



## Activity of natural occurring entomopathogenic fungi on nymphal and adult stages of *Philaenus spumarius*

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### ABSTRACT

The spittlebug *Philaenus spumarius* (Hemiptera: Aphrophoridae) is the predominant vector of *Xylella fastidiosa* (Xanthomonadales: Xanthomonadaceae) in Apulia, Italy and the rest of Europe. Current control strategies of the insect vector rely on mechanical management of nymphal stages and insecticide application against adult populations. Entomopathogenic fungi (EPF) are biological control agents naturally attacking spittlebugs and may effectively reduce population levels of host species. Different experimental trials in controlled conditions have been performed to i) identify naturally occurring EPF on *P. spumarius* in Northwestern Italy, and ii) evaluate the potential for biocontrol of the isolated strains on both nymphal and adult stages of the spittlebug. Four EPF species were isolated from dead *P. spumarius* collected in semi-field conditions: *Beauveria bassiana*, *Conidiobolus coronatus*, *Fusarium equiseti* and *Lecanicillium aphanocladii*. All the fungal isolates showed entomopathogenic potential against nymphal stages of *P. spumarius* ( $\approx 45\%$  mortality), except for *F. equiseti*, in preliminary trials. No induced mortality was observed on adult stage. *Lecanicillium aphanocladii* was the most promising fungus and its pathogenicity against spittlebug nymphs was further tested in different formulations (conidia vs blastospores) and with natural adjuvants. Blastospore formulation was the most effective in killing nymphal instars and reducing the emergence rate of *P. spumarius* adults, reaching mortality levels (90%) similar to those of the commercial product Naturalis®, while no or adverse effect of natural adjuvants was recorded. The encouraging results of this study pave way for testing EPF isolates against *P. spumarius* in field conditions and find new environmentally friendly control strategies against insect vectors of *X. fastidiosa*.

### 1. Introduction

The spittlebug *Philaenus spumarius* (L.) (Hemiptera: Aphrophoridae) is the key vector of the phytopathogenic bacterium *Xylella fastidiosa* Wells et al. (Gammaproteobacteria: Lysobacteraceae) (*Xf*) in Europe (Cavaleri et al., 2019; Cornara et al., 2019, 2018). *Xf* is a xylem-limited bacterium identified as the causal agent of several plant diseases in the American continent (e.g. Pierce's disease on grapevine and citrus variegated chlorosis on citrus), and its recent discovery in the

Mediterranean basin has led to serious phytosanitary concerns. *Xf* was first identified in Europe in 2013 in Apulia (South Italy), and the strain *X. fastidiosa* subsp. *pauca* ST53 was characterized as the causal agent of the olive quick decline syndrome (OQDS), a severe plant disease that led to a dramatic dieback of olive trees in the Italian Region (Almeida, 2016; Cariddi et al., 2014; Saponari et al., 2017). Several *Xf* strains have been then found in different Mediterranean countries (Italy, Spain, France, Portugal, Israel, Lebanon) – although with less severe economic impact than Apulia's scenario – as a result of separate introduction routes of

**Abbreviations:** EPF, Entomopathogenic fungi; *Xf*, *Xylella fastidiosa*; LaB, *Lecanicillium aphanocladii* blastospores; LaC, *Lecanicillium aphanocladii* conidia; Ncf, *Beauveria bassiana* ATCC 74040 Naturalis® conidia applied on nymphs covered by foam; Ncbf, *Beauveria bassiana* ATCC 74040 Naturalis® conidia applied on nymphs not covered by foam; Wtr, water/control.

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**Table 1**  
Experimental design of trial 1 (T1) in 2021.

Code	Fungus	Propagule <sup>†</sup>	Adjuvant	Concentration (propagule/mL)	Replicas	Nymphs/replica
<b>TRIAL 1—2021</b>						
LaB_Ad1	<i>L. aphanocladii</i>	Blastospores	Adjuvant 1 + Tween 80	1 × 10 <sup>8</sup>	3	10
LaB_Ad2	<i>L. aphanocladii</i>	Blastospores	Adjuvant 2 + Tween 80	1 × 10 <sup>8</sup>	3	10
LaB_Wtr	<i>L. aphanocladii</i>	Blastospores	Tween 80	1 × 10 <sup>8</sup>	3	10
LaC_Ad1	<i>L. aphanocladii</i>	Conidia	Adjuvant 1 + Tween 80	1 × 10 <sup>8</sup>	3	10
LaC_Ad2	<i>L. aphanocladii</i>	Conidia	Adjuvant 2 + Tween 80	1 × 10 <sup>8</sup>	3	10
LaC_Wtr	<i>L. aphanocladii</i>	Conidia	Tween 80	1 × 10 <sup>8</sup>	3	10
Myc	<i>L. muscarium</i> Ve6 (Mycotal)	Conidia	—	1 × 10 <sup>8</sup>	3	10
Ad1	—	—	Adjuvant 1	1 × 10 <sup>8</sup>	3	10
Ad2	—	—	Adjuvant 2	1 × 10 <sup>8</sup>	3	10
Wtr <sup>‡</sup>	—	—	Tween 80	—	3	10
<b>TRIAL 2—2022</b>						
LaB	<i>L. aphanocladii</i>	Blastospores	Tween 80	1 × 10 <sup>8</sup>	5	10
LaC	<i>L. aphanocladii</i>	Conidia	Tween 80	1 × 10 <sup>8</sup>	5	10
NCf	<i>B. bassiana</i> ATCC 74040 (Naturalis)	Conidia	Tween 80	1 × 10 <sup>8</sup>	5	10
NCbf	<i>B. bassiana</i> ATCC 74040 (Naturalis)	Conidia	Tween 80	1 × 10 <sup>8</sup>	5	10
Wtr <sup>+</sup>	—	—	Tween 80	—	5	10

<sup>†</sup> blastospores were supplied by Globachem as liquid formulation in Trial 1 and freeze dried spores in Trial 2.

<sup>‡</sup> water treatment = negative control.

different subspecies and strains, some dating back to forty/fifty years ago (Dupas et al., 2023; Moralejo et al., 2020; Soubeyrand et al., 2018). The economic and social consequences of *Xf* in Apulia have already been significant (Italia Olivicola Consorzio Nazionale, 2019; Saponari et al., 2019), and they could increase in the near future, given that the bacterium continues spreading in the Region (Kottelenberg et al., 2021). In addition, the pathogen and its associated diseases could be introduced to other olive-producing areas of the Mediterranean or produce new pathosystems with other susceptible crops, e.g. grapevine (Landa et al., 2020; Schneider et al., 2021, 2020; Sicard et al., 2018).

*Xf* is transmitted from plant to plant by xylem-sap feeding insects, and *P. spumarius* is by far the prominent vector in Europe (Almeida et al., 2005; Bodino et al., 2019; Cornara et al., 2019). Thus, the spittlebug represents a primary target of control strategies to reduce the spread and impact of *Xf* epidemics, given the current lack of curative applications upon persistent systemic infections of crop plants (Bodino et al., 2023; Morelli et al., 2021).

The control of spittlebug populations in Apulia relies on mechanical treatments, such as tillage and mowing, to kill nymphs in spring, and application of insecticides on olive trees against the adult stage (EFSA Panel on Plant Health (PLH) et al., 2019; EU, 2020/1201; Morelli et al., 2021; Sanna et al., 2021). While the effectiveness of agronomic measures in reducing spittlebug populations in the field is demonstrated (EFSA Panel on Plant Health (PLH) et al., 2019; López-Mercadal et al., 2021; Sanna et al., 2021), the impact of insecticidal treatments on vector density remains unclear. Indeed, several issues with the use of insecticides against spittlebugs are currently present, among them: i) few active ingredients available, also due to the banning in EU of some neonicotinoids, the most effective chemical family of insecticide against *P. spumarius* (Dongiovanni et al., 2018); ii) quite low persistence of available insecticides, such as organophosphates and Spinosad (Dáder et al., 2019); iii) high densities of both nymphal and adult stages in semi-natural reservoirs, e.g. grassland, woodland, garrigue (Bodino et al., 2020a, 2019; Cappellari et al., 2022; Cornara et al., 2021); iv) adult spittlebugs exposed to insecticides could suffer sublethal effects and/or active movements (flight), possibly impairing the effectiveness of control measures on the disruption of *Xf* transmission (Bodino et al., 2020b; Lago et al., 2021). Moreover, insecticidal treatments may have not only a significant economic impact but also harmful consequences on non-target arthropods, on microbial biodiversity (Bony et al., 2008; Coll et al., 2011), deep and surface water (Bony et al., 2008), animal and human health (Raheison et al., 2019; Tsakirakis et al., 2014).

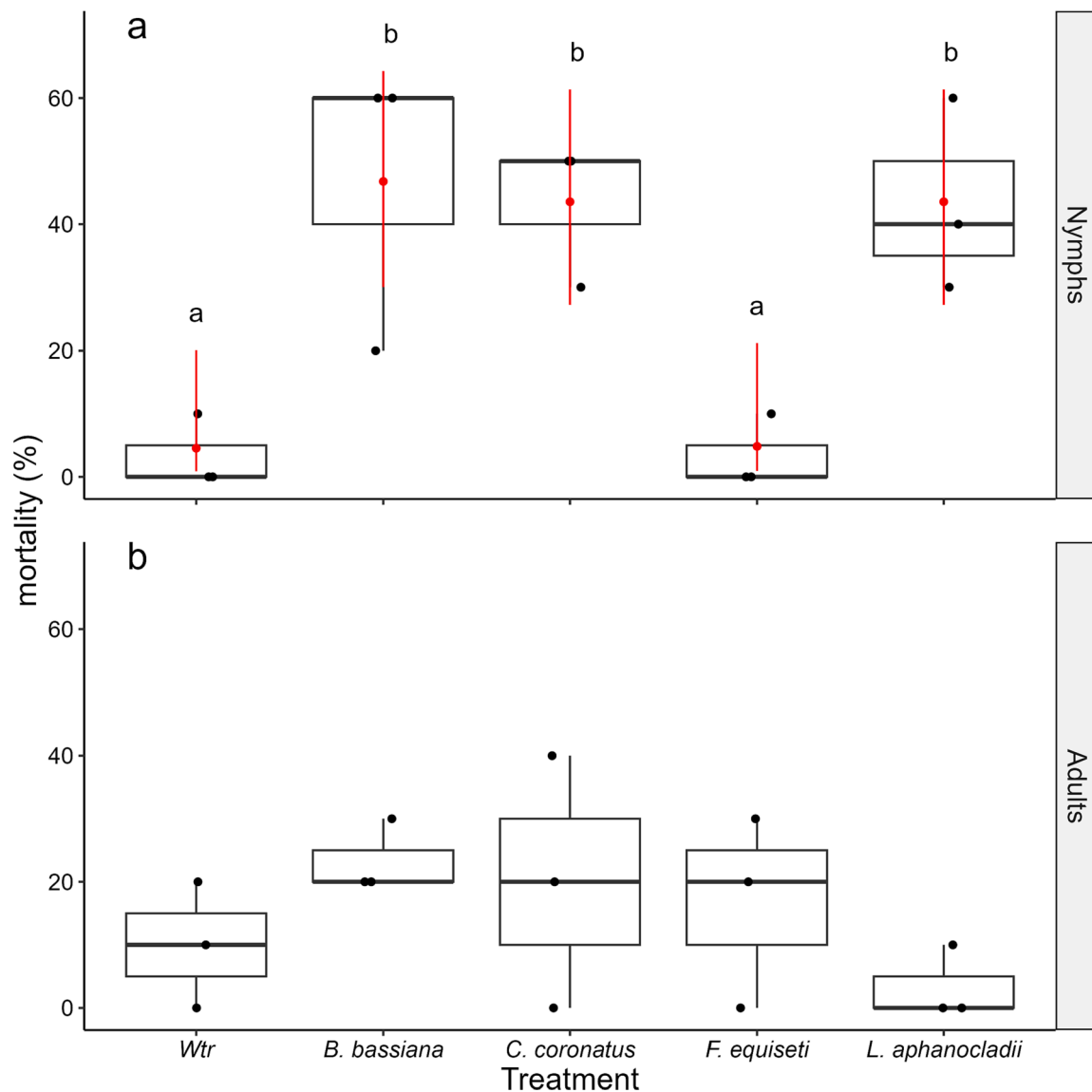
Alternative biological control strategies against *Xf* vectors are currently investigated and their implementation strongly suggested by

EU in the framework of the European “Green Deal”, that requires a transition to fair, healthy and environmentally friendly agricultural systems ([https://ec.europa.eu/info/strategy/priorities-2019–2024/european-green-deal\\_it](https://ec.europa.eu/info/strategy/priorities-2019–2024/european-green-deal_it)). The adverse environmental effects of chemical insecticides have increased the interest in microbiological agents over the years. Among others, entomopathogenic fungi (EPF) stand out as efficient biological control agents that infect the insect hosts by contact through the cuticle. Most, if not all, insect species can be naturally infected by EPF, which exploit them as a nutrient source (Lovett and Leger, 2017). As natural pathogens of insect pests, fungi have been successfully used as biocontrol agents in various crops and over 170 products have been formulated and marketed as mycopesticides (Bamisile et al., 2021; Rondot and Reineke, 2018; Vidal and Jaber, 2015). They are mainly based on fungal propagules to be applied on the vegetation through nebulizing treatments which ensure almost complete coverage and maximize the probability of contact and infection of the target insect.

EPF have been found associated in field conditions to some American *Xf* vectors, e.g. the glassy-winged sharpshooter *Homalodisca vitripennis* (Germar) and *Oncometopia facialis* (Signoret) (Hemiptera: Cicadellidae: Cicadellinae). Various fungi species are known to infect these insects with various efficiency, e.g. *Hirsutella homalodiscae* nom. Prov. (Ascomycota: Hypocreales), *Beauveria bassiana* Balsamo Vuill. (Ascomycota: Hypocreales), *Isaria poprawskii* Caban., J.H. de Leon, Humber, K.D. Murray & W.A. Jones (Ascomycota: Hypocreales). *Metarhizium anisopliae* (Metschn.) Sorokin (Ascomycota: Hypocreales), *Trichothecium roseum* (Pers.) Link (Ascomycota: Hypocreales), although some are probably secondary pathogens or saprobes rather than primary pathogens of the sharpshooter (Boucias et al., 2007; Cabanillas and Jones, 2013; Dara et al., 2008; Kanga et al., 2004; Lietze et al., 2011; Pria Júnior et al., 2008).

