

Project PICTA-KILL



Development of novel formulations for behavior manipulating strategies for the biological control of *Cacopsylla picta*, the vector of apple proliferation

Entwicklung neuartiger Formulierungen für verhaltensmanipulierende Strategien zur biologischen Bekämpfung von *Cacopsylla picta*, dem Überträger der Apfeltriebsucht

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Background

Psyllid pests are distributed all over the world causing damage in different crop and fruit cultures by serving as vector of phytoplasmosis diseases. One of these psyllids damaging european apple trees is the summer apple psyllid (*Cacopsylla picta*).

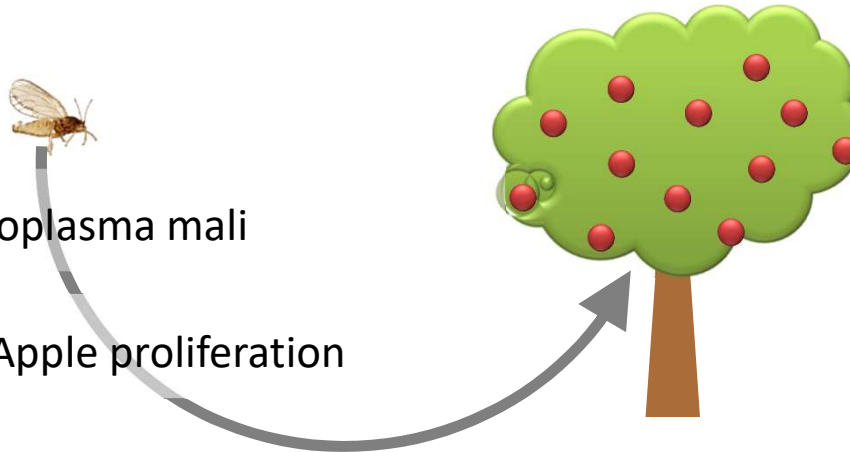
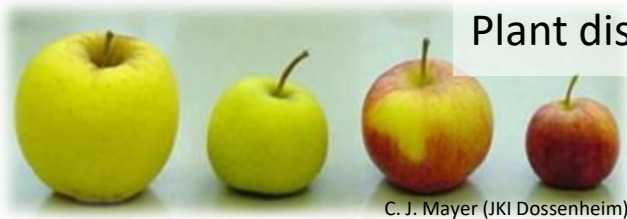


Vector insect: ***Cacopsylla picta***

Causing agent: *Candidatus* Phytoplasma mali

Plant disease: Apple proliferation

Host plant *Malus domestica*



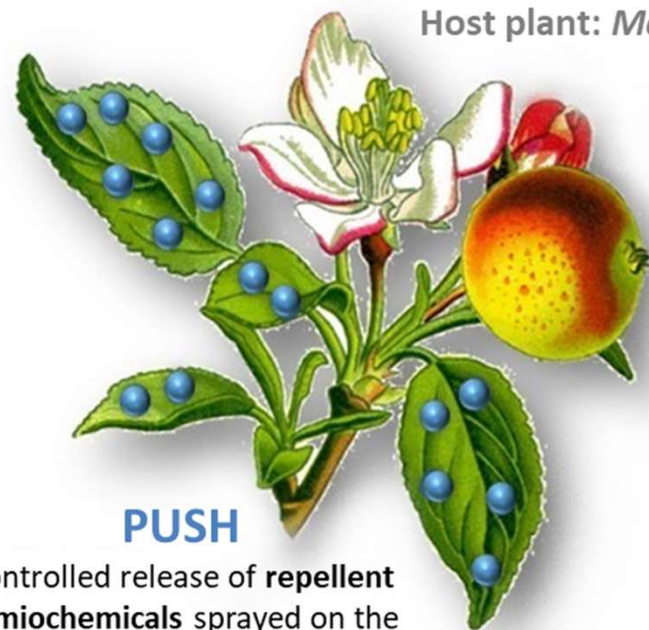
There are no direct measures to combat phytoplasmosis bacteria.

➤ The vector itself has to be controlled.

BUT: The use of chemical insecticides is restricted by low efficacy

OVERALL AIM OF THE PROJECT:

Development of innovative formulations for the release of active compounds of high specificity and low impact on non-target organisms for use in attract-and-kill strategies to combat *Cacopsylla picta* in apple orchards

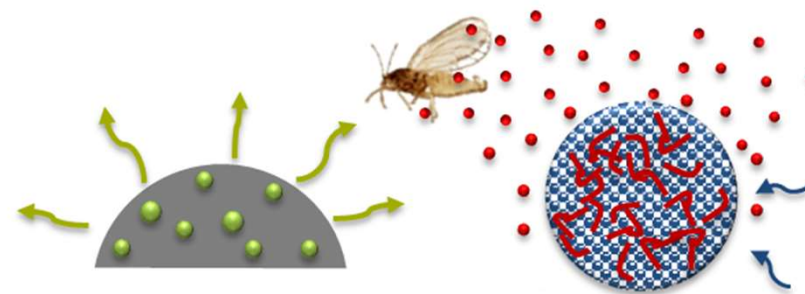


Host plant: *Malus domestica*

PUSH

Controlled release of **repellent semiochemicals** sprayed on the apple tree to push the psyllid pest away from the plant

