Research Article

Received: 13 July 2021 Revised: 18 September 2021 Accepted article published: 15 November 2021 Published online in Wiley Online Library: 3 December 2021

(wileyonlinelibrary.com) DOI 10.1002/ps.6710

Encapsulation of the psyllid-pathogenic fungus *Pandora* sp. nov. inedit. and experimental infection of target insects[1]

Linda C Muskat,[a,b] Louisa M Görg,[c] Pascal Humbert,[a] Jürgen Gross,[c] Jørgen Eilenberg[d] and Anant V Patel[a*]

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 adjuvant 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 adjuvant 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.

Supporting information may be found in the online version of this article.

Keywords: Entomophthorales; encapsulation; biological control; entomopathogenic fungi; virulence; psyllid control

1 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.[1–3] 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.[4–6] In European fruit production, high economic losses are caused by reduced fruit yield and quality and dying plants following phytoplasma infection.[7–9] In Italy, an outbreak of apple proliferation disease caused up to € 100 million losses.[10] 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.[11]

In 2016, an entomophthoralean species of the genus *Pandora* was isolated from infected psyllids collected in a Danish pear

* *Correspondence to: A Patel, Faculty of Engineering, Bielefeld University of Applied Sciences and Mathematics, WG Fermentation and Formulation of Biologicals and Chemicals, Interaktion 1, D-33619 Bielefeld, Germany. E-mail: anant.patel@fh-bielefeld.de*

[1] *A part of this study was presented in an invited talk, entitled 'Fermentation and formulation of* Pandora *sp. nov. inedit. for biological psyllid pest control', at the 2021 International Congress on Invertebrate Pathology and Microbial Control & 53rd Annual Meeting of the Society for Invertebrate Pathology in a session dedicated to progress on entomophthoralean fungi.*

a *Fermentation and Formulation of Biologicals and Chemicals, Faculty of Engineering and Mathematics, Bielefeld University of Applied Sciences, Bielefeld, Germany*

b *Agricultural Entomology, Department of Crop Sciences, Georg-August-University Goettingen, Goettingen, Germany*

c *Institute for Plant Protection in Fruit Crops and Viticulture, Julius Kühn-Institut, Federal Research Institute for Cultivated Plants, Dossenheim, Germany*

d *Department of Plant and Environmental Sciences, University of Copenhagen, Copenhagen, Denmark*

orchard.[12] The species is currently under description as a new species, since it differs from the known *Pandora* species infecting Hemiptera.[13] 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.[12–15] 15 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.[16–20] 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.[16,21–25] Moreover, research regarding pathogenicity and virulence and thus the suitability of entomophthoralean species for pest control strategies of psyllids in orchards is scarce.[26]

The conversion of *Pandora* sp. nov. into a biocontrol agent for psyllid pest control requires a formulation that secures the virulence of the fungus when applied against the target insects. The formulation of living biocontrol agents within a carrier material may improve its 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.[27–30] 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).[31–36] 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 (i) to investigate the maintenance of sporulation duration and intensity of *Pandora* sp. nov. after encapsulation in calcium alginate beads, (ii) to improve and prolong the sporulation by addition of nutritional formulation adjuvants and (iii) 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.

2 MATERIALS AND METHODS

2.1 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.

2.2 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.

2.3 Insects

All insects were collected by the beating tray sampling method.[37] 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 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 × 47.5 × 93 cm BugDorm rearing cages (NHBS, UK). The insect specimens used in bioassays were randomly selected from rearing cages containing one single generation.

2.4 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.

2.5 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.[38] 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*.

2.6 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 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 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 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 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}). All media components were solved 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.

Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

2.7 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.

2.8 Preparation of beads

All bead compositions used in this study are listed in Table 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-solved 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% (Pandora 40) (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 mol L^{-1}) 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.

2.9 Quantification of fungal sporulation from beads

In order to quantify conidial discharge (see Fig. 1(a)), 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.[38] 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.

2.10 Infection bioassay

The laboratory infection bioassays (see Fig. 1(b)), following the recommendations of Hajek *et al*.,[38] 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*.[13] Details on preparation of the PP cups are given in Görg *et al*.[39] 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).

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 three *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).