Scientific literature is rather poor in information on EPF natural occurring on *P. spumarius*. In the past, (Weaver and King, 1954) reported that an epidemic of *Entomophthora aphrophorae* Rostr. was observed in Denmark by Sorauer in 1913. Also, *Zoophthora petchi* was found attacking *P. spumarius* in England (Ben-Ze'ev and Kenneth, 1981) and Holland (Mietkiewski and Van der Geest, 1985) but no further evidence has been reported in literature to date. Only recently, EPF strains isolated from environment and belonging to *Trichoderma* genus have been successfully tested for their pathogenicity against *P. spumarius* although under laboratory conditions (Ganassi et al., 2023).

In light of the above, a gap of knowledge on EPF species naturally occurring on *P. spumarius* needs to be filled. The possibility of handling native EPF strains isolated directly from the target insect could represent



**Fig. 1.** Percentage of mortality observed (boxplots) and predicted by binomial GLM (red point range) for nymphal and adult stages of *Philaenus spumarius* in experimental trials of pathogenic activity of EPF isolates. Treatments sharing the same letter within a facet are not significantly different ( $\alpha = 0.05$ , Tukey-adjusted). Predictions by binomial GLM are not represented for adult stage because the effect of treatment on mortality rate was not significant, i.e., no differences in mortality rate among experimental treatments. Thick horizontal lines represent the median, the boxes the interquartile range and the vertical lines the extent of the variability outside the upper and lower quartiles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

an improvement in terms of both awareness and success rate, and strains isolated from indigenous cadavers warrant study of their biological control potential (Quesada-Moraga et al., 2023; Shang et al., 2022).

Thus, the aims of this study were i) the identification of naturally occurring EPF isolated from *P. spumarius* cadavers in semi-field conditions and ii) the evaluation of biocontrol potential of the strains showing promising pathogenic behaviour against both nymphal and adult stage of the target insect *P. spumarius*.

## 2. Materials and methods

The present study spans over 4 years, from 2019 to 2022, because of both the univoltine life cycle of the target insect and the COVID-19 pandemic restrictions in 2020. In 2019, *P. spumarius* individuals affected by mycosis were recovered, and fungal strains isolated and identified. In late spring-early summer 2019, preliminary tests were carried out using isolates belonging to the most promising fungal species, against both *P. spumarius* nymphs and adults. Trials were resumed

in 2021, after COVID-19-related interruption, and focused on the pathogenic activity of *Lecanicillium aphanocladii* strain versus *P. spumarius* nymphs. Finally, the trials were replicated in spring 2022.

### 2.1. Origin and identification of EPF strains

#### 2.1.1. Fungi from infected *P. spumarius* adults

Fungal strains were isolated from *P. spumarius* adults affected by mycosis and recovered from a semi-field maintenance rearing (e.g. insect-proof greenhouse with grass cover and potted olive plants) in Torino. Four *P. spumarius* cadavers found externally sporulated were carefully collected and each placed in a micro tube with a brush sterilized with alcohol. Small amounts of conidia and of mycelium from cadavers were transferred to Sabouraud dextrose agar (SDA, Oxoid, Milan, Italy) Petri plates, supplemented with penicillin G (0.1 mg/mL) and streptomycin (0.5 mg/mL), and incubated at 25 °C. Pure cultures were obtained by single spore isolation (monosporic cultures), transferred to mycological tubes on Malt Extract Agar (MA, Oxoid) slants, and kept at

**Table 2**

Pathogenic activity of naturally occurring EPF strains against *Philaenus spumarius* nymphs, Nymphal mortality and adult emergence estimates (%) and variance [standard error of the mean (SEM), lower and upper confidence limit 95 % (UCL)] calculated by binomial GLM (actual results reported in the text), Treatments sharing the same letter in group column are not significantly different ( $\alpha = 0.05$ , Tukey-adjusted).

Variable	Treatment	Estimate (%)	SEM	LCL	UCL	group
<b>Nymphal mortality</b>						
	<i>Wtr</i>	4.55	3.68	0.89	20.08	a
	<i>F. equiseti</i>	4.84	3.92	0.95	21.23	a
	<i>C. coronatus</i>	43.55	9.05	27.26	61.35	b
	<i>L. aphanocladii</i>	43.55	9.05	27.26	61.35	b
	<i>B. bassiana</i>	46.77	9.11	30.02	64.29	b
<b>Adult emergence</b>						
	<i>Wtr</i>	87.50	71.06	95.23	b	b
	<i>F. equiseti</i>	86.67	69.41	94.9	b	b
	<i>C. coronatus</i>	50	32.83	67.17	a	a
	<i>L. aphanocladii</i>	40	24.31	58.05	a	a
	<i>B. bassiana</i>	33.33	18.97	51.65	a	a

4 °C for their storage.

### 2.1.2. Molecular identification of fungi

The four fungal strains stored at 4 °C were transferred to MA Petri plates and cultured at 25 °C for 10 days. For each strain, 100 mg of mycelium was harvested and transferred in a micro tube for DNA extraction. Fungal genomic DNA was extracted using DNeasy Plant mini kit (Qiagen, Milan, Italy) in accordance with the manufacturer's instructions. The internal transcribed spacer (ITS) region was used to identify the fungi as proposed by Schoch et al., (2012), PCR was performed by using ITS1 and ITS4 primers (White et al., 1990) and amplicons were sent for purification and sequencing to Eurofins GmbH (Hamburg, Germany). The generated sequences were then analysed using NCBI BLAST database (GenBank) to find the closest match of the sequences for fungal species identification.

## 2.2. Collection and rearing of *P. Spumarius*

Early nymphal stages (1st-2nd) of *P. spumarius* were collected directly in the field during the early Spring (March) of each year of the study. Samplings were carried out on herbaceous cover in a mixed agricultural area near Torino (Chieri: 45.0179 N, 7.787 E). Nymphs were gently collected from the ground vegetation and placed into mesh/plastic containers with excised *Taraxacum officinalis* L. leaves for transportation to the laboratory facilities. Upon arrival at the laboratory, nymphs were transferred to potted *Cichorium intybus* L. (six true leaves growth stage) and *Vicia faba* L. (four true leaves) plants in mesh/plastic cages (Bugdorm BD4S4590) located under controlled conditions in climatic chamber (21 °C and 14:10 h L:D photoperiod). Nymphs were maintained in mass rearings under controlled conditions until they reached the appropriate developmental stage to start the fungal pathogenicity bioassays.

## 2.3. Screening of EPF strains in preliminary trials on *P. spumarius* nymphs and adults

### 2.3.1. Fungal strains and preparation of conidia inoculum

The four fungal strains previously isolated and identified as entomopathogenic species were used in preliminary trials performed in 2019 to assess their potential pathogenic activity against *P. spumarius* nymphs and adults. For each strain, the conidial suspension used in the tests was prepared as follows: fungi were grown on Potato Dextrose Agar (PDA, Oxoid) Petri plates and incubated at 25 °C for 14 days (7 days were sufficient for *Conidiobolus coronatus*); then, conidia were collected by scraping them from the surface of mycelium and filtered through sterile gauze to remove mycelial debris. Conidial suspensions were adjusted

with sterile distilled water with 0.05 v/v Tween 80® (v/v) and vortex-mixed for 2–3 min to obtain the final volume of 15 mL (5 mL per replica, for each strain) and the concentration of  $1 \times 10^8$  conidia/mL ( $2 \times 10^6$  conidia/mL for *C. coronatus*), as reported by other authors for controlled condition trials (González-Mas et al., 2019; Moussa et al., 2021).

The suspensions — except in the case of the biopesticide Mycotal which was used in the condition it was acquired — were freshly prepared on the day of the trials when also conidia viability was determined based on germ tube formation (Cabanillas and Jones, 2013). Conidia suspensions were judged acceptable if the viability was 90 % and above.

### 2.3.2. Experimental setup

The entomopathogenic activity of the four fungal strains previously isolated was tested in 2019 on *P. spumarius* nymphs and adults by direct spraying of conidia suspensions. Nymphal and adult stages were exposed, separately, to five experimental treatments corresponding to the four fungal strains and a negative control (water plus 0.05 v/v Tween 80® (v/v), *Wtr*).

Experimental microcosms (i.e., replicas) consisted in two *V. faba* plants (3 true leaf-age) sown in a single rounded pot (diam, 14 cm) enclosed by a clear plastic cylindrical cage (diam. 14 cm, height 45 cm, two holes with mesh for ventilation at top and side). The cylindrical cage was specifically designed to tightly fit inside the pot to ensure no space allowing insect escape.

Ten *P. spumarius* individuals (2nd-3rd stage nymphs or adults emerged no more than 30 days before, according to the assay) were introduced in each microcosm, after being randomly collected from mass rearings. Three replicas per treatment were performed. Each microcosm was nebulized by aerograph with 5 mL of suspension ( $1 \times 10^8$  conidia/mL =  $5 \times 10^8$  conidia in total) to reach the runoff point on plant leaves.

The fungal treatments were applied 24 h after placing the nymphs on plants, in order to allow nymphs to settle on leaves and stems and produce foams, mimicking natural field condition. Conversely, treatments of adult trials were applied on the plants immediately before placing the insects inside the microcosm. To avoid contamination between treatments, plants were sprayed outside the climatic chamber, left to air dry for 15 min, then returned to the chamber. Treatments were applied in mid-April and mid-June 2019. A data logger placed inside the climatic chamber registered a temperature of  $24.6 \pm 1.5$  °C and  $75.1 \pm 5.6$  % RH during the nymphal experimental trial and  $22.1 \pm 0.65$  °C and  $86.1 \pm 5.1$  % RH during the adult experimental trial.

### 2.3.3. Mortality assessment

After the treatments, microcosms were visually inspected every 2–3 days (i.e. two/three-times per week), assessing the survival rate of insects and collecting dead individuals. Newly emerging adults were also counted, and immediately removed to avoid recounting. When required to correctly assess the number of alive nymphs, foams on plant inside microcosm were gently open with a tiny brush, cleaned in 95 % alcohol between each replica inspection, to avoid possible transfer of fungi between replicas. The inspections were carried out for up to three weeks or when no alive individuals were observed for two inspections in a row, allowing precise estimation of the speed of fungal strain action.

Samples of dead/symptomatic insects were collected to re-isolate the fungal strains. Dead insects were at once placed in 1.5 mL Eppendorf tubes and individually surface sterilized with a small brush dipped in a solution of 1 % sodium hypochlorite and then in distilled sterile water to eliminate sodium hypochlorite residues. Individuals were subsequently stored in Petri plates fitted with moistened filter paper. Insects were incubated at 23 °C for 10 d to encourage mycelium development and daily observation was performed for the presence or absence of mycelium growth and sporulation. Once hyphal outgrowth of presumptive pathogens was detected, a first observation under the stereomicroscope provided an initial assessment of the fungal strain; then, insects were

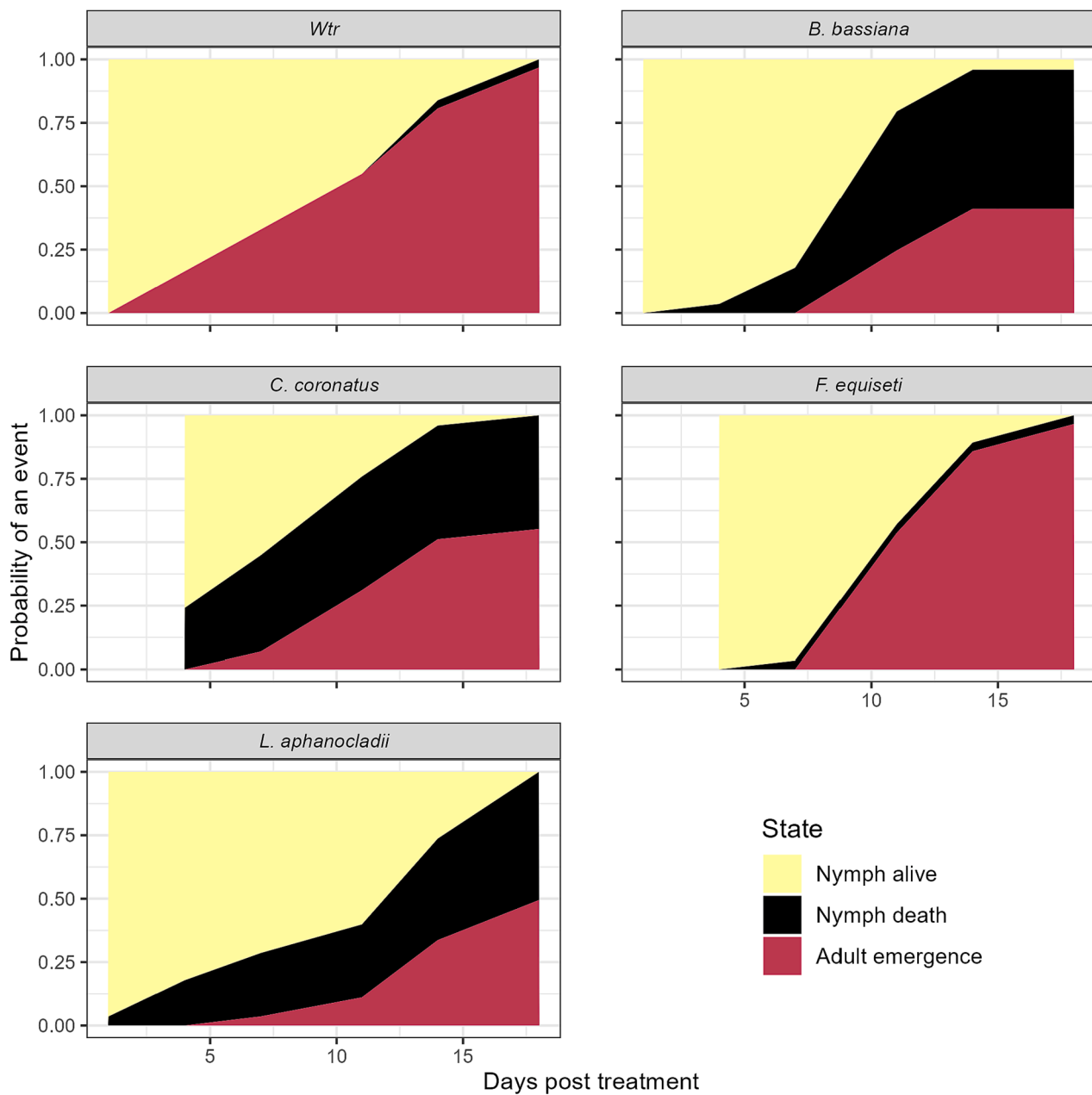


Fig. 2. Survival curves fitted by multi-state Cox Hazard Model, Areas of different colors represent the estimated probability of each *Philaenus spumarius* nymphal state (alive, death, adult emergence) over time after the experimental treatment.

impressed on MA medium supplemented with penicillin G (0.1 mg/mL) and streptomycin (0.5 mg/mL) for fungal re-isolation. Multiplicative structures were observed under optical microscope to confirm the preliminary identification and assess if the fungi isolated from the insects were related to the inoculated species.