Insect pest: *Cacopsylla picta*



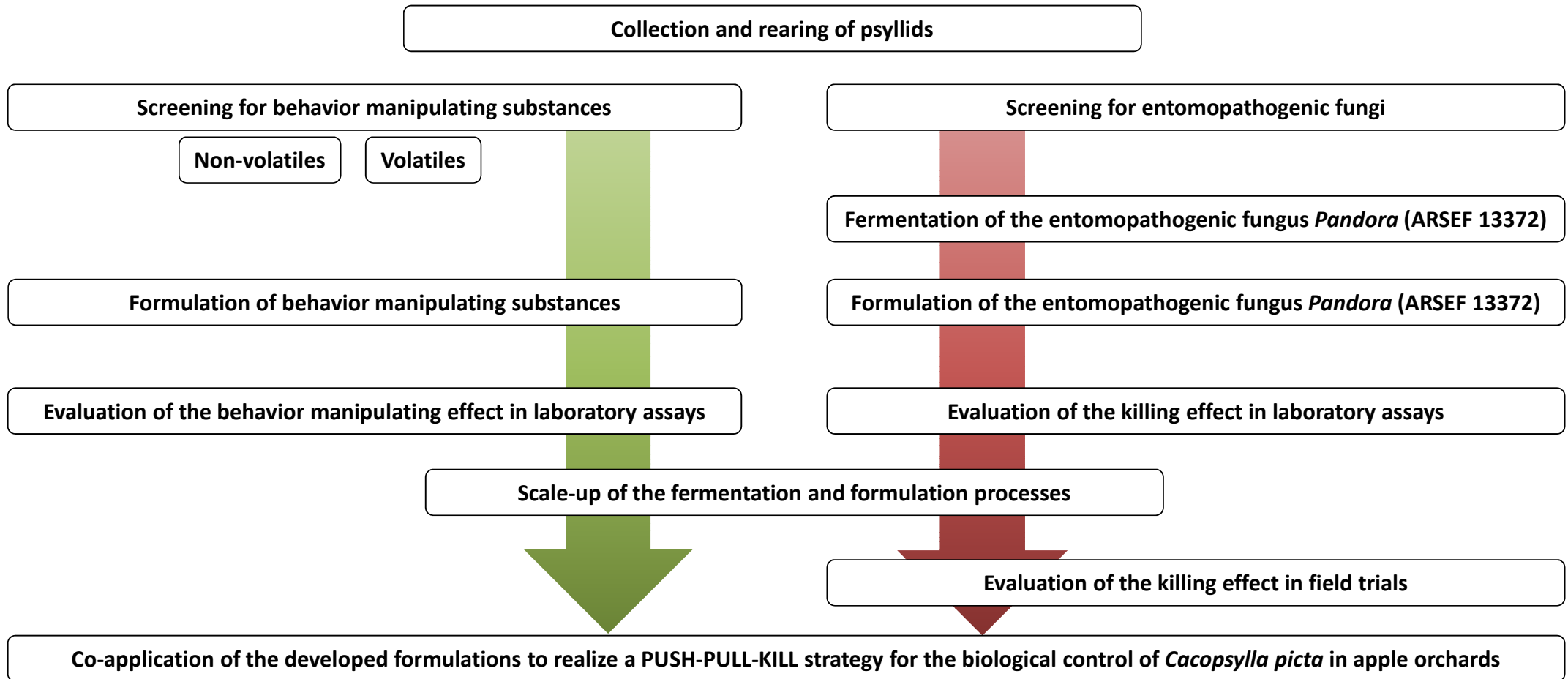
ATTRACT

Controlled release of **β -caryophyllene** to pull the psyllid to the KILL-formulation

KILL

Actively discharged conidia of the entomopathogenic fungus *Pandora* sp. (Entomophthorales)

Project structure

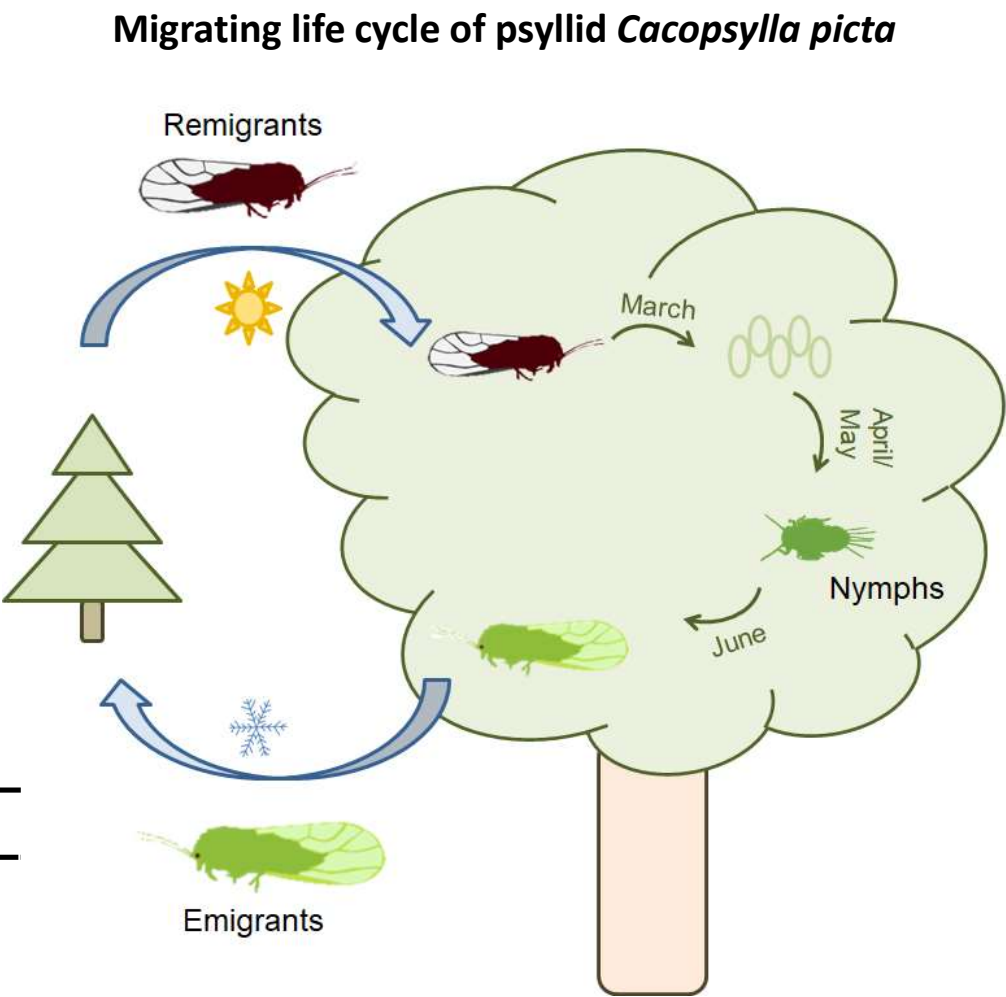


Collection and rearing of psyllids



Collection of *C. picta* remigrants
by beating tray method in extensive
apple orchards in Dossenheim, Germany;
Haiming, Austria and Trentino, Italy

Σ <i>C. picta</i>	♀	♂
2017	29	12
2018	63	19
2019	432	359



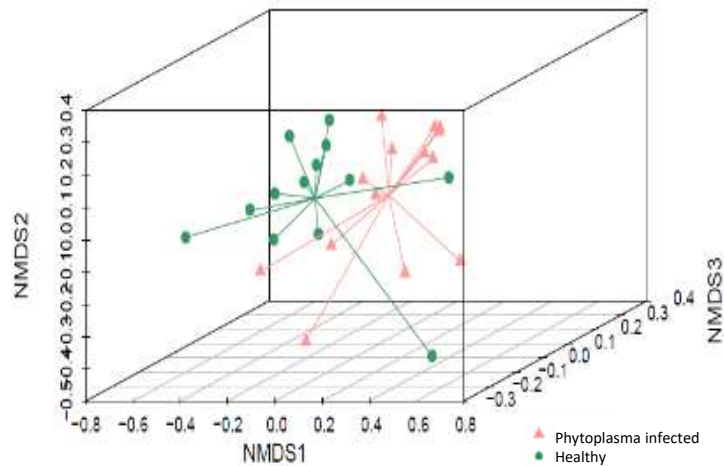
Mating, oviposition and rearing on potted apple plants for one generation

Screening for non-volatile behavior manipulating substances

There is a difference in phloem composition of healthy and phytoplasma infected apple trees.



