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Identification of chemical/biological formulations capable of preventing *Xylella fastidiosa* transmission and disrupting vector feeding

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1. INTRODUCTION

1.1. *Xylella fastidiosa* transmission

The transmission of *X. fastidiosa* is exclusively mediated by xylem-feeding Hemiptera, particularly sharpshooters (Cicadellidae) and spittlebugs (Cercopidae) (Almeida et al., 2005; Cornara et al., 2017). In Europe, the meadow spittlebug *Philaenus spumarius* (Linnaeus, 1758) is recognized as the primary vector and the most important driver of disease spread (Cornara et al., 2017a; Cornara et al., 2017b; Cavalieri et al., 2019; Cornara et al., 2019). This species is widely distributed across the continent, particularly in temperate and humid regions (Saponari et al., 2014; Cornara et al., 2018; Gallego et al., 2023). In addition, *Neophilaenus campestris* and *Philaenus italosignus* have been identified as competent vectors of *X. fastidiosa* (Cavalieri et al., 2019). *Neophilaenus campestris* is also an important vector in the epidemiology of *X. fastidiosa*, as its high dispersal ability and broad distribution throughout the Mediterranean basin (Trkulja et al., 2022).

Philaenus spumarius is a univoltine species that overwinters in the egg stage and depends heavily on herbaceous ground cover for its development. In late winter to early spring, nymphs emerge and feed almost exclusively on ground vegetation, where they remain relatively immobile (Morente et al., 2018a; Morente et al., 2018b; Antonatos et al., 2019; 2020; Bodino et al., 2020). Although nymphs may acquire *X. fastidiosa*, the bacterium is lost during moulting (Cornara et al., 2018a). As individuals reach adulthood in late spring, they become highly mobile and, as herbaceous vegetation progressively dries out, they are forced to disperse from ground cover to surrounding shrubs and trees, such as olive (Bodino et al., 2019). Once infected, adults retain the bacterium and remain infectious throughout their lifetime (Cornara et al., 2018a). This seasonal shift in feeding habitat, from herbaceous vegetation to woody plants, is a key factor in the transmission of *X. fastidiosa*, as it allows infected adults to spread the bacterium to new host plants, making them the main drivers of disease transmission in Mediterranean agroecosystems (Cornara et al., 2016).

1.2. *Xylella fastidiosa* management

At the moment, it is very difficult to control this bacterium directly, as bactericides are unable to penetrate the xylem vessels effectively (EFSA, 2019), although effective methods are currently being developed to combat it (Burbank et al., 2022; Mourou et al., 2025; Rapicavoli et al., 2018). Given the absence of effective curative treatments, management of *X. fastidiosa* relies primarily on prevention, early detection, eradication of infected plants, and limiting vector abundance, movement and inoculation events (EFSA, 2019; Liccardo et al., 2020; Morelli et al., 2021; Burbank, 2022; European Commission, 2024; EPPO, 2025).

Current management strategies for *X. fastidiosa* through vector control can be divided into two main approaches: i) those focused on reducing vector populations and ii) those aimed at limiting transmission, while potentially also causing vector mortality.

Within the first group, ground cover management is one of the most relevant cultural tools, as it directly affects the habitat where the nymphal stages develop. This strategy was extensively addressed in Deliverable 5.2. Practices such as mowing, tillage, and the strategic suppression of spontaneous vegetation can decrease the availability of suitable host plants and alter microhabitat conditions in ways that become unfavorable for *P. spumarius* development. However, these measures are highly dependent on the phenological stage of the insect and on local agroecological conditions, which means that their effectiveness varies across orchards and seasons. For this reason, cover-crop management should be understood not as an isolated tactic but as a preventive measure that reduces the size of the vector population before adults disperse and begin transmitting the bacterium (Sanna et al., 2021; Morente et al., 2022).

Chemical control remains a crucial component of transmission management because it can rapidly reduce vector density and feeding activity when applied at the correct stage. Unlike habitat management, which acts indirectly by limiting vector recruitment, chemical treatments directly target the insect and can be used to suppress both immature and adult populations, thereby reducing the probability of bacterial acquisition and inoculation. Recent studies have shown that efficacy is highly dependent on the active ingredient, application timing, and exposure conditions. In particular, insecticides such as deltamethrin and acetamiprid have shown strong effects on mortality and feeding behaviour in *P. spumarius*, whereas other compounds may produce weaker, more variable, or mainly sublethal effects. This distinction is important because transmission can be reduced not only by killing the vector but also by limiting probing and feeding activity, which are essential for pathogen spread. Moreover, some chemical products may be more effective against nymphs than adults, reinforcing the importance of early-season applications aimed at preventing the emergence of dispersing adults. In this sense, chemical control is most effective when it is precisely timed and integrated with other practices rather than used as a stand-alone response (Dader et al., 2019; Burbank, 2022; Lago et al., 2022; Dongiovanni et al., 2023).

While chemical insecticides can be effective against vector populations, their widespread use raises significant concerns, including environmental contamination, non-target effects on beneficial organisms, development of resistance, and risks to human health (Biondi et al., 2012; Muturi et al., 2017; Song et al., 2017). In this context, and in line with current European policies encouraging the reduction of insecticide use and the transition towards more sustainable agricultural practices, our efforts in this work have been mainly focused on biological control strategies.

Biological control strategies have gained increasing attention as complementary approaches for managing *X. fastidiosa* vectors. Rather than relying on synthetic insecticides, biological strategies aim to suppress the vector through natural enemies or microbial agents (including nematodes, bacteria and fungi), offering a more sustainable long-term solution (Liccardo et al., 2020; Vicente-Díez et al., 2021; Ganassi et al., 2024; McCaw et al., 2023). Entomopathogenic fungi, for example, have shown promising activity against spittlebug vectors. Particularly, *Metarhizium brunneum* and *Beauveria bassiana* have gained considerable attention due to their dual mode of action: direct pathogenicity against insects and endophytic colonization of host plants (Barelli et al., 2016; Vega, 2018; Quesada-Moraga et al., 2020). These fungi not only reduce insect survival but also enhance plant resistance, improve nutrient uptake, and provide protection against other pathogens (Sánchez-Rodríguez et al., 2016; Branine et al., 2019; Quesada-Moraga et al., 2023). Importantly, their endophytic presence can alter insect feeding behaviour and reduce pathogen transmission, even when mortality is not immediate (González-Mas et al., 2019a; Maluta et al., 2023).

Recent studies have demonstrated the effectiveness of *M. brunneum* in reducing *P. spumarius* populations and altering its feeding behaviour, thereby decreasing the risk of *X. fastidiosa* transmission (Yousef-Yousef et al., 2023; Bodino et al., 2024; El-Khoury et al., 2024; Ganassi et al., 2024; Conde-Bravo et al., 2025). A highly useful technique for monitoring and understanding the feeding behaviour of insects under the influence of this fungus is the electrical penetration graph (EPG) method.

EPG technique is a real-time, non-destructive method used to monitor the probing and feeding behaviour of piercing-sucking. In an EPG system, the insect and the host plant form part of an electrical circuit; fluctuations in voltage occur as the insect's stylets penetrate different tissue layers and alternate between salivation and ingestion. These electrical signals or waveforms correspond to specific feeding activities and can be correlated with biological processes such as pathogen acquisition or transmission. By interpreting these patterns, it can identify key behavioural phases involved in the successful transmission of bacteria like *X. fastidiosa* (Walker et al., 2024; Killiny & Fereres, 2025).

This work focuses on assessing the direct and indirect application of EPF as a biological strategy to disrupt vector-plant interactions and reduce the risk of *X. fastidiosa* transmission. Although the initial work plan considered the evaluation of both chemical and biological formulations, we prioritized biological control tools, and specifically EPF, for two main reasons. First, chemical insecticides had already been tested against the main European vector of *X. fastidiosa* (Dongiovanni et al., 2018a, 2018b), whereas the potential of EPF to interfere with vector behaviour, survival and plant-mediated transmission pathways remained less explored. Second, this prioritization was fully aligned with the objectives and expected impact of the BeXyl call, which emphasized the development of sustainable, non-chemical and integrated strategies to prevent and mitigate the spread of *X. fastidiosa* in Europe. By focusing on EPF, the work directly contributes to the generation of

environmentally compatible tools for vector management and supports the transition towards plant protection approaches with reduced reliance on conventional insecticides.

This deliverable is organised into four chapters that together assess the potential of EPF, mainly *M. brunneum* strain EAMa 01/58-Su, to affect *P. spumarius* survival, feeding behaviour, settlement and *X. fastidiosa* transmission. The four chapters follow a progressive experimental logic, from plant-mediated effects of fungal colonisation, to direct fungal application on the vector, to behavioural settlement assays, and finally to transmission experiments with infective insects under controlled conditions. This structure reflects the dual objective of the deliverable: to identify biological formulations capable of disrupting vector-plant interactions and to evaluate whether these effects may contribute to reducing *X. fastidiosa* transmission risk.

2. CHAPTER I: INDIRECT EFFECTS OF ENTOMOPATHOGENIC FUNGI ON MORTALITY AND FEEDING BEHAVIOUR OF *PHILAENUS SPUMARIUS*

This chapter explores the indirect effects of *M. brunneum* on the feeding behaviour and mortality of *P. spumarius*, based on two complementary studies. In both cases, the indirect effect is evaluated via insect feeding on host plants colonized by *M. brunneum*. The first study examines the feeding behaviour of *P. spumarius* on *Sonchus oleraceus*, together with the resulting mortality associated with feeding on fungus-colonized plants (Yousef-Yousef et al., 2023). The second study follows the same approach using olive plants as host, assessing its probing and feeding behaviour in relation to fungal colonization and the subsequent impact on insect survival (Lago et al., 2026).

2.1. Materials and methods

2.1.1. Insect collection and maintenance

Philaenus spumarius individuals were collected from spontaneous vegetation in Villanueva de la Cañada (40°27'15.9"N, 4°00'21.1"W) during the spring period of 2022 and 2023, corresponding to the assays conducted with *S. oleraceus* (Yousef-Yousef et al., 2023) and olive plants (Lago et al., 2026), respectively, and coinciding with the peak abundance of nymphal stages.

Sampling was conducted by direct inspection of herbaceous vegetation for foam masses, which are characteristic of nymphal development. Late-instar nymphs were mainly collected from *Eryngium campestre* L., *Carduus tenuiflorus* L., *Taraxacum* spp. (Wigg), *Plantago* spp., *Borago officinalis* L., and *Sonchus* spp. Collected individuals were carefully transferred to ventilated plastic containers to minimize stress and avoid mechanical damage.

After collection, nymphs were transported to the screenhouse at Instituto de Ciencias Agrarias (ICA-CSIC) (Madrid, Spain). Nymphs were carefully transferred using a fine paintbrush onto *Sonchus oleraceus* plants and reared until adulthood in BugDorm cages (30 × 30 × 30 cm). Insects were maintained under controlled conditions (22-25 °C, 60-70% relative humidity, and a 16:8 h (L:D) photoperiod) until experimental use. Plants were replaced every two to three weeks to ensure optimal rearing conditions.

2.1.2. Fungal strains, culture conditions and inoculum preparation

Entomopathogenic fungal strains were selected based on their documented pathogenicity against hemipteran vectors and their potential compatibility with integrated pest management strategies.

For the *S. oleraceus* trials (Yousef-Yousef et al., 2023), one strain of *B. bassiana* (EABb 01/33-Su) and one strain of *M. brunneum* (EAMa 01/58-Su) from the culture collection of the Department of Agronomy, University of Cordoba (Spain) were used (**Table 1**). *Metarhizium brunneum* was used for the subsequent assessment of feeding behaviour in plants colonised by the fungus, due to its superior colonisation capacity and previous studies conducted on *P. spumarius* (reported in Deliverable 5.2), which demonstrated promising results.

Table 1. Fungal species used in this study

Strain	Fungal Species	Origin	Agroecosystem	Habitat	GenBank Accession Number	Spanish Type Culture Collection Accession Number
EABb 01/33-Su	*B. bassiana*	El Bosque (Cadiz, Spain)	Traditional olive orchard	Soil	FJ972969	21,149
EAMa 01/58-Su	*M. brunneum*	Hinojosa del Duque (Cordoba, Spain)	Wheat crop	Soil	JN900390	20,764

For the olive plants experiment (Lago et al., 2026), the same strain of *M. brunneum* (EAMa 01/58-Su) was selected, due to its significant impact on *P. spumarius* demonstrated in the first assays. This strain was first isolated in 2001 from soil samples collected in Hinojosa del Duque and is currently preserved in the University of Córdoba Entomopathogenic Fungi Collection (Córdoba, Spain) as well as in the Spanish Type Culture Collection (accession number 20764) (**Table 1**). Sequence data corresponding to the ITS region and a gene encoding a secreted insecticidal toxin are available in the GenBank database (accessions KY786030 for ITS and JN900390 for the toxin gene).

For culturing, the strain was maintained on potato dextrose agar (PDA) (Biolife Italiana S.R.L., Italy) in 90 mm Petri dishes and incubated for 15 days at 25 °C under dark conditions. Conidial suspensions were obtained by harvesting conidia from the culture surface and suspending them in sterile aqueous 0.01% Tween 80. The suspensions were subsequently filtered through multiple layers of sterile cheesecloth to eliminate mycelial fragments and then sonicated for 5 min to ensure a uniform inoculum.

Conidial concentration was determined using a hemocytometer, and suspensions were adjusted with 0.01% Tween 80 to a final concentration of 10^8 conidia mL^{-1} for all assays. Prior to use, conidial viability was assessed in liquid Czapek Dox broth supplemented with 1% (w/v) yeast extract, and only suspensions with >97% germination after 24 h were used in the experiments (Yousef et al., 2014).

2.1.3. Plant material and growth conditions

Host plants were selected according to the objectives of each experimental setup and cultivated under controlled or semi-controlled conditions to ensure uniformity.

For the first assay (Yousef-Yousef et al., 2023), sowthistle (*S. oleraceus* L.) was used as the host plant for *P. spumarius*, as it has been previously reported as suitable for continuous indoor rearing of this species (Morente et al., 2018, 2021). *Sonchus oleraceus* plants were grown from seeds sown in pots filled with a mixture of vermiculite (Asfaltex S.A., Barcelona, Spain) and soil substrate (Jiffy substrate, Jiffy Products International BV, Moerdijk, The Netherlands) at a 1:2 ratio. Seeds were first pre-germinated to ensure viability and subsequently transferred into 7 × 7 cm plastic pots containing a mixture of sterilized vermiculite (No. 3, Asfaltex S.A., Barcelona, Spain) and soil (Floragard, Oldenburg, Germany) (1:1). Plants were maintained in an environmental chamber under controlled conditions (25 ± 2 °C; 16:8 h light:dark photoperiod). The growing substrate was sterilized by autoclaving at 121 °C for 20 min, with a 24 h interval between sterilization cycles. Plants were irrigated three times per week, and a nutrient solution (20:20:20 N:P:K; Nutrichem 60 fertilizer, Miller Chemical & Fertilizer Corp., Hanover, PA, USA) was applied at a concentration of 1 g L^{-1}.

For the second assay (Lago et al., 2026), six-month-old healthy potted olive plants (*Olea europaea* L. var. “Arbequina”) were used. Plants were produced in 10 × 10 × 20 cm pots at the greenhouse facilities of the University of Córdoba (Department of Agronomy) and grown for six months until reaching approximately 40 cm in height. They were subsequently transported to the ICA-CSIC, where the study of insect probing and feeding behaviour by means of EPG technique and mortality evaluation were conducted. Olive plants were maintained under greenhouse conditions (24:18 ± 2 °C day:night temperature; 14:10 h light:dark photoperiod; 60-70% relative humidity) and irrigated three times per week until fungal application.

In addition, *S. oleraceus* plants at the 4-5 leaf stage were used for rearing *P. spumarius* nymphs until adult emergence. These plants were cultivated and maintained under the same conditions described for the first assay at ICA-CSIC. No insecticides or fungicides were applied to any of the plants used in the experiments.

2.1.4. Endophytic colonization of Sonchus oleraceus by *Metarhizium brunneum* and *Beauveria bassiana*

The ability of both fungal strains to establish as endophytes was evaluated using two inoculation approaches: soil application and foliar spraying. For each strain, four sets of *S. oleraceus* plants (five plants per set) at the four-leaf stage were arranged. In the first set, leaves were treated with 2 mL of a conidial suspension per plant using an aerograph 27.085 (piston compressor, 23 L/min, 15–50 PSI, 0.3 mm nozzle diameter. The second set served as the foliar control and was sprayed with sterile aqueous 0.1% Tween 80. In the third set, 2 mL of conidial suspension per plant were applied directly to the growing substrate using a pipette, while the fourth set (soil control) received the same volume of sterile aqueous 0.1% Tween 80. To avoid cross-contamination from runoff, the substrate of plants receiving foliar treatments was covered with aluminium foil prior to application (**Figure 1a**).

Following inoculation, both treated and control plants were enclosed in transparent plastic bags and kept in a growth chamber at 25 °C under a 16:8 h light: dark photoperiod for 48 h to facilitate fungal establishment. Endophytic colonization of the phyllosphere was assessed 48 h after treatment (Resquín-Romero et al., 2016; Garrido-Jurado et al., 2017). For this purpose, two leaves per replicate were collected from each treatment and control group and surface-sterilized in 1% sodium hypochlorite for 2 min, followed by two rinses in sterile deionized water (2 min each). Leaves were then air-dried under sterile conditions, and ten fragments (1 cm^2) per leaf were excised with a sterile scalpel (50 fragments per treatment in total) and plated onto Sabouraud Dextrose Agar (SDA) supplemented with 0.5 g L^{-1} chloramphenicol. A new sterile scalpel was used for each plant. Plates were sealed and incubated in darkness at 25 °C for 7 days (**Figure 1b**).

Colonization was quantified as the percentage of leaf fragments showing fungal growth, which was examined under a light microscope (Nikon SMZ800 Model CP-S, Tokyo, Japan) and identified based on morphological characteristics. To verify the effectiveness of surface sterilization, 40 µL samples from the final rinse water were plated onto the same medium and incubated under identical conditions. The same protocol was applied to stem and root samples.

Based on these results, a second experiment was conducted to evaluate the temporal dynamics of endophytic colonization by *M. brunneum* strain EAMa 01/58-Su following foliar application. Ten *S. oleraceus* plants at the four-leaf stage were used. The adaxial and abaxial surfaces of the two basal leaves were sprayed with 2 mL of conidial suspension per plant using

the aerograph. To prevent unintended inoculation, the upper leaves and the soil were protected with plastic bags and aluminium foil, respectively. Control plants were treated in the same manner using sterile aqueous 0.1% Tween 80. Plants were maintained under the same environmental conditions described above.