#### 2.4. Efficacy of a *L. aphanocladii* strain on *P. spumarius* nymphs in pathogenicity bioassays

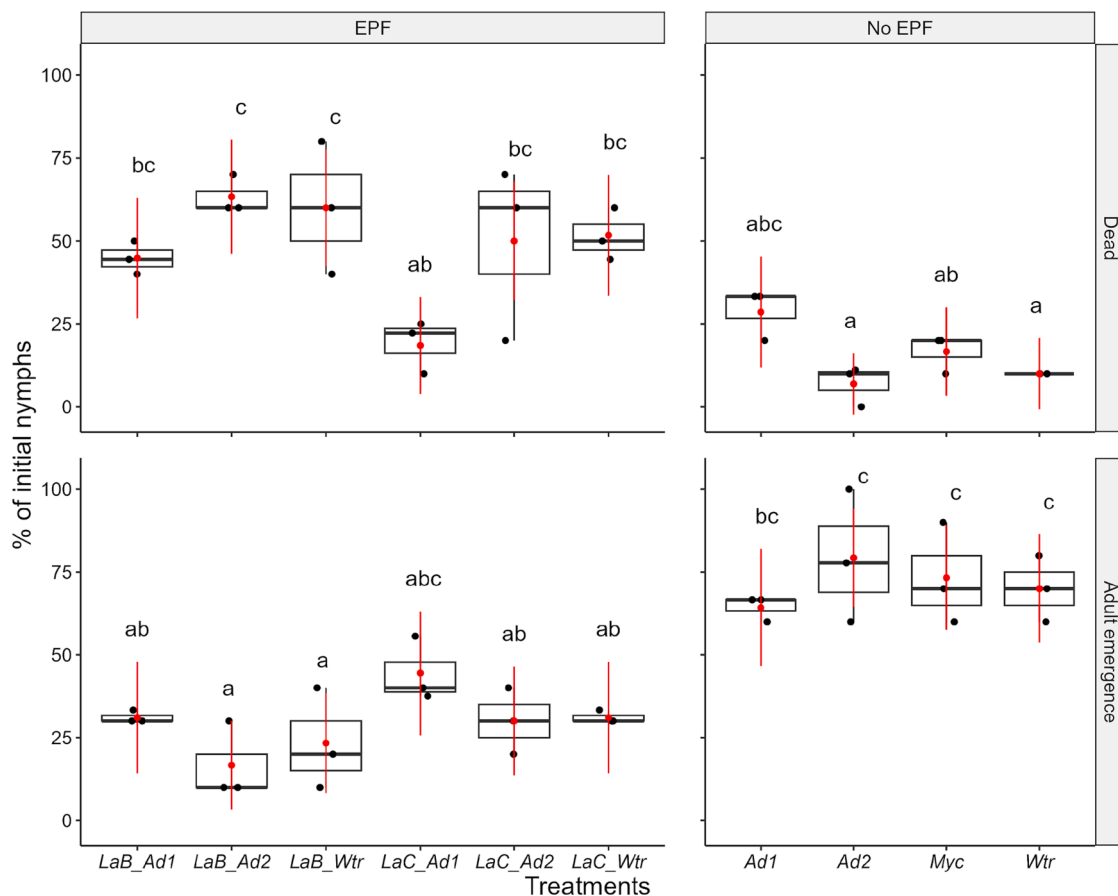
##### 2.4.1. Fungal strains and propagule preparation

Based on the results of preliminary tests, the fungal strain *L. aphanocladii* DSF (code due to its isolation at Department of Agricultural, Forest and Food Sciences - DISAFA - laboratories) was selected and used in pathogenicity bioassays against *P. spumarius* nymphs, both in conidia and blastospore formulations. In addition, *B. bassiana* ATCC 74040 strain from the commercial product Naturalis® (Biogard,

Grassobbio, Bergamo, Italy) and the bio-insecticide Mycotal® (Koppert, Scarborough, Ontario, Canada) based on *Lecanicillium muscarium* Ve6 strain were used as positive controls.

*L. aphanocladii* DSF blastospores obtained from liquid substrate growth of the fungus were provided by the agrochemical company Globachem (Sint-Truiden, Belgium) and kept at 4 °C until use. They were supplied as liquid formulation or freeze-dried spores. The latter were rehydrated in sterile deionized water for 30 min at room temperature before use. Appropriate dilutions of blastospore suspensions were prepared to reach the final concentration of  $1 \times 10^8$  blastospore/mL on the same day of experimental treatments, and added of Tween 80 (0.01 % v/v) for a homogeneous dispersion of the propagules. *L. aphanocladii* DSF conidia suspensions were prepared at DISAFA according to the protocol described above and propagule viability was also determined as previously reported (2.3.1).

*B. bassiana* ATCC 74040 strain was isolated from Naturalis® dried



**Fig. 3.** Percentage of initial nymphs of *Philaenus spumarius* found dead or emerged as adults during the 2021 experimental trial (T1) of *Lecanicillium aphanocladii* DSF efficacy. Data observed (boxplots) and predicted by binomial GLM (red point range) are reported, and treatments sharing the same letter within a row are not significantly different ( $\alpha = 0.05$ , Tukey-adjusted). Thick horizontal lines represent the median, the boxes the interquartile range and the black vertical lines the extent of the variability outside the upper and lower quartiles, Red points and lines represent mean and standard error of the mean, See explanation of abbreviation of treatments in Material and Methods section, (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

powder by streak plate method to obtain a pure culture stored at 4 °C before use; afterwards, a conidia suspension was prepared and adjusted to  $1 \times 10^8$  conidia/mL according to the procedure described for *L. aphanocladii* DSF (2.3.1). Differently, Mycotal® was used as supplied by manufacturer ( $10^{10}$  blastospores/g) and rehydrated in sterile water immediately before use to obtain a suspension of  $10^8$  blastospores/mL.

#### 2.4.2. Experimental treatments and data collection

Pathogenicity bioassays on *P. spumarius* nymphs were carried out, in controlled conditions, in mid-April 2021 (trial 1, T1) and 2022 (trial 2, T2). The experimental design is reported in Table 1. Briefly, in T1 ten experimental treatments were tested, six determined by the combination of fungus (*L. aphanocladii* DSF, La), propagules (conidia, C or blastospores, B) and natural adjuvants, that should help penetrate the insect cuticula, (Ad1 and Ad2, provided by Globachem, composition under business secrecy) to be added to propagule suspensions in order to evaluate their efficacy in synergy with fungal propagules; also, four control levels were tested: Mycotal (Myc), adj1, adj2 and water (Wtr). In T2, *L. aphanocladii* blastospores (LaB) and *L. aphanocladii* conidia (LaC) treatments were compared to *B. bassiana* ATCC 74040 conidia (NCf), used as positive control, and water (negative control, Wtr). All the previously described treatments were applied 24 h after placing the nymphs on plants, in order to allow nymphs to settle on leaves and stems and produce foams. Conversely, an additional treatment was tested to investigate the effect of foam on EPF effectiveness, involving nebulization of *B. bassiana* ATCC 74040 conidia suspension on nymphs

immediately after placing them on plants, before foam formation (NCbf).

The *V. faba* microcosms set up for the treatments are described above (2.3.2). In each microcosm, 10 nymphs of *P. spumarius* were placed, carefully took with brush and placed onto plant leaves from maintenance rearing present at DISAFA. Each treatment was replicated in three (T1) or five (T2) microcosms, for a total of 30–50 individuals (in average 2nd–3rd instar nymphs) for treatment. A data logger placed inside climatic chamber registered hourly climate data for each trial during the experimental periods (mean  $\pm$  SD – 2021:  $22.8 \pm 1.1$  °C and  $79.2 \pm 11.6$  % RH; 2022:  $23.4 \pm 1.6$  °C and  $73.5 \pm 7.6$  % RH).

After the treatments, mortality assessment was performed as previously reported (2.3.3). Dead insects were surface sterilized, as previously reported, and incubated in Petri plates containing filter paper moistened with sterile distilled water to encourage mycelium development and fungal re-isolation on culture medium was performed to assess if the fungi isolated from the cadavers were attributable to *L. aphanocladii* DSF. *B. bassiana* ATCC 74040 and *L. muscarium* Ve6 strains.

#### 2.5. Statistical analysis

The estimates of nymphal survival/mortality rates were calculated using the counts of both the dead nymphs and the adults emerging, being the latter a more reliable proxy of the survival of individuals, given the difficulty in correctly counting the nymphal stages inside the

**Table 3**

T1-2021; *Lecanicillium aphanocladii* DSF efficacy on *Philaenus spumarius* nymphs in 2021 trial (T1), Nymphal mortality and adult emergence estimates (%) and variance [standard error of the mean (SEM), lower and upper confidence limit 95 % (UCL)] calculated by binomial GLM, Treatments sharing the same letter in group column are not significantly different ( $\alpha = 0.05$ , Tukey-adjusted). See explanation of abbreviation of treatments in Material and Methods section.

Variable	Treatment	Estimate (%)	SEM	LCL	UCL	group
<b>Nymphal mortality</b>						
	Ad2	6.90	4.71	0	16.12	a
	Wtr	10.00	5.48	0	20.74	a
	Myc	16.67	6.80	3.33	30.00	ab
	LaC_Ad1	18.52	7.48	3.87	33.17	ab
	Ad1	28.57	8.54	11.84	45.30	abc
	LaB_Ad1	44.83	9.23	26.73	62.93	bc
	LaC_Ad2	50.00	9.13	32.11	67.89	bc
	LaC_Wtr	51.72	9.28	33.54	69.91	bc
	LaB_Wtr	60.00	8.94	42.47	77.53	c
	LaB_Ad2	63.33	8.80	46.09	80.58	c
<b>Adult emergence</b>						
	LaB_Ad2	16.67	6.80	3.33	30.00	a
	LaB_Wtr	23.33	7.72	8.20	38.47	a
	LaC_Ad2	30.00	8.37	13.60	46.40	ab
	LaB_Ad1	31.03	8.59	14.20	47.87	ab
	LaC_Wtr	31.03	8.59	14.20	47.87	ab
	LaC_Ad1	44.44	9.56	25.70	63.19	abc
	Ad1	64.29	9.06	46.54	82.03	bc
	Wtr	70.00	8.37	53.60	86.40	c
	Myc	73.33	8.07	57.51	89.16	c
	Ad2	79.31	7.52	64.57	94.05	c

microcosm. The influence of experimental treatment on survival of *P. spumarius* nymphs was hence inferred by both nymphal mortality (i.e. proportion of nymphs found dead) and adult stage emergence (i.e. total number of adults on total number of nymphs placed into each microcosm before the treatment). The two dependent variables were modelled as end-point data – i.e. nymphal mortality or emergence proportion during the entire duration of trials – using binomial GLMs (glm function in package *stat*). When appropriate, mean bias-reducing adjusted scores approach was used (function *brglmFit* in *brglm2* package. Firth, 1993) to avoid quasi-complete separation due to a category (Treatment) with all (1) or no (0) nymphs dead or emerged. The probability of nymphal status (alive, dead, emerged as adult) through time post treatment as an effect of experimental treatment was analysed by multi-state Cox proportional hazards models (coxph function in package *survival*. Therneau, 2023), with nymphs not found during visual inspection – possibly escaped – as censoring events. Data were tested to confirm they fit model assumptions using the *coxph* function (*survival* package). The end-point proportion of adult stage survival was modelled using a binomial GLM (glm function in package *stats*). All analyses were performed and plots produced using the software R (R Core Team, 2020).

### 3. Results

#### 3.1. Identification of EPF strains isolated from *P. Spumarius*

Four fungal isolates were identified based on rDNA-ITS homology analysis. ITS sequences showed 100 % similarity to *Lecanicillium aphanocladii* (GenBank sequence ID: KC574075.1). 99.8% similarity to *Beauveria bassiana* (GenBank sequence ID: MF872381.1), 99.6 % similarity to *Fusarium equiseti* (GenBank sequence ID: MN559437.1) and 98 % similarity to *C. coronatus* (GenBank sequence ID: AJ345094.1). Given the high level of similarity found, we did not consider testing other gene regions. Furthermore, molecular identification was supported by macroscopic and microscopic analysis of phenotypic characteristics as colony colour, hyphal aspect, multiplicative structure and propagule morphology.

For all the species identified, literature have reported evidence on their entomopathogenic potential (Sharma and Marques, 2018; Souza

et al., 2014; Wrońska et al., 2018; Zhao et al., 2023). Thus, the four fungal strains were studied in preliminary trials to test their efficacy against the target insect *P. spumarius*.

#### 3.2. Pathogenic activity of EPF isolates against *P. Spumarius* nymphs and adults

##### Nymphs.

The viability of conidia used in the tests was comparable between the different strains:  $97.2 \pm 2.2$  % for *B. bassiana*,  $95.8 \pm 4.0$  % for *L. aphanocladii*,  $93.1 \pm 2.5$  % for *C. coronatus* and  $90.2 \pm 4.3$  % for *F. equiseti*.