Phloem sap of healthy and phytoplasma infected apple trees was analyzed after derivatization via gas chromatography coupled with mass spectrometry (GC-MS)



NMDS-Plot, stress =0.10
PERMANOVA df=1, $R^2 = 31.90$, $P < 0.001$ ***

Compounds responsible for the discrimination between healthy and phytoplasma infected apple tree phloem:

Alanine	***
Asparagine	**
Aspartic acid	***
Citric acid	***
Fructose	***
Galactose	***
Glucose	***
Malic acid	***
Maltose	**
Phosphoric acid	***
Serine	***
Shikimic acid	**
Sorbitol	***
Threonine	***

- Phloem sap composition of healthy apple trees differs from phytoplasma infected trees
- Increased amounts of sugars and sugar alcohols in phytoplasma infected trees

➤ Screened compounds =
potential
semiochemicals for
encapsulation

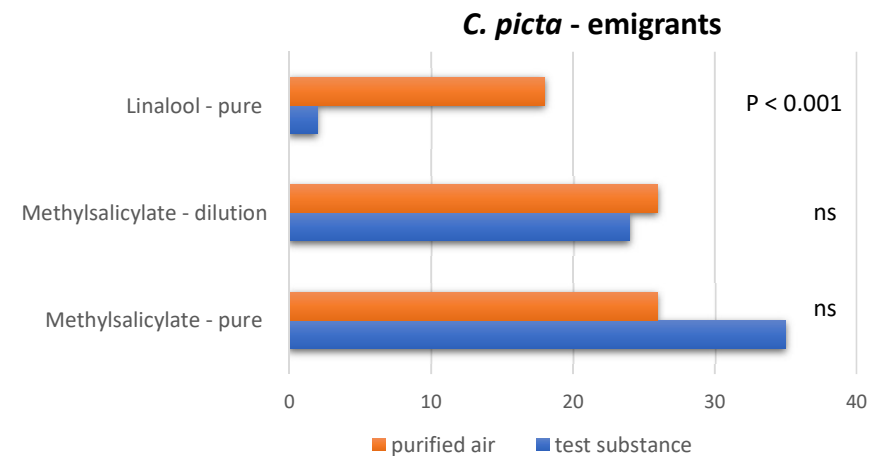
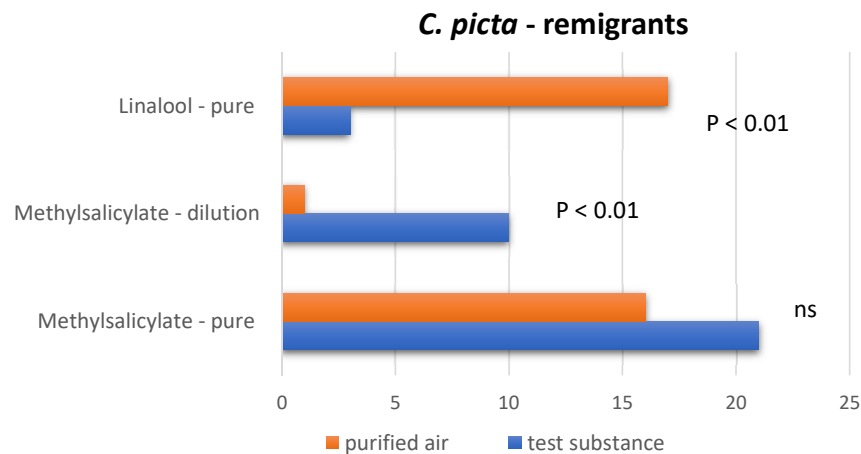
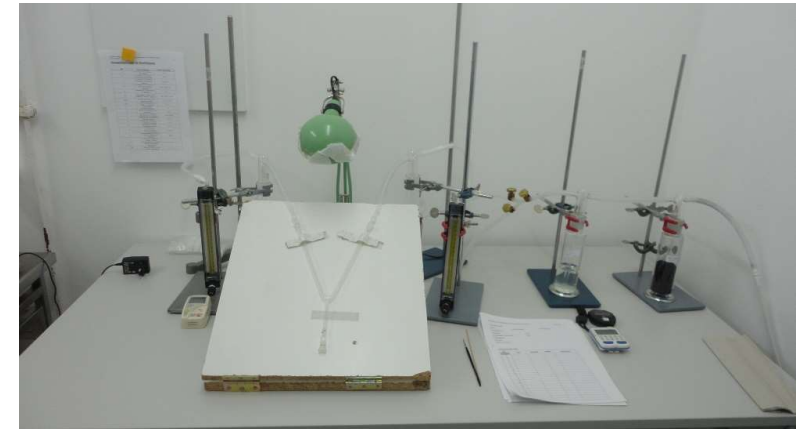


Screening for volatile behavior manipulating substances

Which plant substances manipulate the behavior of *C. picta*?

The effect of different chemical substances (pure and dilutions) on the behavior of the two migratory stages of the psyllids were tested in the Y-tube olfactometer (= two-sided choice test)

- The figures show the number of psyllid individuals walking into the control arm (purified air) or into the test arm (test substance) of the olfactometer
- Pure substances or dilution of 1:1000 were used for experiments.
- The walking activity of the psyllids in the bioassays was very high (between 78 and 100%).



- **Methylsalicylate: only the dilution attracts the remigrants, no effect on emigrants**
- **Linalool: pure substance deters both the remigrants and the emigrants**

Isolation of an entomopathogenic fungus with potential for biological psyllid pest control

- First psyllid-pathogenic Entomophthorales of the genus *Pandora* was isolated from the target insect.
- Genetical differences (ITS2) + first description on psyllid species → novel species

Advantages of the Entomophthorales

- Strict host-specificity
- Fast *speed-to-kill*
- Able to cause epizootics

- **BUT: until today, no entomophthoralean fungus is used in biological pest control**

Challenges

- Difficult to grow *in-vitro* and to be mass-produced
- Contact between fungal propagule and pest insect must be improved.



Pandora growing out of an infected psyllid

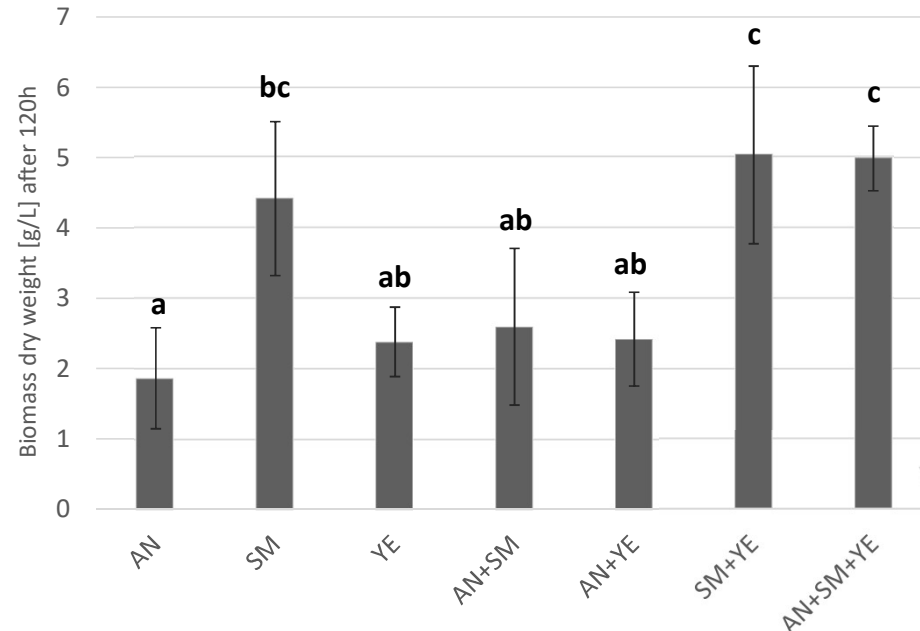
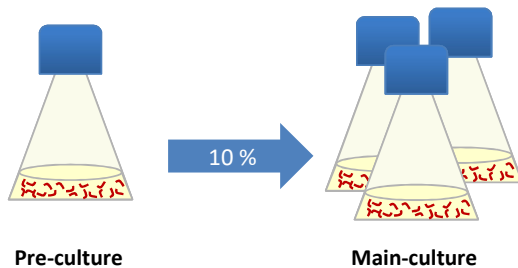
A. H. Jensen

