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Evaluation of the role of biological control agents and agronomic methods to limit *Philaenus spumarius* and other vector species

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1. GENERAL INTRODUCTION

Xylella fastidiosa is a xylem-limited Gram-negative aerobic bacterium belonging to the Xanthomonadaceae family. Within the species, at least six subspecies are recognized: *pauca*, *multiplex*, *fastidiosa*, *sandyi*, and *morus* (Janse & Obradovic, 2010; Mourou et al., 2025).

Several insect species can transmit *X. fastidiosa* between plants by feeding on xylem sap. The vectors are sharpshooter leafhoppers (Hemiptera: Cicadellidae) and spittlebugs (Hemiptera: Aphrophoridae), these latter being the dominant group in Europe (Cornara et al., 2019). Interactions between insects and *X. fastidiosa* are poorly specific, because all xylem-sap feeders are regarded as potential vectors and individual insects may carry more than one *X. fastidiosa* strain. Once acquired, the bacterium attaches to the foregut of the insect and can persist for the entire life of the insect unless shed during moulting. Although *X. fastidiosa* multiplies within the vector, it is restricted to the foregut (in the precibarium), does not colonize hemocoel/haemolymph and is not transmitted to offspring (Krugner et al., 2019; Burbank & Ropert, 2021), so that transmission can be defined as persistent-non circulative.

1.1. *Philaenus spumarius*

The meadow spittlebug *Philaenus spumarius* (Linnaeus, 1758) (Hemiptera: Aphrophoridae) characterized by its ability to produce foam to protect itself during the nymphal stage (Figure 1). This species is widely distributed throughout Europe, and is abundant, especially in temperate and humid regions, where its presence is linked to landscapes with high proportions of forest and cool, humid conditions, although it is almost absent in the warmer, drier areas of the southern Iberian Peninsula. In Europe, it is the main vector of the bacterium *X. fastidiosa*, facilitating its spread among crops and wild species. Therefore, it has the potential to spread it to all other European regions where the pathogen is present (Saponari et al., 2014; Cornara et al., 2018; Gallego et al., 2023).



Figure 1. Different stages of *Philaenus spumarius*: A) egg mass, B) nymph, C) adult.

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However, since *P. spumarius* had never been considered an agricultural pest in Europe before the introduction of *X. fastidiosa*, its biology, ecology and ethology had never been continuously and exhaustively investigated. Currently, several studies have focused on updating knowledge about this insect, paying particular attention to those that can help develop a sustainable and environmentally friendly control strategy to prevent the spread of *X. fastidiosa*. To this end, it has been essential to study its characteristics and life cycle, as well as its preferred host plants (Cornara et al., 2018; Morente et al., 2018a; Di Serio et al., 2019; Morente et al., 2022; Lago et al., 2023).

Philaenus spumarius is a univoltine species, with a single generation per year, exhibiting ovarian parapause and winter diapause in the egg stage (Morente et al. 2018, Antonatos et al. 2020, Bodino et al. 2020). The eggs hibernate until they eclose in early spring. After eclosion, the nymph develops through five nymphal stages, and nymphal development takes 5–8 weeks until they become adults (Weaver and King, 1954; EFSA. 2019). In autumn, mature females lay masses of eggs in dry plant debris on the ground until they die during the winter (Morente et al. 2018b, Dongiovanni et al. 2019, Antonatos et al. 2020) (Figure 2). Among the other recognized vectors in Europe, *Neophilaenus campestris* (Fàllen) is the most common and can be locally more abundant than *P. spumarius*. However, due to lower population density and different associations with host plants (nymphs are strongly associated with gramineous plants, adults prefer conifers) (Di Serio et al., 2019; Antonatos et al., 2021) this species is considered a minor vector.

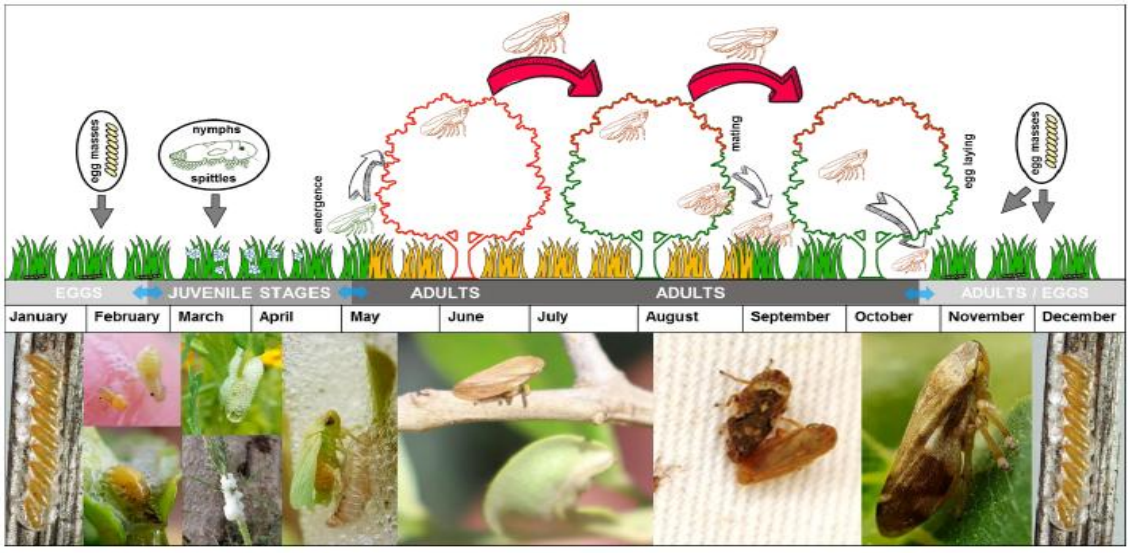


Figure 2. Life cycle of *Philaenus spumarius*, divided into one-year chronology. It also shows how *X. fastidiosa* would be transmitted. Newly emerged adults are *Xylella fastidiosa* free (green insect), they acquire *X. fastidiosa* by feeding on an infected host (in red). The infected vector can then transmit the bacterium to healthy (green) hosts, which in turn become infected. Eggs are not carriers of the bacterium, as transovarial transmission is not possible. Nymphs can also become infected, although they lose infectivity when they moult (from EFSA, 2019).

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1.2. *Xylella fastidiosa* management

In general, the management of *X. fastidiosa* is largely preventive and suppressive rather than curative, with success depending on early detection, rapid regulatory action, and continuous adaptation of integrated strategies (Morelli et al., 2021). Specific measures are aimed at targeting infected plants and/or the bacterium but, being *X. fastidiosa* insect transmitted, vector control is a pillar of the strategy to contain the bacterium (Bodino et al., 2023; Dongiovanni et al., 2023). By focusing on vector management, the number of new *X. fastidiosa* cases can be significantly reduced, limiting economic impact and crop loss (Liccardo et al., 2020; Lago et al., 2021).

A diverse range of approaches have been proposed for *P. spumarius* control:

Chemical control: insecticides can kill both nymphs and adults, although care must be taken with environmental and resistance issues (Dongiovanni et al. 2018a,b; Dáder et al., 2019).

Biological control with arthropods: generalist predators such as spiders or the bug *Zelus renardii*, the egg parasitoid mymarid wasp *Ooctonus vulgatus* and the endoparasitoid fly *Verralia aucta* have shown some efficacy in controlling spittlebug populations (Mesmin et al., 2020; McCaw et al., 2023).

Biological control with microorganisms: entomopathogenic fungi, such as *Beauveria, Metarhizium* and *Lecanicillium* has emerged as promising agents for the control of *P. spumarius* (Yousef-Yousef et al., 2023; Bodino et al., 2024; Conde-Bravo et al., 2025). Entomopathogenic nematodes and their symbiotic bacteria have shown insecticidal properties against nymphs and can also contribute to provide an ecological alternative to chemical insecticides (Vicente-Díez et al., 2021).

Botanical products: Plant-based insecticides and extracts are being studied for sustainable, integrated pest management systems to reduce dependence on chemicals (Rongai et al., 2023; Bedini et al., 2025).

RNA interference (RNAi): a proof-of-concept that dsRNA-based gene silencing can inhibit essential genes in *P. spumarius*, reducing vector viability/prolificity and potentially decreasing transmission has been realized (Parise et al., 2024).

Cultural practices or agricultural management techniques: frequent tillage and mowing of vegetation cover can reduce nymph and adult populations, especially in olive groves, by eliminating breeding and feeding habitats (Sanna et al., 2021).

Ground cover management: cover crop management is a key component of habitat manipulation strategies to reduce *P. spumarius* populations. Morente et al. (2022b) highlighted the effectiveness of managing cover crop species and vegetation composition in olive groves to reduce *P. spumarius* abundance by altering their breeding and feeding habitats. Selective planting of cover crops that are less favourable or antagonistic to the development of spittlebugs, combined with vegetation management, can significantly reduce vector density and the risk of *X. fastidiosa* transmission (Morente et al., 2022b).

Monitoring and Trap-Based Capture: Sticky traps and sweep nets are used to monitor populations and guide timely interventions. Integrating both approaches ensures reliable population estimates and helps optimize management efforts (Dongiovanni et al., 2023).

To date, individual measures have proven insufficient to achieve a satisfactory control of the pathogen spread and, therefore, the integration of several different measures, within a frame

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of an Integrated Pest Management strategy (IPM) is needed. To achieve the best results, it is necessary to both reduce the vector population and disrupt transmission (Gilioli et al. 2023). This deliverable deals with the first objective, the limitation of vector populations.

2. DIRECT AND ENDOPHYTIC PATHOGENICITY OF ENTOMOPATHOGENIC FUNGAL STRAINS (EPF) AGAINST VECTORS OF *XYLELLA FASTIDIOSA*

2.1. Background

Entomopathogenic ascomycetes are among the very few alternatives for the microbial control of piercing-sucking Auchenorrhyncha. This is not only due to their distinctive contact mode of action within entomopathogens (Quesada-Moraga et al., 2023) but also because of the intriguing relationships they form with plants (Quesada-Moraga et al., 2020). These relationships are notable as they facilitate diverse strategic applications aimed at reducing nymph or adult populations of insect vectors of *X. fastidiosa*. Entomopathogenic ascomycetes can be strategically used both for their direct effect as Xf vector microbial control agents and for their indirect effect on these vectors through their partnership with the crop and wild plants (Quesada-Moraga et al., 2022). Hence, several fungal entomopathogenic species can enable the control of *X. fastidiosa* vector nymphs by directly spraying them on the olive cover crops or indirectly through endophytic colonization or priming of the crop weeds. Meanwhile, these fungal biocontrol agents can also be deployed for *X. fastidiosa* adult vector control, both through fungal sprays directly targeting adults and, even more importantly, through exploiting their endophytic or rhizosphere competence behaviour in the olive crop to impair adult fitness.

The following studies aimed at assessing the direct and indirect role of entomopathogenic fungi (EPF) to limit the populations of the two main vectors of *X. fastidiosa* in Europe*, P. spumarius* and *Neophilaenus campestris.* The direct evaluation included the pathogenicity of different EPF strains against adults and nymphs of the vectors under both laboratory and field conditions. The indirect effect was evaluated through the endophytic colonization of herbaceous and olive plants used as a food source by the vectors. Also, the direct pathogenicity effect of selected EPF strains was evaluated against the sharpshooter *Bucephalogonia xanthophis*, known as key vector in *X. fastidiosa* associated with CVC spread in Brazil (Lopes & Krugner, 2016). We tested the entomopathogenic species *Beauveria bassiana* and *Cordyceps javanica*, already present in several commercial products registered in diverse crops for controlling hemipteran vectors in South America (Mascarin & Jaronki, 2016). The results obtained by these studies provide new insights into EPF-based tools for sustainable vector and disease management of diseases associated with *X. fastidiosa*.

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2.2. Direct effect of EPF on spittlebugs

2.2.1. Nymphal stages

2.2.1.1. Laboratory trials

Effect of *Beauveria bassiana* EABb 04/01-Tip, *Lecanicillium. aphanocladii* strain *L. aphanocladii* DSF, and M. brunneum strain EAMa 01/58-Su on survival of *Philaenus spumarius* nymphs

The pathogenicity of three EPF strains (*Beauveria bassiana* strain EABb 04/01-Tip, *Lecanicillium aphanocladii* strain *L. aphanocladii* DSF, and *Metarhizium brunneum* strain EAMa 01/58-Su) against *P. spumarius* nymphs was assessed in P3-UCO laboratory through direct spray application. For that, 2 mL of conidial suspension (1×10^8 conidia/mL) of each strain was sprayed on *Vicia faba* L. plants bearing 3rd–4th instar *P. spumarius* nymphs (10 per plant). Mortality was monitored every two days for two weeks after treatment.

Results showed that nymphs treated with fungal suspensions showed higher mortality compared to the control (***Table 1***). The average mortality recorded for the control group was 26.0 %, while significantly higher mortality levels were observed for *B. bassiana* (54.0 %) and *M. brunneum* (52.0 %), respectively. *L. aphanocladii* caused a lower mortality (34.0 %), which was not statistically different from the control according to the LSD test ($p < 0.05$). In addition, the average survival time (AST) of *P. spumarius* nymphs varied significantly among treatments. The shortest AST values were recorded for *B. bassiana* (8.3 days) and *M. brunneum* (8.86 days), both significantly different from the control and *L. aphanocladii* according to the log-rank test ($p < 0.05$). The control and *Lecanicillium* treatments presented longer survival times (11.22 days and 12.16 days, respectively).

Table 1. Susceptibility of *P. spumarius* nymphs to fungal strains after spraying with conidial suspensions

Treatment*	**Mean mortality (±SE) %**	**AST (±SE; days) ****	**Upper limit**	**Lower limit**
Control	26.0 ± 5.1 A	11.22 ± 0.68 a	12.56	9.88
Beauveria bassiana (EABb 04/01-Tip)	54.0 ± 4.0 B	8.3 ± 0.77 b	9.82	6.78
Lecanicillium aphanocladii (*L. aphanocladii* DSF)	34.0 ± 2.45 A	12.16 ± 0.45 a	13.04	11.28
Metarhizium brunneum (EAMa 01/58-Su)	52.0 ± 14.63 B	8.86 ± 0.77 b	10.36	7.36

** The spray treatment was done with a 1×10^8 conidia/mL suspension (LSD test ($p < 0.05$).*
*** AST: average survival time; these were limited to 14 days.*
Within columns, means with the same letters were not significantly different to each other according to log rank test ($p < 0.05$).

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These results indicate that *B. bassiana* (strain EABb 04/01-Tip) and *M. brunneum* (strain EAMa 01/58-Su) were the most virulent strains against *P. spumarius* nymphs, significantly reducing their survival. The higher mortality and shorter survival times observed suggest a rapid fungal outgrowth development following conidial contact and penetration. In contrast, *L. aphanocladii* exhibited limited pathogenicity under the tested conditions.

Effect of *Lecanicillium aphanocladii* DSF on *P. spumarius* nymphs under laboratory conditions

Lecanicillium aphanocladii DSF strain was isolated from a *P. spumarius* adult affected by mycosis and recovered from a semi-field maintenance rearing in Torino. The *P. spumarius* cadaver found externally sporulated was carefully collected and a small amount of mycelium was transferred to Sabouraud dextrose agar Petri plates, supplemented with penicillin G (0.1 mg/mL) and streptomycin (0.5 mg/mL), and incubated at 25 °C. The pure culture of *L. aphanocladii* DSF was obtained by single spore isolation on Malt Extract Agar.

Pathogenicity bioassays on *P. spumarius* nymphs were carried out according to the following experimental design: in trial 1 (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); also, four control levels were tested: Mycotal (Myc), Ad1, Ad2 and water (Wtr). In trial 2 (T2), *L. aphanocladii* blastospores (LaB) and *L. aphanocladii* conidia (LaC) treatements were compared to *B. bassiana* ATCC 74040 conidia (NCf), used as positive control, and water (negative control, Wtr).

Conidia production for theses La_C, Nat_C, Nat_CNF was carried out at DISAFA. All treatment were 1×10^8 conidia/mL concentrated and added of Tween 80 (0.01 % v/v). *L. aphanocladii* freeze dried blastospores (from liquid substrate fermentation) were provided by Globachem (1×10^{10} blastospores)/g) and kept at 4 °C. On the day of treatment, blastospores were rehydrated in sterile deionized water for 30 min at room temperature and an appropriate dilution was prepared in order to reach the final concentration of 1×10^8 blastospores/mL.

All the treatments were applied 24 h after placing the nymphs on *Vicia faba* plants, in order to allow nymphs to settle on leaves and stems and produce foams. Experimental *V. faba* microcosms (i.e., replicas) consisted in two *V. faba* plants sown in a single rounded pot enclosed by a transparent plastic cylindrical cage with two holes with mesh for ventilation at top and side. In each microcosm, 10 nymphs of *P. spumarius* were placed. 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. After the treatments, microcosms were visually inspected every 2–3 days, assessing the survival rate of insects and collecting dead individuals. Dead insects were surface sterilized and incubated in Petri plates containing filter paper moistened with sterile distilled water to encourage mycelium development.

Results showed that *L. aphanocladii* DSF significantly reduced nymphal survival and adult emergence compared to the control; in particular, *L. aphanocladii* DSF blastospore formulation was significantly more effective than the conidia formulation, with nymphal mortality and adult emergence rates of 90 % and 4 %, respectively (***Figure 3*** and ***Figure 4***). Moreover, 180 dead nymphs were collected during the visual inspections, 77.2 % of which developed mycelium referable to the inoculated fungal species.

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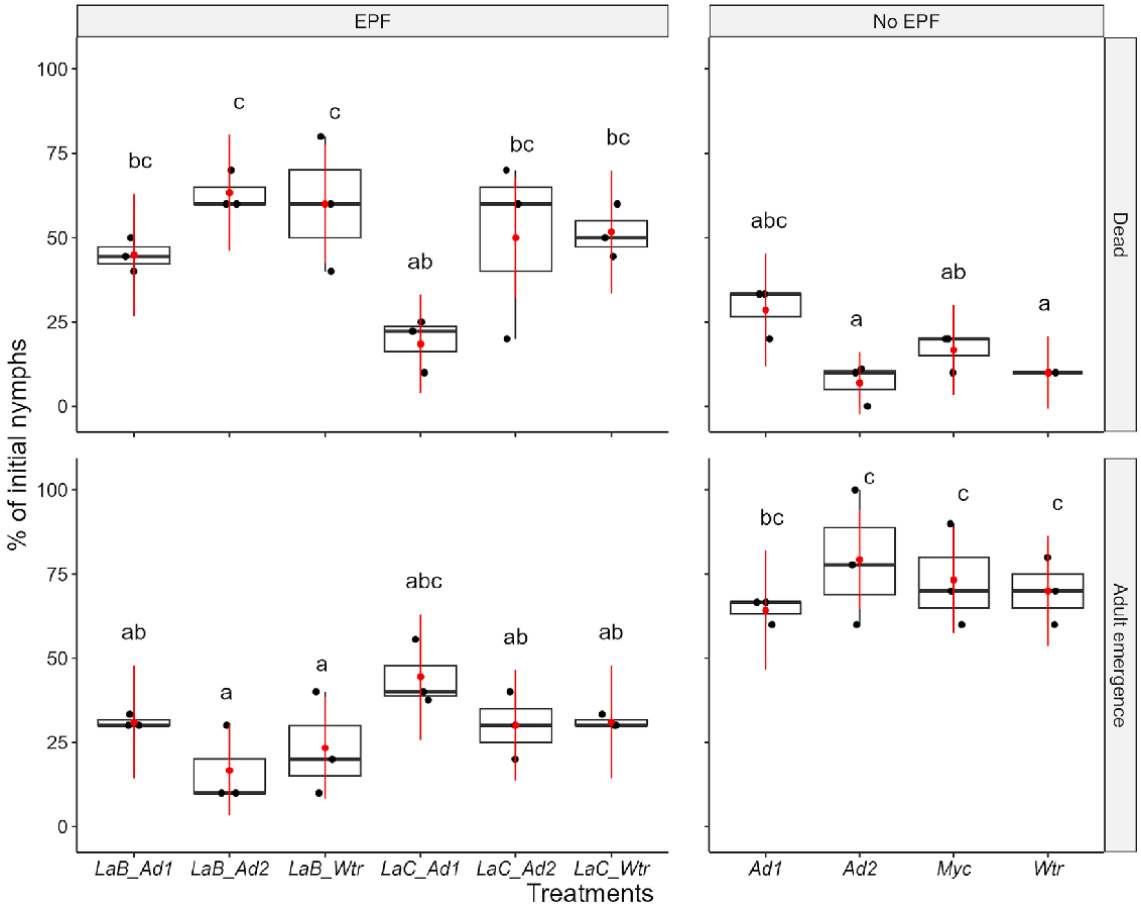


Figure 3. Percentage of initial nymphs of *Philaenus spumarius* found dead or emerged as adults during the 2021 experimental trial (T1) of *Lecanicillium aphanocladii* DSF efficacy. Data observed (boxplots) and predicted by binomial GLM (red point range) are reported, and treatments sharing the same letter within a row are not significantly different (α = 0.05, Tukey-adjusted) (Bodino et al., 2024).

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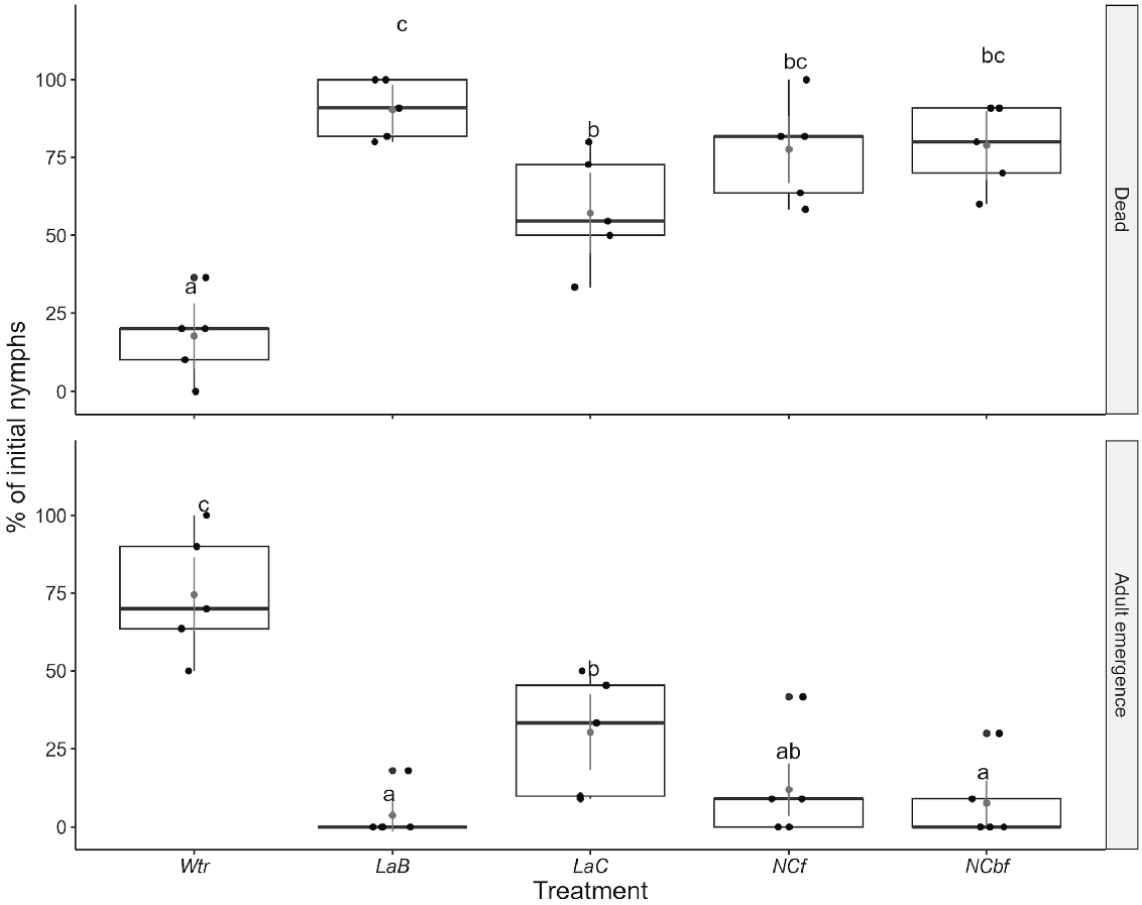


Figure 4. Percentage of initial nymphs of *Philaenus spumarius* found dead or emerged as adults during the 2022 experimental trial (T2) of *Lecanicillium aphanocladii* DSF efficacy. Data observed (boxplots) and predicted by binomial GLM (red point range) are reported, and treatments sharing the same letter within a row are not significantly different (α = 0.05, Tukey-adjusted) (Bodino et al., 2024).

Pathogenicity of a selection of soil-sampled EPF strains on *Philaenus spumarius* nymphs under laboratory conditions.

Twenty EPF strains (***Table 2***) previously isolated from soil samples of vineyards, meadows and woodlands (Piedmont, north-west Italy) were tested under controlled conditions, against *P. spumarius* nymphs through direct spray application. For that, 5 mL of conidial suspension (1×10^8 conidia/mL) of each strain was sprayed on each *V. faba* plant microcosm bearing 3rd–4th instar *P. spumarius* nymphs (10 per plant). Survival rate was monitored 4 and 8 days after the treatment. *Beauveria bassiana* ATCC 74040 was used as positive control.

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Table 2. EPF strains used in pathogenicity bioassays performed by P6-UNITO against *Philaenus spumarius* nymphs.

Strain code	Species
Trial 1	
FV1c	*Metarhizium anisopliae*
Cg1k	*Beauveria bassiana*
BV1a	*Ophiocordyceps heteropoda*
CB2	*Purpureocillium ilacinum*
CaS1d	*Penicillium citrinum*
V2	*Cladosporium sp.*
BP1k	*Metarhizium robertsii*
BP1e	*Metarhizium anisopliae*
PV1d	*Metarhizium anisopliae*
CnV2i	*Metarhizium anisopliae*
NAT	*Beauveria bassiana ATCC 74040*
Trial 2	
Cg2b	*Beauveria pseudobassiana*
CaS1h	*Clonostachys rosea*
MUT	*Lecanicillium aphanocaldii*
CaS1f	*Penicillium citrinum*
Bp1o	*Metarhizium anisopliae*
Bp1l	*Metarhizium anisopliae*
Bp1f	*Metarhizium robertsii*
Bp1r	*Metarhizium robertsii*
Bp1q	*Metarhizium anisopliae*
CaS1a	*Purpureocillium lilacinum*
NAT	*Beauveria bassiana ATCC 74040*

Results showed that 14 out of 20 EPF strains significantly reduced nymphal survival compared to negative control after 8 days from the treatment (*$p < 0.05$, binomial GLM). In ***Figure 5***, the survival rate of *P. spumarius* nymphs in microcosms, after 4 and 8 days from the treatment, is reported. Six strains (CnV2i, BP1q, BP1r, BP1o, BP1k, BP1f) belonging to *Metarhizium* genus were the most active reducing the average nymph survival rate from 2 % to 8 %, comparable to *B. bassiana* ATCC 74040 positive control. Only 5 out of 20 EPF strains proved ineffective (80-90 % survival).

Fungi were successfully reisolated from a total of 195 cadavers with different percentage depending on the strain and ranging from 25 % to 100 %. *Metarhizium anisopliae* CnV2i strain reached the highest value (100 %) (***Figure 6***), and the mycelium emerging from a dead *P. spumarius* nymph is shown in ***Figure 7***.

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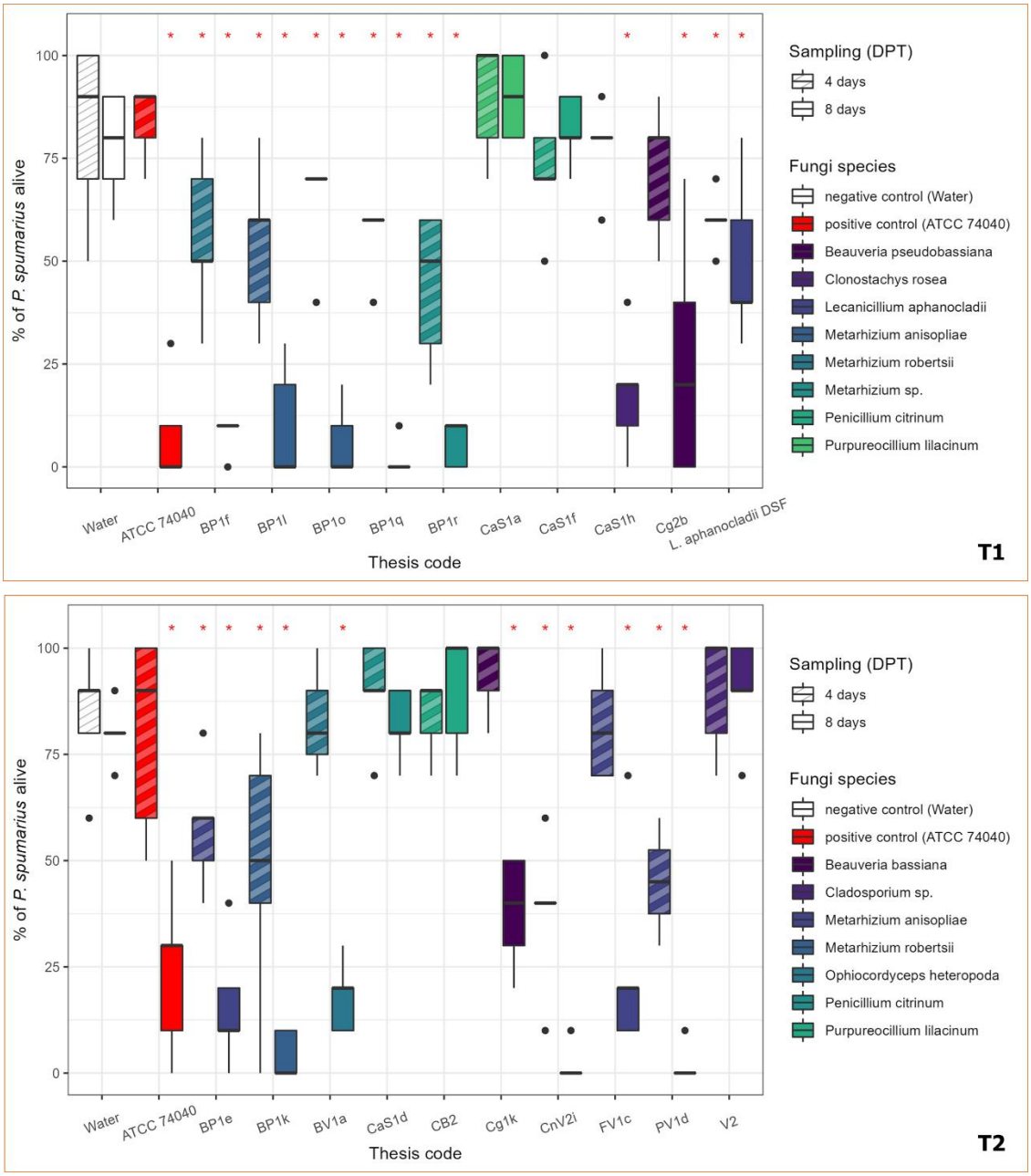


Figure 5. Boxplot of % survival rate of *Philaenus spumarius* nymphs after 4 and 8 days from the treatments, in Trial 1 (T1) and 2 (T2). Red asterisks on top mean that survival rate was significantly lower than negative control (water).

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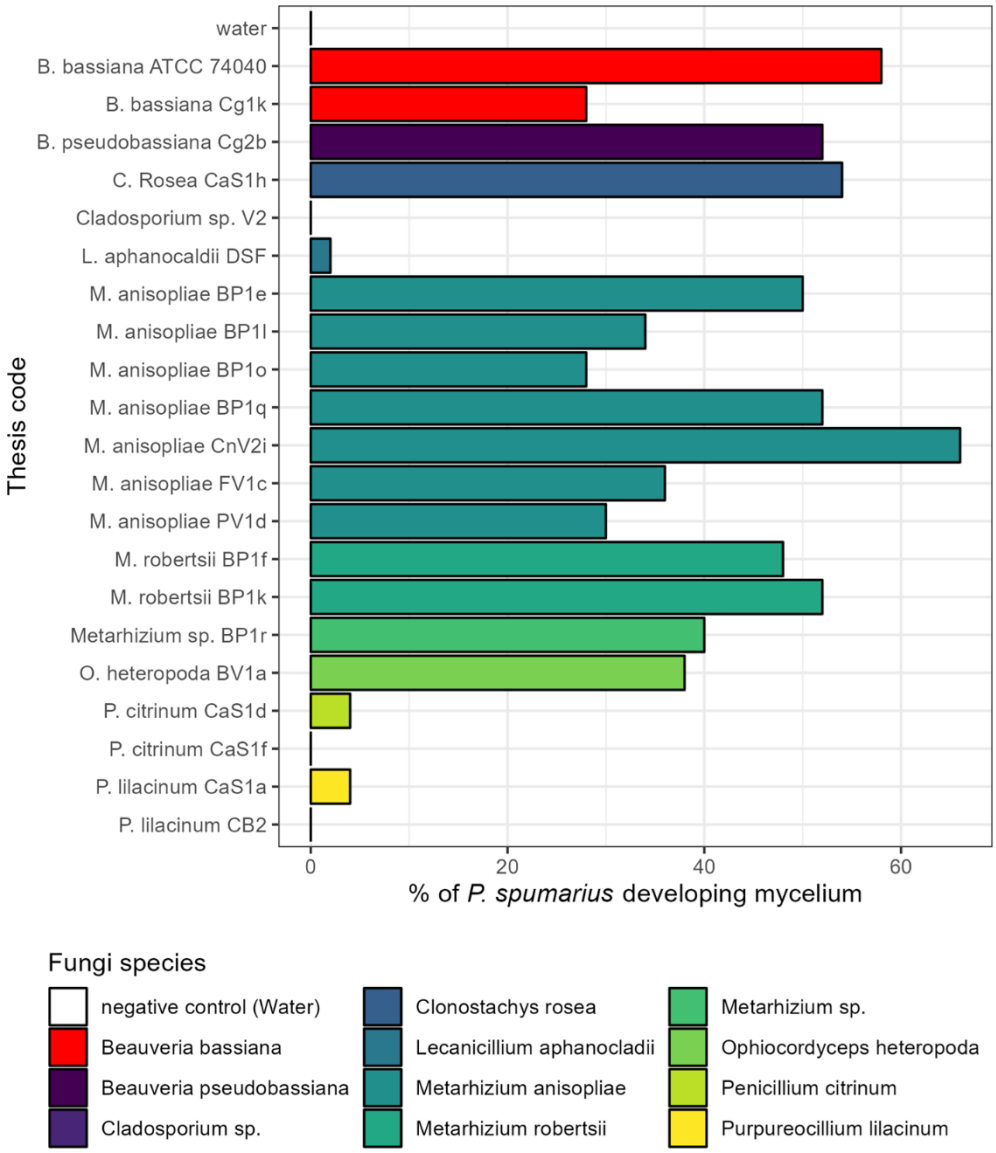


Figure 6. Percentage of *Philaenus spumarius* nymphs developing mycelium referable to the inoculated fungal species on total nymphs

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Figure 7. *Metarhizium anisopliae* CnV2i mycelium emerging from dead *Philaenus spumarius* nymphs

2.2.1.2. Field trials

Efficacy of *Metarhizium. brunneum* EAMa 01/58-Su strain on naturally present nymphal population of *Neophilaenus campestris* in Spain

This experiment aimed to explore and validate the effectiveness of the method for controlling the pierce-sucking spittlebug *N. campestris*, based on the application of fungal suspensions from the EAMa 01/58-Su strain of the EPF *M. brunneum* to the insect over two years to reduce the spittlebug population density. For that, a two-year experiment was conducted in an experimental olive orchard located at the Rabanales University Campus in Cordoba (37° 55' 14.05'' N, 4° 43' 16.31'' W) during early spring in 2023 and 2024. Previously, it was selected the experimental unit of each block consisted of an area of 80 m^2 (10 x 8 m). A randomised block design was applied using two treatments (fungal application and control) and four replicates each, and there was 30 m amongst plots to avoid overlapping, considering that spittlebugs only change the feeding place on the same plant or change the host plant with an adjacent one.