The efficacy of the four EPF strains on *P. spumarius* nymphs (i.e. end-point mortality percentage) was significantly different. Indeed, the probability of nymphal death was significantly higher for individuals exposed to *B. bassiana* (46.7 %, 14/30), *L. aphanocladii* and *C. coronatus* (43.3 %, n = 13/30), compared to *F. equiseti* (3.3 %, 1/30) and control (*Wtr*) (0 %, 0/32) (GLM:  $\chi^2 = 38.73$ , df = 4,  $p < 0.001$ ) (Fig. 1a; Table 2). The hazard ratio of nymphal death (i.e. average risk of death) was significantly higher for nymphs treated with *B. bassiana* (HR = 19.5, CI = 2.8–137), *C. coronatus* (HR = 20.1, CI = 2.76–146) and *L. aphanocladii* (HR = 15.9, CI = 2.13–119) than control (*Wtr*). *Fusarium equiseti* treatment did not significantly increase nymphal death risk compared to control (HR = 1.11, CI = 0.07–17.3), while it showed significantly lower death risk than the other tested EPF species (HR = 14.3–18.0) (Fig. 2).

The probability of adult emergence was significantly lower for individuals exposed to *B. bassiana* (33.3 %, 10/30), *L. aphanocladii* (40 %, n = 12/30) and *C. coronatus* (50 %, n = 15/30), compared to *F. equiseti* (86.7 %, 26/30) and control (*Wtr*) (87.5 %, 28/32) (GLM:  $\chi^2 = 36.92$ , df = 4,  $p < 0.001$ ) (Table 2). Thus, the hazard ratio of adult emergence was significantly lower for individuals treated with *B. bassiana* (HR = 0.69, CI = 0.5–0.96) and *L. aphanocladii* (HR = 0.47, CI = 0.40–0.55) compared to control (*Wtr*). No differences in adult emergence risk were observed between *C. coronatus* (HR = 1.14, CI = 0.94–1.37) and *F. equiseti* (HR = 1.1, CI = 0.82–1.46) compared to the control treatment (*Wtr*) (Fig. 2).

##### Adults.

No significant differences in mortality of adult stage of *P. spumarius* were observed among the four tested EPF strains and compared to the control (*Wtr*) ( $\chi^2 = 7.14$ , df = 4,  $p = 0.13$ ) (Fig. 1b).

For the subsequent pathogenicity bioassays focused on *P. spumarius* nymphs, *L. aphanocladii* DSF strain was selected among the others because judged the most promising in terms of performance and noteworthy for the novelty of the species it belongs to. The strain was named *L. aphanocladii* DSF due to its isolation at Department of Agricultural, Forest and Food Sciences (DISAFA) laboratories.

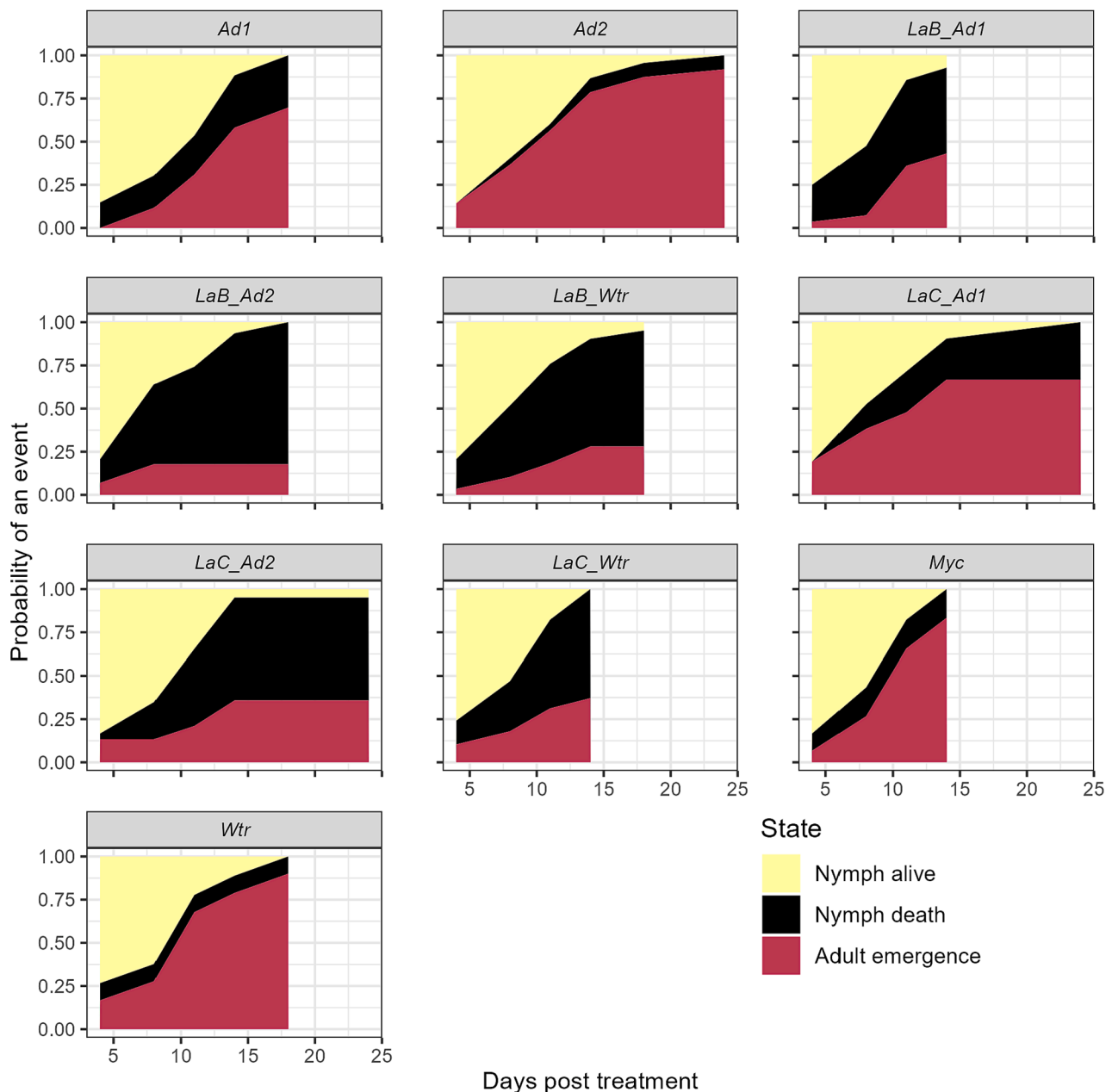
Despite the performance comparable to *L. aphanocladii*, *C. coronatus* and *B. bassiana* strains were not preferred because the latter belongs to a species already well known and present on the market for biocontrol, and the former can cause mycosis in mammals, including humans. Actually, its incidence as a human pathogen remains low but it may represent a risk for immunocompromised individuals (Vilela and Mendoza, 2018).

#### 3.3. *L. aphanocladii* DSF efficacy on *P. spumarius* nymphs

The pathogenicity of *L. aphanocladii* DSF against *P. spumarius* nymphs was assessed under controlled conditions in two separate trials (T1 and T2). The viability of fungal propagules was checked immediately before their use and was of  $95.5 \pm 4.2$  % and  $97.1 \pm 2.2$  % for *L. aphanocladii* blastospores used in T1 and T2, respectively;  $93.8 \pm 3.3$  % and  $94.8 \pm 4.3$  % for *L. aphanocladii* conidia in T1 and T2, respectively;  $97.4 \pm 2.0$  % for *B. bassiana* ATCC 74040 conidia used in T2.

##### T1

The end-point nymphal mortality (i.e. probability of death of a nymph) was overall significantly different among experimental



**Fig. 4.** Survival curves of nymphs *Philaenus spumarius* treated with different *Lecanicillium aphanocladii* formulations in trial T1, fitted by multi-state Cox Hazard Model. Areas of different colors represent the estimated probability of each *P. spumarius* nymphal state (alive, death, adult emergence) over time after the experimental treatment, See explanation of abbreviation of treatments in Material and Methods section.

treatments (GLM:  $\chi^2 = 44.08$ ,  $df = 9$ ,  $p < 0.001$ ), with the EPF-treated microcosms, except for *LaC\_Ad1* treatment, showing higher mortality rates compared to those treated with water (control). The highest nymphal mortality rates were recorded in *LaB\_Ad2* treatment ( $63.3 \pm 8.8\%$ ), although not significantly higher than the other EPF treatments (ranging 44.8–60.0%), again except for *LaC\_Ad1* one ( $18.5 \pm 7.5\%$ ) (Fig. 3 and Table 3), Mycotal *Myc* ( $16.7 \pm 6.8\%$ ) and the adjuvant 1 *Ad1* ( $6.9 \pm 3.3\%$ ) caused significantly less mortality in *P. spumarius* nymphs than *L. aphanocladii* formulations, comparable to that observed in control treatment *Wtr* ( $10.0 \pm 0.0\%$ ). The treatment applied was indeed a significant predictor of nymphal risk of death. Nymphs treated with *L. aphanocladii* formulations were significantly more likely to die compared to control (*Wtr*) (HR EPF treated:control = 4.79–7.94) (Fig. 4; Table 4). The only exception was *LaC\_Ad1* treatment, that did not cause a significant increase in the probability of nymphal death (HR = 2.39, CI = 0.56–10.19). Mycotal did not significantly increase the nymphal death

likelihood compared to control (*Wtr*) (HR = 1.6, CI = 0.35–7.31) (Fig. 4; Table 4).

The adult emergence rate was also significantly different among the tested treatments, with an expected inverse trend compared to nymphal mortality (GLM:  $\chi^2 = 51.29$ ,  $df = 9$ ,  $p < 0.001$ ). All the EPF treatments showed significantly lower emergence rates (16.7–31.0%) than *Wtr* ( $70.0 \pm 0.0\%$ ), *Myc* ( $73.3 \pm 8.1\%$ ), and *Ad1* ( $79.3 \pm 7.5\%$ ) ones (Fig. 3 and Table 3). The results obtained from the end-point adult emergence were confirmed by the over time competing risks analysis on the status of nymphs. Nymphs treated with *LaB\_Ad2*, *LaB\_Wtr*, *LaC\_Ad2* were significantly less likely to become adult than those in control (HR EPF treated:control = 0.25 – 0.54) (Fig. 4; Table 4). Also *Ad1* treatment decreased the likelihood of adult emergence (HR = 0.54, CI = 0.32–0.92), while neither *LaC\_Ad1* nor *Myc* treatments significantly reduced the adult emergence rate (HR *LaC\_Ad1*:control = 0.85, CI = 0.4–1.84; HR *Myc*:control = 1.11, CI = 0.66–1.86).



**Table 4**

T1-2021; Hazard ratios and 95% confidence intervals (95% CI) for transitioning of *Philaenus spumarius* nymphs from alive to dead and alive to adult emergence for experimental treatments respect to control (*Wtr*) and *LaB\_Ad2*, representing minimum and maximum mortality, respectively. See explanation of abbreviation of treatments in Material and Methods section.

Transition	Treatment	With respect to	Hazard ratio	95 % CI		z	p-value		
Alive to dead	<i>Ad1</i>	<i>Wtr</i>	2.06	0.51	8.39	1.01	0.31		
	<i>Ad2</i>	<i>Wtr</i>	0.59	0.10	3.58	-0.58	0.56		
	<i>LaB_Ad1</i>	<i>Wtr</i>	5.77	1.51	22.13	2.56	0.01	**	
	<i>LaB_Ad2</i>	<i>Wtr</i>	7.94	2.21	28.59	3.17	0.00	***	
	<i>LaB_Wtr</i>	<i>Wtr</i>	6.30	1.72	23.04	2.78	0.01	**	
	<i>LaC_Ad1</i>	<i>Wtr</i>	2.39	0.56	10.19	1.17	0.24		
	<i>LaC_Ad2</i>	<i>Wtr</i>	4.79	1.34	17.20	2.40	0.02	*	
	<i>LaC_Wtr</i>	<i>Wtr</i>	6.05	1.67	21.90	2.74	0.01	**	
	<i>Myc</i>	<i>Wtr</i>	1.60	0.35	7.31	0.60	0.55		
	<i>Wtr</i>	<i>LaB_Ad2</i>	0.13	0.03	0.45	-3.17	0.00	***	
	<i>Ad1</i>	<i>LaB_Ad2</i>	0.26	0.11	0.60	-3.15	0.00	***	
	<i>Ad2</i>	<i>LaB_Ad2</i>	0.07	0.02	0.31	-3.57	0.00	***	
	<i>LaB_Ad1</i>	<i>LaB_Ad2</i>	0.73	0.36	1.48	-0.88	0.38		
	<i>LaB_Wtr</i>	<i>LaB_Ad2</i>	0.79	0.42	1.48	-0.73	0.47		
	<i>LaC_Ad1</i>	<i>LaB_Ad2</i>	0.30	0.12	0.74	-2.62	0.01	**	
	<i>LaC_Ad2</i>	<i>LaB_Ad2</i>	0.60	0.33	1.09	-1.68	0.09		
	<i>LaC_Wtr</i>	<i>LaB_Ad2</i>	0.76	0.42	1.37	-0.90	0.37		
	<i>Myc</i>	<i>LaB_Ad2</i>	0.20	0.07	0.56	-3.08	0.00	***	
	Alive to adult	<i>Ad1</i>	<i>Wtr</i>	0.54	0.32	0.92	-2.29	0.02	*
		<i>Ad2</i>	<i>Wtr</i>	0.74	0.41	1.36	-0.96	0.33	
		<i>LaB_Ad1</i>	<i>Wtr</i>	0.52	0.25	1.08	-1.75	0.08	
		<i>LaB_Ad2</i>	<i>Wtr</i>	0.25	0.08	0.74	-2.49	0.01	*
		<i>LaB_Wtr</i>	<i>Wtr</i>	0.28	0.12	0.65	-2.93	0.00	**
		<i>LaC_Ad1</i>	<i>Wtr</i>	0.85	0.40	1.84	-0.40	0.69	
		<i>LaC_Ad2</i>	<i>Wtr</i>	0.30	0.14	0.66	-3.00	0.00	**
		<i>LaC_Wtr</i>	<i>Wtr</i>	0.46	0.21	1.01	-1.94	0.05	
		<i>Myc</i>	<i>Wtr</i>	1.11	0.66	1.86	0.39	0.70	
		<i>Wtr</i>	<i>LaB_Ad2</i>	4.05	1.35	12.17	2.49	0.01	**
		<i>Ad1</i>	<i>LaB_Ad2</i>	2.19	0.76	6.31	1.45	0.15	
		<i>Ad2</i>	<i>LaB_Ad2</i>	3.01	0.99	9.21	1.93	0.05	*
		<i>LaB_Ad1</i>	<i>LaB_Ad2</i>	2.12	0.65	6.86	1.25	0.21	
		<i>LaB_Wtr</i>	<i>LaB_Ad2</i>	1.12	0.32	3.96	0.18	0.86	
		<i>LaC_Ad1</i>	<i>LaB_Ad2</i>	3.46	1.01	11.85	1.98	0.05	*
		<i>LaC_Ad2</i>	<i>LaB_Ad2</i>	1.23	0.36	4.16	0.34	0.74	
<i>LaC_Wtr</i>		<i>LaB_Ad2</i>	1.85	0.55	6.25	0.99	0.32		
<i>Myc</i>		<i>LaB_Ad2</i>	4.48	1.56	12.91	2.78	0.01	**	

**Table 5**

*Lecanicillium aphanocladii* DSF efficacy on *Philaenus spumarius* nymphs in 2022 trial (T2), Nymphal mortality and adult emergence estimates (%) and variance [standard error of the mean (SEM), lower and upper confidence limit 95 % (UCL)] calculated by binomial GLM, Treatments sharing the same letter in group column are not significantly different ( $\alpha = 0.05$ , Tukey-adjusted), See explanation of abbreviation of treatments in Material and Methods section.