Destructive sampling was performed at 2-, 7-, 14-, 21-, and 28-days post-inoculation (dpi), selecting two plants per treatment and control at each time point. From each plant, one treated and one untreated leaf were collected and stored individually in sealed plastic bags. Ten fragments per leaf were analysed for endophytic colonization as previously described. The same procedure was also carried out for stems and roots (**Figure 1b**).

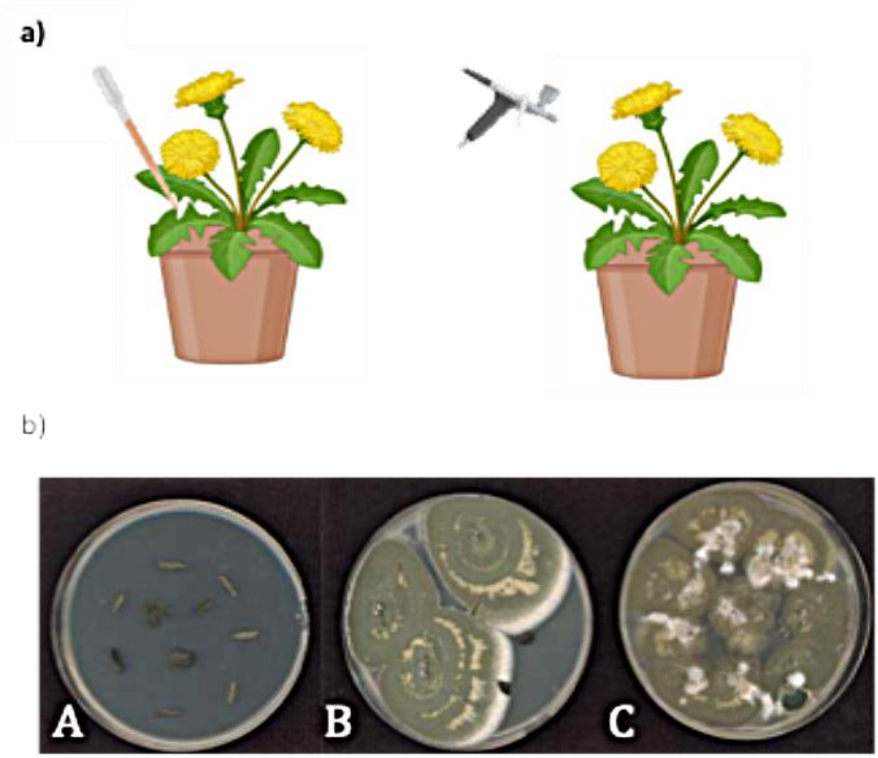


Figure 1. (**a**) Soil drench and foliar spray treatments applied to *Sonchus oleraceus*. (**b**) Growth of *Metarhizium brunneum*.

2.1.5. Effect of endophytic colonization of *Sonchus oleraceus* plants by *Metarhizium brunneum* on the feeding behaviour and mortality of *Philaenus spumarius* adults

Eight-week-old *P. spumarius* adults and *S. oleraceus* plants at the 5-6 leaf stage were used for all EPG assays. Prior to behavioural recordings, insects were deprived of food for 1 h in aerated 1.5 mL tubes, briefly anesthetized with CO_2 (approximately 5 s), and immobilized using a diaphragm pump (Eyela Aspirator A3S; Rikakikai, Tokyo, Japan). Individuals were then tethered to plants following the procedure described by Cornara et al. (2018a, 2018b). In

brief, a 3 cm long, 18 µm diameter gold wire was bent to form a loop, placed on the pronotum, and secured to the leaf surface with a double layer of water-based silver conductive paint (EPG Systems, Wageningen, The Netherlands).

Prior to insect tethering, both adaxial and abaxial surfaces of the two basal leaves were sprayed with a conidial suspension of the *M. brunneum* strain EAMa 01/58-Su, while upper leaves were protected from exposure with aluminium foil. After a 48-h incubation period (25ºC and 16:8 (Light/Dark), as in 1.5 section), two *P. spumarius* adults were attached to each plant: one on the abaxial surface of a directly treated basal leaf, and the other on the abaxial surface of an untreated but endophytically colonized apical leaf. The same protocol was applied to control plants treated with sterile aqueous 0.1% Tween 80.

Feeding behaviour was monitored under laboratory conditions (22-24 °C) using a Giga-8 DC-EPG system (EPG Systems, Wageningen, The Netherlands) with an input resistance of 1 Giga Ohm. Signals were amplified (×100), digitized at 100 samples per second per channel, and recorded using Stylet+ software (EPG Systems, Wageningen, The Netherlands). Voltage levels were adjusted according to the manufacturer's calibration guidelines to ensure signals remained within the ±5 V range supported by the software.

Each plant was used for a single recording session. In each experimental run, 28 *S. oleraceus* plants were evaluated: 14 inoculated with the fungus (basal leaves directly treated and apical leaves endophytically colonized) and 14 control plants (basal leaves sprayed with Tween 80 and apical leaves non-colonized). Recordings lasted 4 h for each insect-plant combination and were conducted inside a Faraday cage to minimize electrical interference. The experiment was repeated over three consecutive days using fresh plants, fungal suspensions, and insects, resulting in a total of 42 plants per treatment.

Six main EPG waveforms (**Figure 2**) were considered following Cornara et al. (2018a, 2018b, 2020): C (stylet pathway phase), Xc (xylem contact/pre-ingestion), Xi (active xylem sap ingestion), R (resting phase associated with xylem feeding), N (interruptions during xylem ingestion), and Xe (occasional behaviour potentially linked to egestion). Waveforms were manually annotated using Stylet+ software. Recordings showing abnormal feeding patterns or interrupted due to insect escape were discarded prior to statistical analysis.

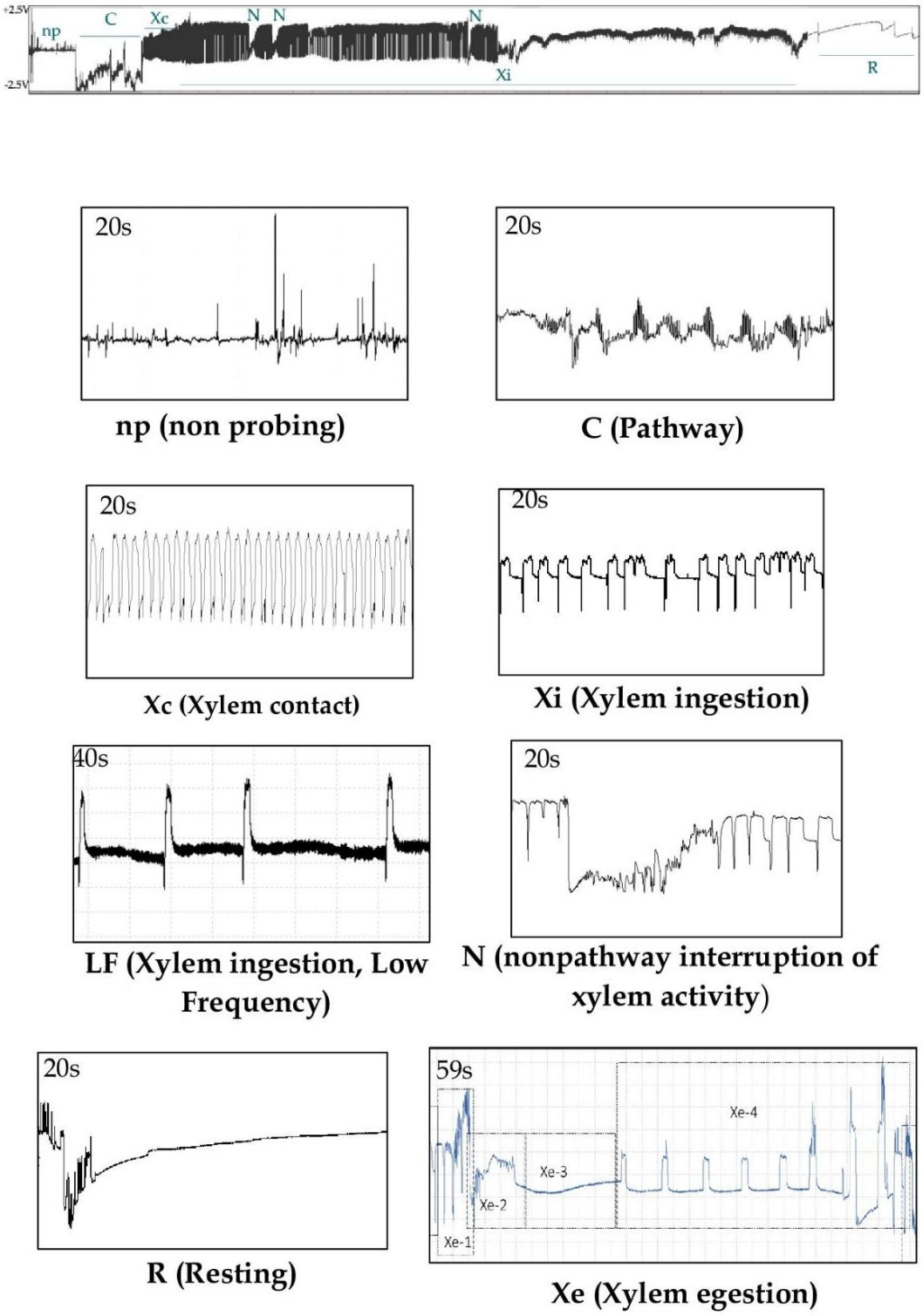


Figure 2. EPG waveforms described in *Philaenus spumarius*.

2.1.6. Endophytic colonization of olive plants by *Metarhizium brunneum*

Plants were treated either with the *M. brunneum* strain EAMa 01/58-Su at a concentration of 1×10^8 conidia mL^{-1} prepared as described in Section 1.2. For foliar treatments, 2 mL of the conidial suspension were applied per plant using an airbrush (model 17,055 AB300; piston compressor delivering 20-23 L min^{-1}, 15-30 PSI, 0.3 mm nozzle diameter). In total, 26 plants received the fungal treatment, and 20 plants were assigned to the control group across both experimental runs (June and October). To restrict inoculation to basal tissues, the apical portion of each plant was covered with a plastic bag during spraying.

A separate set of plants was treated via soil drenching, applying 5 mL of either the conidial suspension or the control solution directly onto the substrate surface. This method included 24 plants treated with *M. brunneum* and 22 control plants.

Following treatment, all plants were maintained for 48 h in growth chambers (25 °C, 16:8 h light: dark photoperiod) to allow fungal establishment within plant tissues. Subsequently, feeding behaviour of *P. spumarius* was evaluated using EPG recordings. Six experimental conditions were defined: (1) fungal-treated sprayed leaves; (2) untreated leaves from fungus-treated plants; (3) control sprayed leaves; (4) untreated leaves from control plants; (5) fungal soil drench treatment; and (6) control soil drench treatment (**Figure 3**). The experiment was conducted twice, in June and October 2023.

Plants were classified as non-colonized and removed from the dataset when the sprayed leaf tested negative for *M. brunneum* using both leaf imprinting and qPCR. In such cases, all associated EPG recordings (from both treated and untreated leaves) were discarded. In contrast, when the sprayed leaf yielded positive results (leaf print and qPCR), the plant was considered successfully exposed to the fungus. If the corresponding non-sprayed leaf tested negative, recordings obtained from insects feeding on that leaf were retained to assess potential indirect or systemic effects of fungal colonization.

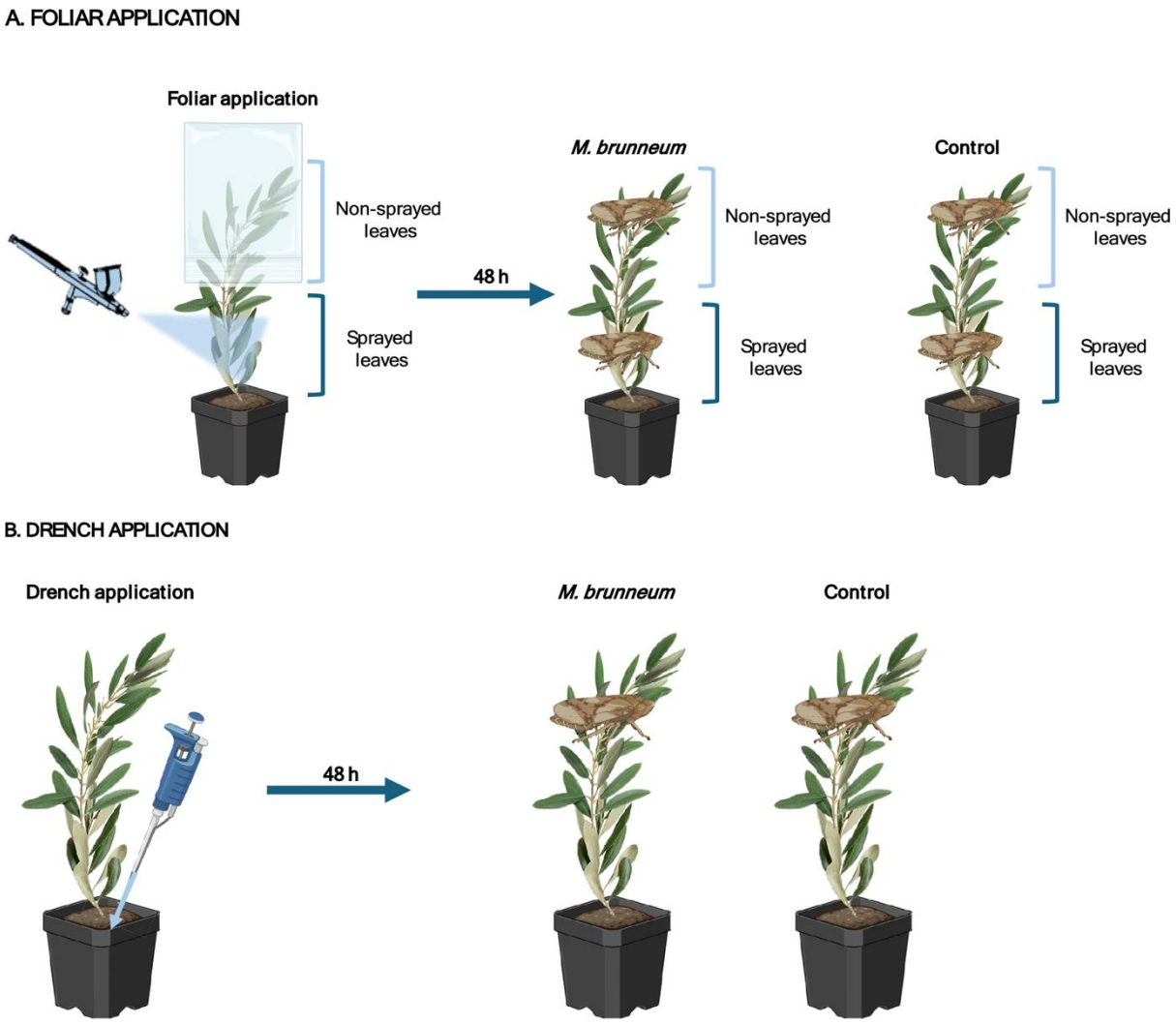


Figure 3. Experimental design for the EPG experiment. *Philaenus spumarius* feeding behaviour was evaluated after (**A**) Foliar application and (**B**) Drench application of *M. brunneum* or control solution (Lago et al., 2026).

2.1.7. *Philaenus spumarius* probing and feeding behaviour on *Metarhizium brunneum* treated and control olive plants

The influence of the six treatments on the feeding behaviour of *P. spumarius* was assessed using the EPG technique, considering each individual insect as the experimental unit. In total, 142 adults (males and females in an approximate 1:1 ratio), aged between 1 week and 3 months, were included across both experimental runs. Prior to recording, insects were anesthetized with CO_2 for 10 s, immobilized using a vacuum device, and connected to the EPG system following the procedure described by Cornara et al. (2018b) (**Figure 4**).



Figure 4. *Philaenus spumarius* individual connected to the EPG system, with the EPG technique in operation.

In the foliar spray treatment, two insects were simultaneously evaluated on the same plant, each assigned to a different leaf: one feeding on a treated leaf and the other on an untreated leaf from the same plant. Insects were initially placed on the adaxial surface but were allowed to move freely across the leaf. Feeding activity was continuously recorded for 4 h using two Giga-4 DC-EPG and two Giga-8 DC-EPG devices (EPG Systems, Wageningen, The Netherlands) placed in a Faraday cage to minimize electrical interference. Signal acquisition and subsequent analyses were performed using Stylet+ software (EPG Systems, Wageningen, The Netherlands).

Feeding behaviour was characterized based on the following waveform types (**Figure 2**): np (non-probing), C (stylet pathway activity within plant tissues), Xc (xylem contact), Xi (xylem sap ingestion), Xe (xylem egestion), N (interruptions during the xylem phase), R (resting), and W (stylet withdrawal). Additional variables were also considered, including the proportion of insects that left the plant during the recording period, the total number of probes (defined as stylet insertion events: C + Xc + Xi + N + R), successful probes (those reaching the xylem), unsuccessful probes (those without Xi), and sustained probes (Xi events lasting longer than 5 min).

2.1.8. Monitoring of *Philaenus spumarius* mortality after 4h of exposure to *Metarhizium brunneum* treated *Sonchus oleraceus* and olive plants

Following the end of the 4 h EPG recordings, mortality of *P. spumarius* adults was evaluated. Insects were transferred onto untreated *S. oleraceus* plants, grouping individuals according to their respective treatments. Spittlebugs were confined on plants using cylindrical plastic mesh cages, with a maximum of three individuals per pot.

After transfer, plants were maintained under semi-natural conditions 24:18 ± 2 °C (day: night) and a 14:10 h light: dark photoperiod. Insect mortality was recorded daily over a period of seven days. At the end of the observation period, both dead and surviving insects were collected, individually stored in 1.5mL tubes for subsequent analysis of fungal colonization.

2.1.9. Detection of *Metarhizium brunneum* in *Sonchus oleraceus* and olive plants after EPG recordings

At the end of the experiments, plant tissues used for EPG recordings were collected to verify endophytic fungal colonization.

In the case of assays with *S. oleraceus*, both basal and apical leaves on which insects had fed were collected to assess endophytic colonisation and to verify the absence of contamination in control plants, as described in Section 1.4. Leaf samples were excised using sterile scissors, placed in sealed plastic bags, and stored at -80 °C until fungal growth assessment.