A total of 3.5 liters of fungal suspension was distributed throughout each selected area for fungal treatment, and the control solution (sterilized water with Tween 80 at 0.01 %) was distributed for the areas selected as control. The spittlebug nymph population was estimated every two days since the day of the inoculation (before the treatment) up to the 8^{th} day after treatment. Data on daily temperatures and relative humidity (RH) were recorded at the agroclimatic station of the Andalusian Institute for Research and Training in Agriculture, Fisheries, Food and Organic Production (IFAPA) in Alameda del Obispo (Córdoba), and no rainfall was recorded during the two experiment periods. Temperature and Relative humidity data are recorded in ***Figure 8*** and ***Figure 9***.

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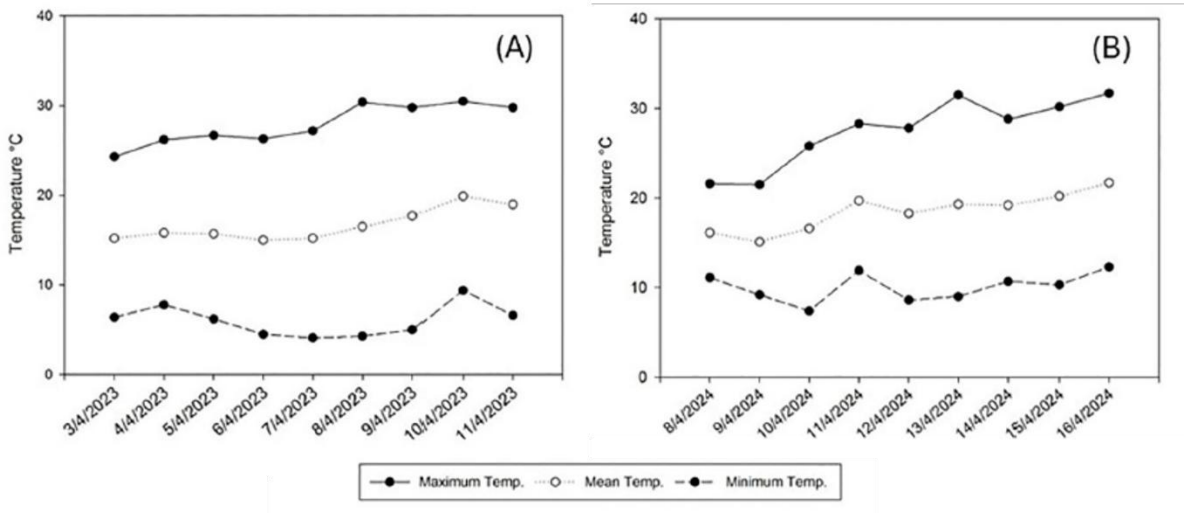


Figure 8. Temperature evolution in Alameda del Obispo (Cordoba) during the trial periods of spring 2023 (A) and spring 2024 (B).

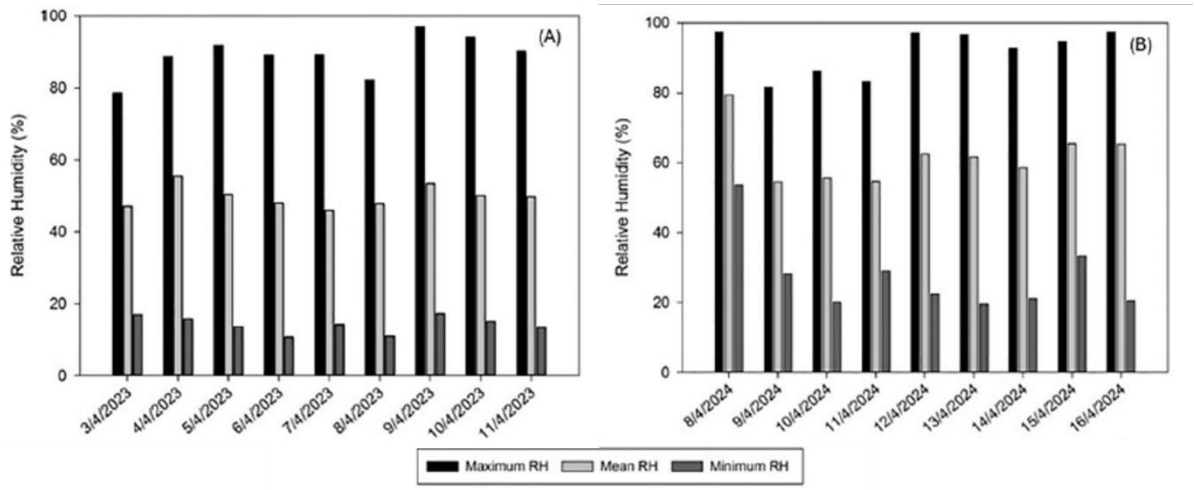


Figure 9. Relative humidity (RH) evolution in Alameda del Obispo (Cordoba) during the trial periods of spring 2023 (A) and spring 2024 (B). No rainfall was recorded during both trial periods.

Results showed that, after spraying the cover crop with the *M. brunneum* strain, the foams produced by *N. campestris* nymphs, the main targets of the treatment, were counted on adventitious plants in the olive grove. A circular frame with a diameter of 0.30 m was used, which was randomly thrown five times within the area of each block, and the foam was counted. The limited mobility of nymphs makes them more susceptible to localised treatments, which increases the likelihood of effective control.

In 2023, a significant reduction in foaming was observed in the treated plots compared to the control over time (b = −1.283, SE = 0.512, p = 0.013; ***Figure 10***a). The average number of foams recorded in the control plots at the end of the experiment was 1.2 foams, while the treated plots showed an average of 0.2 foams, reflecting a reduction of 87.5 %. In 2024, there was a

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reduction in foam, but it was less pronounced and not statistically significant (b = −0.470, SE = 0.492, p = 0.314; ***Figure 10***B). However, the average foam count in the control plots at the end of the experiment was 0.9, compared to an average of 0.2 foams in the treated plots, reflecting a reduction of 76.4 %. Therefore, no significant cumulative or interactive effects were detected for the spittlebugs recorded between the two years (t=−0.430, p=0.665).

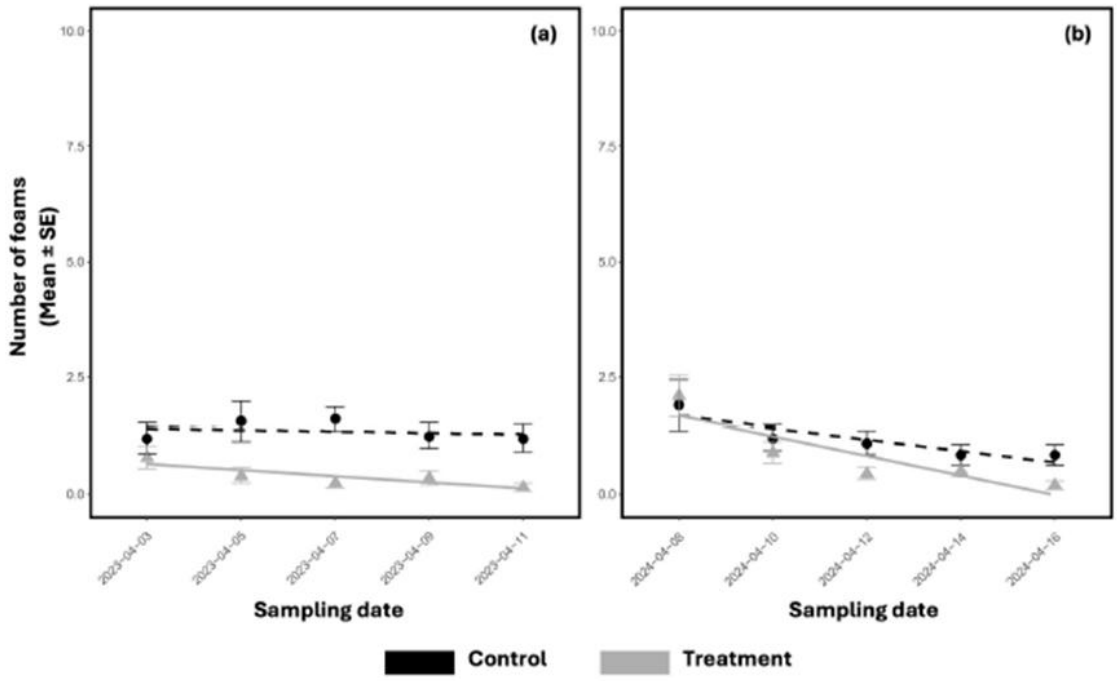


Figure 10. Evolution of the mean number ± SE of *N. campestris* foams counted in spring 2023 (A) and spring 2024 (B) during the test period after spraying with *M. brunneum* EAMa 01/58-Su strain. Lines represent the tendency of nymph and adult populations per block along the time of the sampling date. Black lines with circles represent controls and grey lines with triangles represent treatments. Fungi were applied in treatment plots on the first day of sampling in each year and treatment blocks.

The results of the variation in relative population density following the application of the fungus revealed that both the counted *N. campestris* foams and adult populations decreased during both trials. However, the decrease was not significant for the counted foams in the 2023 and 2024 trials (H = 1.8509, P < 0.1737; H = 3.3827, P < 0.0651, respectively) (***Figure 11***).

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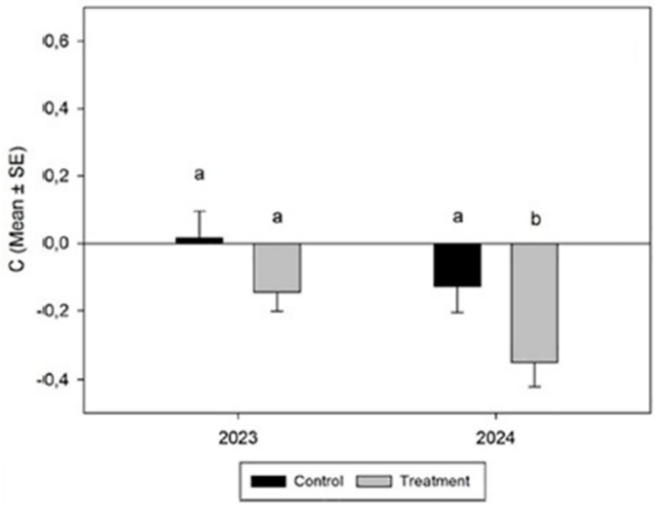


Figure 11. Variation in the relative population density (mean ± SE) of *N. campestris* foams counted in spring 2023 and spring 2024 during the trial period after spraying the cover crop with *M. brunneum* EAMa 01/58-Su strain. This indicator is calculated according to Peveling et al. (40), where the viable population before and after the trial is considered. Negative values of C indicate a decrease in the trial population. Values not sharing the same letter are significantly different at $p \leq 0.05$ (nonparametric Kruskal-Wallis test; alpha=0.05).

Efficacy of *Metarhizium anisopliae* BP1q strain on nymphal populations of *Philaenus spumarius* in Italy

The most promising strain from lab screening *M. anisopliae* BP1q was tested on nymphal populations of the spittlebug in two Xf-infected olive groves in Apulia. The activity of the strain was compared to the commercial EPF product Naturalis® and the physical control method of mowing (mandatory control in infected areas in Apulia). Efficiency of both EPFs was tested applying sequentially one or two treatments. Untreated plots were used as control. A total of six theses were thus tested along a row of one monumental olive orchard, randomized in five blocks (***Figure 12***):

i) one treatment with *M. anisopliae* Bp1q

ii) two treatments with *M. anisopliae* Bp1q

iii) one treatment with Naturalis®

iv) two treatments with Naturalis®

v) mowing

vi) negative control

The conidial formulation of *M. anisopliae* Bp1q was sprayed on cover olive crops at a concentration of 2 x 10^8 conidia/m^2 while the commercial product Naturalis was used according to manufacturer's instruction. The target abundance was evaluated by counting the number of nymphs per sampling unit (0,25 m^2) three times: i) pre-treatment; ii) one-week post-treatment (PostTreatm_1); iii) two weeks post-treatment (PostTreatm_2)

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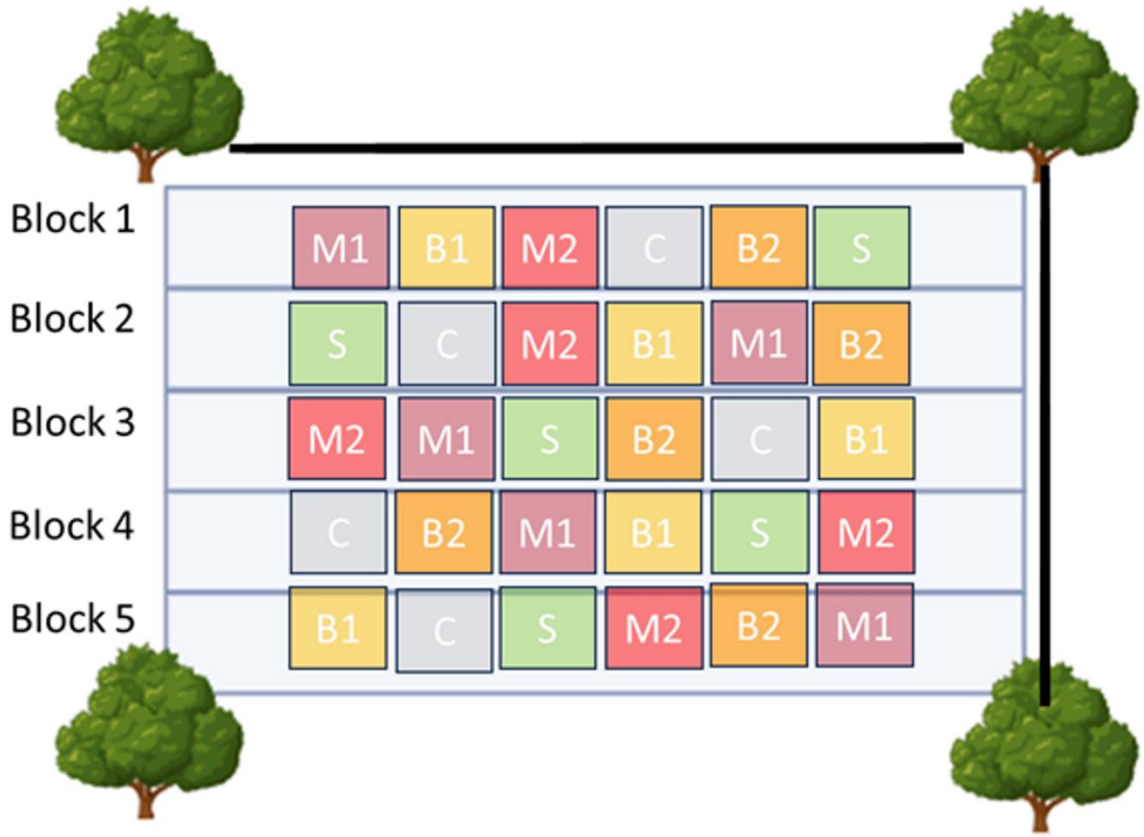


Figure 12. Set up of experimental fields at Cisternino and Crispiano localities in Apulia Region (South-Italy).

Results showed a significant decrease in the number of spittlebug nymphs after one and two treatments of both *M. anisopliae* BP1q and Naturalis® compared to the control (untreated plots), with some differences between the two experimental fields (***Figure 13*** and ***Figure 14***). Specifically, the results showed that already one week after the treatment i) mowing was the most efficient treatment in reducing spittlebug nymphal population (≈8 nymphs/m^2), ii) all EPFs treatments significantly reduced the vector population abundance (≈20–30 nymphs/m^2), except for the Naturalis® two-applications-treated blocks (≈48 nymphs/m^2), compared to control thesis (≈65 nymphs/m^2).

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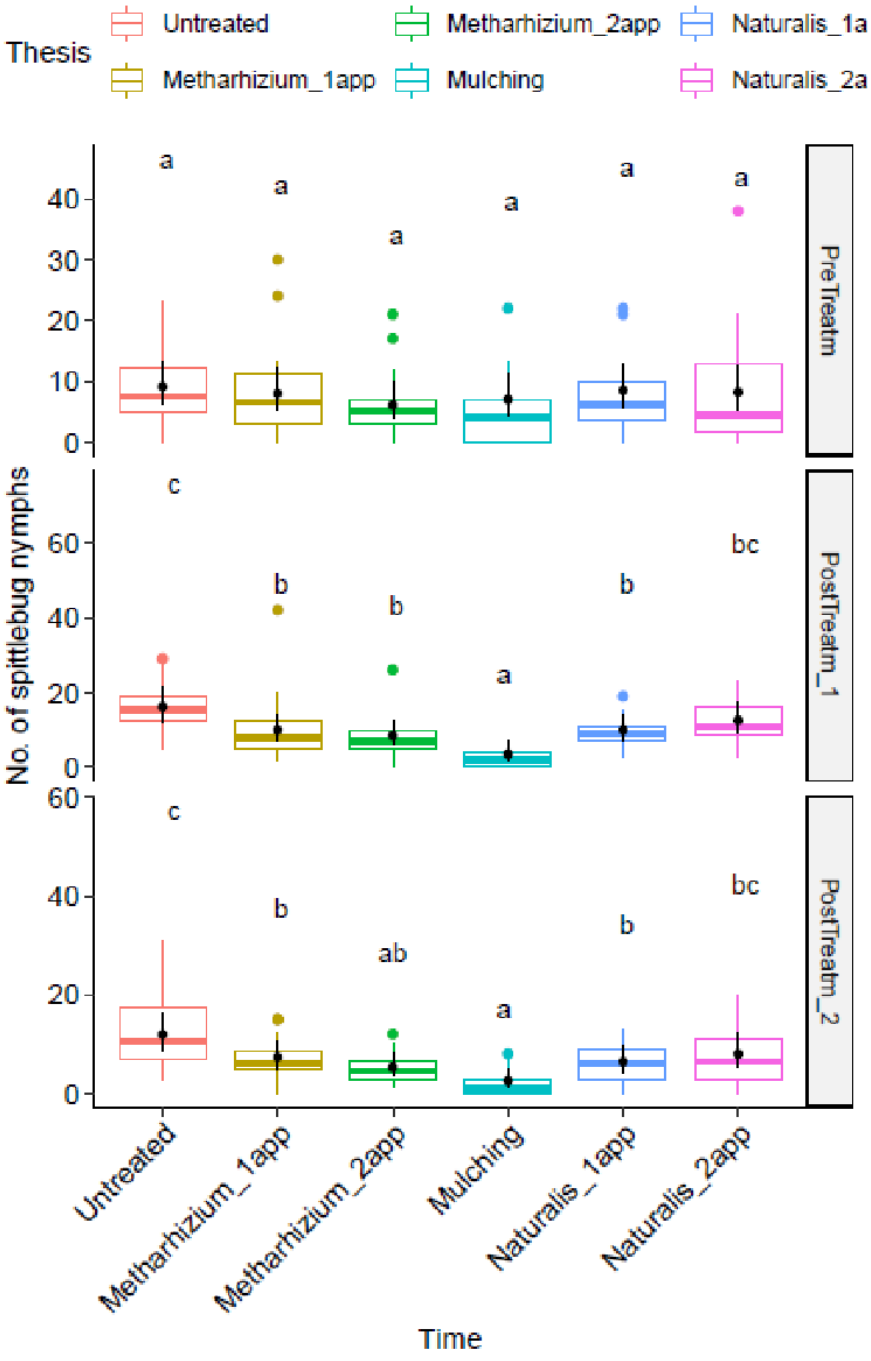


Figure 13. Field at locality Crispiano in Apulia (South-Italy). Number of spittlebug nymphs in 0.25 m^2 plots, according to Thesis and Time (Pre- or Post-Treatment (1-2)). Same letters denote means not significantly different (GLMM model).

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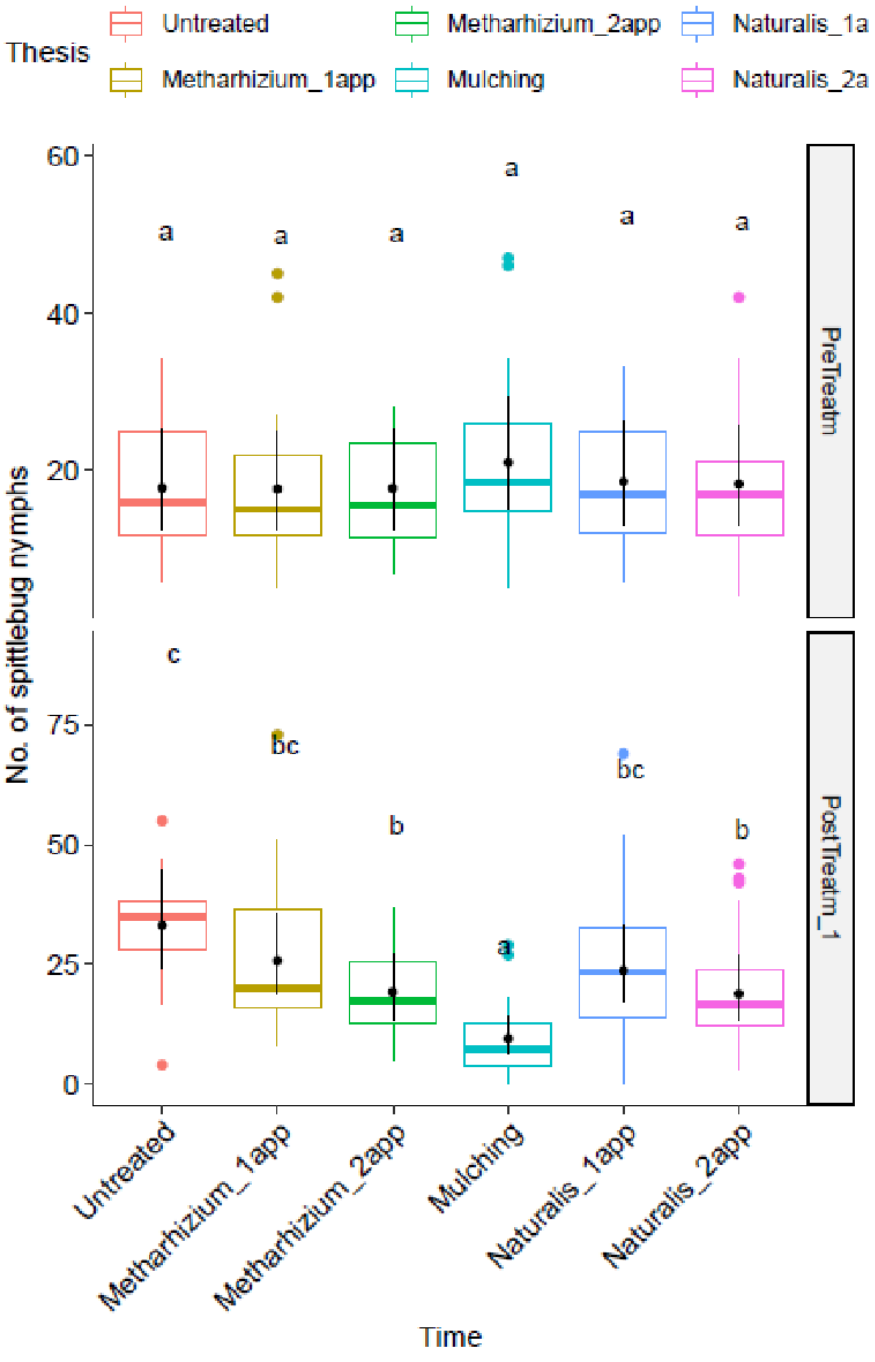


Figure 14. Field at locality Cisternino in Apulia (South-Italy). Number of spittlebug nymphs in 0.25 m^2 plots, according to Thesis and Time (Pre- or Post-Treatment (1-2)). Same letters denote means not significantly different (GLMM model).

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2.2.2. Adult stage

2.2.2.1. Laboratory trials

Effect of EPF strains on *Philaenus spumarius* and *Neophilaenus campestris* adults under laboratory conditions – Spain trials

A total of 25 EPF strains originally isolated from olive agroecosystem and one from wheat crop soil (***Table 3***) were tested against *P. spumarius* adults through direct application.

Table 3. List of entomopathogenic fungal strains from the University of Cordoba obtained from the olive crop agroecosystem evaluated against *P. spumarius* adults

Strain	Fungal Species	Origin	Habitat	Agroecosystem
EABb 04/01-Tip	*B. bassiana*	Ecija (Seville, Spain)	Insect	Iraella luteipes
EABb 08/01-Po	*B. bassiana*	Braganza, Portugal	Insect	Prays oleae
EABb 08/02-Po	*B. bassiana*	Braganza, Portugal	Insect	Prays oleae
EABb 08/03-Po	*B. bassiana*	Braganza, Portugal	Insect	Prays oleae
EABb 08/04-Ep	*B. bassiana*	Jaén, Spain	Insect	Euzophera pinguis
EABb 09/0-Fil	*B. bassiana*	Bobadilla (Malaga, Spain)	plant	Organic olive orchard weed
EABb 10/02-Fil	*B. bassiana*	Bobadilla (Malaga, Spain)	plant	Organic olive orchard weed
EABb 10/19-Fil	*B. bassiana*	Bobadilla (Malaga, Spain)	plant	Organic olive orchard
EABb 10/20 - Fil	*B. bassiana*	Bobadilla (Malaga, Spain)	plant	Organic olive orchard
EABb 10/35-Fil	*B. bassiana*	Adamuz (Cordoba, Spain)	plant	Traditional olive orchard
EABb 10/95-Fil	*B. bassiana*	Bobadilla (Malaga, Spain)	plant	Organic olive orchard
EABb 10/115-Fil	*B. bassiana*	Bobadilla (Malaga, Spain)	plant	Organic olive grove weed
EAMa 10/01-Fil	*M. guizhouense*	Adamuz (Cordoba, Spain)	plant	Traditional olive grove weed
EABb 01/33-Su	*B. bassiana*	El Bosque (Cádiz, Spain)	Soil	Traditional olive orchard
EABb 01/126-Su	*B. bassiana*	Bornos (Cadiz, Spain)	Soil	Olive grove
EAMa 00/19-Su	*M. anisopliae*	Abla (Almeria, Spain)	Soil	Olive grove
EAMa 01/58-Su	*M. brunneum*	Hinojosa del Duque (Cordoba, Spain)	Soil	Wheat crop
EAMa 01/158-Su	*M. guizhouense*	Utrera (Seville, Spain)	Soil	Olive grove
EABb 04/10-Su	*B. bassiana*	Figueras (Gerona, Spain)	Soil	Olive grove
EABb 09/16-Su	*B. bassiana*	Bobadilla (Malaga, Spain)	Soil	Organic olive orchard
EABb 09/23-Su	*B. bassiana*	Bobadilla (Malaga, Spain)	Soil	Organic olive orchard

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Strain	Fungal Species	Origin	Habitat	Agroecosystem
EAMa 09/01-Su	*M. anisopliae*	Valbom de Baixo (Portugal)	Soil	Traditional olive orchard without cover
EALx 09/0 -Su	*Lecanicillium*	Bobadilla (Malaga, Spain)	Soil	Organic olive grove,
EAMb 09/01-Su	*M. brunneum*	Castilblanco de los Arroyos (Seville, Spain)	Soil	Dehesa
EABv 09/01-Su	*B. varroae*	Castilblanco de los Arroyos (Seville, Spain)	Soil	Dehesa
Bb-1333	*B. bassiana*	Greece 1961	Insect	Bactrocera oleae

For that, adults of *P. spumarius* were sprayed with 1 mL of fungal suspensions (1×10^8 conidia mL^{-1}) from each strain per replicate. Before treatment, adult *P. spumarius* were cold-anesthetized. Control insects were treated in the same manner, receiving an equal volume of a sterile aqueous solution containing 0.1 % Tween 80. Three replicates of seven adult *P. spumarius* were used for each fungal strain and control group. Following the spraying procedure, each group of seven adult insects was transferred to a *Sonchus oleraceus* plant. These plants, along with the seven *P. spumarius* adults, were enclosed in individual cages (27 x 27 x 27 cm) and kept under the following controlled conditions: 25 ± 1 °C temperature, 70 ± 5 % relative humidity (RH), and a photoperiod of 16:8 (L:D) hours. Mortality was observed and recorded over 9 days. To determine if the entomopathogenic fungus caused mortality, dead *P. spumarius* adults were removed daily, immediately surface-sterilized for 1 minute in 1 % sodium hypochlorite, rinsed three times in sterile distilled water (1 minute each), placed on sterile wet filter paper in sterile Petri plates, sealed with laboratory film, and incubated at 25 ± 2 °C in darkness, then inspected for fungal outgrowth.

Results showed significant differences in virulence among treatments ($\chi^2 = 167.25$, df = 26, $p < 0.001$). The isolates EAMa 10/01-Fil and EAMa 00/19-Su were the most effective, causing 100 % mortality within nine days, followed by EAMa 01/58-Su, which reached 95.2 % mortality, highlighting its strong pathogenic potential (***Figure 15***).

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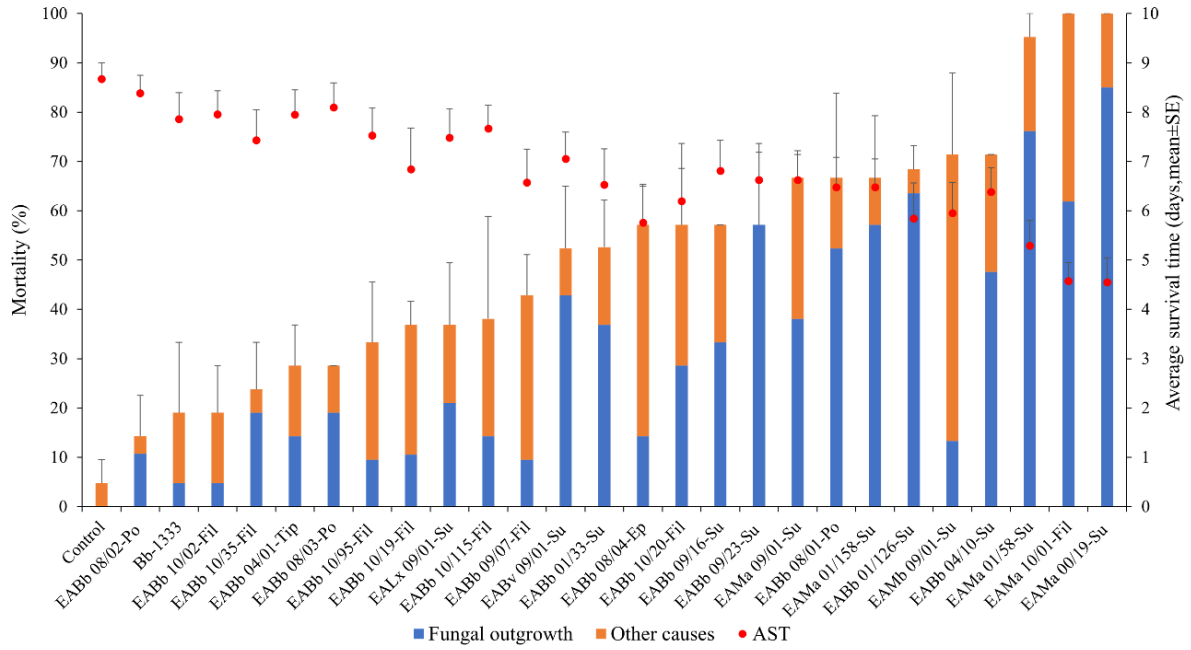


Figure 15. Mortality and fungal growth of *Philaenus spumarius* adults treated with suspensions of 1.0 × 10^8 conidia mL^{-1} applied using an airbrush. The points represent the average survival time (AST), which was limited to 9 days. Data are presented as mean ± standard error.

Based on the results of the initial assay with *P. spumarius*, the nine most virulent strains were selected to assess their efficacy against adult *N. campestris*. These insects were treated under the same methodology, using 1 mL of fungal suspensions (1 × 10^8 conidia mL^{-1}) per replicate. Post-treatment, *N. campestris* adults were placed on *Brachypodium. distachyon* plants and monitored under identical environmental conditions for mortality and fungal outgrowth verification as described for *P. spumarius*.

The results showed significant differences in mortality among treatments ($\chi^2 = 98.86$, df = 9, $p < 0.001$), indicating variability in fungal effectiveness (***Figure 16***). The isolates EAMa 01/58-Su, EAMb 09/01-Su, EAMa 09/01-Su, EAMa 10/01-Fil, and EAMa 01/158-Su were the most effective, each causing over 90 % mortality, with most cadavers showing visible fungal sporulation, confirming infection.

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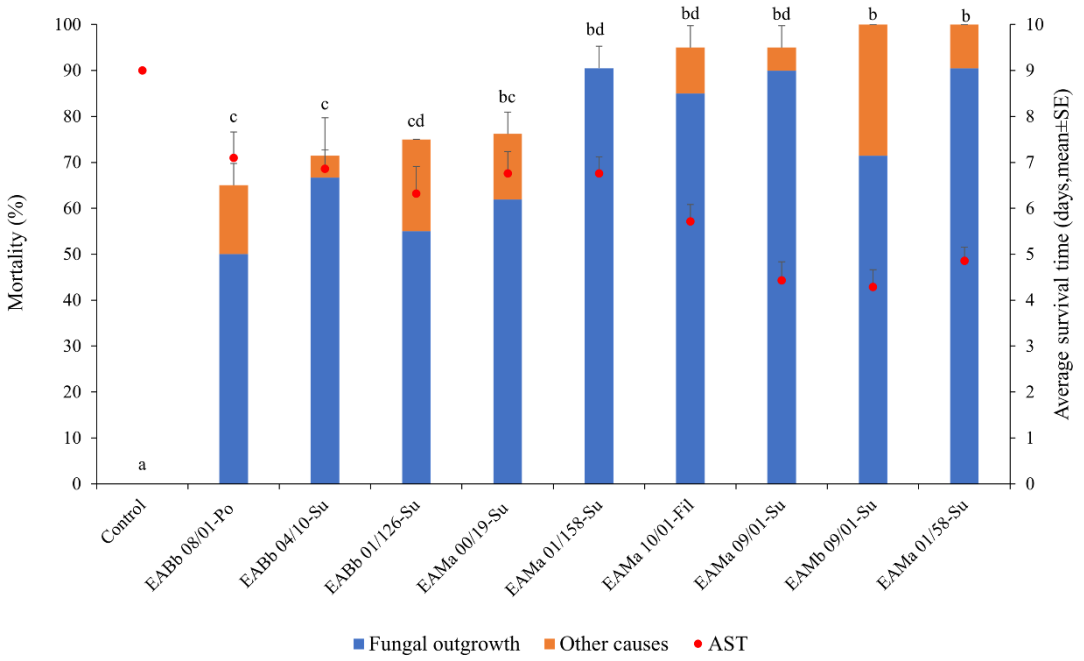


Figure 16. Mortality and fungal growth of *Neophilaenus campestris* adults treated with suspensions of 1.0×10^8 conidia mL^{-1} applied using an airbrush. The points represent the average survival time (AST), which was limited to 9 days. Data are presented as mean ± standard error.

Effect of EPF strains on *Philaenus spumarius* adults under laboratory conditions – Italy trials

Five out of 20 EPF strains were also tested on *P. spumarius* adults. For that, *V. faba* plant microcosms were prepared as follows: 5 plants for each EPF strain with 5 insects per plant. Insects were directly treated in a cage with 1×10^8 conidia/mL suspension and moved to the plants. *Beauveria bassiana* ATCC 74040 was used as positive control. Survival rate was measured at 5 and 11 days after the treatments.