Fermentation of the entomopathogenic fungus *Pandora* (ARSEF 13372)

The novel entomopathogenic fungus was successfully cultivated in submerged shaking culture.

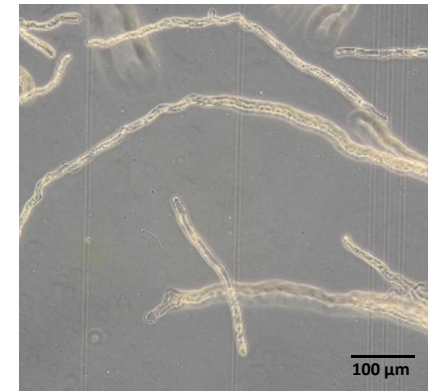
Different complex nitrogen sources were screened for their suitability to grow *Pandora* in submerged shaking culture

- Complex nitrogen sources used in this study were a low cost protein hydrolysate from animal by-products (AN), skimmed-milk from powder (SM) and yeast extract (YE).



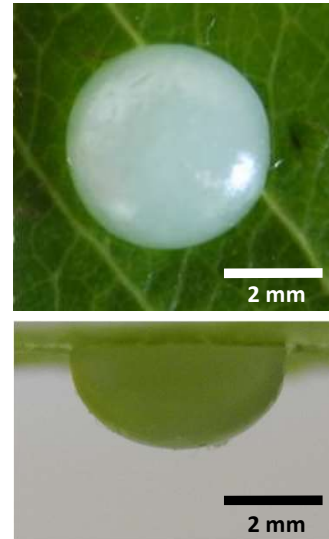
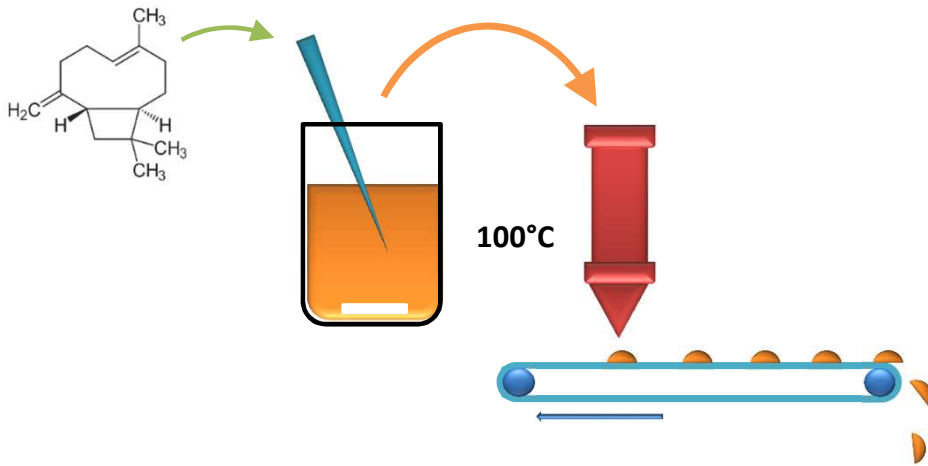
$F_{6,28} = 11.943$; $p < 0.001$; $n=5$; one-way-ANOVA

- A 2-stage fermentation process and medium was established.
- The combination of different nitrogen sources leads to higher biomass production.
- Finely dispersed hyphae of *Pandora* sp. (ARSEF13372) of a mean hyphal length of 550 μm were produced.



Development of hydrophobic formulations as carrier for semiochemicals

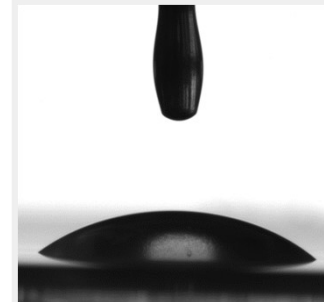
β -caryophyllene → known for its high attractivity to *C. picta*



- A novel formulation based on natural waxes was developed for the slow release of semiochemicals
- Preparable as lens, bead, film or as a paste
- Self-sticking properties on apple leaves

Suitability for entrapment of hydrophobic semiochemicals and for field application

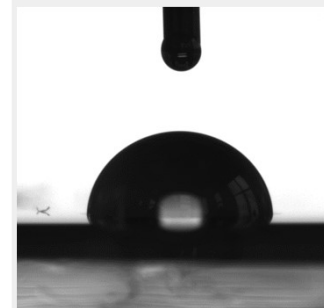
Characterization of the formulation material by contact angle measurements



β -caryophyllene
($\Theta = 39.02^\circ \pm 1.28$)

Low contact angle

- high affinity of β -caryophyllene to the matrix material
- low barrier effect on diffusion-mediated release

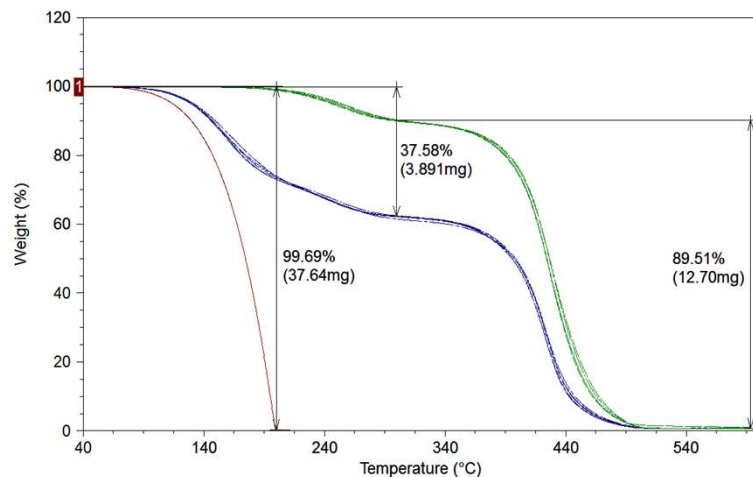


Water
($\Theta = 97.63^\circ \pm 0.63$)

High contact angle

- hydrophobic character
- high water resistance after application

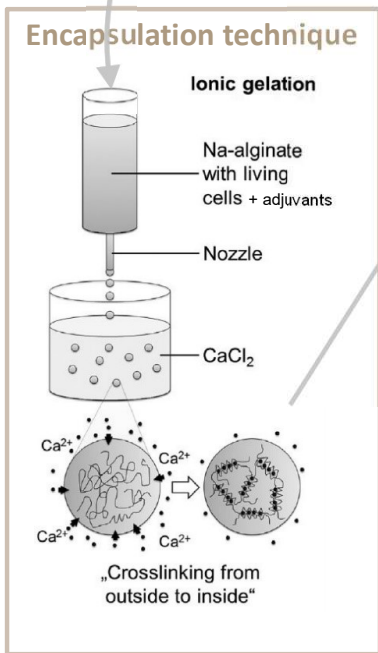
The encapsulation procedure is suitable to formulate a high amount of β -caryophyllene.