In the second assay with olive plants, for those subjected to foliar treatments with *M. brunneum*, one treated (sprayed) and one untreated leaf per plant were sampled separately. For plants receiving soil drench applications, both leaves and roots were collected. Control plants were processed in parallel to confirm the absence of fungal colonization.

To assess the presence of epiphytic fungal propagules on olive leaf surfaces, both the adaxial and abaxial sides of each leaf were “printed” onto 90 mm Petri dishes containing a selective culture medium as described by González-Mas et al. (2019). In the case of soil-treated plants, root imprints were also performed. Plates were incubated in darkness at 25 °C until fungal growth was detected.

Following imprinting, sampled leaves and roots were surface-sterilized using 1% sodium hypochlorite for 2 min, rinsed twice with sterile distilled water, and dried on sterile filter paper. To verify the effectiveness of the sterilization procedure, 100 µL aliquots from the final rinse water were plated individually.

In olive plants, endophytic colonization was further confirmed using molecular techniques. Plant tissues (sprayed and non-sprayed leaves for foliar treatments, and leaves and roots for soil treatments) were surface sterilized as described above and ground to a fine powder in liquid nitrogen using a mortar and pestle. DNA was extracted from 100 mg of tissue using the Plant/Fungi DNA Isolation Kit (Norgen Biotek Corporation), following the manufacturer’s protocol. DNA concentration was quantified with a NanoDrop 2000c spectrophotometer (ThermoFisher Scientific, USA), and all samples were standardized to 20 ng/µL prior to PCR analysis.

Detection and quantification of *M. brunneum* were carried out using species-specific primers targeting the nitrogen response regulator (nrr) gene (Barelli et al., 2018): forward primer 5’-TCA GGC GAT CTC GTG GTA AG-3’ and reverse primer 5’-GGG GTG TAC TTG AGG AAT GGG-3’.

Primers were synthesized commercially (Sigma-Aldrich, USA). Each qPCR reaction (20 µL total olume) contained 1 µL of DNA sample, 10 µL of iTaq Universal SYBR Green Supermix (Bio-Rad, USA), 0.4 µL of each primer (10 µM), and 8.2 µL of PCR-grade water.

To generate calibration curves, genomic DNA from sterile plants was used. Serial dilutions of fungal DNA (ranging from 10 ng/µL to 1×10^{-6} ng/µL) were prepared within a constant plant DNA background (20 ng/µL) to determine amplification thresholds. A detection limit of 1×10^{-4} ng/µL was established, as lower concentrations did not yield amplification. Additional standard curves using fungal DNA alone were constructed to confirm that quantification cycle (Cq) values were independent of plant DNA background.

Quantitative PCR was performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA) following the protocol described by Barelli et al. (2018). The thermal profile included an initial denaturation step at 95 °C for 2 min, followed by 40 amplification cycles (95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s), and a final melting curve analysis from 65 to 95 °C with increments of 0.5 °C every 5 s. Amplification was specific to *Metarhizium* DNA, producing a characteristic melting peak at 82.5 °C. Product specificity and absence of primer-dimer formation were confirmed through dissociation curve analysis, while no amplification was observed in control samples.

2.1.10. Data processing and statistical analysis

- **Endophytic colonization**

Endophytic colonization data were analysed separately for each inoculation method using analysis of variance (ANOVA). Differences among treatments (two fungal strains and control) were evaluated using Tukey's post hoc test ($\alpha = 0.05$) in Statistix®10 (Analytical Software, Tallahassee, USA).

- **Feeding behaviour and subsequent mortality**

Both non-sequential and sequential EPG variables were analysed. Non-sequential parameters included the number of waveform events per insect, total duration of each waveform (min), the proportion of time spent in each waveform relative to the total residence time on the plant, and the mean duration of waveform events. Sequential variables comprised time intervals between key events, such as np to first Xc, np to first Xi, first C to first Xc, first C to first Xi, np to first sustained Xi (> 5 min), first C to first sustained Xi, time to first probe, time to first probe reaching Xi, and time to first sustained Xi event. An EPG recording was considered suitable for analysis only if the insect initiated at least one probing event (i.e., stylet insertion into plant tissue) during the 4 h period and remained on the plant (not escape), or escape only after the first hour. Recordings failing to meet these criteria were excluded.

EPG variables describing the feeding behaviour of *P. spumarius* were obtained using different approaches depending on the assay. In *S. oleraceus*, variables were calculated using the CSIC-UAL v.3.0 EPG Excel workbook (Garzo et al., 2024), obtaining both non-sequential (number of waveform events, total duration, and mean duration per insect) and sequential variables (time intervals between key feeding events), resulting in a total of 36 variables **(**Supplementary Material 1). In olive, EPG waveforms were manually annotated using Stylet+ software (EPG Systems, Wageningen, The Netherlands), and behavioural variables were computed with an updated version of the "XylFeed" Excel macro designed for xylem-feeding insects (Markheiser et al., 2022; available at https://epg.csic.es/excel_workbook). Data from both experimental repetitions (June and October) were pooled for analysis.

Data analysis differed between assays. In *S. oleraceus*, all variables were first tested for normality and homoscedasticity using Shapiro-Wilk and Levene tests, respectively, and log-transformed when necessary. Differences in feeding behaviour related to leaf position (basal vs. apical) were evaluated independently for control and treated plants using Student's t-test or Mann-Whitney U test, depending on data distribution. The effect of *M. brunneum* inoculation on feeding behaviour was then assessed using Linear Models (LMs) or Generalized Linear Models (GLMs; quasipoisson distribution with logit link) as appropriate (Zuur et al., 2009), implemented in R (R Core Team, 2021) using the packages lme4 and nlme. When significant effects were detected, pairwise comparisons were performed using least-square means with the lsmeans package (Lenth, 2016).

In olive, treatment effects on EPG variables were evaluated using Linear Models (LMs; function lm()). When required, data were log-transformed (ln(x + 1)) to improve normality and reduce heteroscedasticity. For non-normally distributed variables, Generalized Linear Models (GLMs) with binomial distribution (glm() from lme4) or negative binomial distribution (glm.nb() from MASS) were applied. To further explore differences between sprayed and non-sprayed leaves within control and treated plants, pairwise comparisons were conducted using Student's t-test or Mann-Whitney U test depending on data distribution. These comparisons were performed even when global models were not significant, in order to detect localized effects.

Mortality analyses were conducted differently depending on the assay. In *S. oleraceus*, mortality data for *P. spumarius* adults were analysed by GLM with binomial distribution and logit link function using JMP 8 software. Treatment comparisons were made using a χ^2 test ($P < 0.05$). Values for average survival times (ASTs) were obtained by Kaplan-Meier survivorship analysis and compared using the log-rank Mantel-Cox significance test calculated with IBM SPSS 25.0 software (SPSS Inc., Chicago, IL, USA).

In olive, mortality rates were analysed using Kaplan-Meier survival analysis, and differences between treatments were assessed using the log-rank Mantel-Cox test after 4 h of exposure. All statistical analyses were conducted in R (R Core Team, version 4.2.2, 2022-10-31).

2.2. Results

2.2.1. Endophytic colonization of *Sonchus oleraceus* by *Metarhizium brunneum* and *Beauveria bassiana*

Statistically significant differences between fungal strains were detected for both application methods, foliar (F_2,15 = 11.10, $P \leq 0.001$) and soil (F_2,15 = 11.50, $P \leq 0.001$). Under both treatment approaches, *M. brunneum* EAMa 01/58-Su exhibited a greater capacity for endophytic colonisation than *B. bassiana* EABb 01/33-Su 48 h after inoculation, with mean recovery rates from leaf tissues of 91.6% and 58.3%, respectively (**Figure 5**).

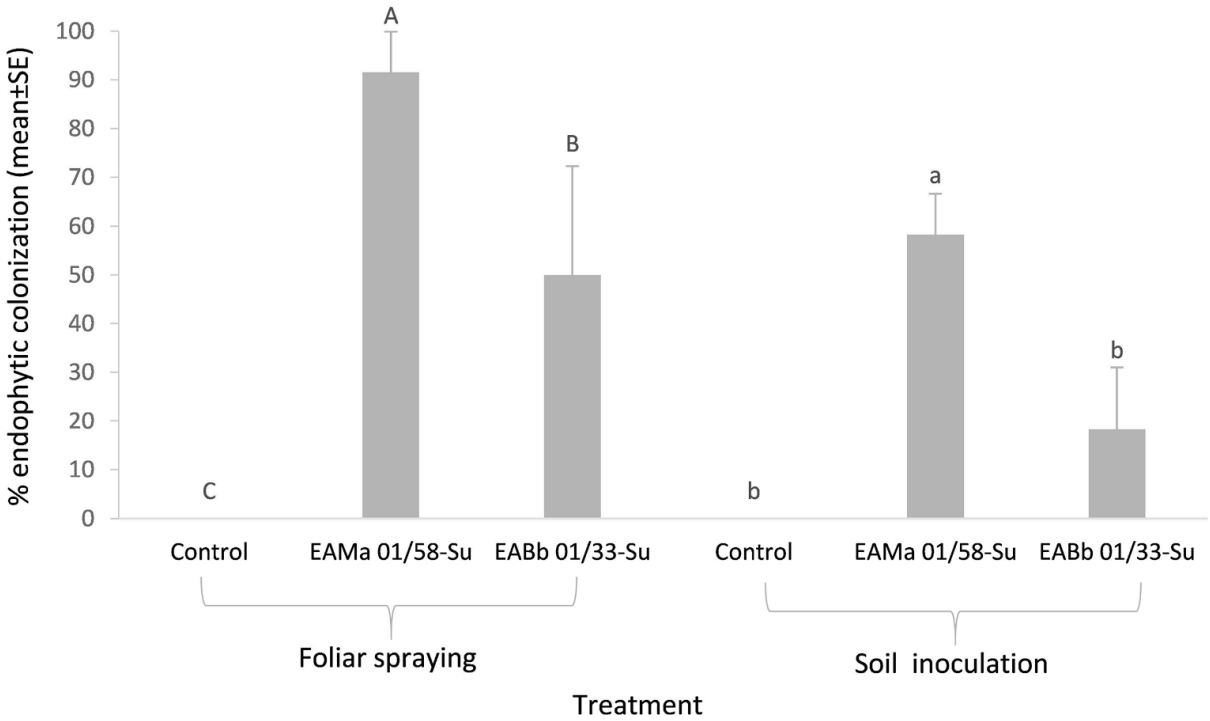


Figure 5. Percentage of *Sonchus oleraceus* leaves endophytically colonized by entomopathogenic fungi 48 h after either foliar spraying or soil inoculation with *M. brunneum* isolate EAMa 01/58-Su or *Beauveria bassiana* isolate EABb 01/33-Su compared with control plants. Within treatments, bars with different upper (foliar) or lower (soil) case letters are significantly different to each other following ANOVA ($p < 0.05$) and Tukey test ($\alpha = 0.05$).

The temporal progression of systemic colonisation of *S. oleraceus* by *M. brunneum* EAMa 01/58-Su was further assessed in both treated and untreated leaves. The fungus was consistently re-isolated from directly treated leaves at high recovery rates up to 14 days post-inoculation (dpi), although a decline was observed over time, from 95% at 2 dpi to 41.2% at 14 dpi (**Figure 6A**). Endophytic colonisation was also detected in untreated leaves, indicating systemic spread, with recovery values ranging from 28.5% at 2 dpi to 10% at 7 dpi (**Figure 6B**),

and dropping to 0% by 14 dpi. No fungal presence was detected in stem or root tissues. Notably, leaves sampled from 7 dpi onwards corresponded to newly developed leaves.

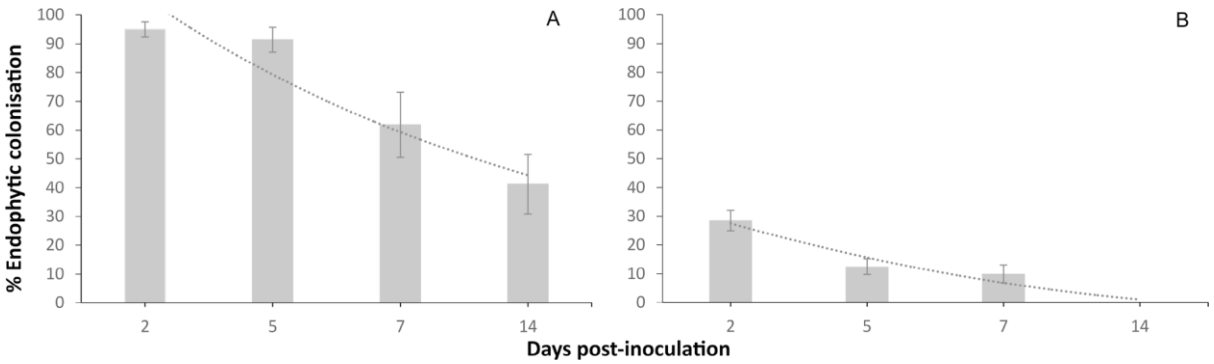


Figure 6. Percentage of *Sonchus oleraceus* plants endophytically colonized by *Metarhizium brunneum* isolate EAMa 01/58-Su 2, 5, 7 and 14 days after spraying the basal leaves with a conidial suspension. (**A**) Timeline of fungal colonization in directly sprayed leaves. (**B**) Timeline of fungal colonization in leaves not treated directly but receiving the fungus endophytically.

2.2.2. Feeding behaviour of *Philaenus spumarius* adults on endophytically colonized *Sonchus oleraceous* plants

A total of 95 valid EPG recordings were obtained and analysed, comprising 25 control leaves sprayed with aqueous Tween 0.1% (basal control, BC), 19 unsprayed control leaves (apical control, AC), 23 leaves sprayed with and endophytically colonised by *M. brunneum* (basal treated, BT), and 28 unsprayed but endophytically colonised leaves (apical treated, AT).

The proportion of directly treated leaves from which *M. brunneum* EAMa 01/58-Su was re-isolated varied across experimental repetitions, ranging from 93% in repetition 1 to 20% in repetition 3. Successful plant colonisation was confirmed by the recovery of the fungus from untreated leaves, with mean recovery rates exceeding 50% in repetition 1 and 40% in repetition 2. Given the variability in fungal recovery among the three repetitions, EPG variables from repetitions 1 and 2 were compared with those from repetition 3 using Linear Models or Generalised Linear Models, depending on residual distribution and homoscedasticity. As no significant differences were detected among repetitions, data from all three were pooled for subsequent analyses.

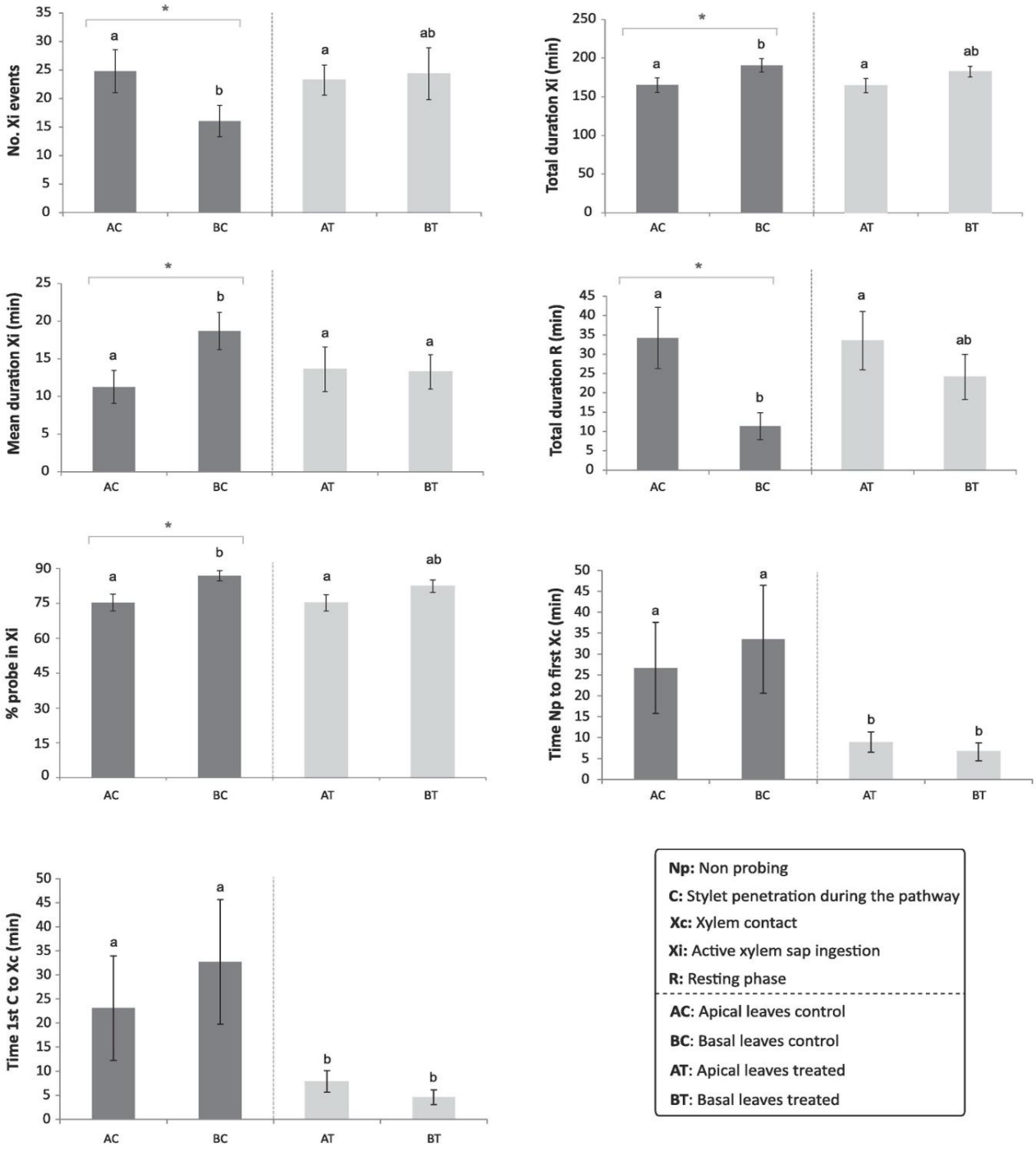


Figure 7. Electrical Penetration Graph (EPG) variables (mean ± SE) influenced by inoculation with *Metarhizium brunneum* (significant differences indicated by different letters; LM and GLM analyses, $P < 0.05$) and by leaf position (apical vs basal) in both control and treated plants (significant differences indicated by asterisks; Student's t-test or Mann-Whitney U test, $P < 0.05$). AT: apical treated leaves; BT: basal treated leaves; AC: apical control leaves; BC: basal control leaves.

