplementary figure 5.1. Conidial discharge by *Pandora* sp. nov. from beads containing different formulation additives at day 4 after bead preparation. All bead matrices based on 1.5% sodium alginate cross-linked in calcium chloride solution (0.1 M) and contain 100 g . L-1 *Pandora* sp. nov. biomass from shaking culture. Alginate = no additional additives; Starch = native maize starch (100 g . L-1); Chi4 = chitin from crab shells (ground; <400 µm; 40 g . L-1); Sm4 = skimmed milk (40 g . L-1). Different letters above bars indicate significant differences based on one-way ANOVA and Tukey post hoc test at $p < 0.05$ (means ± SD, n = 5).

Infection bioassay 1

Supplementary table 1. Infection bioassay 1. Cumulative mortality (± SE) [%] and median survival time [d] of pear psyllid *C. pyri* and summer apple psyllid *C. picta* treated without (Control) or with sodium alginate beads (Blank), 10% *Pandora* sp. nov. biomass and skimmed milk (Sm4) or 10% *Pandora* sp. nov. biomass and skimmed milk+chitin (Sm2Chi2) (10 dpi) are given. Furthermore, the influence of treatment or psyllid species as well as their interaction are described. Estimated marginal means (Emmean) for mean survival time (± SE) [d] and corresponding confidence intervals (CI) from the model are shown for significant factors.

		Kaplan-Meier-Estimates		**Parametric survival regression model[§§§]**		
Treatment	**Species**	**Cumulative mortality ± SE [%]**	**Median survival time [d]**	**Emmean ± SE**		**Lower - upper CI**
Control	*C. pyri*	27.1 ± 6.4	NA[§]	13.0 ± 1.7	ab[§§]	10.1 - 16.7
	C. picta	31.9 ± 7.1	NA	19.1 ± 2.6	cd	14.6 - 24.9
Blank	*C. pyri*	29.3 ± 6.6	NA	14.1 ± 1.9	ac	10.8 - 18.3
	C. picta	20.8 ± 5.9	NA	20.7 ± 2.9	bd	15.7 - 27.4
Sm4	*C. pyri*	75.0 ± 6.3	6	6.5 ± 0.7	e	5.3 - 7.9
	C. picta	48.3 ± 7.3	NA	9.5 ± 1.0	af	7.7 - 11.7
Sm2Chi2	*C. pyri*	58.3 ± 7.1	7	7.3 ± 0.8	ef	5.9 - 9.1
	C. picta	56.2 ± 7.2	10	10.8 ± 1.1	a	8.8 - 13.3

[§] Not available (NA) since cumulative mortality did not reach 50% within a period of 10 days.
[§§] Estimated marginal means followed by the same letter are not significantly different (P > 0.05).
[§§§] A parametric model with loglogistic distribution was used to analyze the effects of main factors and interactions on the mean survival of insects. Model statistics are presented for parametric model simplified by removing non-significant factors due to AIC.

Infection bioassay 2

Supplementary table 2. Infection bioassay 2. Cumulative mortality (± SE) [%] and median survival time [d] of pear psyllid *C. pyri* treated without (Control) or with beads containing 0% (Blank), 10%, 20% or 40% *Pandora* sp. nov. biomass (10 dpi) are given. Estimated marginal means (emmean) for mean time (± SE) [d] and corresponding confidence intervals (CI) from the model are shown for significant factors.

	Kaplan-Meier-Estimates		**Parametric survival regression model[§§§]**		
Treatment	**Cumulative mortality ± SE [%]**	**Median survival time [d]**	**emmean ± SE**		**lower - upper CI**
Control	36.7 ± 8.8	NA[§]	11.9 ± 1.6	a[§§]	9.1 - 15.6
Blank	41.6 ± 9.2	NA	11.2 ± 1.5	ab	8.6 - 14.6
Pandora10	71.4 ± 8.4	5.5	6.4 ± 0.8	c	5.1 - 8.2
Pandora20	89.4 ± 5.8	5	5.3 ± 0.6	c	4.3 - 6.6
Pandora40	75.5 ± 8.0	8	7.0 ± 0.8	bc	5.5 - 8.8

[§] Not available (NA) since cumulative mortality did not reach 50% within a period of 10 days.
[§§] Estimated marginal means followed by the same letter are not significantly different (P > 0.05).
[§§§] A parametric model with loglogistic distribution was used to analyze the effects of main factors and interactions on the mean survival of insects. Model statistics are presented for parametric model simplified by removing non-significant factors due to AIC.

Chapter 6

Sporulation of *Pandora* sp. nov. inedit (ARSEF 13372) under non-saturated humidity and field conditions by co-application with biobased superabsorbents

Linda C. Muskat[1,2], Britta Kais[3], Jürgen Gross[3], Jørgen Eilenberg[4], Anant V. Patel[1]

[1]Bielefeld University of Applied Sciences, Faculty of Engineering and Mathematics, WG Fermentation and Formulation of Biologicals and Chemicals; [2]Georg-August-University Goettingen, Agricultural Entomology, Department of Crop Sciences; [3]Institute for Plant Protection in Fruit Crops and Viticulture, Julius Kühn-Institut, Federal Research Institute for Cultivated Plants; [4]Department of Plant and Environmental Sciences, University of Copenhagen

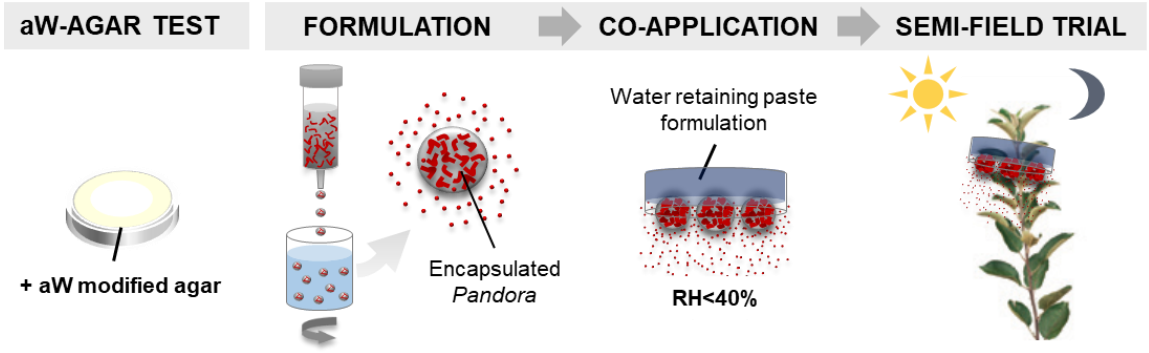
Manuscript prepared for submission

Abstract

Entomophthoralean fungi have high potential for insect pest control due to their high host specificity and their fast speed-to-kill. However, due to low efficacy of hitherto existing formulations in field trials, no preparation based on these fungi has been established for practical use so far. Previous studies have shown that low water availability is the main limiting factor of conidial discharge and germination. In the present study, sporulation of the recently discovered psyllid-pathogenic entomophthoralean fungus *Pandora* sp. nov. inedit. (ARSEF 13372) was not observable under reduced water activity values ($a_W \leq 0.97$). In order to support sporulation of encapsulated *Pandora* sp. under low humidity conditions in above-ground applications in field, a paste-type formulation containing bio-based superabsorbents was developed, which retained water for a prolonged time period. In co-application with the superabsorbent formulation, the otherwise fast-drying capsules were kept sufficiently moist for sporulation for at least 6 days in laboratory trials at low humidity of 30-40%. Using the new formulation, conidial discharge was actually observed under very dry semi-field conditions in summertime, at which trapped conidia numbers correlated with the distance from sporulating capsules, and were ascertained up to 40 cm distance to the sporulation source. Since water availability is a limiting factor for many fungal biocontrol agents, the developed formulation has the potential to improve their efficacy.

Keywords: Entomophthorales, Encapsulation, Biological control, Entomopathogenic fungi, Virulence, Psyllid control, Water activity

Graphical abstract



Introduction

Entomophthoralean fungi have a high potential for insect pest control due to their high host specificity, their fast speed-to-kill and the ability to cause epizootics (Eilenberg and Michelsen 1999; Keller 2007; Pell et al., 2001; Jaques and Patterson 1962; Vega et al., 2012; Eilenberg et al., 2019). Despite their potential, no Entomophthorales-based bioinsecticide has been established and commercialized for practical use so far, due to low efficacy in field trials. Previous studies have shown that dissatisfactory water availability of to date existing formulations under field conditions is the main limiting factor of conidial discharge and germination of a plenty of entomophthoralean species and the main important reason for failure in the field (Delalibera Jr et al., 2006; Hajek et al., 1990). Since conidia are the infective units of these fungi, efficient sporulation is obligatory for successful application of an entomophthoralean fungus in pest control strategies. For some Entomophthorales, germination only occurs in presence of free water (Shimazu, 1977, Hajek et al., 1990) or at least under humidity conditions between 90 and 100% RH (Hall and Papierok, 1982; Glare et al., 1986; Newman and Carner, 1975).

A new entomophthoralean fungus with potential for the control of psyllids, *Pandora* sp. nov. inedit (ARSEF 13372), was isolated from an infected psyllid collected in a Danish pear orchard by Jensen et al. (2018) and is currently under description as a new species. In previous studies, the pathogenicity of *Pandora* sp. nov. has already been demonstrated for several psyllid species such as *Cacopsylla pyri*, *C. pyricola*, *C. picta*, *C. mali*, *C. pruni* and *Trioza apicalis* under laboratory conditions (Jensen 2017; Jensen et al., 2018; Herren, 2018; Görg et al., 2021). As the mass-production of fungal biomass is essential for providing inoculant in a sufficient quantity for large scale field application (Shah and Pell, 2003; Jaronski, 2014), a suitable fermentation medium and process with potential for mass-production of *Pandora* sp. was established by Muskat et al. (2022a). The conversion of *Pandora* into an easily applicable form was realized by encapsulation of hyphal biomass in Ca-alginate capsules additionally providing nutrients for improved sporulation capacity. With this formulation, the two target psyllid species *C. picta* and *C. pyri* were successfully infected by *Pandora* sp. and killed with a mortality up to 89% (Muskat et al., 2022b). Nonetheless, experiments were carried out under optimal humidity conditions in the laboratory. The actual moisture conditions required for growth and sporulation of the new species *Pandora* sp. nov. inedit. (ARSEF 13372) are not known, but suggested to be very high, as for other entomophthoralean species. The overall aim of this study was the development and evaluation of a formulation that compensates for the specific humidity requirements of the encapsulated

entomopathogenic fungus *Pandora* sp. nov. (ARSEF13372) and supports sporulation under low humidity conditions as faced in above-ground applications.

The specific objectives of this study were (1) to identify the water activity values necessary for growth and sporulation of *Pandora* sp. nov., (2) to demonstrate a formulation that maintains a sufficiently high water activity under non-saturated humidity conditions that allows sporulation of *Pandora* sp. nov. (3) to validate the potential of the formulation for improved sporulation under semi-field conditions and (4) evaluate the distance of conidial distribution in a semi-field trial.

Materials & Methods

Chemicals

Chemicals used in this study were acquired either from Carl Roth GmbH (Karlsruhe, Germany) or VWR International GmbH (Darmstadt, Germany). Carboxymethylcellulose was purchased from Dow (Dow Chemical Company, Midland, MI, USA) and Xanthan gum from DuPont (Du Pont de Nemours GmbH, Neu-Isenburg, Germany). Concentrations are given as (w/w), unless otherwise stated.

Fungal isolate

The *Pandora* isolate used in this study originated from an infected *Cacopsylla pyri* specimen collected in a Danish pear orchard (55°50'24.3"N 12°33'46.5"E) by Jensen (2017) and was named KVL 16-44. The strain is deposited in the USDA Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (Ithaca, NY) as ARSEF 13372. The fungus is under description (inedit.) and will here be referred to as *Pandora* sp. nov.

Cultivation of *Pandora* sp. nov.

Pandora sp. nov. was grown on solid medium adapted from Hajek et al. (2012) composed of 4.0% glucose, 2.0% casein and 2.0% agar (Saboraud Dextrose Agar (SDA)) supplemented with 20% of a mixture of 60% egg yolk and 40% fresh skimmed milk (SDAME) on Petri dishes (diameter 90 mm) sealed with Parafilm® and incubated at 18 °C in the dark. To prevent the loss of virulence, the fungus was frequently transferred through the host insect *C. pyri*.

Submerged cultures of *Pandora* sp. nov. were grown following Muskat et al. (2022a) in 100 ml liquid medium in 250 ml shake flasks with four baffles. Briefly, the pre-culture was composed of fresh skimmed milk (10%; w/w), in which the fungus grew for 48 hours at 18 °C and 170 rpm and an amplitude of 20 mm (IKA KS 4000 ic control, Staufen, Germany). The main culture, which was inoculated by 10% (v/v) of the pre-culture and was composed of glucose (26.6 g · L^{-1}), sodium chloride (NaCl; 10 g · L^{-1}), yeast extract (3.33 g · L^{-1}), skimmed milk powder (HEIRLER CENOVIS GMBH, Radolfzell, Germany; 3.33 g · L^{-1}) and a low-cost protein hydrolysate from animal by-products (ANiPept, ANiMOX GmbH, Berlin, Germany, batch No. 1176; 3.33 g · L^{-1}), in which the culture was grown for further 48 h at the same conditions as the pre-culture. All media components were dissolved in ultrapure water and separately autoclaved for 6 min at 121 °C and 2 bar.

Preparation of capsules

All experimental steps were carried out under sterile conditions and all solutions and components were autoclaved for 6 min at 121 °C and 2 bar, unless otherwise stated. Composition and preparation of capsules were adapted from Muskat et al. (2022b). Briefly, finely dispersed hyphae of *Pandora* sp. nov.

were collected from liquid culture by centrifugation (4700 g; 15 min; 18 °C). Sodium alginate was dissolved in ultrapure water to a final concentration of 3.0% and autoclaved for 6 min at 121 °C. The encapsulation suspension was composed of sodium alginate (Manugel GMB, FMC Corporation, PA, USA, batch No. G7708901; 1.5%, w/w), heat-sterilized native corn starch (Maisita, Agrana Beteiligungs-AG, Vienna, Austria; 10%) pre-dissolved skimmed milk powder (4%, w/w) and hyphal material of *Pandora* sp. nov. (10%). For capsule formation, the solution was dripped into a stirred (250 rpm) calcium chloride solution (0.1 M) by using a syringe with a cannula (diameter 2.1 × 0.8 mm, Sterican, B. Braun AG, Melsungen, Germany). Capsules were kept in the solution under stirring for 20 min and were subsequently washed with ultrapure water for 1 min.

Preparation of the water retaining paste formulation

Candelilla wax (CLW; Kahlwax GmbH & Co. KG, Trittau, Germany; batch code: 10-1185; 1.5% w/w) was mixed with sesame oil (from the local organic supermarket) and maintained under stirring at 100 °C for 20 min for sterilization. Carboxymethylcellulose (WALOCEL CRT 60000 GA 07, Dow Chemicals, Batch code: F294H89011, MW: 60000; CMC) was heat-sterilized at 100 °C overnight in an oven. CMC was added to the hot wax oil mixture at a ratio of 1:1 and dispersed by a spatula while cooling the mixture to room temperature for solidification. Xanthan gum (GRINDSTED® Xanthan 80, A45100, Du Pont, Neu-Isenburg, Germany; batch code: 4453438167; 2%; w/w) was dispersed in ultra-pure water and autoclaved at 121 °C and 2 bar for 21 min. 0.5 g of the oil-wax-CMC mixture was spread over the bottom of a Petri dish (Ø = 35 mm) by aid of a spatula and overlayered by 7.5 g of the xanthan gum gel. The Petri dishes were closed with the Petri dish lid, sealed with Parafilm® and maintained at 5 °C in the fridge for at least 24 h and stored there until use.

Determination of water activity

The water activity (a_W value) of the modified media was determined using a water activity meter (LabMASTER-aw, Novasina AG, Lachen, Switzerland) at 25 °C.

Effect of water activity on mycelial growth and sporulation of *Pandora* sp. nov.

In order to examine the water activity demands of *Pandora* sp. nov. for growth and sporulation, a basic solid culture medium, composed of 4.0% glucose, 2.0% casein and 2.0% agar (Saboraud Dextrose Agar (SDA)) supplemented with 10% fresh skimmed milk (SDAM) or water agar (2%; w/w) was modified by addition of glycerol following the method of Hallsworth & Magan (1999). Therefore, 0%/10%/20%/30% (w/w) of the water content of the medium was replaced by glycerol. The a_W value of the medium and the water agar was 0.98/0.99 (0%), 0.96/0.97 (10%), 0.93/0.95 (20%), 0.89/0.91 (30%) (medium/water agar). To determine the effect of water activity on mycelial growth, pieces of mycelium (0.5 cm^2) were placed in the middle of the culture medium (SDAM). Radial mycelium growth was determined daily in two perpendicular directions.

For determination of the water activity effect on sporulation, one capsule per dish, prepared as described above, was placed in the center of glycerol modified water agar plates. The discharged conidia were collected according to the method described in Muskat et al. (2022b). Briefly, the plates with the capsules were invented and placed above a smaller Petri dish (10 mm) filled with 3 ml SDS (0.5%) and incubated at 18 °C for in the darkness for 12 days, as sporulation of *Pandora* sp. nov. is then almost complete (Muskat et al., 2022b). Conidia were quantified in a Fuchs-Rosenthal hemocytometer with 6 replicates per sample.

Sporulation experiments under non-saturated humidity conditions in the laboratory

For determination of sporulation, one capsule per dish was placed on the surface in the center of each paste filled in the Petri dishes. Capsules without the paste were fixed with a drop of pure xanthan gel (2%) on the Petri dish bottom. The dishes were fixed at a height of 3 cm above Petri dishes (Ø = 35 mm) filled with SDS for collection of conidia (**Figure 6.1**). The formulations were incubated at 30-40% room humidity and 21-24 °C for 12 days. Room humidity and temperature was recorded with a datalogger (EBI 20-TH 1, Ebro Electronic™, Xylem Analytics Germany Sales GmbH & Co. KG, Weilheim, Germany) throughout the experiment. The SDS was replaced every 24 h. The collected conidia were quantified in a Fuchs-Rosenthal hemocytometer with 6 replicates per sample.

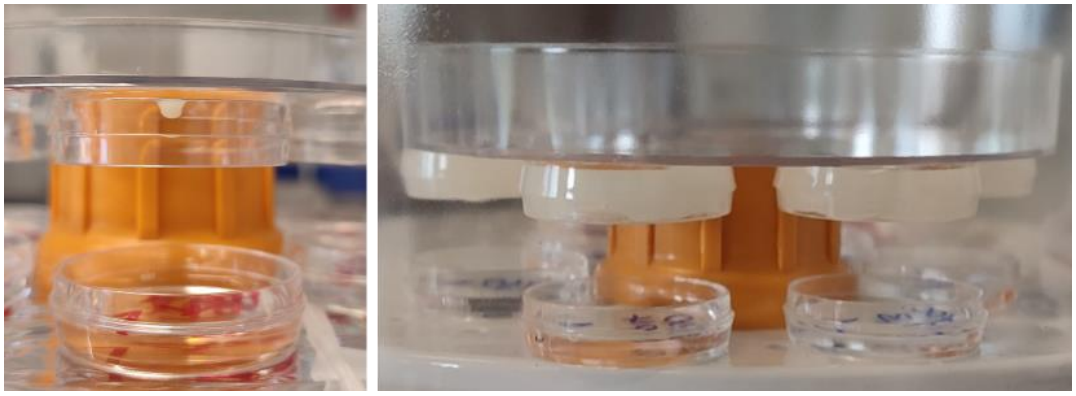


Figure 6.1. Experimental set-up of the sporulation experiment under non-saturated humidity conditions. *Pandora* capsules were fixed in Petri dishes without (left) or with the paste formulation (right) above a SDS solution for conidia collection and were incubated at 30 – 40% room humidity and 22 – 24 °C for 12 days.

Semi-field sporulation trial

For testing the effect of the superabsorbent paste on fungal sporulation under field conditions, semi-field trials have been conducted. Two 175 x 175 x 175 cm net cages (Aerarium Nets, Bern, Switzerland) with sun and rain shelters were built on experimental field at the Julius Kühn-Institut (Dossenheim, Germany). One potted pear tree (*Pyrus communis* L. cv. 'Williams Christ' grown on cv. *P. communis* 'Kirchensaller Mostbirne' rootstock) was placed in each net cage. Before starting the experiment, the capsules were pre-incubated on the water retaining paste formulation in Petri dishes (Ø = 35 mm) for three days. The dishes containing the paste formulation and the pre-incubated capsules were fixed to the top of the potted pear tree with the dish opening facing downwards. Five petri dishes were fixed to each tree, considering in each case the different cardinal directions. To determine sporulation, glass slides (Thermo Scientific, Braunschweig, Germany) were placed at regular intervals of 5, 20, and 40 cm from each sporulation source (Fig. 6.5A). The experiment was conducted in warm September 2020. Humidity and temperature were recorded with data loggers (DS1923-F5, Hygrochron Temp/Luftfeuchte, Elektronik Fuchs, Weingarten, Germany) throughout the experiment. After 72 hours, the glass slides were recollected. The conidia attached to slides were scanned and counted with a digital microscope (VHX 7000, Keyence ® Deutschland, Neu-Isenburg, Germany).

Statistical analysis

Statistical analysis of mycelial growth and sporulation of *Pandora* sp. nov. was carried out using the software SPSS Statistics V25.0 (SPSS, Chicago, IL). All data are given as mean values ± standard deviations (SD). Data for conidial discharge were checked for normality and homogeneity of variance using Shapiro–Wilk and Levene test. Mean numbers of discharged conidia were tested for significant

differences by one-way ANOVA followed by a Tukey post hoc range test and Bonferroni correction. If the criteria for variance homogeny and normal distribution were not met, data have been calculated by nonparametric Kruskal-Wallis test followed by Bonferroni for multiple comparisons with one treatment. The number of discharged conidia over time was compared with a repeated measures ANOVA, with time and treatment as independent variables. The sphaericity of the matrix assumption was assessed with the Mauchly sphericity test. If the outcome of the test was significant, the Greenhouse–Geisser adjustment was used to correct for violations of sphericity. The level of significance was set to $p < 0.05$. All experiments were carried out with at least 5 replicates.