Results showed that 3 strains belonging to the genus *Metarhizium* (*M. anisopliae* BP1q, *M. robertsii* BP1f, *M. robertsii* BP1k) were able to kill 75 % of *P. spumarius* adults (***Figure 17***). The strain *M. anisopliae* BP1q was chosen to be used under field conditions for *P. spumarius* nymphal control in Apulia.

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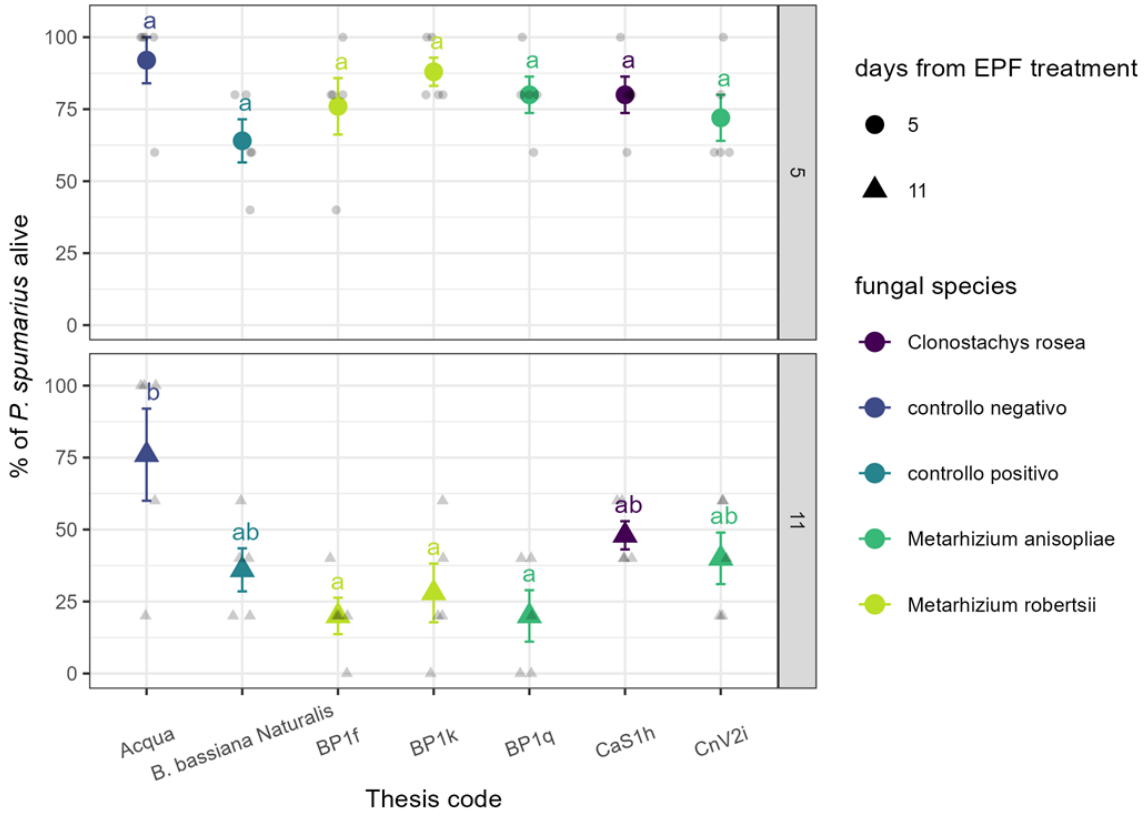


Figure 17. Survival rate (%) of *Philaenus spumarius* adults after 5 and 11 days from the treatments.

2.2.2.2. Field trials

Efficacy of *Metarhizium brunneum* EAMa 01/58-Su strain on naturally present adult population of *Neophilaenus campestris* in Spain

Adult specimens of *N. campestris* were captured using a 0.35 m diameter entomological sleeve. Each sample consisted of ten sweep nettings on the surface of the cover crop in each test block, with a total of three replicates per block. The captured adults were stored in a sealed plastic bag for later identification.

Additionally, all live insects captured with entomological nets during the trials underwent a selection process to unravel the possible impact of the fungal spray. *N. campestris* insects were visually identified and separated from other insects using 40× magnification binoculars. The selected insects were then superficially disinfected by immersing them in a 5 % sodium hypochlorite solution for 1 minute and then rinsed with sterile deionised water for 1 minute. Under sterile conditions, the insects were placed in humid chambers, which consisted of a moist filter paper disc placed inside a Petri dish with 1 ml of sterile deionised water. All insect plates were incubated at 25 ± 1 °C for 20 days. Subsequently, insects with fungal outgrowth on their surface were recorded.

Results showed that adult spittlebug population, in 2023, a reduction in the treated adult spittlebug population was detected over time, compared to the control. However, it was not statistically significant (b=−0.663, SE=0.372, p=0.078; ***Figure 18***a). Like foams, the average

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catch in the control plots at the end of the experiment was 6.08 adults, compared to 1.8 in the treated plots, representing a reduction of 71.2 %. In 2024, although a decrease in the adult population was observed in the treated plots compared to the controls, it was not statistically significant (b = −0.301, SE = 0.335, p = 0.368; ***Figure 18***b). The average catch in the control plots at the end of the experiment was 9.2, while in the treated plots it was 3.2, representing a reduction of 83.7 %. When comparing the decline in adults in both years, no significant effects of treatment or its interaction with date and year were detected (t = −1.030, p = 0.304), suggesting that there are no clear cumulative or interactive effects on adult populations.

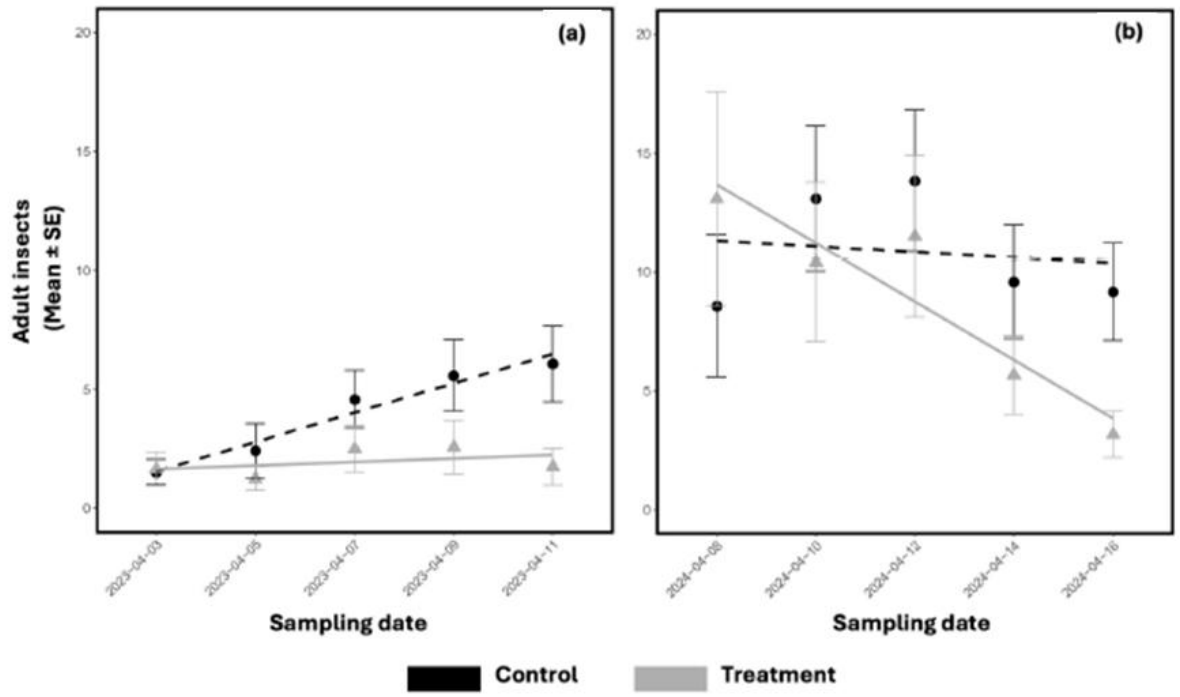


Figure 18. Evolution of the mean number ± SE of the adults captured in spring 2023 (A) and spring 2024 (B) during the test period after spraying with *Metarhizium brunneum* EAMa 01/58-Su strain. Lines represent the tendency of nymph and adult populations per block along the time of the sampling date. Black lines with circles represent controls and grey lines with triangles represent treatments. Fungi were applied in treatment plots on the first day of sampling in each year and treatment blocks.

The results of the relative density variation for *N. campestris* adults showed significant differences in both trials, 2023 and 2024 (H = 8.4397, P < 0.0017; H = 10.5082, P < 0.0012, respectively) (***Figure 19***).

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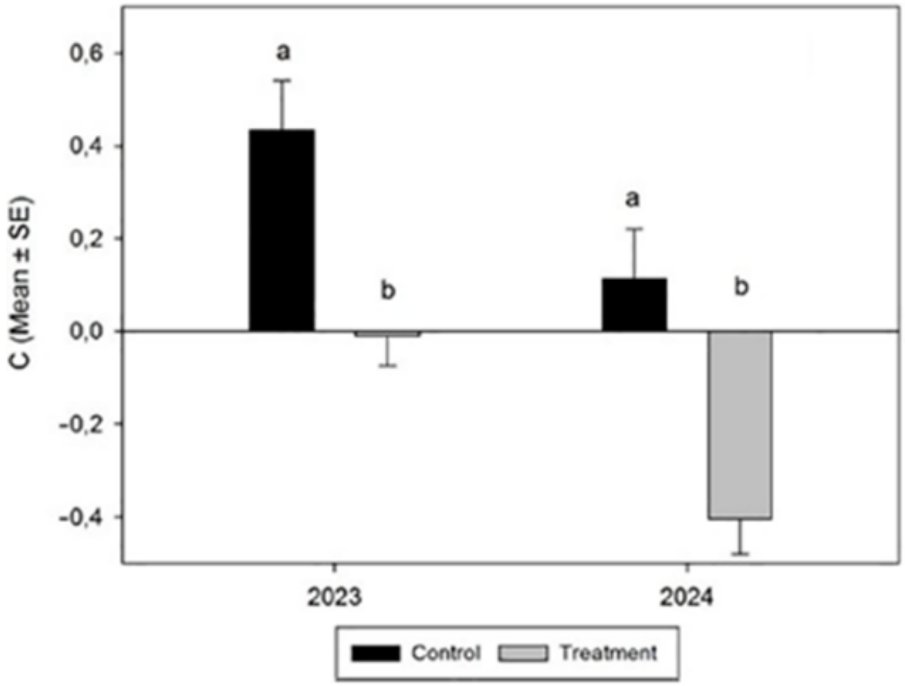


Figure 19. Variation in the relative population density (mean ± SE) of *Neophilaenus campestris* adult population counted in spring 2023 and spring 2024 during the trial period after spraying the cover crop with *Metarhizium brunneum* EAMa 01/58-Su strain. This indicator is calculated according to Peveling et al., where the viable population before and after the trial is considered. Negative values of C indicate a decrease in the trial population. Values not sharing the same letter are significantly different at $p \leq 0.05$ (nonparametric Kruskal-Wallis test; alpha=0.05).

Field persistence of *Metarhizium brunneum* EAMa 01/58-Su strain

Before fungal treatment, three entirely random soil samples were collected using a soil corer (5 cm in diameter) to a depth of 15 cm to determine the natural presence of entomopathogenic fungi in the soil. Following fungal treatment, three trees were randomly chosen from each of the subfields (twelve in total) to evaluate the presence of inoculum in the soil. Soil samples were collected immediately after the treatment and then monthly from beneath the canopy, following the same procedure as previously described, for a duration of four months post-treatment application. To quantify the conidial density in each sample, the count of colony-forming units (CFU) per gram of dry soil was calculated by employing Sabouraud Chloramphenicol Agar medium in petri dishes. One gram of the thoroughly mixed soil sample was combined with 9 ml of sterile distilled water and agitated using a rotary shaker (P-selecta shakers, Barcelona, Spain) for 60 minutes. After homogenization, three dilutions were created for each sample (1:10, 1:100, 1:1000), and 100-µl aliquots were evenly spread onto the medium. The plates were incubated at 25 °C for 10 days, and colony-forming units were counted.

Results showed that no native *Metarhizium* strains were present in the soil before soil application. After spring treatments with *M. brunneum* EAMa 01/58-Su, the fungus persisted for up to 3–4 months, with variable concentrations depending on the year. The highest values were recorded in April 2025 (1.98×10^6 conidia/g soil) and April 2023 (3.69×10^5), while in

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2024, concentrations declined rapidly and became undetectable by May (***Figure 20***). This limited persistence coincided with unusually high rainfall early that year, suggesting a negative effect of excess precipitation on fungal survival.

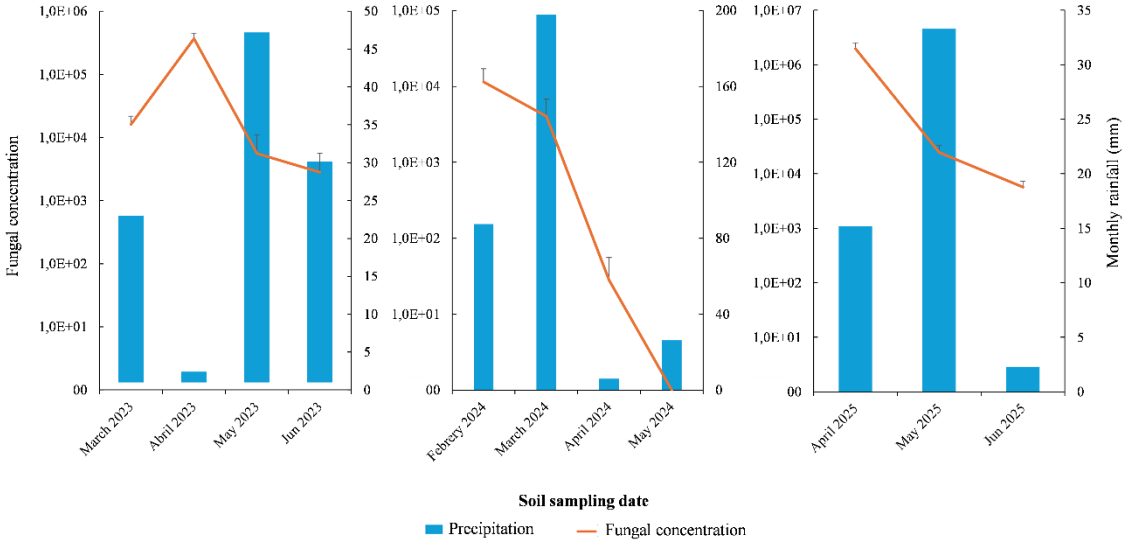


Figure 20. Time course of *Metarhizium brunneum* EAMa 01/58-Su strain concentration in soil after treatments (line) and monthly precipitation (bars) registered during the period 2022-2025.

2.3. Endophytic colonization potential of EPF

2.3.1. Spain trials

2.3.1.1. Endophytic colonization of adventitious plants by Metarhizium brunneum *EAMa 01/58-Su strain*

This study aimed to assess the potential colonization of the most common adventitious species found in olive groves. Initially, colonization percentage was evaluated in the plant species *Sinapis alba* (L.), *Lolium rigidum* (Gaud.) and *Brachypodium distachyon* (L.), using three inoculation methods (foliar spray, seed dressing, and soil drenching). Subsequently, the plant species and inoculation method combination that resulted in the highest colonization percentages were selected. Once the plant species and inoculation method were chosen, the effect of the time elapsed between treatment application and plant sowing in the treated soil on fungal colonization was evaluated.

2.3.1.2. Inoculation methods and isolation of Metarhizium brunneum *from the plant tissues of the three adventitious plants*

Inoculation with the fungal suspension of *M. brunneum* strain EAMa 01/58-Su was performed using three different methods: seed dressing, soil drenching, and foliar spraying. A total of 216 pots were assessed across two experimental replicates, with 12 pots per plant species assigned to each inoculation method (12 seed dressing, 12 soil drenching, and 12 foliar spraying, both fungus-treated and untreated), resulting in 72 pots per plant species. Control treatments without fungal inoculation were carried out in parallel with the inoculated ones.

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Fungal re-isolation was periodically assessed at 21, 28, 50, and 70 days after sowing (DAS). Three plants per species, inoculation method, and sampling date were collected, and plant tissues were divided into three parts: leaf, stem, and root. Each tissue type was placed in an individual Petri dish containing Glucose Sabouraud Chloramphenicol Agar (GSCA). Leaf samples were cut into ten fragments of approximately 1 cm^2, whereas stem and root tissues were sectioned into ten segments of about 1 cm in length. For *L. rigidum* and *B. distachyon*, only leaf and root segments were analyzed. All plant tissues were surface sterilized in 1 % NaClO for 1 minute and rinsed twice with sterile deionized water for 1 minute each. Subsequently, tissues were cut using sterile scalpels and scissors in a laminar flow chamber and placed in the prepared culture dishes. The dishes were incubated at 25 ± 2 °C for 10–15 days before evaluation. The effectiveness of surface sterilization was verified by plating 100 µl aliquots of the final rinse water on GSCA and incubating them at 25 °C for one week, yielding no fungal growth. Fungal colonization rates were determined by recording the number of plant tissue fragments showing fungal outgrowth.

Results showed that fungal colonization was significantly affected by plant species ($F_{2,9}$=3.09, P=0.0470) and DAS ($F_{3,9}$=3.36, P=0.0195). The colonization percentage ranged from 10.8±2.3 % in plant species *B. distachyon* to 6.0±1.6 % in species *S. alba*. Species *B. distachyon* and *L. rigidum* did not differ significantly from each other, with *L. rigidum* showing a colonization value of 9.3±2.1 % (***Figure 21***A). Regarding colonization percentages of the three plant species over time, a progressive decline was observed, decreasing from 14.3±2.9 % at 21 DAT to 5.2±2.3 % at 70 DAT (***Figure 21***B). Although no significant differences were detected among treatments ($F_{2,9}$=1.17, P=0.3119) or sampled tissues ($F_{2,9}$= 1.15, P=0.3198), colonization percentage was slightly higher in the soil drenching treatment in the plant species *L. rigidum* (***Figure 21***C). This treatment was therefore selected for subsequent experiments.

2.3.1.3. Effect of the pre-sowing interval on the Lolium rigidum colonization by Metarhizium brunneum

Following the results of the previous section, *L. rigidum* was selected as the sole plant species for further experiments. Soil treatment with *M. brunneum* was performed as described previously. The experiment included different intervals between soil treatment and seed sowing (***Figure 22***). In scenario A, sowing occurred on the same day as soil treatment, and roots and leaves were collected 21 days later. In scenario B, seeds were sown 30 days after soil treatment, and sampling was conducted 21 days post-sowing (51 days after treatment). In scenario C, sowing took 60 days after soil treatment, and samples were collected 21 days after sowing (81 days after treatment). Root and leaf fragments were surface disinfected as previously described. Fungal outgrowth of *M. brunneum* was checked after 10 days of incubation at 25 ± 2 °C.

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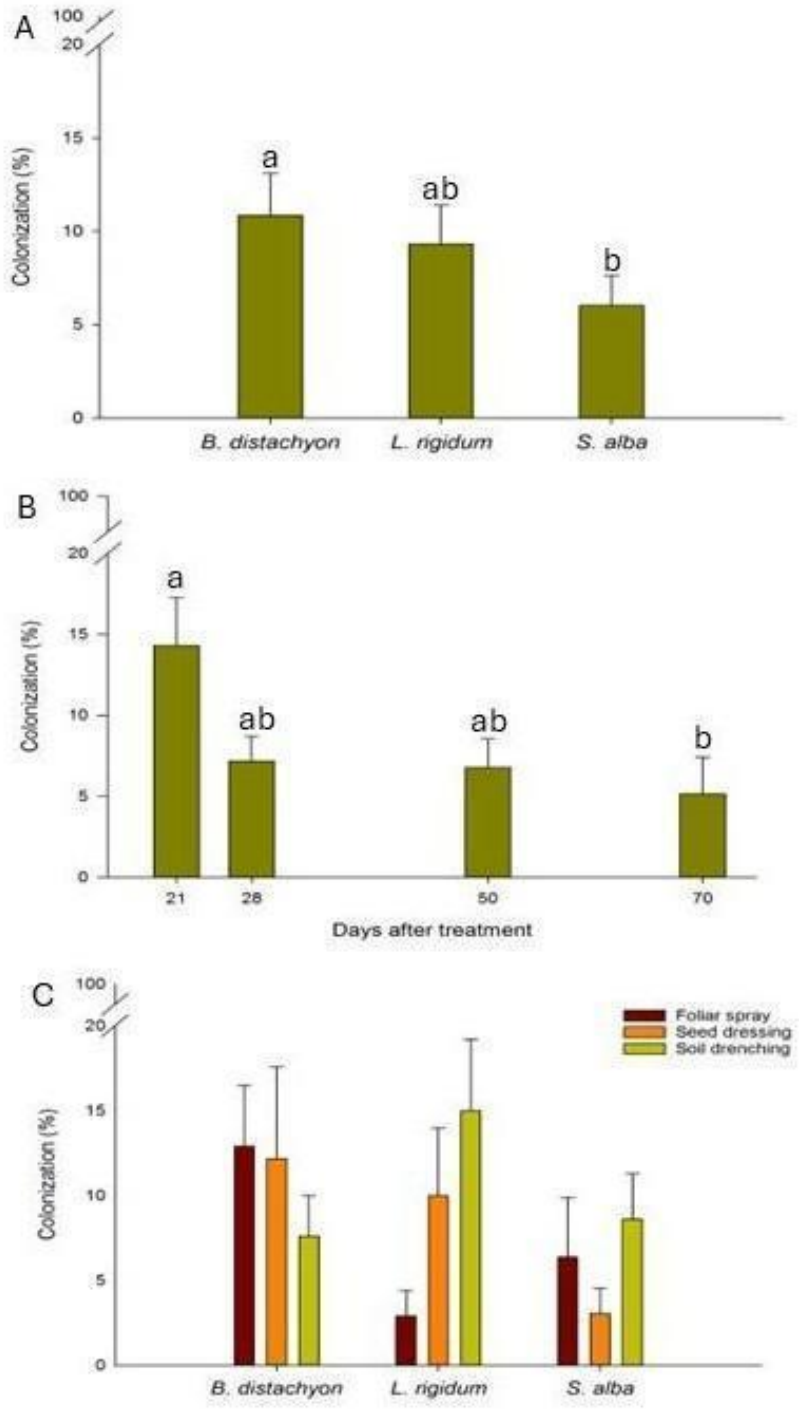


Figure 21. Colonization of adventitious plants by *M. brunneum* EAMa 01/58-Su strain. A) Total colonization (%) of the three plant species; B) Total colonization (%) of the three plant species at 21, 28, 50, and 70 days after sowing; C) Percentage of colonization of the three plant species according to the treatment type (foliar spray, seed dressing, or soil drenching). Different letters indicate significant differences ($p < 0.05$) according to Tukey's HSD test.

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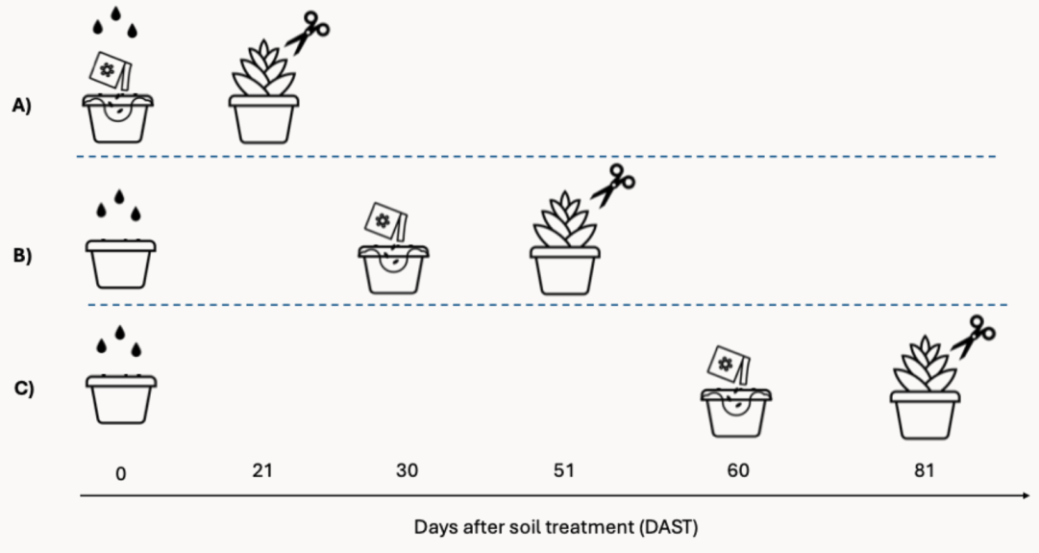


Figure 22. Scheme of treatments carried out in the assay. In scenario A, sowing was performed on the same day as the soil treatment with *Metarhizium brunneum* strain, and root and leaf fragments were collected 21 days after soil treatment. In scenario B, sowing occurred 30 days after soil treatment, and plant material was collected 21 days post-sowing (51 days after soil treatment). In scenario C, sowing took place 60 days after soil treatment, with plant material harvested 21 days after sowing (81 days after soil treatment).

Fungal biomass in plant tissues was quantified by qPCR. Genomic DNA was extracted from each tissue sample using the Clean-Easy™ PCR Purification Kit (Canvax Biotech, Córdoba, Spain), following the manufacturer's protocol. DNA concentration was measured with a Nanodrop 2000C spectrophotometer (Thermo Scientific, USA). Quantification of *M. brunneum* DNA used the SensiFAST™ SYBR & Fluorescein Kit (Meridian Bioscience) in 10 µl reactions containing 2× Mix, primers nrrf (5'-TCAGGCGATCTCGTGGTAAG-3') and nrrr (5'-GGGGGGTGTACTTGAGGAATGGG-3'), water, and 1 µl of plant DNA (Barelli et al. 2018). Thermal cycling comprised 95 °C for 3 min, then 39 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 10 min, followed by melting curve analysis (65–95 °C, 0.5 °C increments). Ct values and standard curves were generated using CFX Manager 1.1 (BioRad). The standard curve was built from five serial dilutions of *M. brunneum* genomic DNA (4.2 to 0.000042 ng), whereas 0.00003 ng of plant DNA was used as reference. Fungal load was expressed as the ratio of fungal to plant DNA (µg g^{-1}).

Additionally, 1 g of substrate was collected from the top 0–2 cm of soil at the begining of the experiment and at the end of each scenario. Each sample was mixed with 10 ml sterile deionized water and shaken at 12 rpm for 90 min. Aliquots (0.1 ml) from five serial dilutions were plated on SDAC with dodine and incubated at 25 ± 2 °C for 10–15 days. Colony-forming units (CFUs) were then counted after visual identification of *M. brunneum* colonies.

Results showed that, *M. brunneum* was not detected in the soil of the control pots. The fungus was detected in the pots throughout the entire experiment. The initial concentration on the day of treatment was $1.8x10^6$ CFU/g of soil, which decreased to $6.8x10^4$ CFU/g of soil by day 81 DAST (***Figure 23***Figure 21).

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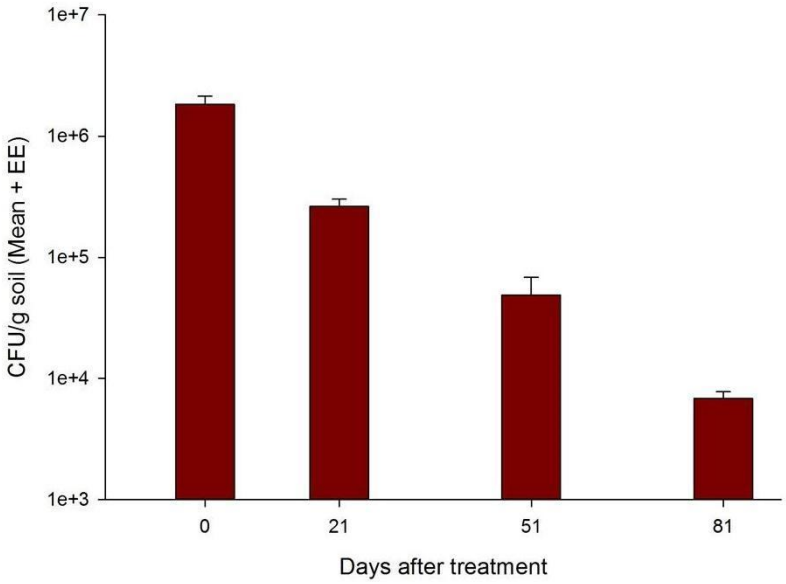


Figure 23. Persistence of *Metarhizium brunneum* EAMa 01/58-Su strain in the soil of pots where L. rigidum plants were sown at different intervals after fungal soil treatment. Day 21, 51 and 81 correspond to scenarios A, B and C, respectively.

Table 4. Presence *of Metarhizium brunneum* EAMa 01/58-Su strain in L. rigidum plants sown at different intervals after fungal soil treatment

Scenario[1]	Sample	Colonization (%)[2]	Amount of DNA (µg/g sample)[2]
A	Root	58.3 ± 11.2 b	4.4 ± 2.4 ab
	Leaf	0.0 ± 0.0 a	24.7 ± 1.9 c
B	Root	12.5 ± 4.2 a	0.4 ± 0.2 a
	Leaf	64.2 ± 5.1 b	5.9 ± 0.9 b
C	Root	14.2 ± 5.8 a	0.1 ± 0.0 a
	Leaf	15.0 ± 4.7 a	0.8 ± 0.2 a

** Colonization and DNA quantity in the control samples were zero.*
[1]A) sowing was performed on the same day as the soil treatment with M. brunneum strain, and root and leaf fragments were collected 21 days after soil treatment. B) sowing occurred 30 days after soil treatment, and plant material was collected 21 days post-sowing (51 days after soil treatment). C) sowing took place 60 days after soil treatment, with plant material harvested 21 days after sowing (81 days after soil treatment).
[2]Same letters in the same column per cultivar indicate that there were no significant differences in the test of Tukey HDS (P≤0.05).

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Statistical analysis revealed significant differences between treatments ($F_{1,12}$ =118.46, P<0.0001), days after soil treatment (DAST) ($F_{1,12}$=7.57, P=0.0012) and their interaction ($F_{2,12}$=7.56, P=0.0012). Besides, significant differences were found in the interaction DAST and tissue ($F_{2,12}$=39.96, P<0.0001) and the interaction between treatment, DAST and tissue ($F_{2,12}$=39.96, P<0.0001). In scenario A, where sowing occurred on the same day as the treatment application, the percentage of root colonization (58.3 %) was significantly higher than that of the leaf (0.0 %) at 21 DAST (Table 3). In scenario B, where sowing occurred 30 days after the fungal treatment, colonization values were reversed between sampled tissues, with significantly higher colonization in the leaf (64.2 %) compared to the root (12.5 %) at 51 DAST. Finally, in scenario C, where sowing occurred 60 days after the soil treatment, colonization percentages in root and leaf were similar, with values of 14.2 % and 15.0 %, respectively (***Table 4***).

2.3.2. Italy trials

2.3.2.1. Evaluation of Cichorium intybus endophytic colonisation by selected EPF strains

To assess the capability of six pathogenic EPF strains to colonize *Cichorium intybus* plant tissues and behave as endophytes, three *C. intybus* plants per strain were sprayed with a $1x10^8$ conidia/mL suspension in P6-UNITO laboratory.

Colonization of plant tissues was assessed, after leaf surface sterilization, on selective medium supplemented with antibiotics, at 3-, 7- and 14-days post treatment. *M. anisopliae* CnV2i, *Clonostachys rosea* Cas1h and *Purpureocillium lilacinum* CB2 were able to colonize effectively plant tissues, with more than 50% of the analysed leaves colonized 14 days after the treatment (***Figure 24***).

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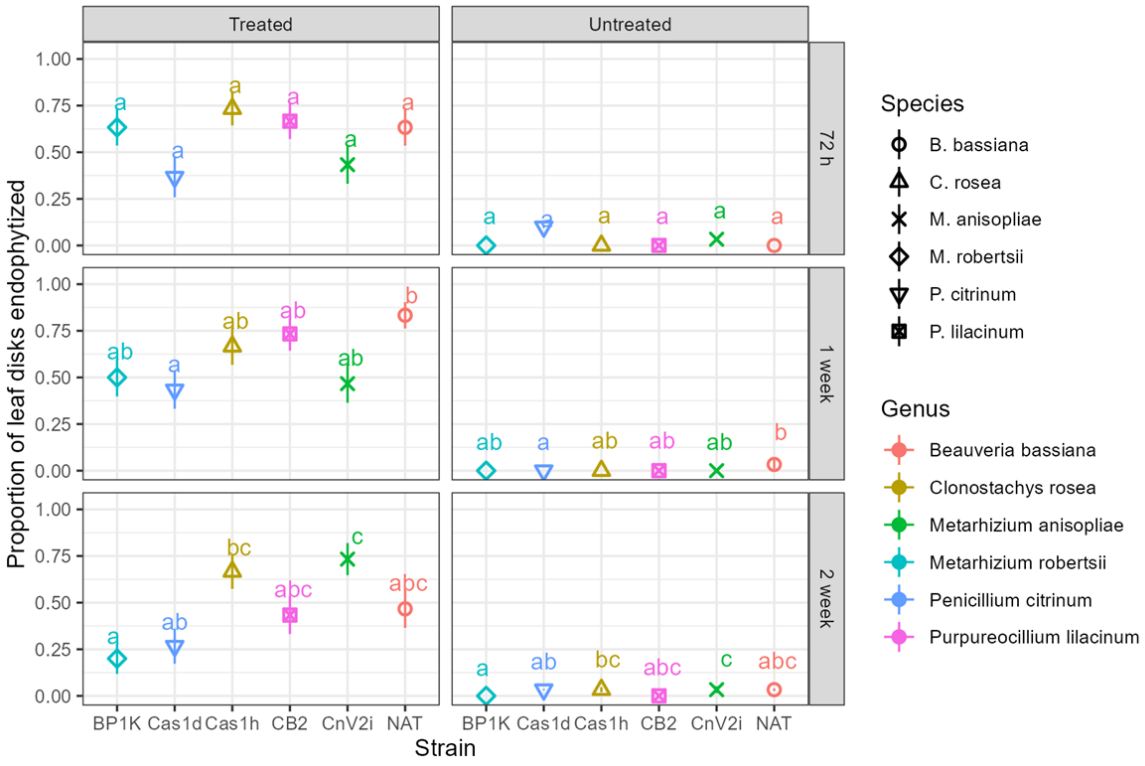


Figure 24. Endophytic colonization of treated and untreated leaves of Cichorium intybus by six EPF strains after 72 h, 1 and 2 weeks after the treatment.

2.4. Indirect effect of EPF on spittlebugs

2.4.1. *Neophilaenus campestris*

2.4.1.1. Effect of endophytic colonization of Lolium rigidum *plants by* Metarhizium brunneum *on the* Neophilaenus campestris *survival*

Insects of *N. campestris* were captured in the experimental olive orchard located at the Rabanales University Campus in Cordoba (37° 55' 14.05'' N, 4° 43' 16.31'' W). Due to limited insect availability, only soil treatment with *M. brunneum* was conducted as previously described. The bioassay was performed following the protocol detailed in the prior subheading.

Lolium rigidum plants treated with the fungus *M. brunneum* via soil drenching reached a colonization percentage of 36.7 % (***Figure 25***A). Significant differences in total mortality were found between the control and the soil drenching treatment [$\chi2(1) = 23.75$, $p<0.001$] with values of 13.3 % and 73.3 %, respectively (***Figure 25***B). The AST of *N. campestris* fed on endophytically colonized plants and fed with plant control was 5.9 and 6 days, respectively (***Figure 25***B). The soil drenching treatment caused a significant reduction of the survival ratio of *N. campestris* when compared to the control (***Figure 25***C).