Table 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

Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

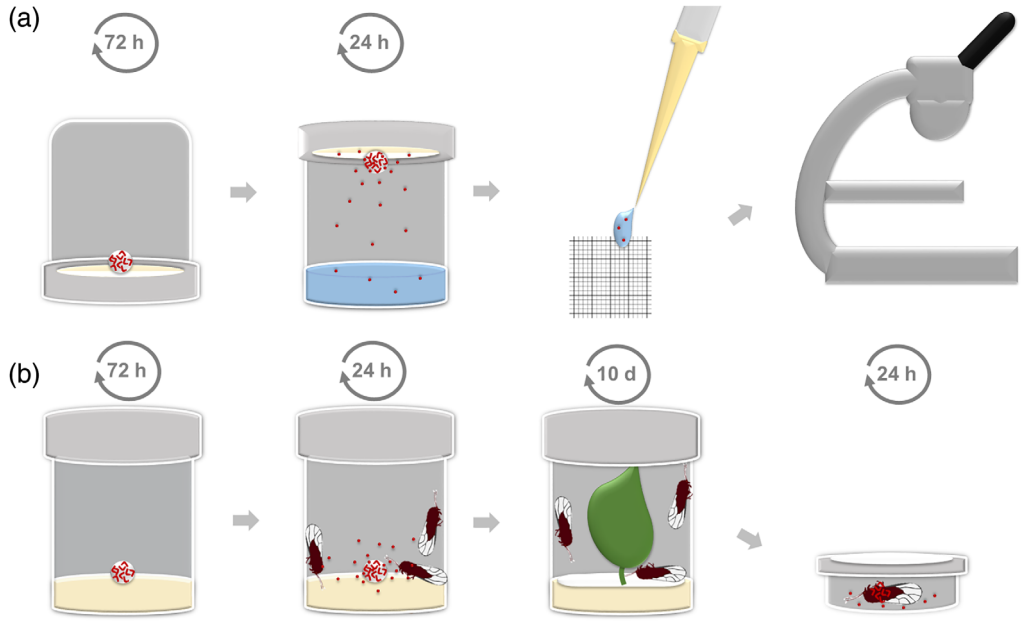

Figure 1. Experimental setup of quantification of discharged conidia (a) and infection bioassays with *Pandora* sp. nov. beads (b).

2.11 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, USA). 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 adjuvants (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 sphericity 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'[40] and 'survminer'[41] packages for time-to-event curves. Analysis of time-to-event data (event: death) for censored data according to *Kaplan–Meier method*[43] was performed using the *survfit* function of the 'survival' package.[42] 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 *et al.*[43] 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[42] 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[44] and *P*-value adjustment by the method of *Tukey* (Supplementary Tables S1 and S2). 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.[45]

3 RESULTS

3.1 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 Fig. 2.

The number of conidia discharged from the beads was significantly affected by the formulation adjuvants ($F_{2,177} = 986.751$; $P < 0.001$), the time ($F_{6.946,1229.449} = 757.302$; $P < 0.001$) and the interaction of formulation adjuvants and time ($F_{13.892,1229.449} = 184\,022$; $P < 0.001$). Within the first 24 h, only few conidia were discharged from all beads (Fig. 3). Sporulation from starch beads increased distinctly at day 2 of incubation and reached a plateau with a maximum of 6.59×10^5 ($\pm 1.94 \times 10^5$) conidia per bead and day at day 3 followed by a constant regression in conidia numbers. Sporulation from Sm4 beads increased rapidly up to 2.1×10^6 ($\pm 3.73 \times 10^5$) conidia per bead and reached a single peak sporulation event at day 4 after bead preparation. After 12 days, only few conidia were discharged from all bead variations compared to conidia numbers during peak sporulation within the first 6 days after bead preparation.

By the addition of skimmed milk (4%; w/w), the total number of conidia discharged from the beads (9.57×10^6) was significantly increased by 2.95-fold compared to starch beads (3.25×10^6) ($X^2 = 12.500$; df = 2; $P < 0.001$; *Bonferroni*).