Results

Effect of water activity on mycelial growth and sporulation of *Pandora* sp. nov.

Water activity (a_W) of the medium had a significant effect on growth speed of *Pandora* sp. nov. over time ($F_{5,15}$=303,13; p<0.001; *Greenhouse-Geisser*), as shown in **Figure 6.2**. Water activity itself had a significant effect on mycelial growth ($F_{3,76}$ = 933,046; p<0.001; *Bonferroni*). Mycelial growth was slower at a_W=0.96 compared to a_W=0.98, and totally suppressed at a_W≤0.93.

As shown in **Figure 6.3A**, also sporulation from capsules was significantly affected by water activity (Chi^2 = 102,790; df = 3; p<0.05; Bonferroni). At an a_W value of 0.99, 4.36 x 10^6 (±9.35 x 10^5; n=5) conidia were discharged per capsule during 12 days experimental time, which was significantly more than at all other water activities (p<0.001). Under these saturated conditions, sporulation and growth of *Pandora* sp. nov. from the capsules was clearly visible (**Figure 6.3B**). At a marginally reduced water activity of 0.97, only a few and less than 1.25 x 10^4 (±2.4 x 10^4; n=5) conidia per capsule were discharged, but mycelial growth from the capsules was observable (**Figure 6.3C**). At water activity values ≤0.95, neither conidial discharge (**Figure 6.3A**, nor mycelial growth was noticeable (**Figure 6.3D**).

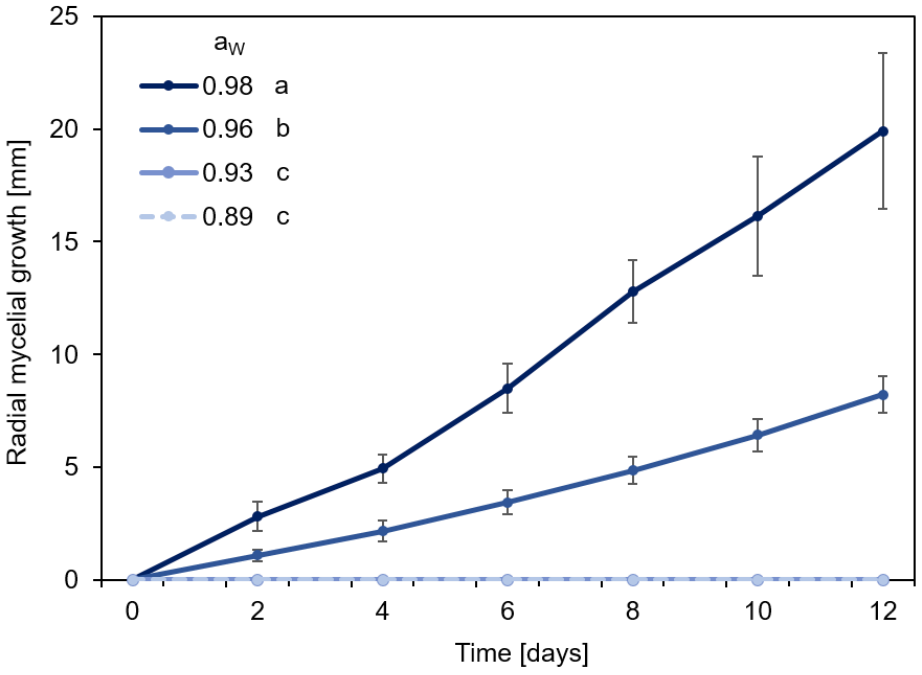


Figure 6.2. Effect of water activity on radial mycelial growth during 12 days. Different letters in the legend indicate significant differences according to RM-ANOVA followed by a Greenhouse-Geisser post-hoc test at $p < 0.05$. Means ± SD, n = 5.

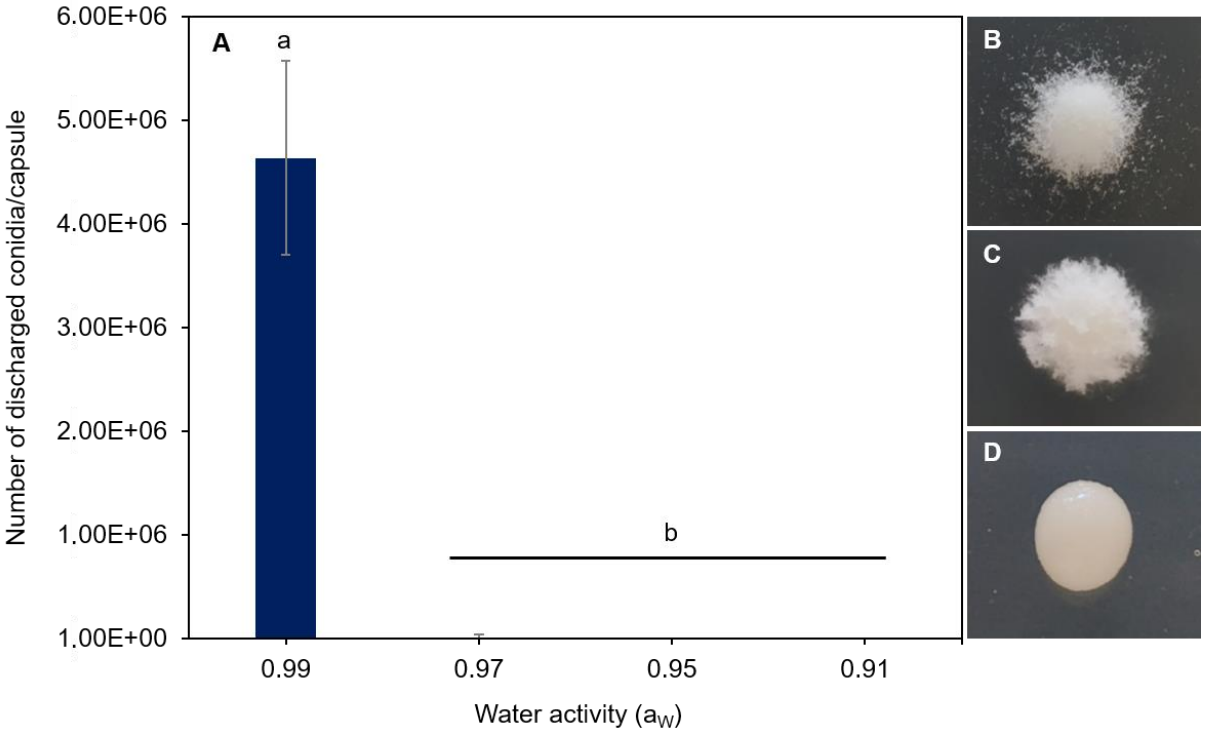


Figure 6.3 Effect of water activity (a_W) on total conidia numbers released by encapsulated *Pandora* sp. nov. during 12 days of incubation. Pictures of the capsules after 4 days of incubation at aW 0.99 (B), aW 0.97 (C) and aW ≤ 0.95 (D). Different letters above bars indicate significant differences according to Kruskall-Wallis followed by Bonferroni post-hoc test ($p < 0.05$). Means ± SD, n = 5.

Sporulation of *Pandora* sp. nov. under non-saturated humidity conditions

Co-application of the capsules with the paste formulation supported growth and sporulation of encapsulated *Pandora* sp. nov. under non-saturated humidity conditions in the laboratory for 7 days at a room humidity between 30 and 40 % (**Figure 6.4**). Compared to raw capsules, which dried under the present conditions and did not release any conidia, significantly more conidia were discharged by encapsulated *Pandora* sp. nov. when co-applied with the paste formulation ($F_{1,58}$ = 734,951; $p<0.001$; *Games-Howell*).

A peak sporulation event was observable at day 4 with the highest conidial numbers of 6.96×10^5 ($\pm 3.08 \times 10^5$) conidia/capsule on day 4. The total number of conidia released from the capsules during the 7 days within sporulation was detectable was 1.79×10^7 ($\pm 3.21 \times 10^6$).

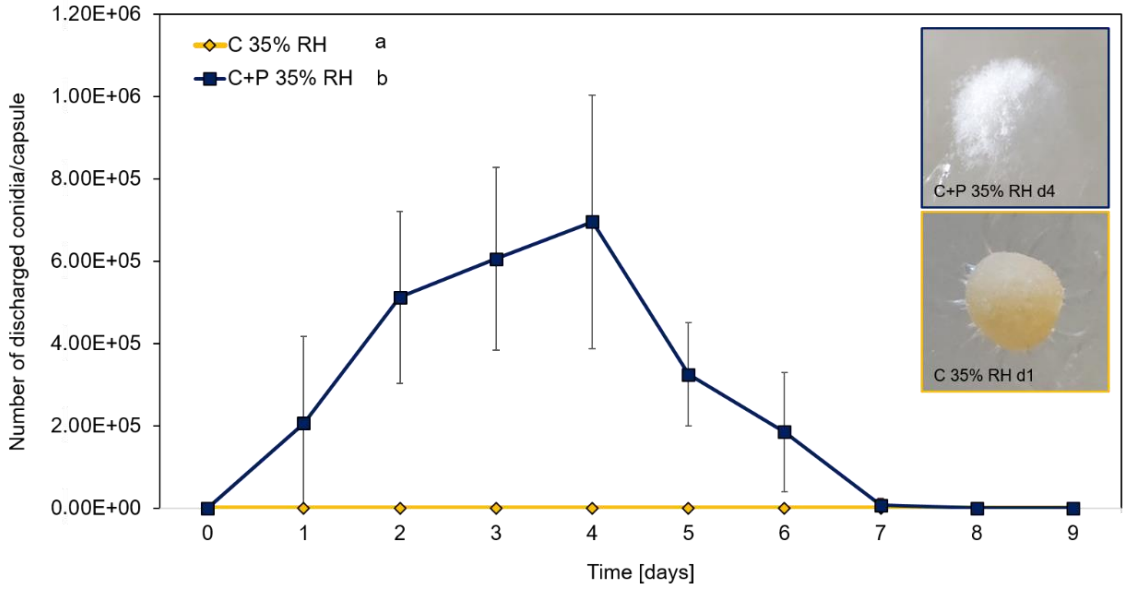


Figure 6.4 Sporulation of *Pandora* sp. nov. from capsules (C) and from capsules co-applied with a biobased superabsorbent past-type formulation (C+P) under non-saturated humidity conditions (30–40% RH) within 9 days. Different letters in the legend indicate significant differences according to RM-ANOVA and Games-Howell correction at $p < 0.01$. Means ± SD, n = 5.

Sporulation under semi-field conditions

In the semi-field trial conducted in September 2020, capsules co-applied with the water retaining paste formulation were fixed at the top of pear trees. Mean temperature during the experiment was 19.5 °C (min 11 °C; max 28 °C) and mean humidity was 64% (min 32%; max 100%).

Pandora sp. nov. was able to sporulate under these application conditions. Sporulation was determined by collection of conidia on glass slides fixed at 5, 20 and 40 cm distance from the sporulation source. Most conidia of *Pandora* sp. nov. were collected at the lowest distance, but conidia were observable on all glass slides. The results from two tents are shown in **Figure 6.5B**. At the lowest distance of 5 cm, 2730 (±566) conidia per cm^2 were counted. At 20 cm distance 362 (±85) conidia per cm^2 were found and at 40 cm distance 90 (±16) condia/cm^2 were counted.

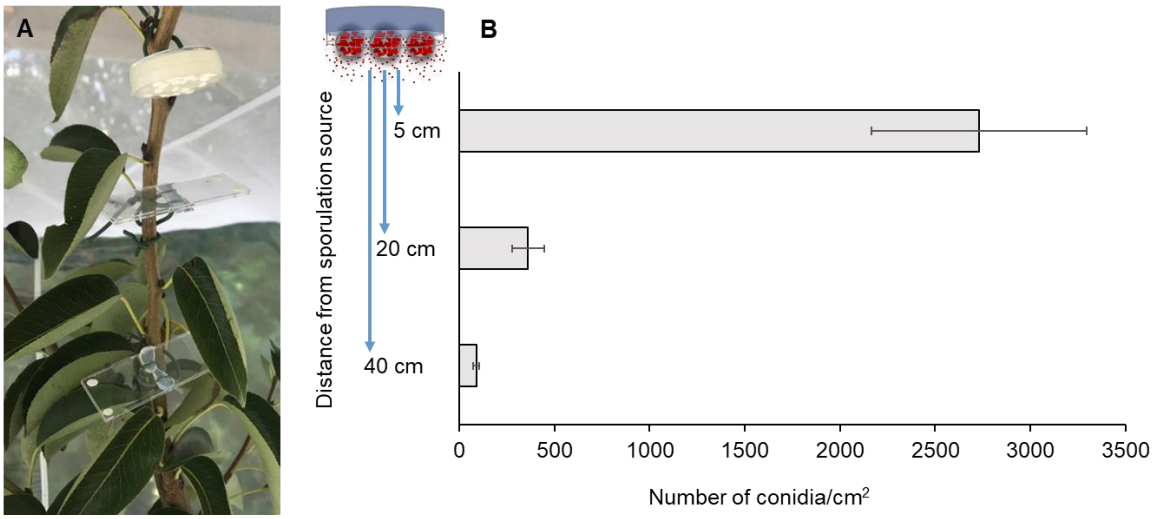


Figure 6.5 A: Experimental set-up of the semi-field trial. The Petri dish containing 20 *Pandora* capsules and the water retaining paste formulation were fixed at the top of a pear tree. Glass slides were fixed below at different distances to the Petri dish for conidia collection. **B:** Number of conidia collected on the glass slides fixed at different distances from the Petri dish containing the paste and the *Pandora* capsules as sporulation source during 72 h. Mean of two different tents + SD. n = 2.

Discussion

Water activity (a_w) effect on growth and sporulation

Several studies on entomophthoralean sporulation have reported that sporulation is associated with high humidity conditions and often occurs during night time when relative humidity (RH) and leaf wetness is high enough (Hemmati et al., 2001 a; b; 2002; Milner & Bourne, 1982; Yu et al., 1995; Nielsen & Hajek, 2006). To be more precise, most entomophthoralean species have shown conidial discharge only when RH was higher than 90% (Glare et al., 1986). The individual humidity needed for growth and sporulation depends on the entomophthoralean genus and species: sporulation by *Entomophthora aphidis* and *E. thaxteriana* is only possible at >90% RH (Wilding, 1969), by *Neozygites tanajoae* at >96% RH (Delalibera Jr et al., 2006) and for *Erynia* sp. (=*Pandora* sp.) at >91% RH (Millstein et al., 1982). Only a few Entomophthorales were found to be capable to sporulate at humidities below 90%, such as *Entomophaga maimaiga* (Hajek et al., 1990) and *Entomophthora muscae* (Kramer, 1980). In general, also in these species, lower humidity conditions lead to depressions in growth, sporulation, and germination of spores or formation of appressoria (Magalhães et al., 1991). However, the specific water activity (a_w) value needed for growth and sporulation of *Pandora* sp. nov. inedit. (ARSEF 13372) was not yet known. To the best of our knowledge, the effect of reduced water activity on mycelial growth on solid media and on sporulation from Ca-alginate capsules had not been reported for any *Pandora* species, and definitely not for the psyllid-pathogenic species used in the present study.

In order to compare the results from the listed studies with those from the present one, where the water activity value was investigated instead of RH, the comparability needs to be explained: the definition of a_w is $a_w = p_{sample}/p_{water}$, where p_{sample} is the partial vapor pressure in equilibrium with the tested material or solution and p_{water} is the partial vapor pressure of pure water at the same temperature. In the present study, glycerol was selected as a_w modifier, as it is known for its a_w stability under different temperatures (Hallsworth & Magan, 1999). The relative humidity of air in equilibrium with a material or solution is also called the Equilibrium Relative Humidity (ERH) expressed in %. It is equal to water activity according to ERH = a_w x 100%. Therefore, the results from studies investigating RH are comparable to the present study investigating a_w.

In the present study it was found that efficient sporulation by *Pandora* sp. nov. was only possible when a_w value was 0.99, but mycelial growth was possible under reduced a_w of 0.97 from capsules, but not observable at 0.94. Our results are similar to those made by Glare et al. (1986) who found that *Zoophthora phalloides* was able to grow from *Myzus persicae* cadavers at 98% RH, but no more at 94% and sporulation was only possible at saturated humidity conditions (100%). This can be explained by the active mode of conidial discharge of *Pandora* and other Entomophthorales. Page & Humber (1973) discussed that medium osmotic pressure of the environment directly affects the conidiophore´s and the spore's turgor pressure, which is needed, as described for the case of *Conidiobolus coronatus* and other fungi for spore discharge and germination (Inglis et al., 2001; Webster & Weber, 2007). Thus, in some species such as *Condiobolus* sp., *Zoophthora* and *Pandora*, free water is needed for the generation of a high turgor pressure in the conidiophores, which is required for the ballistic spread of the conidia (Latgé et al., 1989).

Sporulation of *Pandora* sp. nov. by co-application with the paste formulation in the laboratory

In biological control strategies, efficient sporulation of the entomopathogenic fungus is required simultaneously with target insect abundancy. Moreover, very short viability and infectivity of the discharged conidia is suggested (Yendol, 1968; Brobyn et al., 1985; 1987; Carruthers et al., 1988; Hajek et al., 1990; Uziel & Kenneth, 1991; Griggs et al., 1999). Relying on nocturnal sporulation with sufficient ambient humidity, which may be sufficient for the natural maintenance of the species, is not an option for controlling the target insect. Therefore, persistence and sustainability of sporulation is required to thoroughly provide viable conidia when the target insect is present.

Few attempts have been made addressing the humidity problem of the Entomophthorales. Pell et al. (1993) designed a trap for co-application of *Zoophthora radicans* with attracting semiochemicals in order to realize an attract-and-kill strategy for the control of *Plutella xylostella*. Sufficient moisture remained high within the trap in order to enable efficient sporulation of the fungus by a wick connected to a water reservoir placed in the central arena of the trap. Another more practical option for technical application is the use of formulated entomopathogenic fungi: Zhou et al. (2009) developed a granular broomcorn millet formulation supplemented with synthetic polyacrylate superabsorbent polymers for improved sporulation capacity of *Pandora nouryi* under non-saturated humidity conditions. Unfortunately, solid state fermentation was required and polyacryl derivatives of synthetic origin were used, which are manufactured in a toxic preparation process and are hardly biodegradable and hence are not approved for organic agriculture.

A more environmentally friendly alternative to non-biodegradable polyacryl-based materials are biobased polymers with a high water sorption capacity (Chen et al., 2022). Derivatives of starch or cellulose have gained attention in recent years for different agricultural applications, in particular for improved water capacity of soil (Demitri et al., 2013). The paste-type formulation tested in the present study containing cellulose and xanthan as polymers of high water sorption capacity is biodegradable. Moreover, these biopolymers are registered for pesticide applications even in organic agriculture (Speiser et al., 2022).

The biopolymer-based paste formulation provides a water activity value of 0.99–1.0 to the co-applied capsules, which was found to be sufficiently high for sporulation of *Pandora* sp. nov. By co-application, sporulation under non-saturated humidity conditions of 30-40% RH was enabled for at least 6 days. A peak sporulation event, typical for *Pandora* sporulation (Muskat et al., 2022b) was observable from the capsules co-applied with the paste under reduced RH.

Furthermore, the results are similar to a previous study by Muskat et al. (2022b), where a sporulation peak on day 4 was also observable for the same encapsulated fungus, where the experiments were conducted under saturated humidity conditions. Moreover, the total number of discharged conidia under non-saturated humidity conditions of 30-40% RH ($1.79x10^7$) during the 7 days of conidial discharge was as high as under saturated conditions, where $9.57x10^6$ ($\pm 8.34x10^5$) conidia/capsule were reported during the 12 days conidia were released from capsules of the same composition. Thus, the paste formulation is capable to compensate for unsuitable environmental humidity conditions by providing a satisfactory water activity to the encapsulated fungus and thereby enabling efficient sporulation.

However, the sporulation duration under non-saturated humidity conditions was shorter compared to the previous experiment, where some conidia were observable even on day 12, whereas sporulation in the present study was finished on day 7. This can be attributed to the drying of the paste. Experiments were carried out at a constant humidity regime of 30-40% RH. Under application conditions in the field, it is suggested that under alternating humidity conditions at the place of application in the field, the formulation will be rewetted during night time and thereby spend moisture for a prolonged period.

As the surface of the paste formulation provides conditions similar to free water, it serves as an optimal medium to enable germination of primary conidia. This will further enable a prolongation of the sporulation from the co-formulant of *Pandora* capsules with the paste.

In nature, even very short sporulation durations from cadavers of less than 3 days were almost sufficient to cause epizootics in insect populations (Aoki et al., 1981; Kalsbeek et al., 2001; Wraight et al., 2003; Li et al., 2006). Hence, the sporulation duration of 6 days observed even under constantly low humidity conditions (30–40% RH) should be effective for infection of the target insect and the initiation of an epizootic.

Sporulation of *Pandora* sp. nov. under semi-field conditions

The ability of an entomophthoralean fungus for sufficient sporulation simultaneously with the insect´s abundance at the place of application in the fruit tree is essential for infection. Moreover, also the distance of conidial discharge will affect the frequency and intensity of contact between the fungus and the target host insect. A wider distance of conidial discharge will encounter more insects and increase the ability to initiate epizootics (Six & Mullens, 1997). In nature, the Entomophthorales are known to induce the so called ´summit disease´ in their infected insect host, forcing the insect moving to high or exposed sites shortly before death (Roy et al., 2006; Elya et al., 2018). Due to the exposed position, the actively discharged spores spread over a large area. Those airborne conidia can further be transported by wind over long distances (Weseloh & Andreadis 1992; Dwyer et al., 1998; Hemmati et al., 2001a; b; Keller, 2007). Besides the humid microenvironment, the paste also has the benefit of a high adhesive performance and thus enables an overhead application. Thereby the formulation enabled fixation of the sporulation source at a high and exposed place in the plant, in order to simulate the natural death orientation and supports a sporulation of the fungus over a wide area by simulating the naturally occurring summit disease.

As even under very dry conditions in the laboratory, conidia were released by encapsulated *Pandora* sp. nov. due to co-application with the water retaining paste formulation, it was not surprising that sporulation was also observable in the semi-field trial under alternating humidity conditions.