- **Effect of fungal colonisation on the feeding behaviour of *Philaenus spumarius***

Feeding behaviour on *M. brunneum*-treated and untreated plants showed similar patterns in non-sequential EPG variables associated with Np, C, Xc, R and Xe (**Figure 7**). For the xylem ingestion phase (Xi), no overall treatment effect was observed in terms of the number or duration of events. However, when considering both leaf position and treatment, Xi events tended to be more frequent in insects feeding on basal treated leaves (BT) compared with basal control leaves (BC) (t-ratio = −1.866, df = 91, *P* = 0.06) (**Figure 7**).

Fungal colonisation influenced the time required to reach xylem tissues. Specifically, the intervals from Np to first Xc (F = 5.21, df = 3, *P* = 0.002) and from first C to first Xc (F = 5.23, df = 3, *P* = 0.002) were shorter in insects feeding on treated plants compared with controls (**Figure 7**; **Supplementary Material 1**). No significant differences were found in probe-related variables, including total probing time, time to first probe, and percentage of probes (**Supplementary Material 1**).

- **Effect of leaf position on the feeding behaviour of *Philaenus spumarius***

In control plants, leaf position significantly affected several Xi-related variables (BC vs AC). Insects feeding on apical control leaves (AC) exhibited a higher number of Xi events (t = 2.12, df = 91, *P* = 0.04), but with lower total and mean duration of Xi (total duration: W = 149, *P* = 0.04; mean duration: t = −2.45, df = 37.60, *P* = 0.02), as well as a lower proportion of probing time spent in Xi (W = 134, *P* = 0.01), compared with those feeding on basal control leaves (BC). Additionally, the total duration of R was greater in AC than in BC (W = 337, *P* = 0.02) (**Figure 7**).

No significant differences were observed in the remaining sequential or non-sequential EPG variables between basal and apical leaves in control plants, and no effect of leaf position was detected for any EPG variable in treated plants (**Supplementary Material 1**).

2.2.3. Detection of *Metarhizium brunneum* in the olive plants

The presence of active propagules of *M. brunneum* on the surface of sprayed leaves (epiphytic occurrence) was confirmed in all olive plants 52 h after foliar application using the leaf print microbiological method. In contrast, internal colonisation of these leaves (endophytic presence) was verified by qPCR in 65.38% of the plants (**Table 2**). Only EPG data from plants confirmed as endophytically colonised were retained for statistical analysis.

For non-sprayed leaves from foliar-treated plants, fungal propagules were detected on the leaf surface in 16.67% of cases based on microbiological analysis (**Table 2**). However, these samples tested negative by qPCR, suggesting that positive leaf print results were likely due to superficial contamination. Anyhow, the corresponding EPG data were excluded from the analysis. Endophytic colonisation of non-sprayed leaves was confirmed by qPCR in 8.33% of the plants (**Table 2**). Two additional non-sprayed samples could not be analysed due to

technical issues, and therefore no data were available for these plants. No evidence of *M. brunneum* colonisation was detected in control plants (**Table 2**).

In plants subjected to soil drench application, no endophytic colonisation by *M. brunneum* was detected.

Table 2. Proportion of *Metarhizium brunneum* epiphytic and endophytic colonized olive leaves 52 h after foliar spray

Treatment		Number of samples	Epiphytic colonization: Microbiological analyses (leaf print)			Endophytic colonization: Molecular analyses (qPCR)		
			Positives	Negatives	%Positives	Positives	Negatives	%Positives
Control	Sprayed	20	0	20	0	0	20	0
	Non sprayed	20	0	20	0	0	20	0
M. brunneum	Sprayed	26	26	0	100	17	9	65.38
	Non sprayed	24	4	20	16.67	2	22	8.33

2.2.4. *Philaenus spumarius* probing and feeding behaviour on *Metarhizium brunneum* treated olive plants

A total of 97 valid EPG recordings were obtained from plants confirmed to be endophytically colonised: 17 corresponding to *P. spumarius* feeding on control leaves sprayed with 0.01% aqueous Tween 80, 20 on non-sprayed control leaves, 16 on *M. brunneum*-sprayed leaves, 12 on non-sprayed leaves from *M. brunneum*-treated plants, 15 on *M. brunneum* drench-treated plants, and 17 on control drench-treated plants. No significant differences were detected between sprayed and non-sprayed control leaves for any of the EPG variables analysed, indicating that neither leaf position nor Tween 80 influenced the feeding behaviour of *P. spumarius* in olive plants (**Supplementary Material 2**). Overall, foliar application of *M. brunneum* significantly affected the frequency of several waveform events, with more pronounced effects observed in directly sprayed leaves than in non-sprayed leaves from treated plants.

The number of probing events was significantly higher in insects feeding on *M. brunneum*-sprayed leaves compared with controls (**Figure 8A**; **Supplementary Material 2**). Likewise, the number of unsuccessful probes, defined as probes in which the insect did not reach the xylem, was also significantly greater in sprayed leaves (**Figure 8B**; **Supplementary Material 2**). In parallel, the number of np events was significantly increased in *M. brunneum*-sprayed leaves relative to controls (**Figure 9A**; **Supplementary Material 2**).

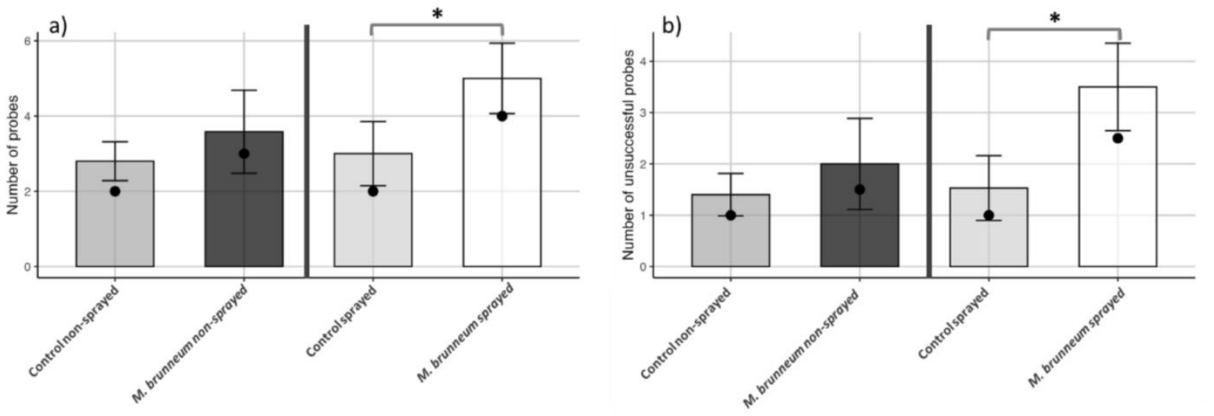


Figure 8. Number of probes (**A**) and unsuccessful probes (**B**) performed by *Philaenus spumarius* on control non-sprayed, *M. brunneum* non-sprayed, control sprayed, and *M. brunneum* sprayed leaves ($N = 65$). Bars represent means ± standard errors; dots indicate medians. Black horizontal lines show pairwise comparisons within each application method (non-sprayed on the left, sprayed on the right). Asterisks (*) indicate significant differences ($p < 0.05$).

Although no differences were found in the total duration of np between sprayed and control leaves (**Figure 9B**; **Supplementary Material 2**), a significant difference emerged when comparing sprayed leaves with non-sprayed control leaves (**Supplementary Material 2**).

In contrast, the number of N events was significantly reduced in *M. brunneum*-sprayed leaves compared with sprayed controls, and also in non-sprayed leaves from treated plants compared with their respective controls (**Figure 9C**). Consistently, both the total and relative duration of N were lower in treated plants, with the strongest reduction observed in sprayed leaves (**Figure 9D**; **Supplementary Material 2**). Similarly, the total duration of Xc was significantly shorter in *M. brunneum*-sprayed leaves than in both sprayed and non-sprayed control leaves (**Figure 9E; Supplementary Material 2**).

The time required to the first probe with Xi was significantly longer in *M. brunneum*-sprayed leaves compared with non-sprayed control leaves (**Figure 10; Supplementary Material 2**). A similar pattern was observed for the time to the first sustained Xi event, with the longest delays recorded in sprayed leaves, differing significantly from both non-sprayed control leaves and non-sprayed leaves from treated plants (**Figure 9**; **Supplementary Material 2**). Although only two sequential variables showed statistically significant differences, all of them followed a consistent trend indicating delayed access to xylem in insects feeding on sprayed leaves (**Supplementary Material 2**). No differences were detected between non-sprayed leaves from treated plants and control leaves.

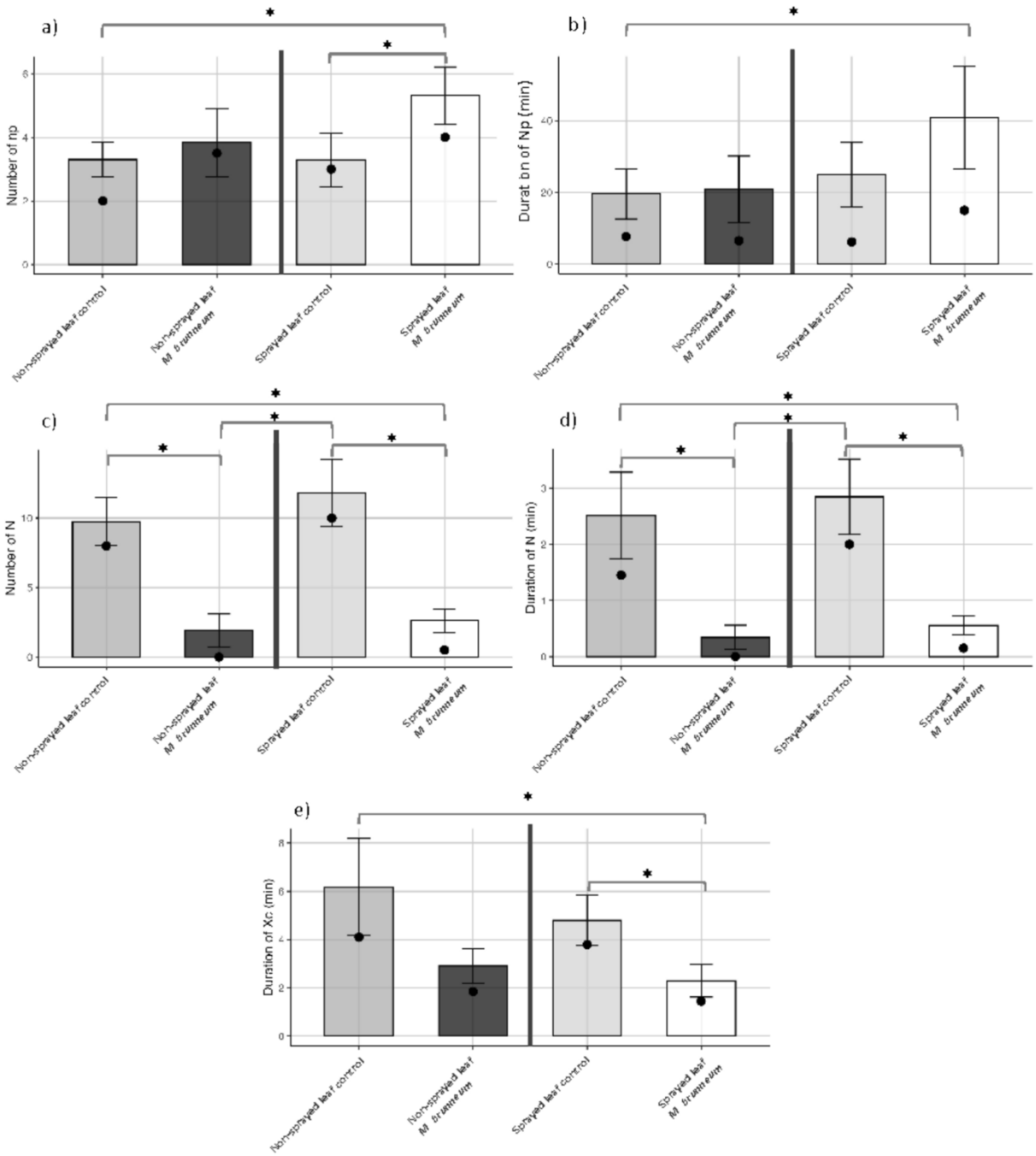


Figure 9. **(A)** Number of np events; **(B)** Duration of np events; **(C)** Number of N; **(D)** Duration of N; and **(E)** Duration of Xc performed by *Philaenus spumarius* on control non-sprayed, *Metarhizium brunneum* non-sprayed, control sprayed, and *M. brunneum* sprayed leaves. *N* = 65. Bars represent mean values ± standard error; dots indicate medians. Asterisk (*) indicates statistically significant differences ($p < 0.05$).

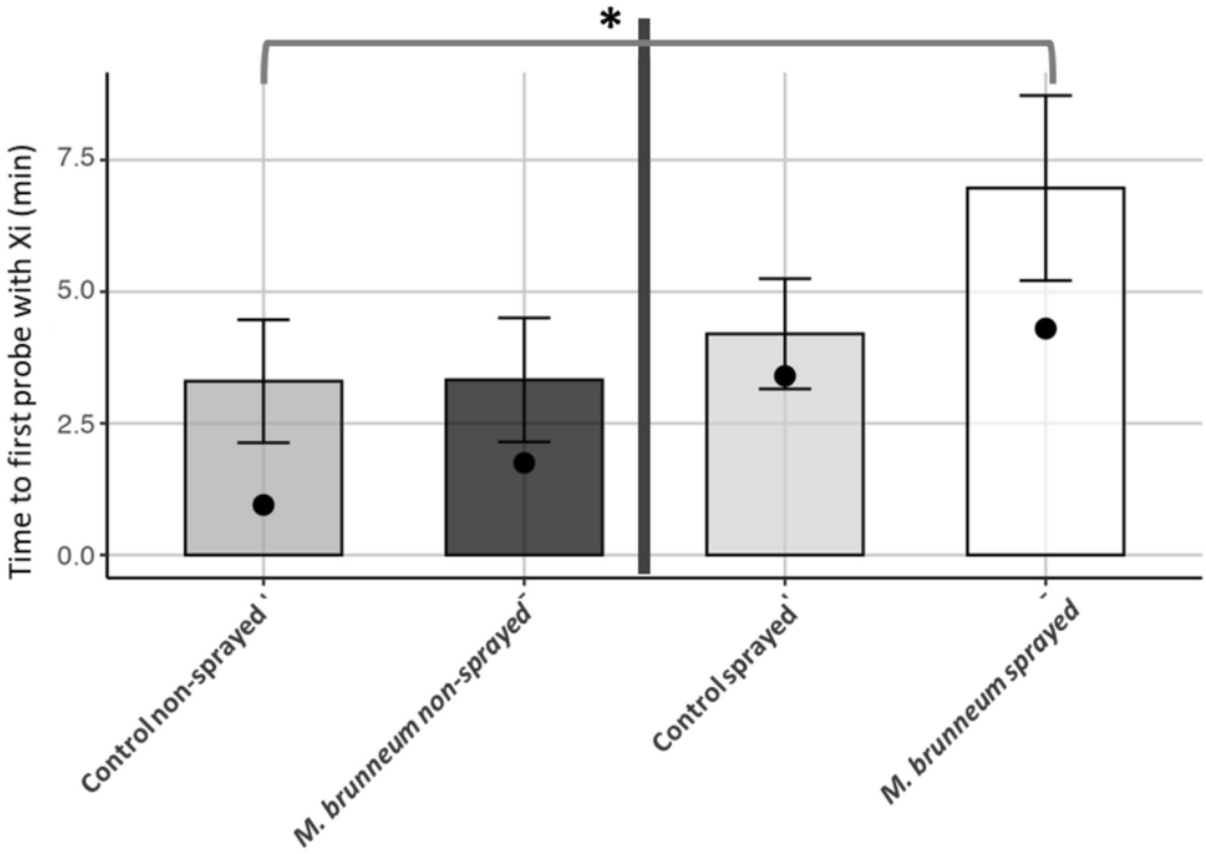


Figure 10. Time to first probe with Xi performed by *Philaenus spumarius* on olive leaves from control non-sprayed, *Metarhizium brunneum* non-sprayed, control sprayed, and *M. brunneum* sprayed plants $N = 65$. Bars represent mean values ± standard error; dots indicate medians. Asterisk (*) indicates statistically significant differences ($p < 0.05$).

Xe waveforms (xylem egestion events associated with Xf transmission) were rarely observed, with only two events recorded in separate recordings, both on sprayed control leaves. The R waveform also occurred at very low frequency, being detected in one recording from *M. brunneum*-sprayed leaves, one from non-sprayed leaves of treated plants, two from sprayed controls, and six from non-sprayed control leaves; therefore, only descriptive (non-statistical) analysis was performed. No additional significant differences were found among treatments for the remaining EPG variables (**Supplementary Material 2**).

No significant effects of *M. brunneum* were observed on the feeding behaviour of *P. spumarius* in plants subjected to soil drench application. All raw EPG data are provided in **Supplementary Material 3**.

2.2.5. Survival of *Philaenus spumarius* after exposure to treated sowthistle and olive plants

Mortality responses differed markedly between experimental systems. In both cases, after the 4 h feeding recordings, insects were transferred to untreated *S. oleraceus* plants. In *S. oleraceus*, *P. spumarius* adults feeding on plants colonised by *M. brunneum* showed significantly higher mortality than those feeding on control plants. Mortality reached 53.8% in insects feeding on directly treated leaves (BT) compared with 13.6% in control leaves (BC; treated with Tween 0.1%), while insects feeding on untreated but endophytically colonised leaves (AT) exhibited 48.2% mortality compared with 18.1% in their respective controls (AC). Fungal outgrowth was observed only in a proportion of dead insects from directly treated leaves (18% in BT), whereas most dead individuals did not show fungal development after incubation, and no outgrowth was detected in insects that had fed on untreated but colonised leaves. In contrast, in olive, *P. spumarius* adults exposed to *M. brunneum*-treated or control plants showed no significant differences in survival among treatments, with overall very low mortality. Only two deaths were recorded, occurring after exposure to a non-sprayed control leaf and a non-sprayed leaf from a *M. brunneum*-treated plant.

2.3. Conclusions

The results of these studies indicate that *M. brunneum* strain EAMa 01/58-Su represents a promising candidate for incorporation into sustainable management strategies targeting *P. spumarius*. The fungus was able to successfully establish as an endophyte in both *S. oleraceus* and olive plants, demonstrating its capacity to colonize relevant host species under different conditions.

Exposure of *P. spumarius* individuals to colonized plants resulted in a measurable impact on insect performance. After a short feeding period (4 h), a significant increase in mortality was observed in insects feeding on colonized *S. oleraceus*, whereas this effect was not detected in olive plants.