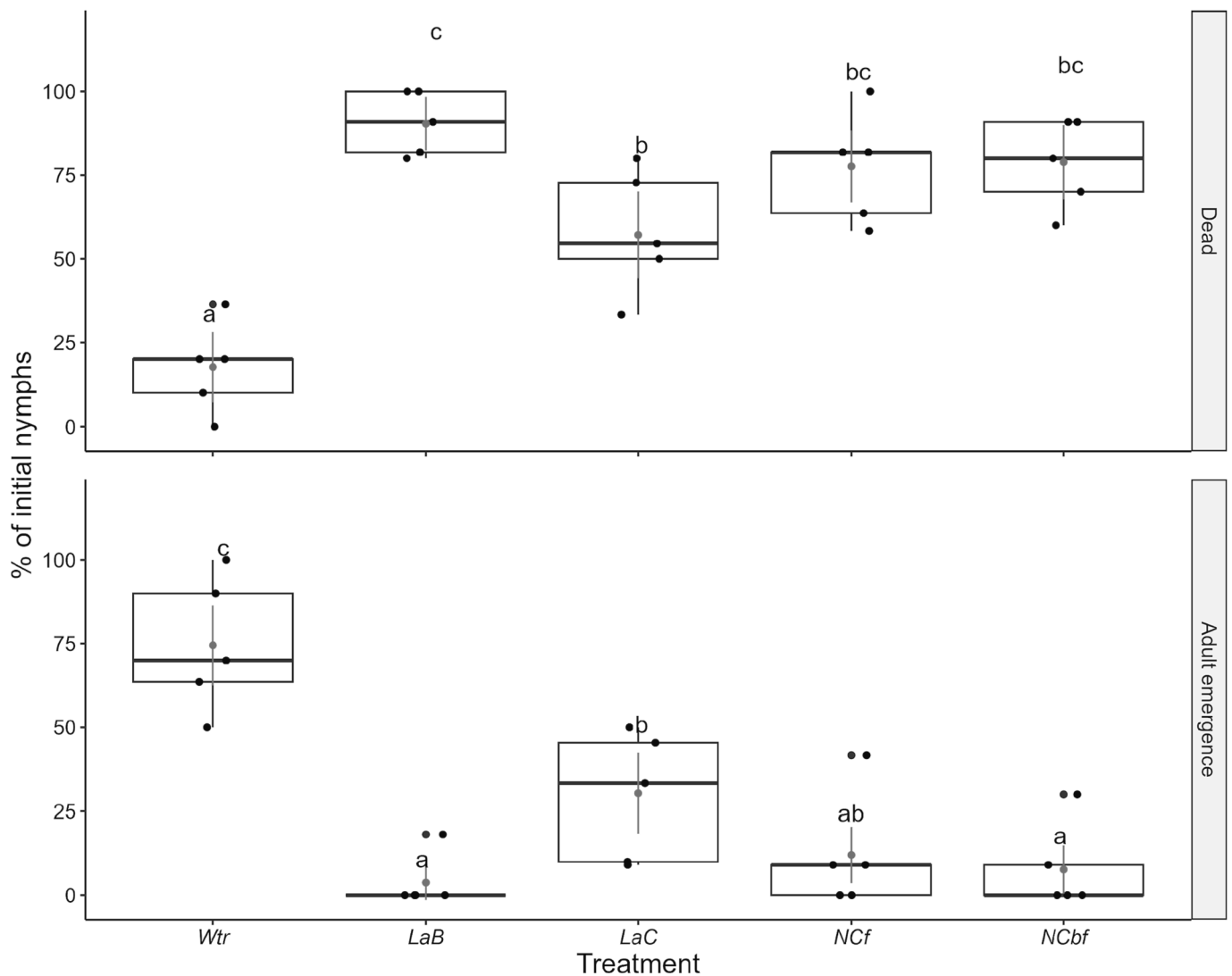
Variable	Treatment	Estimate (%)	SEM	LCL	UCL	group
<b>Nymphal mortality</b>	<i>Wtr</i>	17.65	5.34	7.18	28.11	a
	<i>LaC</i>	57.14	6.61	44.18	70.10	b
	<i>NCf</i>	77.59	5.48	66.85	88.32	bc
	<i>NCbf</i>	78.85	5.66	67.75	89.95	bc
	<i>LaB</i>	90.38	4.09	82.37	98.40	c
<b>Adult emergence</b>	<i>Wtr</i>	74.51	6.10	62.55	86.47	c
	<i>LaC</i>	30.36	6.14	18.31	42.40	b
	<i>NCf</i>	12.07	4.28	3.69	20.45	ab
	<i>NCbf</i>	7.69	3.70	0.45	14.93	a
	<i>LaB</i>	3.85	2.67	-1.38	9.07	a

A total of 156 dead nymphs were collected during the visual inspections, and all were prepared for fungal reisolation. *L. aphanocladii* was successfully reisolated from approximately half of them (n = 65, 47.8 %), all collected from fungal treatments. A single dead nymph collected from Mycotal-treated microcosm did not develop *L. muscarium* mycelium.

**T2**

The end-point nymphal mortality was significantly different among experimental treatments (GLM:  $\chi^2 = 41.77$ , df = 4,  $p < 0.001$ ), with all the EPF-treated microcosms showing higher mortality rates of nymphs compared to those treated with water (control) (Table 4). Namely, nymphs sprayed with *L. aphanocladii* blastospores (*LaB*) showed the highest mortality rate ( $91.1 \pm 4.3$  %, 47 dead/52 exposed) and were significantly more likely to die during the assay than both control (*Wtr*) ( $16.6 \pm 6.0$  %, 9/51) and *L. aphanocladii* conidia (*LaC*) ( $59.3 \pm 8.4$  %, 32/54) (Table 5). The two *B. bassiana* treatments (*NCf*:  $81.4 \pm 6.4$  %, 44/55; *NCbf*:  $81.3 \pm 6.4$  %, 41/51) did not significantly differ for the nymphal mortality rate among them and from both *L. aphanocladii* formulations (*LaB* and *LaC*) (Fig. 5, Table S1). The hazard ratios of death were significantly higher for all the individuals treated with EPF strains compared to control (*Wtr*) (HR EPF-treated:control = 4.9–14.1) (Table 4), although *LaC*-treated nymphs were significantly less likely to die than those treated with the other fungal propagules (HR *LaC*:other EPF treatments = 0.30–0.44) (Table 3). The progression of nymphal mortality was significantly different among experimental treatments, with nymphs treated with EPFs reaching 50 % nymphal mortality within 3–4 days post treatment (CI 95 % 1–4 days), except for *LaC* that reached 50 % mortality significantly later (7 days post treatment, CI 95 % 7–9 days) (Fig. 6).

As expected, the probability of adult emergence followed an inverse pattern, hence significantly lower for individuals exposed to all EPF treatments [*LaB* ( $3.8 \pm 4.1$  %, 2/47), *NCbf* ( $7.8 \pm 5.8$  %, n = 4/51), *NCf*



**Fig. 5.** Percentage of initial nymphs of *Philaenus spumarius* found dead or emerged as adults during the 2022 experimental trial (T2) of *Lecanicillium aphanocladii* DSF efficacy, Data observed (boxplots) and predicted by binomial GLM (red point range) are reported, and treatments sharing the same letter within a row are not significantly different ( $\alpha = 0.05$ , Tukey-adjusted), Thick horizontal lines represent the median, the boxes the interquartile range and the vertical lines the extent of the variability outside the upper and lower quartiles, Red points and lines represent mean and standard error of the mean, See explanation of abbreviation of treatments in Material and Methods section, (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

( $12.7 \pm 6.9\%$ , 7/55) and LaC ( $31.5 \pm 9.7\%$ ,  $n = 17/54$ ) compared to control (Wtr) ( $74.5 \pm 9.4\%$ , 38/51) (GLM:  $\chi^2 = 37.63$ ,  $df = 4$ ,  $p < 0.001$ ) (Fig. 5, Table 5, Table S1). LaB and NCbf adult emergence rates were also significantly lower than LaC, The over time competing risk analysis on the status of nymphs confirmed that nymphs were less likely to emerge as adults when treated with EPF, in particular LaB one (HR LaB:control = 0.1, CI = 0.02–0.47) (Table 4). The progression of adult emergence through time post treatment highlighted that the emergence rate was quite constant, although different, for both Wtr and LaC treatments, while for LaB, NCf and NCbf the few adults were emerging immediately after and within one week from the treatment (Fig. 6).

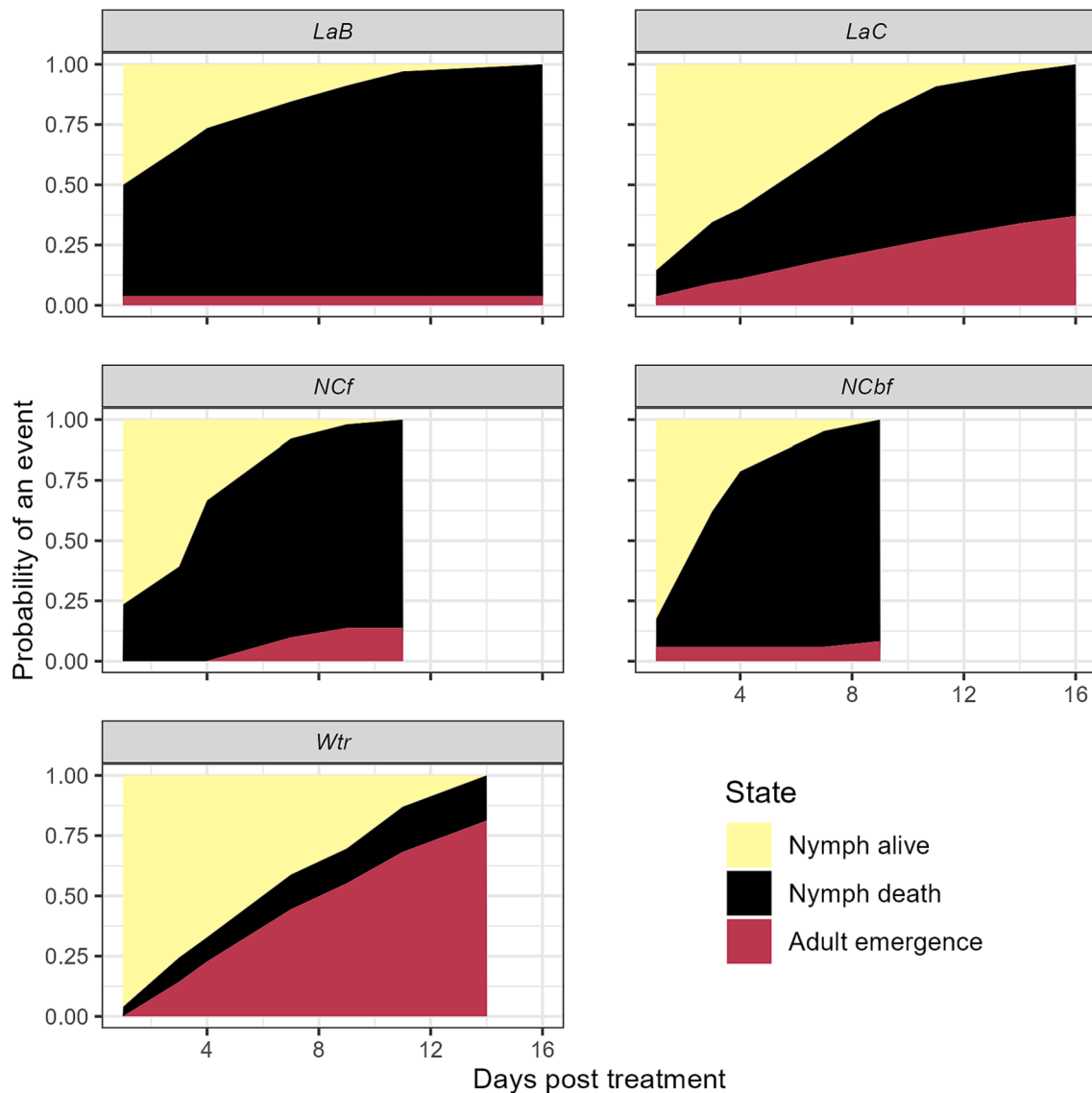
One-hundred-eighty dead nymphs were collected during the visual inspections, 77.2 % of which developed mycelium referable to the inoculated fungal species. Ten dead nymphs were collected from negative control (Wtr), and did not show any fungal growth. The symptoms of nymphs affected by mycosis due to *L. aphanocladii* DSF or *B. bassiana* ATCC 74040 were clearly visible under the stereomicroscope (Fig. 7), *B. bassiana* ATCC 74040 emerging from the cadaver covered the insect with a white and rather compact mycelium while *L. aphanocladii* DSF formed

a loose, cobweb-like mycelium of an off-white colour.

#### 4. Discussion

Entomopathogenic fungi (EPF) play an important role in the natural regulation of insect populations and they have been recognized as important agents in biocontrol strategy.