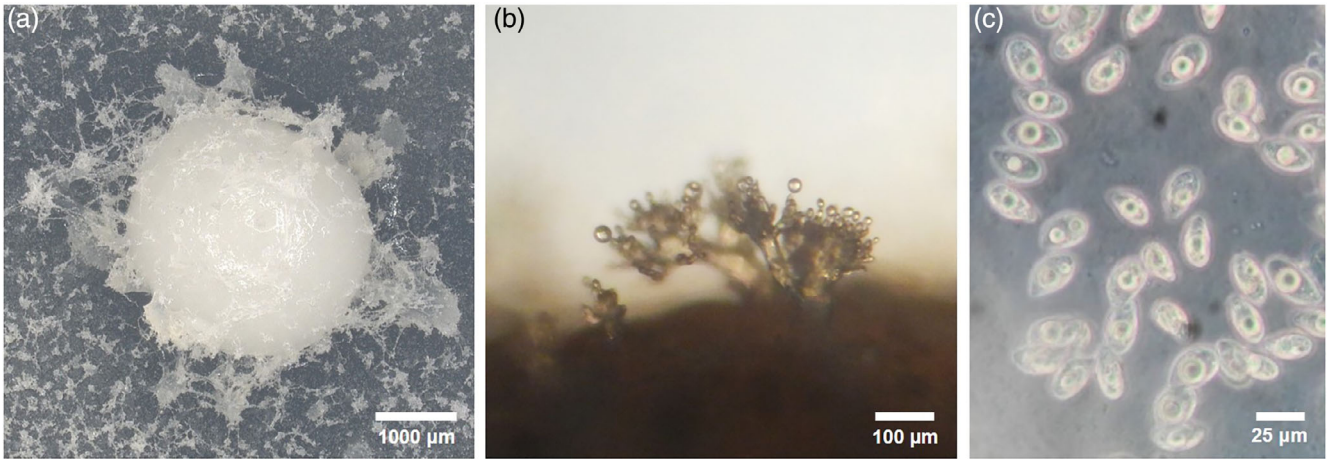

Figure 2. *Pandora* sp. nov. growing from calcium alginate beads after 6 days of incubation on water agar plates (A). Conidiophores are observable on the bead surface within the first 24 h (B). Actively discharged conidia from encapsulated *Pandora* sp. nov. (C).

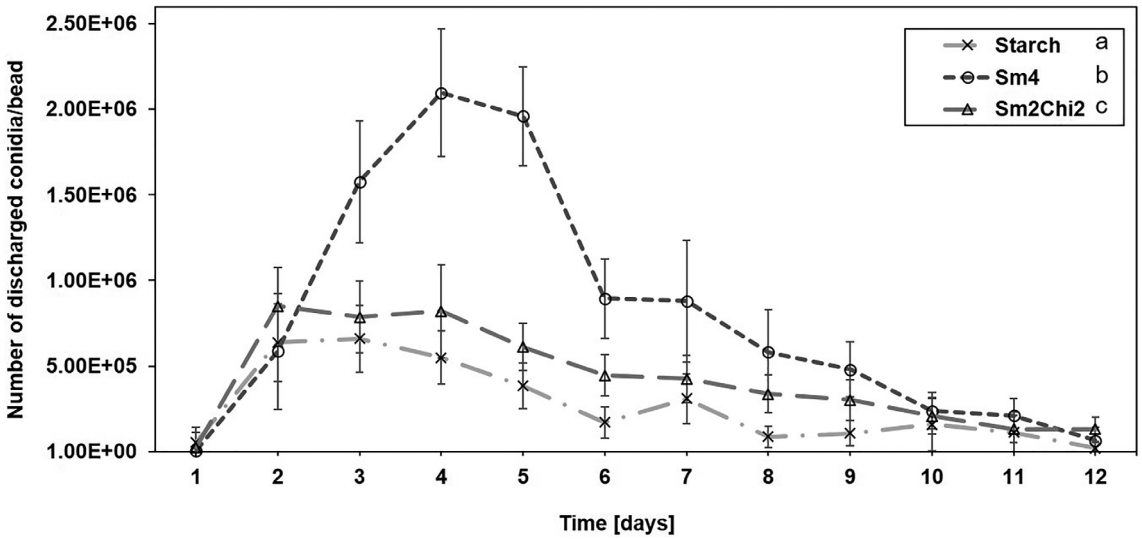

Figure 3. Duration and intensity of conidial discharge by *Pandora* sp. nov. from different beads within the first 12 days after bead preparation. Different letters in the legend indicate significant differences according to RM-ANOVA with *Games-Howell post hoc test* at $P < 0.05$. Means ± SD, n = 5.

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 (5.30×10^6) by 1.63-fold compared to beads without nutritional adjuvants ($P = 0.231$; *Bonferroni*), but much less than beads containing skimmed milk ($P < 0.001$).

3.2 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*; Fig. 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*).

3.3 Infection bioassay for evaluation of pathogenicity of formulated *Pandora* sp. nov. on *Cacopsylla picta* and *Cacopsylla pyri*

Sm2Chi2 beads produced significantly more conidia ($3 \times 10^4 \pm 7\text{x}10^3$ conidia) than Sm4 beads ($1 \times 10^4 \pm 1\text{x}10^3$) 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 Fig. 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 S1). 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) (Fig. 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, Fig. 5).