Studies on the distance of conidia discharged are rare: Six & Mullens (1997) found the conidia of *Entomophthora musca* and *E. schizophorae* from *Musca domestica* cadavers are only discharged over a distance of 0 – 8.75 cm in a chamber under still air. Carruthers (1982) found a wider range up to 34 cm when fly cadavers were fixed at a height of 50 cm in the field. The observations of Carruthers (1982) are similar to the present study, as even at a distance of 40 cm from the sporulation source, conidia were found. In view of the planned application of the fungus *Pandora* sp. nov. in psyllid pest control strategies in pear, apple and other fruit trees in middle Europe, the results are promising. In commercial apple and pear plantations, the trees that needs to be protected are of a mean height of 2 – 4 m. Thus,

when the fungus is able to release its conidia over a distance of at least 40 cm, the application of only a few capsule/paste formulations should be sufficient to cause infections in target insects. Nevertheless, the lethal dose of the fungus against different psyllid species needs to be determined. To improve the effectiveness of the formulation, it should be combined with an attractant in order to lure the target insect close to the formulation (attract-and-kill strategy), as in a low distance more conidia were counted.

Conclusion

As known for many entomopathogenous fungi applied as biocontrol agents, moist conditions are essential for effective use of fungi in microbial control against insects. Thus, in this study we demonstrated that sporulation by *Pandora* sp. nov. inedit. can be enabled under non-saturated humidity conditions by co-application with a specific water absorbing biobased polymer formulation. Our findings present for the first time a strategy of using biobased polymers of huge water absorbency to enable efficient sporulation of an Entomophthorales in the field. These promising results will pave the way for further developments. What should be addressed in the future is the scale-up of the formulation, selection of an appropriate application method the efficiency of the formulation in killing target insects.

Author contributions

LCM conceptualized the experiments, designed and conducted the experiments in the laboratory, analyzed the data, visualized the graphics and wrote the manuscript, which is not yet revised. BK designed and conducted the experiments in the semi-field trial, analyzed the data and added all material and method information on the semi-field trial in the manuscript. JG conceptualized the experiments from the semi-field trial. JE edited the manuscript. AP conceptualized the experiments in the laboratory and acquired funding of the project.

References

Aoki J (1981) Pattern of conidial discharge of an *Entomophthora* species ("*grylli*" type) (Entomophthorales: Entomophthoracea) from infected cadavers of *Mamestra brassicae* L. (Lepidoptera: Noctuidae). Appl Entomol Zool 16(3):216-224

Brobyn PJ, Wilding N, Clark SJ (1985) The persistence of infectivity of conidia of the aphid pathogen *Erynia neophidis* on leaves in the field. Ann Appl Biol 107(3):365-376

Brobyn PJ, Wilding N, Clark SJ (1987) Laboratory observations on the effect of humidity on the persistence of infectivity of conidia of the aphid pathogen *Erynia neoaphidis*. Ann Appl Biol 110:579–584

Carruthers RI (1982) The Biology and Ecology of *Entomophthora muscae* in the Onion Agroecosystem." Ph.D. dissertation, Michigan State University, East Lansing, MI

Carruthers RI, Feng Z, Ramos ME, Soper RS (1988) The effect of solar radiation on the survival of *Entomophaga grylli* (Entomophthorales: Entomophthoraceae) conidia. J Invertebr Pathol 52(1):154-162

Chen J, Wu J, Raffa P, Picchioni F, Koning CE (2022) Superabsorbent Polymers: From long-established, microplastics generating systems, to sustainable, biodegradable and future proof alternatives. Prog Polym Sci 125:101475

Delalibera Jr I, Demétrio CG, Manly BF, Hajek AE (2006) Effect of relative humidity and origin of isolates of *Neozygites tanajoae* (Zygomycetes: Entomophthorales) on production of conidia from cassava green mite, *Mononychellus tanajoa* (Acari: Tetranychidae), cadavers. Biol Control 39(3):489-496

Demitri C, Scalera F, Madaghiele M, Sannino A, Maffezzoli A (2013) Potential of cellulose-based superabsorbent hydrogels as water reservoir in agriculture. Int J Polym Sci 13: 435073

Dwyer G, Elkinton JS, Hajek AE (1998) Spatial scale and the spread of a fungal pathogen of gypsy moth. Am Nat 152:485–494

Eilenberg J, Michelsen V (1999) Natural host range and prevalence of the genus *Strongwellsea* (Zygomycota: Entomophthorales) in Denmark. J Inv Pathol 73:189–198

Eilenberg J, Saussure S, Fekih IB, Jensen AB, Klingen I (2019) Factors driving susceptibility and resistance in aphids that share specialist fungal pathogens. Curr Opin Insect Sci 33:91–98

Elya C, Lok TC, Spencer QE, McCausland H, Martinez CC, Eisen M (2018) Robust manipulation of the behavior of *Drosophila melanogaster* by a fungal pathogen in the laboratory. eLife 7:e34414

Glare TR, Milner RJ, Chilvers GA (1986) The effect of environmental factors on the production, discharge, and germination of primary conidia of *Zoophthora phalloides* Batko. J Invertebr Pathol 48(3):275-283

Görg L, Eilenberg J, Jensen AB, Jensen AH, Gross J (2021) Pathogenicity against hemipteran vector insects of a novel insect pathogenic fungus from Entomophthorales (*Pandora* sp. nov. inedit.) with potential for biological control. J Invertebr Pathol 183(3):107621

Griggs MH, Vandenberg JD, Sawyer AJ (1999) Effect of relative humidity on viability of primary conidia of *Zoophthora radicans*. J Invertebr Pathol 73:315–320

Hajek AE, Carruthers RI, Soper RS (1990) Temperature and moisture relations of sporulation and germination by *Entomophaga maimaiga* (Zygomycetes: Entomophthoraceae), a fungal pathogen of *Lymantria dispar* (Lepidoptera: Lymantriidae). Environ Entomol 19(1):85-90

Hajek AE, Papierok B, Eilenberg J (2012) Methods for study of the Entomophthorales. In: Lacey LA (ed) Manual of techniques in invertebrate pathology, 2nd Edition, Academic Press, Amsterdam, pp 285-316.

Hall R, Papierok B (1982) Fungi as biological control agents of arthropods of agricultural and medical importance. Parasitology 84(4):205-240

Hallsworth JE, Magan N (1999) Water and temperature relations of growth of the entomogenous fungi *Beauveria bassiana*, *Metarhizium anisopliae*, and *Paecilomyces farinosus*. J Invertebr Pathol 74(3):261-266

Hemmati F, McCartney HA, Clark SJ, Deadman ML (2001a) Conidial discharge in the aphid pathogen *Erynia neoaphidis*. Mycol Res 105(6):715-722

Hemmati F, Pell J, Mccartney H, Deadman M (2001b) Airborne concentrations of conidia of *Erynia neoaphidis* above cereal fields. Mycol Res 105(4):485-489.

Hemmati F, Pell J, Mccartney H, Deadman M (2002) Aerodynamic diameter of conidia of *Erynia neoaphidis* and other entomophthoralean fungi. Mycol Res 106(2):233-238

Herren P (2018) Conidial discharge of *Pandora* sp., a potential biocontrol agent against *Cacopsylla* spp. in apple and pear orchards. MSc thesis, Department of Plant and Environmental Sciences, University of Copenhagen

Inglis GD, Goettel MS, Butt TM, Strasser H (2001) Use of hyphomycetous fungi for managing insect pests. In: Butt TM, Jackson C, Magan N (ed) Fungi as biocontrol agents: progress problems and potential, CABI Publishing, Wallingford, Oxon, UK, pp 23 - 69

Jaques RP, Patterson NA (1962) Control of the apple sucker, *Psylla mali* Schmidb., by the fungus *Entomophthora sphaerosperma* (Fresenius). Can Entomol 94:818–825

Jaronski ST (2014) Mass production of entomopathogenic fungi: State of the art. In: Morales-Ramos JA, Guadalupe Rojas M, Shapiro-Ilan DI (ed) Mass production of beneficial organisms, Elsevier Inc., Amsterdam, pp 357-413

Jensen AH (2017) A new insect pathogenic fungus from Entomophthorales with potential for psyllid control. MSc thesis, Department of Plant and Environmental Sciences, University of Copenhagen

Jensen AH, Gross J, Jensen AB, Gallinger J, Eilenberg J, (2018) A new insect pathogenic fungus from Entomophthorales with potential for psyllid control. Mitt Dtsch Ges Allg Angew Entomol 21:283-286

Kalsbeek V, Pell JK, Steenberg T (2001) Sporulation by *Entomophthora schizophorae* (Zygomycetes: Entomophthorales) from housefly cadavers and the persistence of primary conidia at constant temperatures and relative humidities. J Invertebr Pathol 77(3):149-157

Keller S (2007) Arthropod-pathogenic Entomophthorales: Biology, ecology, identification. Office for Official Publications of the European Communities.

Kramer JP (1980) The house-fly mycosis caused by *Entomophthora muscae*: Influence of relative humidity on infectivity and conidial germination. J N Y Entomol Soc 88(4):236–240

Latgé JP, Perry DF, Prévost MC, Samson RA (1989) Ultrastructural studies of primary spores of *Conidiobolus, Erynia* and related Entomophthorales. Canad J Bot 67(9):2576-2589

Li W, Xu WA, Sheng CF, Wang HT, Xuan WJ (2006). Factors affecting sporulation and germination of *Pandora nouryi* (Entomophthorales: Entomophthoraecae), a pathogen of *Myzus persicae* (Homoptera: Aphididae). Biocontrol Sci Technol 16(6):647-652

Magalhães BP, Humber RA, Shields EJ, Roberts DW (1991) Effects of environment and nutrition on conidium germination and appressorium formation by *Zoophthora radicans* (Zygomycetes: Entomophthorales): a pathogen of the potato leafhopper (Homoptera: Cicadellidae). Environ Entomol 20(5):1460-1468

Millstein JA, Brown GC, Nordin GL (1982) Microclimatic humidity influence on conidial discharge in *Erynia* sp. (Entomophthorales: Entomophthoraceae), an entomopathogenic fungus of the alfalfa weevil (Coleoptera: Curculionidae). Environ Entomol 11(6):1166-1169

Milner RJ, Bourne J (1983) Influence of temperature and duration of leaf wetness on infection of *Acyrthosiphon kondoi* with *Erynia neoaphidis*. Ann Appl Biol 102(1):19-27

Milner RJ, Holdom DG, Glare TR (1984) Diurnal patterns of mortality in aphids infected by entomophthoran fungi. Entomol Exp Appl 36(1):37-42

Mullins DE (1985) Chemistry and physiology of the haemolymph. In: Kerkut GA, Gilbert LI (ed) Comprehensive Insect Physiology, Biochemistry and Pharmacology, Pergamon, Oxford, pp 355–399

Muskat LC, Przyklenk M, Humbert P, Eilenberg J, Patel AV (2022a) Fermentation of the psyllid-pathogenic fungus *Pandora* sp. nov. inedit. (Entomophthorales: Entomophthoraceae), Biocontrol Sci Technol 32(5):564-585

Muskat LC, Görg LM, Humbert P, Gross J, Eilenberg J, Patel AV (2022b) Encapsulation of the psyllid-pathogenic fungus *Pandora* sp. nov. inedit. and experimental infection of target insects. Pest Manag Sci 78:991-999

Newman GG, Carner GR (1975) Environmental factors affecting conidial sporulation and germination of *Entomophthora gammae*. Environ Entomol 4(4):615-618

Nielsen C, Hajek AE (2006) Diurnal pattern of death and sporulation in *Entomophaga maimaiga*-infected *Lymantria dispar*. Entomol Exp Appl 118(3):237-243

Olsen NL, Herren P, Markussen B, Jensen AB, Eilenberg J (2019) Statistical modelling of conidial discharge of entomophthoralean fungi using a newly discovered *Pandora* species. PloS One 14(5):e0215914

Page RM, Humber RA (1973) Phototropism in *Conidiobolus coronatus*. Mycologia 65(2):335-354

Pell JK, Eilenberg J, Hajek AE and Steinkraus DC (2001) Biology, ecology and pest management potential of Entomophthorales. In: Butt TM, Jackson C, Magan N (ed) Fungi as biocontrol agents: progress problems and potential, CABI Publishing, Wallingford, Oxon, UK, pp 71–154

Pell JK, Macaulay EDM, Wilding N (1993) A pheromone trap for dispersal of the pathogen, *Zoophthora radicans* Brefeld (Zygomycetes: Entomophthorales) amongst populations of the diamondback moth, *Plutella xylostella* L. (Lepidoptera: Yponomeutidae). Biocontrol Sci Technol 3:315–320

Roy HE, Steinkraus DC, Eilenberg J, Hajek AE, Pell JK (2006) Bizarre interactions and endgames: Entomopathogenic fungi and their arthropod hosts. Annu Rev Entomol 51:331-357

Shah P, Pell J (2003) Entomopathogenic fungi as biological control agents. Appl Microbiol Biotechnol 61:413-423

Shimazu M (1977) Factors affecting conidial germination of *Entomophthora delphacis* Hori (Entomophthorales: Entomophthoraceae). Appl Entomol Zool 12(3):260-264

Six DL, Mullens BA (1997) Distance of conidial discharge of *Entomophthora muscae* and *Entomophthora schizophorae*. J Invertebr Pathol 67:253–258

Speiser B, Tamm L, Leschenne V, Kretzschmar U, Brändle I, Maurer V, Schneider C, Dittmann M, Berger T (2022) Betriebsmittelliste 2022 für den ökologischen Landbau in der Schweiz, Forschungsinstitut für biologischen Landbau FiBL, Frick, Switzerland

Steinkraus DC, Slaymaker PH (1994) Effect of temperature and humidity on formation, germination, and infectivity of conidia of *Neozygites fresenii* (Zygomycetes: Neozygitaceae) from *Aphis gossypii* (Hemiptera: Aphididae). J Invertebr Pathol 64:130–137

Uziel A, Kenneth RG (1991) Survival of primary conidia and capilliconidia at different humidities in *Erynia* (subgen. *Zoophthora*) spp. and in *Neozygites fresenii* (Zygomycotina: Entomophthorales), with special emphasis on *Erynia radicans*. J Invertebr Pathol 58(1):118-126.

Vega FE, Goettel MS, Blackwell M, Chandler D, Jackson MA, Keller S, Koike M, Maniania NK, Monzón A, Ownley BH, Pell JK, Rangel DEN, Roy HE (2009) Fungal entomopathogens: new insights on their ecology. Fungal Ecol 2:149–159

Watson DW, Petersen JJ (1993b) Seasonal activity of *Entomophthora muscae* (Zygomycetes: Entomophthorales) in *Musca domestica* L. (Diptera: Muscidae) with reference to temperature and relative humidity. Biol Control 3:182–190

Webster J, Weber R (2007) Introduction to fungi. Cambridge University Press, Cambridge

Weseloh RM, Andreadis TG (1992) Epizootiology of the fungus *Entomophaga maimaiga*, and its impact on gypsy moth populations. J Invertebr Pathol 59(2):133-141

Wilding N (1969) Effect of humidity on the sporulation of *Entomophthora aphidis* and *E. thaxteriana*. Trans Br Mycol Soc 53:126–130

Wilding N (1970) *Entomophthora* conidia in the air-spora. J Gen Microbiol 62:149–157

Wraight SP, Galaini-Wraight S, Carruthers RI, Roberts DW (2003) *Zoophthora radicans* (Zygomycetes: Entomophthorales) conidia production from naturally infected *Empoasca kraemeri* and dry-formulated mycelium under laboratory and field conditions. Biol Control 28(1):60-77

Yendol WG (1968) Factors affecting germination of *Entomophthora* conidia. J Invertebr Pathol 10(1):116-121

Yu Z, Nordin GL, Brown GC, Jackson DM (1995) Studies on *Pandora neoaphidis* (Entomophthorales: Entomophthoraceae) infectious to the red morph of tobacco aphid (Homoptera: Aphididae). Environ Entomol 24:962–966

Zhou X, Feng MG (2009) Sporulation, storage and infectivity of obligate aphid pathogen *Pandora nouryi* grown on novel granules of broomcorn millet and polymer gel. J Appl Microbiol 107(6):1847-1856

Chapter 7

Image analysis-based quantification of fungal sporulation by automatic conidia counting and gray value correlation

Linda C. Muskat[1,2]; Yannic Kerkhoff[3,4]; Pascal Humbert[1]; Tim W. Nattkemper[3]; Jørgen Eilenberg[5]; Anant V. Patel[1]

[1]Bielefeld University of Applied Sciences, Faculty of Engineering and Mathematics, WG Fermentation and Formulation of Biologicals and Chemicals; [2]Georg-August-University Goettingen, Agricultural Entomology, Department of Crop Sciences; [3]University of Bielefeld, Faculty of Technology, Biodata Mining Group; [4]Free University of Berlin, Institute of Chemistry and Biochemistry; [5]University of Copenhagen, Department of Plant and Environmental Sciences, Insect Pathology and Biological Control

Published in: MethodsX, 2021, 8, 101218

DOI: 10.1016/j.mex.2021.101218

Abstract

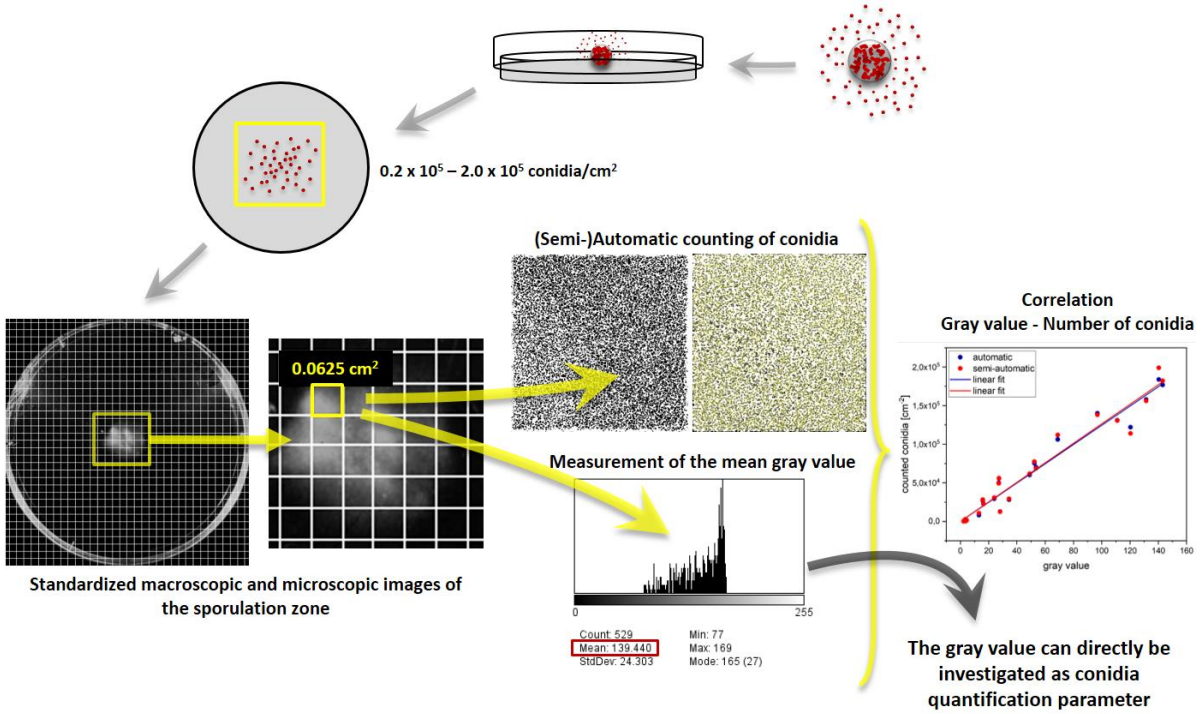
The present work describes a new computer-assisted image analysis method for the rapid, simple, objective and reproducible quantification of actively discharged fungal spores which can serve as a manual for laboratories working in this context. The method can be used with conventional laboratory equipment by using bright field microscopes, standard scanners and the open-source software ImageJ. Compared to other conidia quantification methods by computer-assisted image analysis, the presented method bears a higher potential to be applied for large-scale sample quantities. The key to make quantification faster is the calculation of the linear relationship between the gray value and the automatically counted number of conidia that has only to be performed once in the beginning of analysis. Afterwards, the gray value is used as single parameter for quantification. The fast, easy and objective determination of sporulation capacity enables facilitated quality control of fungal formulations designed for biological pest control.

Highlights

- Rapid, simple, objective and reproducible quantification of fungal sporulation suitable for large-scale sample quantities.
- Requires conventional laboratory equipment and open-source software without technical or computational expertise.
- The number of automatically counted conidia can be correlated with the gray value and after initial calculation of a linear fit, the gray value can be applied as single quantification parameter.

Keywords: (Semi-)Automatic conidia counting, Computer-assisted sporulation quantification, Entomopathogenic fungi

Graphical abstract



Introduction

The formulation of living biocontrol agents as capsules improves their applicability, shelf life and storability and prolongs the sporulation duration after field application by serving as a "microfermenter" formulation (Przyklenk et al., 2017). When considering entomopathogenic fungi for use in biological control, one of the most important issues is a reliable method for quantification of virulent conidia formed by encapsulated fungal cells.

Fungi of the order of Entomophthorales bear an exceptional high potential for biological insect pest control because of their narrow host range and fast speed-to-kill (Pell et al., 2001) and the genus *Pandora* contains several species with potential for biological control. Conidia, the asexual spores which are the infection units of these fungi, are actively discharged and dispersed into the environment. After landing, the conidia of *Pandora* sp. stick, by aid of mucoid substances and a partly detached cell wall, to surfaces like the host insect cuticle (Keller, 1991; Olsen et al., 2019). Although the role of this mucous layer is not yet completely clear, it makes classical collection and counting of the conidia using common surfactants like polysorbates, e.g. Tween 80, difficult or even impossible.

A standardization of a novel observation method to determine the meaningful parameter of sporulation capacity of *Pandora* spp. is necessary to enable a routine quality control for biocontrol formulations containing living entomophthoralean fungal cells.