The present research represents one of the first work on EPF genera and species naturally attacking *P. spumarius*, vector of the noxious phytopathogenic bacterium *X. fastidiosa*. We successfully isolated four different strains of EPF naturally infecting *P. spumarius* in the Piemonte Region (Northwestern Italy), and belonging to the species *L. aphanocladii*, *B. bassiana*, *C. coronatus* and *F. equiseti*. Preliminary experimental trials involving direct nebulization of conidia on nymphal stages, on a single potted plant, showed promising pathogenic activity of the isolates (43–46 % mortality), except for *F. equiseti* (3 % mortality). Conversely, preliminary trials on adults of *P. spumarius* revealed very low mortality rates of EPF-treated individuals and no significant differences compared to those exposed to control treatment (water). Similar differences in EPF



**Fig. 6.** Survival curves of nymphs *Philaenus spumarius* treated with different *Lecanicillium aphanocladii* formulations in trial T2, fitted by multi-state Cox Hazard Model. Areas of different colors represent the estimated probability of each *P. spumarius* nymphal state (alive, death, adult emergence) over time after the experimental treatment, See explanation of abbreviation of treatments in Material and Methods section.

lethal activity against nymphal and adult stage were not observed in previous studies involving sharpshooters (Cicadellidae: Proconiini) (Cabanillas and Jones, 2013; Lietze et al., 2011), although differences in setup, target species, and fungal strains could play a role in the different outcomes. Notably, the observed different pathogenicity level of fungal strains against *P. spumarius* nymphs and adults could be influenced also by the different spraying method of propagules employed (nymphs: direct, adult: indirect), due to logistic difficulties given by the different mobility of the insect stages. This may have reduced the probability of propagule-insect contact.

Given the low efficiency against adult stage of the isolated fungal strains, and the need of a control tool able to prevent the emergence of adults spreading *Xf*, subsequent bioassays were performed on nymphal stages, as a more suitable insect-stage target. Furthermore, the choice to focus on preimaginal stages was done also considering the epidemiological roles of the different life stages of *P. spumarius*. Nymphs have no relevant role in *Xf* transmission to the crop, and all the mortality caused to this stage will likely lower the adult densities in olive groves and thus the *Xf* spread likelihood. Furthermore, given that *P. spumarius* adults may acquire and inoculate *Xf* soon after colonization of olive trees

(Bodino et al., 2021; Cornara et al., 2017), even timely EPF treatments on adult stage could be not very effective in reducing *Xf* spread on the crop. Also, nymphs are characterised by low mobility, high densities in a single vegetation compartment (herbaceous cover), and they are present during a relatively humid and cool period of the year (early Spring) (Bodino et al., 2019; Morente et al., 2018). These traits make nymphs likely the best biological stage for application of EPF.

Among fungal isolates, *L. aphanocladii* DSF was considered the most promising strain to be further investigated because showed low rates of nymphal survival (43 %) and adult emergence (40 %), similar to those observed in spittlebugs treated with *B. bassiana*, a fungal species well recognized as an essential source of myco-pesticides used for numerous insect pest all over the world (Soliman et al., 2022), and it is a new species for the biological product market, although already found in nature as an entomopathogenic species (Nedveckyté et al., 2021; Zhou et al., 2020).

*Lecanicillium* sp., along with *Beauveria* sp., *Metarhizium* sp. and *Isaria* sp. are natural enemies of insect pests and widely studied for use in biocontrol and reducing the need for synthetic pesticides. Representative strains of the genus *Lecanicillium* are already used as active

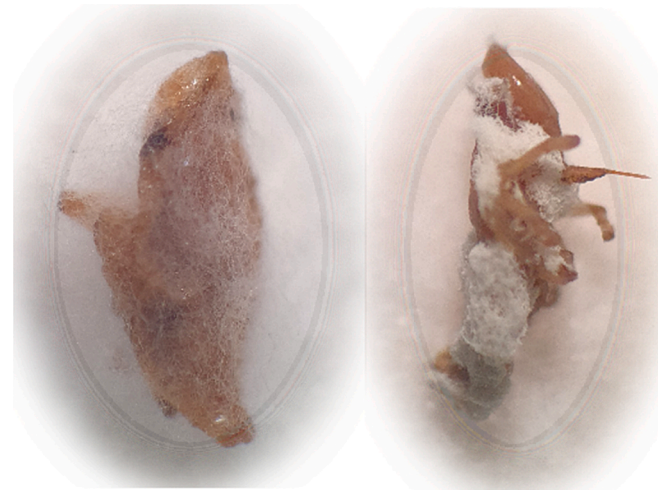


Fig. 7. *Lecanicillium aphanocladii* (a) and *Beauveria bassiana* (b) mycelium emerging from dead *Philaenus spumarius* nymphs collected in trial T2.

ingredients of several mycoinsecticides (Bamisile et al., 2021; Goettel et al., 2008). Totally, about 15 *Lecanicillium* spp.-based commercial preparations have been developed (Faria and Wraight, 2007), as Mycotal® and Lecatech®, formulated with *L. muscarium* and *Lecanicillium lecanii*, respectively. Currently, no products based on *L. aphanocladii* propagules are present on the market.

In nature, this species was initially isolated from fungi, such as *Agaricus* sp. and *Sphaerotheca* sp., and insects, as mosquito larvae. *Bombyx mori* (L.) and *Trialeurodes vaporariorum* Westwood and it is considered a cosmopolitan species (Zare and Games, 2003). More recently, *L. aphanocladii* has been found affecting the adult stage of the leaf-miner horse-chestnut pest *Cameraria ohridella* Descha & Dimic and it showed effective properties and suitability for field application trials (Nedveckytė et al., 2021). Moreover, it was found active against *Frankliniella occidentalis* Pergande, together with several other *Lecanicillium* spp. (Zhou et al., 2020).

In greenhouse bioassays. *L. aphanocladii* DSF efficacy was measured based on nymphal survival/adult emergence rate of *P. spumarius*, with different combinations of propagules (conidia or blastospores) and adjuvants (adjuvant 1 and 2), compared to both negative control (water) and a commercial product based on *L. muscarium* (Mycotal®) which finally resulted not effective on *P. spumarius* nymphs. All experimental treatments with *L. aphanocladii* DSF, except for conidia formulation with adjuvant 1, significantly increased nymphal mortality and reduced adult emergence rate compared to the controls, water and adjuvants alone. Apart for the lower mortality rate caused by *L. aphanocladii* DSF conidia formulation with adjuvant 1, no significant differences between the other *L. aphanocladii* DSF formulations were observed in efficacy against *P. spumarius*. A second trial was carried out to compare the two *L. aphanocladii* DSF formulations (conidia and blastospores) with the commercial product Naturalis® based on a *B. bassiana* ATCC 74040 strain. Even if all the EPF treatments (*L. aphanocladii* DSF and *B. bassiana* ATCC 74040) significantly reduced nymphal survival and adult emergence compared to the control, *L. aphanocladii* DSF blastospore formulation was significantly more effective than the conidia formulation, with nymphal mortality/adult emergence rates (mortality: 90 %; emergence: 4 %) even higher, although not significantly than those observed in *B. bassiana* treatments.

Despite their well-known potential, few authors have tested the efficacy of blastospores, compared with conidia, in pathogenicity bioassays. Blastospores are proposed as suitable contact mycoinsecticide in spray applications for their ability to germinate and infect insects more rapidly than conidia (6 h versus 16–24 h). At the same time, blastospores are less tolerant to environmental stresses than conidia, even though

faster germination may reduce the exposure period to deleterious environmental stresses after application (Jaronski and Mascarín, 2017). For these reasons, we investigated and compared the pathogenic action of both *L. aphanocladii* DSF propagules. Furthermore, blastopore-based mycoinsecticides of *Lecanicillium* spp. are currently produced (Mycotal® and Vertalec®).

The selection process of a mycoinsecticide must also evaluate the potential of fungal strain to form stable propagules that can be economically mass-produced (Jaronski and Mascarín, 2017). Stability in storage is critical in most commercial situations, although in some cases provision of fresh mycoinsecticide to the user is feasible. Blastospores are considered less amenable to storage conditions and also more sensitive to desiccation than conidia. Nevertheless, cost-effective methods for producing high concentrations of desiccation-tolerant blastospores of *Lecanicillium* and *Beauveria* have been already successfully achieved and reported (Jaronski and Mascarín, 2017). From our experience, freeze dried blastospores remained stable for months without losing vitality but it was necessary to store them at 4 °C which is inconvenient for industry.

In the same trials, we tested also for the influence of spittlebug foam on effectiveness of *B. bassiana* ATCC 74040 conidia treatment on nymphal stages. No differences in mortality or adult emergence rate were observed between nymphs treated when covered by foam or “naked”, i.e. with no foam. The results, although preliminary, showed that the presence of foam seemed to neither hinder nor favour the pass through of *B. bassiana* hydrophobic conidia. The composition of spittlebug foam is not well-known, but it is similar to the exudate of host plants, although the nymphs secrete several chemical components, such as glycopeptides and proteoglycans, as well as metabolites with likely defensive/repellent function towards predators (Chen et al., 2018; del Campo et al., 2011; Mello et al., 1987). Foam could act as a physical barrier against conidia, but, since nymphs tend to move quite frequently out of their foam, they may come into contact with conidia nonetheless. On the other hand, the wet conditions of the insect inside the foam, may help the germination of conidia. Potential effect of foam on EPF should be explored in further studies.

The results obtained in this study represent one of the first evidence on EPF genera and species attacking *P. spumarius*. The initial recovery of *P. spumarius* individuals affected by mycosis was a fundamental step towards a good chance of success. Native strains naturally infecting target insects can be quite promising in the development of biocontrol agents. Recently, Wang et al., (2022) isolated four EPF strains from infected *Ostrinia furnacalis* highly pathogenic to larvae, Dara et al., (2008) isolated native EPF strains from both arthropod cadavers and environmental sources with similar virulence to *Homalodisca coagulata* individuals, showing a natural control ability. In addition, it is imperative to carefully choose environmentally competent fungal strains that are able to persist in the host environment for the required infection period (Quesada-Moraga et al., 2023). Some authors highlighted that the virulence of indigenous EPF strains can be higher than that of commercial strains and their effectiveness is largely dependent on genetic variability and adaptation to host-related environmental factors (Ou et al., 2019; Thube et al., 2022; Wu et al., 2021), Boucias et al., (2007) started from a field-survey to recover fungi associated to the spittlebug *H. vitripennis*, and described strains belonging to *H. homalodisciae* species as the most predominant, Foieri et al., (2017) observed the natural occurrence of genus *Pandora* on spittlebug pests (Hemiptera: Cercopidae) of pasture grasses, although no isolation of fungal pure cultures from individuals affected by mycosis was achieved. The inability (or scarce ability) to grow and sporulate *in vitro* is a major limitation of some EPF, especially belonging to the order of *Entomophthorales*, which makes them unsuitable for possible use. Many species have never been cultured *in vitro* because of their nutritional fastidiousness (Jaronski and Mascarín, 2017).

Data collected from climatic chamber assays are quite encouraging even if trials were carried out under controlled conditions, optimal for

fungus development in terms of temperature and RH. However, we avoided *in vitro* conditions used by other authors where insects were directly dipped in conidial suspension for a specified time (Cabanillas and Jones, 2013; Ganassi et al., 2023), and the experimental set up was closer to environmental conditions, with conidial suspensions nebulized in the microcosms, which more closely mimics the conidia carried by air currents and deposited on the cuticle of the insects. Pathogenicity of EPF strains were tested by direct spraying of fungal propagules on *V. faba* leaves in microcosms colonised by *P. spumarius* to ensure almost complete coverage and maximize the probability of contact and infection of the target insects. The low mortality of nymphs detected in the negative control (Wtr) highlighted a good set up of the trials with good environmental conditions for *P. spumarius* survival.

## 5. Conclusion

In this study, the biological control potential of *L. aphanocladii* towards *P. spumarius* nymphs was successfully investigated. Certainly, the results obtained in this study require validation in field assays in order to confirm the potential of *L. aphanocladii* DSF against *P. spumarius* nymphs. Environmental conditions are of course a key determinant of biopesticide efficacy.

In addition, due to the relative slowness of EPF in acting, further studies should be focused on the capacity of *L. aphanocladii* DSF in spreading among insects (e.g. mycosis-affected individuals stuck to the leaves which can transmit the fungus to healthy individuals) and persistence in the environment (e.g. propagule resistance to UV and fungal aptitude for endophytic colonisation of target insect host plants).

Although the European objectives for eco-sustainability and organic farming are accelerating the introduction of microbial pesticides, their use remains limited and new efforts should be made by the scientific community to test the efficacy of new strains. This study represents a first attempt in answering the question if entomopathogenic fungi may represent an alternative or be synergic to natural insecticides to achieve effective control of spittlebug populations in agroecosystems threatened by *X. fastidiosa*.

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### CRedit authorship contribution statement

**Bodino Nicola:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization. **Barbera Riccardo:** Writing – review & editing, Methodology, Investigation, Data curation. **González-Mas Natalia:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Demichelis Stefano:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Bosco Domenico:** . **Dolci Paola:** Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

### Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Bosco Domenico reports financial support and equipment, drugs, or supplies were provided by Globachem Discovery Ltd, Bosco Domenico reports financial support was provided by Horizon Europe Food Bioeconomy Natural Resources Agriculture and Environment].

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jip.2024.108078>.