3.4 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 ($\chi^2 = 33.04$, df = 4, $P < 0.0001$; *Kruskal–Wallis*). Pandora20 beads produced $4 \times 10^4 \pm 2 \times 10^3$ conidia. This was significantly more (z = 3.49, $P = 0.001$; *Dunn–Bonferroni*) than for Pandora40 beads that

Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

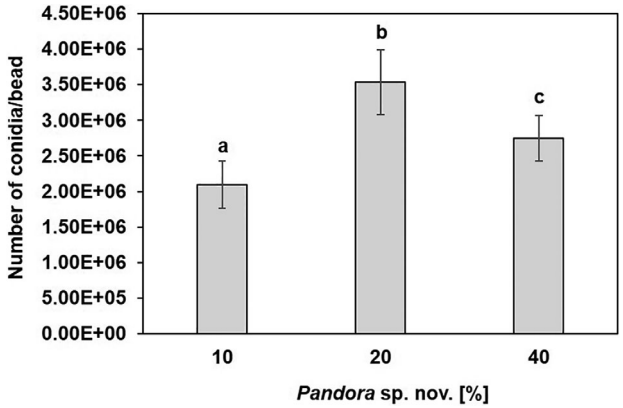

Figure 4. Effect of *Pandora*. sp. nov. concentration in beads on conidial discharge during day 4 after bead preparation. Different letters above bars indicate significant differences based on *Kruskal-Wallis* and *Games-Howell post-hoc range test* at $P < 0.05$. Means ± SD, n = 5.

produced $3 \times 10^3 \pm 5 \times 10^2$ conidia). Both Pandora20 and Pandora40 beads produced significantly more conidia than Pandora10 beads ($z = 2.39$, $P = 0.03$; *Dunn–Bonferroni*), that produced $1 \times 10^4 \pm 2 \times 10^3$ conidia. Beads without *Pandora* sp. nov. material did not produce any conidia.

Treatment with distinct bead formulations with varying *Pandora* sp. nov. concentrations had a highly significant effect on the mean survival time of *C. pyri* ($\chi^2 = 31.25$, df = 4, $P < 0.0001$; *survreg*; Supplementary Table S2). 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 (Fig. 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 (Fig. 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. 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.

4 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.[46–50] 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.*,[50] 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 adjuvant compared to beads without nutritional additives. This is in accordance with the observation of Shah *et al.*[51] 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.[52–54] 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: (i) Prolonged sporulation duration, and (ii) 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,[55,56] 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 17 Hypocreales species or strains on Asian

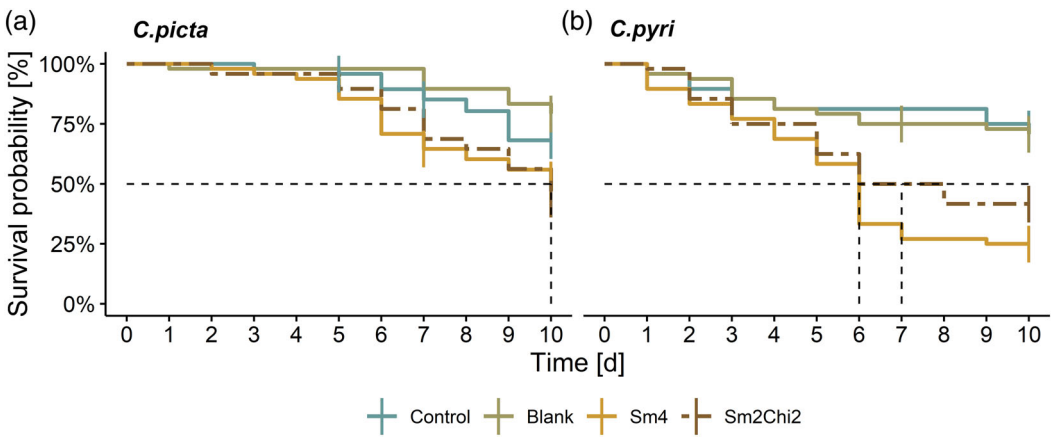

Figure 5. Survival [%] for psyllids a) *C. picta* (n = 48/treatment and b) *C. pyri* (n = 48/treatment) 10 days after exposure to beads containing *Pandora* sp. nov. visualized by *Kaplan–Meier* curves. The dashed black lines indicate the median survival time. Individuals which escaped or died due to handling mishaps were marked as censored events, indicated by tick marks on the *Kaplan–Meier* curve.[43]