Calculation of encapsulation efficiency by thermogravimetric analysis

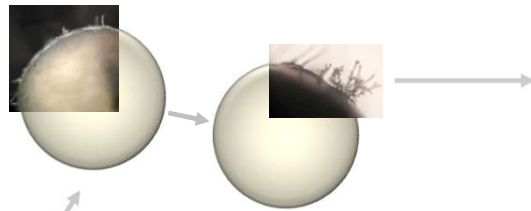
- **Successful entrapment of β -caryophyllene within the matrix**
- **Encapsulation efficiency = 92.12 % (± 0.4809 ; n=5)**

Formulation of the entomopathogenic fungus *Pandora* (ARSEF 13372)



Formulation within Ca-alginate beads

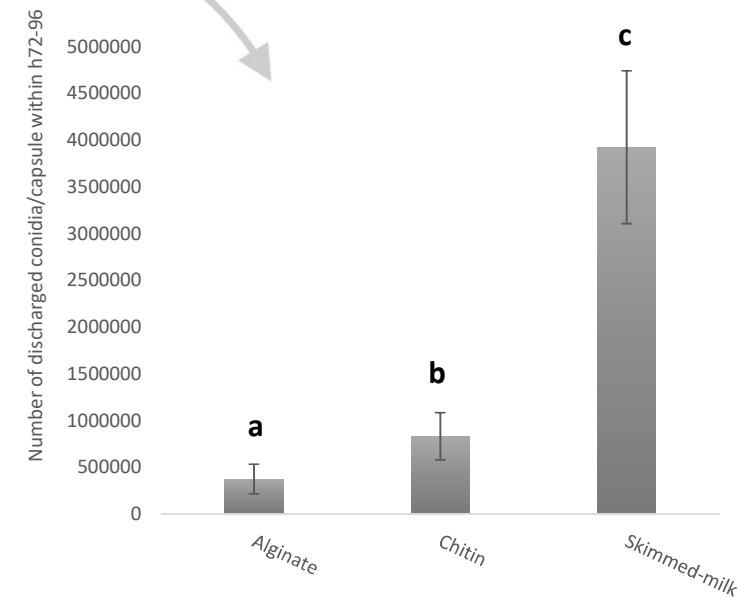
Outgrowth and conidial discharge



100 % r.h. 18°C



Conidia discharged from beads were collected and counted to investigate the effect of different formulation additives on sporulation intensity.

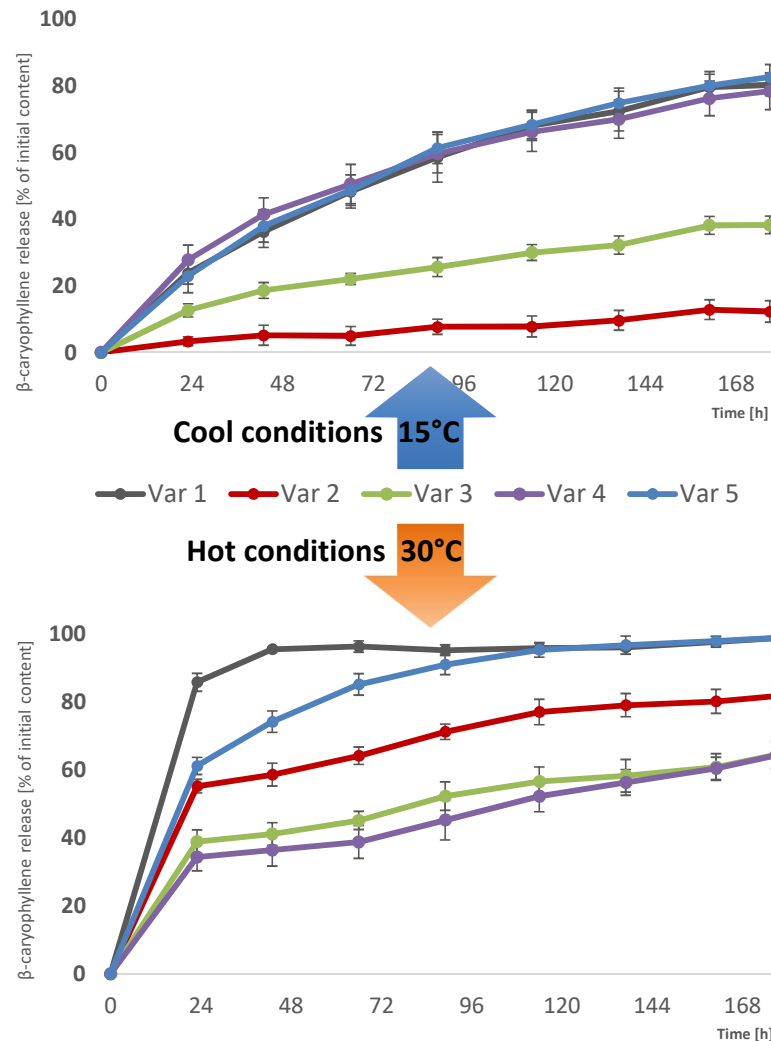


$F_{4,295} = 624,836$; $p < 0.001$; $n = 5$; one-way ANOVA

- Finely dispersed hyphae of the novel *Pandora* species produced in submerged culture were encapsulated in biodegradable hydrogel beads.
- *Pandora* is able to grow and sporulate from the beads.
- Nutrient addition increase the number of discharged conidia.

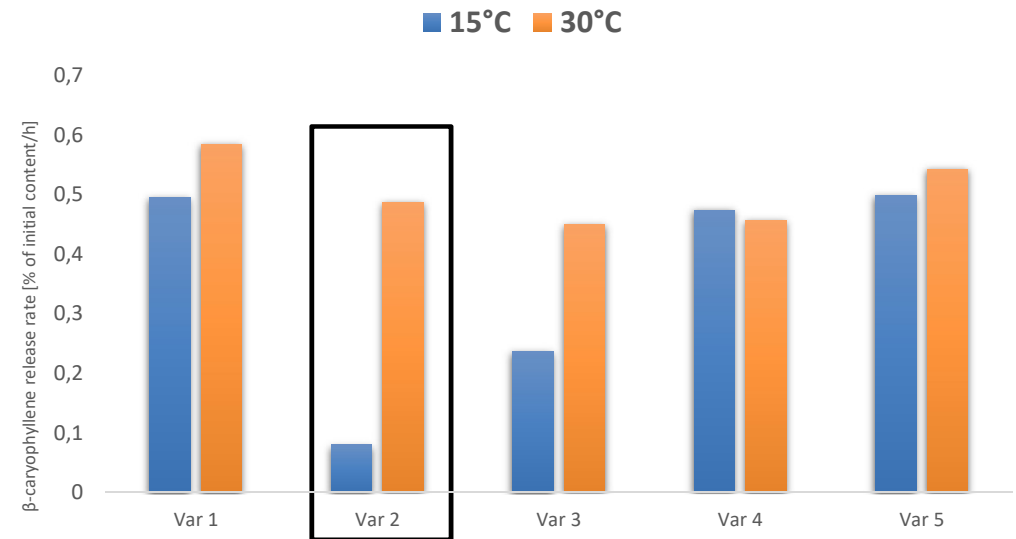
Temperature effects on β -caryophyllene release from different formulations

Release of β -caryophyllene from different formulations was investigated under hot and cold temperature conditions to find a formulation suitable for long-term release of the attractant under field conditions.