## References

- Almeida, R.P.P., 2016. Can Apulia's olive trees be saved? *Sci.* 353, 346–348. <https://doi.org/10.1126/science.aaf9710>.
- Almeida, R.P.P., Blua, M.J., Lopes, J.R.S., Purcell, A.H., 2005. Vector transmission of *xylella fastidiosa*: applying fundamental knowledge to generate disease management strategies. *Ann. Entomol. Soc. Am.* 96, 775–786. [https://doi.org/10.1603/0013-8746\(2005\)098\[0775:VTOXFA\]2.0.CO;2](https://doi.org/10.1603/0013-8746(2005)098[0775:VTOXFA]2.0.CO;2).
- Bamisile, B.S., Akutse, K.S., Siddiqui, J.A., Xu, Y., 2021. Model application of entomopathogenic fungi as alternatives to chemical pesticides: prospects, challenges, and insights for next-generation sustainable agriculture. *Front. Plant Sci.* 12, 741804.
- Ben-Ze'ev, I., Kenneth, R.G., 1981. *Zoopthora radicans* and *zoopthora petchi* sp. nov. [zygomycetes: entomophthorales], two species of the "sphaerosperma group" attacking leaf-hoppers and frog-hoppers [hom.]. *Entomophaga* 26, 131–142. <https://doi.org/10.1007/BF02375027>.
- Bodino, N., Cavalieri, V., Dongiovanni, C., Plazio, E., Saladini, M.A., Volani, S., Simonetto, A., Fumarola, G., Carolo, M.D., Porcelli, F., Gilioli, G., Bosco, D., 2019. Phenology, seasonal abundance and stage-structure of spittlebug (hemiptera: aphrophoridae) populations in olive groves in Italy. *Sci Rep.* 9, 17725. <https://doi.org/10.1038/s41598-019-54279-8>.
- Bodino, N., Cavalieri, V., Dongiovanni, C., Saladini, M.A., Simonetto, A., Volani, S., Plazio, E., Altamura, G., Tauro, D., Gilioli, G., Bosco, D., 2020a. Spittlebugs of Mediterranean olive groves: host-plant exploitation throughout the year. *Insects* 11, 130. <https://doi.org/10.3390/insects11020130>.
- Bodino, N., Cavalieri, V., Dongiovanni, C., Simonetto, A., Saladini, M.A., Plazio, E., Gilioli, G., Molinatto, G., Saponari, M., Bosco, D., 2020b. Dispersal of *Philaenus spumarius* (hemiptera: aphrophoridae), a vector of *xylella fastidiosa*, in olive grove and meadow agroecosystems. *Environ Entomol.* 50, 267–279. <https://doi.org/10.1093/ee/veaa140>.
- Bodino, N., Cavalieri, V., Pegoraro, M., Altamura, G., Canuto, F., Zicca, S., Fumarola, G., Almeida, R.P.P., Saponari, M., Dongiovanni, C., Bosco, D., 2021. Temporal dynamics of the transmission of *xylella fastidiosa* subsp. *pauca* by *Philaenus spumarius* to olive plants. *Entomol Gen.* 41, 463–480. <https://doi.org/10.1127/entomologia/2021/1294>.
- Bodino, N., Cavalieri, V., Dongiovanni, C., Saponari, M., Bosco, D., 2023. Bioecological traits of spittlebugs and their implications on the epidemiology and control of *xylella fastidiosa* epidemic in Apulia (southern Italy). *Phytopathology*®. <https://doi.org/10.1094/PHYTO-12-22-0460-IA>.
- Bony, S., Gillet, C., Bouchez, A., Margoum, C., Devaux, A., 2008. Genotoxic pressure of vineyard pesticides in fish: field and mesocosm surveys. *Aquat. Toxicol.* 89, 197–203. <https://doi.org/10.1016/j.aquatox.2008.06.017>.
- Boucias, D.G., Scharf, D.W., Breaux, S.E., Purcell, D.H., Mizell, R.E., 2007. Studies on the fungi associated with the glassy-winged sharpshooter *homalodisca coagulata* with emphasis on a new species *hirsutella homalodiscae* nom. prov. *BioControl* 52, 231–258. <https://doi.org/10.1007/s10526-006-9019-3>.
- Cabanillas, H.E., Jones, W.A., 2013. Pathogenicity of *isaria poprawskii* (ascomycota: hypocreales: cordycipitaceae) against the glassy-winged sharpshooter, *homalodisca vitripennis* (hemiptera: cicadellidae), under laboratory conditions. *Crop Prot.* 50, 46–52. <https://doi.org/10.1016/j.cropro.2013.03.007>.
- Cappellari, A., Santoiemma, G., Sanna, F., D'Ascenzo, D., Mori, N., Lami, F., Marini, L., 2022. Spatio-temporal dynamics of vectors of *xylella fastidiosa* subsp. *pauca* across heterogeneous landscapes. *Entomol Gen.* 42, 515–521. <https://doi.org/10.1127/entomologia/2022/1427>.
- Cariddi, C., Saponari, M., Boscia, D., Stradis, A.D., Loconsole, G., Nigro, F., Porcelli, F., Potere, O., Martelli, G.P., 2014. Isolation of a *xylella fastidiosa* strain infecting olive and oleander in Apulia. *Italy. J. Plant Pathol.* 96, 425–429. <https://doi.org/10.4454/JPP.V96I2.024>.
- Cavalieri, V., Altamura, G., Fumarola, G., di Carolo, M., Saponari, M., Cornara, D., Bosco, D., Dongiovanni, C., 2019. Transmission of *xylella fastidiosa* subspecies *pauca* sequence type 53 by different insect species. *Insects* 10, 324. <https://doi.org/10.3390/insects10100324>.
- Chen, X., Meyer-Rochow, V.B., Fereres, A., Morente, M., Liang, A.-P., 2018. The role of biofoam in shielding spittlebug nymphs (insecta, hemiptera, cercopidae) against bright light. *Ecol Entomol.* 43, 273–281. <https://doi.org/10.1111/een.12496>.
- Coll, P., Le Cadre, E., Blanchart, E., Hinsinger, P., Villenave, C., 2011. Organic viticulture and soil quality: a long-term study in southern France. *Appl. Soil Ecol.* 50, 37–44. <https://doi.org/10.1016/j.apsoil.2011.07.013>.
- Cornara, D., Saponari, M., Zeilinger, A.R., de Stradis, A., Boscia, D., Loconsole, G., Bosco, D., Martelli, G.P., Almeida, R.P.P., Porcelli, F., 2017. Spittlebugs as vectors of

- xylella fastidiosa* in olive orchards in Italy. *J. Pest Sci.* 90, 521–530. <https://doi.org/10.1007/s10340-016-0793-0>.
- Cornara, D., Bosco, D., Fereres, A., 2018. *Philaenus spumarius*: when an old acquaintance becomes a new threat to European agriculture. *J. Pest Sci.* 91, 957–972. <https://doi.org/10.1007/s10340-018-0966-0>.
- Cornara, D., Morente, M., Markheiser, A., Bodino, N., Tsai, C.-W., Fereres, A., Redak, R. A., Perring, T.M., Lopes, J.R.S., 2019. An overview on the worldwide vectors of *xylella fastidiosa*. *Entomol. Gen.* 39, 157–181. <https://doi.org/10.1127/entomologia/2019/0811>.
- Cornara, D., Panzarino, O., Santoiemma, G., Bodino, N., Loverre, P., Mastronardi, M.G., Mattia, C., de Lillo, E., Addante, R., 2021. Natural areas as reservoir of candidate vectors of *xylella fastidiosa*. *Bulletin of the Georgian Academy of Sci. Insectology* 74, 173–180.
- Dáder, B., Viñuela, E., Moreno, A., Plaza, M., Garzo, E., del Estal, P., Fereres, A., 2019. Sulfoxaflo and natural pyrethrin with piperonyl butoxide are effective alternatives to neonicotinoids against juveniles of *Philaenus spumarius*, the European vector of *xylella fastidiosa*. *Insects* 10, 225. <https://doi.org/10.3390/insects10080225>.
- Dara, S.K., McGuire, M.R., Ulloa, M., Kaya, H.K., 2008. Evaluation and molecular characterization of beaveria bassiana for the control of the glassy-winged sharpshooter (homoptera: cicadellidae) in California. *J. Entomol. Sci.* 43, 241–246. <https://doi.org/10.18474/0749-8004-43.2.241>.
- de Faria, M.R., Wraight, S.P., 2007. Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. *Biol. Control* 43, 237–256. <https://doi.org/10.1016/j.biocontrol.2007.08.001>.
- del Campo, M.L., King, J.T., Gronquist, M.R., 2011. Defensive and chemical characterization of the froth produced by the cecropid *Aphrophora cribrata*. *Chemoecology* 21, 1–8. <https://doi.org/10.1007/s00049-010-0059-x>.
- Dongiovanni, C., Di Carolo, M., Fumarola, G., Tauro, D., Altamura, G., Cavaliere, V., 2018. Evaluation of Insecticides for the Control of Juveniles of *Philaenus spumarius* L., 2015–2017. *Arthropod Manag. Tests* 43. <https://doi.org/10.1093/amt/tsy073>.
- Dupas, E., Durand, K., Rieux, A., Briand, M., Pruvost, O., Cuntly, A., Denancé, N., Donnadieu, C., Legendre, B., Lopez-Roques, C., Cesbron, S., Ravigné, V., Jacques, M.-A., 2023. Suspicions of two bridgehead invasions of *xylella fastidiosa* subsp. multiplex in France. *Commun. Biol.* 6, 1–13. <https://doi.org/10.1038/s42003-023-04499-6>.
- EFSA Panel on Plant Health (PLH), Bragard, C., Dehnen-Schmutz, K., Serio, F.D., Gonthier, P., Jacques, M.-A., Miret, J.A.J., Justesen, A.F., MacLeod, A., Magnusson, C.S., Milonas, P., Navas-Cortés, J.A., Pottting, R., Reignault, P.L., Thulke, H.-H., van der Werf, W., Civera, A.V., Yuen, J., Zappalà, L., Boscia, D., Chapman, D., Gilioli, G., Krugner, R., Mastin, A., Simonetto, A., Lopes, J.R.S., White, S., Abrahantes, J.C., Delbianco, A., Maiorano, A., Mosbach-Schulz, O., Stancanelli, G., Guzzo, M., Parnell, S., 2019. Update of the scientific opinion on the risks to plant health posed by *xylella fastidiosa* in the EU territory. *EFSA J.* 17, e05665.
- EU 2020/1201, Commission Implementing Regulation (EU) 2020/1201 of 14 August 2020 as regards measures to prevent the introduction into and the spread within the Union of *Xylella fastidiosa* (Wells et al.), 02020R1201.
- Firth, D., 1993. Bias reduction of maximum likelihood estimates. *Biometrika* 80, 27–38. <https://doi.org/10.1093/biomet/80.1.27>.
- Foieri, A., Pedrini, N., Toledo, A., 2017. Natural occurrence of the entomopathogenic genus *Pandora* on spittlebug pests of crops and pastures in Argentina. *J. Appl. Entomol.* 142, 363–370. <https://doi.org/10.1111/jen.12476>.
- Ganassi, S., Domenico, C.D., Altomare, C., Samuels, G.J., Grazioso, P., Cillo, P.D., Pietrantonio, L., De Cristofaro, A., 2023. Potential of fungi of the genus *trichoderma* for biocontrol of *Philaenus spumarius*, the insect vector for the quarantine bacterium *xylella fastidiosa*. *Pest Manag. Sci.* 79, 719–728. <https://doi.org/10.1002/ps.7240>.
- Goettel, M.S., Koike, M., Kim, J.J., Aiuchi, D., Shinya, R., Brodeur, J., 2008. Potential of *Lecanicillium* spp for management of insects nematodes and plant diseases. *J. Invertebr. Pathol. Special Issue for SIP* 98, 256–261. <https://doi.org/10.1016/j.jip.2008.01.009>.
- González-Mas, N., Ortega-García, L., Garrido-Jurado, I., Dembilio, O., Jaques, J.A., Quesada-Moraga, E., 2019. Which came first: the disease or the pest? is there a host mediated spread of beaveria bassiana (ascomycota: hypocreales) by invasive palm pests? *J. Invertebr. Pathol.* 162, 26–42. <https://doi.org/10.1016/j.jip.2019.01.007>.
- Jaronski, S., Mascarin, G., 2017. Mass production of fungal entomopathogens. *Microb. Control Insect Mite Pests* 141–155.
- Kanga, L.H.B., Jones, W.A., Humber, R.A., Jr, D.W.B., 2004. Fungal pathogens of the glassy-winged sharpshooter *Homalodisca coagulata* (Homoptera: Cicadellidae), *Fla. Entomol.* 87, 225–228. [https://doi.org/10.1653/0015-4040\(2004\)087\[0225:FPOTGS\]2.0.CO;2](https://doi.org/10.1653/0015-4040(2004)087[0225:FPOTGS]2.0.CO;2).
- Kottelenberg, D., Hemerik, L., Saponari, M., van der Werf, W., 2021. Shape and rate of movement of the invasion front of *xylella fastidiosa* spp. *paucis* in Puglia. *Sci. Rep.* 11, 1061. <https://doi.org/10.1038/s41598-020-79279-x>.
- Lago, C., Morente, M., Heras-Bravo, D.D., las Martí-Campoy, A., Rodríguez-Ballester, F., Plaza, M., Moreno, A., Fereres, A., 2021. Dispersal of *neophilaenus campestris*, a vector of *xylella fastidiosa*, from olive groves to over-summering hosts. *J. Appl. Entomol.* 145, 648–659. <https://doi.org/10.1111/jen.12888>.
- Landa, B.B., Castillo, A.I., Giampetruzzi, A., Kahn, A., Román-Écija, M., Velasco-Amo, M. P., Navas-Cortés, J.A., Marco-Noales, E., Barbé, S., Moralejo, E., Coletta-Filho, H.D., Saldarelli, P., Saponari, M., Almeida, R.P.P., 2020. Emergence of a plant pathogen in Europe associated with multiple intercontinental introductions. *Appl. Environ. Microbiol.* 86 <https://doi.org/10.1128/AEM.01521-19>.
- Lietze, V.-U., Mizell, R.F., Boucias, D.G., 2011. Transmission of the mycopathogen, *hirsutiella* spp., to nymphs and adults of the glassy-winged sharpshooter, *homalodisca vitripennis* (=coagulata), in the greenhouse. *Fla. Entomol.* 94, 106–108.
- López-Mercadal, J., Delgado, S., Mercadal, P., Seguí, G., Lalucat, J., Busquets, A., Gomila, M., Lester, K., Kenyon, D.M., Ruiz-Pérez, M., Paredes-Esquivel, C., Miranda, M.A., 2021. Collection of data and information in Balearic Islands on biology of vectors and potential vectors of *xylella fastidiosa* (GP/EFSA/ALPHA/017/01). *EFSA Support. Publ.* 18, 6925E. <https://doi.org/10.2903/sp.efsa.2021.EN-6925>.
- Lovett, B., Leger, R., 2017. *The insect pathogen.*, in: *the fungal kingdom*. ASM Press, Washington, DC, pp. 925–943.
- Mello, M.L.S., Pimentel, E.R., Yamada, A.T., Storopoli-Neto, A., 1987. Composition and structure of the froth of the spittlebug. *Deois* Sp. *Insect Biochem.* 17, 493–502. [https://doi.org/10.1016/0020-1790\(87\)90009-6](https://doi.org/10.1016/0020-1790(87)90009-6).
- Mietkiewski, R., Van der Geest, L., 1985. Notes on entomophthoraceous fungi infecting insects in the Netherlands. *Entomol. Ber.* 45, 190–192.
- Moralejo, E., Gomila, M., Montesinos, M., Borrás, D., Pascual, A., Nieto, A., Adrover, F., Gost, P.A., Seguí, G., Busquets, A., Jurado-Rivera, J.A., Quetglas, B., García, J., de, D., Beidas, O., Juan, A., Velasco-Amo, M.P., Landa, B.B., Olmo, D., 2020. Phylogenetic inference enables reconstruction of a long-overlooked outbreak of almond leaf scorch disease (*xylella fastidiosa*) in Europe. *Commun. Biol.* 3, 1–13. <https://doi.org/10.1038/s42003-020-01284-7>.
- Morelli, M., García-Madero, J.M., Jos, Á., Saldarelli, P., Dongiovanni, C., Kovacova, M., Saponari, M., Baños Arjona, A., Hackl, E., Webb, S., Compant, S., 2021. *Xylella fastidiosa* in olive: a review of control attempts and current management. *Microorganisms* 9, 1771. <https://doi.org/10.3390/microorganisms9081771>.
- Morente, M., Cornara, D., Plaza, M., Durán, J.M., Capiscol, C., Trillo, R., Ruiz, M., Ruz, C., Sanjuan, S., Pereira, J.A., Moreno, A., Fereres, A., 2018. Distribution and relative abundance of insect vectors of *xylella fastidiosa* in olive groves of the iberian peninsula. *Insects* 9, 175. <https://doi.org/10.3390/insects9040175>.
- Moussa, A., Maixner, M., Stephan, D., Santoiemma, G., Passera, A., Mori, N., Quaglino, F., 2021. Entomopathogenic nematodes and fungi to control hyalastes obsoletus (hemiptera: auchenorrhyncha: cixiidae). *BioControl* 66, 523–534. <https://doi.org/10.1007/s10526-020-10076-1>.
- Italia Olivicola Consorzio Nazionale, 2019. *Xylella*, lo studio: 4 milioni di alberi morti e improduttivi, 50mila ettari desertificati, perso il 10% dell'olio italiano.
- Nedveckyte, I., Pečiulyte, D., Būda, V., 2021. Fungi associated with horse-chestnut leaf miner moth *cameraria ohridella* mortality. *Forests* 12, 58.
- Ou, D., Zhang, L.-H., Guo, C.-F., Chen, X.-S., Ali, S., Qiu, B.-L., 2019. Identification of a new *cordyceps javanica* fungus isolate and its toxicity evaluation against asian citrus psyllid. *Microbiologyopen* 8, e00760.
- Pria Júnior, W.D., Lacava, P.T., Messias, C.L., Azevedo, J.L., Lacava, P.M., 2008. Bioassay assessment of metarhizium anisopliae (metchnikoff) sorokin (deuteromycota: hyphomycetes) against oncometopia facialis (signoret) (hemiptera: cicadellidae). *Braz. J. Microbiol.* 39, 128–132. <https://doi.org/10.1590/S1517-83822008000100027>.
- Quesada-Moraga, E., González-Mas, N., Yousef-Yousef, M., Garrido-Jurado, I., Fernández-Bravo, M., 2023. Key role of environmental competence in successful use of entomopathogenic fungi in microbial pest control. *J. Pest Sci.*
- R Core Team, 2020. *R: A Language and Environment for Statistical Computing*, R Foundation for Statistical Computing, Vienna, Austria.
- Raherison, C., Baldi, I., Pouquet, M., Berteaud, E., Moesch, C., Bouvier, G., Canal-Raffin, M., 2019. Pesticides exposure by air in vineyard rural area and respiratory health in children: a pilot study. *Environ. Res.* 169, 189–195. <https://doi.org/10.1016/j.envres.2018.11.002>.
- Rondot, Y., Reineke, A., 2018. Endophytic beaveria bassiana in grapevine *Vitis vinifera* (L.) reduces infestation with piercing-sucking insects. *Biol. Control* 116, 82–89. <https://doi.org/10.1016/j.biocontrol.2016.10.006>.
- Sanna, F., Mori, N., Santoiemma, G., D'Ascenzo, D., Scotillo, M.A., Marini, L., 2021. Ground cover Management in Olive Groves Reduces Populations of *Philaenus spumarius* (hemiptera: aphrophoridae), vector of *xylella fastidiosa*. *J. Econ. Entomol.* 114, 1716–1721. <https://doi.org/10.1093/jeet/toab116>.
- Saponari, M., Boscia, D., Altamura, G., Loconsole, G., Zicca, S., D'Attoma, G., Morelli, M., Palmisano, F., Saponari, A., Tavano, D., Savino, V.N., Dongiovanni, C., Martelli, G. P., 2017. Isolation and pathogenicity of *xylella fastidiosa* associated to the olive quick decline syndrome in southern Italy. *Sci. Rep.* 7, 17723. <https://doi.org/10.1038/s41598-017-17957-z>.
- Saponari, M., Giampetruzzi, A., Loconsole, G., Boscia, D., Saldarelli, P., 2019. *Xylella fastidiosa* in olive in Apulia: where we stand. *Phytopathology* 109, 175–186. <https://doi.org/10.1094/PHYTO-08-18-0319-FI>.
- Schneider, K., van der Werf, W., Cendoya, M., Mourits, M., Navas-Cortés, J.E.A., Vicent, A., Lansink, A.O., 2020. Impact of *xylella fastidiosa* subspecies *paucis* in European olives. *Proc. Natl. Acad. Sci.* 117, 9250–9259. <https://doi.org/10.1073/pnas.1912206117>.
- Schneider, K., Mourits, M., van der Werf, W., Lansink, A.O., 2021. On consumer impact from *xylella fastidiosa* subspecies *paucis*. *Ecol. Econ.* 185, 107024 <https://doi.org/10.1016/j.ecolecon.2021.107024>.
- Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., Fungal Barcoding Consortium Fungal Barcoding Consortium Author List Bolchacova, E., Voigt, K., Crous, P.W., Miller, A.N., Wingfield, M.J., Aime, M.C., An, K.-D., Bai, F.-Y., Barreto, R.W., Begerow, D., Bergeron, M.-J., Blackwell, M., Boekhout, T., Bogale, M., Boonyuen, N., Burgaz, A.R., Buyck, B., Cai, L., Cai, Q., Cardinali, G., Chaverri, P., Coppins, B.J., Crespo, A., Cubas, P., Cummings, C., Damm, U., de Beer, Z.W., de Hoog, G.S., Del-Prado, R., Dentinger, B., Diéguez-Uribeondo, J., Divakar, P.K., Douglas, B., Duenas, M., Duong, T.A., Eberhardt, U., Edwards, J.E., Elshahed, M.S., Fliiegerova, K., Furtado, M., García, M.A., Ge, Z.-W., Griffith, G.W., Griffiths, K., Groenewald, J.Z., Groenewald, M., Grube, M., Gryzenhout, M., Guo, L.-D., Hagen, F., Hambleton, S., Hamelin, R.C., Hansen, K., Harrold, P., Heller, G., Herrera, C., Hirayama, K., Hirooka, Y., Ho, H.-M., Hoffmann, K., Hofstetter, V., Högnabba, F., Hollingsworth, P.M., Hong, S.-B.,