Compared to manual counting of discharged conidia, which is a subjective and time-lasting procedure, automatized image analysis is quicker and can improve the comparability of results. Nielsen et al. (2001) developed a method for the characterization of conidial size and shape from microscopic images of the conidia of different *Pandora* species. Korsnes et al. (2016) accelerated counting of conidia from spore

trap samples by automatized detection and identification of conidia of *Pandora neoaphidis*. Bonner et al. (2003) established a semi-automatized method for the identification and counting of conidia of *P. neoaphidis* by selection of conidia based on the gray scale for shape recognition. These publications demonstrate that automatized detection and counting of fungal spores is a subject of relevance, with regards to biological control and also, it is relevant for understanding the biology of entomophthoralean fungi as well as other fungi. These published methods make counting faster, easier and more objective but so far they have solely been proven useful for small sample quantities and from microscopic images.

The aim of the present study was the development of a rapid, simple and objective method for the quantification of discharged conidia of the encapsulated entomophthoralean fungus *Pandora* sp. nov. (ARSEF11372), which has potential for psyllid control. Our starting assumption and hypothesis: We assumed that conidia that were dispersed onto a smooth surface would reflect diffuse light and we suggested that this emission would correlate with the number of discharged conidia. By calculating the correlation of the gray value as magnitude of the reflected light with the automatically counted number of conidia, we aimed to develop a method to accelerate the speed of conidia quantification.

Method details

Fungal strain

The fungus *Pandora* sp. nov. (ARSEF 13372) used in this work was isolated from an infected pear psyllid (*Cacopsylla pyri*) collected from a Danish pear orchard in 2016 by the Eilenberg-group (Department of Plant and Environmental Sciences, University of Copenhagen). The fungus is a novel, not yet named species of the entomophthoralean genera *Pandora* (Jensen, 2017).

Cultivation of the entomopathogenic fungus *Pandora* sp. nov. (ARSEF13372)

The fungus was grown as described by Hajek et al. (2012) on Saboraud Dextrose Agar (SDA) supplemented with 20% of a mixture of egg yolk (60%) and fresh skimmed milk (40%) (SDAME) on Petri dishes (diameter 90 mm) sealed with parafilm® and incubated at 18°C in the dark. To maintain the culture, mycelia plugs (0.5 cm^2) were cut and transferred to fresh media, when the mycelium reached the boarder of the plate. The fungus used for experiments was transferred less than 3 times.

Production of hyphal material of *Pandora* sp. nov. (ARSEF13372) in liquid culture

The cultivation method was adapted from Shah et al. (1998). First, in order to transfer the fungus from the solid media into liquid culture, three pieces of mycelia (0.5 cm^2) grown on SDAME agar plates were cut with a scalpel and transferred to 100 ml of a pre-culture medium composed of 10% skimmed milk in ultra-pure water (MilliQ) in 250-ml shaking flasks with 4 baffles and incubated at 18°C and 180 rpm with an amplitude of 20 mm in the dark for 48 hours. The main-culture, composed of dextrose (1.6%, w/v), yeast extract (1%, w/v), sodium chloride (NaCl; 0.9%, w/v) and skimmed-milk powder (10%, w/v) was inoculated with 10% (v/v) of the pre-culture and incubated at the same conditions for 72 h.

Encapsulation of the fungus within calcium alginate beads

The hyphal material from the liquid shaking culture was separated from the medium by centrifugation (4500 rpm; 10 min; 18°C) and washed twice in NaCl solution (0.9%) before encapsulation within calcium alginate beads. The formulation composition was adapted from Shah et al. (1998) with some modifications. The formulation solution was composed of sodium alginate (1.5%, w/w), maize starch

(10%, w/w), NaCl (0.9%, w/w) and hyphal material of *Pandora* sp. nov. (ARSEF 13372) (10%, w/w). After mixing the components in a beaker by magnetic stirring at 250 rpm, the mixture was dripped by means of a 20 ml syringe (Braun, Germany) equipped with a needle (0.90 x 40 mm; Braun, Germany) from a height of 10 cm into a stirred (250 rpm) calcium chloride solution (0.1 M). The formed beads were gelled for 20 min in the cross-linking solution and afterwards washed with a NaCl solution (0.9%) for 30 sec. All encapsulation steps were carried out at room temperature (22-24°C). The beads size was 4.44 mm (±0.24) diameter and the initial water activity (a_W) was about 0.955.

Conidia collection

Freshly prepared calcium alginate beads containing *Pandora* sp. nov. were transferred to Petri dishes, filled with 20 ml water agar (2 %, w/v). The distance between the agar and the lid of the Petri dish on which the conidia were collected in this experiment was 7 mm.

The Petri dishes were sealed with Parafilm® and incubated at 18°C in the dark. After 72 hours, the conidia collected on the lids of the Petri dishes were used as sample within the next experimental steps for sporulation quantification, which are shown in **Figure 7.1**.

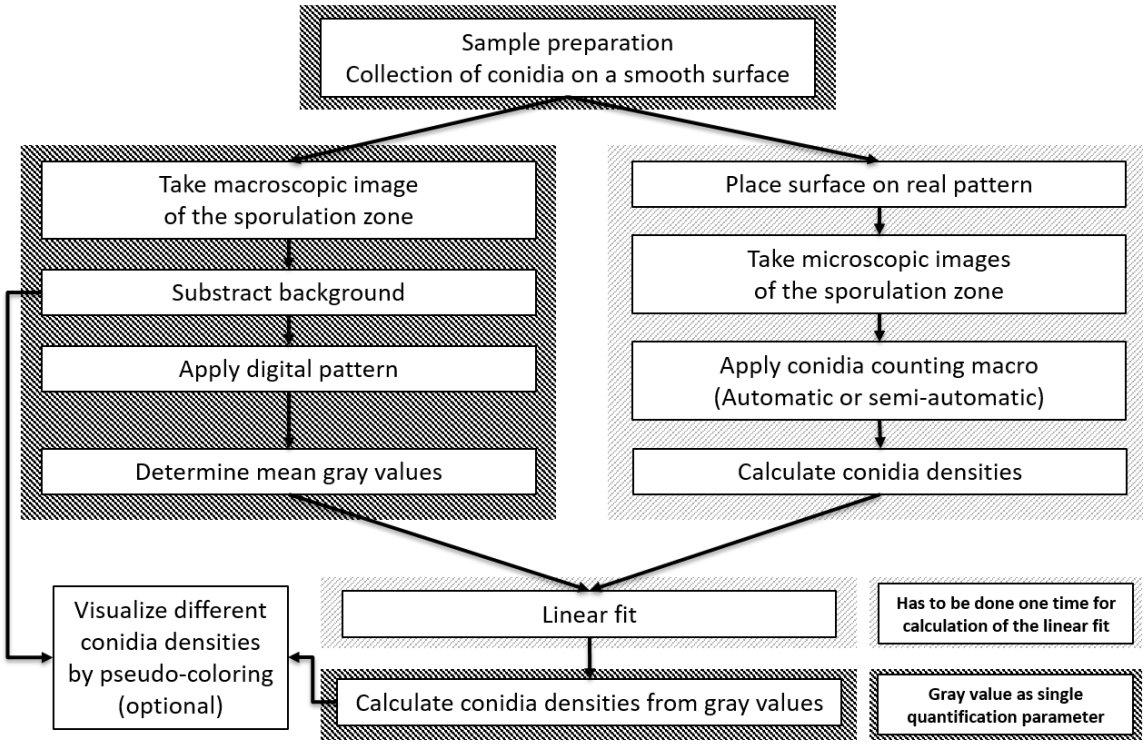


Figure 7.1. Workflow of the sporulation quantification. The sample must be prepared by collecting conidia on a smooth surface. The surface with the sporulation zone has to be placed on a grid pattern and microscope images have to be taken. One of the provided macros must be applied to get the conidia densities and fit them against the mean gray values of the corresponding digital grid from macroscopic images. The fit allows to calculate conidia densities from the gray values and allows optional pseudo-coloring for visualization purposes.

Sample preparation

The method is suitable for fungi or other organisms that disperse their conidia or any other actively discharged units into the environment. The discharged units must be identifiable by bright field microscopy.

A Petri dish lid, a glass slide or any other kind of smooth surface used for the collection of particles must be clear, clean and dry. We suggest cleaning a surface with an alcoholic solvent and a lint-free paper tissue, but gently to avoid creating scratches. The blanks should be prepared the same way. If the

sample collection surface is wet after the collection of conidia, e.g. under high humidity conditions like required for the sporulation of the fungus used in this work, it should be dried prior to the image generation.

Macroscopic image generation of sporulation zone

The surface for particle collection (e.g. Petri dish lid) is placed in a macroscopic camera system, e.g. scanning or gel reading apparatus. The sporulation zone is checked for disturbing scratches and the parameters are adjusted so that a clear picture of the sporulation zone can be recorded. (**Figure 7.2, A**). In addition, an image of the empty camera system is taken using the same parameters as with the surface as blank. All images should be saved as 8-bit images (e.g. tif format).

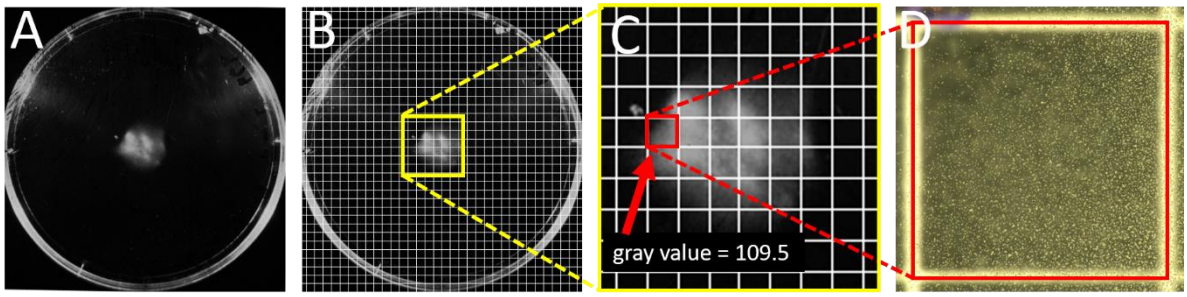


Figure 7.2 Generation of gray value and corresponding microscopic sporulation image. A) Macroscopic image of the conidia collected on a Petri dish lid after background subtraction. B) Background corrected images overlaid with a digital grid for separation of the sporulation zone into disjunct areas. C) Gray value measurement of a selected grid cell. D) Microscopic image of the selected grid cell for conidia counting.

Image processing software ImageJ

To perform the following image processing steps, the open-source software ImageJ (v1.52p, Fiji) is required. We recommend the download of Fiji, which is a version of ImageJ with some useful pre-installed plug-ins. Further information on software download, plug-in options and theoretical background are available on the ImageJ homepage (https://imagej.nih.gov/ij/index.html; https://imagej.net/Welcome)

Macroscopic image correction and determination of the mean gray values

Empty background image subtraction is carried out using the function Image Calculator (Process>Image Calculator…) from the open-source software ImageJ (v1.52p, Fiji) so that the image of the empty gel reading apparatus (blank) is subtracted from the images taken with a surface. The background corrected images are used to overlay a digital grid (Analyze>Tools>Grid) (**Figure 7.2, B**). This way of separation of the sporulation zone into disjunct areas is achieved (**Figure 7.2, C**). It should be noted that in “Set Measurements” (Analyze>Set Measurements…) the “Mean gray value” checkbox must be activated when repeating the experiment. Finally, the areas are marked (“Rectangle”) and the gray values can be read out (Ctrl+M). For each area, the average gray value g has to be stored.

Microscopic image generation of the sporulation zone

The surface on which the particles are collected must be placed on a grid pattern (which corresponds to the digital pattern) and both are inspected with a bright field microscope. Certain areas of the sporulation zone are selected and an image (8-bit format) will be recorded. Afterwards, the resulting image areas will be identified on the macroscopic images from the scanning or gel reading apparatus

(**Figure 7.2, C & D**). In our set-up, grids of 2.5 x 2.5 mm were used. Only grids containing particles will be considered for further analysis.

Automatic and semi-automatic counting of conidia

In the following, the process of automatic conidia counting (ACC) is described. Afterwards, the semi-automatic conidia counting (SACC) method ACC will be described, which is a minor variation of the ACC.

First, all of the microscopic images of the sporulation zone areas are investigated manually and the inside of the grid cells are cropped ("Rectangle" & "Duplicate") and saved as a stack of image patches. Next, one of the provided ImageJ macros will be applied to the stack. To this end, the macros have to be provided as .txt file and executed via the standard ImageJ functionality (Plugins>Macros). Please note that the "Adjustable Watershed" algorithm has to be installed, i.e. the Adjustable_Watershed.class has to be in the ImageJ folder "Plug-ins". The result of this process is a stack of binary image patches with segmented spores, corresponding to the input image stack.

In all patches of the binary stack, the number of particles (white blobs) is determined with the Particle Analyzer (Analyze>Analyze Particles) of ImageJ. The check-box "Summarize" has to be activated to get the number of particles on each image and other parameters like size and shape that can be analyzed, if the corresponding check-boxes at "Set Measurement" are activated.

To perform the semi-automatic conidia counting (SACC), the same macro is applied as in the ACC before with the distinction that the intensity threshold t of the images can be manually adjusted. Pixels with an intensity value below the threshold t are regarded as background and shown in blue. Pixels with an intensity value above the threshold are regarded as conidia and keep their original intensity values. The intensity threshold t has to be adjusted until spores and background are best separated. Afterwards, by call Process>Find Maxima… and selection of "Above lower threshold" and "Preview point selection" the local intensity maxima will be shown which should correspond to the conidia. By variation of the option "Prominence" in the "Find Maxima" box the number of detected maxima (conidia) in the result can be adjusted. Lower "Prominence" will result in more maxima, higher "Prominence" will result in less maxima. Now, a suitable "Prominence" value has to be found for a noticeable number of particles or by selection of "Segmented Particles" at "Output type:" to get a segmented binary image like in the automatic macro. The segmented binary image can then be analyzed by the Particle Analyzer.

Calculation of a linear fit between automatically counted conidia and gray value

To analyze the resulting data for correlation, first, all of the conidia densities are computed from the number of conidia counted within all of the specific areas. Next, the gray values (x-axis) are plotted against the corresponding conidia densities C (y-axis) and a linear fit will be carried out with some arbitrary basic data analysis software like R or any other suitable. The result formula provides the final function to estimate the conidia density represented by a specific observed gray value g.

Visualization of different conidia densities by pseudo-coloring

For visualization, the macroscopic 8-bit images from the scanning or gel reading apparatus are opened and the ImageJ function Image>Lookup Tables>6 shades is applied. The image of the sporulation zone will be pseudo-colored to represent areas with different conidia densities (see **Figure 7.6** for an example).

Method validation

Two image analysis workflows as previously described were developed in order to automatize the counting of conidia from bright field microscopic images. To determine the accuracy and precision of both workflows, 14 image crops of 500 x 500 µm2 containing different numbers of conidia were counted manually and (semi)-automatic. The results were plotted and a linear fit was applied, as a linear correlation was expected (**Figure 7.3**).

As expected, both workflows showed a high correlation (R2 = 0.989 and 0.999) and therefore high accuracy and a high precision (automatic workflow underestimated the conidia number by 1%; semi-automatic workflow overestimated by 3 %). Both workflows were therefore considered to be as reliable as manual counting but at the same time considerably faster. The semi-automatic workflow is a bit more time-consuming (approximately 1 minute per image) than the fully automatic workflow (due to manual setting of parameters), however it is more precise in detecting conidia in clusters as seen by comparing the middle of **Figure 7.3, B** and **7.3, D.**

Both workflows were then applied to a data set of 25 microscopy bright field images of 2.5 x 2.5 mm2 containing different amounts of conidia in order to determine the conidia density (number of conidia per cm2). The counted conidia densities were then plotted against the gray values (8-bit, 0-255) of the corresponding area of the sporulation zone to determine the correlation between conidia density and resulting gray value. A linear fit was applied as a linear correlation was expected (**Figure 7.4**).

While with both workflows the conidia densities determined showed little deviation, the linear fit was mostly identical for both (slope = 1247 and 1264). The correlation (R2 = 0.959 and 0.948) and therefore the precision was very high and proves a high correlation between the conidia density and the gray value of the sporulation zone. This indicates that it is possible to determine the conidia density by analyzing the gray values of the sporulation zone respectively.

To proof this, a third data set of 5 bright field microscope images of 2.5 x 2.5 mm^2 containing different amount of conidia was analyzed for validation. The theoretical conidia density *C* of the gray values *g* from 1 to 175 was calculated using the fits of **Figure 7.4** simplified to:

$$C[cm^{-2}] = 1250 \pm 60 \cdot g - 100 \pm 3650 \qquad (1)$$

and plotted against the corresponding gray values, resulting in a prediction range for conidia density. The conidia of the correlation and validation image sets were counted by the automatic and semi-automatic workflow and plotted (**Figure 7.5**) to compare the calculated and the actual conidia densities. The counted conidia from the previous correlation data set were also plotted.

Figure 7.5 shows that the counted conidia densities of the validation data set have a similar distribution like that of the correlation data set. All counted conidia densities increase in linear manner with the gray values of the corresponding areas in the sporulation zone. **Figure 7.5** also shows that the prediction model of equation 1 only includes approximately 50% of the counted conidia densities. The other half of the counted conidia densities are outside auf the error interval. To tackle this issue, the error interval of the model of equation 1 was expanded:

$$C[cm^{-2}] = 1250 \pm 250 \cdot g \pm 8000 \qquad (2)$$

The error interval of the model of equation 2 is now broader and this includes approximately 90% of the counted conidia densities of the correlation data set and 100% of the counted conidia densities of the validation data set. The new model is therefore less precise but has a higher accuracy. It can therefore be assumed that if equation 2 is used to calculate the conidia densities from gray values, the true value lies within the calculated range in 90% of cases.

Equation 2 was therefore used to determine the theoretical conidia densities of two additional sporulation zones (**Figure 7.4**) to allow a quantitative comparison.

The 8-bit images were pseudo-colored by the 6 shades look-up-table make the zones with different gray values and thus different conidia densities respectively more distinguishable for the observer. It shows that the outer area of the first sporulation zone (**Figure 7.6, A**) contains about $5x10^4$ conidia per cm^2 and two areas with approximately 4-fold higher conidia densities of about $1.9x10^5$ conidia per cm^2 (**Figure 7.6, B**). The overall conidia density of the second sporulation zone **(Figure 7.6, C**) is significantly lower than that of the first. It contains a small area (blue) with a 2-fold higher conidia density ($1x10^5$) (**Figure 7.6, D**) than the outer area of the zone (approximately $5x10^4$) which is just half the density of the yellow zones of **Figure 7.6, B.**

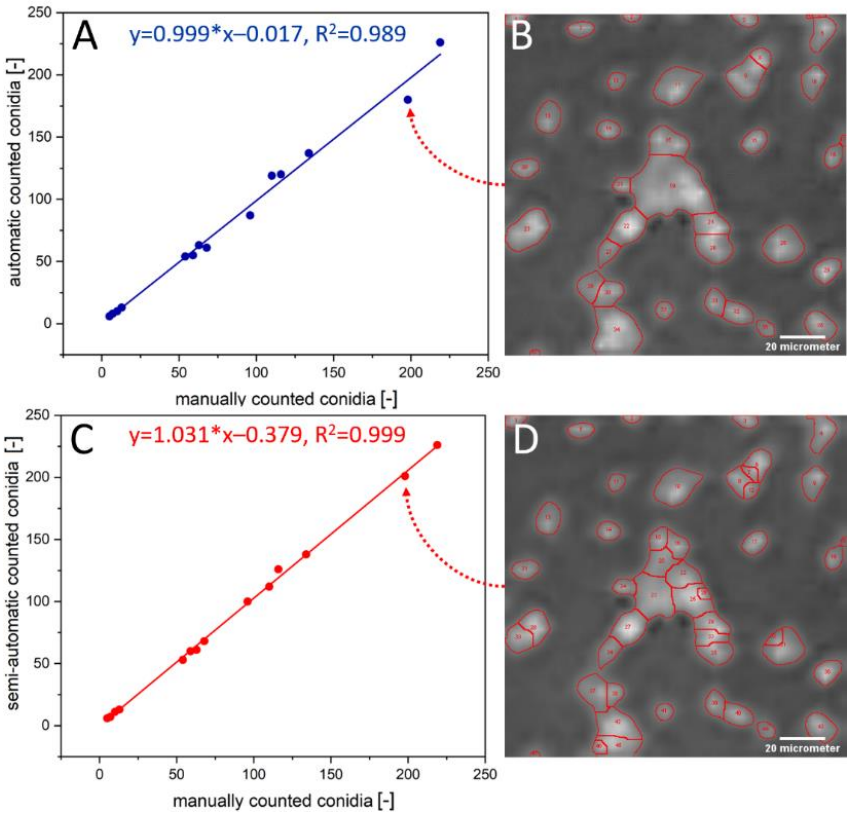


Figure 7.3 Correlation between (semi-)automatically and manually counted conidia. A) Plot of the automatically and manually counted conidia including linear fit with slope of 0.999 and R^2 = 0.989. B). Visualization of the automatic performed segmentation and counting on an example image crop (scale 20 µm) containing single conidia and clusters. The big cluster shows low degree of segmentation (underestimation) and thus leads to a higher deviation from the manually counted conidia number (red arrow). C) Plot of the semi-automatically and manually counted conidia including a linear fit with slope of 1.031 and R^2 = 0.999. D) Visualization of the semi-automatically performed segmentation and counting on an example image crop (scale 20 µm) containing single conidia and clusters. Here, the big clusters were further segmented after manual inspection for more reliable results (red arrow).