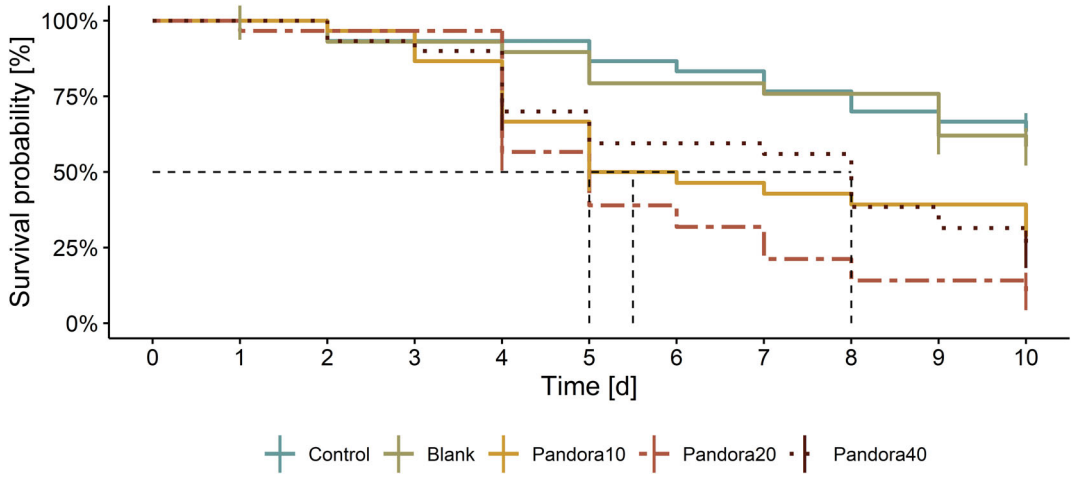

Figure 6. Survival [%] for pear psyllid *C. pyri* (n = 30/treatment) 10 days past inoculation visualized by *Kaplan–Meier* curves. The dashed black lines indicate the median survival time. Individuals which escaped or died due to handling mishaps were marked as censored events, indicated by tick marks on the *Kaplan–Meier* curve.[43]

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.[57] In the same experiment, we hypothesized that chitin may serve as a virulence-enhancing formulation adjuvant for improved infection efficiency of *Pandora* sp. nov. as reported for other entomopathogenic fungi.[33,58,59] 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 Fig. S1). 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 (i) a faster metabolization of the nutrients due to more viable fungal cells, or (ii) self-metabolization of the fungus due to a lack of nutrients, or (iii) release of toxic components by the fungus e.g. ammonium, or (iv) 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,[12–15] 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 with 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).[60] 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 labor-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.[61]

5 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.[50,62,63] 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.

CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Weintraub PG and Beanland L, Insect vectors of phytoplasmas. *Annu Rev Entomol* **51**:91–111 (2006). https://doi.org/10.1146/annurev.ento.51.110104.151039.
2 Jarausch B, Tedeschi R, Sauvion N, Gross J and Jarausch W, Psyllid vectors, in *Phytoplasmas: Plant Pathogenic Bacteria, Volume 2, Transmission and Management of Phytoplasma- Associated Diseases*, ed. by Bertaccini A, Weintraub PG, Rao GP and Mori N. Springer, Singapore, pp. 53–78 (2019). https://doi.org/10.1007/978-981-13-2832-9_3.
3 Gallinger J, Zikeli K, Zimmermann MR, Görg LM, Mithöfer A, Reichelt M *et al.*, Specialized 16SrX phytoplasmas induce diverse morphological and physiological changes in their respective fruit crops. *PLoS Pathog* **17**:e1009459 (2021). https://doi.org/10.1371/journal.ppat.1009459.
4 Carraro L, Loi N, Ermacora P, Gregoris A and Osler R, Transmission of pear decline by using naturally infected *Cacopsylla pyri* L, in *XVII International Symposium Virus and Virus-Like Diseases of Temperate Fruit Crops*, Vol. **472**, pp. 665–668 (1997). https://doi.org/10.17660/ActaHortic.1998.472.89.
5 Frisinghelli C, Delaiti L, Grando MS, Forti D and Vindimian ME, *Cacopsylla costalis* (Flor 1861), as a vector of apple proliferation in Trentino. *J Phytopathol* **148**:425–431 (2000). https://doi.org/10.1046/j.1439-0434.2000.00403.x.
6 Seemüller E and Schneider B, '*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 (2004). https://doi.org/10.1099/ijs.0.02823-0.
7 Bovey R, *Apple Proliferation Disease*. Stations fédérales d'essais agricoles, Lausanne (1961).
8 Civolani S, The past and present of pear protection against the pear psylla, *Cacopsylla pyri* L, in *Insecticides - Pest Engineering*, ed. by Perveen F. InTech, Rijeka, pp. 385–408 (2010).
9 Seemüller E, Carraro L, Jarausch W and Schneider B, Apple proliferation phytoplasma, in *Virus and Virus-like Diseases of Pome and Stones Fruits*, ed. by Hadidi A, Barba M, Candresse T and Jelkmann W. The American Phytopathological Society, St. Paul, MI, pp. 67–76 (2011). https://doi.org/10.1094/9780890545010.014.
10 Strauss E, Phytoplasma research begins to bloom. *Science* **325**:388–390 (2009). https://doi.org/10.1126/science.325_388.
11 Jarausch B and Jarausch W, Psyllid vectors and their control, in *Phytoplasmas: Genomes, Plant Hosts and Vectors*, ed. by Weintraub PG and Jones P. New York, USA: CABI, pp. 250–271 (2009). https://doi.org/10.1079/9781845935306.0000.
12 Jensen AH, Gross J, Jensen AB, Gallinger J and Eilenberg J, A new insect pathogenic fungus from Entomophthorales with potential for psyllid control. *Mitt Dtsch Ges Allg Angew Entomol* **21**:283–286 (2018).
13 Görg L, Eilenberg J, Jensen AB, Jensen AH and Gross J, 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**:107621 (2021). https://doi.org/10.1016/j.jip.2021.107621.
14 Jensen AH, A new insect pathogenic fungus from Entomophthorales with potential for psyllid control, MSc thesis, Department of Plant and Environmental Sciences, University of Copenhagen (2017).
15 Herren P, 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 (2018).
16 Pell JK, Eilenberg J, Hajek AE and Steinkraus DC, Biology, ecology and pest management potential of Entomophthorales, in *Fungi as Biocontrol Agents: Progress, Problems and Potential*, ed. by Butt TM, Jackson C and Magan N. New York, USA: CABI, pp. 71–154 (2001).
17 Eilenberg J and Philipsen H, The occurrence of Entomophthorales on the carrot fly (*Psila rosae* F.) in the field during two successive seasons. *Entomophaga* **33**:135–144 (1988). https://doi.org/10.1007/BF02372648.
18 Nielsen C, Eilenberg J, Harding S, Oddsdottir E and Haldorsson G, Geographical distribution and host range of Entomophthorales infecting the green spruce aphid *Elatobium abietinum* Walker in Iceland. *J Invertebr Pathol* **78**:72–80 (2001). https://doi.org/10.1006/jipa.2001.5045.
19 Filotas MJ, Hajek AE and Humber RA, Prevalence and biology of *Furia gastropachae* (Zygomycetes: Entomophthorales) in populations of forest tent caterpillar (Lepidoptera: Lasiocampidae). *Can Entomol* **135**:359–378 (2003).
20 Vega FE, Goettel MS, Blackwell M, Chandler D, Jackson MA, Keller S *et al.*, Fungal entomopathogens: new insights on their ecology. *Fungal Ecol* **2**:149–159 (2009). https://doi.org/10.1016/j.funeco.2009.05.001.
21 Barta M and Cagáň L, Aphid-pathogenic Entomophthorales (their taxonomy, biology and ecology). *Biologia* **61**:543–616 (2006). https://doi.org/10.2478/s11756-007-0100-x.
22 Jaronski ST, Mass production of entomopathogenic fungi: state of the art, in *Mass Production of Beneficial Organisms*, ed. by Morales-Ramos JA, Rojas MG and Shapiro-Ilan DJ. Elsevier Inc., Amsterdam, pp. 357–415 (2013). https://doi.org/10.1016/B978-0-12-391453-8.00011-X.
23 Shah P and Pell J, Entomopathogenic fungi as biological control agents. *Appl Microbiol Biotechnol* **61**:413–423 (2003). https://doi.org/10.1007/s00253-003-1240-8.
24 Wilding N and Latteur G, The Entomophthorales - problems relative to their mass production and their utilisation. *Meded Fak Landbouwwet R U Gent* **52**:159–164 (1987).
25 Wraight S, Jackson M and De Kock S, Production, stabilization and formulation of fungal biocontrol agents, in *Fungi as Biocontrol Agents: Progress, Problems and Potential*, ed. by Butt TM, Jackson C and Magan N. New York, USA: CABI, pp. 253–287 (2001).
26 Gross J, Gallinger J and Görg LM, Interactions between phloem-restricted bacterial plant pathogens, their vector insects, host plants, and natural enemies mediated by primary and secondary plant metabolites. *Entomol Gen* (2021). https://doi.org/10.1127/entomologia/2021/1254.
27 Chen C, Ye S, Wang D, Hatting JL and Yu X, Alginate embedding and subsequent sporulation of in vitro-produced *Conidiobolus thromboides* hyphae using a pressurised air-extrusion method. *Biol Control* **69**:52–58 (2014). https://doi.org/10.1016/j.biocontrol.2013.10.016.
28 Shah PA, Aebi M and Tuor U, Method to immobilize the aphid-pathogenic fungus *Erynia neoaphidis* in an alginate matrix for biocontrol. *Appl Environ Microbiol* **64**:4260–4263 (1998). https://doi.org/10.1128/AEM.64.11.4260-4263.1998.

29 Shah P, Aebi M and Tuor U, Effects of constant and fluctuating temperatures on sporulation and infection by the aphid-pathogenic fungus *Pandora neoaphidis*. *Entomol Exp Appl* **103**:257–266 (2002). https://doi.org/10.1046/j.1570-7458.2002.00980.x.