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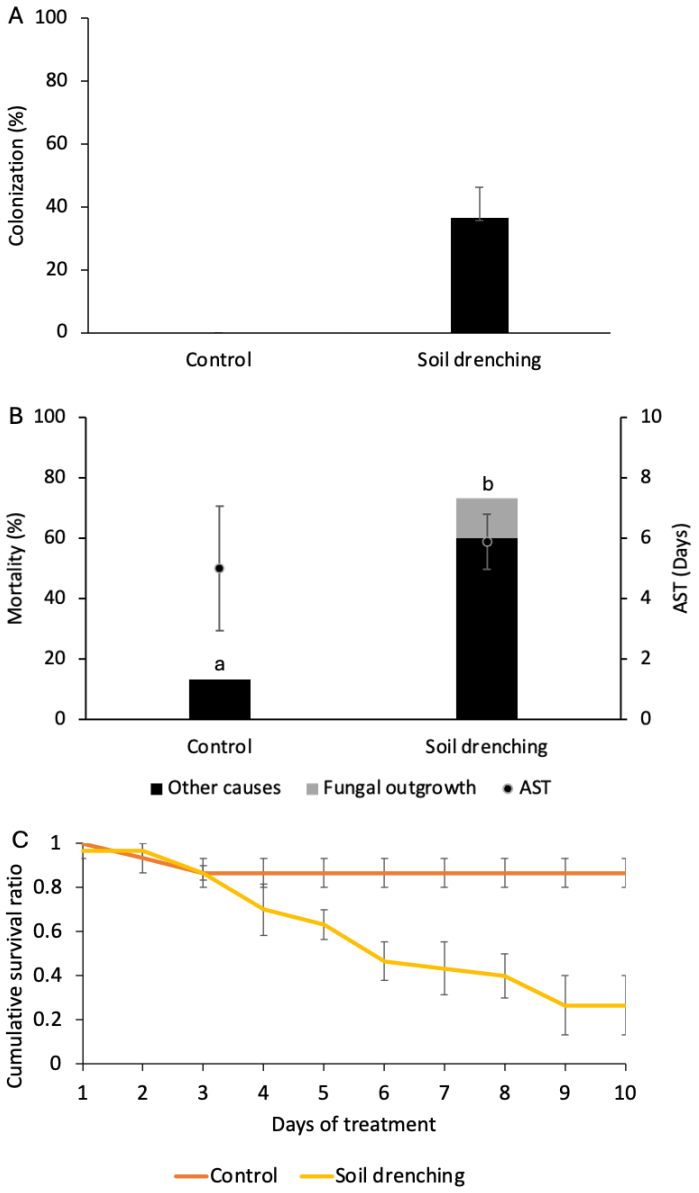


Figure 25. A) Colonization of *Lolium rigidum* plants by *Metarhizium brunneum* EAMa 01/58-Su strain after treatment with soil drenching. B) *N. campestris* adult mortality (%) with fungal outgrowth (black bars) and due to other causes (grey bars) of *N. campestris* fed on *M. brunneum*-colonized plants treated by soil drenching and fed on control plants. Different letters indicate significant differences ($p < 0.05$) according to the generalized linear model. The dots represent average survival time (AST), which were limited to 10 days. The data are presented as the mean ± standard error. C) Cumulative survival ratio (mean ± SE) of *N. campestris* adults fed on *M. brunneum*-colonized plants treated by soil drenching and fed on control plants.

2.4.1.2. Effect of endophytic colonization of Lolium rigidum *plants by* Metarhizium. guizhouense *on the* Neophilaenus campestris *behaviour*

Neophilaenus campestris adult host preference in response to *M. guizhouense* endophytic colonization was evaluated by a choice experiment conducted under semi-controlled conditions. Four sets of stiff ryegrass plants (*L. rigidum*) were used. Each set consisted of a

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mesh cage (90 x 60 x 60 cm) containing eight plants: four plants previously inoculated with *M. guizhouense* at 1×10^8 conidia/mL and four control plants (Figure 17). For the treatments, the entire aerial part of each 15-days-old *L. rigidum* plant was sprayed with 2 mL of the conidial suspension at 10^8 conidia mL^{-1} using an aerograph 27085 (piston compressor of 23 L/min, 15–50 PSI and a 0.3 mm nozzle diameter, Artesanía Latina S.A., Spain). Control plants were treated in the same way but sprayed with sterile water with 0.01 % Tween 80. After that, they were transferred to an environmental chamber for 48 hours at 25 ± 2 °C, a 16-h light: 8-h dark regime, and 70 % RH. Insect preference was assessed 48 hours after inoculation. Plants were placed into the mesh cages in a circular pattern, alternating *M. guizhouense* treated plants with control plants, at equal distances from each other (***Figure 26***). Into each cage, a 90 mm Petri dish containing twenty-four insects was introduced in the center of the cage to disperse simultaneously. *N. campestris* position was monitored at 1, 2, 3, 4, 24 and 48 hours after release by counting individuals placed on each plant. To avoid positional bias, the mesh cage will be turned over in the final measurement.

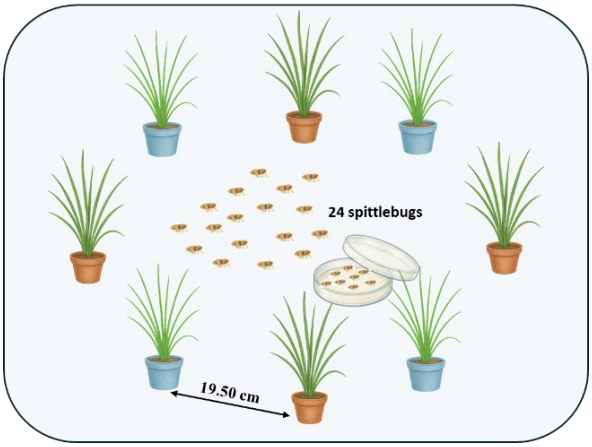


Figure 26. Design of the experimental arena used for *N. campestris* choice experiment under semi-controlled conditions. The set-up consisted of one large mesh cage (80 mm x 60 mm x 60 mm) with alternated four *M. guizhouense* foliar sprayed plants and four control plants. Brown pots represent *M. guizhouense* foliar sprayed plants, and blue pots represent control plants. One Petri plate (90 mm diameter) containing 24 spittlebugs were placed on the centre of the cage at a distance of 19.50 cm from each plant Insect position on treated/control plant or off the plant was recorded 1, 2, 3, 4, 24 and 48 hours after release after release.

Results showed that, plants used in the choice experiment, whose aerial part was sprayed with 2 mL of *M. guizhouense* fungal suspension of 10^8 conidia/mL, a percentage of 60.00 ± 4.08 % was observed in leaf tissues 48 hours after treatment. Results show a significant preference of *N. campestris* for *M. guizhouense*-endophytically colonized plants compared to control plants both at 1 and 2 hours after release ($\chi^2_1 = 10.97$; $p = 0.00$, $\chi^2_1 = 5.78$; $p = 0.01$, respectively). Then, it was observed that insects actively explored the experimental area, visiting both control and colonized plants without a clear preference. Therefore, as exposure time increased, insect preference became less pronounced, with individuals settling and feeding indistinctly on both control and endophytically colonized plants ($\chi^2_1 = 1.20$; $p = 0.27$, $\chi^2_1 = 0.75$; $p = 0.38$, and $\chi^2_1 = 0.15$; $p = 69$ at 3, 4, 24, and 48 h respectively) (***Figure 27***).

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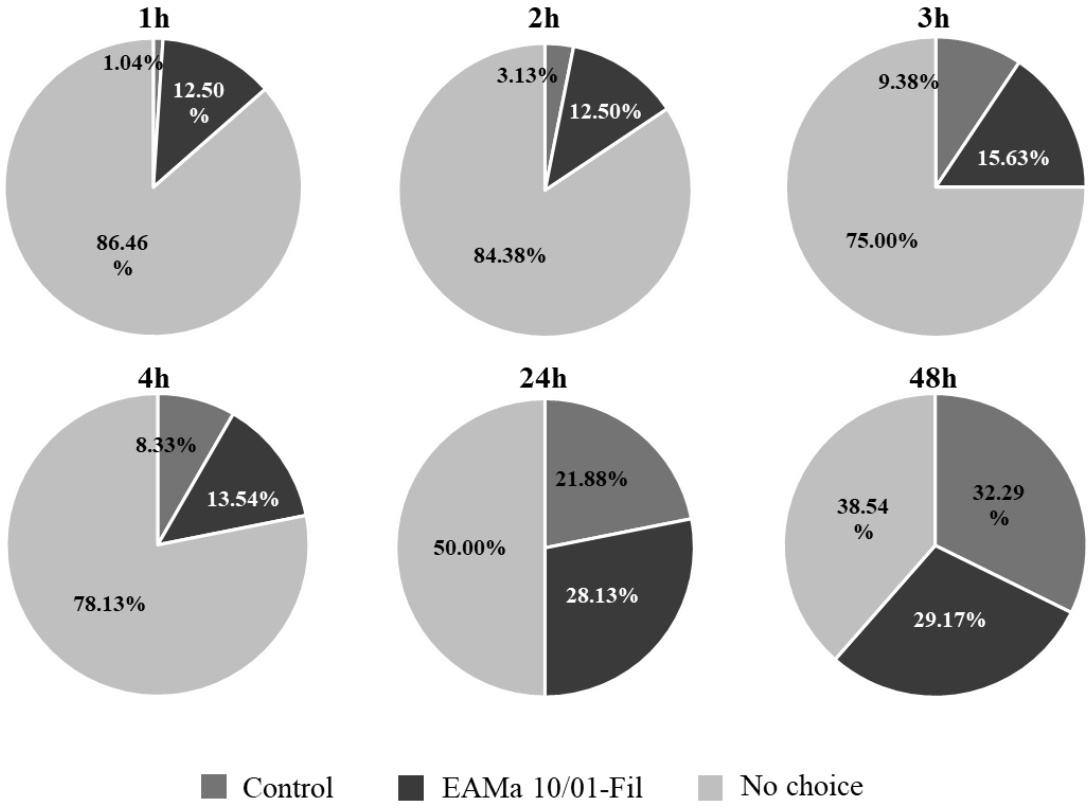


Figure 27. Percentage of *Neophilaenus campestris* adults (N=24) choosing *Metarhizium guizhouense* endophytically-colonized plants. Spittlebugs behaviour was observed once every hour for four hours, at 24 h, and the final establishment was recorded at 48 h. (Pearson correlation, $\chi^2_1 = 10.97$; p = 0.00, $\chi^2_1 = 5.78$; p = 0.01, $\chi^2_1 = 1.51$; p = 0.21, $\chi^2_1 = 1.20$; p = 0.27, $\chi^2_1 = 0.75$; p = 0.38, and $\chi^2_1 = 0.15$; p = 69 at 1, 2, 3, 4, 24, and 48 h respectively).

2.4.2. *Philaenus spumarius*

2.4.2.1. Effect of endophytic colonization of Cichorium intybus *plants by different EPF on the survival and oviposition efficiency of* Philaenus spumarius *adult females*

The strains *M. anisopliae* CnV2i and *P. lilacinum* CB2 selected during endophytic colonization trials described in page 35 were tested for their potential effect on oviposition of *P. spumarius* females. Thirteen endophytically colonized *C. intybus* plants were set up for each EPF strain and insects were exposed at 72 h post EEF treatment. *P. spumarius* female survival rate was measured weekly, and oviposition rate checked every 15 days for two months. A fair percentage of colonization was recorded (13.3 % after 14 days and 54.0 % after 30 days for *P. lilacinum* CB2, and 63.3 % after 14 days and 30.0 % after 30 days for *M. anisopliae* CnV2i), the oviposition timing was not delayed compared the negative control and the treatments did not affect the number of eggs laid by *P. spumarius* females (***Figure 28***). It is noteworthy that the strain *M. anisopliae* CnV2i decreased the longevity of adult females, probably due to a direct pathogenicity effect. The trial will be repeated next year with a modification of the experimental set up. In particular, the idea is to expose the *P. spumarius* females to

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endophytically colonized *C. intybus* plants early in the season, during oogenesis and not late immediately before the oviposition.

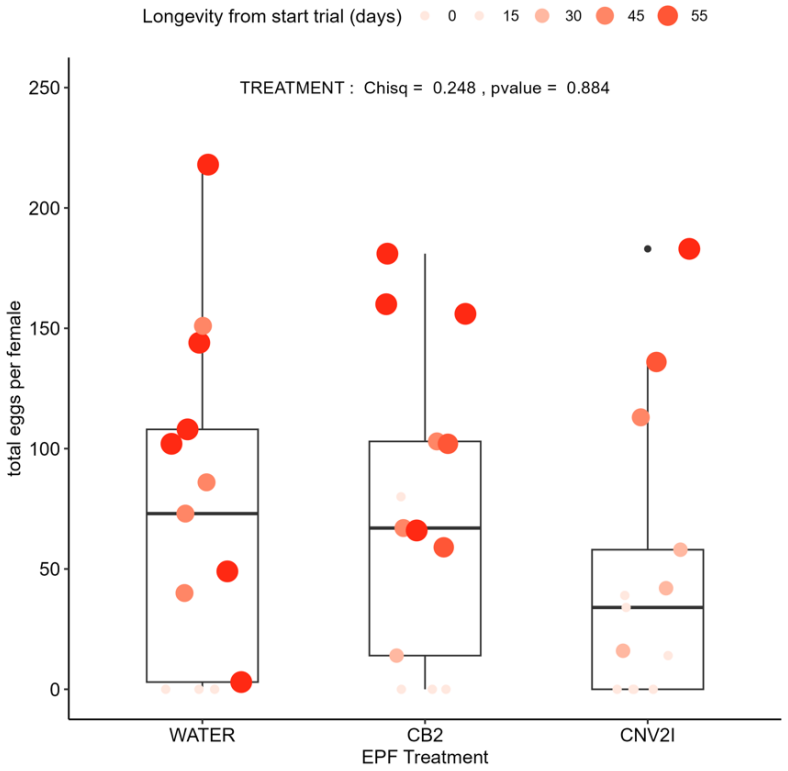


Figure 28. Effects of endophytic colonization of *Cichorium intybus* plants by two EPF strains on egg oviposition by *Philaenus spumarius* in controlled conditions.

2.5. Direct pathogenicity of EPF against the South American sharpshooter *Bucephalogonia xanthophis*

2.5.1. Materials and Methods

2.5.1.1. Plants, sharpshooters rearing, and inoculum of EPF

A healthy colony of the sharpshooter *Bucephalogonia xanthophis* obtained from adults collected on plants of *Lagerstroemia indica* L. and *Duranta erecta* L. in Piracicaba (Brazil) was maintained on *Vernonia condensata* Baker in a greenhouse, as described by Marucci et al. (2003). Plants of *V. condensata* were obtained from stem cuttings in pots with a commercial substrate made from pine bark, peat, and vermiculite (Tropstrato HT, Vida Verde, Mogi-Mirim, Brazil). During the experiments, adult sharspshooters (1-2 weeks old) were maintained on plants of *Ocimum basilicum* L. (basil) with a height of 20-30 cm, obtained from seeds in pots with Tropstrato HT.

The entomopathogenic isolates, *C. javanica* (ESALQ 1296) and *B. bassiana* (ESALQ 1296), were selected for the pathogenicity study, and both belong to the collection "Sérgio Batista Alves" at Laboratory of Pathology and Microbial Control of Insects, in the Department of Entomology

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and Acarology, located at "Luiz de Queiroz" College of Agriculture - University of São Paulo (ESALQ/USP), São Paulo, Piracicaba, Brazil. Fungal suspensions were prepared as described by Alves and Moraes (1998) and adjusted to a concentration of 2 x 10^7 conidia mL^{-1} in distilled water with 0.02% Silwet®. Only suspensions with germination rate above 90% were used in the assays (Oliveira et al., 2015).

2.5.1.2. Pathogenicity trial

To evaluate the pathogenicity of EPF strains against the sharpshooter *B. xanthophis*, an experiment was conducted on sharpshooter adults caged on basil plants by foliar application of the following treatments: 1) *B. bassiana* (ESALQ 1432); 2) C. *javanica* (ESALQ 1296); and 3) control (distilled water + 0.02% Silwet®). The experiment followed a completely randomized design, with six replicates per treatment and the experimental unit was comprised of 10 sharpshooter adults (1-2 weeks old) per plant, confined in a clear acetate tube cage. Two milliliters of the fungal suspension were sprayed per plant with an air compressor and an airbrush (Vonder®), until total coverage of both adaxial and abaxial leaf surfaces and stems. Between applications, the airbrush was sanitized with 70% alcohol and distilled water. The experiment was conducted in a climate-controlled room at 25 ± 3°C, 70% RH, and a 12:12 h L:D cycle. The mortality rates were assessed for 12 days after the treatments, and the dead insects were transferred to a humid chamber to confirm fungal infection by sporulation.

For survival analysis, a survival model was fit to the data using the 'survfit' function from the 'survival' package, and the graphs were visualized using Kaplan-Meier survival curves (Therneu 2021; Kalbfleisch & Prentice, 2011). The statistical analysis of mortality was performed using a generalized linear model with a binomial distribution. When the data did not fit the binomial model, a quasibinomial model was adjusted. To assess the adequacy of the model, the heteroscedasticity of normalized weight-residuals was verified. The analyses were performed using the R statistical program (version 4.4.1).

To assess the evolution of fungal colonization in *B. xanthophis*, four insects per treatment were analyzed at 9, 18, 24, 30, 48, and 72 h post-inoculation. The insects were then fixed in paraformaldehyde for 24 h and placed on stubs for gold metallization (Baltec SCD050 metalizer) for 200 s, followed by visualization and image digitalization in a scanning electron microscope (SEM) (JEOL JSM IT300 LV, Tokyo, Japan) at 20 kV.

2.5.2. Results

Both isolates of *B. bassiana* (ESALQ 1432) and *C. javanica* (ESALQ 1296) showed pathogenicity to the sharpshooter *B. xanthophis*, causing average mortalities of 48 and 43%, respectively, which were not statistically different from each other, but were higher than that observed for the control (distilled water + 0.02% Silwet) (12%) ($F_{2,28} = 7.27$, $P < 0.05$) (***Figure 29***). Based on the sharpshooter survival analysis, the median time after application required for 50% mortality to occur was estimated at 12 days for the *B. bassiana* isolate ESALQ 1432. The other fungus treatment (*C. javanica* isolate ESALQ 1296) did not reach the median survival within the observation period (***Figure 30***).

The analysis of dead insects placed in the humid chamber confirmed infection and sporulation by *B. bassiana* and *C. javanica*, whereas the scanning electron microscopy analysis allowed for the detailed examination of the external surface of the sharpshooter adults, revealing

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distinct phases of fungal colonization on the head, thorax, and abdomen. Up to 24 h after application, only adhesion of fungal conidia to the insect cuticle was observed (***Figure 31***). The formation of germ tubes from the conidia were visible at 30 h after application, marking the start of the germination process for *B. bassiana* and *C. javanica* treatments (***Figure 31***). Hyphae formation and penetration on different parts of the insect body (e.g. head, thorax and intersegmental areas of the abdomen) was evident after 72 h (***Figure 32***). Even in the most sclerotized regions, such as the legs, fungus germination and colonization were observed. Conidial extrusion occurred after 5 days from the beginning of colonization.

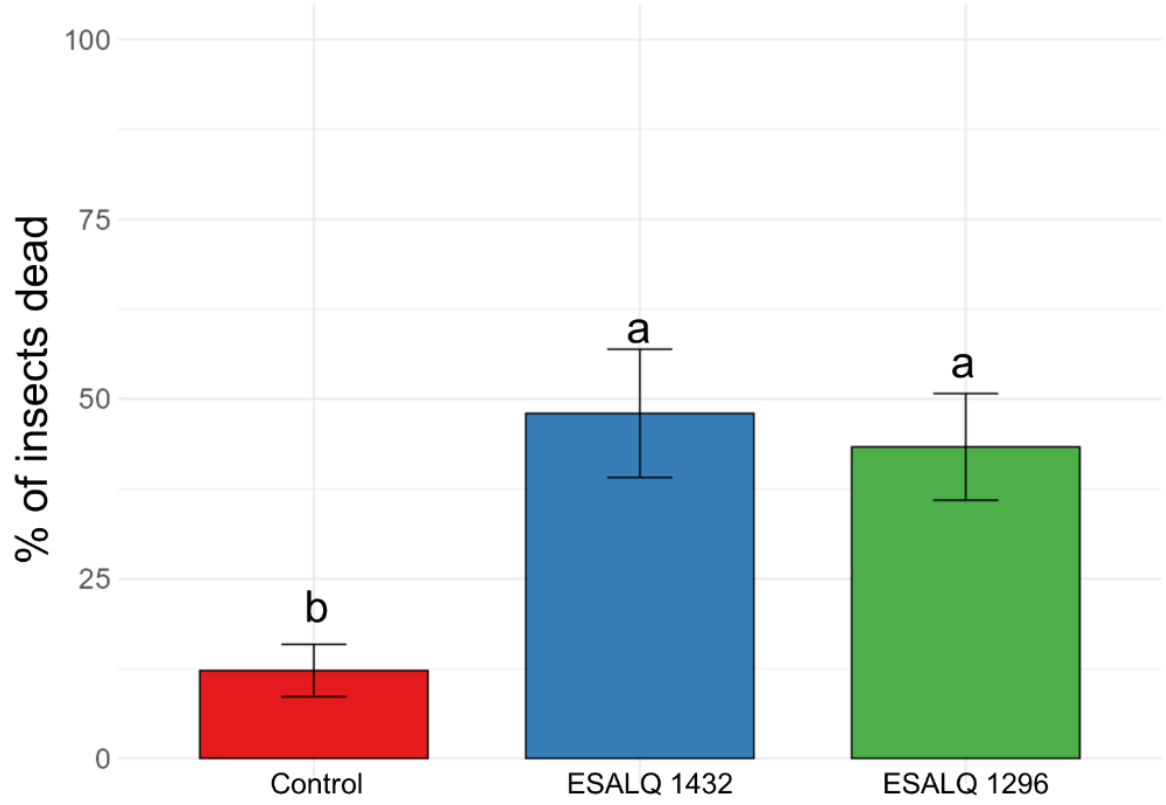


Figure 29. Mortality of *Bucephalogonia xanthophis* from Control (distilled water + 0.02% Silwet®), *Beauveria bassiana* (ESALQ 1432), and *Cordyceps javanica* (ESALQ 1296) treatments. Bars with different letters indicate statistical differences among the treatments by one-way ANOVA, Tukey's honestly significant difference (HSD) test ($\alpha < 0.05$).

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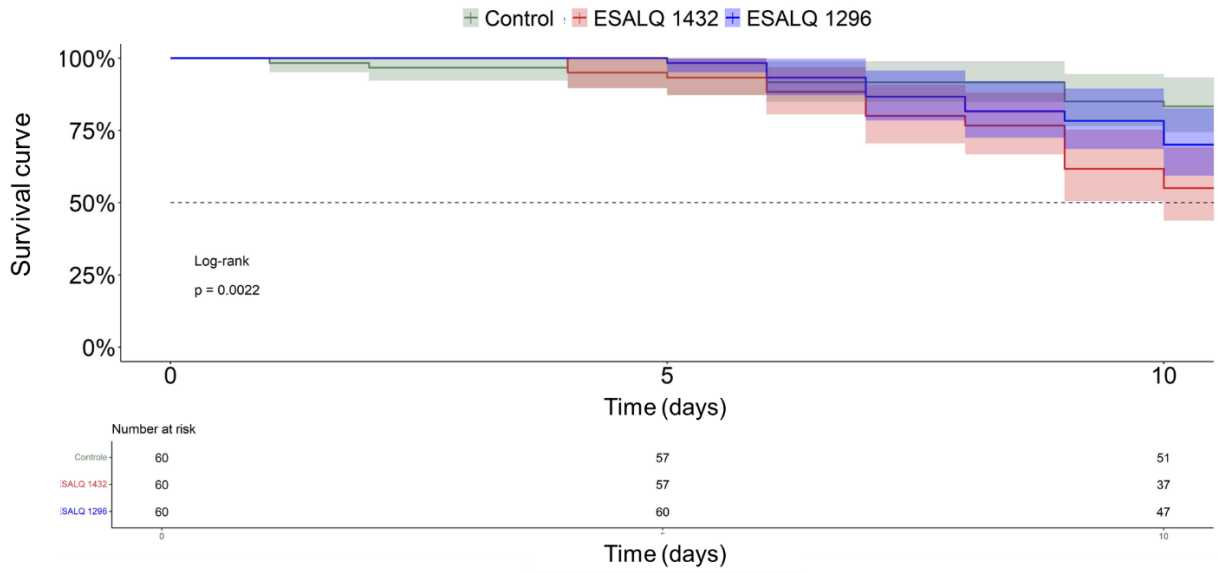


Figure 30. Survival curves graph of *Bucephalogonia xanthophis* during ten days after spray application of the treatments *Beauveria bassiana* (ESALQ 1432; red), and *Cordyceps javanica* (ESALQ 1296; blue), and control (distilled water with 0.02% Silwet®; green). The dashed line indicates the time point at which 50% mortality was reached in the *B. bassiana* treatment.

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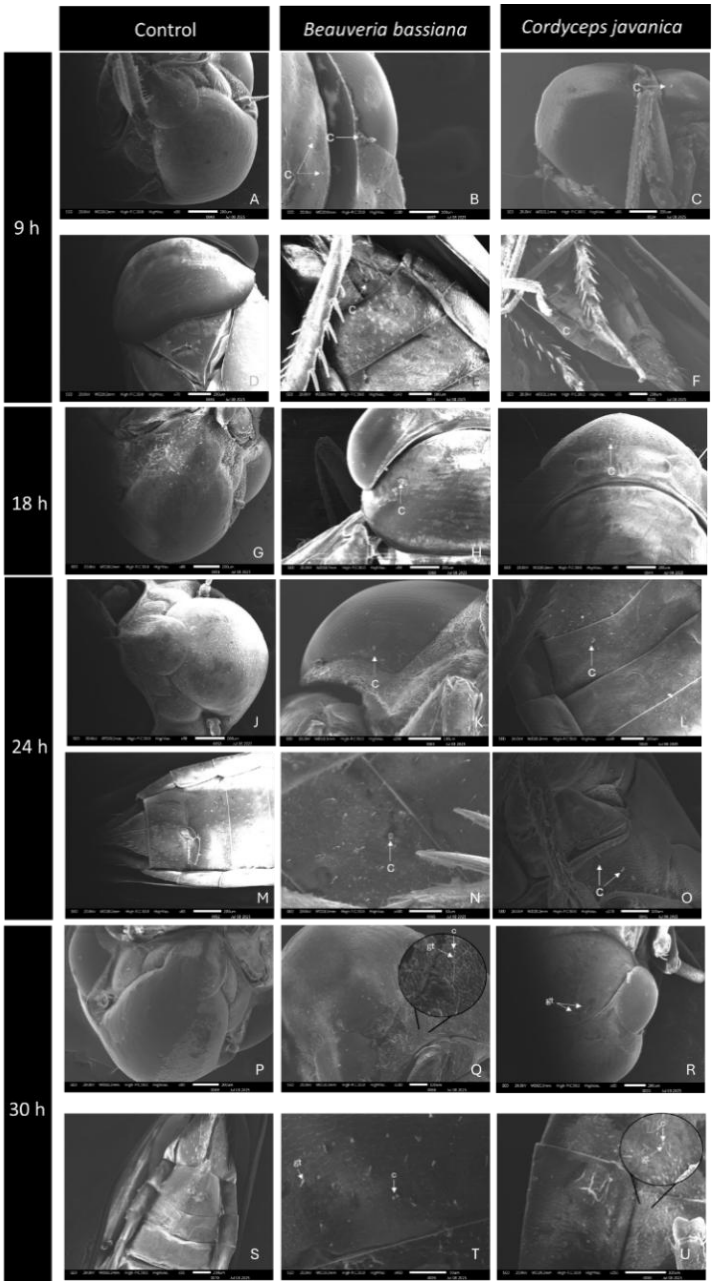


Figure 31. Evolution of colonization of the sharpshooter *Bucephalogonia xanthophis* by *Beauveria bassiana* (strain ESALQ 1432) and *Cordyceps javanica* (strain ESALQ 1296), compared to the control (distilled water with 0.02% Silwet®) at 9, 18, 24 and 30 h after foliar application. Nine hours after colonization were observed :(A) Ventral view of the head in the control treatment showing non-germination conidia; (B e C) Dorsal view of the head in *B. bassiana* and *C. javanica* treatments, respectively, showing no germination; (D) Dorsal view of head and thorax in the control treatment with absence of fungal conidia; (E and F) Ventral abdominal view showing non-germinated conidia for *B. bassiana* and C*. javanica*, respectively. Eighteen hours after inoculation were observed the following images: (G) Absence of conidia in the control treatment; (H and I) Head dorsal view with non-germinated conidia in both treatments. After 24 hours from the inoculation: (J) Absence of conidia was observed in the control treatment; (K – L) Presence of non-germinated conidia in *B. bassiana* and *C. javanica*; (M) Absence of conidia fungi on the control treatment; (N-O) Ventral abdominal view with non-conidia for *B. bassiana* and *C. javanica*. After 30 days from the inoculation: (P and S) Absence of conidia in control treatment; (Q and R) Conidia germination and tube germinative formation was observed in the insect on both treatments, *B. bassiana* and *C. javanica*; (T and U) Conidia germination occurrence on the abdominal ventral view in *B. bassiana* and *C. javanica*, respectively. (c) conidia and tg (tube germination).

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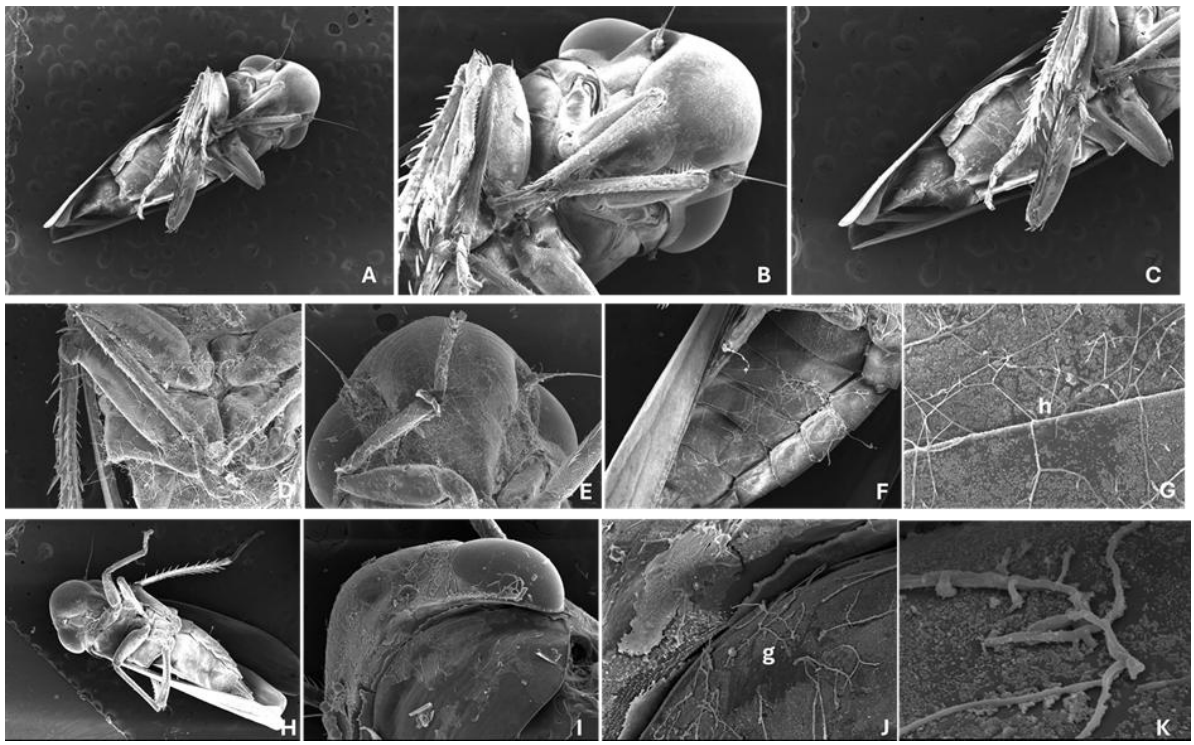


Figure 32. Images of *Bucephalogonia xanthophis* adults after 72 h of application. A: Ventral view of the insect in the control treatment. (B and C): Ventral view of the control treatment showing the head and abdomen, respectively. (D, E, F): Ventral view of the insect in the *Beauveria bassiana* treatment; thorax and legs showing the onset of sporulation, head, and abdomen. (G) Division of the abdominal segment with entry of the fungal hyphae. (H) Ventral view of the insect showing the early sporulation of *Cordyceps javanica.* (I) Dorsal view of the thorax with the presence of hyphae. (J) Germination of the entomopathogenic fungus. (K) Penetration of the fungus into the insect's cuticle. g: Germination; h: Hypha.

2.6. Conclusions

Entomopathogenic fungi (EPF) are a promising option for managing *X. fastidiosa* vectors like *P. spumarius* and *N. campestris*. Across extensive laboratory and field evaluations, several EPF species and strains — primarily *B. bassiana*, *M. brunneum*, *M. anisopliae*, *M. guizhouense* and *L. aphanocladii* — exhibited strong pathogenicity against both nymphs and adults of the vector species. Notably, *M. brunneum* EAMa 01/58-Su and *M. anisopliae* BP1q consistently achieved high mortality rates and reduced survival times in separate trials in both controlled and field conditions in Spain and Italy, supporting their use in crops. Selected EPF strains could effectively complement other control measures such as mowing, which remains the most effective physical control for spittlebug nymph populations.

Several EPF strains also showed endophytic and rhizosphere abilities in key olive agroecosystem plants. For instance, *M. brunneum* efficiently colonized *Lolium rigidum* and other herbaceous hosts, with success depending on plant species, tissue, and timing of soil inoculation. Endophytic colonization increased adult *N. campestris* mortality and possibly influenced host preference, suggesting indirect ways EPF may impact vector fitness and behaviour. Studies in Italy found EPF strains – *M. anisopliae* CnV2i and *P. lilacinum* CB2 – colonize *C. intybus* leaves and possibly affecting longevity of *P. spumarius* females feeding on

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them, although oviposition rates were unaffected. Complementary assays conducted in Brazil confirmed that *B. bassiana* and *C. javanica* are pathogenic to the sharpshooter *B. xanthophis*, a key *X. fastidiosa* vector in South America, demonstrating the broader applicability of EPF-based strategies across pathosystems.

Overall, the results confirm that EPF represent an effective option for direct biocontrol of nymphs and adults; moreover, results suggest that they also have indirect plant-mediated impacts – by endophytic or rhizosphere associations – on survival, fitness and behaviour of adult spittlebugs. Taken together, these findings provide a strong scientific foundation for integrating EPF into sustainable, ecologically based IPM strategies targeting *X. fastidiosa* vectors, while also identifying key strains for further optimization, formulation development, and large-scale field validation.

3. EVALUATION OF THE PREVALENCE AND EFFICACY OF PARASITOIDS ASSOCIATED WITH *PHILAENUS SPUMARIUS*

3.1. Background

Biological control approaches exploiting natural enemies offer interesting sustainable promise in managing populations of *P. spumarius*, possibly adding up to current strategies relying on mechanical (tillage) and chemical (insecticide) interventions. Several arthropods are known to feed on spittlebugs, although the majority are generalist predators, with a low potential efficiency in suppressing the insect vector populations (Benhadi-Marín et al. 2020; Lahbib et al. 2022). Current knowledge of natural enemies that are instead specifically associated with spittlebugs—and that may hold potential as biological control agents—remains limited (Mesmin et al., 2020; Molinatto et al., 2020). Among the potential biological agents are egg parasitoids, which specifically target the egg stage of the vector's life cycle. By parasitising and killing the eggs, these natural enemies prevent the emergence of adult vectors before they become epidemiologically significant in the transmission of *X. fastidiosa*. The egg parasitoid *Ooctonus vulgatus* Haliday (Hymenoptera: Mymaridae) has been recently found associated to *P. spumarius* eggs in Corsica, with parasitism rates up to 69% (Mesmin et al., 2020), highlighting its potential effectiveness as a biocontrol agent of the spittlebug populations. This study aimed to assess the presence and prevalence of *O. vulgatus* in different Regions of Italy. In addition, the study investigated some key biological traits of this egg parasitoid, with a view to better understanding its potential role in sustainable management programmes of targeting *P. spumarius* populations.