Selection of an insect behavior matching formulation

Assumption: Psyllid flight activity increases with temperature.

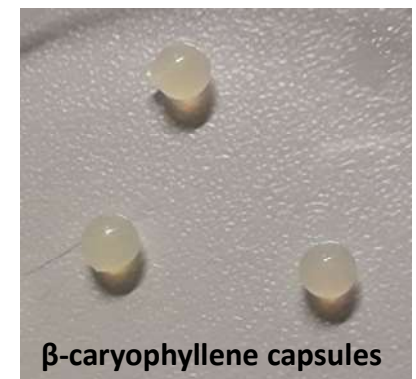
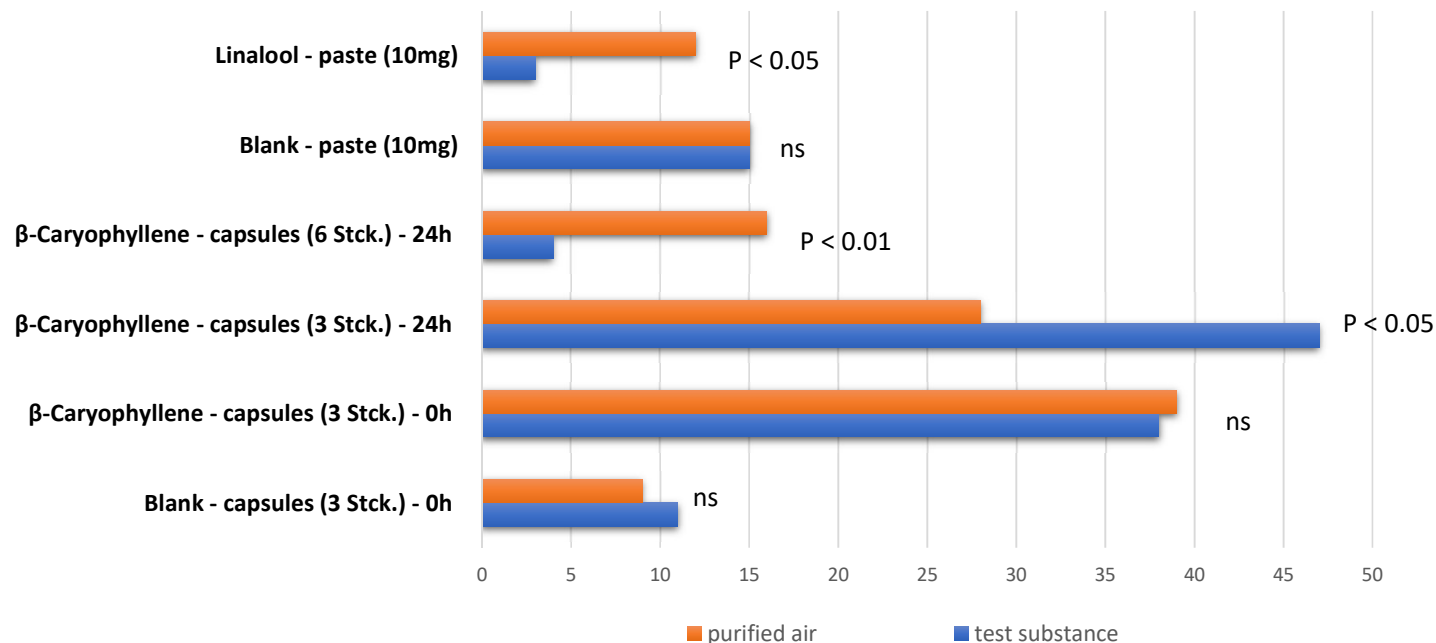


One of the formulations shows a favourable release profile for the control of psyllids under application-oriented conditions:
High amount of β -caryophyllene released during insect peak activity
Slowed release during night time to save semiochemical depot

Evaluation of the insect behavior manipulating effect in the lab

Different formulations of linalool and β -caryophyllene manipulate the behavior of the emigrants.

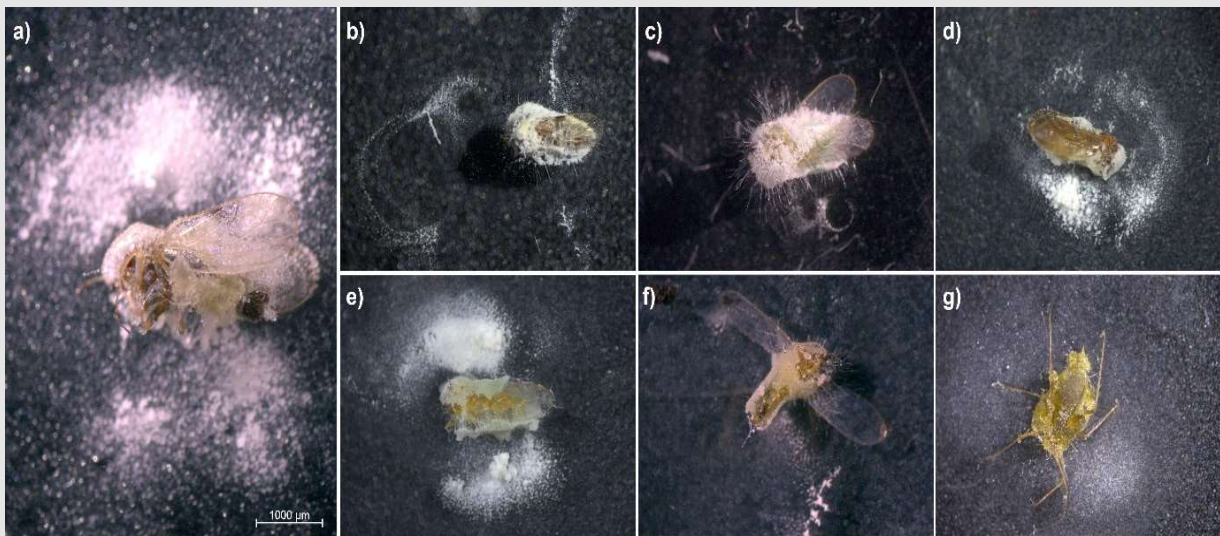
- The figure shows the number of emigrants walking into the control arm (purified air) or into the test arm (test substance) of the olfactometer
- The walking activity of the psyllids in the bioassays was very high (between 87 and 100%)