30 Zhou X, Su Y and Liu H, A floatable formulation and laboratory bioassay of *Pandora delphacis* (Entomophthoromycota: Entomophthorales) for the control of rice pest *Nilaparvata lugens* Stål (Hemiptera: Delphacidae). *Pest Manag Sci* **72**: 150–154 (2016) https://doi.org/10.1002/ps.3981.

31 Przyklenk M, Vemmer M, Hanitzsch M and Patel A, A bioencapsulation and drying method increases shelf life and efficacy of *Metarhizium brunneum* conidia. *J Microencapsul* **34**:498–512 (2017). https://doi.org/10.1080/02652048.2017.1354941.

32 Burges HD, Formulation of mycoinsecticides, in *Formulation of Microbial Biopesticides*, ed. by Burges HD. Springer, Dordrecht, pp. 131–185 (1998). https://doi.org/10.1007/978-94-011-4926-6_4.

33 Gerding-Gonzalez M, France A, Sepulveda ME and Campos J, Use of chitin to improve a *Beauveria bassiana* alginate-pellet formulation. *Biocontrol Sci Technol* **17**:105–110 (2007). https://doi.org/10.1080/09583150600937717.

34 Krell V, Jakobs-Schoenwandt D, Vidal S and Patel AV, Cellulase enhances endophytism of encapsulated *Metarhizium brunneum* in potato plants. *Fungal Biol* **122**:373–378 (2018). https://doi.org/10.1016/j.funbio.2018.03.002.

35 Hallmann J, Gutberlet V, Jakobs-Schönwandt D, Vorlop KD, Müller J and Patel AV, Effect of additives on the efficacy of microencapsulated *Hirsutella rhossiliensis* controlling *Heterodera schachtii* on sugar beets. *Biol Control* **128**:40–47 (2019). https://doi.org/10.1016/j.biocontrol.2018.09.005.

36 Vemmer M, Schumann M, Beitzen-Heineke W, French BW, Vidal S and Patel AV, Development of a CO_2 -releasing coformulation based on starch, *Saccharomyces cerevisiae* and *Beauveria bassiana* attractive towards western corn rootworm larvae. *Pest Manag Sci* **72**:2136–2145 (2016). https://doi.org/10.1002/ps.4245.

37 Müther J and Vogt H, Sampling methods in orchard trials: a comparison between beating and inventory sampling, in *IOBC/WPRS Bulletin, Proceedings of the 6th International Conference on Integrated Fruit Production; 2004 Sept 26-30; Baselga di Piné, Italy*, Vol. **28**, p. 481 (2005).

38 Hajek AE, Papierok B and Eilenberg J, Methods for study of the Entomophthorales, in *Manual of Techniques in Invertebrate Pathology*, 2nd edn, ed. by Lacey LA. Academic Press, Amsterdam, pp. 285–316 (2012). https://doi.org/10.1016/B978-0-12-386899-2.00009-9.

39 Görg LM, Jensen AH, Eilenberg J and Gross J, Developing a new biological control strategy for *Cacopsylla* spp. with the novel entomopathogenic fungus *Pandora* sp., in *IOBC/WPRS Bulletin, Proceedings of the 9th* meeting of the Working Groups "Pheromones and other semiochemicals in integrated production" and "Integrated Protection of Fruit Crops", 2019, Jan 20-25, Lisbon, Portugal, Vol. **146**, pp. 88–90 (2019).

40 Wickham H, Chang W, Henry L, Pedersen TL, Takahashi K, Wilke C *et al.*, *ggplot2: Elegant Graphics for Data Analysis*. RStudio, New York, NY (2019).

41 Kassambara A, Kosinski M, Biecek P & Fabian S. Survminer: Drawing Survival Curves using 'ggplot2', R package version 0.3, p. 1 (2017).

42 Therneau T & Lumley T Survival: survival analysis, including penalised likelihood. R Package Version 2. R Found. Stat Comput (2009).

43 Jager KJ, van Dijk PC, Zoccali C and Dekker FW, The analysis of survival data: the Kaplan–Meier method. *Kidney Int* **74**:560–565 (2008). https://doi.org/10.1038/ki.2008.217.

44 Lenth R, Singmann H, Love J, Buerkner P and Herve M, Emmeans: estimated marginal means, aka least-squares means. *R Package Version* **1**: 3 (2018).

45 Pohlert T, PMCMRplus: calculate pairwise multiple comparisons of mean rank sums extended. *R Package Version* **1**:4 (2020).

46 Aoki J, 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 (1981). https://doi.org/10.1303/aez.16.216.

47 Kalsbeek V, Pell JK and Steenberg T, 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 (2001). https://doi.org/10.1006/jipa.2001.5012.