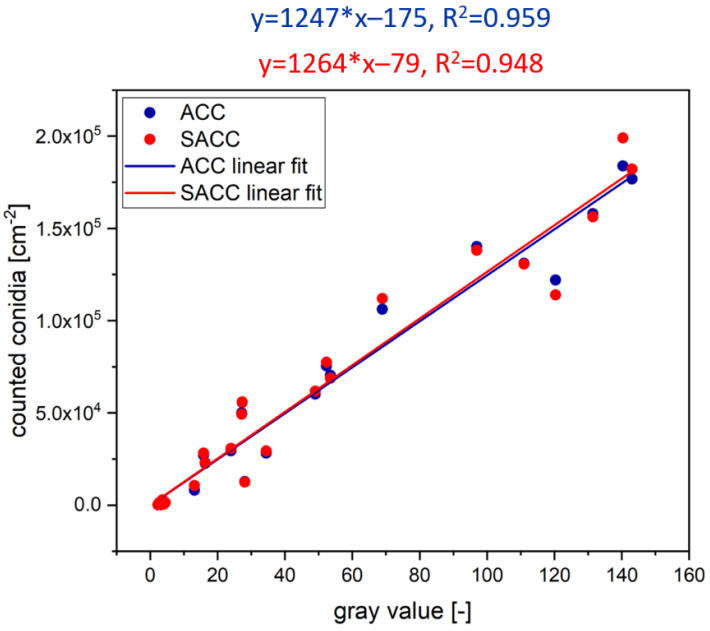


Figure 7.4 Correlation of the (semi-)automatically counted conidia on microscope images and gray values of the corresponding areas of the sporulation zone. The linear fit of the automatic workflow has a slope of 1247, a y 0 of -175 and an R^2 of 0.959. The linear fit of the semi-automatic workflow has a slope of 1264, a y 0 of -79 and an R^2 of 0.948. The error of the slope for both fits is approximately ±60 and the error of the y 0 for both fits is approximately ±3650.

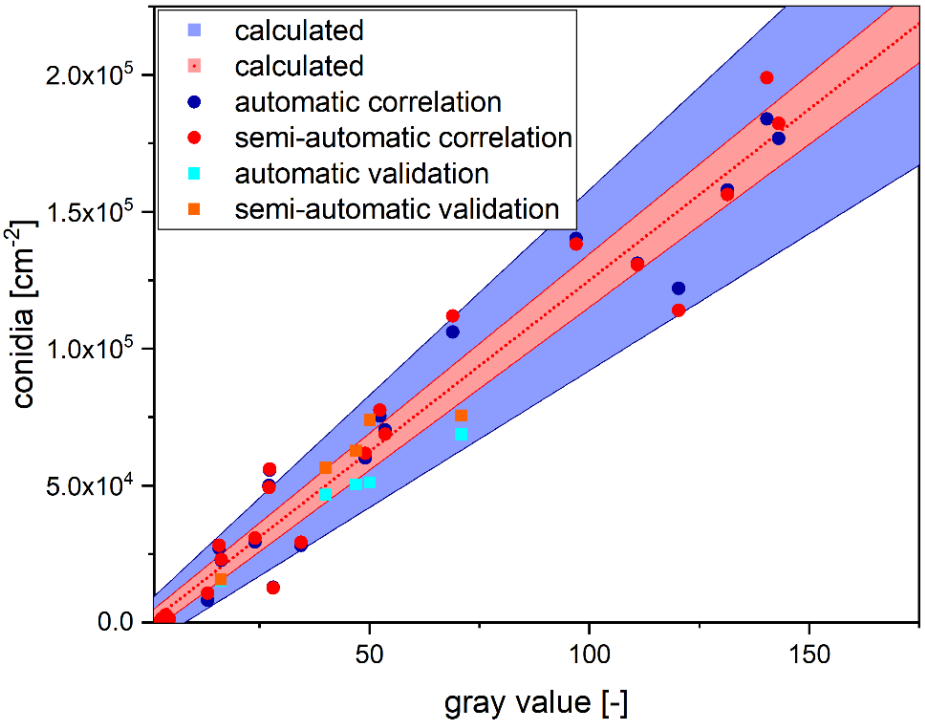


Figure 7.5 Comparison of conidia densities calculated from gray values and (semi-)automatic counted conidia densities. Blue and red dots represent the conidia densities counted in the correlation data set. Cyan and orange squares represent the conidia densities counted in the validation date set. The red area represents the theoretical conidia densities calculated from gray values by Eq. 1 . The blue area represents the theoretical conidia densities calculated from gray values by Eq. 2 .

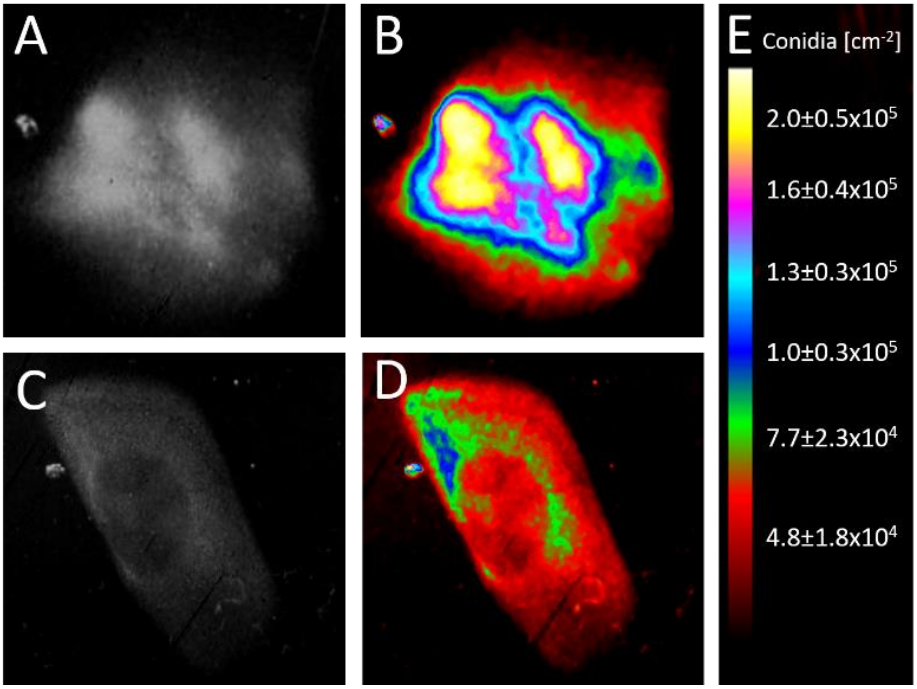


Figure 7.6 Gray value image and calculated conidia densities of sporulation zones. A) 8-bit gray value image of the first sporulation zone. B) 6 shades pseudo-coloring of the first sporulation zone. C) 8-bit gray value image of the second sporulation zone. D) 6 shades pseudo-coloring of the second sporulation zone. E) Color scale of the pseudo-coloring with corresponding conidia densities C[cm −2] calculated from gray values g by Eq. 2.

Limits of the method and discussion

Working with living fungal cells bears some error sources that can negatively affect the sporulation quantification by the developed method. In the present work, the conidia density on the Petri dish lid was insufficiently low to be detected by the developed method within the first 48 hours of sporulation from capsules. As far as the detectable conidia number threshold is not reached, the automatized counting can be applied but not the quantification by the gray value. Moreover, there is an upper limit of the conidia number quantifiable by the developed method based on a maximum gray value threshold. Furthermore, at high conidia densities, conidia are tending to stick together. The problem of segmenting sticking conidia was solved by the application of the watershed algorithm (see (Semi-)Automatic counting of conidia in the methodology). Another error source, also existing for other fungi, is the germination of conidia under suitable conditions. As shown in **Figure 7.7**, after application of the watershed algorithm, the germ-tubes (**Figure 7.7, A**) are separated and counted as single particles (**Figure 7.7, B**).

In the special case of the Entomophthorales, another point worth noting is the formation of secondary conidia when landing on a surface not suitable for germination. Each primary conidium can form just one secondary conidium, so a secondary conidium represents a primary conidium having landed. The leaving remnant of the primary conidium can, however, cause a potentially increase in the gray value signal. In the case of *Pandora*, the error sources of germination and higher order conidia could be eliminated by a shorter sample collection time about 4 hours during high sporulation rate intervals to avoid germination into secondary conidia (Eilenberg, personal observations). In case of a few secondary conidia present, the strong difference in intensity of the secondary conidia and the remnants from primary conidia can be clearly distinguished and it is unlikely that remnants will interfere with the analysis (**Figure 7.7, C**). Nevertheless, the influence of remnants on the gray value should be evaluated.

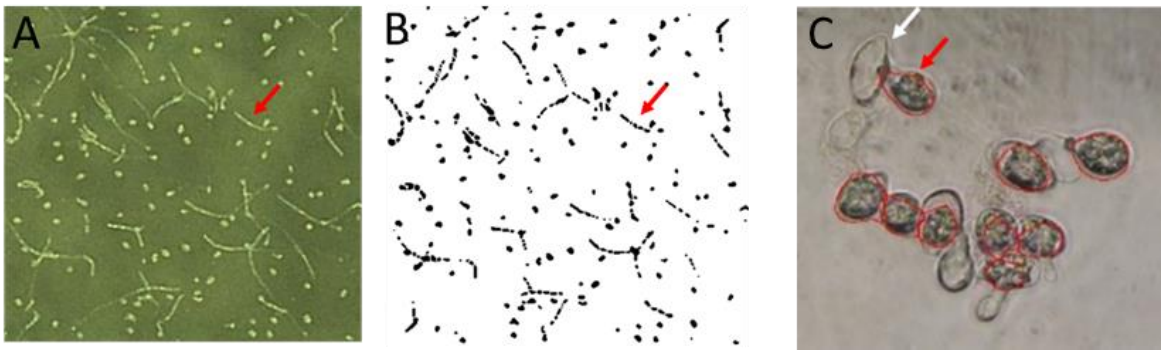


Figure 7.7 A & B The germ-tubes of the conidia are separated by the watershed algorithm and counted as single particles.**C** Primary conidia forming secondary conidia (red arrow) and leaving remnants (white arrow). Due to low intensity, the remnants are ignored and only the secondary conidia are counted (red outlines).

Conclusion

Compared to manual counting of discharged conidia which is a subjective and time-wasting procedure, automatized image analysis improves the comparability of results. Existing computer-assisted methods for conidia quantification are generally not well suited for large-scale sample quantities like it is required for the development of biopesticides based on fungi actively discharging their infective conidia into the

environment. The aim of this study was the development of a method for faster and more precise high-throughput quantification of fungal sporulation by computer-assisted image analysis. It was demonstrated that the correlation between the gray value of the sporulation zone, as magnitude of the reflected light by conidia and the actual number of automatically counted conidia can be applied for a comparable quantification of conidial discharge. To give consideration to conidia counting, automatic and semi-automatic image analysis were tested for their practicability. Automatic counting has proved as less subjective and easier for the experimenter, but semi-automatic counting shows higher correlation with the actual number of conidia. To eliminate one of the main error sources of sticking conidia packages, the watershed algorithm was applied for conidia separation. The linear relationship between the number of conidia and the gray value can be investigated for calculation within the range of $0.2x10^5$ to $2.0x10^5$ conidia/cm^2. The statistical power of the developed quantification method was validated by comparing the automatized counting results with manual counting to demonstrate the applicability of the developed method. A modified protocol also provides the opportunity to make conidia densities more visible and comparable by more obvious pseudo-coloring. The present method can be performed with low cost, conventional laboratory equipment and the open-source software ImageJ that enables the adaptability of the method by other scientists. Furthermore, the developed method could be combined with established methods for shape and pattern recognition, e.g. those presented by Nielsen et al. (2001) or Ranzato et al. (2007) and might be adapted for other fungi or microscopic particles as well. It will also prove useful to quantify spores produced in solid state fermentation on technical scale and provide a reliable option for quality control of these bioprocesses. This study demonstrates the high potential of the connection between classical biological techniques and automatized image analysis and should be confirmed by further publications like the present one to make these methods usable for every scientist.

Acknowledgements

This research was supported by means of the German Federal Ministry of Food and Agriculture (BMEL) as part of the project PICTA-KILL (project number 28-1-49.004-15). Financial support by a DFG-publication grant is kindly acknowledged. Finally, we want to thank Michaela Klöcker for providing access to the microscope equipment, Till Karwen for support during the laboratory work and Johannes Brikmann for critical revision of the manuscript.

Author contributions

Linda Muskat and **Yannic Kerkhoff** contributed equally. **Linda Muskat:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – Original draft, Visualization **Yannic Kerkhoff:** Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – Original draft, Visualization **Pascal Humbert:** Project administration, Writing Review & Edit **Tim W. Nattkemper:** Supervision, Writing Review & Edit **Jørgen Eilenberg:** Resources, Supervision, Writing Review & Edit **Anant V. Patel:** Supervision, Writing Review & Edit, Funding acquisition

References

Bonner TJ, Pell JK & Gray SN (2003) A novel computerised image analysis method for the measurement of production of conidia from the aphid pathogenic fungus *Erynia neoaphidis*. FEMS Microbiology Letters 220(1): 75-80.

Hajek AE, Papierok B & Eilenberg J (2012) Methods for study of the Entomophthorales. In Lacey LA (ed.) Methods in Insect Pathology, Academic Press Amsterdam, 2nd Edition: 285-316.

Herren P (2018) Conidial discharge of Pandora sp., a potential biocontrol agent against *Cacopsylla* spp. in apple and pear orchards. MSc thesis. Department of Plant and Environmental Sciences, University of Copenhagen, Denmark.

Jensen AH (2017) A new insect pathogenic fungus from Entomophthorales with potential for psyllid control. MSc thesis. Department of Plant and Environmental Sciences, University of Copenhagen, Denmark.

Keller S (1991) Arthropod-pathogenic Entomophthorales of Switzerland. II. *Erynia*, *Eryniopsis*, *Neozygites*, *Zoophthora* and *Tarichium*. Sydowia 43: 39-122.

Korsnes R, Westrum K, Fløistad E & Klingen I (2016) Computer-assisted image processing to detect spores from the fungus *Pandora neoaphidis*. MethodsX 3: 231-241.

Nielsen C, Sommer C, Eilenberg J, Hansen KS & Humber RA (2001) Characterization of aphid pathogenic species in the genus *Pandora* by PCR techniques and digital image analysis. Mycologia 93(5): 864-874.

Olsen NL, Herren P, Markussen B, Jensen AB & Eilenberg J (2019) Statistical modelling of conidial discharge of entomophthoralean fungi using a newly discovered *Pandora* species. PloS One 14(5): e0215914.

Pell JK, Eilenberg J, Hajek AE & Steinkraus DC (2001) Biology, ecology and pest management potential of Entomophthorales. In Butt TM, Jackson C and Magan N (ed.) Fungi as biocontrol agents: progress, problems and potential, CABI: 71-154.

Przyklenk M, Vemmer M, Hanitzsch M & Patel A (2017) A bioencapsulation and drying method increases shelf life and efficacy of *Metarhizium brunneum* conidia. Journal of Microencapsulation 34(5): 498-512.

Ranzato M, Taylor PE, House JM, Flagan RC, LeCun Y & Perona P (2007) Automatic recognition of biological particles in microscopic images. Pattern Recognition Letters 28(1): 31-39.

Shah, P A, & Pell, J K (2003) Entomopathogenic fungi as biological control agents. Applied Microbiology and Biotechnology 61(5-6): 413-423.

Chapter 8

General discussion and conclusion

Decades of widespread use of pesticides in crop protection have led to several problems. Moreover, favored by climate change and globalization, insect populations are threatening to flourish and new invasive pests are spreading. This increasing insect pest pressure on farmers, which threatens their livelihood, the crop plant, contradicts the increasingly stringent efforts to reduce pesticide use. Psyllids are a major risk to global fruit and vegetable production due to their role as vector insects for phytoplasmas (Weintraub & Beanland, 2006; Hogenhout et al., 2008). A psyllid causing economic damage in European apple production is *Cacopsylla picta*, the vector of *Candidatus* Phytoplasma mali, the causing agent of apple proliferation (Seemüller & Schneider, 2004; Jarausch et al., 2019). As there are no direct measures to combat phytoplasmas in the plant, the only measure is the control of the vector insect. In the face of the increasing popularity of organically grown food and the efforts of politics to reduce the application of pesticides, the demand for alternative environmentally friendly and target specific insecticides increases. Supported by the EU (European Parliament, 2009) and the European Commission's "Green Deal" (European Commission, 2022), the conditions for new developments are not only ideal and contemporary at present, but will become indispensable for the next years and decades when pesticides have been reduced by 50% by 2030 and thereafter in the sense of the “Green Deal”.

To satisfy the demand for alternative control options, new active ingredients and delivery systems and their incorporation into control strategies needs to be explored. By combining semiochemicals for behavioral manipulation of insect pests with biobased killing components, such as entomopathogenic fungi, high specificities for the target insect and improved efficacy of the killing effect can be achieved in so-called attract-and-kill strategies.

Embedded in this context, the present work aimed to develop formulations that can be applied in attract-and-kill strategies for the biological control of psyllids as phytoplasma vectors in fruit and vegetable crops. The focus was set on the development of a new delivery system for semiochemicals, in particular β-caryophyllene as an attractant for *Cacopsylla picta* and for the new entomophthoralean fungus *Pandora* sp. nov. inedit (ARSEF 13372) as a kill compound.

In the following section, the results of the present work are discussed in a general context and suggestions for improvements and prospects for future research are given. It has to be noted that a detailed discussion regarding each topic is given in the corresponding chapter to avoid redundancy.

ATTRACT

A wide range of formulation materials and methods for different application scenarios of semiochemicals have been developed during the last decades, which are reviewed in **Chapter 1** and in Muskat & Patel (2022).

With the aim of slow release of β-caryophyllene as semiochemical for insect behavior manipulation, a novel oleogel based formulation was developed in **Chapter 2**. As oleogel engineering technology is in its early stages, oleogels are not yet established for agricultural applications. To the best of the author´s knowledge, this work is the first attempt to design an ethylcellulose-candelilla wax-hybrid oleogel for the slow and controlled release of semiochemicals. This assumption is, moreover, supported and reinforced in view of the fact that this new formulation was classified as worthy of invention and a patent application was filed based on this work (Patel, Muskat & Humbert (2019). DE 10 2019 119 888 A1; Patel, Muskat & Humbert (2021). WO 2021/013917 A1).

During the developmental work in **Chapter 2**, special emphasize was laid on suitability for (1) semiochemical entrapment and release and (2) application in agriculture. Therefore, important material and formulation characteristics and properties were analyzed and modified by the formulation composition to adjust the gel to the intended application and for the active ingredient β-caryophyllene. This started with the selection of the formulation materials and additives, which are all biobased and biodegradable, which is important when released in the environment for agricultural applications, and are mainly registered for organic agriculture (Speiser et al., 2022).

A general drawback of oleogels is the hot-melt preparation procedure needed for most oleogel formations, which is unsuitable when encapsulating highly volatile and chemically instable substances (Mao et al., 2020), such as semiochemicals. The first developmental step was to address this drawback and make the oleogel suitable for volatile encapsulation. Therefore, the sol-gel temperature of the gel was lowered by addition of octyldodecanol as a softener, resulting in increased encapsulation efficiency. In a broader context, this finding is interesting for all oleogels being designed for encapsulation of heat-sensitive active compounds. Furthermore, it must be mentioned that, to the best of the author's knowledge, the method of high-pressure capillary rheometry has not yet been employed to analyse the gelation process of oleogels.

With regard to application on plant leaves, the oil leakage typical for oleogels, in which the oil is physically entrapped by hydrophobic interactions between the oil and the oleogelator, such as EC oleogels (Davidovich-Pinhas et al., 2015), poses a problem for the plant. Therefore, in the next developmental step, candelilla wax (CLW) was added as a second oleogelator and functional additive. Such hybrid oleogelator systems were earlier postulated to improve the properties of oleogels (Pakseresht, & Mazaheri Tehrani, 2022). The additive had two positive effects on the material properties, namely reduced oil loss from the gel and an increased mechanical stability, which will become important in agricultural application during shipping, storage and technical application.

Another interesting feature of the second oleogelator CLW became apparent when analysing the release behavior from the formulation with different CLW contents. Based on the reduced oil loss, it was hypothesized that the candelilla wax crystals would also act as a barrier to the diffusion of β-caryophyllene. This hypothesis was only partially verified: at a candelilla wax content between 1 and 4%, the release of bCAR was significantly slowed down compared to the gel without CLW. Surprisingly, the release increased with increasing wax content compared to gels with lower wax content, and the release from the gel with 16 % wax was as fast as from the wax-free gel. This result was unexpected as it is generally assumed that the higher the crystallinity, the slower the diffusion of molecules through a material (Yoon et al., 2017; Scher et al., 1977; Frank et al., 2005), including oil diffusion through

crystalline matrices (Nelis et al., 2021). A simple explanation is based on a change in the solubility of the various gel compounds, including β-caryophyllene (Hansen et al. 2007). In the discussion of **Chapter 2** this phenomenon of a refastened release from gels with higher wax contents was explained with a more aggregated and crystalline state in which the non-crystalline β-caryophyllene is located in the interstitial spaces allowing it to diffuse unhindered. Ongoing experiments should elucidate the nature and strength of the interaction between β-caryophyllene and the wax crystals.

Release from common semiochemical formulations increases with temperature (Nielsen et al., 2019). Most of these formulations do not contain a compound which is meltable within the temperature range present at the place of application. The starting hypothesis of **Chapter 3** was that the second oleogelator CLW acts as a thermo-responsive additive. Consequently, temperature must have an impact on the release behavior from the formulations containing CLW by melting and crystallization of CLW as a diffusion barrier. Some variations of the developed formulation exhibit a very slow release at temperatures below the melting point of the second oleogelator candelilla wax and as suspected, a shift in the increased release correlating with wax melting was detectable. Such a release behavior is highly attractive for target insects whose flight activity increases at a specific temperature. Insect flight activity increases with temperature, but each insect has its individual peak flight temperature range (Taylor, 1963; Goller & Esch, 1990; Hall, 2009). Fine-tuning the release of the semiochemical from the formulation to the activity of a specific target insect would be an interesting new challenge. The present work has provided a basis for such further developments. The gel represents a prototypical matrix formulation that can be used and further modified. To achieve the goal of a temperature range that matches with any insects activity that are active at lower temperatures, the CLW can be replaced, e.g. by other natural waxes with lower and narrower melting temperature ranges.

Another highly interesting feature of the formulation are the self-adhesive properties on plant leaves. Rainfastness is still an issue for spray-applied formulations in agriculture (Taylor & Matthews, 1986; Knight et al., 2004). For the analysis of the adhesive behavior of such large formulations like the oleogel developed in the present study on plant leaves, no method was available. Therefore, a new method was adapted from classical sticker research and established for investigating the adhesion strength of the gel on apple leaves by dynamic mechanical analysis in a shear stress experiment. The results from the experiment indicate that the oil leaking from the formulation serves as a sticker up to an optimum but then serves as a lubricant when oil-leakage becomes too high. The sticking was further negatively correlated with the stiffness of the gel, due to a reduced contact area between the two surfaces (**Chapter 3**).