In addition to its effects on survival, *M. brunneum* colonization interfered with several feeding behavioural patterns of *P. spumarius*. Notably, some of these behaviours are directly linked to the processes of *X. fastidiosa* acquisition and inoculation (Cornara et al., 2020), indicating that the fungus may influence vector competence even in the absence of increased mortality.

Taken together, these findings support the potential of *M. brunneum* EAMa 01/58-Su as a sustainable control tool. Its application, for instance through the treatment of ground vegetation in crops at risk of *X. fastidiosa*, could contribute to vector management while reducing reliance on agrochemicals and avoiding the need for ground cover removal.

3. CHAPTER II: EFFECT OF DIRECT APPLICATION OF *METARHIZIUM BRUNNEUM* ON *PHILAENUS SPUMARIUS* FEEDING BEHAVIOUR AND SURVIVAL

Following the findings of Chapter I, which demonstrated the effect of *M. brunneum* on how plant colonization (in *S. oleraceus* and *O. europaea*) influences its feeding behaviour and subsequent mortality, this chapter aims to further investigate the impact of direct fungal application on the proper body of the insect. Specifically, we assessed how the direct treatment of *P. spumarius* adults with *M. brunneum* affects both its survival and its feeding behaviour, the latter evaluated on *S. oleraceus* and olive plants 72 hours after application.

3.1. Materials and methods

3.1.1. *Philaenus spumarius* collection and maintenance

Philaenus spumarius individuals used in this study were obtained from meadows in an unmanaged area in Villanueva de la Cañada (40°27’18.28”N, 4°00’20.57”W). The area was characterized by the presence of *Quercus ilex* L. together with a diverse herbaceous layer dominated by *Eryngium campestre* L., *Carduus tenuiflorus* L., *Plantago* spp., and *Sonchus* spp. Field surveys were performed during April and May 2025, with sampling focused on herbaceous host plants. Late-instar nymphs were collected on *E. campestre* L. and *C. tenuiflorus* L. Plant material bearing nymphs was cut and stored in plastic bags for transport to the greenhouse at ICA-CSIC for rearing.

Upon arrival, nymphs were individually relocated using a fine paintbrush onto *S. oleraceus* plants. Each plant was infested with twenty individuals and kept inside insect-proof BugDorm cages (90 × 60 × 60 cm). Rearing took place under greenhouse conditions (25.47 ± 0.3 °C and 62.1 ± 0.5% relative humidity) at ICA-CSIC. The nymphs were maintained on these plants until reaching the adult stage. After emergence, adults remained under the same environmental conditions and continued feeding on fresh *S. oleraceus*. Host plants were replaced periodically, approximately every two to three weeks, to ensure an adequate food supply, avoid overcrowding, and maintain appropriated rearing conditions.

3.1.2. Fungal strain and inoculum preparation

Inoculum preparation was performed as described in Chapter I (Section 1.2). Only conidial suspensions of *M. brunneum* (EAMa 01/58-Su) with >97% germination after 24 h were used (Yousef et al., 2014).

3.1.3. Plant material

Sonchus oleraceus plants at the 4-5 leaf stage were used for rearing *P. spumarius* nymphs until adult emergence, as well as for maintaining *P. spumarius* adults. In addition, *S. oleraceus* plants at the same 4-5 leaf stage were used to study insect probing and feeding behaviour using the electrical penetration graph technique (EPG) and to subsequently assess insect mortality.

Sonchus oleraceus plants were grown in a climatic chamber (24:20°C day: night temperature; 16:8h light: dark photoperiod) at ICA-CSIC. Seeds were pre-germinated and transplanted as described in Chapter 1, Section 1.3, and plants were grown under the same conditions.

Olive plants were additionally used to evaluate insect probing and feeding behaviour by means of the EPG technique and to subsequently assess insect mortality. Six-month-old healthy olive plants (*Olea europaea L. var. "Arbequina"*) were produced in 10 × 10 × 20 cm pots at the greenhouse facilities of the UCO. Olive plants were maintained under controlled greenhouse conditions (24:18 ± 2°C day: night temperature, 14:10 h light: dark photoperiod; 60-70% relative humidity and then transported to ICA-CSIC, where EPG recordings were conducted.

3.1.4. Experimental design

Philaenus spumarius adults raised in the ICA-CSIC greenhouse facilities (1.5- and 3.0-month old) were used for experiments, which were conducted between June and July 2025. Each day, approximately 22 insects were selected, anesthetized by cold exposure for 20 minutes, and subsequently immobilized using a vacuum device. Individuals were then connected to a gold wire using silver conductive paint as previously described Cornara et al. (2018b) (**Figure 11**).



Figure 11. *Philaenus spumarius* individuals were first immobilized using a vacuum device and subsequently connected to a gold wire using silver conductive paint.

Once tethered, insects were treated with 196.6 ± 3.8 µL of either a control solution (0.01% Tween 80) or a fungal suspension of *M. brunneum* strain EAMa 01/58-Su at a concentration of 1×10^8 conidia mL^{-1} (as described in Section 1.2), applied using a glass atomiser. For each daily replicate, 11 insects were assigned to the control treatment and 11 to the fungal treatment. In total, 191 insects received the control solution and 192 insects were treated with the fungal suspension (**Figure 12a**).

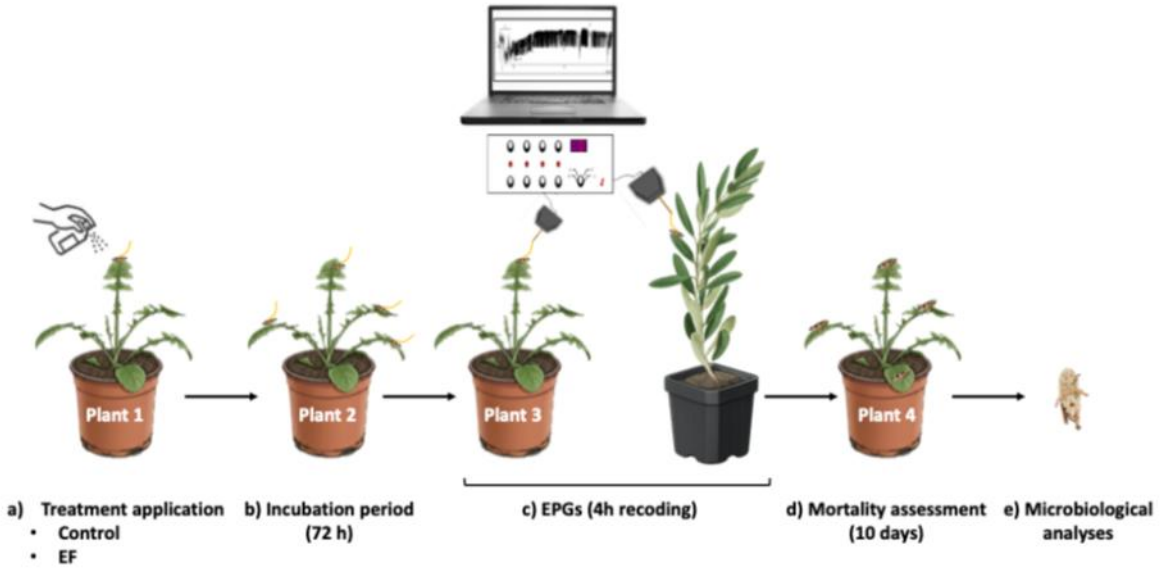


Figure 12. Experimental design: (**A**) Insect treatment (Control solution: 0.01% Tween 80%; EF: *Metarhizium brunneum* (10^8 conidia mL^{-1}) 0.01% Tween 80%), (**B**) incubation period, (**C**) experimental EPGs in olive and *Sonchus oleraceous*, (**D**) mortality assessment, and (**E**) detection of the fungus.

Following treatment, insects were transferred to untreated *S. oleraceus* plants and maintained for 72 h under controlled environmental conditions (24:20 ± 2 °C day: night temperature; 14:10 h light: dark photoperiod). This 72-h period corresponded to the incubation phase of the fungal infection and was conducted on untreated plants to assess the direct effects of the fungal treatment on the insect, avoiding any potential secondary exposure through feeding on fungus-treated plants. Before treatment, insects were connected to a gold wire using silver conductive paint Ted Pella, no. 16062; Pelco → Colloidal Silver (Ted Pella, Redding, CA, USA). Although insects remained attached to the gold wire, they were not connected to any other object, allowing unrestricted movement on the leaf throughout this period. Mortality was assessed daily during these 72 h (**Figure 12b; Figure 13**).



Figure 13. At 72 h incubation period, insects remained attached to the wire but were allowed to move freely.

After the 72-h incubation period, insects holding the gold wire were connected on to a copper nail using silver conductive paint and then connected to the EPG probe following the procedure described by Cornara et al. (2018b) (**Figure 4; 12c**). Feeding behaviour was then recorded continuously for 4 h using either Giga-4 DC-EPG or Giga-8 DC-EPG devices (EPG Systems, Wageningen, The Netherlands) (**Figure 14**). Recordings on *S. oleraceus* and olive plants were not performed simultaneously but were conducted in separate experimental EPG sessions. All recordings were carried out inside a Faraday cage to minimise electrical noise. One insect per plant was placed in the case of *S. oleraceus* and two insects per plant were placed in olive plants. Individuals were initially placed on the adaxial leaf surface but were allowed to move freely during the recording period.

After the 4 h of EPG recordings, insects were individually transferred to new untreated *S. oleraceus* plants and maintained in a greenhouse under controlled conditions (25.5 ± 1.5 °C and 62 ± 2% relative humidity) for up to 10 days post-treatment. Mortality was monitored daily during this period (**Figure 12d**) (**Figure 15**). Subsequently, microbiological analyses were conducted on all individuals, to determine the presence or absence of *M. brunneum* (**Figure 12e**).

To confirm fungal infection as the cause of death, dead individuals were surface-sterilized, incubated under controlled conditions, and examined for fungal growth to verify *M. brunneum* infection as described in Chapter I (Quesada-Moraga et al., 2006).



Figure 14. Experimental setup for EPG recordings on *Sonchus oleraceous* and olive plants.



Figure 15. Mortality assessment following EPG recordings.

3.1.5. Data processing and statistical analysis

Only EPG recordings from control insects in which *M. brunneum* was not detected, and from treated insects in which the fungus was confirmed, were included in the feeding behaviour analyses. EPG recordings from insects that escaped from the plant were not considered in the analysis. The same criteria set out in Chapter I were applied when selecting valid recordings.

Signal acquisition and waveform analysis were performed using Stylet+ software (EPG Systems, Wageningen, The Netherlands). EPG waveforms were manually annotated, and behavioural variables were calculated using an updated version of the "XylFeed" Excel macro specifically developed for xylem-feeding insects (Markheiser et al., 2022; available at https://epg.csic.es/excel_workbook). Both sequential and non-sequential parameters were considered, as previously described in Chapter I (Yousef-Yousef et al., 2023; Lago et al., 2026) and the waveforms considered for the analysis were the same as those described in Lago et al. (2026), except for Xe, which was never observed in our EPG recordings. Data obtained from *S. oleraceus* and olive plants were analysed separately.

To evaluate the effects of treatments on the feeding behaviour of *P. spumarius*, all EPG variables were tested for normality and homogeneity of variance using the Shapiro Wilk and Levene tests, respectively, implemented in IBM SPSS Statistics (version 30). When necessary, data were transformed using the natural logarithm [ln(x + 1)] or square root transformation [(x + 1)] to meet model assumptions. Normally distributed data were analysed using Student's *t*-test, whereas non-normally distributed data were analysed using the non-parametric Mann-Whitney *U* test.

Mortality and escape rates were analysed using contingency table analysis (χ^2 test). When expected frequencies were lower than 5, Fisher's exact t-test was applied. Analyses were conducted with a 95% confidence level using StatView 4.01 software (Abacus Concepts, 1990). Comparisons between treatments (control *vs*. fungal treatment) were based on the total number of dead insects and escaped individuals relative to the total number of treated insects or the number of insects used in EPG recordings.

Finally, survival analyses were performed using the Kaplan-Meier method, and differences between treated and untreated insects were assessed using the log-rank (Mantel-Cox) test calculated with IBM SPSS software (SPSS Inc., Chicago, IL, USA).

3.2. Results

3.2.1. Feeding behaviour of *Philaenus spumarius* 72 h after direct exposure to *Metarhizium brunneum* on *Sonchus oleraceous*

A total of 90 EPG recordings were obtained 72 h after fungal application, comprising 48 insects treated with the control solution and 42 insects treated with *M. brunneum*. After fungal confirmation, there were 86 recordings (46 control and 40 treated with *M. brunneum*).

During the recordings, 6 control insects and 2 treated insects escaped, resulting in invalid recordings. The proportion of escaped insects was higher in control individuals than in those treated with *M. brunneum* (X^2: 1.649 *P*= 0.199). After data filtering, 57 valid EPG recordings were retained and analysed, corresponding to 27 control individuals and 30 *M. brunneum*-treated individuals.

Both sequential and non-sequential variables were assessed as previously described (Lago et al., 2026) (**Supplementary Material 4**). Regarding the number of events—defined as the frequency with which insects performed a given waveform—no statistically significant differences were detected across any of the variables analysed (**Figure 16**).

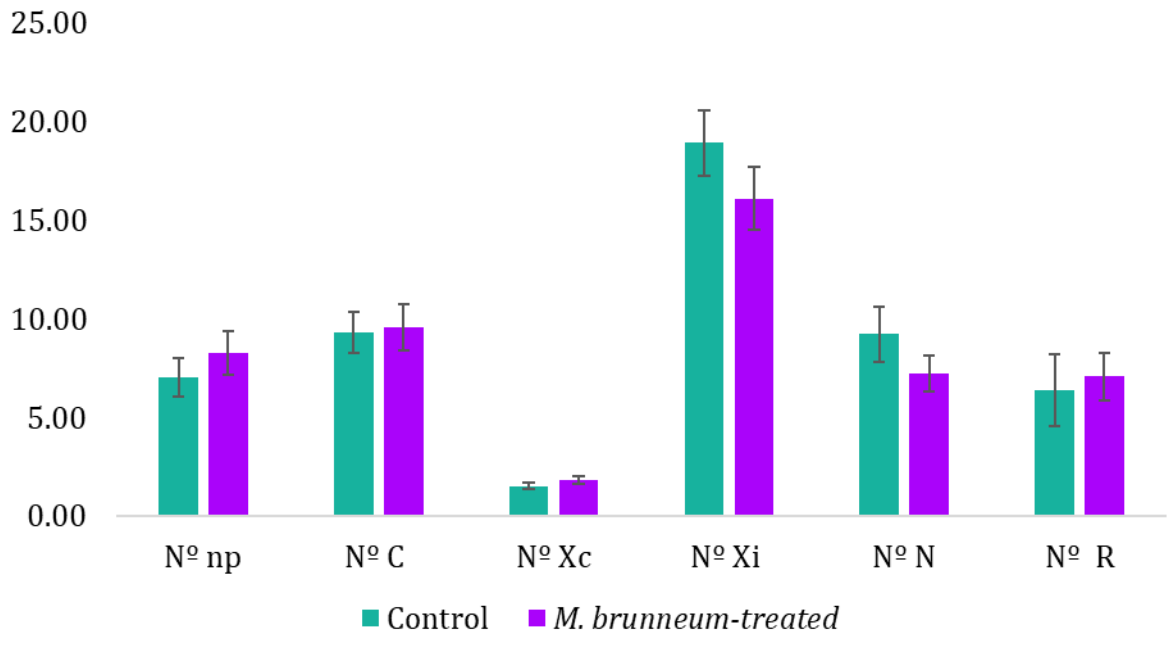


Figure 16. Number of times represented as mean ± standard error a of given waveform along the 4h recording.

An important aspect for evaluating the effect of the fungus is the total duration of each waveform, understood as the total number of minutes during which the insect is engaged in a given activity over the entire recording period. The total duration of the resting phase and the total duration of the xylem contact phase were significantly higher in treated than in control individuals. In contrast, the xylem ingestion and pathway phases were longer in control individuals (although no statistically significant differences were found). (**Figure 17**; **Supplementary Material 4**).

By converting these total durations into percentages, it is possible to gain an overview of the time spent by the insect in each behavioural activity along the total recording time, as shown in **Figure 18**. This figure indicates that, regardless of treatment, insects spent most of the recording period engaged in xylem ingestion, while the resting, pathway, and non-probing phases account for less than one-third of the time allocated to xylem ingestion.

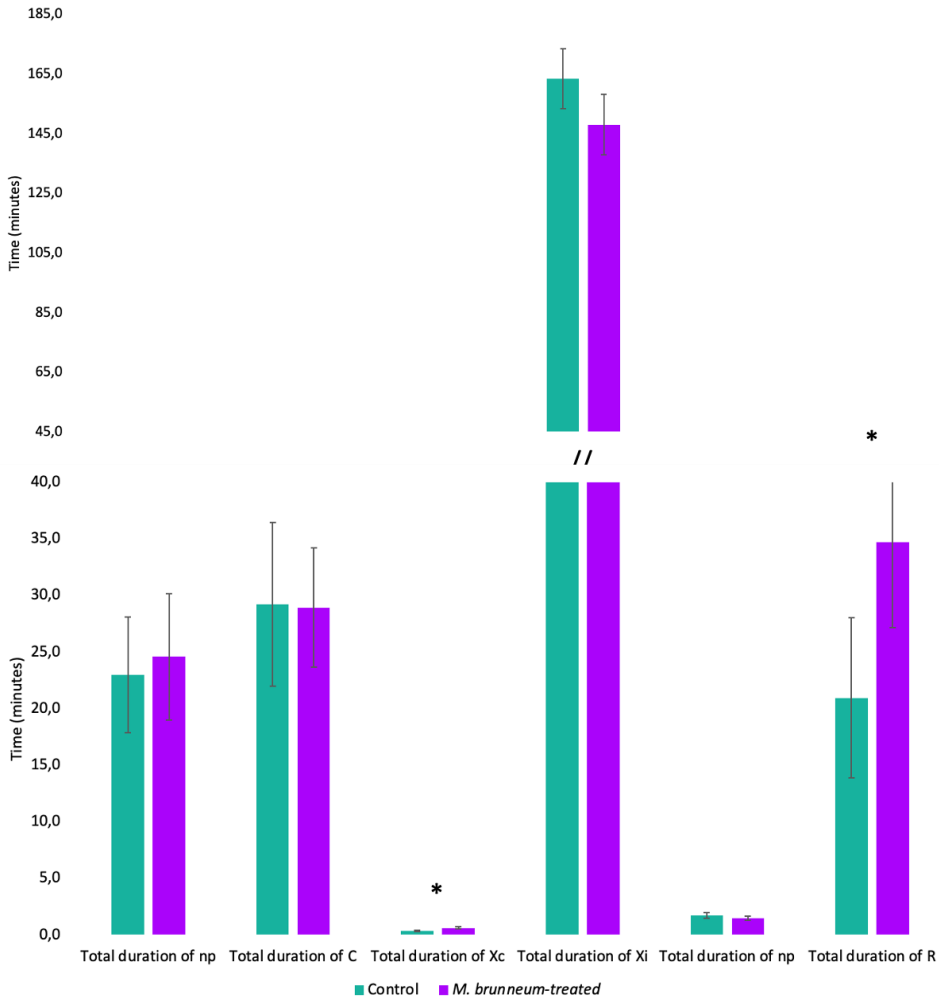


Figure 17. Total duration of the analysed variables during a 4-hour recording period. Statistical differences analysed with Student's *t*-test and Mann-Whitney *U* test.