3.2. Presence and prevalence of egg parasitoids associated to *Philaenus spumarius* in Northwestern Italy

3.2.1. Materials and Methods

The presence and prevalence of egg parasitoids in the field were estimated using sentinel eggs in ten sites across two Northwestern Regions of Italy: Liguria and Piedmont. Some preliminary work was carried out during the 2022/23 autumn/winter season in Northwestern

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Italy to test different methodologies of exposition of sentinel eggs, whose results are reported together with those obtained during the Project's period. However, considering the poorly understood biology of *O. vulgatus*, and no previously reported or tested methods to systematically collect the parasitoid, the sentinel eggs setups, the exposure periods, and the sampling sites were adjusted each year based on the most successful results from the previous season, in order to maximize the likelihood of catching the egg parasitoid.

Sentinel eggs preparation

Sentinel eggs to be exposed for parasitisation in the field were obtained by oviposition of *P. spumarius* females using three different protocols: (i) oviposition in mass rearing cages, (ii) oviposition following a parapause-breaking protocol (Morente et al., 2021), and (iii) oviposition directly in experimental sites in field conditions.

Mass rearings of the spittlebug (i) were setup each year using *P. spumarius* adults collected in fallows in July–September. Insects were placed and reared with a female-biased sex ratio (2♀:1♂) in mesh/plastic cages (BugDorms-2S400) with potted host plants (*Vicia faba*, *Vitis vinifera*, *Corylus avellana*, *Picea abies*), and barley straw was provided for oviposition. Straw was collected in mid-October (≈30 days of exposition in mass rearing), *P. spumarius* eggs counted under stereomicroscope, divided into 50-egg groups placed in Petri dishes (⌀ 10 cm) with moist filter paper, then stored in a climate chamber (5±1 °C; 14:10 L:D) until field placement. The parapause-breaking protocol (ii) (Morente et al., 2020, 2021) enabled earlier sentinel egg exposure by treating newly emerged adults in April, which produced eggs by late June; these eggs were collected and stored as described above for field use. For field oviposition (iii), *P. spumarius* adults were isolated in cages for oviposition directly at experimental sites, as described below.

Sentinel eggs exposure (Sites, setups and timing)

The surveyed sites were selected in different agroecosystems (olive grove, vineyard, fallow), in localities where *P. spumarius* density was known to be high. The presence of egg parasitoids was monitored from September to January using sentinel eggs for two autumn/winter seasons (2023/24 and 2024/25). A preliminary experimental trial was conducted during 2022/23 autumn/winter.

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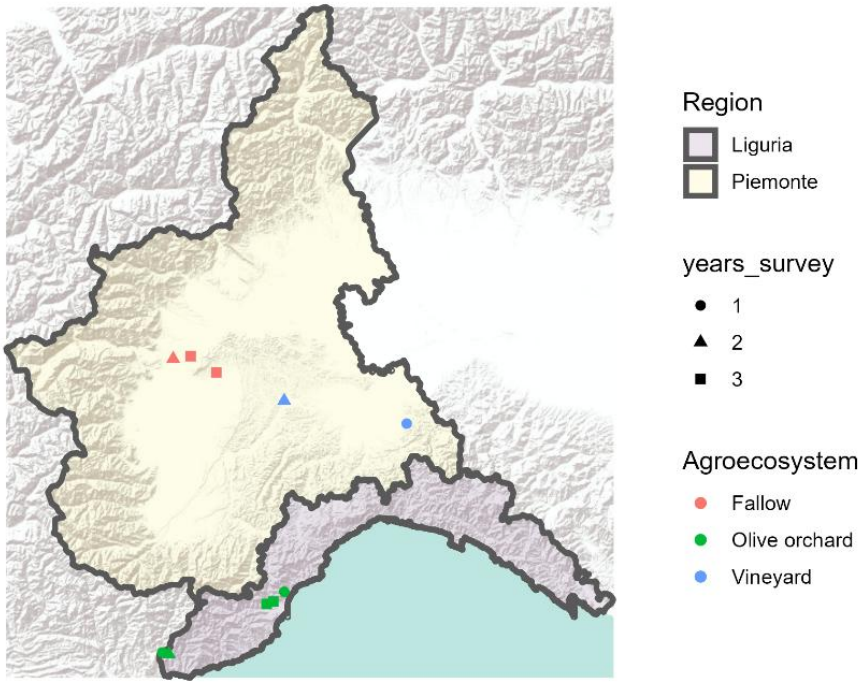


Figure 33. Map of the surveyed sites for egg parasitoids in Northwestern Italy.

Three different exposition setups were used to place sentinel eggs in the field and monitor the presence and prevalence of egg parasitoids: (a) cylinder (SE_C), (b) tent (SE_T), and (c) sentinel rearing (SE_R). The first two setups allowed the exposition of eggs obtained by laboratory mass rearings (i and ii), while the latter allowed the oviposition directly in the field (iii). SE_C was a cylindrical net isolator (ø = 15 cm, height = 40 cm, net width = 5 mm) containing a pot (ø =12 cm) with straw containing approximately 50–100 eggs placed on wet filter paper over a mixture of moist peat and perlite. SE_T consisted of a plastic food tray (18x10x4 cm) straw containing approximately 50 eggs placed on wet filter paper over a mixture of moist peat and perlite and covered with a polycarbonate sheet as a roof to protect the eggs from the rain. SE_R setup was used to expose freshly oviposited eggs in the experimental sites. 15 adults (10♀:5♂) of *P. spumarius* were enclosed in a cylindrical nylon cages (ø = 45 cm, height = 50 cm, (Di Serio et al. 2019)), together with four single-potted host plants (*Vicia faba*, *Cichorium intybus*, *Olea europaea*, *Picea abies*), and straw as oviposition substrate. At the bottom of the SE_R was present a layer of potting soil (height = 10 cm) to allow root development of plants outside their pots, ensuring a longer life span of host plants. Three replicas of SE_C and SE_T, and two SE_R replicas per site per year were exposed. Eggs in SE_C and SE_T setups were replaced in December, allowing to have data on potential flight period of egg parasitoids during two exposure periods: November to mid-December and mid-December to January. Additionally, eggs obtained from *P. spumarius* females induced to oviposit through the parapause-breaking protocol were exposed in one tent and one cylinder per site during the second year only, covering the period from September to November, when *P. spumarius* eggs naturally occur in the field. Eggs directly laid in the field by encaged insects (SE_R setup) were exposed from September to December.

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Figure 34. Different exposure setups used during egg parasitoid surveys in Northwestern Italy: a) Cylinder (SE_C), b) Tent (SE_T), c) Rearing (SE_R).

At the end of each exposure period, sentinel eggs were retrieved, counted under a stereomicroscope (6×-45×). Counted eggs were then transferred to Petri dishes (ø 9 cm) with wet filter paper and honey drops, and maintained in cold chamber (6 °C, 8:16 L:D). To assess parasitisation rate, Petri dishes with sentinel eggs were exposed to spring conditions in climatic chamber (18±2 °C; photoperiod 14:10 L:D) and checked every two days for newly emerged wasps. Upon emergence, the number of parasitoids was recorded, individuals were sexed and removed from the Petri dish.

Identification of parasitoid specimens

Emerged adult parasitoids were morphologically identified using the keys of Triapitsyn (2010) using a stereomicroscope (Leitz, 150–200x). Three specimens per locality and year were identified using COI sequences through PCR-based molecular techniques (Mesmin et al. 2020).

3.2.2. Results

Sentinel eggs exposed in field conditions in different agroecosystems in Northwestern Italy allowed to obtain a several hundreds of egg parasitoid over a three-year survey (Table 1). All the emerging parasitoids were identified as *O. vulgatus* Haliday (Hymenoptera: Mymaridae) using both morphological and molecular identification. Different exposition methods (i.e. Setups) were tested, with mass rearings of *P. spumarius* adults left directly in the field for oviposition (SE_R) being the most parasitised, with about 650 adult parasitoids emerging from this setup type. The sex ratio was always quite strongly female-biased (> 2:1 ♀:♂), and the preimaginal development time – once eggs were exposed to spring climatic conditions (18±2 °C, 14:10 L:D) – was very variable (24.8±10.9 days). Most of the adult parasitoids (≈93 %) were found from eggs exposed in autumn (late September – mid December), compared to those exposed in winter period (late December – January). Parasitoids were found from sentinel eggs exposed both in olive groves, vineyards and fallows, although with highly variable numbers and distribution.

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Table 5. Summary of sentinel egg exposed, retrieved and egg parasitoids emerged during the three-year survey in Northwestern Italy.

Year	No. eggs exposed	No. eggs retrieved	No. parasitoid emerged				Sex ratio
			Cylinder	Tent	Rearing	Tot	
2022	1500	800	19	57	17	92	4.3:1
2023	-	8046	6	2	627	635	3:1
2024	-	2039	-	-	25	25	2:1

More in detail, during the preliminary year of the survey (2022/23), sentinel eggs were exposed using SE_C and SE_T setups only. A total of ninety-two egg parasitoids (sex ratio 4.3:1 ♀♀:♂♂) emerged from egg masses retrieved in four out of five sites surveyed. The total parasitisation rate – based on the total number of eggs retrieved – was 10.3%, while the mean efficiency rate (i.e., parasitoids emerged/eggs in parasitised setup replica) was 17.28%. In 2023/24, sentinel eggs were exposed with all the three setups described above. A total of 636 egg parasitoids emerged, 628 of them from eggs exposed in SE_R setups from seven out of eight sites. The average parasitisation rate (adult parasitoid emerging/total eggs retrieved from a setup) was 3.21% in Piedmont and 24% in Liguria, while the efficiency rate (adult parasitoid emerging/total eggs retrieved from parasitised setups only) was 7.8% in Piedmont and 29.7% in Liguria (Figure 35). In 2024/25, only sentinel eggs were exposed using SE_R setup only. The number of emerging adult *O. vulgatus* was, however, very low (n = 25), and from three out of eight sites only. Considering the two sites in which egg parasitoids were found alive only, the parasitisation rate recorded was 4.9 % in Piedmont site and 2.59% in Liguria site, while the efficiency rate was 6.35% and 16.1% in Piedmont and Liguria, respectively.

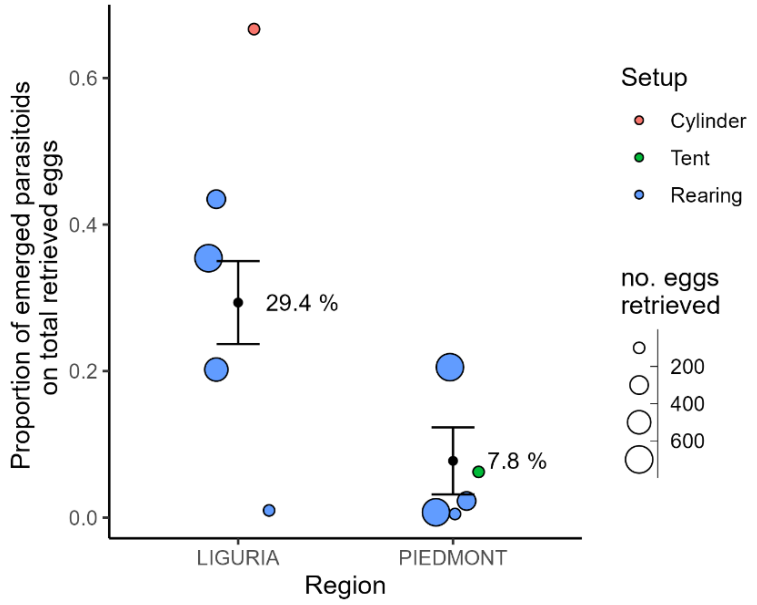


Figure 35. Mean realized parasitisation rate of *Philaenus spumarius* eggs (i.e. proportion of sentinel eggs parasitised) during 2023/24 survey in Northwestern Italy.

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3.3. Parasitism rate and biological traits of the egg parasitoid *Ooctonus vulgatus*

3.3.1. Materials and Methods

3.3.1.1. Parasitisation trials

The parasitisation rate of *P. spumarius* eggs by *O. vulgatus* emerging from field surveys in Northwestern Italy was evaluated under laboratory conditions using as a proxy the number of parasitoids emerging from field-exposed sentinel eggs. During each observation session of emergences from sentinel eggs in Petri dish, pairs of newly emerged parasitoids (one male and one female) were collected, transferred in new Petri dishes (mating arena, ø 5 cm) containing wet filter paper and small drops of honey. The Petri dishes were sealed with Parafilm to prevent parasitoid escape and maintained under controlled conditions (18±2 °C; 14:10 L:D) for 24 hours to allow mating. After mating, females and males were separated in different Petri dishes together with other individuals of the same sex (maintenance arena, ø 9 cm) and kept in cold conditions (6 °C, 14:10 L:D).

A series of biological assays were conducted with parasitoid emerging from sentinel eggs in 2022/23 and 2023/24 to evaluate the parasitism potential of *O. vulgatus* females on i) eggs of different insect species and ii) eggs at different developmental stages. Each trial included both no-choice and double-choice tests. No-choice tests involved exposure to one of four egg categories: i) *P. spumarius* eggs, ii) *Euscelidius variegatus* (Hemiptera: Cicadellidae) as alternative host, iii) “fresh” *P. spumarius* eggs, and iv) “old” *P. spumarius* eggs. The distinction between “fresh” and “old” eggs followed the criteria described by Witsack (1973): “fresh” eggs have the embryo in diapause, while “old” eggs are non-diapause, developed eggs displaying a black operculum and nearing hatching. Two configurations of double-choice trials allowed *O. vulgatus* females to select between two egg types: i) developmental stage, “fresh” versus “old” *P. spumarius* eggs, or ii) host species, *P. spumarius* versus *E. variegatus* eggs. Additionally, the parasitisation capability of *O. vulgatus* on frozen *P. spumarius* eggs was evaluated. Eggs laid during winter and stored at 6 °C (14:10 L:D) were frozen for two days at −80 °C and then offered to *O. vulgatus* females within 20 minutes of removal from the freezer. The *P. spumarius* eggs used in these experiments were obtained from mass rearings conducted between October and December and maintained in a climatic chamber (6 °C, 14:10 L:D); eggs were categorized as either “fresh” or “old” before exposure to fertilized *O. vulgatus* females. *E. variegatus* eggs were obtained on *Avena sativa* leaved from a continuous mass rearing maintained at IPSP-CNR (Turin, Italy). During parasitisation trials involving the exposition of *E. vulgatus* eggs, the stalk of leaf containing eggs was inserted into a 2 mL Eppendorf tube filled with water and sealed with Parafilm, to preserve the leaf turgor throughout experimentation. Across all experimental protocols, approximately 10 eggs of each category were exposed to one *O. vulgatus* females for seven days in the parasitisation arena (ø 9 cm) in controlled conditions (18±2 °C; 14:10 L:D) (***Figure 36***).

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Figure 36. *Ooctonus vulgatus* female displaying drumming search behaviour on *Philaenus spumarius* egg mass.

3.3.1.2. Biological traits assays

Longevity was estimated using freshly emerged (<24h) *O. vulgatus* individuals (n = 100) from field-exposed sentinel eggs. Pairs of insects (one male and one female) were placed in 5-cm Petri dishes and maintained under controlled conditions (18±2 °C; 14:10 L:D). Survival was monitored and recorded daily until the death of both individuals. Throughout the trial, moisture levels and honey availability were regularly checked and maintained. The ovigeny strategy of *O. vulgatus* females was evaluated by dissection of 16 individuals, either within 24 hours from emergence or one-week post-emergence. Dissections were performed using entomological needles under a stereomicroscope (Leica MZ6 60×).

3.3.2. Results

3.3.2.1. Parasitisation trials

Emergence rate, a proxy of parasitism efficiency, obtained during the several parasitisation trials was very low. In preliminary work (2022/23), seven *O. vulgatus* (1.3:1 ♀♀:♂♂ sex ratio) emerged from parasitisation trials on *P. spumarius* eggs (parasitisation rate of replicas with at least one parasitoid emerged: 7 emergences/47 eggs exposed = 14.9 %; per-trial parasitisation rate: 7/224 = 3.1 %). In 2023/24 trials, *O. vulgatus* adults emerged from “fresh” eggs of *P. spumarius* only. Five egg parasitoid adults (1.5:1 ♀♀:♂♂ sex ratio) emerged from “fresh” eggs of a single replica (i.e., parasitisation arena) (per-replica parasitisation rate: 5 emergences/12 eggs exposed = 41.7 %; overall parasitisation rate on “fresh” *P. spumarius* eggs during double-choice trial: 5/130 = 3.8 %). A single adult parasitoid emerged from *P. spumarius* eggs exposed to parasitoid female during a no choice trial with “fresh” egg. No parasitoids emerged from the “frozen” eggs or “old” eggs in any trial. No parasitism trials were performed in 2024/25 because of the small number of adult parasitoids emerging from field-exposed sentinel eggs. The preimaginal development of *O. vulgatus* in the host eggs was in average 30 days (16 to 40 days).

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3.3.2.2. Biological traits assays

The adult longevity of *O. vulgatus* was estimated in 9.8 ± 4.7 days (n = 111), with females living on average four days longer than males.

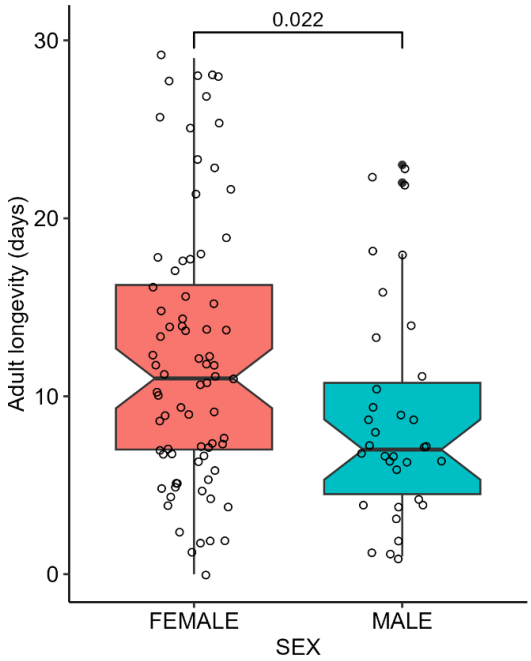


Figure 37. Adult longevity of *Ooctonus vulgatus.*

The oviposition strategy of O. vulgatus females was assessed through ovary dissections (***Figure 38***A). The dissections revealed that *O. vulgatus* is a quasi-proovigenic species; in fact, females emerge with an average of 23.13 mature eggs in the abdomen, and this number increases significantly after one week, reaching an average of 28.71 eggs (***Figure 38***B).

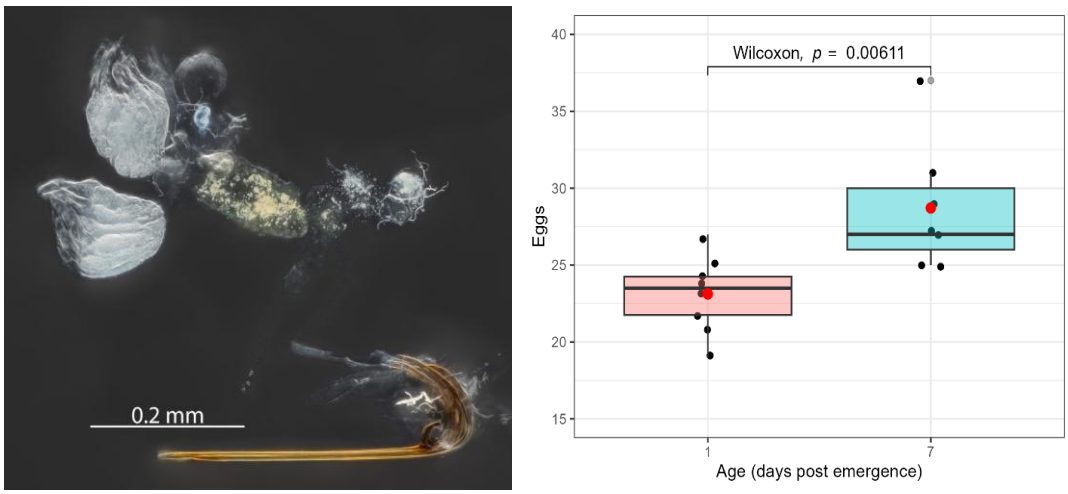


Figure 38. A) photo of dissected reproductive organs of *Ooctonus vulgatus* female; B) No. of developed eggs (full size) in ovary of females emerged one or seven days before the dissection.

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3.4. Presence and prevalence of parasitoids associated to *Philaenus spumarius* in South Italy (Apulia)

3.4.1. Materials and Methods

3.4.1.1. Presence and prevalence of adult parasitoids of Philaenus spumarius

Table 6. Sites where egg sampling was conducted in Apulia (Italy), 2022–2024

Site	Province	Coordinates (latitude, longitude)	Winter sampling period		
			2022-2023	2023-2024	2024-2025
Acquarica	Lecce	39°52'43.28"N, 18°13'40.05"E			x
Alessano	Lecce	40°21'49.6"N, 18°17'57.6"E			x
Alessano	Lecce	39°52'43.74"N, 18°18'18.76"E			x
Brindisi	Brindisi	40°39'0.51"N, 17°49'18.22"E			x
Brindisi	Brindisi	40°33'56.99"N, 17°55'58.20"E			x
Casarano	Lecce	40° 1'37.74"N, 18°12'36.54"E			x
Casarano	Lecce	40°1'34.838''N, 18°12'8.762''E			x
Cisternino	Brindisi	40°45'25.2"N, 17°27'17.6"E	x	x	
Cerignola	Foggia	41°25'10.72"N, 15°50'53.19"E			x
Cursi	Lecce	40°24'47.65''N, 18°5'39.127''E			x
Galatina	Lecce	40°13'39.62"N, 18° 4'59.36"E			x
Gallipoli	Lecce	40°01'27.9"N, 18°01'45.1"E	x	x	
Latiano	Brindisi	40°36'5.88"N, 17°42'22.24"E			x
Lecce	Lecce	40°21'49.6"N 18°17'57.6"E	x	x	
Manduria	Taranto	40°25'0.76"N, 17°39'13.43"E			x
Mariotto	Bari	41°01'60.0''N, 16°32'18.0''E			x
Mariotto	Bari	41° 1'46.93"N, 16°32'45.94"E			x
Montemesola	Taranto	40°34'27.90"N, 17°20'47.67"E			x
Montemesola	Taranto	40°34'31.41"N, 17°21'3.17"E			x
Nardò	Lecce	40°13'38.01"N, 18° 2'40.51"E			x
Otranto	Lecce	40° 5'29.76"N, 18°26'59.80"E			x
Ruvo di Puglia	Bari	41° 1'10.33"N, 16°29'57.44"E			x
Ruvo di Puglia	Bari	41° 1'3.12"N, 16°29'56.95"E			x
Santeramo in Colle	Bari	40°46'00.0"N, 16°44'52.1"E	x	x	
Squinzano	Lecce	40°28'30.4''N, 18°05'39.4''E			x
Squinzano	Lecce	40°26'58.58"N, 18° 3'8.03"E			x
Trepuzzi	Lecce	40°24'47.65''N, 18°5'39.127''E			x
Ugento	Lecce	39°55'31.6''N, 18°10'24.1''E			x
Ugento	Lecce	39°54'43.91"N, 18°10'6.06"E			x
Valenzano	Bari	41°01'39.2"N, 16°54'17.5"E	x	x	
Veglie	Lecce	40°20'26.398''N, 17°54'21.193''E			x
Vernole	Lecce	40°19'08.7''N, 18°19'49.8''E			x

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Sampling of plant residues under natural conditions

During the first two years (winter 2022-2023 and 2023-2024), sampling was carried out at five sites located in the provinces of Brindisi, Lecce and Bari (Table 6). During winter 2024–2025, field surveys were extended to twenty-seven additional sites in the provinces of Lecce, Brindisi, Bari and Foggia, as reported in ***Table 6***. Each year, sampling was performed at the end of the winter (January-February), when plant residues and straw lying on the ground near host plants, known to generally shelter spittlebugs prior to egg hatching, were collected. Straw was first inspected with naked eye to search for egg masses and subsequently examined under a stereomicroscope (10-20x) to count the number of eggs. Egg masses recovered from straw were placed in Petri dishes lined with moist filter paper and maintained at room temperature. From mid-February to late March, the dishes were inspected daily to record egg hatching and the possible emergence of parasitoids. Emerging nymphs and parasitoids were collected from the Petri dishes and stored in 70% ethanol at -20°C until identification.

Using straw bundles or spy cages for oviposition

During the autumn-winter periods of 2023-2024 and 2024-2025, adults of *P. spumarius* were collected by sweep netting from late September to first days of November across several sites located in *Xylella*-free area known for high vector population densities. Most individuals were collected in olive groves and on ground vegetation, where adults typically aggregate at the end of the season. Collected adults were transferred into entomological cages (Bugdorm, 45 x 45 x 90 cm) containing *Catharanthus roseus* plants and an oviposition substrate consisting of wheat and barley straw, including leaf sheaths in 2023-2024 or spy cages (steel-grid wrapped straw of the same composition) in 2024-2025 to promote oviposition. Approximately 1600 adults were introduced each year (winters 2023–2024 and 2024–2025), corresponding to four release events of 400 individuals each (***Figure 39***A). The cages containing *P. spumarius* adults (both female and male) were kept inside a greenhouse to protect them from adverse weather conditions and to ensure a suitable microclimate for oviposition.

Between mid-November and mid-December, the straw substrate (2023-2024) or spy cages (2024-2025) were transferred to five open field locations: Gallipoli (LE), Lecce (LE), Cisternino (BA), Santeramo in Colle (BA) and Valenzano (BA), to act as traps for egg parasitoids. Straw bundles or spy cages were placed close near shrub vegetation (***Figure 40***), primarily *Pistacia lentiscus* and *Myrtus communis* or other shrubs occurring in areas where *P. spumarius* adults were typically abundant. In both years, the straw bundles or spy cages contained the egg-laden straw were retrieved between January and February for detailed examination. In the laboratory, the straw was first inspected visually for characteristic signs of egg masses and subsequently examined under a stereomicroscope to count the eggs. After counting, straw pieces containing eggs were placed in Petri dishes lined with filter paper to prevent the loss of detached eggs and were monitored for parasitoids emergence from mid-February to late March.

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Figure 39. A) Entomological cages containing a *Catharanthus roseus* plant together with small 'spy cages' equipped with straw as an oviposition substrate; B) Arrangement of 'spy cages' inside the entomological cage; C) Adult of *P. spumarius* feeding on C. roseus; D) Female of *P. spumarius* ovipositing on straw within the 'spy cage'.



Figure 40. Straw-filled monitoring cages used by *P. spumarius* females for oviposition, placed at the base of shrubs.

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Table 7. Sites where adults of *Philaenus spumarius, Neophilaenus campestris* and *Philaenus italosignus* were sampled in Apulia (Italy), 2020–2022

Site	Province	Coordinates (latitude, longitude)
Alberobello	Bari	40°46'33.6"N, 17°12'25.7"E
Alberobello	Bari	40°47'25.5"N, 17°14'01.2"E
Alberobello	Bari	40°46'20.4"N, 17°14'30.5"E
Canosa	Barletta-Andria-Trani	41°10'06.3"N, 16°02'37.1"E
Castellana Grotte	Bari	40°50'15.6"N, 17°30'48.0"E
Castellana Grotte	Bari	40°53'34.3"N, 17°09'09.1"E
Castellana Grotte	Bari	40°51'22.9"N, 17°13'07.7"E
Castellaneta	Taranto	40°36'10.6"N, 16°56'35.9"E
Crispiano	Taranto	40°36'37.5"N, 17°22'10.3"E
Cutrofiano	Lecce	40°06'57.7"N, 18°14'36.6"E
Erchie	Brindisi	40°25'09.8"N, 17°46'37.6"E
Felline	Lecce	39°55'10.4"N, 18°06'33.6"E
Galatina	Lecce	40°15'31.2"N, 18°06'41.7"E
Gallipoli	Lecce	40°01'25.1"N, 18°01'52.3"E
Gallipoli	Lecce	40°00'39.7"N, 18°02'52.9"E
Gallipoli	Lecce	40°01'01.2"N, 18°03'29.6"E
Latiano	Brindisi	40°34'12.5"N, 17°45'10.1"E
Latiano	Brindisi	40°35'57.5"N, 17°40'43.4"E
Locorotondo	Bari	40°45'50.0"N, 17°18'35.2"E
Locorotondo	Bari	40°45'32.0"N, 17°17'45.9"E
Manduria	Taranto	40°24'35.5"N, 17°39'25.1"E
Martina Franca	Taranto	40°44'36.9"N, 17°15'17.7"E
Martina Franca	Taranto	40°43'28.9"N, 17°14'15.8"E
Massafra	Taranto	40°34'01.6"N, 17°03'52.5"E
Massafra	Taranto	40°37'32.2"N, 17°10'32.2"E
Massafra	Taranto	40°35'06.1"N, 17°05'41.6"E
Monopoli	Bari	40°56'40.0"N, 17°11'50.1"E
Monopoli	Bari	40°56'03.9"N, 17°16'53.6"E
Monopoli	Bari	40°51'15.9"N, 17°18'10.2"E
Monopoli	Bari	40°52'39.8"N, 17°13'48.8"E
Mottola	Taranto	40°40'57.9"N, 17°08'29.9"E
Mottola	Taranto	40°36'35.3"N, 17°03'55.2"E
Noci	Bari	40°48'12.9"N, 17°09'30.8"E
Noci	Bari	40°48'37.6"N, 17°05'32.1"E
Oria	Brindisi	40°29'01.0"N, 17°39'03.0"E
Ostuni	Brindisi	40°43'35.7"N, 17°30'48.0"E
Polignano	Bari	40°54'54.3"N, 17°10'53.7"E
Polignano	Bari	41°01'39.9"N, 17°09'23.8"E
San Vito dei Normanni	Brindisi	40°38'54.2"N, 17°45'20.7"E
Statte	Taranto	40°32'49.9"N, 17°14'27.6"E
Valenzano	Bari	41°01'39.2"N, 16°54'17.5"E
Ugento	Lecce	39°55'01.1"N, 18°12'03.0"E

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3.4.1.2. Presence and prevalence of adult parasitoids of Philaenus spumarius

Over a three-year period, from 2020 to 2023, adults of *P. spumarius*, *N. campestris*, and *Philaenus italosignus* Drosopoulos et Remane were collected during summer-autumn surveys in 42 olive groves located across several provinces (Table 7). All specimens were collected using sweep-net following the protocol of Bodino et al. (2019).

Adult spittlebugs were dissected under a stereomicroscope and any Pipunculidae larva found was stored in 70% ethanol at –20 °C for subsequent morphological and molecular analyses.

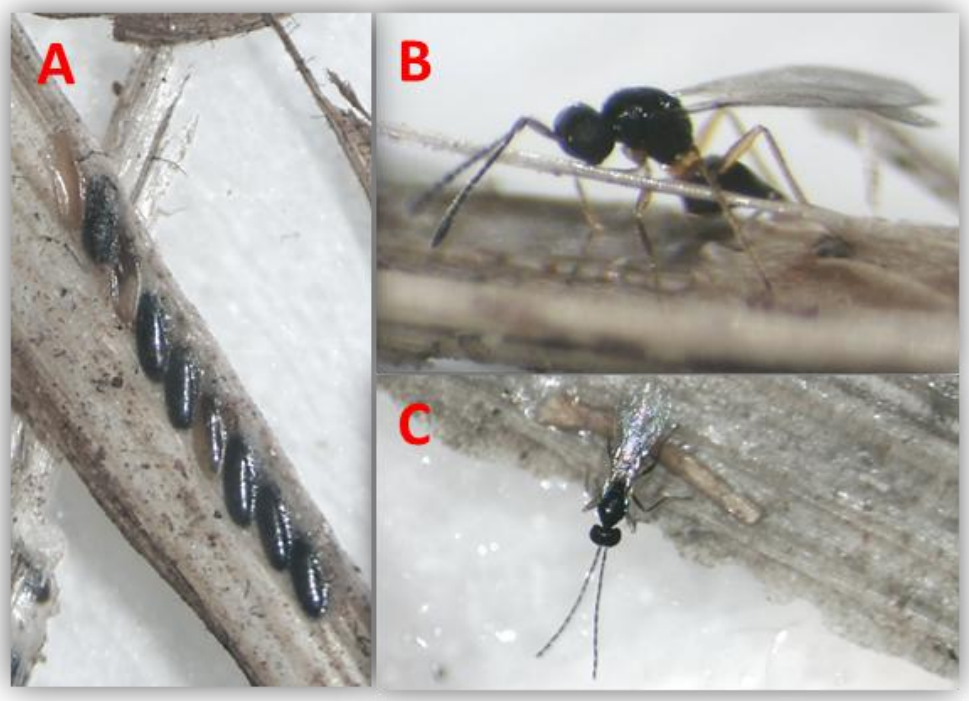
3.4.2. Results

3.4.2.1. Presence and prevalence of adult parasitoids of Philaenus spumarius

Sampling of plant residues in natural conditions

During 2022-2023, parasitised eggs were recovered exclusively from straw samples collected at the Valenzano and Gallipoli sites, with parasitism rates of 24.5% and 11.9%, respectively. In the remaining three sites, none of egg masses identified in the collected plant residues showed any signs of parasitism. Overall, the level of parasitism was very low, with an average value of 5.8% (Table 3).

Parasitoids emergence occurred between late February and early April, and all individuals displayed homogeneous morphological characteristics and were initially assigned to the genus *Ooctonus*. A total of eight specimens, four from the Valenzano and four from Gallipoli, were sent to partner P6-UNITO for further identification. All individuals were identified as *Ooctonus vulgatus* (Hymenoptera: Mymaridae) (Figure 41), based on morphological characters (Haliday, 1833) and molecular analysis.



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Figure 41. A) *P. spumarius* eggs parasitised by *Ooctonus vulgatus*; B-C) Adult individuals of *O. vulgatus*.

During winter 2023–2024, the same sites monitored during the previous winter (2022–2023) were surveyed again. At the Valenzano site, the number of detected egg masses was higher than in the previous year, but the parasitism rate was lower (12.9%). In Gallipoli, the number of collected egg masses was lower than in the previous monitoring period, and none of them were parasitised. Conversely, at the Cisternino site, 7.1% of the 113 eggs examined were found to be parasitised. In Lecce the number of eggs recorded was comparable to the previous year, a marked reduction in egg abundance was observed in the Santeramo in Colle area; in both sites, no parasitised eggs were detected.

All emerging parasitoids were identified as O. vulgatus*, with the exception of four individuals of an unidentified wasp that emerged from eggs collected in Valenzano, corresponding to a parasitism rate of 6.7%. Overall, during the 2023–2024 winter season, the level of parasitism by* O. vulgatus *remained very low, with an average rate of 9.4% (***Table 8***). During the 2024–2025 survey, despite the expansion of the monitoring network to a larger number of sites and the detection of* P. spumarius *eggs at nearly all locations, albeit with variable densities, no parasitised eggs were recorded (*

Using straw bundles or spy cages for oviposition

Throughout the first monitoring period, when straw bundles were placed from mid-November to December at the base of shrubs, where *P. spumarius* adults were typically captured, only a limited number of eggs were recorded (***Table 10***). In the following year (2024-2025), the use of spy cages resulted in a higher number of recorded eggs; however, no parasitoid emergence was detected in either period. All eggs recovered from cages yielded *P. spumarius* only.