The self-sticking behavior will enable application of the gel on the plant without additional surfactants. Application of attractant semiochemicals directly on the crop plant could be interesting when benefical parasitoids or predators should be attracted to the plant. When incorporating repellents, the plant can be protected from pest insects in push-pull strategies.

To conclude, the new formulation type has two special features, not offered by classical semiochemical formulations: (1) self-sticking properties on leaves and (2) temperature-responsible material properties enabling a thermo-triggered release. Moreover, in comparison to commercialized passive dispensers made from plastics, or active aerosol dispensers the developed formulation is biodegradable and cheap and easy to prepare.

In addition, **Chapters 2 and 3** provide a set of methods that enable analysis of soft materials such as oleogels, but also hydrogels, with emphasize on material properties suitable for application in agriculture. It includes: the important parameter of encapsulation efficiency, which was analyzed by Thermogravimetric Analysis (TGA); the mechanical stability, which is important in view of shipping, storage and technical application; self-adhesive properties of macroformulations on plant leaves; oil-loss, which should be reduced to not to damage the plant or other material that gets in contact with the formulation and the thermal stability analyzed by Differencial Scanning Calorimetry (DSC) which is important for (1) application under alternating thermal conditions and (2) analysis of thermo-responsiveness of the gel. Thus, the **Chapters 2 and 3** can be used as a manual for material characterizations for future development approaches in this field.

What is missing so far and should be investigated in future studies are the storability, scalability and the application technique for the developed formulation and, maybe most important, the amount of semiochemical required for perception and behavior manipulation in any target insect.

In view of storability, it has to be proven, whether the low risk for oxidation known from oleogels (Shi et al., 2014; O´Sullivan et al., 2016) is sufficient to protect chemically instable compounds such as β-caryophyllene and other semiochemicals from oxidation.

Another point is the scale-up. As the gel exhibits thermoplastic properties, it can be processed by classical thermal methods used in plastics processing, such as extrusion in single or double screw extruders for preparation of larger granules or beads or even layers or strips. For the preparation of smaller sprayable particles, spray chilling of the oleogel melt is an option, which should be tested in future studies, when a spray application is desired, and a smaller depot is acceptable.

The application by a turn table technique utilized for granules is an option. Another idea is the application via drones, which is a growing field of engineering research in the course of a more automatized and digitalized agriculture. A current drawback of drones is a limited payload weight (Rejeb et al., 2022). The formulation developed could be suitable for use in drones, as the self-adhesive properties mean that no spray broth or adhesive is required, thus saving weight.

Crucial for commercialization of the new delivery system for semiochemicals are the cost calculations. One kg of the gel with a β-caryophyllene loading of 30% and without CLW costs ~35 €. For calculation of costs per hectare, it is essential to know about the semiochemical concentration perceptible by the target insect to achieve the desired behavior manipulating effect. Pheromones are effective in very low concentrations due to the presence of specialized receptors to provoke a desired effect on the insect (Deisig et al. 2014; Fleischer & Krieger 2018; Renou 2014). On the contrary, allelochemicals, such as β-caryophyllene used in the present study are often needed in much higher concentrations to be perceptible to the target insect and their effect can change depending on the concentration. The exact β-caryophyllene amount perceptible by *C. picta* has yet to be determined. Mayer et al. (2008a) was able to trap *C. picta* in a trap releasing β-caryophyllene from a polyethylene dispenser. Thus, there is evidence that the attraction of *C. picta* is possible even under dilution of the released β-caryophyllene in the field in presence of the host plant. In the present work, the β-caryophyllene releasing formulation was demonstrated to attract the model organism *Aphidius colemani* when trained with the formulation and then tested in an olfactometer experiment (**Chapter 2**). Nevertheless, for future developments, in

particular in view of the fine-tuned or triggered release of any semiochemical, it will be important to know about the concentration range that is needed for the attraction of a specific insect species. What is further missing are greenhouse and field studies for validation of the attractive effect of the formulation under application conditions.

KILL

Entomopathogenic fungi of the Entomophthorales are generally considered to be host specific with a narrow host range (Eilenberg and Michelsen 1999; Jensen et al. 2001; Keller 2007; Pell et al. 2001), are known for their fast speed-to-kill (Pell et al. 2001) and for their ability to cause epizootics (Jaques and Patterson 1962; Keller 2007; Pell et al. 2001). Due to these advantages, the Entomophthorales have been recognized as promising biocontrol agents for a long time. Despite their potential they have not yet been successfully introduced into pest management strategies.

The entomophthoralean fungus *Pandora* sp. nov. inedit. (ARSEF 13372) was isolated from infected *Cacopsylla* spp. collected in a Danish pear orchard by Jensen (2017). The fungus is not yet named but based on morphological characteristics of the conidia and DNA sequences of the ITS-region, the fungus was found to be a new entomophthoralean species (Jensen et al., 2018) which is currently under description (Eilenberg et al. unpubl.) and will be named *Pandora cacopsyllae* (J. Eilenberg, personal communication).

In fact, as this fungus was a new species which is currently under description, the results from this work, in particular **Chapter 4 – 7** present the first reports on the approach to convert the new fungus *Pandora* sp. nov. inedit. (ARSEF 13372) into a biocontrol agent for psyllid pest control.

The present work addressed the major obstacles of Entomophthorales as biocontrol agents, namely (1) the difficulty to grow an entomophthoralean fungus in vitro and to be mass produced, (2) the short sporulation duration known from infected sporulating cadavers and mycelial mats, (3) the high humidity requirements for sufficient sporulation and (4) the contact failure between the fungus and the target insect.

For successful large-scale field application of an entomopathogenic fungus the mass-production of fungal biomass is essential for providing inoculant in a sufficient quantity (Jaronski, 2014; Shah & Pell, 2003).

The Entomophthorales have often been reported to be difficult to grow in vitro (Eilenberg et al., 1992; Hajek & St. Leger, 1994; Hajek et al., 2012; Jaronski, 2014; Latgé, 1981; Papierok & Hajek, 1997; Pell et al., 2001). For the Entomophthorales that have been cultivated in vitro it was observed that the best medium for cultivation of the Entomophthorales is species and even strain dependent. Since *Pandora* sp. nov. was a completely new fungus which not yet have been tested to be grown in liquid culture this issue was crucial to address in **Chapter 4.**

The aim of **Chapter 4** needs to take the next step of formulation (**Chapter 5**) into account, in order to select a suitable biomass to be produced: conidia, resting spores or hyphal material (Latgé et al., 1983). Conidia of *Pandora* spp. are surrounded by a sticky mucous layer which makes them difficult to collect (Eilenberg et al., 1986; Hajek et al., 2012; Olsen et al., 2019). Furthermore, entomophthoralean conidia

are sensitive to UV and desiccation and suffer from a short viability. Moreover, when using conidia they have to be sprayed when the target insect is present and needs to hit the insect directly. Resting spores are not described for *Pandora* species and have not yet been observed in the present or other studies for *Pandora* sp. nov. When storage is required and lag time due to asyncronous activation of resting spores after application are tolerable (Latgé et al., 1983), this cell type could be a promising option for other Entomophthorales, such as *Entomophaga maimaiga* (Hajek & Roberts, 1991; Hajek et al., 2001) or Conidiobolus obscurus (Latgé et al., 1983). In this work the decision fell on hyphal material. The application of hyphal material enables growth and sporulation over a prolonged duration in the field and imitates natural infection from sporulating cadavers (Hajek et al., 2012). Furthermore, hyphal material can be produced in liquid shaking culture (Hajek et al., 2012), which in general enables short process times and is easily scalable (Jackson, 1997; Jaronski, 2014). Thus, the aim of **Chapter 4** was the production of hyphal material of *Pandora* sp. nov. in liquid culture.

It has been previously reported from other entomophthoralean species belonging to the phylogenetic *Erynia* – *Pandora* – *Furia* genera clade (Gryganskyi et al., 2013) and thereby being phylogenetically near to *Pandora* sp. nov. can be cultured in complex nitrogen sources, such as protein hydrolysates in combination with glucose as carbon source (Li et al., 1993; Freimoser et al., 2000; Wraight et al., 2003; Leite et al., 2003; 2005; Hajek et al., 2012).

In the present study, fastest growth, in combination with high biomass production, was found to be best when skimmed milk (SM) was combined with yeast extract (YE) and animal-based protein hydrolysate (AN). The reasons are discussed in detail in **Chapter 4**. Nevertheless, this result is in well accordance with other studies investigating media screening, who found SM and YE were suitable media components for cultivation of phylogenetically related species such as *P. neoaphidis* (Russell and Paterson, 1982; Latgé et al., 1983; Li et al., 1993) and *Furia* sp. (Leite et al., 2005). Moreover, the selection of complex nutrient sources, such as protein hydrolysates instead of defined media components is an option to achieve reduced fermentation costs, which fits well with the current trend in fermentation research (de Lima et al., 2021; Iwanicki et al., 2020; Mascarin et al., 2018).

One common problem when culturing filamentous microorganisms under submerged culture conditions is the occurrence of conglomerated mycelium forming pellet-like structures (Cox et al., 1998; Cui et al., 1998), as observed in the present study. When such pellets reach sizes up to several mm (Cox et al., 1998), they are unsuitable for encapsulation in small-sized beads (Krell et al., 2018; Patel et al., 2011). By modification of fermentation parameters, such as increased osmolality (Wucherpfennig et al., 2011), increased osmolality in combination with increased viscosity of the medium (Krell et al., 2018; Wucherpfennig et al., 2011) or variation of the stirring speed (Cui et al., 1998; Patel et al., 2011), the formation of pellets can be prevented.

To the best of the author´s knowledge and as fermentation studies on the Entomophthorales in general are scarce, there have not been made any attempts on targeted production of finely dispersed mycelium of an Entomophthorales in submerged culture suitable for subsequent formulation.

In the present study the pellets formed by *Pandora* in shaking culture were successfully reduced by the simple measure of increased media osmolality by addition of sodium chloride. Beyond, Differencial

Scanning Calorimetry (DSC) is introduced in this chapter as a new method for determining the media osmolality via the lowered crystallization temperature of the medium.

Furthermore, the pellet quantification by software-supported image analysis as performed in **Chapter 4** enables, similar to the automatized counting of conidia presented in **Chapter 7** a more objective analysis method compared to manual analysis of cell structures or morphologies.

The scalability of the fermentation of *Pandora* sp. nov. was proven in a stirred tank bioreactor, where four times more biomass was produced compared with the shaking culture in flasks. This can be explained by improved pO_2 and nutrient supply and is discussed in detail in **Chapter 4**. However, after 48 h, the fungal cells attached to the vessel wall at the medium-air interface and formed a growing mycelial ring. This mycelium was not collected for biomass dry weight determination but was probably viable. Hence, the biomass yield is not represented by the growth kinetic curve and is supposed to be higher. This can be the starting point for further improvements and optimizations of the fermentation. The idea of Nolan (1990; 1991) who tested differently charged Teflon disks for the selective production of *Entomophaga aulicae* protoplasts under mass fermentation conditions could also be a promising technical solution for inhibition of hyphae or mycelium attachment to bioreactor vessels and stirrers.

To conclude, the results from **Chapter 4** demonstrate that the new fungus *Pandora* sp. nov. can be grown in liquid culture in common fungal media components and the highest biomass production was observable in the media containing skimmed milk (SM) and the cultivation is scalable. Thus, the first obstacle of supposed difficult cultivation had been successfully met.

The next step on the way to establishment of *Pandora* sp. nov. as a biocontrol agent was the formulation of the produced living biomass. The specific aim in **Chapter 5** was to overcome the short and inconsistent sporulation described from infected cadavers or mycelial mats of entomophthoralean fungi (Aoki, 1981; Kalsbeek, 2001; Olsen et al., 2019), in order to improve infection probability after field application.

Ca-alginate beads were selected as formulation material and method as it has some preferable properties for the encapsulation of living cells, such as moderate temperatures and conditions during the encapsulation process and the ease to co-formulate the cells with beneficial additives (Nussinovitch, 2010; Vemmer & Patel, 2013).

Skimmed milk, which has proven to increase biomass of *Pandora* sp. nov. in liquid culture, incorporated as a nutrient source also increased conidial numbers discharged by encapsulated *Pandora* sp. nov. from the Ca-alginate beads compared to beads without nutrients. Increased number of infection units by addition of nutritional additives to formulations is well known from other fungi (Przyklenk et al., 2017; Krell et al., 2018) including entomophthoralean fungi (Shah et al., 1999).

Moreover, the sporulation duration of 12 days observed form the beads was much longer compared to cadavers or mycelial mats: For the same fungus investigated in the present study a peak sporulation event <50 h and a maximal sporulation duration of 125 h when mycelia mats were incubated under humid conditions was observed by Olsen et al. (2019).

Besides the question for sporulation duration and intensity, the virulence to the target insects is another essential characteristic, which needed to be investigated. In the case of the Entomophthorales research

regarding pathogenicity and virulence of entomophthoralean fungi to psyllid species is scarce (Gross et al., 2022). This makes the results of the infection trials even more relevant. The significant reduction in survival time of 5-6 days for *C. pyri* and 9-10 days for *C. picta* treated with the formulated *Pandora* sp. nov. inedit. further demonstrate the potential of the fungus to be applied in biological psyllid pest control.

The results from the infection bioassays of the present study – in particular in view of *C. pyri*, - further support the fast speed-to-kill and the high host specificity of the Entomophthorales. The survival time in *C. pyri* of 5-6 days is much faster in comparison to other psyllid pathogenic hypocrealean fungi. The screening of 17 Hypocreales species and strains on Asian citrus psyllid *Diaphorina citri* revealed a median survival time of 6 to 10 days whereas for eight fungal species or strains the mortality rate did not even reach 50% within 10 days (Ausique et al., 2017). In the second bioassay the mortality in *C. pyri* reached 89%.

The fact that *Pandora* sp. nov. was isolated from a psyllid collected in pear orchards in combination with the higher mortality and shorter median survival time in pear psyllid *C. pyri* supports the advantage of the high host specificity in the Entomophthorales.

At this point, however, it must be mentioned - somewhat mitigating this assumption - that these differences in mortality cannot be confirmed by the study of Görg et al. (2021), who found a similar speed-to-kill of 5 days in both species, but a higher mortality of 83.3% in *C. picta* compared to of 70.9% in C. pyri.

However, during the 5 - 10 days until death the psyllids can act as vectors of phytoplasmas. Nevertheless, according in the sense of DeBach (1964) who postulated that biological control is “the action of parasites, predators, or pathogens in maintaining another organism´s density at a lower average than would occur in their absence”, the application of *Pandora* sp. nov. would be a promising measure for psyllid population reduction, not in the least as there is currently no insecticide registered for psyllid pest control in organic agriculture.

In order to clarify the underlying causes of the inconsistent results on mortality, even under controlled laboratory conditions where, for example, sporulation failure due to unfavourable conditions can be widely ruled out as a reason for failure, and the 100% mortality not achieved in any study, although the experimental cups were relatively small, requires further investigation. Are there differences in susceptibility of individual psyllids? Affected by age or gender? Are there resistances in the psyllid against *Pandora*? Is there a failure of germination in *Pandora* conidia? Is this involved in a strategy of *Pandora* sp. nov. and the Entomophthorales in general to avoid destroying the entire host insect population?

In this chapter it was further hypothesized that chitin may serve as a virulence-enhancing formulation additive, as it plays a crucial role during the percutaneous infection process in many entomopathogenic fungi (Charnley & St. Leger, 1991; Charnley, 2003). *Pandora* sp. nov. was found to release significantly more conidia from beads containing chitin compared to beads without this additive ($P < 0.001$; Tukey post hoc test; Chapter 5, Supplementary figure 5.1), which indicates that the fungus can metabolize chitin. Hence, the chitin would be an interesting alternative nutrient source compared to skimmed milk which is of high risk for contaminations. The chitin as a virulence enhancing additive hypothesis was not verified. Besides the chitinolytic enzyme activity, proteases and lipases are involved in successful host

insect cuticle penetration and are considered as the most important virulence factors of entomopathogenic fungi (Charnley and St. Leger, 1991; Humber, 2008). Moreover, these enzymes are known to be differentially expressed or up-regulated in the presence of the host insect cuticle and during the infection progress (Xu et al., 2006; Grell et al., 2011; Malagocka et al., 2015). The addition of such components as virulence enhancing formulation additives to induce enzymes involved in the infection process should be taken into account in future studies in order to improve the infection efficacy of encapsulated entomopathogenic fungi.

What is currently still missing is drying and evaluation of the storability of the formulated *Pandora* sp. nov. and the scale-up of the formulation process to enable broad field application of the KILL formulation.

The Ca-alginate bead production used in this study required dripping into the cross-linking solution which is simple and enables a low particle size distribution, but suffers from the formation of comparatively large beads (1 – 4 mm) and a limited production capacity (Przyklenk, 2017). The scale-up of the Ca-alginate bead production can be achived by the use of dripping devices with increased number of needles and pressure to increase the flow rate or by droplet breaking systems. The droplet breaking method, which has proven best for the technical high-throughput production of Ca-alginate beads is the so called JetCutting technology. JetCutting is a technique with a comparatively high bead production rate and allows for the production of smaller beads (<1 mm), which are suitable for technical spray application (Prüsse et al., 2008; Przyklenk, 2017). Another option would be the transfer of the bead production into an extrusion process on one- or twin-screw extruders. In this process, the Na-alginate solution containing the fungal cells and additives can be extruded into the cross-linking solution, forming a filament, which is then cut into cylindrically shaped granules followed by subsequent spheronization to produce round beads, which is an option when a larger bead size is afforded.

To conclude the results from **Chapter 5**, the formulation of *Pandora* sp. nov. within calcium alginate beads had at least two beneficial effects: (1) prolonged sporulation duration compared to cadavers or mycelial mats and (2) increased conidia production due to nutrient addition. Thus, the well-known problem of the Entomophthorales of very short sporulation capacities observable from cadavers was overcome.

Since conidia are the infective units of entomophthoralean fungi, efficient sporulation is obligatory for successful application of an entomophthoralean fungus in pest control strategies. As most Entomophthorales are only able to sporulate under saturated or saturation near humidity conditions, the lack of water availability is the main limiting factor of conidial discharge and conidia germination of a plenty of entomophthoralean species and, thus, one of the main reasons for failure in the field (Glare et al., 1986; Hajek et al., 1990; Delalibera Jr et al., 2006).

Therefore, in **Chapter 6** the humidity problem was addressed. First of all, the specific humidity requirements of *Pandora* sp. nov. for sporulation needs to be identified. As expected, *Pandora* sp. nov. was only able to sporulate when water activity was 0.99, at 0.97 no sporulation was observable. Since *Pandora* sp. nov. could still grow at water activities of 0.97 and 0.95, it can be assumed that the inhibitory effect is not due to a toxic effect of the glycerol which was used to set the a_W values. The observations are in well accordance with Glare et al. (1986) who found that *Zoophthora phalloides* was able to grow from *Myzus persicae* cadavers at 98% RH, but no more at 94% and sporulation was only possible at

saturated humidity conditions (100%). The comparability of RH and a_w is explained in the discussion of Chapter 6.

The high humidity requirements are explainable by the active mode of conidial discharge of *Pandora* spp. and other Entomophthorales: osmotic pressure from the environment is crucial for the generation of the turgor pressure in the conidiophores required for the ballistic spread of the conidia (Page & Humber, 1973; Latgé et al., 1989; Inglis et al., 2001; Webster & Weber, 2007).

Nevertheless, when aiming at establishment of a fungus with such high humidity requirements like *Pandora* sp. nov. the demand of the fungus needs to be satisfied. The present work provides one possible solution in form of a paste-type formulation. Co-application with a paste-type formulation allowed encapsulated *Pandora* sp. nov. to sporulate under very dry humidity conditions of 30 – 40% RH in the laboratory for at least 6 days. A peak sporulation event, typical for *Pandora* sporulation (**Chapter 5**) was also observable from the beads co-applied with the paste. The total number of discharged conidia from the paste-bead-co-application under non-saturated humidity conditions of 30 – 40% RH ($1.79x10^7$) during the 7 days was as high as under saturated conditions, where $9.57x10^6$ ($\pm 8.34x10^5$) conidia/bead were reported during the 12 days conidia were released from beads of the same composition. Thus, the paste formulation is capable to compensate for unsatisfactory environmental humidity conditions by providing a satisfactory water activity to the encapsulated fungus and thereby enable efficient sporulation.

Compared to the other few attempts that have been made to address the humidity problem of the Entomophthorales, the paste-formulation base on carboxymethylcellulose and xanthan and, thus, will be biodegradable, other than polyacryl based superabsorbents (Zhou et al., 2009). Another advantage is, that the paste was sufficient to maintain a high water activity and for sporulation even when applied in an open environment, which makes the application of the fungus able without a protective trap into which the target insects would have to enter to get in contact with the fungus (Pell et al., 1993). The paste enabled fixation of the sporulation source at a high and exposed position in the plant, in order to simulate the natural death orientation and supports a sporulation of the fungus over a wide area by simulating the naturally occurring summit disease. The actively discharged spores spread over a large area can further be transported by wind (Weseloh & Andreadis 1992; Dwyer et al., 1998; Hemmati et al., 2001; Keller, 2007).

As the surface of the paste formulation provides conditions similar to free water, it serves as an optimal medium to enable germination of primary conidia. This will further enable a prolongation of the growth and sporulation from the co-formulant of *Pandora* beads with the paste.

The developed paste can also serve as a universal carrier formulation for co-application with other biocontrol agents with high moisture requirements, including other entomopathogenic fungi, but also nematodes. This assumption is further supported by the fact that this new formulation was also found to be according to the invention and a patent application was filed based on this work (Muskat & Patel, 2022. EP 22 159 230.6.).