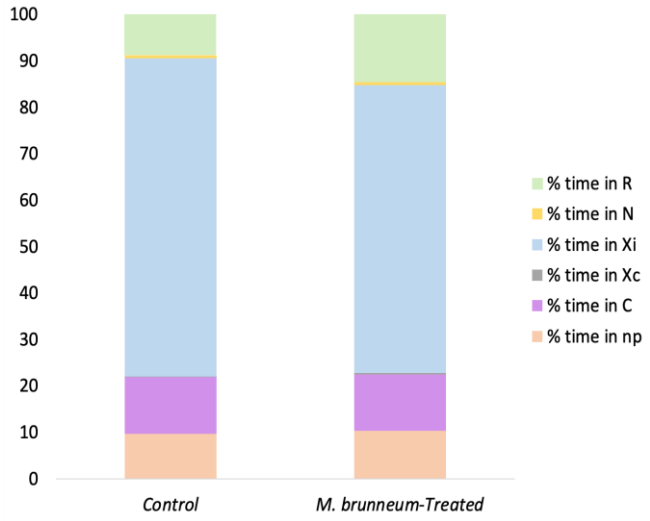


Figure 18. Percentage distribution of the total recording time.

Finally, sequential variables, defined as those describing the time between different events, were analysed (**Figure 19; Supplementary Material 4**). Overall, the time required for insects to reach xylem contact and to initiate ingestion was significantly shorter in treated than in control individuals. A similar pattern was observed for sustained xylem ingestion events (Xi sustained, defined as durations longer than 5 minutes). The variable "time to first probe with Xi" did not show statistically significant differences according to a non-parametric test (Mann–Whitney); however, a noticeable decrease was observed in treated individuals compared to controls.

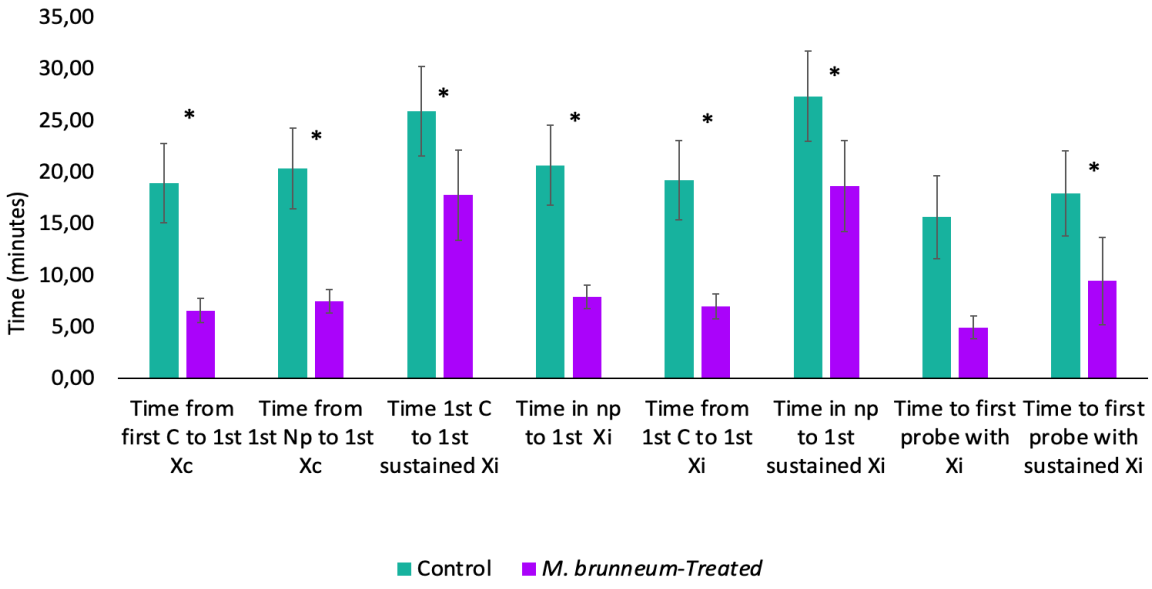


Figure 19. Differences in sequential variables between control individuals and those treated with *M. brunneum*. Student's *t*-test and Mann-Whitney *U* test was performed (**Supplementary Material 4**).

3.2.2. Feeding behaviour of *Philaenus spumarius* 72 h after direct exposure to *Metarhizium brunneum* in olive plants

A total of 73 EPG recordings were obtained 72 h after treatment application, including 40 control and 33 treated with *M. brunneum*. As in the case of *S. oleraceus* (Section 2.1), only control individuals in which no fungal presence was detected and treated individuals in which *M. brunneum* was confirmed were included in the analyses, resulting in 56 EPG valid recordings, comprising 33 insects treated with the control solution and 23 insects treated with *M. brunneum*. During the recording, 11 control insects and 5 treated insects escaped, which is translated into invalid recordings. Control individuals showed a 12% higher escape rate than *M. brunneum*-treated insects (X^2: 1.381 p= 0.240). After data filtering, 36 valid EPG recordings were retained, corresponding to 19 control individuals and 17 *M. brunneum*-treated individuals.

The same analyses were carried out as in the case of the feeding behaviour evaluation in *S. oleraceus* (section 2.1). The table with the EPG variables studied in olive can be found in **Supplementary Material 5.** Concerning the number of events, the same as in the analysis conducted on *S. oleraceus*, no significant differences were observed among the variables analysed (**Figure 20**), although in this case a general trend was noted, with a higher number of all waveform types in the control group compared to the treated group.

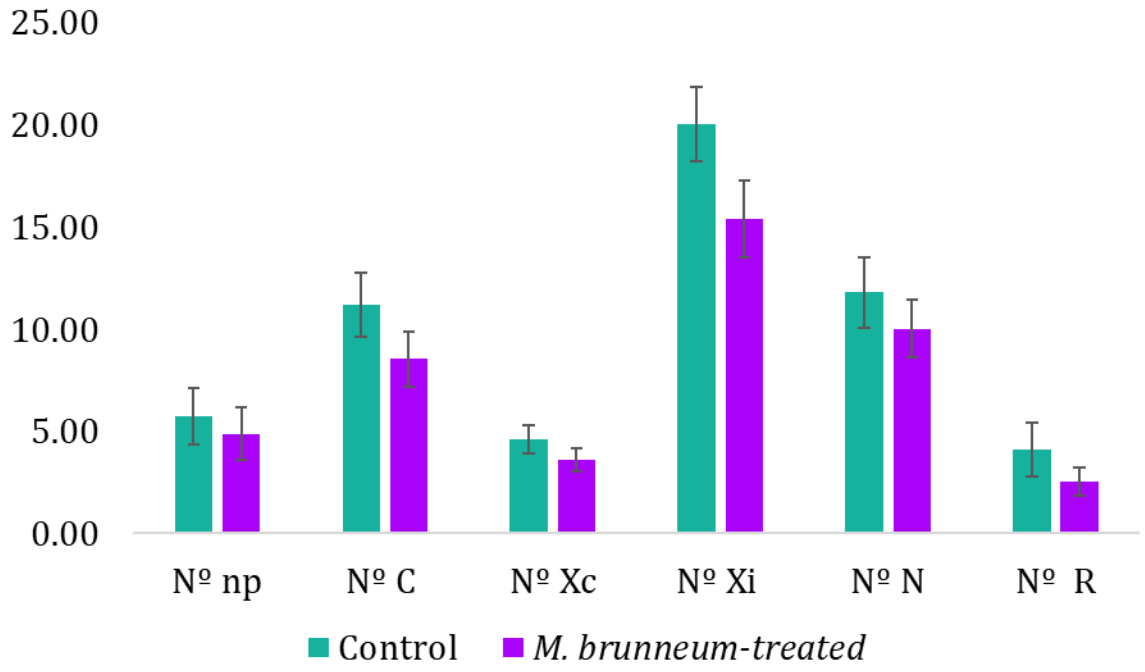


Figure 20. Differences in the number of events (mean ± standard error) between treated and control individuals.

Regarding the duration of these events, Figure 21 shows the mean duration of each event for treated and control individuals. A trend can be observed (although not statistically significant) indicating that treated individuals spend less time in np, C, and N phases, and more time in Xi. In contrast, the duration of xylem contact, and the duration of the resting phase are significantly shorter in treated individuals compared to controls.

As in section 2.1, the percentage of time that insects spend in each phase was calculated (**Figure 22**). As observed in the recordings on *S. oleraceus*, insects spend most of the 4-hour recording period engaged in xylem ingestion. In this case, control insects spend more time in the non-probing phase, accounting for more than one-third of the time spent in Xi.

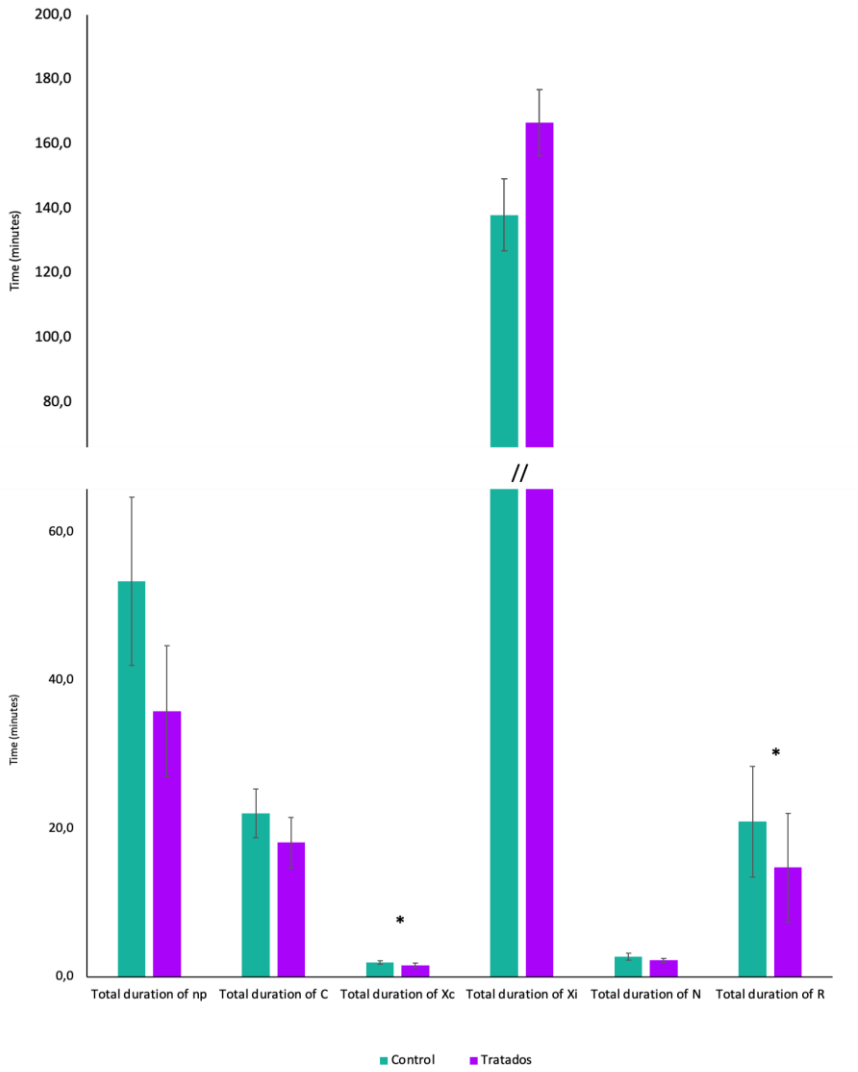


Figure 21. Total duration of the main events during a 4-hour recording period. Student's T test was conducted.

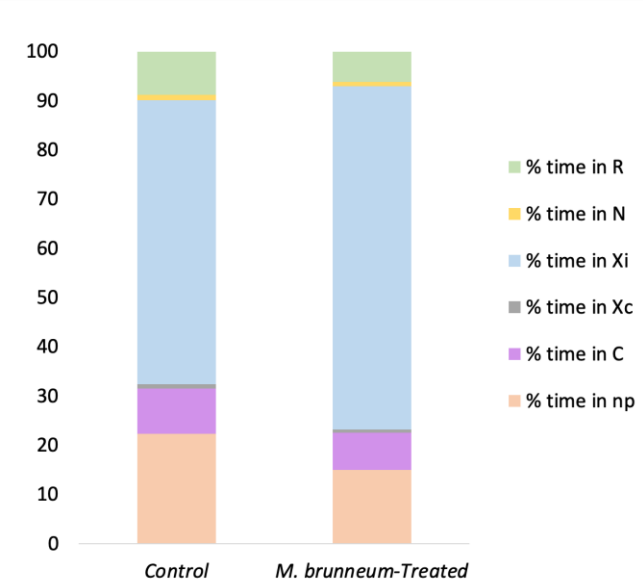


Figure 22. Percentage distribution of the total recording time.

In this case, no significant differences were observed in the sequential variables. However, a trend similar to that observed in insects whose EPG recordings were conducted on *S. oleraceus* can be noted: the time to contact the xylem is reduced in treated individuals, the same as for the time taken to initiate xylem ingestion (**Figure 23; Supplementary Material 5**).

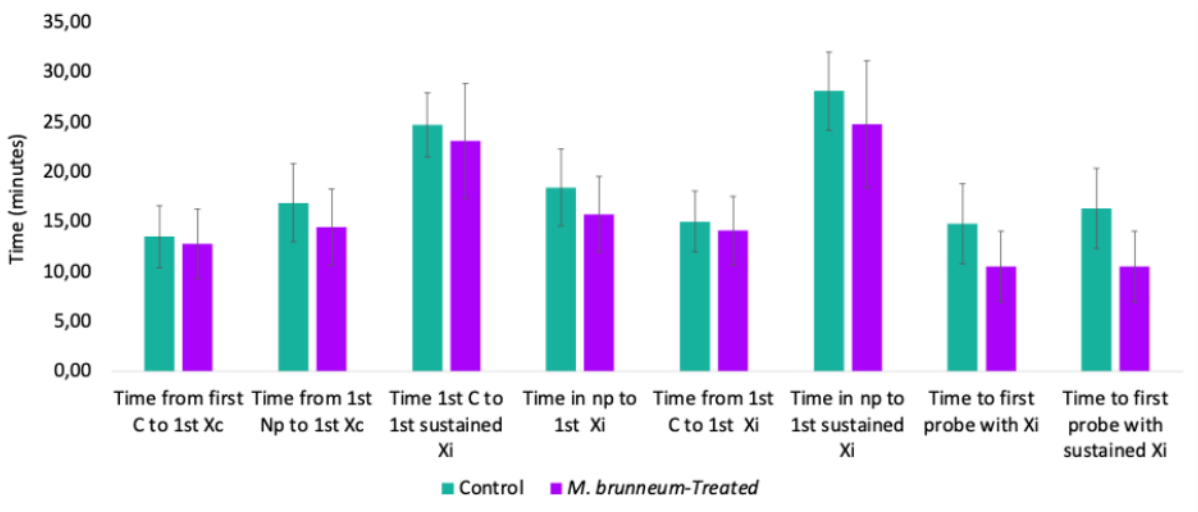


Figure 23. Differences in sequential variables between individuals treated with *Metarhizium brunneum* and control individuals. Statistical differences analysed with *Student*'s T test.

3.2.3. Survival of *Philaenus spumarius* after direct exposure to *Metarhizium brunneum* over a 10-day period

Insects directly treated with the *M. brunneum* suspension exhibited significantly higher mortality compared to those treated with the control solution. Mortality reached 48.0% in treated insects versus 21.3% in the control in assays conducted with *S. oleraceus*, and 40.23% versus 7.5% in treated and control insects, respectively, in assays conducted with olive plants. However, no statistically significant differences were detected in corrected mortality between experiments performed on *S. oleraceus* and those on olive (**Figure 24; Table 3**). Additionally, only insects that show fungal outgrowth after *M. brunneum* treatment and once the EPG experiment had finished were considered in the analyses (**Figure 27**).

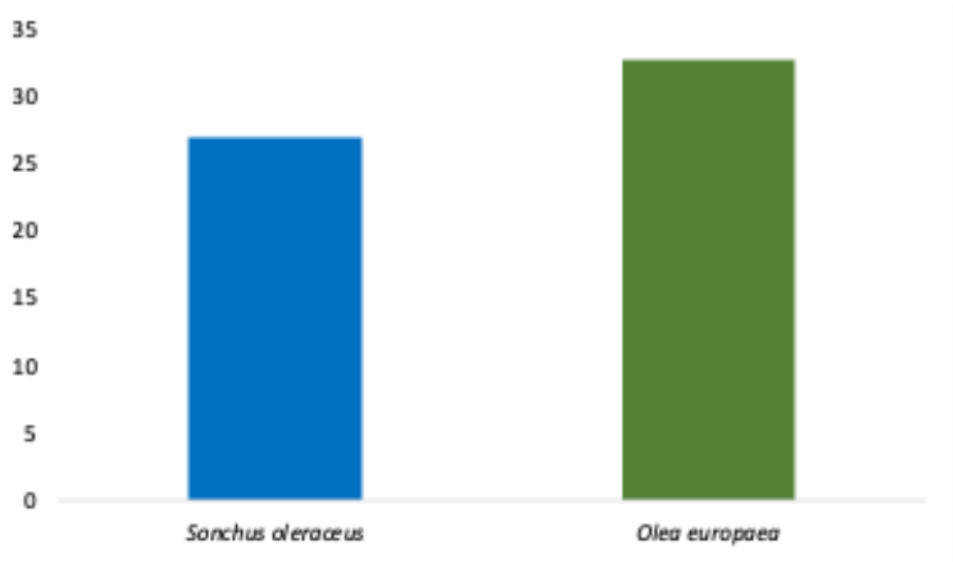


Figure 24. Percentage of corrected mortality in *Sonchus oleraceous* and olive. No statistically significant differences were found (χ^2= 0.099, *P* = 0.753).