Table 9).

Table 8. Rates of parasitised eggs recorded during the 2022-2023 and 2023-2024 winter seasons.

Site	2022-2023			2023-2024		
	N. eggs collected	**N. parasitoids emerged**	**Parasitism rate (%)**	**N. eggs collected**	**N. parasitoids emerged**	**Parasitism rate (%)**
Valenzano	49	12	24.5	193	25	12.9
Santeramo in Colle	31	0	0	2	0	0
Cisternino	160	0	0	113	8	7.1
Lecce	36	0	0	32	0	0
Gallipoli	67	8	11.9	12	0	0
Total	**343**	**20**	**5.8**	**352**	**33**	**9.4**

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Using straw bundles or spy cages for oviposition

Throughout the first monitoring period, when straw bundles were placed from mid-November to December at the base of shrubs, where *P. spumarius* adults were typically captured, only a limited number of eggs were recorded (***Table 10***). In the following year (2024-2025), the use of spy cages resulted in a higher number of recorded eggs; however, no parasitoid emergence was detected in either period. All eggs recovered from cages yielded *P. spumarius* only.

Table 9. Number of eggs recorded during the winter of 2024-2025 at the monitored sites.

Site	Province	Coordinates (latitude, longitude)	N. eggs collected
Acquarica	Lecce	39°52'43.28"N, 18°13'40.05"E	10
Alessano	Lecce	40°21'49.6"N, 18°17'57.6"E	202
Alessano	Lecce	39°52'43.74"N, 18°18'18.76"E	194
Brindisi	Brindisi	40°39'0.51"N, 17°49'18.22"E	32
Brindisi	Brindisi	40°33'56.99"N, 17°55'58.20"E	43
Casarano	Lecce	40° 1'37.74"N, 18°12'36.54"E	94
Casarano	Lecce	40°1'34.838''N, 18°12'8.762''E	191
Cerignola	Foggia	41°25'10.72"N, 15°50'53.19"E	64
Cursi	Lecce	40°24'47.65''N, 18°5'39.127''E	154
Galatina	Lecce	40°13'39.62"N, 18° 4'59.36"E	91
Latiano	Brindisi	40°36'5.88"N, 17°42'22.24"E	166
Manduria	Taranto	40°25'0.76"N, 17°39'13.43"E	159
Mariotto	Bari	41°01'60.0''N, 16°32'18.0''E	12
Mariotto	Bari	41° 1'46.93"N, 16°32'45.94"E	5
Montemesola	Taranto	40°34'27.90"N, 17°20'47.67"E	8
Montemesola	Taranto	40°34'31.41"N, 17°21'3.17"E	0
Nardò	Lecce	40°13'38.01"N, 18° 2'40.51"E	133
Otranto	Lecce	40° 5'29.76"N, 18°26'59.80"E	143
Ruvo di Puglia	Bari	41° 1'10.33"N, 16°29'57.44"E	122
Ruvo di Puglia	Bari	41° 1'3.12"N, 16°29'56.95"E	38
Squinzano	Lecce	40°28'30.4''N, 18°05'39.4''E	97
Squinzano	Lecce	40°26'58.58"N, 18° 3'8.03"E	56
Trepuzzi	Lecce	40°24'47.65''N, 18°5'39.127''E	31
Ugento	Lecce	39°55'31.6''N, 18°10'24.1''E	61
Ugento	Lecce	39°54'43.91"N, 18°10'6.06"E	78
Veglie	Lecce	40°20'26.398''N, 17°54'21.193''E	18
Vernole	Lecce	40°19'08.7''N, 18°19'49.8''E	0
Total			**2202**

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Table 10. Number of eggs recorded during two winter periods (2023-2024 and 2024-2025) using straw bundles or spy cages.

Site	N. eggs collected	
	2023-2024	2024-2025
Valenzano	5	58
Santeramo in Colle	12	75
Cisternino	25	156
Lecce	54	137
Gallipoli	67	194
Total	**163**	**620**

3.4.2.2. Presence and prevalence of adult parasitoids of Philaenus spumarius

All parasitised adult individuals of *P. spumarius*, *N. campestris* and *P. italosignus* collected at different sites in Apulia between 2020 and 2022 were parasitised by Pipunculidae. Second-instar larvae were identified based on the morphology of the posterior spiracular plate following the method of Benton (1975) (***Figure 42***2). Nucleic acids were extracted from all dipteran larvae using a CTAB-based extraction protocol (EPPO, 2021). Mitochondrial cytochrome c oxidase subunit I (COI) from seven larvae collected from each of the three spittlebug species was amplified using primers LCO1490 and HCO2198 (Folmer et al., 1994) and sequenced by Macrogen Europe via Sanger sequencing. The resulting sequences were compared with those available in the BOLD Systems database (https://www.boldsystems.org/). Molecular identification of larvae was then performed following the protocol of Molinatto et al. (2020) using species-specific primers targeting *Verralia aucta* ITS2 (nuclear internal transcribed spacer 2) in qPCR and COI in conventional PCR. All individuals recorded were assigned to *V. aucta* (***Figure 43***3).

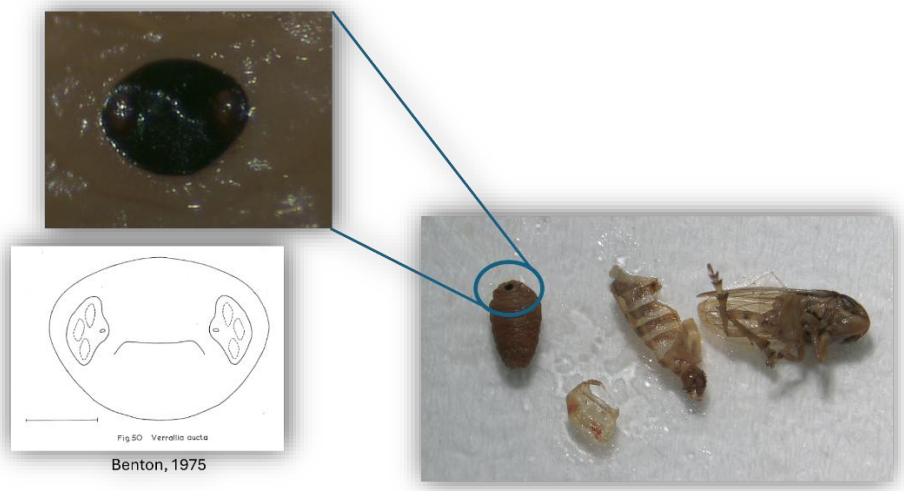


Figure 42. Morphological identification of second-instar larvae of *Verrallia aucta*.

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Table 11. Rates of parasitised individuals of spittlebug species by Verralia aucta during the survey years.

Spittlebugs	Year 2020			Year 2021			Year 2022		
	N. individuals captured	N. parasitised individuals	Parasitism rate (%)	N. individuals captured	N. parasitised individuals	Parasitism rate (%)	N. individuals captured	N. parasitised individuals	Parasitism rate (%)
P. spumarius	309	6	1.9	711	39	5.5	264	0	0
N. campestris	383	52	13.6	662	32	4.8	359	15	4.2
P. italosignus	0	0	0	62	2	3.2	0	0	0

Parasitisation levels varying across years and host species. In 2020, N. campestris *exhibited the highest parasitism rate (13.6%), whereas* P. spumarius *was only marginally affected (1.9%). In 2021, the percentage of parasitised adults in each spitllebug species was very low, although all species were parasitised, with values ranging between 3.2% (*P. italosignus*) to 5.5% (*P. spumarius*). In the final sampling year (2022), only individuals of* N. campestris *were parasitised with a parasitisation rate of 4.2% (*

Table 11).

Overall, parasitism rates were 2.5% in *P. spumarius*, 7.5% in *N. campestris*, and 1.1 in *P. italosignus* (***Figure 44***4), indicating a limited level of parasitisation by *V. aucta* on the spittlebug populations examined.



Figure 43. *Verralia aucta* individuals observed under a stereomicroscope.

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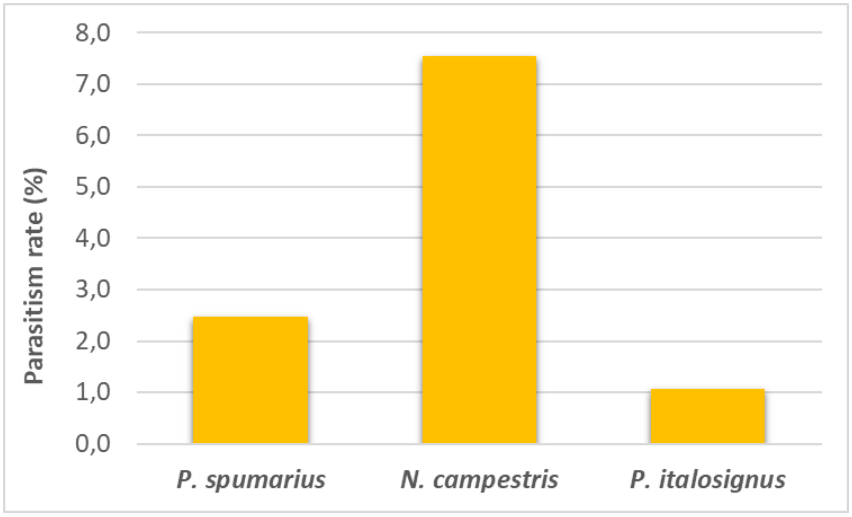


Figure 44. Rates of parasitised individuals of spittlebug species by Verrallia aucta recorded over the three-year period (2020-2022).

3.5. Conclusions

Parasitoids associated to spittlebugs were investigated during multi-year field surveys across different regions of Italy, with a focus on their potential as biological control agents of *X. fastidiosa* vectors. Field surveys using different sentinel eggs setups and direct egg sampling revealed that the mymarid *O. vulgatus* is the dominant— and essentially sole—egg parasitoid natural enemy of *P. spumarius* in Northwestern and Southern Italy. Although the species was consistently detected, parasitism levels were highly variable and generally low, rarely exceeding 10–25% even in the most favourable sites. Among the different sentinel setups tested, only field-oviposition cages with P. spumarius individuals yielded substantial parasitoid emergence, whereas static tent and cylinder setups proved largely ineffective, demonstrating that methodological constraints can significantly limit detection of egg parasitoid activity.

Laboratory experiments highlighted some biological traits of *O. vulgatus*, including a strongly female-biased sex ratio, relatively short adult longevity, quasi-proovigenic ovigeny, and a preference for "fresh" *P. spumarius* eggs. However, parasitisation success in laboratory assays remained low, not allowing the setup of continuous mass rearings. Also, *O. vulgatus* showed no ability to parasitise older or frozen eggs, indicating a narrow temporal window of host suitability. These characteristics, combined with the observed low field prevalence, suggest limited capacity of this species to regulate spittlebug populations at levels relevant for vector control.

Surveys of adult parasitoids revealed the big-headed fly *V. aucta* parasitising *P. spumarius*, *N. campestris*, and *P. italosignus*, with parasitism rates between 1–13% depending on year and host species. These values further underline that natural parasitism pressure on spittlebug populations is weak under current ecological conditions in Italy.

Overall, the evidence indicates that, despite the presence of specific parasitoids, their contribution to the natural suppression of *P. spumarius* populations appears limited. Their low parasitism efficiency and inconsistent presence across sites and years constrain their potential use as standalone biological control agents. Nevertheless, the findings also highlight

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that ecological factors—such as microhabitat availability, phenological synchrony, and landscape structure—should be investigated further to understand how parasitoid impact might be enhanced and possibly complement other biological control strategies for X. fastidiosa vectors in Europe.

4. COVER CROP MANAGEMENT

4.1. Background

The management of *P. spumarius*, the main vector of *X. fastidiosa* in Mediterranean agroecosystems, remains a priority due to its wide distribution and high transmission efficiency. Current evidence confirms that the suppression of juvenile stages—characterized by limited mobility and strict association with herbaceous vegetation—is the most effective method of reducing population density before adults emerge and can acquire and spread the bacterium (Wilson et al., 2020). To support the correct timing of control measures, life cycle models based on degree-day have been developed to predict egg hatching and early nymphal development (Lago et al. 2023; Gilioli et al. 2024), which represent first step to predict the best timing to adopt control actions during the most vulnerable life stages of spittlebugs.

Conventional management practices such as soil tillage, milling, and vegetation mowing are commonly implemented during spring to disrupt nymphal development. Tillage, when applied at later nymphal instars (III–IV), can achieve population reductions close to 100% (Dongiovanni et al., 2019). Mowing also contributes to lowering nymph densities, although regrowth dynamics and the persistence of basal rosette species limit its overall effectiveness (Dongiovanni et al., 2019; Sanna et al., 2021). Despite their efficacy, these practices present environmental drawbacks: tillage accelerates soil erosion and compaction and reduces microbial biodiversity (Gomez et al. 2009; Sastre et al., 2017), while mowing alone cannot ensure sufficient vector control. This highlights the need for more pest-specific, sustainable and integrated strategies.

Ground cover management using selected plant species offers a promising alternative. *P. spumarius* nymphs are extremely polyphagous, however, studies in Apulia and Spain demonstrated that some plant species are preferentially selected (Dongiovanni et al., 2019; Bodino et al., 2020; Morente at al. 2022). By modifying the floristic composition of the herbaceous layer, it may be possible to reduce the availability of suitable host plants for nymphs, while simultaneously providing agroecological benefits such as improved soil fertility, nutrient cycling, and erosion control (Lopez et al., 2021). This approach is consistent with push-pull principles, where trap plants attract ovipositing females but do not allow nymph development, and repellent or unsuitable plants reduce nymph survival (Cook et al., 2007). Because adults are highly mobile and polyphagous, the selection of plant species must consider both oviposition preference and nymphal viability (Morente et al., 2022).

Previous studies identified several candidate species suitable for cover crop-based vector suppression in laboratory and semi-field conditions. *Anthriscus cerefolium* operates as a trap plant, attracting oviposition but being lethal for nymphal stages; *Diplotaxis tenuifolia* does not favour oviposition but induces moderate mortality; and *Sinapis alba* shows toxic effects at specific developmental stages (Morente et al. 2022).

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Building on these findings, the present study conducted field trials in Spain to assess the suitability of several plant species as ground cover in crops highly susceptible to *X. fastidiosa*, such as olive, grapevine and citrus. Additional greenhouse assays were performed in Spain to evaluate other plant species as potential trap or non-host plants for *P. spumarius*.

In parallel, mixtures of Cruciferae, Fabaceae, and Poaceae were tested as winter cover crops in olive groves in South Italy (Apulia), together with the effect of mulching applied on late nymphal instar of spittlebugs. The functional traits of the selected plant families present complementary agronomic functions: legumes contribute nitrogen fixation, crucifers enhance nutrient mobilization and may suppress soilborne pathogens, and grasses stabilize soil structure and organic biomass (Jiayu et al., 2025).

The overall objective was to determine whether the modification of herbaceous cover in different crops using selected plant species and their mixtures could effectively reduce juvenile vector abundance compared with spontaneous vegetation, mowing, and conventional tillage. The results will inform the development of more sustainable Integrated Pest Management (IPM) strategies tailored to limit the spread of *X. fastidiosa* in high-risk cropping systems.

4.2. Materials and methods

4.2.1. Survival of *Philaenus spumarius* nymphs on selected cover crop plant species under greenhouse conditions

4.2.1.1. Collection and maintenance of nymphs of Philaenus spumarius

Nymphs of *P. spumarius* were collected in the Spring of 2024 in Villanueva de la Cañada, Madrid (Spain) (40°36'40.7"N 4°15'47.5"W). To locate them, we checked rosette-shaped plants, where *P. spumarius* nymphs are usually found in the beginning of Spring. Most nymphs were found on thistle *Sonchus oleraceus* and other plants species of the Asteraceae family. Plants showing typical foams, most frequently *Sonchus oleraceus* and other Asteraceae, were cut and collected. Most of the nymphs collected were on the 2-3rd nymphal stage. The plant and foams were collected and stored in a bag and taken to the greenhouse facilities at ICA-CSIC. In the greenhouse, the nymphs were transferred with a brush to their preferred host plant, which in our case was *S. oleraceus*. The nymphs remained on *S. oleraceus* plants until the assay was started. A temperature of 24–20 °C light/dark and artificial lighting with a photoperiod of 16 h:8 h (L/D) was maintained during the assay. Nymphs in stages N4 and N5 were selected for this assay.

The mortality of *P. spumarius* nymphs was assessed on different plant species, using *S. oleraceus* as a susceptible control. Thus, the plant species tested were *Moricandia moricandioides*, *Poa pratensis*, *Papaver rhoeas* and *Salvia verbenaca* (Figure 45). All seeds were provided by Cantueso Natural Seeds, Cordoba, Spain, https://cantuesoseeds.com/en/home/.

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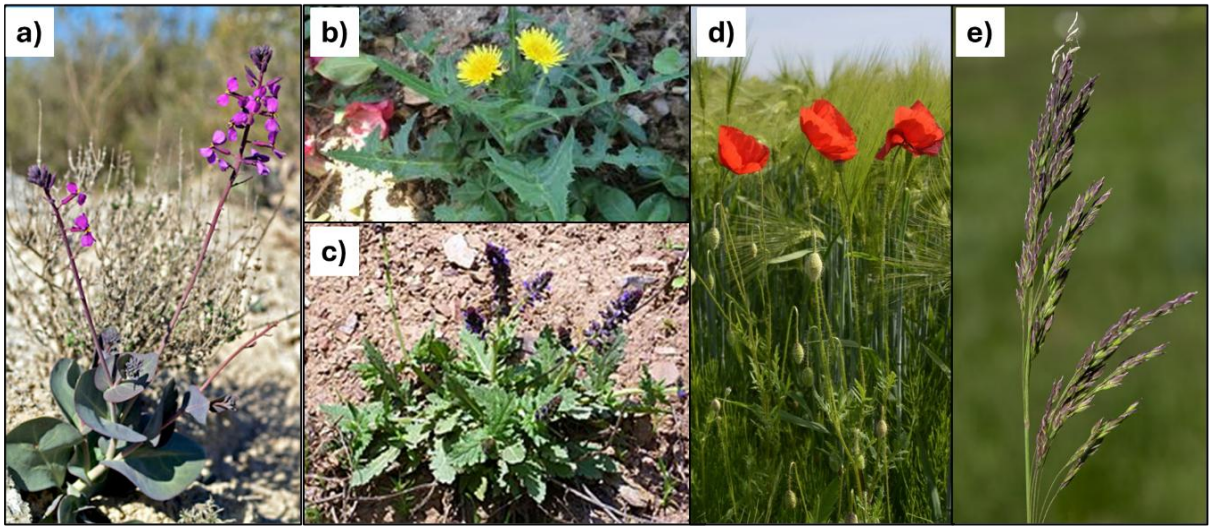


Figure 45. *a) Moricandia moricandioides,* b) *Sonchus oleraceus,* c) *Sallvia verbenaca*, d) *Papaver rhoeas,* e) *Poa pratensis.*

4.2.1.2. Nymphal survival on selected plant species

Three N4-N5 instar nymphs were placed on a single plant that was covered by a ventilated plastic cage. (Figure 46). Six replicates (cages) per plant species were conducted in each assay, which were repeated twice, once in April 2024 and again in May 2024 (thus 12 replicates per plant species in total). In the first repetition we used *M. moricandioides*, *S. oleraceus*, *S. verbenaca* and *P. pratensis*, and in the second replicate *P. pratensis* was replaced by *P. rhoeas*. All nymphs were raised in each plant species at a temperature of 24–20 °C light/dark (L/D) and a photoperiod of 16 h:8 h (L/D) as described above. The mortality of *P. spumarius* nymphs was evaluated daily over a period of 14 days.

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Figure 46. This image shows one of the treatments in this assay (*P. pratensis*), with six replicates. The cylinders are placed in all replicates.

4.2.2. Efficacy of ground cover vegetation to reduce the population density of *Philaenus spumarius* in different crops in Spain

4.2.2.1. Experimental design, plant selection and location of field assays

The assays were conducted in different locations in Spain during 2023-2024 and 2024-2025. Ground covers were sown in the fall and nymph sampling was conducted in the following spring. The locations and experimental design varied from year to year because of different reasons (low population density, poor germination of seeds, flooding and other difficulties observed). A total of five experimental fields, all of them commercial fields were provided by farmers except the one in Areeiro (Estacion Experimental de Areeiro, Diputación de Pontevedra). We conducted three trials per year and one was repeated in two consecutive years. These locations had different climatic and environmental conditions. Field plots with different crops were initially selected in each location: grapevine (Pontevedra, Cenicientos), olive (Valdepielagos) and citrus (Valencia). (Figure 47).

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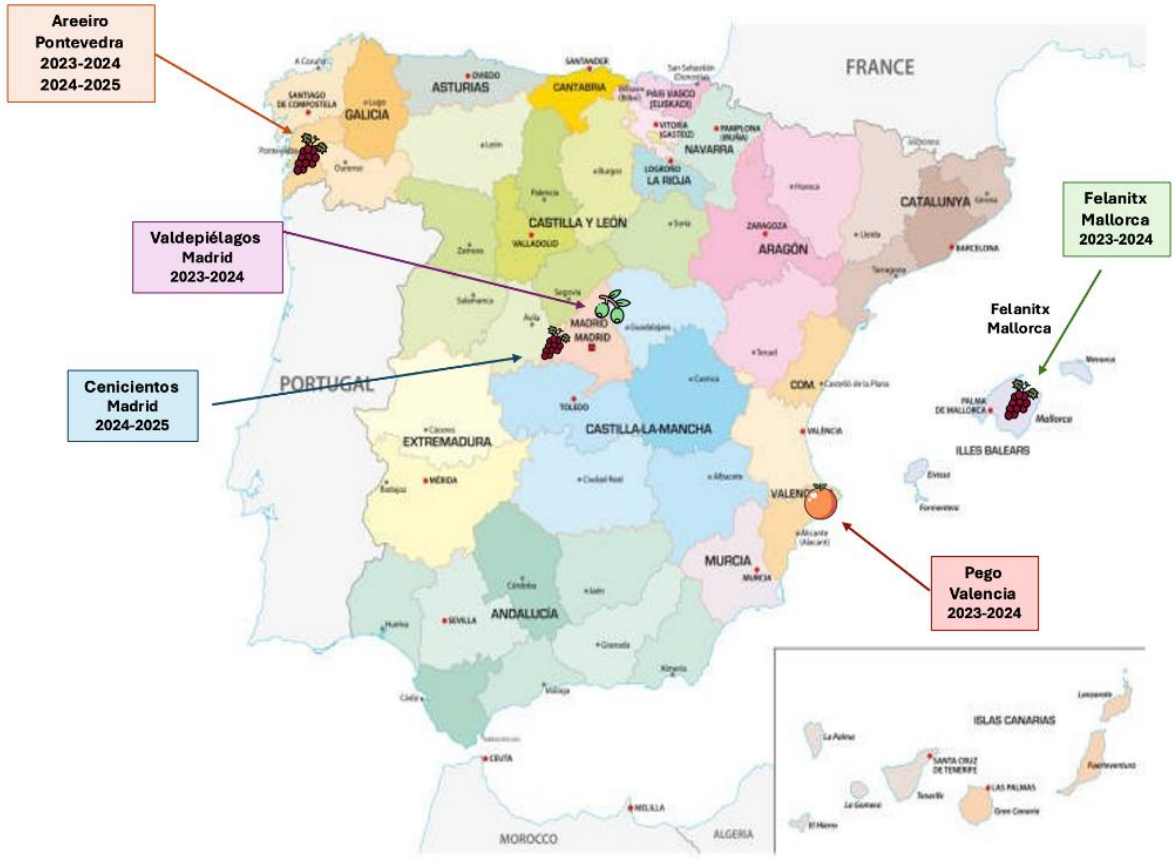


Figure 47. Map of the different experimental fields in assessment periods.

Each experimental field had a specific experimental design in each location, with different treatments and plot size depending on the acreage provided by the farmer. Also, a specific sowing dose and a combination of plant species tested varied depending on the availability of seeds. Before sowing, an assessment of the population density of *P. spumarius* adults in the area was carried out in the fall, using an entomological sweep net. For each sample, a total of 10 double sweeps were collected and a total of 10 samples per field were obtained.

The plant species selected for these field assays were the ones that had most promising according to the results of the laboratory and greenhouse trials conducted by Morente et al., 2022 which were: *A. cerefolium*, *D. tenuifolia*, and *S. alba* (Figure 48a, b, c). Furthermore, *M. moricandioides* (Figure 48d) was also selected in the Cenicientos field trial because it induced a high mortality of P*, spumarius* nymphs according to our greenhouse survival assays. All seeds were provided by by Cantueso Natural Seeds, Cordoba, Spain, https://cantuesoseeds.com/en/home/.

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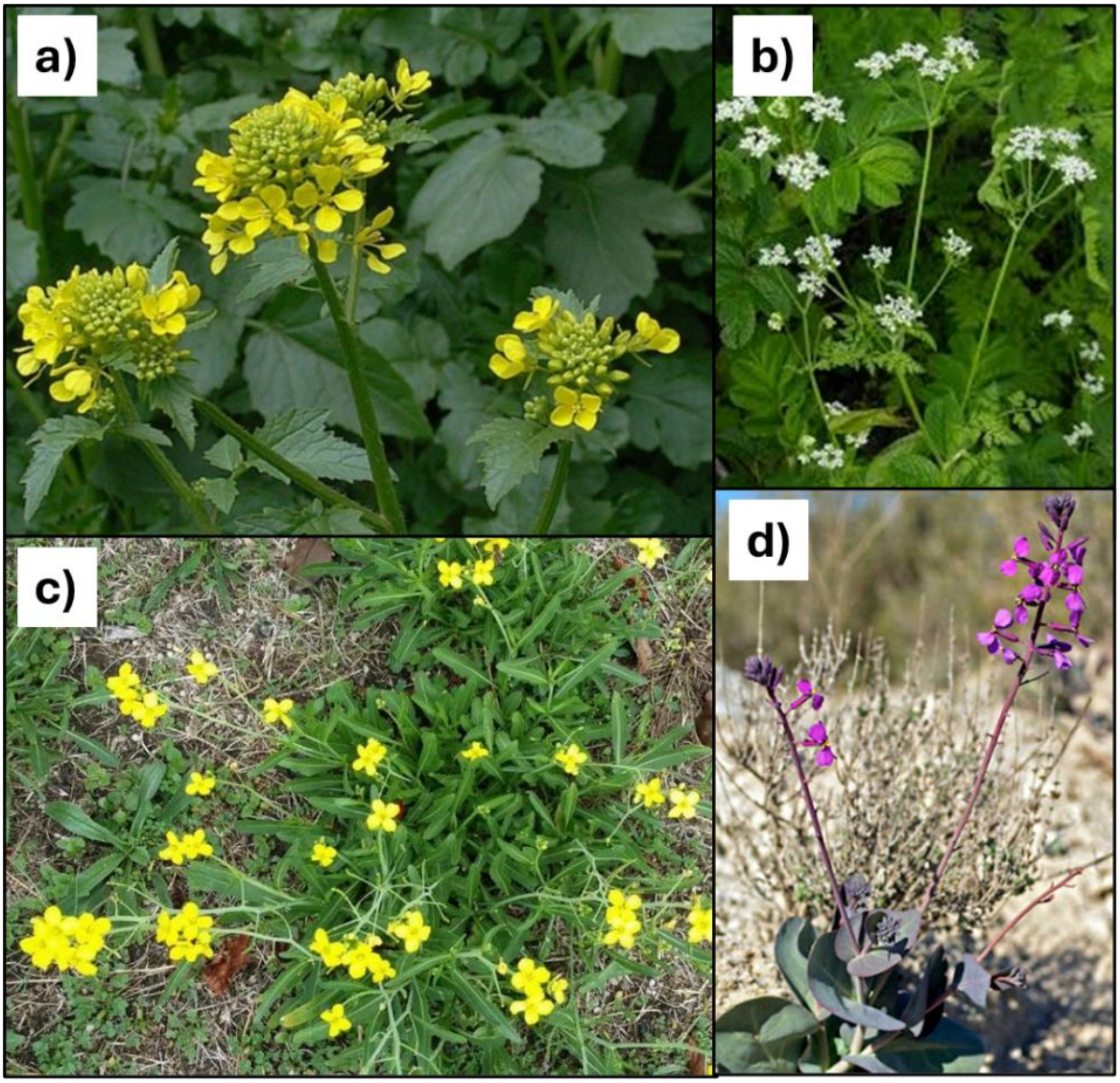


Figure 48. a) *Sinapis alba*, b) *Anthriscus cerefolium*, c) *Diplotaxis tenuifolia*, d) *Moricandia moricandioides.*

In the first period, 2023–2024, the experimental designs and locations were the following:

Experimental plot 1

A sweet orange commercial field in **Pego, Valencia** (38°52'11.86"N; 0° 4'39.91"W), where cover crops of *D. tenuifolia*, *A. cerefolium* and *S. alba* were alternated according to the experimental design shown in ***Figure 49***. The seeds were sown on 22nd of January, 2022 in winter (22nd January, 2024).at a dose of 30 kg/ha for *A. cerefolium*, 20 kg/ha for *S. alba*, and 2 kg/ha for *D. tenuifolia*. The study carried out in Pego was conducted during the 2023–2024 period, when a prolonged drought occurred, causing both the ground cover and the spontaneous vegetation and nearby crops to dry out. Thus, sampling of nymphs during spring was not possible, and not results were obtained.

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Figure 49. Experimental design and image of the field in Pego, Valencia.

Experimental plot 2

An olive grove in **Valdepielagos**, Madrid (40°45'04"N; 3°28"30"W), where rows of spontaneous vegetation were combined with rows of *A. cerefolium* and with a mixture of *D. tenuifolia* and *S. alba* according to the experimental design shown in Figure 11. Sowing was carried out on the 24th of November 2023, and the sowing density used was 30 kg/ha of *A. cerefolium*, 20 kg/ha of *S. alba*, and 2 kg/ha of *D. tenuifolia* (***Figure 50***).



Figure 50. Experimental design and image of the field in Valdepielagos, Madrid.

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Experimental plot 3

A grapevine plot in **Areeiro Experimental Station**, Pontevedra (42°24'13.19"N; 8°40'17.47"O), where the plot was divided into two lateral rows with a mixture of *D. tenuifolia* and *S. alba* and two rows of *A. cerefolium* interspaced with the spontaneous vegetation. This experimental design was used to locate the repellent/non preferred plants on the borders of the plot and trap plants intercropped between the spontaneous vegetation. The sowing density used was the same as in the plot in Valdepielagos, Madrid (30 kg/ha of *A. cerefolium*, 20 kg/ha of *S. alba*, and 2 kg/ha of *D. tenuifolia*) (***Figure 51***). Sowing was done on 8th September 2023



Figure 51. Experimental design and image of the field in Areeiro, Pontevedra. The lines between streets are grapevines. All rows have a width of 3 m. The rows sown with the *D. tenuifolia* and *S. alba* mixture (rows 1 and 10) are 23.5 m and 33 m long, respectively. The rows sown with *A. cerefolium* (rows 4 and 7) each measure 25 m in length. The rows with spontaneous vegetation measure 25 m (rows 2, 3, 5 and 6), 27 m (row 8), and 33 m (row 9) in length.

In the second season, 2024–2025, the experimental designs were:

Experimental plot 3

The same plot as in the previous year in **Areeiro**, Pontevedra, maintaining the same experimental design, the same treatments, and the same sowing density as previously described (***Figure 51***). Sowing date was October 14th, 2024.

Experimental plot 4

A grapevine plot in **Cenicientos**, Madrid (40°15'29.1"N;4°30'46.8"W) was selected in the fall of 2024 to conduct further assays in the Central region of Spain. Rows sown with a mix of seeds were alternated with rows of spontaneous vegetation according to the experimental design shown in Figure 52. The seed mixture consisted of: *S. alba* (32 kg/ha), *D. tenuifolia* (3.2 kg/ha), *A. cerefolium* (48 kg/ha) and *M. moricandia* (4.8 kg/ha). Sowing was carried out on 30th October 2024.

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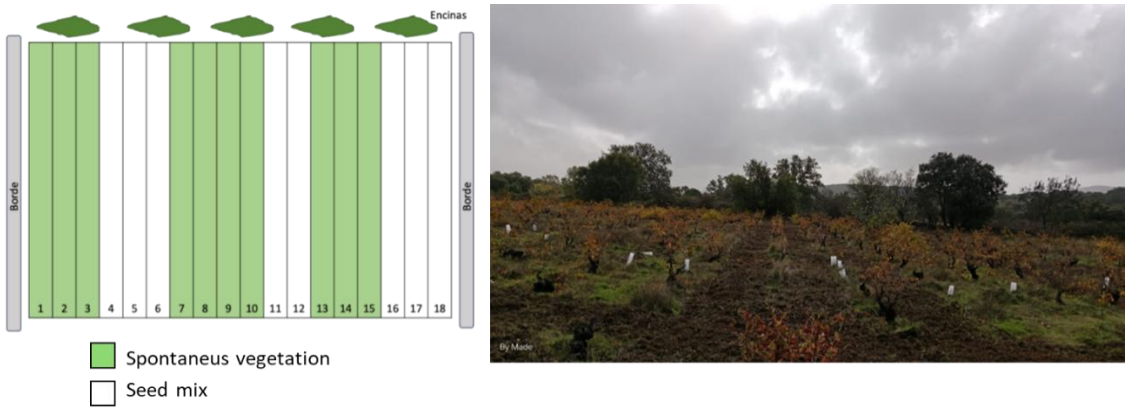


Figure 52. Experimental design and image of vineyard in Cenicientos, Madrid. All rows are 2 m wide and 50 m long.

Experimental plot 5

Another vineyard in **Felanitx,** Mallorca (39° 30' 14.60"N; 3° 9' 27.26' W) where double rows of plant covers consisting of *A. cerefolium* and others of *S. alba* were sown, leaving rows with natural vegetation as a control (Figure 53). The seeds were sown between 15th and 18th October 2024, and the sowing density was 20 kg/ha of *S. alba* and 30 kg/ha of *A. cerefolium*.

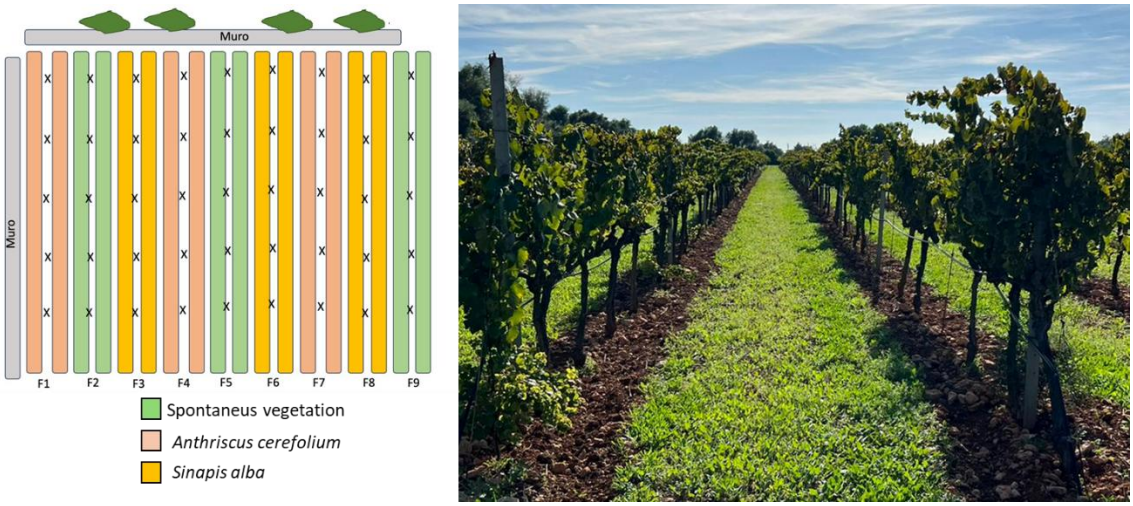


Figure 53. Experimental design and image of vineyard in Felanitx, Mallorca. All rows were double rows with a total length of 160 m. Each double row consists of two adjacent single rows: one 2.25 m wide and the other 2.75 m wide.