- **Attractant effect:** caused by 3 capsules of β -caryophyllene evaporated for 24 h
- **Repellent effect:** caused by 6 capsules of β -caryophyllene evaporated for 24 h and the paste of linalool

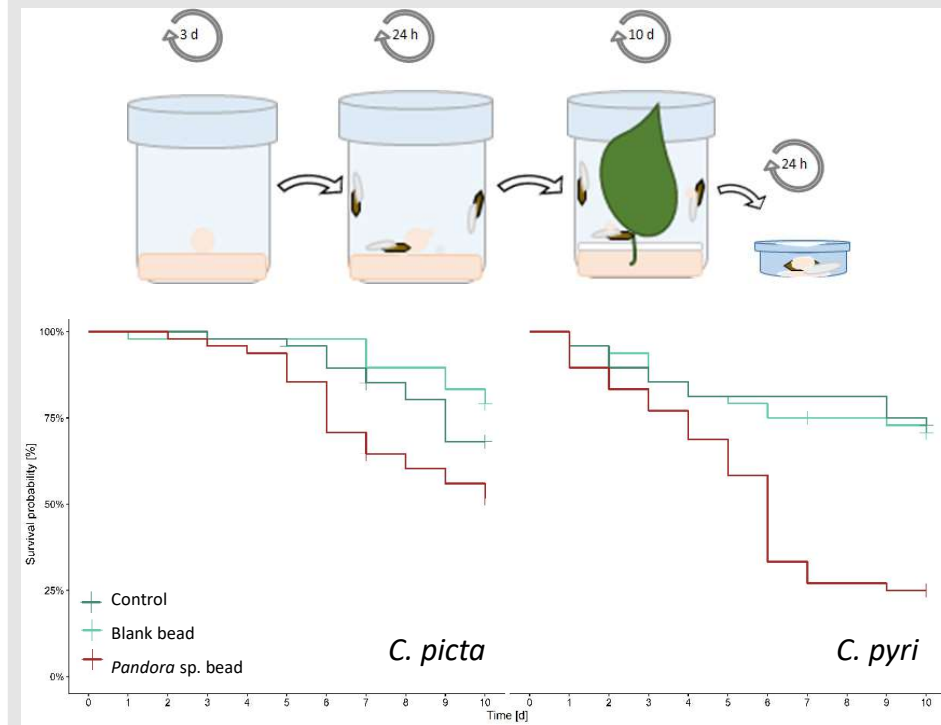
Evaluation of the killing effect in the lab

Pandora (ARSEF 13372) conidia are pathogenic for different phloem feeding pest insects.



- *Pandora* (ARSEF 13372) conidia successfully infect and kill (a-e) Psyllidae *C. pyri*, *C. pyricola*, *C. picta*, *C. pruni* and *C. peregrina*; (f) Triozidae *Trioza apicalis* and (g) Aphididae *Acyrthosipon pisum*

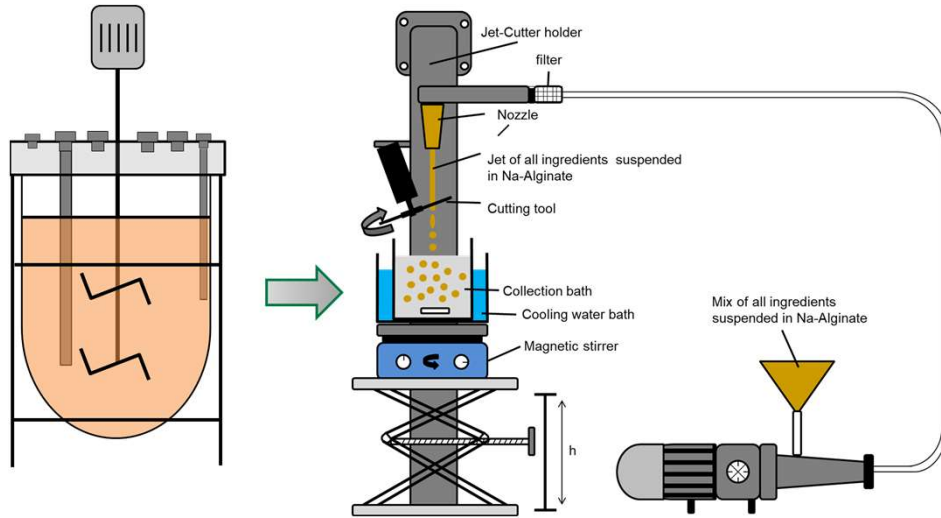
The developed KILL-formulation of encapsulated *Pandora* (ARSEF 13372) is lethal for psyllid pests.



- Decreased survival probability of psyllids exposed to *Pandora* sp. bead formulations.

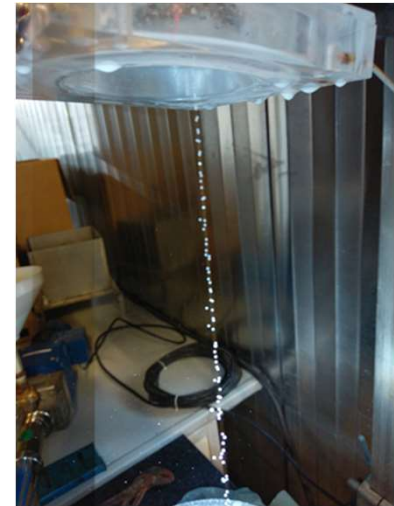
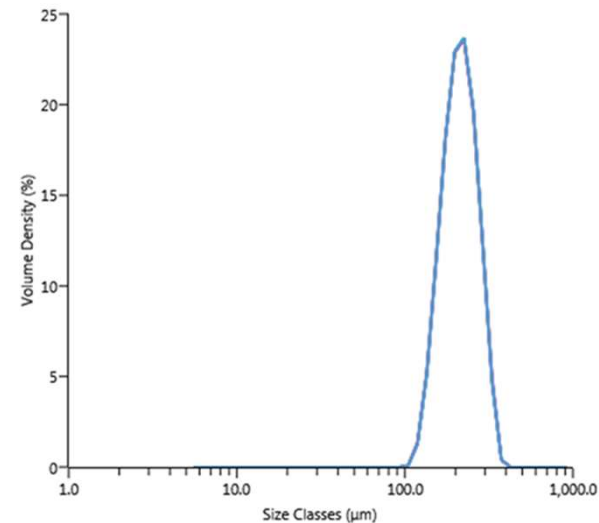
- The suitability of the developed KILL-formulation with encapsulated *Pandora* sp. for psyllid pest control was demonstrated in the lab.

Scale-up – Fermentation and formulation of *Pandora* sp. on technical scale



Schematic drawing of the liquid fermentation and bead production with the jet-cutter. The fermentation medium consisted of 3% glucose, 1% NaCl, 0.33% yeast extract, 0.33% Anipept and 0.33% skim milk powder. The encapsulation matrix was prepared by mixing 1.5% sodium alginate with 20% corn starch, 4% skimmed-milk powder and fungal biomass.