In addition to corrected mortality, insect survival was monitored daily for 10 days after treatment. Kaplan–Meier survival analysis revealed significant differences between *M. brunneum* treatments and controls in both *S. oleraceus* (**Figure 25**) and olive assays (**Figure 26**) (**Table 3**).

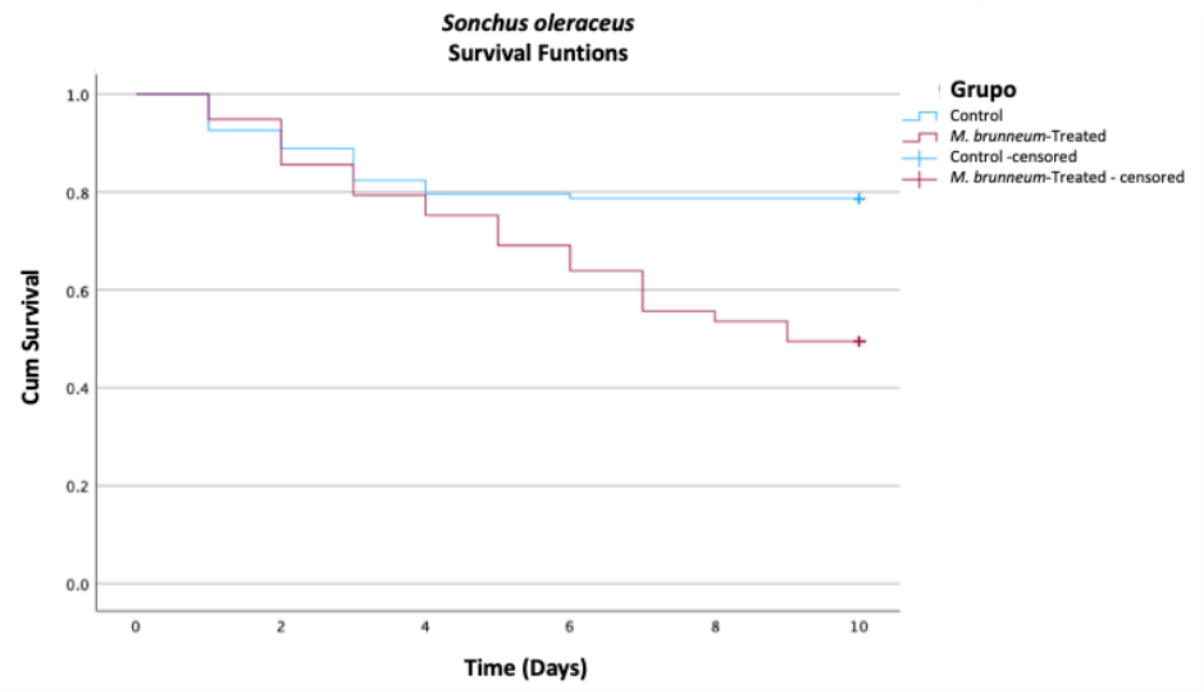


Figure 25. Kaplan–Meier survival curves of insects recorded on *S. oleraceus*, showing the proportion of surviving individuals over time for treated and control groups. Log-rank test: $\chi^2 = 15.705$, $P = < 0.001$

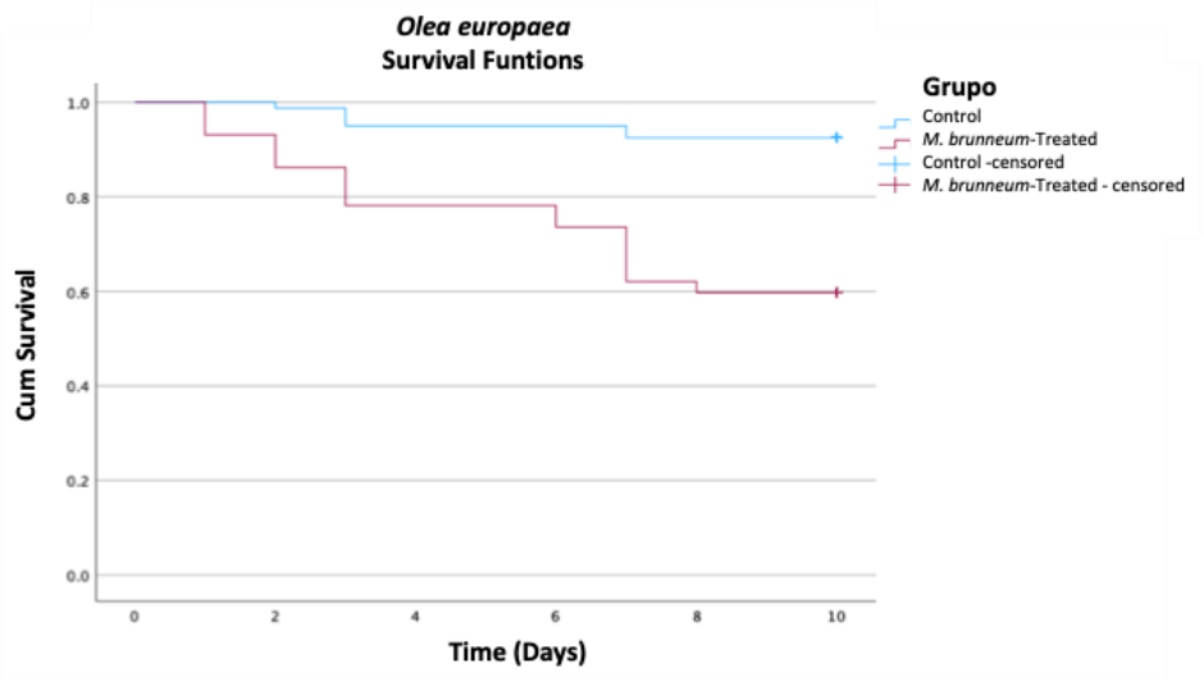


Figure 26. Kaplan–Meier survival curves of insects recorded on olive plants, showing the proportion of surviving individuals over time for treated and control groups. Log-rank test: $\chi^2 = 24.044$, $P = < 0.001$.



Figure 27. *Philaenus spumarius* specimens collected after the EPG experiment and incubated under controlled conditions to promote fungal outgrowth. Insects from the control treatment are shown at the top, while insects from the *Metarhizium brunneum* treatment are shown at the bottom.

Furthermore, the analysis showed significant differences between insects that fed on *S. oleraceus* and those that fed on olive plants during EPG recordings. Specifically, mortality in control groups was significantly lower in olive assays compared to *S. oleraceus* assays (**Table 3**).

Table 3. Pairwise comparisons of survival distributions among experimental groups (Log-rank Mantel–Cox test).

(Log-rank mantel–cox test) Comparison	χ^2	*P*-value
Control *S. oleraceus* vs treated *S. oleraceus*	15.704	<0.001
Control *S. oleraceus* vs control *O. europaea*	6.953	0.008
Control *S. oleraceus* vs treated *O. europaea*	6.788	0.009
Treated *S. oleraceus* vs control *O. europaea*	37.047	<0.001
Treated *S. oleraceus* vs treated *O. europaea*	1.760	0.185
Control *O. europaea* vs treated *O. europaea*	24.044	<0.001

3.3. Conclusions

Control insects showed a higher proportion of escape events in the Electrical Penetration Graph (EPG) recordings compared to insects treated with *M. brunneum*. This difference may be associated with decreased activity level in treated individuals following 72 hours of exposure to *M. brunneum*.

Direct application of *M. brunneum* also had a significant impact on the feeding behaviour of *P. spumarius* on *S. oleraceus*, accelerating access to the xylem and leading to earlier initiation of the first xylem ingestion compared to untreated control insects. A comparable trend was observed in olive, where treated insects also tend to show modified feeding dynamics in the same way; however, these differences were not statistically significant. Notably, in olive, xylem ingestion events were also longer in treated than in untreated insects. Overall, this consistent directional pattern across both host plants may reflect a compensatory response aimed at mitigating physiological stress associated with fungal colonisation.

Mortality was significantly higher in insects treated with *M. brunneum* compared to the control, with the effect becoming more pronounced from day 4 onwards. These results indicate that *M. brunneum* may be considered an effective component under an integrated pest management strategy for *P. spumarius*.

4. Chapter III: Effect of *Metarhizium brunneum* on settlement behaviour of *Philaenus spumarius* in *Sonchus oleraceous* treated plants

4.1. Materials and methods

4.1.1. Experimental materials and conditions: insect collection and maintenance, fungal cultures and inoculum preparation, and plant growth conditions

Insects were collected in Villanueva de la Cañada, Madrid (40°27'15.9"N, 4°00'21.1"W) and maintained as described in Chapter I, Section 1.1. Fungal inoculum was prepared following the protocol detailed in Chapter I, Section 1.2. Plant material used in the assay was grown under the conditions specified in Chapter I, Section 1.3.

To evaluate if the *M. brunneum* treatment affect the settlement behaviour of *P. spumarius* adults, we conducted a series of non-choice and free-choice assays under semi-field conditions in a greenhouse at ICA-CSIC (Madrid, Spain) under natural light conditions and with a maximum temperature controlled to not exceed 25ºC in June 2024 using *Sonchus oleraceus*

as experimental plant species (5 to 6-leaf stage). The strain of *M. brunneum* EAMa 01/58-Su from the culture collection of UCO were used.

Adaxial and abaxial leaf surfaces of *S. oleraceus* plants were sprayed with 2 mL of the conidial suspension of the strain EAMa 01/58-Su 48 h before the experiments. After inoculation, treated and control plants were covered with transparent plastic shielding bags and maintained in a growth chamber at 25ºC and a photoperiod of 16:8 (Light/Dark) to promote fungal colonization. To confirm that the phyllosphere of inoculated plants had been successfully endophytically colonized by the fungus, samples of leaves were collected once the experiments had finished to determine endophytic colonization.

4.1.2. Non-choice settlement assays

The preference of *P. spumarius* to settle on EAMa 01/58-Su inoculated and non-inoculated *S. oleraceus* plants was assessed at different time periods after insect release. For that, a single EAMa 01/58-Su inoculated or non-inoculated *S. oleraceus* plant was placed inside an insect-proof cage (0.25x0.25x0.25 m) (**Figure 28**).

Insects used in the experiment were transferred from the rearing cages to Eppendorf tubes (one Eppendorf tube per cage) with a mouth vacuum aspirator. Thereafter, they were gently released inside cages after a starvation period of 1 hour. A total of three *P. spumarius* individuals per cage were released. The number of insects settled on either EAMa 01/58-Su inoculated or non-inoculated *S. oleraceus* plants was counted at different time intervals (1, 2, 3 and 4 h). Each non-choice assay was replicated twice, using three cages for each treatment and a new set of insects for each replicate.

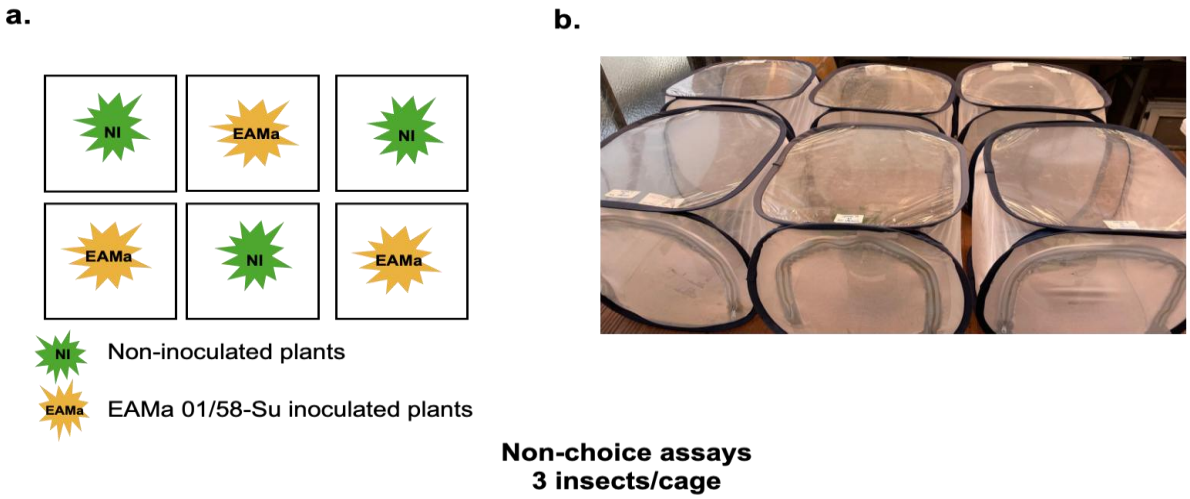


Figure 28. Experimental setup to evaluate settling behaviour of *Philaenus spumarius* on EAMa 01/58-Su inoculated or non-inoculated *Sonchus oleraceous* plants under non-choice experiments.

4.1.3. Free-choice settlement assay

In this case, the preference of *P. spumarius* adults to settle either on EAMa 01/58-Su inoculated or non-inoculated *S. oleraceus* plants was tested at the same different periods under free choice conditions: A total of eight test plants, four EAMa 01/58-Su inoculated and four non-inoculated *S. oleraceus* plants, were alternated in a circle inside a 1x1x1 m cage equipped with an insect proof mesh net (**Figure 29**). The insects were handled as previously described, placing 24 adult individuals in a Falcon tube (one per cage, from which they were released. The number of insects remaining on each treatment was counted at different time intervals (1, 2, 3 and 4h). Each free-choice assay was replicated twice, using four cages with a new set of insects for each replicate

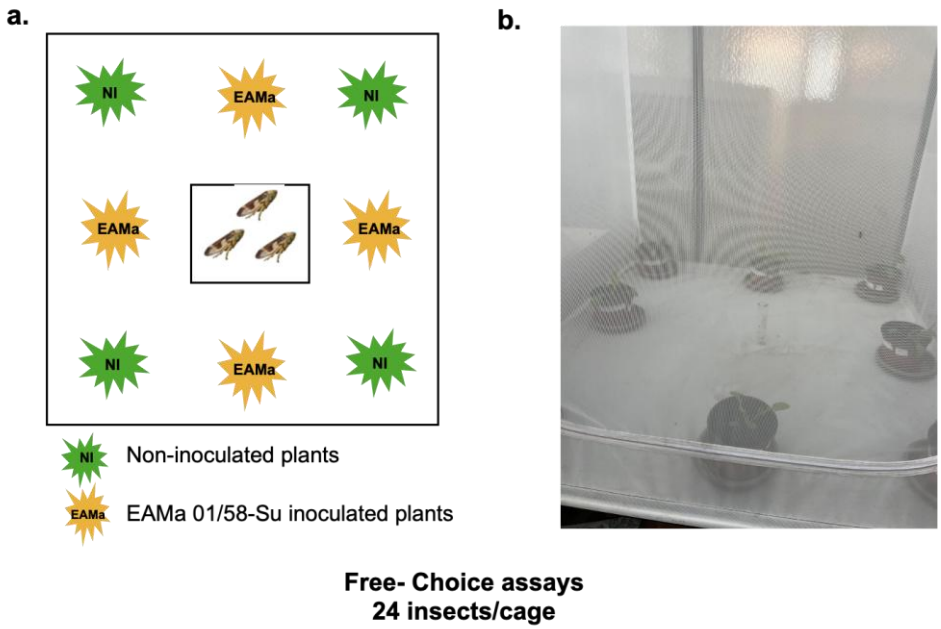


Figure 29. Experimental setup to evaluate settling behaviour of *Philaenus spumarius* on EAMa 01/58-Su inoculated or non-inoculated *Sonchus oleraceous* plants under free-choice experiments.

In both sets of assays, the settling rate for each treatment and time interval was calculated as the mean percentage of insects that remained on the test plant.

4.1.4. Data analysis

To assess the effect of EAMa 01/58-Su strain of *M. brunneum* over time on insect settling behaviour, generalized linear mixed models (GLMM) with a Poisson distribution were applied separately for the free-choice and non-choice experiments. Specifically, we evaluated the percentage of insects settled on each plant relative to the total number of insects released. In both experiments, the model included "treatment" and "time" as fixed effects, with "cage"

as a random effect to account for the grouping of plants within each cage. Post-hoc pairwise comparisons between treatments at each time point were conducted using estimated marginal means (emmeans) with a Tukey adjustment to control for multiple comparisons. Statistical analysis was conducted with R software (R Core Team (2024) R version 4.4.1 (2024-06-14 ucrt); URL https://www.R-project.org/).

4.2. Results

In the free-choice experiment (**Figure 30a**) where insects were allowed to select between EAMa 01/58-Su inoculated or non-inoculated plants, the percentage of insects settling on plants increased over time for both treatments, with no statistically significant differences between treatments. In the non-choice experiment (**Figure 30b**), where insects were confined to a single plant (either EAMa 01/58-Su inoculated or non-inoculated), settlement rates raised up 24h after insect release. Although the differences between treatments were not statistically significant, a trend was observed, indicating that insects may be less likely to settle on EAMa 01/58-Su inoculated plants when no alternative was available.

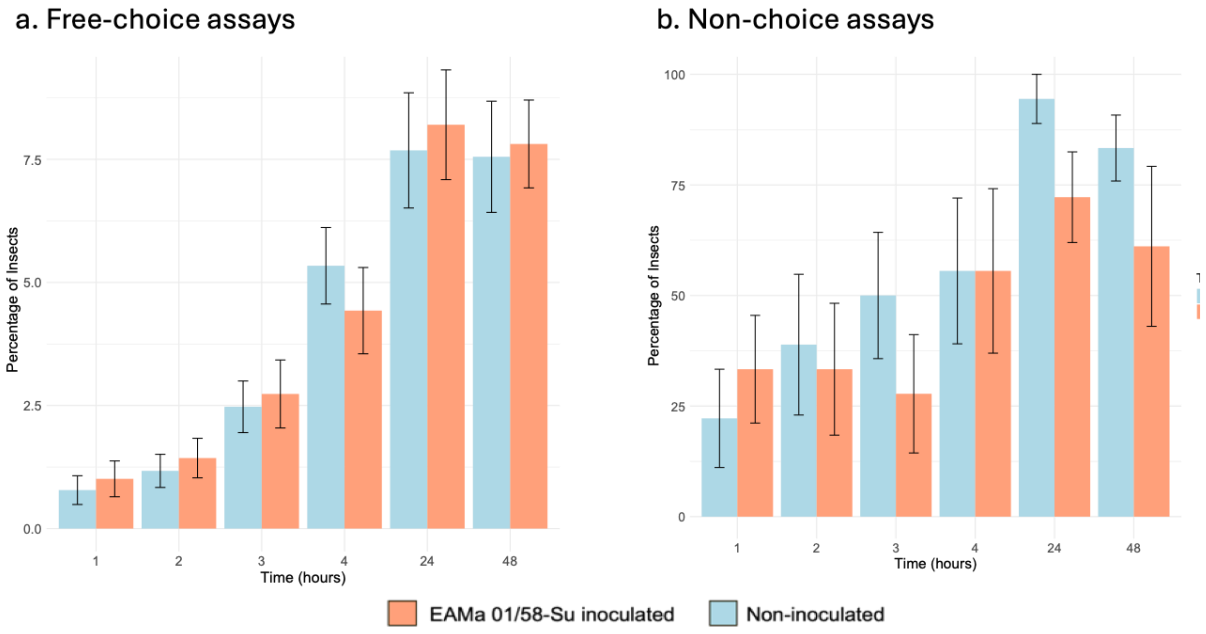


Figure 30. Percentage of *Philaenus spumarius* settled on EAMa 01/58-Su inoculated and non-inoculated plants over time (1-4h and 24h, 48h) under (**A**) free- choice and (**B**) non-choice conditions. Bars represent mean values and whiskers show standard errors.