Quantification of conidia, the infective units of *Pandora* sp. nov., is crucial when investigating the beneficial effects of formulation additives, such as nutrients. Manual counting of conidia is a time-wasting and a non-objective procedure. Moreover, in the special case of *Pandora* spp., a mucous layer

surrounds the conidia, let them stick on the host insect cuticle (Keller, 2007; Olsen et al., 2019) and on various other surfaces. Although the role of this mucous layer is not yet completely clear, it makes classical collection and counting of the conidia using common surfactants difficult or even impossible. Driven by the problem of difficult conidia collection in combination with the desired acceleration of conidia quantification led to the development of a new computer-assisted image analysis method for the rapid, simple, objective and reproducible quantification of discharged conidia (**Chapter 7**). As the developed method can be performed with conventional laboratory equipment and the open-source software ImageJ it can be used as a manual for quantification of other cells. There are some points to be taken into account, the cells should be collected on a clean and smooth surface and should not be layered. A drawback is the limiting range of 0.2 $\times 10^5$ to 2.0 $\times 10^5$ conidia/cm^2. Thus, at lower numbers, the method based on the gray value is not applicable, the automatized counting from microscopic images can be performed. Image-analysis based methods can support laboratory research as mentioned before in **Chapter 4** where pellet-like structures of mycelia grown in liquid shaking culture were analyzed based on an image-analysis based method. Thus, besides the improvements during laboratory work by automatization and digitalization, such a method can be further developed as a standardized method to determine the meaningful parameter of sporulation to enable a routine quality control for biocontrol formulations containing living biocontrol agents.

Besides the drying, storage, scale-up and application of the developed formulations, a final hurdle on the way to commercialization will be the registration. The narrow host range of the Entomophthorales makes them favorable candidates for utilization as biocontrol agents (Barta and Cagáň, 2006), as it reduces the risk for non-target organisms, as long as beneficial insects are excluded from the narrow host range.

Releasing a living biocontrol agent is always associated with a potential risk (Scheepmaker et al., 2019; Köhl et al., 2019). An advantage long assumed for the Entomophthorales was their inability to produce toxic secondary metabolites, as they kill their host insect by exhausting nutrients and destroying the host insect´s organs due to tissue colonisation with little or no use of toxins (Humber 1984; Shah & Pell, 2003). However, this assumption needs to be revised, since it has recently be found by Boyce et al. (2019) that the so called summit disease, the manipulation of the host insect, is mainly controlled by fungus-derived neurogenic metabolites and endotoxins, such as psilocybin. With these new findings, combined with the possibility of mass production, there are new opportunities to use entomophthoralean fungi for the biotechnological production of secondary metabolites that may be useful for humans. Nevertheless, the secondary metabolites pose a risk to humans if the fungus and its metabolites come into direct contact with the crop plant.

The attract-and-kill approach offers a way to separate the fungus from the crop by attracting the insect out of the plantation. Both formulations could be placed near to the protected plantation in the boarder areas to attract and infect the inflying psyllids on their way to their summer host plant.

Another imaginable application scenario aims for the application of both formulations within the orchard early in the season when no fruits can come into contact with the fungus. The best option would be to apply both formulations on two different height levels. The KILL formulation at a higher level and the ATTRACT formulation on the level below. As shown in **Chapter 6**, the developed and co-applied *Pandora* bead/paste formulation released conidia over a distance of at least 40 cm. This wide-spread

distribution of conidia below the formulation when applied at a higher position in the fruit tree canopy creates a broad possible infection area below. Here would be a suitable place where the ATTRACT formulation releasing β-caryophyllene should be applied. For this application scenario further developmental work is needed to produce both formulations in a spreadable form by a reduced particle size of the *Pandora* beads, a reduced viscosity of the paste formulation which can then be used as a sticking agent and a reduced particle size of the oleogel beads, e.g. by preparation by spray-cooling.

From the current point of view, the co-formulation of the attractant compound β-caryophyllene with the killing compound *Pandora* sp. nov. within one formulation is not an option. First, the two active ingredients have opposite formulation requirements: *Pandora* sp. nov. demands for high humidity for sufficient sporulation (**Chapter 6**), while the release of β-caryophyllene is suppressed when the formulation matrix swells from moisture (Daems et al., 2016). Second, green leaf volatiles and thus semiochemicals have been found to inhibit sporulation and growth of entomophthoralean fungi (Brown et al., 1995; P.A. Shah, unpublished data). Moreover, the separation of the two active ingredients in individual formulations offers new opportunities for the modular use of both components in pest control strategies.

As mentioned in **Chapter 1**, the studies by Martini et al. (2020), Chow et al. (2019) and George et al. (2020a) reinforce the assumption that attract-and-kill is a promising tool for integrated or biological pest management of psyllids, as demonstrated for *D. citri.* Furthermore, to expand the attract-and-kill approach, repellent semiochemicals could be added in order to protect the plant from the vector insect in a push-pull-kill strategy (Cook et al., 2006). Therefore, formulated repellent semiochemicals can be applied at the protected fruit crop. Gallinger et al. (2019) was successful in repelling *C. pruni* with semiochemical blends applied in dispensers on prune trees and demonstrated that the behavior of *C. pruni* can be manipulated in the field by repellent semiochemical mixtures. Those repellent semiochemicals can also be directly applied on the plant in formulations such as the oleogel based formulation developed in the present work (**Chapter 2**).

As it has been reported that a combination of more than one cue is more effective in attracting an insect or generally influencing its behavior, other stimuli should be considered in addition to the attractive volatiles. As illustrated in the state-of-the-art (**Figure 1.4**), different stimuli types can be elements of attract-and-kill or push-pull-kill strategies (Zhang et al., 2013; Eigenbrode et al., 2016; Gregg et al., 2018). New insights into colour preferences of various psyllids, such as *D. citri* (Hall et al., 2007; Wenninger et al., 2009; Sétamou et al., 2014) and pear psyllid *C. pyri* (de Jorge et al., 2019), offer the option to add visual cues in form of preferred colours, e.g. in traps or by colouring the formulation or add UV reflectants (George et al., 2020b). In the case of the oleogel formulation, the addition of lipophilic pigments will work well. Whether the insects also accept or prefer the colored formulations should be investigated in future studies. Also phagostimulants can be added as arresting element in order to arrest the attracted psyllid near to the kill compound, e.g. *Pandora* sp. nov., to improve infection. Based on the studies of Gallinger & Gross (2018) and Görg et al. (2021), who investigated the role of phloem composition on host plant preferences, the preferred sugar and amino acid composition of the target insects *C. pruni* and *C. picta* can be included as a phagostimulant medium. The challenge will be the co-formulation of lipophilic semiochemical compounds and hydrophilic compounds under maintenance

of a desired release of the semiochemical and accessability for the insect and prolonged water retention of the hydrophilic phagostimulant medium.

To conclude, the present thesis provides a set of novel delivery systems for successful application of biopesticides. The formulation and release of active agents with different physico-chemical properties have been investigated in this highly interdisciplinary work at the edge between mycology, biotechnology, bioprocess engineering, chemical ecology, material science and formulation technology. With the oleogel-based formulation developed and patented in this work, a completely new and innovative semiochemical formulation has been introduced, which has a high potential to become an universal delivery system for semiochemicals. Thus, this work will contribute to the establishment of oleogels for agricultural applications. In the second part of this thesis, the major obstacles of the Entomophthorales as biocontrol agents have been successfully addressed investigating a new psyllid-pathogenic *Pandora* species. Based on the present work, the new fungus can be mass-produced and sufficient sporulation can be achieved due to formulation and co-application with the patented paste type formulation based on biopolymers of high water sorptive capacity. Due to their versatility, as the developed formulations can be used as a basic matrix for a variety of active compounds, they have a high potential to be applied in innovative modular attract-and-kill and push-pull-kill strategies for the control of psyllids and other target insects. Thus, this work contributes to a more sustainable and environmentally friendly agriculture by providing alternative control options.

References

Aoki J (1981) Pattern of conidial discharge of an *Entomophthora* species ("*grylli*" type) (Entomophthorales: Entomophthoracea) from infected cadavers of *Mamestra brassicae* L. (Lepidoptera: Noctuidae). Applied Entomology and Zoology 16(3): 216-224.

Ausique JJ, D'Alessandro CP, Conceschi MR, Mascarin GM & Delalibera Jr I (2017) Efficacy of entomopathogenic fungi against adult *Diaphorina citri* from laboratory to field applications. Journal of Pest Science 90(3): 947-960.

Barta M & Cagáň LU (2006) Aphid-pathogenic Entomophthorales (their taxonomy, biology and ecology). Biologia 61(21): 543-616.

Boyce GR, Gluck-Thaler E, Slot JC, Stajich JE, Davis WJ, James TY, Cooley JR, Panaccione DG, Eilenberg J, De Fine Licht HH, Macias AM, Berger MC, Wickert KL, Stauder CM, Spahr EJ, Maust MD, Metheny AM, Simon C, Kritsky G, Hodge KT, Humber RA, Gullion T, Short DPG, Kijimoto T, Mozgai D, Arguedas N, Kasson MT (2019) Psychoactive plant-and mushroom-associated alkaloids from two behavior modifying cicada pathogens. Fungal Ecology 41: 147-164.

Brown GC, Prochaska GL, Hildebrand DF, Nordin GL & Jackson DM (1995) Green leaf volatiles inhibit conidial germination of the entomopathogen *Pandora neoaphidis* (Entomophthorales: Entomophthoraceae). Environmental Entomology 24(6): 1637–1643.

Bruce TJ& Pickett JA (2011) Perception of plant volatile blends by herbivorous insects–finding the right mix. Phytochemistry 72(13): 1605-1611.

Charnley AK & St. Leger RJ (1991) The role of cuticle-degrading enzymes in fungal pathogenesis in insects. In Cole GT & Hoch HC (ed.) The fungal spore and disease initiation in plants and animals. Springer Science & Business Media, New York: 267-286.

Charnley AK (2003) Fungal pathogens of insects: Cuticle degrading enzymes and toxins. Advances in Botanical Research 40: 241-321.

Chow A, Czokajlo D, Patt JM & Sétamou M (2019) Development and field validation of a beta-cyfluthrin-based 'attract-and-kill'device for suppression of Asian citrus psyllid (Hemiptera: Liviidae) on residential citrus. Journal of Economic Entomology 112(6): 2824-2832.

Cook SM, Khan ZR & Pickett JA (2006) The use of push-pull strategies in integrated pest management. Annual Review of Entomology 52: 375-400.

Cox PW, Paul GC & Thomas CR (1998) Image analysis of the morphology of filamentous microorganisms. Microbiology 144(4): 817–827.

Cui YQ, Okkerse WJ, Van der Lans RGJM & Luyben KCA (1998) Modeling and measurements of fungal growth and morphology in submerged fermentations. Biotechnology and Bioengineering 60(2): 216–229.

De Jorge BC, Hummel HE & Gross J (2019) Investigating the role of leaf color and plant semiochemicals on the behaviour of *Cacopsylla pyri*. IOBC-WPRS Bulletin 146: 5-9.

de Lima IJ, Carneiro Leão MP, da Silva Santos AC, da Costa AF & Tiago PV (2021) Production of conidia by entomopathogenic isolates of *Fusarium caatingaense* on different vegetable substrates. Biocontrol Science & Technology 31(2): 206–218.

DeBach P (1964) The scope of biological control. In DeBach P (ed.) Biological Control of Insect Pests and Weeds. Chapman and Hall Ltd., London: 3-20.

Deisig N, Dupuy F, Anton S & Renou M (2014) Responses to pheromones in a complex odor world: Sensory processing and behavior. Insects 5(2): 399–422.

Delalibera Jr I, Demétrio CG, Manly BF & Hajek AE (2006) Effect of relative humidity and origin of isolates of *Neozygites tanajoae* (Zygomycetes: Entomophthorales) on production of conidia from cassava green mite, *Mononychellus tanajoa* (Acari: Tetranychidae), cadavers. Biological Control 39(3): 489-496.

Dwyer G, Elkinton JS & Hajek AE (1998) Spatial scale and the spread of a fungal pathogen of gypsy moth. American Naturalist 152(3): 485–494.

Eigenbrode SD, Birch ANE, Lindzey S, Meadow R & Snyder WE (2016) A mechanistic framework to improve understanding and applications of push-pull systems in pest management. Journal of Applied Ecology 53(1): 202-212.

Eilenberg J, Bresciani J & Latgé JP (1986) Ultrastructural studies of primary spore formation and discharge in the genus *Entomophthora*. Journal of Invertebrate Pathology 48: 318-324.

Eilenberg J, Michelsen V (1999) Natural Host Range and Prevalence of the Genus *Strongwellsea* (Zygomycota: Entomophthorales) in Denmark. Journal of Invertebrate Pathology 73(2): 189–198.

Eilenberg J, Wilding N & Bresciani J (1992) Isolation in vitro of *Strongwellsea castrans* (Fungi: Entomophthorales) a pathogen of adult cabbage root flies, *Delia radicum* (Dipt.: Anthomyiidae) Entomophaga 37(1): 65-77.

European Commission (2022) https://ec.europa.eu/commission/presscorner/detail/en/ip_22_3746; Accessed 07/21/2022.

European Parliament (2009) Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009 establishing a framework for Community action to achieve the sustainable use of pesticides. Official Journal of the European Union 309: 71-86.

Fleischer J & Krieger J (2018) Insect pheromone receptors – key elements in sensing intraspecific chemical signals. Frontiers in Cellular Neuroscience 12: 425.

Frank A, Rath SK & Venkatraman SS (2005) Controlled release from bioerodible polymers: effect of drug type and polymer composition. Journal of Controlled Release 102(2): 333-344.

Freimoser FM, Grundschober A, Aebi M & Tuor U (2000) In vitro cultivation of the entomopathogenic fungus *Entomophthora thripidum*: Isolation, growth requirements and sporulation. Mycologia 92(2): 208–215.

Gallinger J & Gross J (2018) Unraveling the host plant alternation of *Cacopsylla pruni*–adults but not nymphs can survive on conifers due to phloem/xylem composition. Frontiers in Plant Science 9: 484.

Gallinger J, Dippel C & Gross J (2019) Interfering host location of *Cacopsylla pruni* with repellent plant volatiles. IOBC WPRS Bulletin 146: 10-12.

George J, Lapointe SL, Markle LT, Patt JM, Allan SA, Setamou M, Rivera MJ, Qureshi JA & Stelinski LL (2020a) A multimodal attract-and-kill device for the Asian citrus psyllid *Diaphorina citri* (Hemiptera: Liviidae). Insects 11(12): 870.

George J, Paris TM, Allan SA, Lapointe SL & Stelinski LL (2020b) UV reflective properties of magnesium oxide increase attraction and probing behavior of Asian citrus psyllids (Hemiptera: Liviidae). Scientific Reports 10(1): 1-10.

Glare TR, Milner RJ & Chilvers GA (1986) The effect of environmental factors on the production, discharge, and germination of primary conidia of *Zoophthora phalloides* Batko. Journal of Invertebrate Pathology 48(3): 275-283.

Goller F & Esch H (1990) Comparative study of chill-coma temperatures and muscle potentials in insect flight muscles. Journal of Experimental Biology 150(1): 221-231.

Görg LM, Gallinger J & Gross J (2021) The phytopathogen '*Candidatus* Phytoplasma mali' alters apple tree phloem composition and affects oviposition behavior of its vector *Cacopsylla picta*. Chemoecology 31(1): 31-45.

Görg LM, Eilenberg J, Jensen A, Jensen A & Gross J (2021) Pathogenicity against hemipteran vector insects of a novel insect pathogenic fungus from Entomophthorales (*Pandora* sp. nov. inedit.) with potential for biological control. Journal of Invertebrate Pathology 183: 107621.

Gregg PC, Del Socorro AP & Landolt PJ (2018) Advances in attract-and-kill for agricultural pests: Beyond pheromones. Annual Review of Entomology 63: 453-470.

Grell MN, Jensen AB, Olsen PB, Eilenberg J & Lange L (2011) Secretome of fungus-infected aphids documents high pathogen activity and weak host response. Fungal Genetics and Biology 48(4): 343-352.

Gross J, Gallinger J & Görg LM (2021) Interactions between phloem restricted bacterial plant pathogens, their vector insects, host plants, and natural enemies mediated by primary and secondary plant metabolites. Entomologia Generalis 42(2): 185-215.

Gryganskyi AP, Humber RA, Smith ME, Hodge K, Huang B, Voigt K & Vilgalys R (2013) Phylogenetic lineages in Entomophthoromycota. Persoonia: Molecular Phylogeny and Evolution of Fungi 30(1): 94–105.

Hajek AE & Roberts DW (1991) Pathogen reservoirs as a biological control resource: Introduction of *Entomophaga maimaiga* to North American gypsy moth, *Lymantria dispar*, populations. Biological Control 1(1): 29–34.

Hajek AE & St. Leger RJ (1994) Interactions between fungal pathogens and insect hosts. Annual Review of Entomology 39(1): 293–322.

Hajek AE, Carruthers RI & Soper RS (1990) Temperature and moisture relations of sporulation and germination by *Entomophaga maimaiga* (Zygomycetes: Entomophthoraceae), a fungal pathogen of *Lymantria dispar* (Lepidoptera: Lymantriidae). Environmental Entomology 19(1): 85-90.

Hajek AE, Papierok B & Eilenberg J (2012) Methods for study of the Entomophthorales. In Lacey LA (ed.) Methods in Insect Pathology, Academic Press Amsterdam, 2nd Edition: 285-316.

Hajek AE, Wheeler MM, Eastburn CC & Bauer LS (2001) Storage of resting spores of the gypsy moth fungal pathogen, *Entomophaga maimaiga*. Biocontrol Science & Technology 11(5): 637–647.

Hall DG (2009) An assessment of yellow sticky card traps as indicators of the abundance of adult *Diaphorina citri* (Hemiptera: Psyllidae) in citrus. Journal of Economic Entomology 102(1): 446–452.

Hall DG, Hentz MG & Ciomperlik MA (2007) A comparison of traps and stem tapsampling for monitoring adult Asian citrus psyllid (Hemiptera: Psyllidae) in citrus. Florida Entomologist 90(2): 327-334.

Hansen CM (2007) Hansen solubility parameters: A user's handbook, CRC Press, Boca Raton, Florida, USA.

Hemmati F, Pell J, Mccartney H & Deadman M (2001b) Airborne concentrations of conidia of *Erynia neoaphidis* above cereal fields. Mycological Research 105(4): 485-489.

Hogenhout SA, Oshima K, Ammar ED, Kakizawa S, Kingdom HN & Namba S (2008) Phytoplasmas: Bacteria that manipulate plants and insects. Molecular Plant Pathology 9(4): 403-423.

Huang W, Reyes-Caldas P, Mann M, Seifbarghi S, Kahn A, Almeida RP, Béven L, Heck M, Hogenhout SA & Coaker G (2020) Bacterial vector-borne plant diseases: Unanswered questions and future directions. Molecular Plant 13(10): 1379-1393.

Humber RA (1984) Foundations for an evolutionary classification of the Entomophthorales (Zygomycetes). In Wheeler Q & Blackwell M (ed.) Fungus-insect relationships: Perspectives in ecology and evolution. Columbia University Press: 166-183.

Humber RA (2008) Evolution of entomopathogenicity in fungi. Journal of Invertebrate Pathology 98(3): 262-266.

Inglis GD, Goettel MS, Butt TM & Strasser H (2001) Use of hyphomycetous fungi for managing insect pests. In Butt TM, Jackson C, Magan N (ed.) Fungi as biocontrol agents: Progress, problems and potential, CABI Publishing, Wallingford, Oxon, UK: 23.

Iwanicki NSA, Delalibera Junior I, Eilenberg J & De Fine Licht HH (2020) Comparative RNAseq analysis of the insect-pathogenic fungus *Metarhizium anisopliae* reveals specific transcriptome

signatures of filamentous and yeast-like development. G3: Genes, Genomes, Genetics 10(1): 2141–2157.

Jackson MA, Dunlap CA & Jaronski ST (2010) Ecological considerations in producing and formulating fungal entomopathogens for use in insect biocontrol. BioControl 55(1): 129-145.

Jaques RP & Patterson NA (1962) Control of the apple sucker, *Psylla mali* Schmidb., by the fungus *Entomophthora sphaerosperma* (Fresenius). The Canadian Entomologist 94(8): 818-825.

Jarausch B, Tedeschi R, Sauvion N, Gross J, Jarausch W (2019) Psyllid Vectors. In Bertaccini A, Weintraub PG, Rao GP, Mori N (ed) Phytoplasmas: Plant Pathogenic Bacteria - II, vol 92. Springer Singapore, Singapore: 53–78

Jaronski ST (2014) Mass production of entomopathogenic fungi: State of the art. In Morales-Ramos JA, Guadalupe Rojas M & Shapiro-Ilan DI (ed.) Mass production of beneficial organisms, Elsevier Inc., Amsterdam: 357-413.

Jensen AH (2017) A new insect pathogenic fungus from Entomophthorales with potential for psyllid control, MSc thesis, Department of Plant and Environmental Sciences, University of Copenhagen, Denmark.

Jensen AB, Thomsen L & Eilenberg J (2001) Intraspecific variation and host specificity of *Entomophthora muscae* sensu stricto isolates revealed by random amplified polymorphic DNA, universal primed PCR, PCR-restriction fragment length polymorphism, and conidial morphology. Journal of Invertebrate Pathology 78(4): 251-259.

Jensen AH, Gross J, Jensen AB, Gallinger J & Eilenberg J (2018) A new insect pathogenic fungus from Entomophthorales with potential for psyllid control. Mitteilungen der Deutschen Gesellschaft für allgemeine und angewandte Entomologie 21: 283-286.

Kalsbeek V, Pell JK & Steenberg T (2001) Sporulation by *Entomophthora schizophorae* (Zygomycetes: Entomophthorales) from housefly cadavers and the persistence of primary conidia at constant temperatures and relative humidities. Journal of Invertebrate Pathology 77(3): 149-157.

Keller S (2007) Systematics, taxonomy and identification. In Keller S (ed.) Arthropod-pathogenic Entomophthorales: Biology, ecology, identification. Office for Official Publications of the European Communities: 110-132.

Knight AL, Larsen TE & Ketner KC (2004) Rainfastness of a microencapsulated sex pheromone formulation for codling moth (Lepidoptera: Tortricidae). Journal of Economic Entomology 97(6): 1987-1992.

Köhl J, Booij K, Kolnaar R & Ravensberg WJ (2019) Ecological arguments to reconsider data requirements regarding the environmental fate of microbial biocontrol agents in the registration procedure in the European Union. BioControl 64(5): 469-487.

Krell V, Jakobs-Schönwandt D, Vidal S & Patel AV (2018) Encapsulation of *Metarhizium brunneum* enhances endophytism in tomato plants. Biological Control 116: 62-73.

Krell V, Jakobs-Schönwandt D, Vidal S & Patel AV (2018) Cellulase enhances endophytism of encapsulated *Metarhizium brunneum* in potato plants. Fungal Biology 122(5): 373–378.