Particle size distribution of beads produced with the jet-cutter was analyzed using the Mastersizer 3000 (Malvern Panalytical)
➤ Mean particle size was 212 μm (D_{50})



Bead production with the jet-cutter



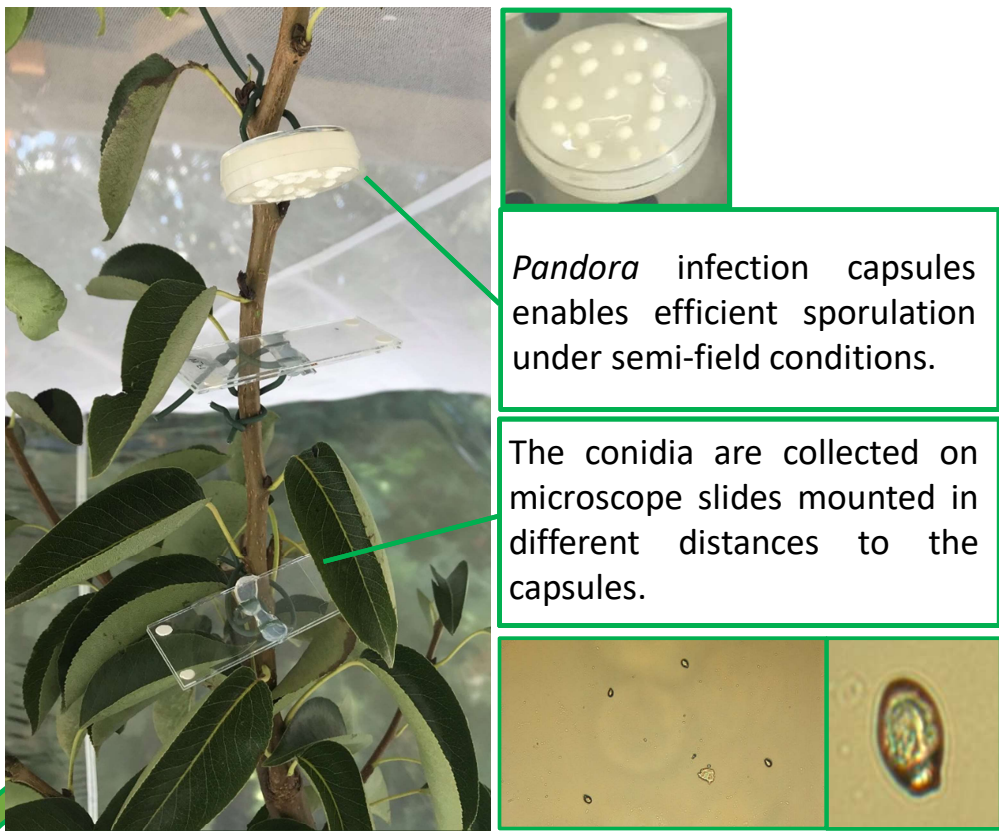
Jet-cut KILL-beads



Pandora growing and sporulating from jet-cut beads

- The liquid fermentation process was successfully upscaled from a 250 ml flask to a 10 L fermenter for mass-production of *Pandora* sp. (ARSEF 13372) on low costs (0.26€/L)
- Scale-up of the formulation process was realized by encapsulation of *Pandora* sp. (ARSEF 13372) in Ca-alginate beads using the jet-cutter technology

Efficacy tests in semi-field trials 2020 with *Pandora* (ARSEF 13372) infection capsules

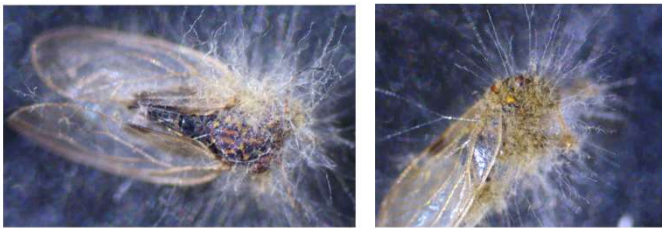


Pandora infection capsules enables efficient sporulation under semi-field conditions.

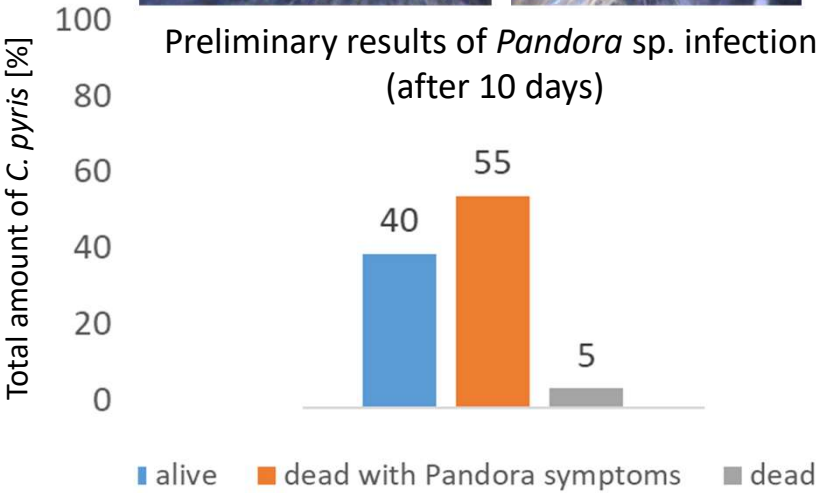
The conidia are collected on microscope slides mounted in different distances to the capsules.



The tests are done in isolated net cages with potted pear trees.



Preliminary results of *Pandora* sp. infection (after 10 days)



Pandora is sporulating from the capsules under field conditions

Cacopysylla pyri becomes infected

Summary

- In this project, novel formulations based on volatile semiochemicals and an insect-killing component were developed.
- A completely new formulation was developed for the controlled release of β -caryophyllene as an attractant for *C. picta*.
- A previously unknown fungus (*Pandora* sp.) was isolated from the target insect, which kills the target insect faster and more specifically than other known insect-pathogenic fungi.
- The host spectrum of the fungus was investigated and the potential of the fungus to control other closely related psyllids was recognized.
- The prerequisite for the utilization of this fungus was created by establishing a fermentation and formulation process with potential for mass-production.
- The effectiveness of the KILL formulation has already been proven in laboratory tests with *C. picta*.
- An image analysis-based method was developed for the fast and objective quantification of fungal sporulation from beads by automatic conidia counting.
- The kill-concept was already proved in semi-field experiments. The tests of the attract-and-kill approach are still under development.

Outlook

We are only just beginning to identify and formulate behavior-modifying substances with the aim of driving harmful insects away from crops and attracting and killing them by means of biological agents. By combining repellents for dispersal with attract-and-kill systems to form so-called push-pull-kill systems, new paths of non-chemical plant protection will be taken. With these innovative selective and sustainable methods, the biodiversity of non-target organisms is to be better protected and thus preserved for future generations.

Thank you for your attention!