48 Wraight SP, Galaini-Wraight S, Carruthers RI and Roberts DW, *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 (2003). https://doi.org/10.1016/S1049-9644(03)00035-5.

49 Li W, Xu WA, Sheng CF, Wang HT and Xuan WJ, Factors affecting sporulation and germination of *Pandora nouryi* (Entomophthorales: Entomophthoraecae), a pathogen of *Myzus persicae* (Homoptera: Aphididae). *Biocontrol Sci Technol* **16**:647–652 (2006). https://doi.org/10.1080/09583150500532840.

50 Olsen NL, Herren P, Markussen B, Jensen AB and Eilenberg J, Statistical modelling of conidial discharge of entomophthoralean fungi using a newly discovered *Pandora* species. *PLoS One* **14**:e0215914 (2019). https://doi.org/10.1371/journal.pone.0215914.

51 Shah PA, Aebi M and Tuor U, Production factors involved in the formulation of *Erynia neoaphidis* as alginate granules. *Biocontrol Sci Technol* **9**:19–28 (1999). https://doi.org/10.1080/09583159929875.

52 Hua L and Feng MG, 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 (2003). https://doi.org/10.1016/S0378-1097(03)00711-0.

53 Zhou X and Feng MG, Improved sporulation of alginate pellets entrapping *Pandora nouryi* and millet powder and their potential to induce an aphid epizootic in field cages after release. *Biol Control* **54**:153–158 (2010). https://doi.org/10.1016/j.biocontrol.2010.04.007.

54 Zhou X, Guo K, Mao SF, He W and Zhu YY, Modeling analysis on sporulation capacity, storage and infectivity of the aphid-specific pathogen *Conidiobolus obscurus* (Entomophthoromycota: Entomophthorales). *Mycoscience* **55**:21–26 (2014). https://doi.org/10.1016/j.myc.2013.03.005.

55 Coremans P, Note preliminaire sur l'isolement d'entomophthora en Belgique. *Bull Soc Fr Mycol Méd* **5**:153–156 (1976).

56 Latgé J, Silvie P, Papierok B, Remaudière G, Dedryver C and Rabasse J, Advantages and disadvantages of *Conidiobolus obscurus* and of *Erynia neoaphidis* in the biological control of aphids, in *Proceedings of a Meeting of the EC experts' Group: Aphid Antagonists, Portici, Italy, 1982, Nov 23–24*, ed. by Cavalloro R and Balkema AA. CEC, Rotterdam, pp. 20–32 (1983).

57 Ausique JJS, D'Alessandro CP, Conceschi MR, Mascarin GM and Delalibera I Jr, Efficacy of entomopathogenic fungi against adult *Diaphorina citri* from laboratory to field applications. *J Pest Sci* **90**:947–960 (2017). https://doi.org/10.1007/s10340-017-0846-z.

58 Charnley AK, Fungal pathogens of insects: cuticle degrading enzymes and toxins. *Adv Bot Res* **40**:241–321 (2003). https://doi.org/10.1016/S0065-2296(05)40006-3.

59 Fang W, Leng B, Xiao Y, Jin K, Ma J, Fan Y *et al.*, Cloning of *Beauveria bassiana* chitinase gene Bbchit1 and its application to improve fungal strain virulence. *Appl Environ Microbiol* **71**:363–370 (2005). https://doi.org/10.1128/AEM.71.1.363-370.2005.

60 Ben Fekih I, Jensen AB, Boukhris-Bouhachem S, Pozsgai G, Rezgui S, Rensing C *et al.*, Virulence of two entomophthoralean fungi, *Pandora neoaphidis* and *Entomophthora planchoniana*, to their conspecific (*Sitobion avenae*) and heterospecific (*Rhopalosiphum padi*) aphid hosts. *Insects* **10**:54 (2019). https://doi.org/10.3390/insects10020054.

61 Muskat LC, Kerkhoff Y, Humbert P, Nattkemper TW, Eilenberg J and Patel AV, Image analysis-based quantification of fungal sporulation by automatic conidia counting and gray value correlation. *MethodsX* **8**:101218 (2021). https://doi.org/10.1016/j.mex.2021.101218.

62 Delalibera I Jr, Demétrio CG, Manly BF and Hajek AE, 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**:489–496 (2006). https://doi.org/10.1016/j.biocontrol.2006.08.003.

63 Hajek AE and Carruthers RI, Soper RS temperature and moisture relations of sporulation and germination by *Entomophaga maimaiga* (Zygomycetes: Entomophthoraceae), a fungal pathogen of *Lymantria dispar* (Lepidoptera: Lymantriidae). *Environ Entomol* **19**:85–90 (1990). https://doi.org/10.1093/ee/19.1.85.