4.2.2.2. Sampling

All experimental plots were sampled once every two weeks during the period when nymphs are present (March-June). First, we recorded the number of foams per plant, the number of nymphs per foam, and the position (low, medium, or high) in the ground vegetation in a sampling unit.

The sampling unit differed in some plots. In 2024, the sampling unit in all plots was 100 × 25 cm (***Figure 54***). Three sample units randomly distributed over each row were selected per

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sampling date. In 2025, the sampling scheme varied to accommodate the number of samples to the size of the plots: five sample units per row were used in Felanitx, while the entire row was sampled for the presence of foams in the Areeiro field, which was much shorter (only 25 m long instead of 160 m long in Felanitx). In addition, the percentage of ground cover vegetation was assessed by visual inspection in each row and plot. In all cases, sampling was non-destructive: after visual detection of foams, the number of nymphs per foam were counted after gently removing the foam with the tip of a fine brush and then were left on their host plants.



Figure 54. Sampling ground vegetation (sampling unit of 30x 100 cm)

Given that the sampling scheme for each experimental field was different, the results obtained were always referred in terms of the number of nymphs per square meter.

4.2.2.3. Statistical Analysis

To evaluate the overall density of nymphs across locations and cover crop treatments, a generalized linear mixed model (GLMM) was fitted using the glmmTMB function with a negative binomial (nbinom2) error distribution and log link. The model included cover_crop as a fixed factor and location/block, year, and rowID as random effects. Sampling area was included as an offset (log(area)). Model significance was assessed using Type III Wald χ^2 tests (car::Anova), and post-hoc pairwise comparisons were performed using Tukey's adjustment (emmeans). Estimated marginal means and standard errors were used to compute means (±SE) per cover crop type, and significance letters were obtained using the multcompView package.

Site specific analysis: for Felanitx, nymph density data were analyzed with a GLMM including cover_crop as a fixed effect and block/rowID as random effects, using a negative binomial distribution. To assess temporal variation, additional models were fitted separately for each sampling date. For each date, a GLMM was run with cover_crop as a fixed factor and block/rowID as random effects. Post-hoc Tukey comparisons and estimated marginal means were obtained for each model. Data from Areeiro were analyzed first across both years (2024–2025) using a GLMM with cover_crop as fixed effect and random intercepts for year and block/rowID. Subsequently, separate analyses were conducted by year to evaluate interannual differences. For both 2024 and 2025, additional GLMMs were fitted independently for each sampling date following the same model structure as in Felanitx. For

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Cenicientos, a GLMM with a negative binomial distribution was used to test for differences among cover crop types: Post-hoc pairwise comparisons (Tukey) were performed, and mean densities (±SE) were calculated for each treatment.

4.2.2.4. Spatial Analysis by Distance Indices (SADIE)

The spatial distribution of *P. spumarius* nymphs was studied using the SADIE software (v.1.22) (Perry, 1995). The data obtained were arranged in coordinates, with the X axis representing the row and the Y axis representing the vine number. SADIE calculates the distance to regularity (D), that is the minimum value of a total distance that *P. spumarius* inidividuals (in this case) have to move, so that the population are dispersed as regularly as possible. The spatial pattern of data was described by the index of aggregation, **Ia**, the probability value of Ia, **Pa**, the positive patch cluster index, **Vi**, and the negative gap cluster index, **Vj** (Perry, 1999). The value of Ia indicates whether or not the sample is aggregated, being aggregated if Ia>1 (Pa <0.5), a spatialy random sample if Ia=1 (Pa≈0.5) and a regular sample if Ia <1 (Pa≈1). The probability that the null hypothesis of spatial randomness is true and the arrangement is significantly aggregated at the 5% level is denoted as Pa (Perry et al., 1996, 1999). Contour maps were drawn using the Kriging interpolation method with the flow value of each unit, representing values of v close to the sample mean (v=0) with a blue dotted line, those above the mean vi > 1.5 with thick red lines (peaks) and vj <1.5 with thick blue lines (troughs), and v values close to unity (vi ≈1, vj ≈ -1) with finer solid blue lines. Using the clustering index of each sample unit (v), point and circle maps were created, with values of v>0 marked in red and v <0 in blue. Both maps were created and superimposed using the SURFER (Surfing Mapping System) v.9.0 programme, which allows for the graphical representation of patches and gaps.

4.2.3. Field evaluation of green manure and cover crop mixtures for controlling juvenile populations of spittlebugs in Apulia

Six field trials were carried out in the Apulia region (southern Italy) during the winter-spring periods of three consecutive years (2022-2023, 2023-2024, and 2024-2025). The trials were conducted in sites previously identified as hosting high populations density of *P. spumarius*. The objective was to assess the suitability of commonly used green manure species (i.e., cover crops) for reducing density of juvenile stages of *P. spumarius*.

During the 2022-2023 season, two trials were set up: one in Santeramo in Colle (BA) (Trial A) and one in Locorotondo (BA) (Trial B). In each trial, the following cover crop treatment were sown at the specified seed rates reported as following:

1) *Cruciferae* mixture: total 15 kg/ha (*Sinapis alba* (5 kg/ha)*, Brassica juncea* (5 kg/ha)*, Raphanus sativus* (5 kg/ha));
2) Fabaceae mixture: total 15 kg/ha (*Trifolium incarnatum* (7.5 kg/ha) *Medicago sativa* (7.5 kg/ha));
3) Mixture of *Cruciferae* (15 kg/ha) + *Fabaceae* (15 kg/ha) + *Lolium italicum* (10 Kg/ha).

All treatments were compared with undisturbed natural ground vegetation and with ploughed soil, all conditions under mulched and non-mulched conditions (***Table 12***).

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During the winter-spring period of 2023-2024, two additional field trials were performed in Gallipoli (Le) (Trial C) and Cisternino (Br) (Trial D). These trials followed the same experimental design adopted in the Trial A (2022-2023); with the inclusion of an additional treatment consisting of *Phacelia tanacetifolia*. As in the other treatments, the effects of the cover crop were evaluated under two conditions: (i) undisturbed *P. tanacetifolia* plots, and (ii) plots mulched when juvenile *P. spumarius* reached the 3rd-4th instars. All four trials performed in 2022-2023 and in 2023-2024 were arranged in a randomized block design with five replicates per treatments and plots of 20 m^2.

Table 12. Description of the treatments evaluated in field trials A, B, C and D.

Treatment	Trials			
	Santeramo	Locorotondo	Gallipoli	Cisternino
Undisturbed ground vegetation	x	x	x	x
Undisturbed ground vegetation + mulching	x	x	x	x
Sowing Cruciferae	x	x	x	x
Sowing Cruciferae + mulching	x	x	x	x
Sowing Fabaceae	x	-	x	x
Sowing Fabaceae + mulching	x	-	x	x
Sowing (Grasses + Cruciferae + Fabaceae)	x	x	x	x
Sowing (Grasses + Cruciferae + Fabaceae) + mulching	x	x	x	x
Sowing Phacelia	-	-	x	x
Sowing Phacelia + mulching	-	-	x	x
Plouging	x	x	x	x

During the winter-spring of 2024-2025, two large-scale field trials were established: one in Squinzano (LE) (Trial E) and the others in Andria (BAT) (Trial F). These trials aimed to validate the cover crop mixture that had shown the best performance in previous four trials. Specifically, a mixture composed of Poaceae, Cruciferae and Fabaceae at the same rates used in the previously trials, was tested in large plots of 880 m^2 (Squinzano) and 800 m^2 (Andria). Each treatment was replicated twice, and the mixture was compared with tilled soil when *P. spumarius* juveniles reached the 3rd–4th instars.

Across all six trials, the sowing of each cover crop was carried out in December following careful soil preparation. Sowing was done manually, and the seeds were subsequently incorporated superficially into the soil (***Figure 55*** and ***Figure 56***).

A single survey was carried out in each trial during the spring, 5-6 days after mulching or ploughing of the ground vegetation, which was performed when *P. spumarius* juveniles had reached the 3rd-4th instars. To quantify the abundance of juvenile spittlebugs, a quadrat sampling method was used, employing a rectangular metal frame of 0.25 m^2 (100 cm × 25 cm). Within each quadrat, both vegetation and soil surfaces were visually inspected to detect spittle masses and nymphs. For each quadrat, the number of spittle masses and the number of individuals contained within each mass were recorded. All individuals were identified to

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species level (*P. spumarius* and *N. campestris*). In trials A to D, five quadrats were randomly placed within each plot. In the two validations trials (E and F), sixty transect were examined per plot to account for the larger plot size.



Figure 55. Manual sowing and incorporation of the seeds into the soil.

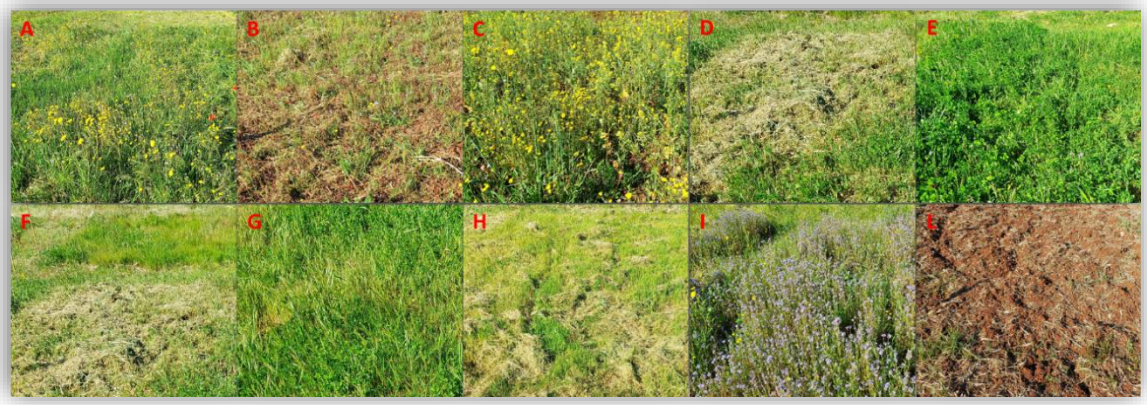


Figure 56. Picture of experimental plots in trial D: *A) Undisturbed natural ground vegetation; B) Undisturbed natural ground vegetation + mulching; C) Sowing Cruciferae; D) Sowing Cruciferae + mulching; E) Sowing Fabaceae; F) Sowing Fabaceae + mulching; G) Sowing Poaceae, Cruciferae and Fabaceae in mixture; H) Sowing Poaceae, Cruciferae, Fabaceae in mixture + mulching; I) Sowing Phacelia tanacetifolia; L) Ploughing*

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4.3. Results

4.3.1. Survival of *Philaenus spumarius* nymphs on selected cover crop plant species under greenhouse conditions

The data from the first experimental repetition was discarded due to the unusually high mortality observed in the control treatment (*S. oleraceus*). In the second repetition, mortality after 24 hours reached 50% in *S. verbenaca* and 61% in both *M. moricandioides* and *P. rhoeas*, while remaining 0% in the control (*S. oleraceus*). Mortality in *S. oleraceus* stayed at 0% until day 10, and after it increased to 6%. In contrast, *M. moricandioides* reached 100% mortality by this time, and *P. rhoeas* 94%, while *S. verbenaca* showed intermediate values (72%) (***Figure 57***). These findings suggested that species such as *M. moricandioides*, *S. verbenaca*, *P. pratensis*, and *P. rhoeas* may be suitable candidates for cover crop management due to their consistent effects on nymph mortality. Among them, *M. moricandioides* was selected to be included in the seed mixture used in the Cenicientos field assay sown in the fall 2024.

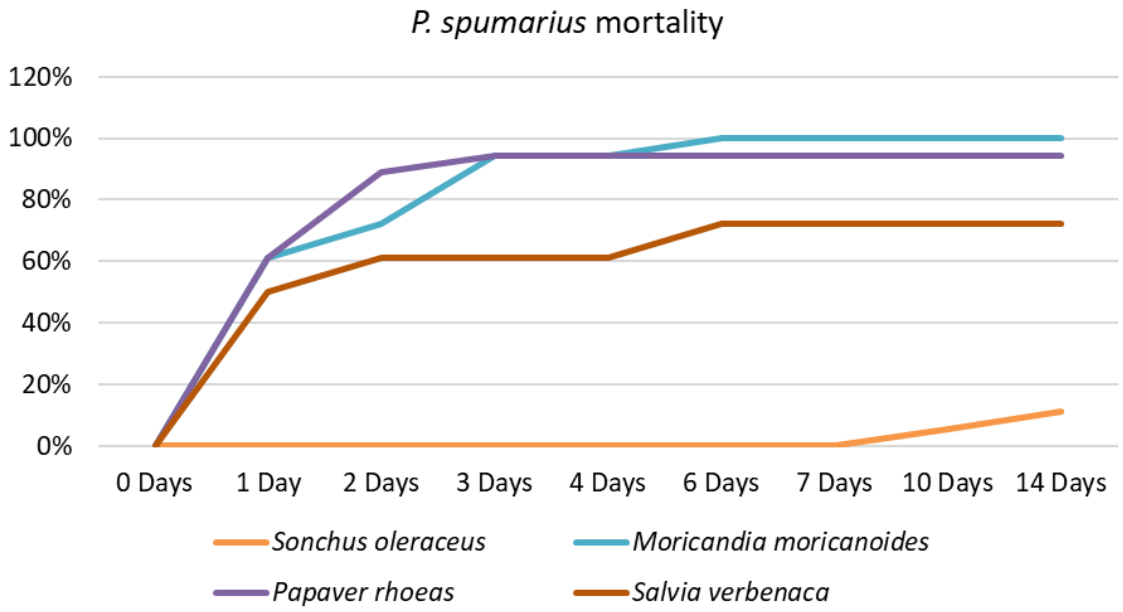


Figure 57. Mortality of *P. spumarius* in different plant species grown under greenhouse conditions.

4.3.2. Efficacy of ground cover vegetation to reduce the population density of *Philaenus spumarius* in different field crops in Spain

4.3.2.1. Experiments conducted in 2023-2024

Pego, Valencia

No nymphs nor adults of *P. spumarius* were captured during sampling conducted in spring of 2024. This result is likely attributable to the severe drought affecting the area at the time of the study. As no captures were made in 2023–2024 or at the start of the 2024–2025 campaign, this location was excluded from further analysis.

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Valdepiélagos, Madrid

Germination began around 19 December, approximately one month after sowing. The first nymphs were detected on 15 March along field borders, including *P. spumarius* and *N. campestris*, but none within the experimental plots. Due to the very low nymph densities, the site was not included in further assessments and the Valdepielagos experimental site was discarded.

Areeiro, Pontevedra

Germination was first observed on 23 October 2023. Only *A. cerefolium* and *S. alba* germinated; *D. tenuifolia* failed to do so and was therefore excluded from the analysis. Spittlebug population density was rather low along all sampling dates in spring 2024. Although no statistically significant differences were detected among treatments, nymph densities tended to be lower in rows sown with *S. alba* than in those with *A. cerefolium* or spontaneous vegetation (Figure 58a). Over time, nymph density increased in spontaneous vegetation and *A. cerefolium* rows, while it decreased in *S. alba*, potentially due to the toxic effects associated with certain phenological stages of *S. alba*, as reported by Morente et al. (2022) (Figure 58b). Overall, *S. alba* appeared to be the most promising species selected for this site.

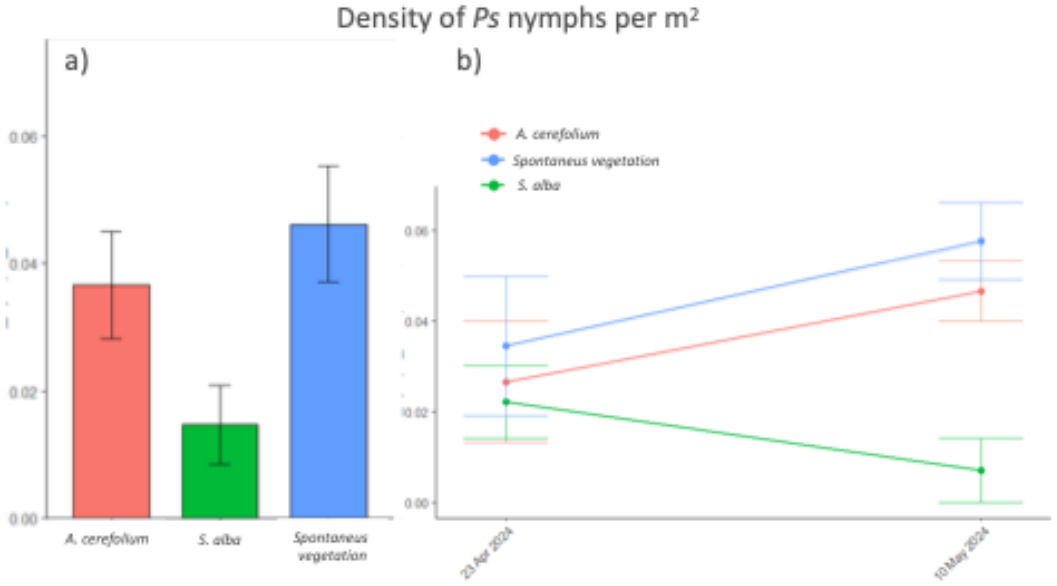


Figure 58. Density of *P. spumarius* nymphs in different cover crop in Areeiro, Pontevedra (2024), (a) overall total densities (nymphs/m2) and (b) densities (nymphs/m2) by date.

4.3.2.2. Experiments conducted in 2024-2025

Areeiro, Pontevedra

In 2025 *D. tenuifolia* didn't germinate at all, so the comparisons were made between spontaneous vegetation, *S. alba* and *A. cerefolium*. Contrary to the previous year, nymph densities were lowest in *A. cerefolium* rows, although differences were not statistically

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significant (Figure 59a). Nymph densities increased across all treatments throughout spring (Figure 59b). Other potential vectors of *X. fastidiosa* were also detected, including *Aphrophora alni*, *N. campestris* and *Cicadella viridis*.

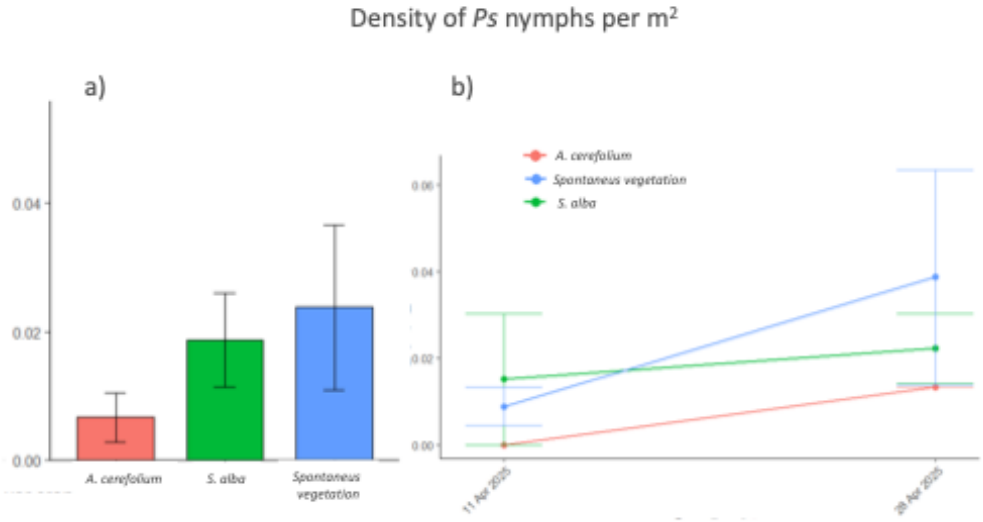


Figure 59. Density of P. spumarius nymphs in different cover crops in Areeiro, Pontevedra (2025), (a) overall total densities (nymphs/m2) and (b) densities (nymphs/m2) by date.

Felanitx, Mallorca

Germination started on 30 October, two weeks after sowing. Nymph densities were significantly lower in rows with *S. alba* than in those with spontaneous vegetation, while *A. cerefolium* displayed intermediate densities (Figure 60a). Density declined over time in all treatments except *A. cerefolium*, which showed a temporary increase during the second sampling date (Figure 60b). *N. campestris* individuals were also recorded.

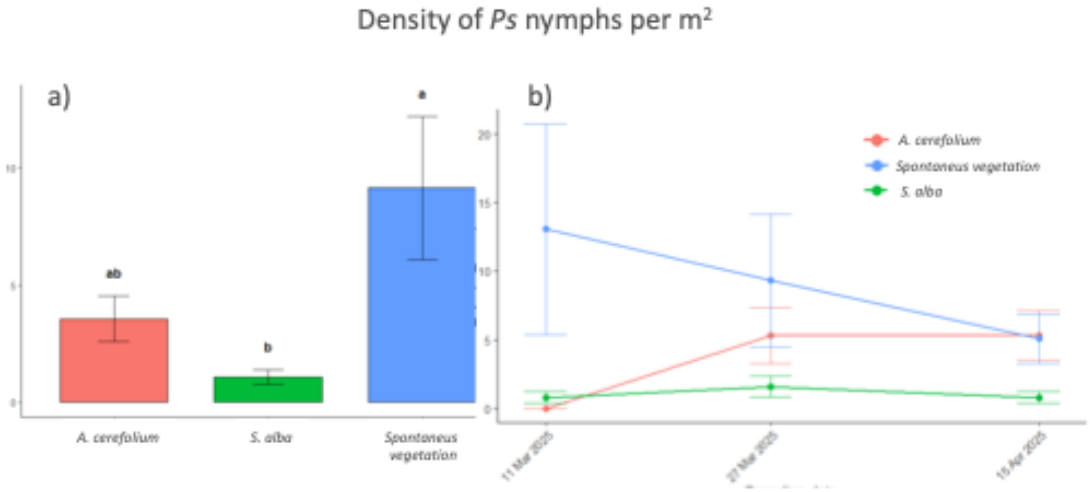


Figure 60. Density of *P. spumarius* nymphs in different cover crop in Felanitx, Mallorca. (a) overall total densities (nymphs/m2) and (b) densities (nymphs/m2) by date.

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Spatial Analysis of the distribution of nymphs in each experimental field

A SADIE (Spatial Analysis by Distance Indices) analysis conducted on the final sampling date (15 April) in the Felanitx farm revealed an aggregated distribution (Ia = 1.476). Clustering indices (Vi = 1.270; Vj = –1.477) indicated that aggregation occurred predominantly near the wooded boundaries of the plot, which may act as reservoir for *P. spumarius*, consistent with previous findings (Cornara et al., 2021) (Figure 61). Actually, the borders located in the North and West of the experimental plot were close to wild olives and oak trees.

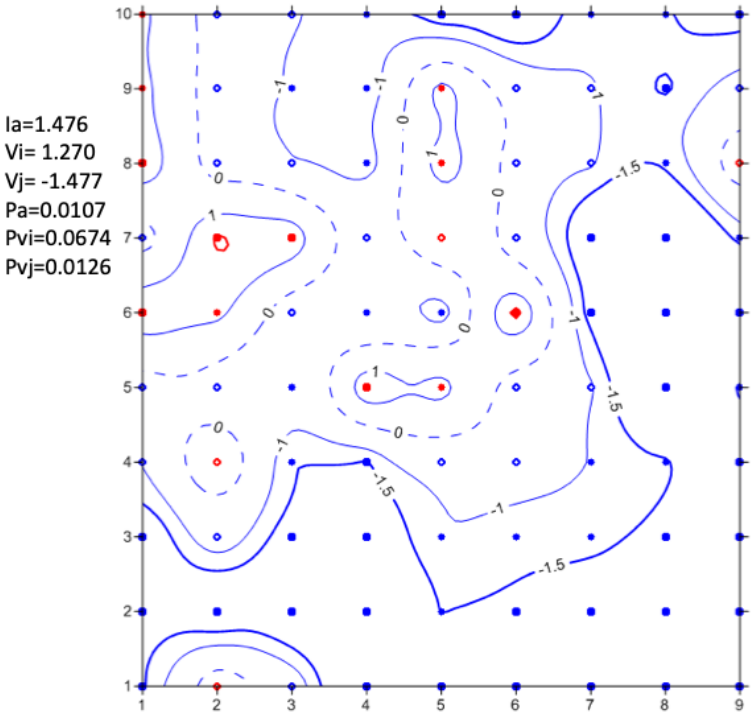


Figure 61. Classed post map of the spatial distribution of mean number of *P. spumarius* nymphs on April 15th in Felanitx.

Cenicientos, Madrid

Due to the uneven and poor germination of the species selected for the field study, the only plant species that was actually considered for analysis was *A. cerefolium*. The presence of *A. cerefolium* resulted in a significant reduction in the population density of *P. spumarius* compared to the density observed in areas with spontaneous vegetation, as demonstrated for both sampling dates (Figure 62).

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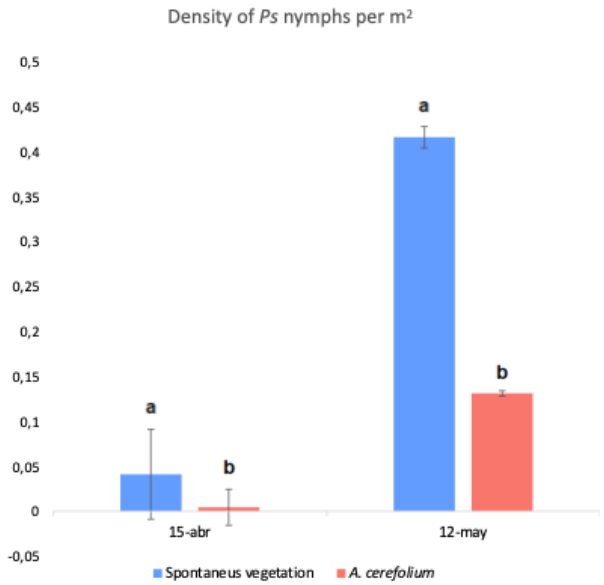


Figure 62. Density of P. spumarius nymphs in different cover crops in Cenicientos, Madrid in two sampling dates.

Spatial Analysis of the distribution of nymphs in each experimental field

Spatial analyses were performed for both sampling dates (Figure 63 and Figure 64). In each case, an aggregated distribution of nymphs was detected, with aggregation indices exceeding 1 (Ia = 1.599 on 15th April; Ia = 3.277 on 12nd May). On 15th April, the Vi (1.662) and Vj (-1.663) values indicated the locations of patches, which, similar to the Felanitx plot, were concentrated near wooded areas surrounding the experimental field (Figure 633).

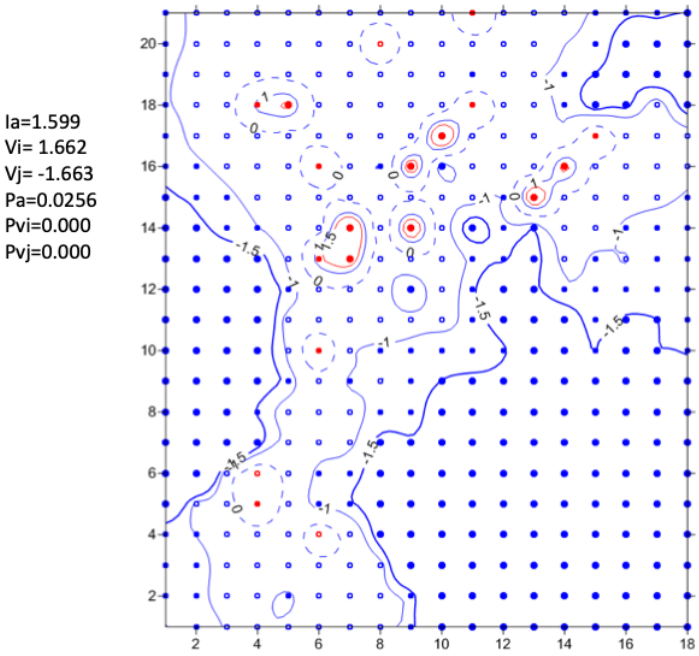


Figure 63. Classed post map of the spatial distribution of mean number of *P. spumarius* nymphs on April 15th in Cenicientos.

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By 12nd May, (Figure 64), the patches had expanded across the plot (Vi = 3.466; Vj = –3.173). In addition to those near the wooded borders (mainly oak trees), new patches appeared in more central areas. Nevertheless, patch density remained higher at the edges than in the central areas. Some locations without data points were observed; these reflect missing data for technical or logistical reasons and do not represent true gaps in nymph distribution. Additionally, several rows present in the previous sampling were not available for resampling—specifically rows –6 to –1—and were replaced with rows of spontaneous vegetation due to these sampling limitations. Overall, the pattern continued to show a tendency for patches to cluster closer to the edges of the plot.

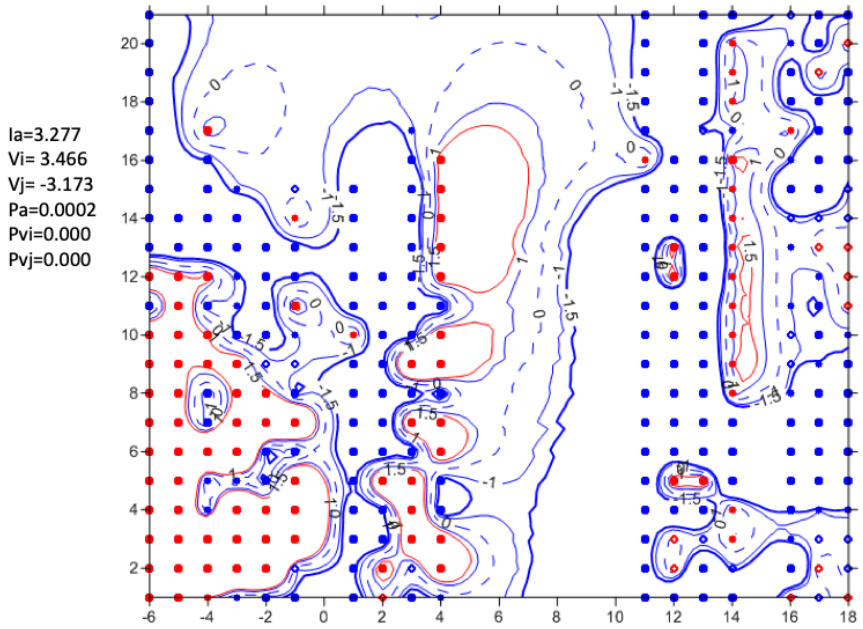


Figure 64. Classed post map of the spatial distribution of mean number of *P. spumarius* nymphs on May 12nd in Cenicientos.

Taken together, all field trials demonstrate **the effectiveness of the germinated plants, *A. cerefolium* and *S. alba* to reduce *P. spumarius* population densities in the field**, as previously shown under indoor conditions (Morente et al., 2022). Different field layouts were tested to evaluate whether specific row arrangements could enhance efficacy, given previous reports suggesting improved performance when certain species are positioned at plot borders (Cook et al., 2007). However, in these trials, no evidence indicated that plot arrangement influenced performance.

A limiting factor identified was the sowing density in some plots, particularly in Cenicientos, where germination was irregular and most of the seeds failed to germinate except those of *A. cerefolium*. To address this problem, additional replicates and higher seed densities are being used in the 2025–2026 assays (Figure 65). Sowing for the 2025 season has already been completed in all plots: in Areeiro on 9th September, in Felanitx on 24th September, and in Cenicientos on 13th November. The sowing density of both *A. cerefolium* and *S. alba* will be increased.

Based on evidence supporting tilling and regular mowing as effective management methods (Sanna et al., 2021), a new treatment will be introduced consisting of tilled rows without sowing, in addition to rows of spontaneous vegetation, *A. cerefolium*, and *S. alba*. Considering

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the characteristics of the three plots, the next experimental design will include at least three blocks, with all row treatments arranged randomly to ensure statistical robustness.

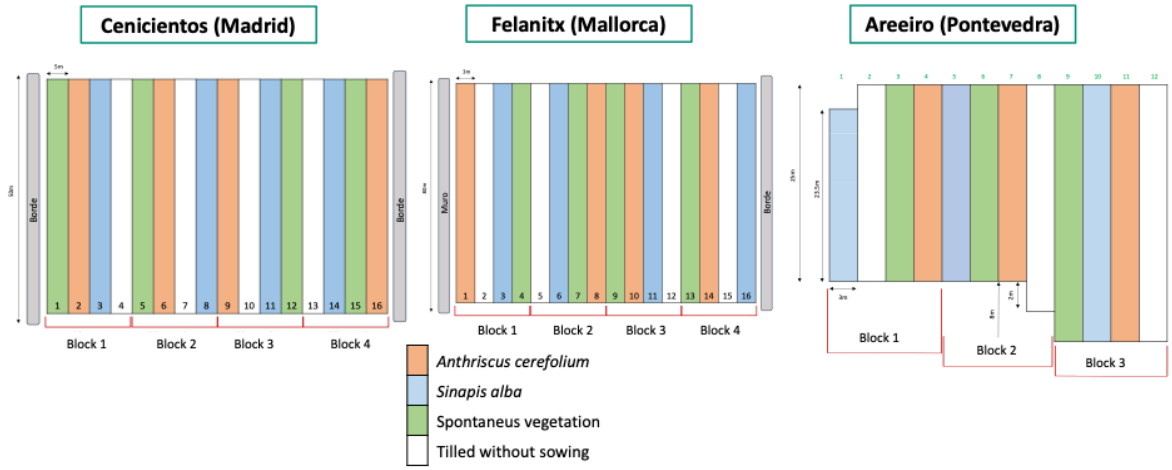


Figure 65. Experimental design of the plots in Cenicientos, Felanitx and Areeiro for the 2025-2026 trials.

4.3.3. Field evaluation of green manure and cover crop mixtures for controlling juvenile populations of spittlebugs in Apulia

Trial A – Santeramo in Colle (BA)

The average number of spittle masses and nymphs in natural, undisturbed ground vegetation plots were 18.5 and 22.9 per square meter, respectively (Table 13). A significant reduction was observed under the same conditions when plots were mulched in spring, with mean values of 2.8 spittle masses and 3.9 nymphs per square meter. After ploughed plots, that exhibit an Abbott index closed to 100%, the mulched plots showed the highest Abbott's index values, equal to 84.9% and 83.1%, for spittle masses and nymphs, respectively (Figure 66).

Among the cover crop, the best results was obtained with the mixture of Cruciferae + Fabaceae + Poaceae, with a mean number of 8.3 spittle masses and 7.5 nymphs per square meter (Table 13), and an Abbott's index value of 64% and 59.7%, respectively (Figure 666). Lower effects were recorded when sowing Fabacee or Crucifereae alone, with mean densities of approximately 10 spittle masses and 13.5 nymphs per square meters (Table 13), and an effectiveness values of 39.6% (sowing Cruciferae) and 45.4% (sowing Fabaceae) for spittle masses, and 35.5% (sowing Cruciferae) and 41.9% (sowing Fabaceae) for nymphs (Figure 66).

In all cover-crop treatments, the introduction of mulching further enhanced efficacy. The strongest improvement was observed in plots sown with Fabaceae and subsequently mowed, which reached Abbott's index values of 77% for spittle masses and 79.7% for nymphs (Figure 66). Regarding *N. campestris*, densities were very low across all treatments (<1.3 spittle masses and nymphs/m^2), preventing the detection of significant differences among the various soil management strategies (Table 13).

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Table 13. Number of spittle masses and nymphs/m^2 of *P. spumarius* and *N. campestris* in Trial A (2023).