- Hosaka, K., Houbraken, J., Hughes, K., Huhtinen, S., Hyde, K.D., James, T., Johnson, E.M., Johnson, J.E., Johnston, P.R., Jones, E.B.G., Kelly, L.J., Kirk, P.M., Knapp, D.G., Kõljalg, U., Kovács, G.M., Kurtzman, C.P., Landvik, S., Leavitt, S.D., Liggenstoffer, A.S., Liimatainen, K., Lombard, L., Luangsa-ard, J.J., Lumbsch, H.T., Maganti, H., Maharachchikumbura, S.S.N., Martin, M.P., May, T.W., McTaggart, A.R., Methven, A.S., Meyer, W., Moncalvo, J.-M., Mongkolsamrit, S., Nagy, L.G., Nilsson, R.H., Niskanen, T., Nyilasi, I., Okada, G., Okane, I., Olariaga, I., Otte, J., Papp, T., Park, D., Petkovits, T., Pino-Bodas, R., Quaedvlieg, W., Raja, H.A., Redecker, D., Rintoul, T.L., Ruibal, C., Sarmiento-Ramírez, J.M., Schmitt, L., Schüller, A., Shearer, C., Sotome, K., Stefani, F.O.P., Stenroos, S., Stielow, B., Stockinger, H., Suetrong, S., Suh, S.-O., Sung, G.-H., Suzuki, M., Tanaka, K., Tedersoo, L., Telleria, M.T., Tretter, E., Untereiner, W.A., Urbina, H., Vágvölgyi, C., Vialle, A., Vu, T.D., Walther, G., Wang, Q.-M., Wang, Y., Weir, B.S., Weiß, M., White, M.M., Xu, J., Yah, R., Yang, Z.L., Yurkov, A., Zamora, J.-C., Zhang, N., Zhuang, W.-Y., Schindel, D., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. *Proc. Natl. Acad. Sci.* 109, 6241–6246. <https://doi.org/10.1073/pnas.1117018109>.
- Shang, J., Tang, G., Lu, M., Wang, C., 2022. Host and environmental sensing by entomopathogenic fungi to infect hosts. *Curr. Clin. Microbiol. Rep.* 9, 69–74. <https://doi.org/10.1007/s40588-022-00185-z>.
- Sharma, L., Marques, G., 2018. *Fusarium*, an entomopathogen—a myth or reality? *Pathogens* 7, 93.
- Sicard, A., Zeilinger, A.R., Vanhove, M., Scharrel, T.E., Beal, D.J., Daugherty, M.P., Almeida, R.P.P., 2018. *Xylella fastidiosa*: insights into an emerging plant pathogen. *Annu. Rev. Phytopathol.* 56, 181–202. <https://doi.org/10.1146/annurev-phyto-080417-045849>.
- Soliman, A., Nada, M., Gad, A., 2022. Evaluation of the effects of the entomopathogenic fungus *beauveria bassiana* (ascomycota: hypocrales) on some histological and physiological parameters for the green bug *Nezara viridula* (L.) (Hemiptera: pentatomidae). *Alex. Sci. Exch. J.* 43, 229–238.
- Soubeyrand, S., de Jerphanion, P., Martin, O., Saussac, M., Manceau, C., Hendrikx, P., Lannou, C., 2018. Inferring pathogen dynamics from temporal count data: the emergence of *xylella fastidiosa* in France is probably not recent. *New Phytol.* 219, 824–836. <https://doi.org/10.1111/nph.15177>.
- Souza, R.K., Azevedo, R.F., Lobo, A.O., Rangel, D.E., 2014. Conidial water affinity is an important characteristic for thermotolerance in entomopathogenic fungi. *Biocontrol Sci. Technol.* 24, 448–461.
- Therneau, T.M., 2023. A Package for Survival Analysis in R.
- Thube, S.H., Pandian, R.T.P., Babu, M., Josephraj Kumar, A., Mhatre, P.H., Kumar, P.S., Kumar, B.N., Hegde, V., Chavan, S.N., 2022. Evaluation of a native isolate of *metarhizium anisopliae* (metschn.) sorokin TMBMA1 against tea mosquito bug, *Helopeltis theivora* infesting cocoa (*Theobroma cacao* L.). *Biol. Control* 170, 104909.
- Tsakirakis, A.N., Kasiotis, K.M., Charistou, A.N., Arapaki, N., Tsatsakis, A., Tsakalof, A., Machera, K., 2014. Dermal & inhalation exposure of operators during fungicide application in vineyards, evaluation of overall performance. *Sci. Total Environ.* 470–471, 282–289. <https://doi.org/10.1016/j.scitotenv.2013.09.021>.
- Vidal, S., Jaber, L.R., 2015. Entomopathogenic fungi as endophytes: plant–endophyte–herbivore interactions and prospects for use in biological control. *Curr. Sci.* 109, 46–54.
- Vilela, R., Mendoza, L., 2018. Human pathogenic entomophthorales. *Clin. Microbiol. Rev.* 31.
- Wang, X., Ding, X., Fu, K., Guo, W., Zhan, F., Yuan, Z., Jia, Z., Zhou, L., Jiang, X., Osman, G., et al., 2022. Molecular identification and efficacy of entomopathogenic fungi isolates against larvae of the asian corn borer *ostrinia furnacalis* (lepidoptera: crambidae) in Xinjiang, China. *J. Appl. Microbiol.* 133, 2979–2992.
- Weaver, C., King, D., 1954. Meadow spittlebug *Philaenus leucophthalmus* (L.). *Ohio Agric. Exp. Stn. Res. Bull.* 741, 1–99.
- White, T.J., Bruns, T., Lee, S., Taylor, J., et al., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protoc. Guide Methods Appl.* 18, 315–322.
- Wronska, A.K., Boguś, M.I., Kaczmarek, A., Kazek, M., 2018. Harman and norharman, metabolites of entomopathogenic fungus *conidiobolus coronatus* (entomophthorales), disorganize development of *Galleria mellonella* (lepidoptera) and affect serotonin-regulating enzymes. *PLoS One* 13, e0204828.
- Wu, S., Toews, M.D., Castrillo, L.A., Barman, A.K., Cottrell, T.E., Shapiro-Ilan, D.I., 2021. Identification and virulence of *Cordyceps javanica* strain wf GA17 isolated from a natural fungal population in sweetpotato whiteflies (hemiptera: aleyrodidae). *Environ. Entomol.* 50, 1127–1136.
- Zare, R., Games, W., 2003. *Lecanicillium aphanocladii*. [Descriptions of Fungi and Bacteria]. *Descriptions of Fungi and Bacteria. Sheet 1563*. doi: 10.1079/DFB/20056401563.
- Zhao, X., Chai, J., Wang, F., Jia, Y., 2023. Optimization of submerged culture parameters of the aphid pathogenic fungus *Fusarium equiseti* based on sporulation and mycelial biomass. *Microorganisms* 11, 190.
- Zhou, Y., Zou, X., Zhi, J., Xie, J., Jiang, T., 2020. Fast recognition of *Lecanicillium* spp., and its virulence against *Frankliniella occidentalis*. *Front. Microbiol.* 11, 561381.