Latgé JP (1981) Comparaison des exigences nutritionnelles des Entomophthorales. Annales de Microbiologie 132(3): 299–306.

Latgé JP, Perry DF, Prévost MC & Samson RA (1989) Ultrastructural studies of primary spores of *Conidiobolus*, *Erynia*, and related Entomophthorales. Canadian Journal of Botany 67(9): 2576-2589.

Latgé JP, Silvie P, Papierok B, Remaudière G, Dedryver C & Rabasse J (1983) Advantages and disadvantages of *Conidiobolus obscurus* and of *Erynia neoaphidis* in the biological control of aphids. Aphid Antagonists, AA Balkema, Rotterdam: 20-32.

Leite LG, Alves SB, Batista Filh A & Roberts DW (2005) Simple, inexpensive media for mass production of three entomophthoralean fungi. Mycological Research 109(3): 326–334.

Leite LG, Alves SB, Batista Filho A & Roberts DW (2003) Effect of salts, vitamins, sugars and nitrogen sources on the growth of three genera of entomophthorales: *Batkoa*, *Furia* and *Neozygites*. Mycological Research 107(7): 872–878.

Li Z, Butt TM, Beckett A & Wilding N (1993) The structure of dry mycelia of the entomophthoralean fungi *Zoophthora radicans* and *Erynia neoaphidis* following different preparatory treatments. Mycological Research 97(11): 1315–1323.

Małagocka J, Grell MN, Lange L, Eilenberg J & Jensen AB (2015) Transcriptome of an entomophthoralean fungus (*Pandora formicae*) shows molecular machinery adjusted for successful host exploitation and transmission. Journal of Invertebrate Pathology 128: 47-56.

Mao L, Lu Y, Cui M, Miao S & Gao Y (2020) Design of gel structures in water and oil phases for improved delivery of bioactive food ingredients. Critical Reviews in Food Science & Nutrition 60(10): 1651-1666.

Martini X, Hoyte A, Mafra-Neto A, Aksenov AA, Davis CE & Stelinski LL (2020) Progress toward an attract-and-kill device for Asian citrus psyllid (Hemiptera: Liviidae) using volatile signatures of citrus infected with Huanglongbing as the attractant. Journal of Insect Science 20(6): 25.

Mascarin GM, Kobori NN, Jackson MA, Dunlap CA & Delalibera Jr I (2018) Nitrogen sources affect productivity, desiccation tolerance and storage stability of *Beauveria bassiana* blastospores. Journal of Applied Microbiology 124(3): 810–820.

Muskat LC & Patel AV (2022) Innovations in semiochemical formulations. Entomologia Generalis 42(2): 231–249.

Muskat LC, Patel AV (2022) Carrier material for living biologicals and preparation thereof. EP 22 159 230.6.

Nelis V, De Neve L, Danthine S, Dewettinck K, Martins J C & Van der Meeren P (2021) Oil diffusion in fat crystal matrices: Characterization by NMR relaxometry and diffusometry. European Journal of Lipid Science & Technology 123(4): 2000237.

Nielsen MC, Sansom CE, Larsen L, Worner SP, Rostás M, Chapman RB, Butler RC, de Kogel WJ, Davidson MM, Perry NB & Teulon DA (2019) Volatile compounds as insect lures: Factors affecting release from passive dispenser systems. New Zealand Journal of Crop and Horticultural Science 47(3): 208-223.

Nolan RA (1990) Enhanced hyphal body production by *Entomophaga aulicae* protoplasts in the presence of a neutral and a positively charged surface under mass fermentation conditions. Canadian Journal of Botany-Revue Canadienne De Botanique 68(2): 2708–2713.

Nolan RA (1991) Influence of a negatively charged surface (teflon disk) on *Entomophaga aulicae* protoplast morphogenesis under mass fermentation conditions. Canadian Journal of Botany-Revue Canadienne De Botanique 69(11): 2578–2581.

Nussinovitch A (2010) Polymer macro- and micro-gel beads: Fundamentals and applications. Springer, New York.

Olsen NL, Herren P, Markussen B, Jensen AB & Eilenberg J (2019) Statistical modelling of conidial discharge of entomophthoralean fungi using a newly discovered *Pandora* species. PloS One 14(5): e0215914.

O'Sullivan CM, Barbut S & Marangoni AG (2016) Edible oleogels for the oral delivery of lipid soluble molecules: Composition and structural design considerations. Trends in Food Science & Technology 57: 59-73.

Page RM & Humber RA (1973) Phototropism in *Conidiobolus coronatus*. Mycologia 65(2): 335-354.

Pakseresht S & Mazaheri Tehrani M (2022) Advances in multi-component supramolecular oleogels - A review. Food Reviews International 38(4): 760-782.

Papierok B & Hajek AE (1997) Fungi: Entomophthorales. In Lacey LA (ed.) Manual of techniques in insect pathology. Academic Press, San Diego, CA: 187-212.

Patel AV, Jakobs-Schönwandt D, Rose T & Vorlop KD (2011) Fermentation and microencapsulation of the nematophagous fungus *Hirsutella rhossiliensis* in a novel type of hollow beads. Applied Microbiology and Biotechnology 89(6): 1751–1760.

Patel AV, Muskat LC, Humbert P (2019) Neue Formulierung auf Basis eines Oleogels insbesondere zur Freisetzung von volatilen Komponenten und Verfahren zu dessen Herstellung. DE 10 2019 119 888.7.

Pell JK, Eilenberg J, Hajek AE & Steinkraus D (2001) Biology, ecology and pest management potential of Entomophthorales. In Butt TM, Jackson C and Magan N (ed.) Fungi as biocontrol agents: progress, problems and potential, CABI: 71-154.

Pell JK, Macaulay EDM and Wilding N (1993) A pheromone trap for dispersal of the pathogen, *Zoophthora radicans* Brefeld (Zygomycetes: Entomophthorales) amongst populations of the diamondback moth, *Plutella xylostella* L. (Lepidoptera: Yponomeutidae). Biocontrol Science & Technology 3(3): 315–320.

Prüsse U, Bilancetti L, Bučko M, Bugarski B, Bukowski J, Gemeiner P, Lewińska D, Manojlovic V, Massart B, Nastruzzi C, Nedovic V, Poncelet D, Siebenhaar S, Tobler L, Tosi A, Vikartovská A & Vorlop KD (2008) Comparison of different technologies for alginate beads production. Chemical Papers 62(4): 364-374.

Przyklenk M, Vemmer M, Hanitzsch M & Patel AV (2017) A bioencapsulation and drying method increases shelf life and efficacy of *Metarhizium brunneum* conidia. Journal of Microencapsulation 34(5): 498-512.

Przyklenk M (2017) Encapsulation of *Metarhizium brunneum* as basis for an attract-and-kill strategy to control herbivorous soil insects, Doctoral dissertation, Faculty of Biology, University of Bielefeld, Germany.

Rejeb A, Abdollahi A, Rejeb K & Treiblmaier H (2022) Drones in agriculture: A review and bibliometric analysis. Computers and Electronics in Agriculture 198: 107017.

Renou M (2014) Pheromones and general odor perception in insects. In Mucignat-Caretta C (ed.) Neurobiology of chemical communication, CRC Press, Taylor & Francis, Boca Raton, FL: 23–55.

Russell R & Paterson M (1982) Effects of variations in nitrogen and carbon sources on the physiology of growth of *Erynia radicans*, a potential mycoinsecticide. Biotechnology Letters 4(2): 115–120.

Scheepmaker JW, Busschers M, Sundh I, Eilenberg J & Butt TM (2019) Sense and nonsense of the secondary metabolites data requirements in the EU for beneficial microbial control agents. Biological Control 136: 104005.

Scher HB (1977) Microencapsulated pesticides. In Scher H (ed.) Controlled Release Pesticides. ACS Publications, Washington, DC, USA: 126-144.

Seemüller E & Schneider B (2004) '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri' and '*Candidatus* Phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. International Journal of Systematic and Evolutionary Microbiology 54(4): 1217–1226.

Sétamou M, Sanchez A, Saldaña RR, Patt JM & Summy R (2014) Visual responses of adult Asian citrus psyllid (Hemiptera: Liviidae) to colored sticky traps on citrus trees. Journal of Insect Behavior 27(4): 540-553.

Shah P & Pell JK (2003) Entomopathogenic fungi as biological control agents. Applied Microbiology and Biotechnology 61(5): 413-423.

Shah PA, Aebi M & Tuor U (1999) Production factors involved in the formulation of *Erynia neoaphidis* as alginate granules. Biocontrol Science & Technology 9(1): 19-28.

Shi R, Zhang Q, Vriesekoop F, Yuan Q & Liang H (2014) Preparation of organogel with tea polyphenols complex for enhancing the antioxidation properties of edible oil. Journal of Agricultural and Food Chemistry 62(33): 8379-8384.

Speiser B, Tamm L, Leschenne V, Kretzschmar U, Brändle I, Maurer V, Schneider C, Dittmann M, Berger T (2022) Betriebsmittelliste 2022 für den ökologischen Landbau in der Schweiz, Forschungsinstitut für biologischen Landbau FiBL, Frick, Switzerland 2022.

Taylor LR (1963) Analysis of the effect of temperature on insects in flight. The Journal of Animal Ecology: 99-117.

Taylor N & Matthews GA (1986) Effect of different adjuvants on the rainfastness of bendiocarb applied to Brussels sprout plants. Crop Protection 5(4): 250-253.

Vemmer M & Patel AV (2013) Review of encapsulation methods suitable for microbial biological control agents. Biological Control 67(3): 380-389.

Webster J & Weber R (2007) Introduction to Fungi. Cambridge University Press.

Weintraub PG & Beanland L (2006) Insect vectors of phytoplasmas. Annual Review of Entomology 51: 91-111.

Wenninger EJ, Stelinski LL & Hall DG (2009) Role of olfactory cues, visual cues, and mating status in orientation of *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) to four different host plants. Environmental Entomology 38(1): 225-234.

Weseloh RM & Andreadis TG (1992) Epizootiology of the fungus *Entomophaga maimaiga*, and its impact on gypsy moth populations. Journal of Invertebrate Pathology 59(2): 133-141.

Wraight SP, Galaini-Wraight S, Carruthers RI & Roberts DW (2003) *Zoophthora radicans* (Zygomycetes: Entomophthorales) conidia production from naturally infected *Empoasca kraemeri* and dry-formulated mycelium under laboratory and field conditions. Biological Control 28(1): 60–77.

Wucherpfennig T, Hestler T & Krull R (2011) Morphology engineering – osmolality and its effect on *Aspergillus niger* morphology and productivity. Microbial Cell Factories 10(1): 58.

Xu J, Baldwin D, Kindrachuk C & Hegedus DD (2006) Serine proteases and metalloproteases associated with pathogenesis but not host specificity in the Entomophthoralean fungus *Zoophthora radicans*. Canadian Journal of Microbiology 52(6): 550-559.

Yoon JY, Yang CY & Kim JD (2017) Sustainable delivery of a sex pheromone with an ester wax to disrupt *Grapholita molesta* mating. Macromolecular Research 25(4): 374-380.

Zhang Z, Sun X, Luo Z, Gao Y & Chen Z (2013) The manipulation mechanism of “push–pull” habitat management strategy and advances in its application. Acta Ecologica Sinica 33(2): 94-101.

Zhou X & Feng MG (2009) Sporulation, storage and infectivity of obligate aphid pathogen *Pandora nouryi* grown on novel granules of broomcorn millet and polymer gel. Journal of Applied Microbiology 107(6): 1847-1856.

Author information

List of publications

Peer-reviewed publications

Muskat, L.C. & Patel, A.V. (2022). Innovations in semiochemical formulations. Entomologia Generalis, 42(2): 231–249. https://doi.org/10.1127/entomologia/2021/1230

Muskat, L.C., Jiang L., Rostás M., Patel A.V. (2022) Development of a self-sticking oleogel formulation for the slow release of semiochemicals. Macromolecular Materials and Engineering, 307, 2200276. https://doi.org/10.1002/mame.202200276

Muskat, L.C., Przyklenk, M., Humbert, P., Eilenberg, J. and Patel, A.V. (2022) Fermentation of the psyllid-pathogenic fungus *Pandora* sp. nov. inedit. (Entomophthorales: Entomophthoraceae), Biocontrol Science & Technology, 32:5, 564-585. https://doi.org/10.1080/09583157.2022.2035680

Muskat, L.C., Görg, L.M., Humbert, P., Gross, J., Eilenberg, J. and Patel, A.V. (2022). Encapsulation of the psyllid-pathogenic fungus *Pandora* sp. nov. inedit. and experimental infection of target insects. Pest Management Science, 78: 991-999. https://doi.org/10.1002/ps.6710

Muskat, L.C., Kerkhoff, Y., Humbert, P., Nattkemper, T.W., Eilenberg, J., & Patel, A.V. (2021). Image analysis-based quantification of fungal sporulation by automatic conidia counting and gray value correlation. MethodsX, 8, 101218. https://doi.org/10.1016/j.mex.2021.101218

Patents

Patel, A.V., Muskat, L.C., Humbert P. (2019). New formulation based on an oleogel, especially for the release of volatile components and a process for its production. WO2021013917A1

Muskat, L.C., Patel A.V. (2022) Carrier material for living biologicals and preparation thereof. EP 22 159 230.6.

Oral Presentations

Muskat, L.C., Görg, L.M., Przyklenk, M., Kais, B., Humbert, P., Gross, J., Eilenberg, J., Patel, A.V. (2021). Entwicklung einer Formulierung für die biologische Bekämpfung von Psylliden mit dem entomopathogenen Pilz *Pandora* sp. nov. (ARSEF13372). 62. Deutsche Pflanzenschutztagung; Göttingen, Germany/virtual

Muskat, L.C., Patel, A.V. (2021). Entwicklung einer neuen Formulierung für die temperatur-gesteuerte Freisetzung von Semiochemikalien zur Verhaltensmanipulation von Schad- und Nutzinsekten. 62. Deutsche Pflanzenschutztagung; Göttingen, Germany/virtual

Kais, B., Muskat, L.C., Kind, S., Görg, L.M., Eilenberg, J., Patel, A.V., Gross, J. (2021). Wirksamkeitstests mit *Pandora* sp. nov. (ARSEF 13372) - Formulierungen zur Psyllidenbekämpfung in Halbfreilandversuchen. 62. Deutsche Pflanzenschutztagung; Göttingen, Germany/virtual

Muskat, L.C., Görg, L.M., Kais, B., Przyklenk, M., Gross, J., Eilenberg, J., Patel, A.V. (2021). Fermentation and formulation of *Pandora* sp. nov. for biological psyllid pest control. Invited Talk at the 2021 International Congress on Invertebrate Pathology and Microbial Control & 53rd Annual Meeting of the Society for Invertebrate Pathology; Merida, Mexico/virtual

Muskat, L.C., Görg, L.M., Kais, B., Przyklenk, M., Gross, J., Eilenberg, J., Patel, A.V. (2021). Development of Attract-and-Kill formulations for the biological control of psyllid pests in fruit orchards. 23. Jahrestagung Arbeitskreis Biologischer Pflanzenschutz (DPG); virtual

Muskat, L.C., Humbert, P., Eilenberg, J., Patel, A.V. (2019). Development of formulations to control psyllid pests in fruit orchards with the entomopathogenic fungus *Pandora* sp. 52nd Annual Meeting of the Society for Invertebrate Pathology & 17th Meeting of the IOBC-WPRS Working Group "Microbial and Nematode Control of Invertebrate pests"; Valencia, Spain

Muskat, L.C., Humbert, P., Görg, L.M., Gross, J., Dippel, C., Schulke, J., Patel, A.V. (2019). Slow release of semiochemicals for biological psyllid pest control. 46th Controlled Release Society Annual Meeting; Valencia, Spain

Muskat, L.C., Humbert, P., Görg, L.M., Gross, J., Dippel, C., Przyklenk, M., Beitzen-Heineke, E., Beitzen-Heineke, W., Herren, P., Jensen, A.H., Jensen, A.B., Eilenberg, J., Patel, A.V. (2019). Fermentation and Formulation of the entomopathogenic fungus *Pandora* sp. (ARSEF 13372) for the biological control of *Cacopsylla picta*. 22. Jahrestagung Arbeitskreis Biologischer Pflanzenschutz (DPG); Bielefeld, Germany

Muskat, L.C., Humbert, P., Kerkhoff, Y., Nattkemper, T.W., Eilenberg, J., Patel, A.V. (2018). Development of a computer-assisted method for the quantification of discharged conidia of an entomopathogenic fungus with potential for biological psyllid pest control. 61. Deutsche Pflanzenschutztagung; Hohenheim, Germany

Muskat, L.C., Humbert, P., Görg, L. M., Gross, J., Dippel, C., Przyklenk, M., Beitzen-Heineke, E., Beitzen-Heineke, W., Herren, P., Jensen, A.H., Jensen, A.B., Eilenberg, J., Patel, A.V. (2018). Fermentation and formulation of the entomopathogenic fungus *Pandora* sp. (ARSEF13372) for biological control of *Cacopsylla picta*. 61. Deutsche Pflanzenschutztagung; Hohenheim, Germany

Poster presentations

Muskat, L.C. & Patel, A.V. (2021). Automatische Quantifizierung der Sporulation entomopathogener Pilze mittels computergestützter Bildanalyse. Digitale Innovationen: Fachkongress für eine nachhaltige Entwicklung, Bielefeld, Germany

Muskat, L.C., Kerkhoff, Y., Humbert, P., Nattkemper, T. W., Eilenberg, J., Patel, A.V. (2021). Automatische Quantifizierung der Sporulation entomopathogener Pilze mittels computergestützter Bildanalyse. 62. Deutsche Pflanzenschutztagung; Göttingen/virtual *awarded*

Muskat, L.C., Kais, B., Gross, J., Patel, A.V. (2021). Biobasierte Superabsorber für die verbesserte Sporulation des entomopathogenen Pilzes *Pandora* sp. nov. (ARSEF13372) bei geringer Luftfeuchtigkeit. 62. Deutsche Pflanzenschutztagung; Göttingen, Germany/virtual

Muskat, L.C., Jakobs-Schönwandt, D., Brikmann, J., Schöning, S., Hüsgen, B., Schröder, C., Patel, A.V. (2021). Entwicklung neuer Extrusions- und Trocknungverfahren für biobasierte Granulate. 62. Deutsche Pflanzenschutztagung; Göttingen/virtual

Muskat, L.C., Humbert, P., Görg, L.M, Kais, B., Beitzen-Heineke, E., Beitzen-Heineke, W., Przyklenk, M., Jensen, A.H., Dippel, C., Eilenberg, J., Gross, J., Patel, A.V. (2020). Development of novel formulations for behavior manipulating strategies for the biological control of *Cacopsylla picta*, the vector of apple proliferation. Innovationstage 2020 digital, BLE; virtual

Muskat, L.C., Humbert, P., Görg, L.M., Gross, J., Dippel, C., Schulke, J., Patel, A.V. (2019). Development of hydrophobic formulations for the slow release of semiochemicals. 26th International Conference on Bioencapsulation; Strasbourg, France *awarded*

Przyklenk, M., Beitzen-Heineke, E., Muskat, L.C., Görg, L.M., Humbert, P., Dippel, C., Gross, J., Patel, A.V., Beitzen-Heineke, W. (2019). Liquid fermentation and encapsulation of the entomophthoralean fungus *Pandora* sp. 26th International Conference on Bioencapsulation; Strasbourg, France

Muskat, L.C., Humbert, P., Görg, L.M., Gross, J., Dippel, C., Schulke, J., Patel, A.V. (2019). Slow release of semiochemicals for biological psyllid pest control. 52nd Annual Meeting of the Society for Invertebrate Pathology & 17th Meeting of the IOBC-WPRS Working Group "Microbial and Nematode Control of Invertebrate pests”; Valencia, Spain *awarded*

Muskat, L.C., Humbert, P., Kerkhoff, Y., Nattkemper, T.W., Eilenberg, J., Patel, A.V. (2019). Development of a computer-assisted method for the quantification of discharged conidia of an entomopathogenic fungus. 52nd Annual Meeting of the Society for Invertebrate Pathology & 17th Meeting of the IOBC-WPRS Working Group "Microbial and Nematode Control of Invertebrate pests”; Valencia, Spain

Muskat, L.C., Humbert, P., Görg, L.M., Gross, J., Dippel, C., Schulke, J., Patel, A.V. (2019). Slow release of semiochemicals for biological psyllid pest control. 46th Controlled Release Society Annual Meeting; Valencia, Spain

Muskat, L.C., Humbert, P., Görg, L.M., Gross, J., Dippel, C., Przyklenk, M., Beitzen-Heineke, E., Beitzen-Heineke, W., Herren, P., Jensen, A.H., Jensen, A.B., Eilenberg, J., Patel, A.V. (2018). Das Projekt PICTA-KILL - Entwicklung neuartiger Formulierungen für verhaltensmanipulierende Strategien zur biologischen Bekämpfung von *Cacopsylla picta*, dem Überträger der Apfeltriebsucht. 61. Deutsche Pflanzenschutztagung; Hohenheim, Germany

Muskat, L.C., Humbert, P., Görg, L.M., Gross, J., Dippel, C., Przyklenk, M., Beitzen-Heineke, E., Beitzen-Heineke, W., Herren, P., Jensen, A.H., Jensen, A.B., Eilenberg, J., Patel, A.V. (2018). The project PICTA-KILL - Novel strategies for biological psyllid pest control. 50th Annual Meeting of the Society for Invertebrate Pathology; San Diego, California, USA

Muskat, L.C., Humbert, P., Görg, L.M., Gross, J., Dippel, C., Przyklenk, M., Beitzen-Heineke, E., Beitzen-Heineke, W., Herren, P., Jensen, A.H., Jensen, A.B., Eilenberg, J., Patel, A.V. (2017). The project PICTA-KILL - Novel strategies for biological psyllid pest control. Tropentag 2017; Bonn, Germany

Declarations

I, hereby, declare that this Ph.D. thesis has not been presented to any other examining body either in its present or a similar form.

Furthermore, I also affirm that I have not applied for a Ph.D. at any other higher school of education.

Bielefeld, 22nd July 2022

Linda Muskat

I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorised aid.

Bielefeld, 22nd July 2022

Linda Muskat