Treatment	*P. spumarius*		N. campestris	
	Number of spittle masses	Number of nymphs	Number of spittle masses	Number of nymphs
Undisturbed	18.5 a	22.9 a	1.2 a	1.3 a
Undisturbed + mowing	2.8 cd	3.9 bc	0.3 a	0.3 a
Cruciferae	11.2 ab	14.8 ab	0.5 a	0.5 a
Cruciferae + mowing	4.8 bcd	6.4 bc	0.3 a	0.3 a
Fabaceae	10.1 bc	13.3 ab	1.3 a	1.3 a
Fabaceae + mowing	4.3 bcd	4.7 bc	0.0 a	0.0 a
Mix	7.5 bcd	8.3 bc	0.7 a	0.7 a
Mix + mowing	5.6 bcd	6.7 bc	0.7 a	0.7 a
Ploughing	0.7 d	0.7 c	0.0 a	0.0 a

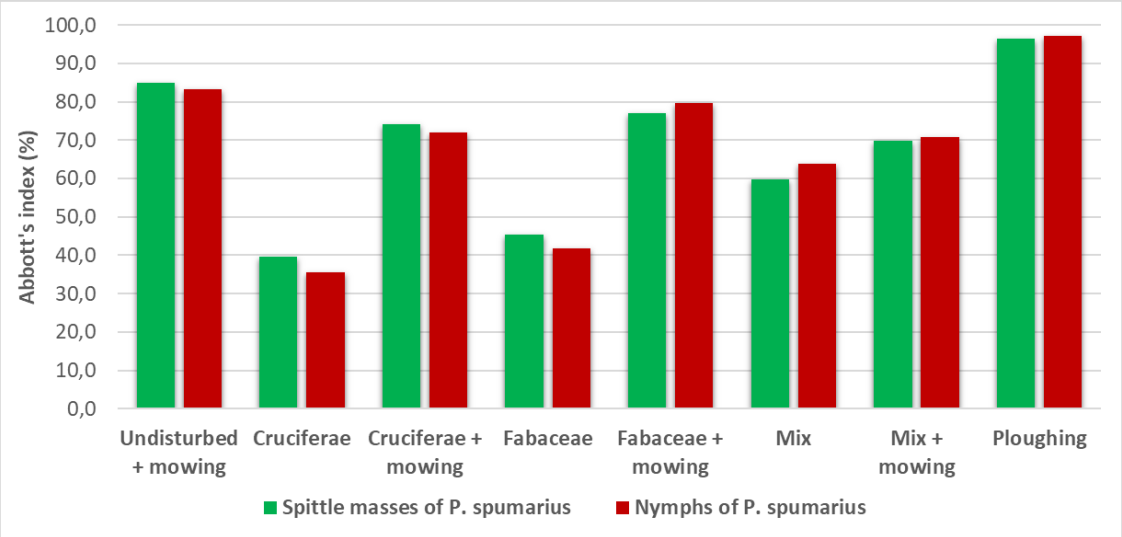


Figure 66. Abbott's index (%) determined on mean number of spittle masses and nymphs of *P. spumarius* (Trial A).

Trial B – Locorotondo

The density of *P. spumarius* in undisturbed spontaneous vegetation plots was 6.8 spittle masses/m^2 and 7.3 nymphs/m^2 (Table 14). Under these conditions of low population density, no significant effects were detected in most treatments, except in ploughed plots and in plots sown with the mixture of Poaceae, Cruciferae, Fabaceae combined with mowing. In both treatments almost all nymphs died, resulting in Abbott indices higher than 94% (Figure 67).

No improvement was detected in the treatments subjected to mulching compared with the undisturbed and self-sown treatments. This lack of effect was likely due to the floristic composition being dominated by rosette-forming species, within which the juveniles nest and could escape the mechanical action of mulching. For *N. campestris*, mean densities of spittle masses and nymphs were very low across all treatments (Table 14), preventing the detection of significant differences among the various soil management strategies.

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Table 14. Number of spittle masses and nymphs/m^2 of *P. spumarius* and *N. campestris* in Trial B (2023).

Treatment	*P. spumarius*		N. campestris	
	Number of spittle masses	Number of nymphs	Number of spittle masses	Number of nymphs
Undisturbed	6.8 a	7.3 a	0.3 a	0.3 a
Undisturbed + mowing	6.7 a	8.8 a	0.1 a	0.1 a
Cruciferae	3.9 a	4.8 a	0.4 a	0.4 a
Cruciferae + mowing	2.0 a	2.3 a	0.5 a	0.5 a
Mix	5.1 a	6.5 a	0.1 a	0.1 a
Mix + mowing	0.4 b	0.4 b	0.0 a	0.0 a
Ploughing	0.1 b	0.1 b	0.0 a	0.0 a

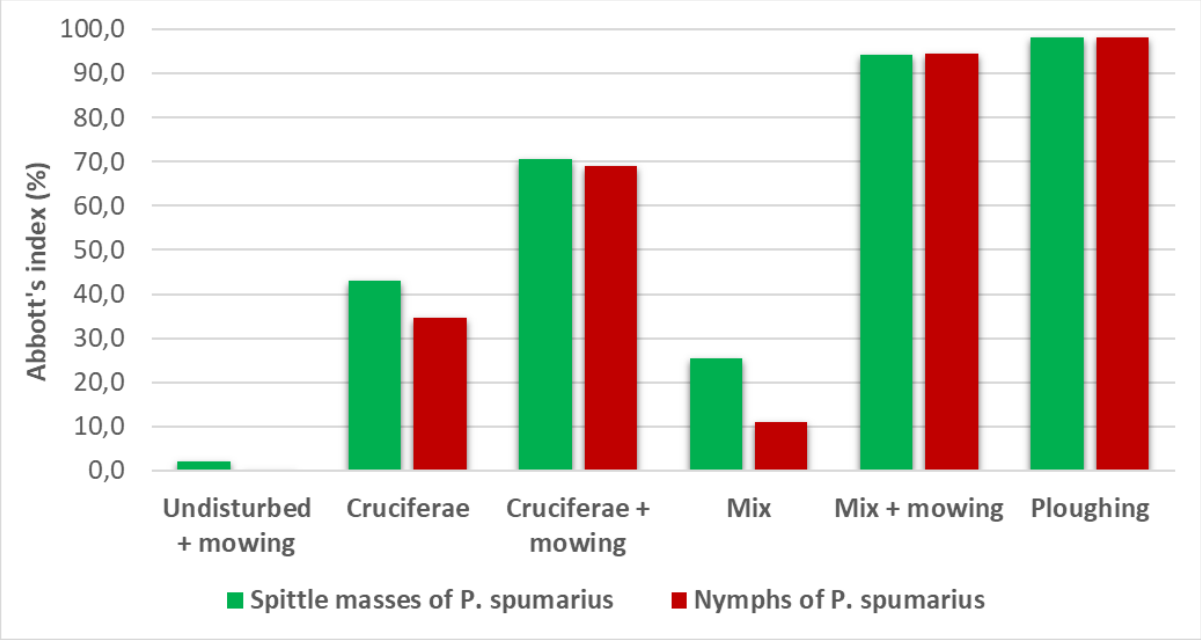


Figure 67. Abbott's index (%) determined on mean number of spittle masses and nymphs of *P. spumarius* (Trial B).

Trial C – Gallipoli (LE)

Plots with undisturbed spontaneous vegetation showed the highest densities of *P. spumarius*, with average values of 15.8 spittle masses/m^2 and 19.4 nymphs/m^2 (Table 15). No effects were observed when sowing Cruciferae and Fabaceae alone, as the numbers of spittle masses and nymph were similar to those recorded in undisturbed vegetation. Conversely, the mixture composed of Poaceae, Cruciferae and Fabaceae significantly reduced (p=0.05) *P. spumarius* densities, achieving an efficacy of 64.1% for spittle masses and 67.4% for nymphs (Figure 68). Across all treatments, mulching further improved effectiveness. Among the mulched treatments, the lowest reduction was observed in plots sown with Fabaceae, where densities

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reached 8.9 spittle masses and 9.6 nymphs per square meters. The best results were obtained in plots sown with the mixture of Cruciferae, Fabaceae and Poaceae, which showed mean values of 2.4 spittle masses and 3.0 nymphs per square meter, an even better performance than that observed in tilled plots, where 6.6 spittle masses and 7.0 nymphs/m^2 were recorded (Table 15). As a consequence, the Abbott's indices for mowing treatments were markedly higher than those obtained for the same species without mowing, ranging from 43.7% (Fabaceae + mowing) to 84.5% (Poaceae-Cruciferae-Fabaceae mixture + mowing) for spittle masses, and from 50.9% (Fabaceae + mowing) to 84.6% (Poaceae-Cruciferae-Fabaceae mixture + mowing) for nymphs (Figure 68). Regarding *N. campestris*, densities were very low across all treatments (<3.2 spittle masses and nymphs/m^2; Table 15) preventing the detection of significant differences among the soil-management strategies adopted.

Table 15. Number of spittle masses and nymphs/m^2 of *P. spumarius* and *N. campestris* in Trial C (2024).

Treatment	Number of spittle masses of *P. spumarius*/m^2	Number of nymphs of *P. spumarius*/m^2	Number of spittle masses of *N. campestris*/m^2	Number of nymphs of *N. campestris*/m^2
Undisturbed	15.8 a	19.4 a	0.4 a	0.4 a
Undisturbed + mowing	5.7 ab	6.3 bc	0.4 a	0.6 a
Cruciferae	14.6 a	16.3 ab	0.8 a	1.1 a
Cruciferae + mowing	4.0 b	4.2 bc	0.9 a	1.3 a
Fabaceae	13.1 ab	14.3 ab	2.1 a	2.4 a
Fabaceae + mowing	8.9 ab	9.6 b	0.6 a	0.7 a
Mix	5.7 b	6.3 bc	2.3 a	3.2 a
Mix + mowing	2.4 b	3.0 c	1.1 a	1.9 a
Phacelia	7.4 ab	7.9 b	1.3 a	1.8 a
Phacelia + moving	8.3 ab	8.8 b	0.1 a	0.1 a
Ploughing	6.6 ab	7.0 b	1,8 a	2,0 a

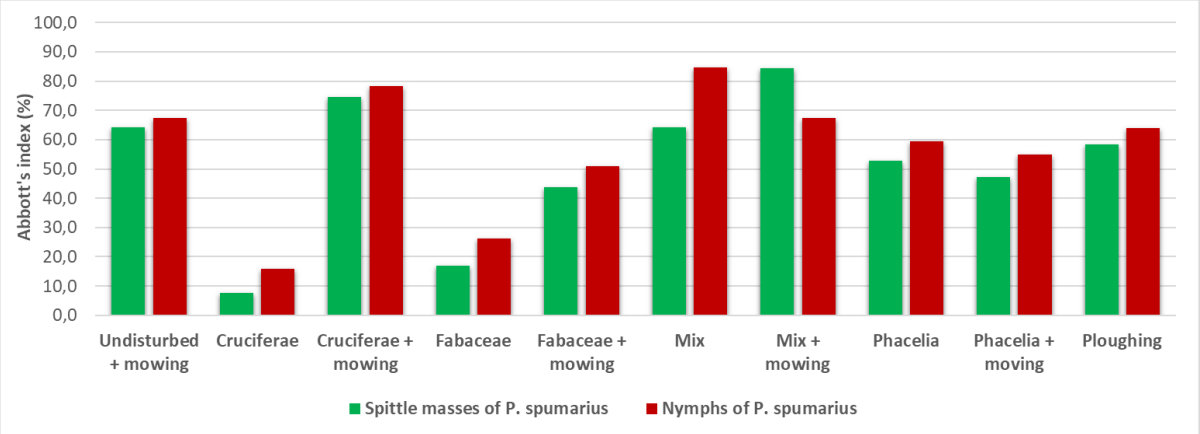


Figure 68. Abbott's index (%) determined on mean number of spittle masses and nymphs of *P. spumarius* (Trial C).

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Trial D – Cisternino (BR)

The highest densities of *P. spumarius*, with average values of 28.7 spittle masses/m^2 and 34.5 nymphs/m^2, were recorded in plots with undisturbed spontaneous vegetation (Table 16). The introduction of cover crops led to significant reductions in the densities of spittle masses and nymphs (p=0.05) in almost all treatments, except in plots sown with Cruciferae alone. In the remaining sown plots, excluding Cruciferae, values ranged from 3.9 (Cruciferae, Fabaceae, Poaceae in mixture) to 11.2 (Fabaceae) spittle masses/m^2 and from 4.1 (Cruciferae, Fabaceae, Poaceae in mixture) to 13.5 (Fabaceae) nymphs/m^2 (Table 16). Abbott's indices ranged from 50% in the treatment with Fabaceae alone to 82.6% in the Poaceae-Cruciferae-Fabaceae mixture for nymphs (Figure 699).

The addition of mowing further increased the effectiveness of the treatments. Abbott's indices ranged from 70.1% (Cruciferae + mowing) to 83.2% (Cruciferae, Fabaceae, Poaceae mixture+mowing) for spittle masses, and from 70.2% (Cruciferae + mowing) to 82.4% (Cruciferae, Fabaceae, Poaceae mixture+mowing) for nymphs (Figure 69). Ploughing was the most effective treatment overall, with Abbott's indices close to 100%, completely eliminating nymphs of *P. spumarius* (Figure 69). *N. campestris* showed very low densities (<1.7 individuals/m^2; Table 16), preventing an assessment of the effects of the different management strategies.

Table 16. Number of spittle masses and nymphs/m^2 of *P. spumarius* and *N. campestris* in Trial D (2024).

Treatment	Number of spittle masses of *P. spumarius*/m^2	Number of nymphs of *P. spumarius*/m^2	Number of spittle masses of *N. campestris*/m^2	Number of nymphs of *N. campestris*/m^2
Undisturbed	28.7 a	34.5 a	1.2 a	1.2 a
Undisturbed + mowing	9.7 b	11.2 b	0.0 a	0.0 a
Cruciferae	19.6 ab	23.6 ab	0.9 a	0.9 a
Cruciferae + mowing	6.7 bc	8.1 bc	0.4 a	0.4 a
Fabaceae	11.2 b	13.5 b	0.8 a	0.9 a
Fabaceae + mowing	4.8 bc	5.2 bc	0.0 a	0.0 a
Mix	3.9 bc	4.1 bc	1.7 a	1.7 a
Mix + mowing	3.7 bc	4.8 bc	0.4 a	0.4 a
Phacelia	8.0 bc	8.8 bc	0.1 a	0.1 a
Phacelia + moving	3.5 bc	3.5 bc	0.1 a	0.3 a
Ploughing	0.3 c	0.3 c	0.0 a	0.0 a

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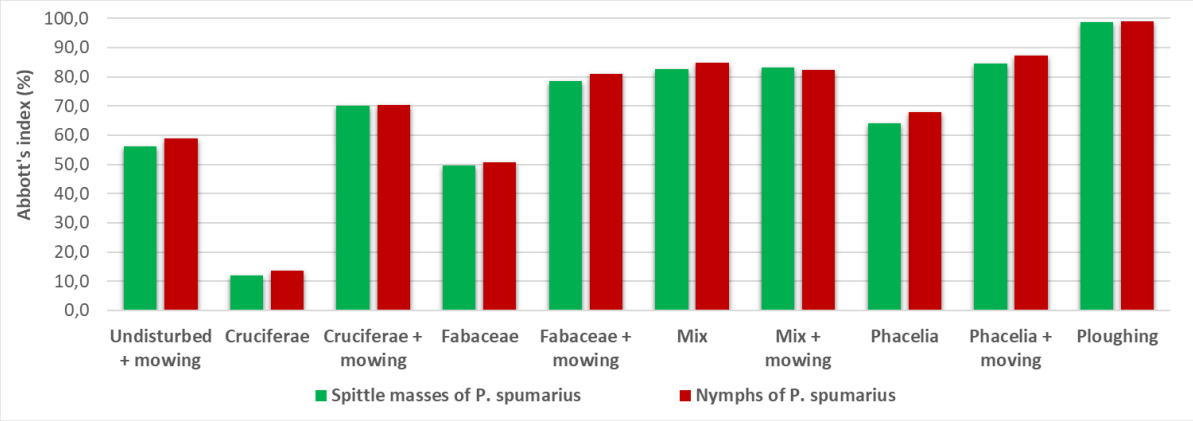


Figure 69. Abbott's index (%) determined on mean number of spittle masses and nymphs of *P. spumarius* (Trial D).

Trial E – Squinzano (LE)

This trial aimed to validate on a large scale the effectiveness of the cover crop that had shown the highest performance in previous trials. Although the experimental site was known to host high populations of *P. spumarius*, the survey revealed unexpectedly low densities in plots with undisturbed ground vegetation, with mean values of 4.0 spittle masses and nymphs per m² (Table 17). These unusually low values are likely attributable to the anomalous climatic conditions occurring during the early stages of *P. spumarius* development. In the first days of March until mid-March, temperatures constantly increased, but were subsequently followed by periods of heavy rainfall and a sharp temperature drop in the first week of April (Figure 71). Such fluctuations may have created unfavourable conditions for egg hatching or may have caused mortality in the early juvenile stages, resulting in the reduced densities observed during the survey.

Under these conditions, sowing the Poaceae, Cruciferae, Fabaceae mixture resulted in a 40% reduction in spittle mass and nymph densities (Figure 71), while no individuals or spittle masses were recorded in the ploughed plots. However, these differences were not statistically significant (p = 0.05), due to the overall low population levels observed.

For *N. campestris*, densities were even lower (<0.4 individuals/m²; Table 17), preventing reliable conclusions regarding the effectiveness of the agronomic strategies applied.

Table 17. Number of spittle masses and nymphs/m² of *P. spumarius* and *N. campestris* (2025) in Trial E.

Treatment	**Number of spittle masses of *P. spumarius*/m²**	**Number of nymphs of *P. spumarius*/m²**	**Number of spittle masses of *N. campestris*/m²**	**Number of nymphs of *N. campestris*/m²**
Undisturbed	4.0 a	4.0 a	0.4 a	0.4 a
Mix	2.4 a	2.4 a	0.0 a	0.0 a
Ploughing	0.0 a	0.0 a	0.0 a	0.0 a

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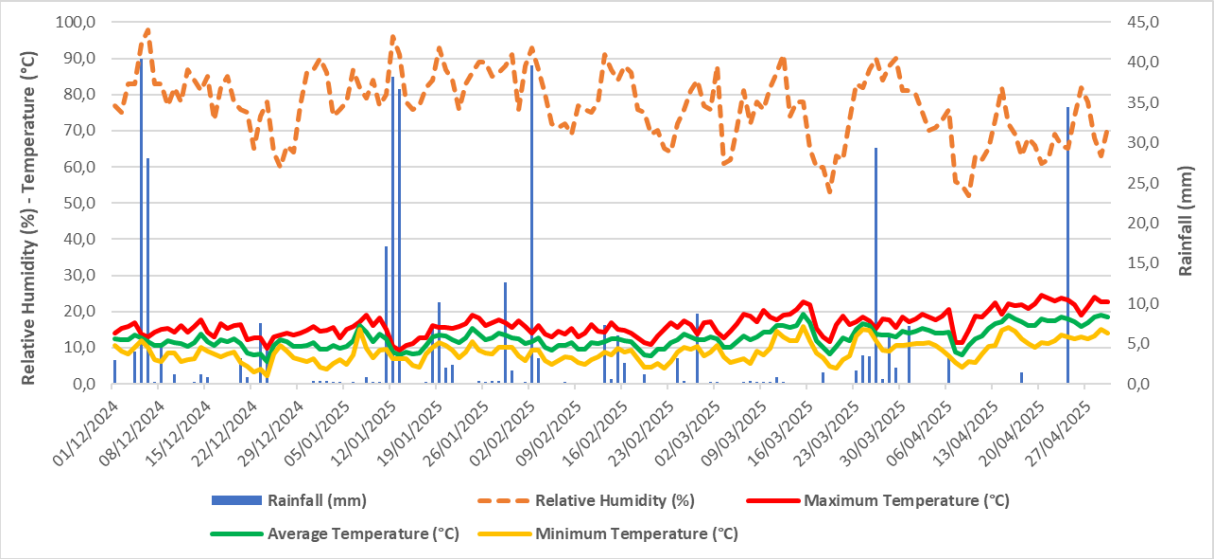


Figure 70. Meteorological trend recorded during the trial period in Squinzano (source: Puglia Civil Protection).

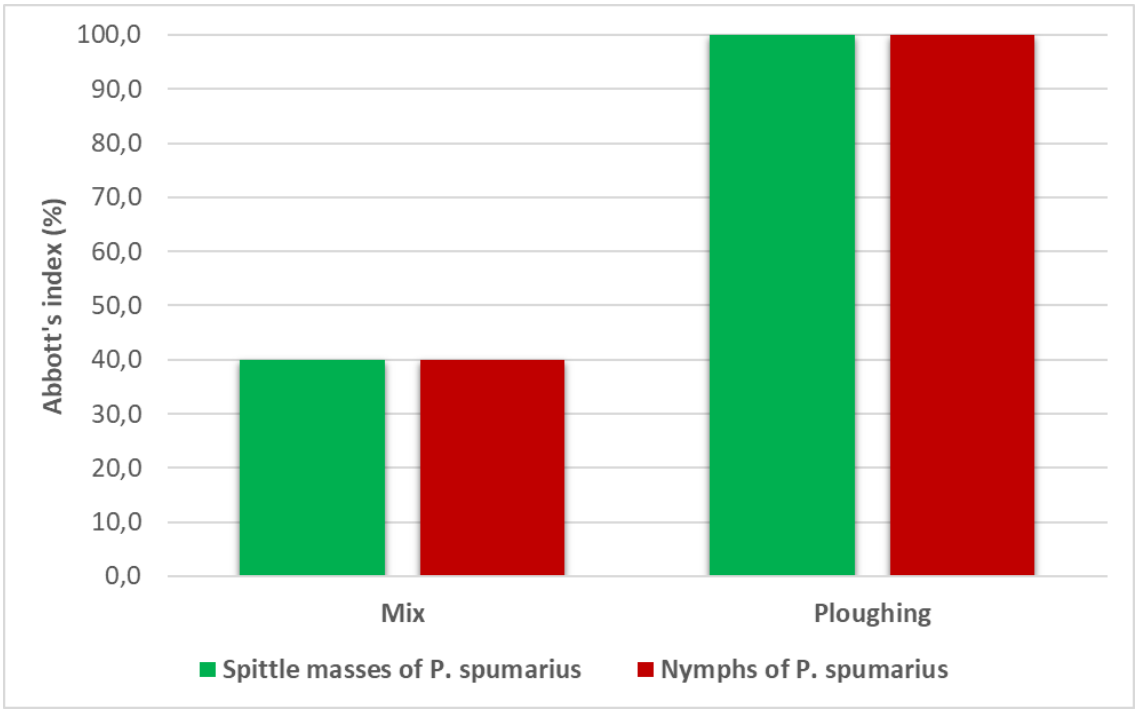


Figure 71. Abbott's index (%) determined on mean number of spittle masses and nymphs of *P. spumarius* (Trial E).

Trial F – Andria (BAT)

Very low densities of *P. spumarius* spittle masses and nymphs, 3.0 and 4.0 per m², respectively (***Table 18***), were observed in plots with undisturbed ground vegetation. This low population density, was unexpected given that the site was selected because it normally hosts remarkably high nymphal abundances, could be attributed to the climatic anomalies

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occurring during the early stages of the vector's life cycle. In particular, the alternation of heavy rainfall in the second half of March, combined with increasing temperatures in early March, followed by a sharp drop in temperature during the first week of April (Figure 72), likely created un unfavourable condition for the hatching and survival of juvenile *P. spumarius*, resulting in a marked population decline.

Under these conditions of very low *P. spumarius* density, sowing the Poaceae, Cruciferae, Fabaceae mixture resulted in reductions of 55.6% in spittle masses and 33.3% in nymphs compared with undisturbed vegetation (***Figure 73***), whereas ploughing eliminated both spittle masses and nymphs. However, the low overall population levels prevented the detection of statistically significant differences among treatments (p = 0.05). In contrast to *P. spumarius*, *N. campestris* showed high abundance. In plots with undisturbed vegetation, mean densities reached 61.0 spittle masses and 64.0 nymphs per square meter (***Table***). Under these conditions, both soil-management strategies led to significant reductions (p = 0.05) in juvenile populations. Sowing the cover crop reduced spittle masses by 93.4% and nymphs by 87.5%, whereas ploughing eliminated all spittle masses and nymphs, as confirmed by an Abbott's index of 100% (Figure 74).

Table 18. Number of spittle masses and nymphs/m^2 of *P. spumarius* and *N. campestris* in Trial F (2025).

Treatment	Number of spittle masses of *P. spumarius*/m^2	Number of nymphs of *P. spumarius*/m^2	Number of spittle masses of *N. campestris*/m^2	Number of nymphs of *N. campestris*/m^2
Undisturbed	3.0 a	4.0 a	61.0 a	64.0 a
Mix	1.3 a	2.7 a	4.0 b	8.0 b
Ploughing	0.0 a	0.0 a	0.0 b	0.0 b

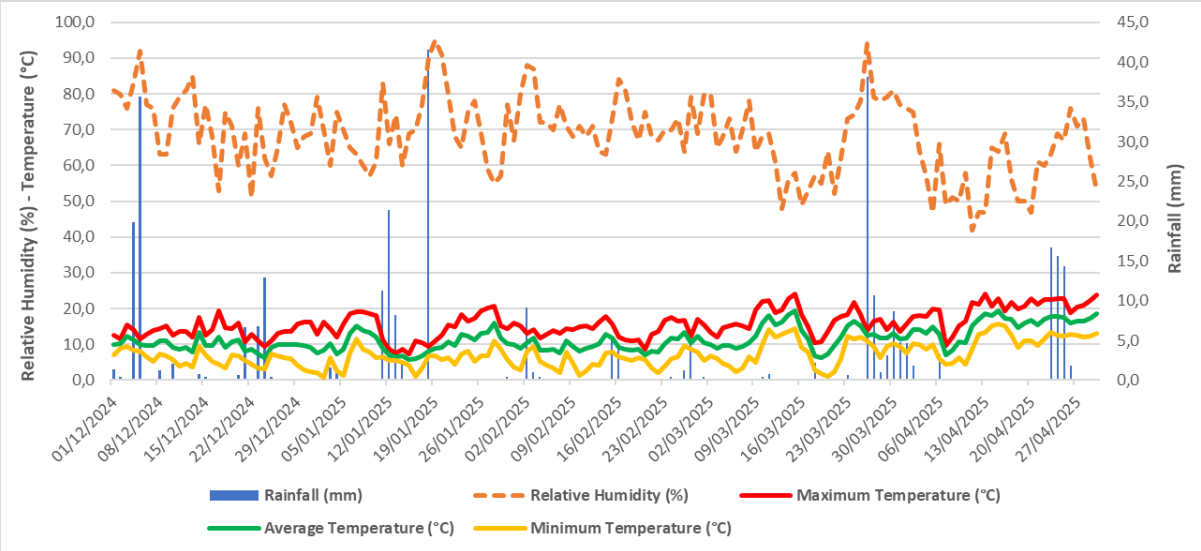


Figure 72. Meteorological trend recorded during the trial period in Andria (source: Puglia Civil Protection).

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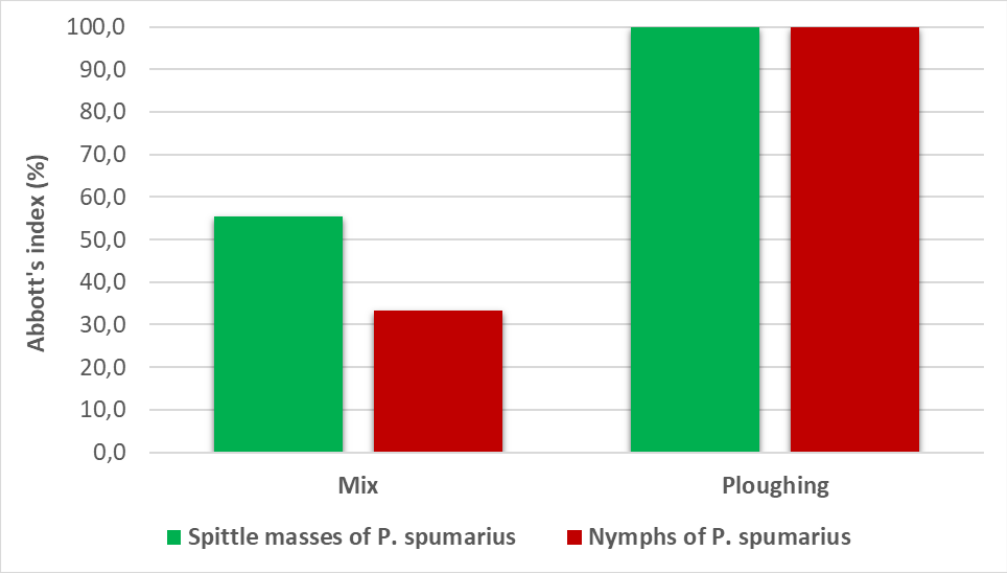


Figure 73. Abbott's index (%) determined on mean number of spittle masses and nymphs of *P. spumarius* (Trial F).

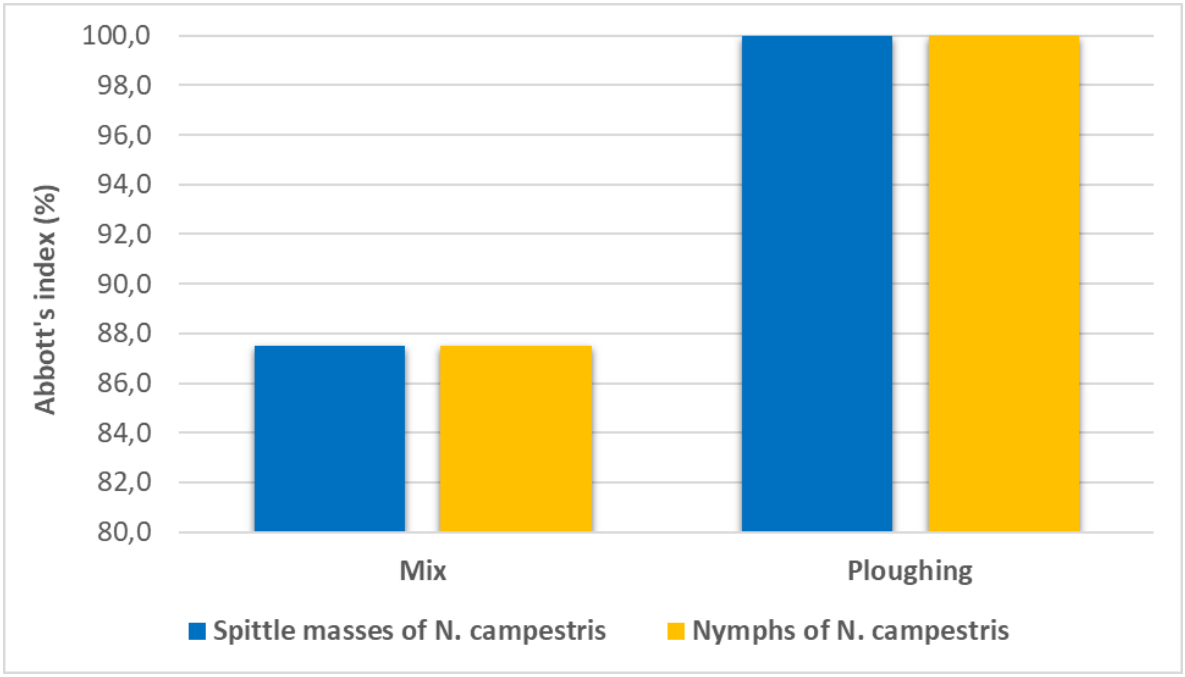


Figure 74. Abbott's index (%) determined on mean number of spittle masses and nymphs of *N. campestris* (Trial F).

4.4. Conclusions

The studies carried out across Spain and Italy demonstrate that cover crops represent a promising, sustainable, and scalable strategy to reduce juvenile populations of *P. spumarius* and other xylem-feeding vectors of *X. fastidiosa*. Greenhouse assays identified several plant species eliciting high nymphal mortality, pointing to the candidates for field evaluation. Field trials in Spain consistently showed that sowing *S. alba* and *A. cerefolium* can lower *P. spumarius* nymph densities compared with spontaneous vegetation in different crops, though

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their relative performance varied by site and year due to differences in germination success, local vegetation, and environmental conditions. Spatial analyses further revealed aggregated nymph distributions, typically concentrated near wooded field borders, highlighting the importance of landscape context in vector dynamics. Apulian (South Italy) field trials showed that mixtures of Poaceae, Cruciferae and Fabaceae repeatedly outperformed single-species sowings, suggesting that floristic diversity strengthens the suppressive effect on vector populations. Mowing systematically enhanced cover-crop efficacy by reducing habitat suitability for nymphal development, while spring ploughing remained the most effective management option overall, achieving near-complete suppression of spittlebugs across locations. Importantly, extremely low nymph densities observed in some sites during 2024–2025 underscored the strong influence of anomalous climatic conditions on population dynamics, occasionally limiting the statistical detection of treatment differences.

Beyond the interesting results on vector suppression capabilities, the studies presented here confirm that cover crops also provide agronomic and ecological benefits. In fact, their use improves soil structure and fertility, limits erosion, enhances water infiltration, and promotes biodiversity, making them a sustainable alternative to repeated mechanical disturbance, supporting their integration into sustainable olive orchard and vineyard management. The methodological refinements planned for the 2025–2026 season in Spain – including increased sowing density, additional replicates, and the introduction of tilled-row controls – will strengthen the robustness of future comparisons. Overall, the combined evidence demonstrates that strategically selected cover crops, especially when used in mixture and complemented with mowing, can substantially reduce juvenile spittlebug populations while promoting ecosystem health. This integrated soil- and vegetation-management approach represents a viable, environmentally sound component of vector management programmes and contributes to long-term strategies for mitigating the spread of *X. fastidiosa*.

5. PUBLICATIONS FROM THIS DELIVERABLE

The following publications have been compiled until 15/12/2025 for deliverable D5.2:

- Conde-Bravo, J., Garrido-Jurado, I., Fernández-Bravo, M.C., Yousef-Yousef, M., Quesada-Moraga, E. 2025. Targeting the *Xylella fastidiosa* spittlebug vector *Neophilaenus campestris* in the olive cover crops with the entomopathogenic fungus *Metarhizium brunneum*. Frontiers in Insect Science, 5. 10.3389/finsc.2025.1579244
- Yousef-Yousef, M., Morente, M., González-Mas, N., Fereres, A., Quesada-Moraga, E., Moreno, A. 2023. Direct and indirect effects of two endophytic entomopathogenic fungi on survival and feeding behaviour of meadow spittlebug *Philaenus spumarius*. Biological Control, 186: 105348. https://doi.org/10.1016/j.biocontrol.2023.105348
- Bodino N., Barbera R., González-Mas N., Demichelis S., Bosco D., Dolci P., 2024. Activity of natural occurring entomopathogenic fungi on nymphal and adult stages of *Philaenus spumarius*. Journal of Invertebrate Pathology 204, 108078. 10.1016/j.jip.2024.108078
- Bodino N, Cavalieri V, Dongiovanni C, et al (2023) Bioecological Traits of Spittlebugs and Their Implications for the Epidemiology and Control of the *Xylella fastidiosa* Epidemic in Apulia (Southern Italy). Phytopathology® 113:1647–1660. https://doi.org/10.1094/PHYTO-12-22-0460-IA

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- Lago C., Yousef Yousef M., Fereres A., Quesada-Moraga E., Moreno A., Gonzalez-Mas N. Epiphytic and endophytic colonization of olive by *Metarhizium brunneum* modifies the feeding behavior of the *Xylella fastidiosa* vector *Philaenus spumarius*. Accepted for publication.
- Volpe F, Cavalieri V, Iodice L, Martel G, Tortorici F, Bosco D, Dongiovanni C, Bodino N. Prevalence and biocontrol potential of a native egg parasitoid associated to *Philaenus spumarius* (Hemiptera: Aphrophoridae) in Italy: a potential biocontrol agent for the key vector of *Xylella fastidiosa* in Europe. In preparation.

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