4.3. Conclusion

Overall, EAMa 01/58-Su inoculation did not significantly affect insect settlement, although insects showed a tendency to reduce their settling rate on plants inoculated by EPF under non-choice conditions.

5. CHAPTER IV: EPIPHYTIC AND ENDOPHYTIC EFFECTS OF ENTOMOPATHOGENIC FUNGI ON MORTALITY OF *PHILAENUS SPUMARIUS* AND *XYLELLA FASTIDIOSA* TRANSMISSION

Transmission experiments with *X. fastidiosa* ST53 (strain De Donno; CFBP 8402) were performed on the herbaceous plant periwinkle (*Catharanthus roseus* (L.) G.Don), and on olive plantlets (*Olea europaea* L. cv Leccino) at Li Foggi, Gallipoli (Lecce province), inside the *X. fastidiosa* infected area. In this case, entomopathogenic fungal strains that showed the highest percentage of mortality in the *P. spumarius* nymphs were used in the bioassays, EAMa 01/58-Su and EABb 04/01-Tip, belonging to *Metarhizium brunneum* and *Beauveria bassiana* species respectively. *Philaenus spumarius* adult mortality caused by the contact with the residual fungal propagules over the plant and/or endophytic colonization of the plant was recorded at the end of the inoculation access period (IAP) (one week).

5.1. Materials and methods

For the herbaceous host experiments, 20 one-month-old seedlings of periwinkle plants were used per treatment in the case of the *B. bassiana* EABb 04/01-Tip insect transmission assay and 15 periwinkle plants per treatment in the case of the *M. brunneum* EAMa 01/58-Su insect transmission assay. For the olive experiments, 20 one-year-old (approximately 30 cm in height) greenhouse-reared olive plants (*Olea europaea* L. cv Leccino) were used per treatment in *Beauveria* and *Metarhizum* assays.

P. spumarius at an adult stage were collected in olive groves located in a *X. fastidiosa* free area from Puglia region (Italy). Then, acquisition was performed by confining on branches of field-grown infected olive trees. Individuals were caged in groups of 20–100 per branch for an acquisition access period (AAP) of 96 h. Before setting the experiments, 15–20 specimens were assessed by qPCR to confirm the presence of the bacterium. These insects were used for the transmission experiments once they were confirmed to have acquired the bacteria.

The entire part of the plants was sprayed either with a conidial suspension of the relevant strain adjusted at 1.0×10^8 conidia mL^{-1} or with control solution helped by an aerograph (2 mL) (**Figure 31**). Seventy-two hours after spraying, the *Xylella*-infected *Philaenus* adults were transferred to the plants. In the case of the periwinkle plants treated with the EAMa 01/58-

Su strain, two spray applications were performed. A second treatment was applied 19 days after the first spraying, and 4 h after this treatment the insects were transferred to the plants.

The insects were transferred onto receptor plants in groups of five per plant for an IAP of one week. Plants were maintained in a climatic chamber under control conditions throughout the IAP (**Figure 32**). After the IAP, all the insects were collected from the cages (either dead or alive individuals) for testing by qPCR an estimation of the bacterial concentration (CFU/head) in the positive specimens following the methodology explained by Cavalieri et al. (2019). At the end of each AAP and IAP experiment, data on survival of the insects were also recorded.



Figure 31. Periwinkle spray treatment helped by an aerograph.



Figure 32. Cages and climatic chambers used to contain the *Philaenus spumarius* adults during the *Xylella fastidiosa* insect transmission to the plants.

Since *X. fastidiosa* only colonizes the foregut and does not systemically spread into the body, only the head of the insect should be used for DNA extraction. On the other side, DNA extraction from insect bodies and qPCR was performed as well to detect the possible presence of the fungus in the insect and to ascertain the cause of their death in this case. DNA of the insect bodies was extracted by placing individually in a 2-mL tube with 2 tungsten carbide beads (for a maximum of 15–20 s at a frequency of 24 cycles s–1, in a Mill300 mixer/Tissue Lyser II (Qiagen) prior to extraction using the CTAB DNA based-extraction protocol (EPPO PM7/24). The quality of the DNA was checked, and concentration was estimated using a NanoDrop 2000 C Spectrophotometer. The DNA was diluted as appropriate and assessed by qPCR to confirm the presence of the fungus (Landa et al., 2013; Barelli et al., 2018).

Endophytic colonization was confirmed by microbiological methods as explained in Chapter I (Resquín-Romero et al., 2016; Garrido-Jurado et al., 2017).

5.2. Results

The remaining inoculum of *M. brunneum* EAMa 01/58-Su over the periwinkle plants caused the mortality of the 33.33 % (20/60) of the *P. spumarius* adults found, 40.00 % (35/75) of the insects not found were considered dead, while the mortality of the insects from the control treatment was 13.73 % (7/51). *Metarhizium brunneum* was not detected in any of the insects from the control periwinkle plants. However, it was detected in 75.00 % (30/60) of the sampled insects (alive and dead) in the *M. brunneum* sprayed plants. The presence of *M. brunneum* was detected in 95 % (19/20) of the found cadavers.

No *M. brunneum* outgrowth was observed in any of the control samples examined or from the plated rinse water. In contrast, it was detected in 33.33 % (5/15) of the sampled leaves from *M. brunneum* sprayed plants.

When *B. bassiana* EABb 04/01-Tip was assayed, it was observed a 22.47 % (20/89) of mortality of the *P. spumarius* adults found, 31.00 % (31/100) if the insects not found were considered dead, while the mortality of the insects from the control treatment was 7.53 % (7/93). The detection of the *B. bassiana* in the sampled insects still needs to be performed.

No *B. bassiana* outgrowth was observed in any of the control samples examined or from the plated rinse water. However, *B. bassiana* was not found when the samples from the fungal sprayed plants were processed due to the high presence of other endophytic microorganisms.

In the case of the olive experiments, we observed a mortality of the 6.90 % (6/87) of the *P. spumarius* adults found on the *M. brunneum* sprayed plants, 19.00 % (19/100) if the insects that were not found were considered dead, while the mortality of the insects from the control treatment was 11.25 % (9/80). *Metarhizium brunneum* was not detected in any of the insects from the control olive plants. However, it was detected in 4.60 % (4/87) of the sampled insects (alive and dead) from *M. brunneum* sprayed plants. The presence of *M. brunneum* was detected in 0.17 % (1/6) of the found cadavers. Related to the *B. bassiana* experiment, we observed that a mortality of the 2.04% (2/98) of the *P. spumarius* adults found, 4.00 % (4/100) of the insects not found were considered dead, while the mortality of the insects from the control treatment is 6.90 % (6/87). The detection of the *B. bassiana* in the sampled insects still needs to be performed.

Regarding the *X. fastidiosa* infectivity of *P. spumarius* used for the olive transmission tests, it was detected a 23.71 % (23/97) and 13.95 % (12/86) of PCR-positive specimens when *P. spumarius* from *Beauveria* treated and control plants were analysed, respectively. In the olive-*Metarhizium* experiment, 17.24 % (15/87) and 20.00 % (16/80) of PCR-positive

specimens were detected when *P. spumarius* from *Metarhizium* was treated and control plants were analysed, respectively.

Results from *Xylella* transmission experiment performed on *B. bassiana* EABb 04/01-Tip treated periwinkle plants showed that the *Xylella* transmission rate was lower when receptor plants had previously been treated with *B. bassiana* conidial suspension (65.00 %, 13/20) compared to that observed in the non-treated plants (85.00 %, 17/20). Results showed a reduction in *Xylella* incidence of 20.00 % because of *B. bassiana* treatment. However, no significant differences between treatments were observed when the statistical analysis was performed (n=40; $\chi 2=2.13$, $P = 0.144$).

In the case of the *X. fastidiosa*-transmission experiment performed on *M. brunneum* EAMa 01/58-Su treated periwinkle plants, no significant differences in the *X. fastidiosa*-transmission rate were discerned between control (93.33 %, 14/15) and *M. brunneum*-treated plants (100.00 %, 15/15) (Fisher's exact test (two-sided $P = 1.000$) (**Figure 33**).

Similarly, in olive plants, no differences were detected among treatments, either with *M. brunneum* EAMa or *B. bassiana* EABb 04/01-Tip.

Results from the *X. fastidiosa*-transmission experiment performed on olive plants treated with *B. bassiana* showed that the *X. fastidiosa* transmission rate was 42.11 % (8/19) in receptor plants previously treated with *B. bassiana* conidial suspension compared to 31.58 % (6/19) in non-treated plants; however, no significant differences were observed between treatments (n = 38; $\chi^2 = 0.45$; $P = 0.50$).

Results from the *M. brunneum*-treated olive plants showed a *X. fastidiosa* transmission rate of 35.00 % (7/20) in treated plants compared to 40.00 % (8/20) in non-treated plants; however, no significant differences were observed between treatments (n = 40; $\chi^2 = 0.11$; $P = 0.74$).

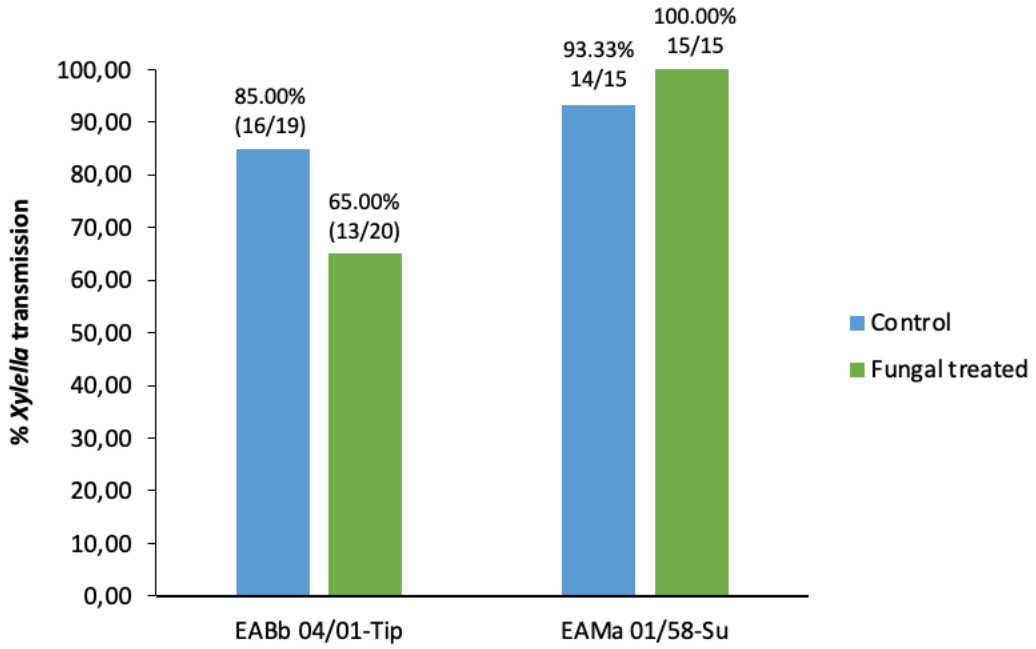


Figure 33. *Xylella fastidiosa* transmission by *Philaenus spumarius* to periwinkle plants endophytically colonized by the entomopathogenic fungus *Beauveria bassiana* EABb 04/01-Tip and *M. brunneum* EAMa 01/58-Su (dark green bars) and control plants (blue bars). No significant differences were observed for *Xylella* transmission when the plants were treated with the EABb 04/01-Tip *B. bassiana* strain (n=40; χ^2=2.13, *P* = 0.144) nor when the plants were treated with the EAMa 01/58-Su *M. brunneum* strain (n=30; χ^2=1.03).

5.3. Conclusion

The results of the *X. fastidiosa* transmission experiments should be interpreted cautiously, as further temporal replicates and larger-scale trials are needed to confirm their robustness. The lack of significant effects may reflect either true biological patterns or experimental variability. Additional experiments are therefore required to clarify the role of fungal colonization in transmission dynamics.

6. GENERAL DISCUSSION

Entomopathogenic fungi constitute a group of microorganisms with strong potential for microbial insect pest control due to their numerous attributes and their complex modes of action (Quesada-Moraga et al., 2020, Quesada-Moraga et al., 2022).

In this deliverable, the evaluation of endophytic colonisation by *M. brunneum* and *B. bassiana* in *S. oleraceus*, and of *M. brunneum* in *O. europaea*, was primarily carried out to determine how fungal establishment within the host plant influences the feeding behaviour of *P. spumarius*. This study provides the first evidence of successful colonisation of olive tissues by an entomopathogenic fungi. The work assessed both the indirect effects of fungal colonisation on *P. spumarius*, including host preference or repellence towards colonised *S. oleraceus* plants, feeding behaviour, and mortality after feeding on colonised plants, as well as the direct pathogenic effect of *M. brunneum* following fungal application to the insect, with mortality evaluated ten days after treatment. Direct effects were explored using *M. brunneum* due to previous studies which demonstrated a greater pathogenic effect of this fungus against *P. spumarius* compared with *B. bassiana* (Yousef-Yousef et al., 2023).

The feeding and development of *P. spumarius* are closely linked to the presence of fresh vegetation (Wise et al., 2006). Therefore, the successful establishment of entomopathogenic ascomycetes (EA) within host plants is essential to ensure insect exposure to fungal propagules and to evaluate their indirect effects on vector behaviour and survival. In *S. oleraceus*, *M. brunneum* showed greater endophytic colonisation efficiency than *B. bassiana* following both foliar spraying and soil application. Moreover, *M. brunneum* achieved high colonisation rates in directly sprayed leaves after 14 days and progressively colonised non-sprayed leaves systemically.

In contrast, colonisation in *O. europaea* was more limited than that observed in other both arable and horticultural herbaceous crops (Raya-Díaz et al., 2017; Gonzalez-Mas et al., 2019b; Cuenca-Medina et al., 2025).

Active fungal propagules were detected epiphytically on 100% of sprayed olive leaves 52 h after application, while molecular analyses confirmed endophytic colonisation in sprayed leaves and, to a lesser extent, in distant non-sprayed leaves, demonstrating the capacity of *M. brunneum* to translocate within olive tissues, a capacity not previously reported in woody crops except for young seedling of radiata pine (Brownbridge et al., 2012). However, drench applications failed to establish endophytic colonisation in olive plants, unlike previous results obtained in herbaceous hosts. This lower colonisation efficiency observed in olive compared with herbaceous crops may be associated with intrinsic characteristics of woody species, including secondary growth, vascular cambium activity, plant-associated microbiota, and the production of secondary metabolites that may restrict fungal establishment (Martins et al., 2018; Moloiyane & Nchu, 2019; Novelli et al., 2019; Bettenfeld et al., 2021; González-Mas et al., 2021; Ponchon et al., 2022; Vergani et al., 2024).

Nevertheless, even transient colonisation may provide important advantages for biological control because endophytic fungi persist more effectively within plant tissues than on leaf surfaces (Vega et al., 2008; Quesada-Moraga et al., 2014), while also inducing systemic physiological and defensive changes in the host plant. Indeed, García-Espinoza et al. (2023) demonstrated that *M. brunneum* can activate jasmonic acid, salicylic acid, and ethylene-related defence pathways in colonised plants, potentially affecting herbivore fitness and behaviour. In both *S. oleraceus* and olive, fungal colonisation was temporary, as previously described for *M. brunneum* and other entomopathogenic fungi such as *B. bassiana* (Landa et al., 2013; Garrido-Jurado et al., 2017; Ullrich et al., 2017; Koch et al., 2018). This transient aspect highlights the importance of synchronising fungal applications with periods of peak adult abundance of *P. spumarius* in ground vegetation, particularly considering the asynchronous emergence of adults in the field (Weaver and King, 1954). Furthermore, considering the strong pathogenic effect observed after direct fungal application to the insect, timing applications to coincide with population peaks may be especially important under field conditions, where both the direct effects of fungal contact and the indirect effects mediated through plant colonisation could act simultaneously, potentially enhancing the overall impact on *P. spumarius* populations and consequently reducing the risk of *X. fastidiosa* infection spread (Vega et al., 2008; Gurulingappa et al., 2010; Quesada-Moraga et al., 2006;2014; Bodino et al., 2024).

The indirect effects of fungal colonisation on the feeding behaviour of *P. spumarius* differed substantially depending on the host plant species.

In *S. oleraceus*, insects were still able to feed efficiently on *M. brunneum*-colonised plants despite some significant modifications in EPG variables. Control insects preferentially fed on basal leaves, exhibiting longer xylem ingestion (Xi) events, fewer Xi events, and shorter resting periods compared with apical leaves. Since xylem tension increases towards apical tissues, feeding on apical leaves may represent a greater energetic cost (Hacke and Sperry, 2001; Zimmermann, 1978). Interestingly, *M. brunneum* colonisation reduced these positional differences, suggesting that fungal presence modified the feeding dynamics across the plant.

Non-sequential EPG variables were not affected by the presence of *M. brunneum* in the colonized plant, suggesting that fungal colonization does not disrupt feeding or enhance potential for *X. fastidiosa* transmission. Waveforms corresponding to the pathway phase (C), xylem contact (Xc), interruption of xylem ingestion (N) and resting (R) are not associated with bacterial transmission to the host plant. Nevertheless, waveforms of xylem ingestion (Xi) and fluid egestion (Xe) are related to acquisition and inoculation of *X. fastidiosa* respectively (Cornara et al., 2020). The presence of *M. brunneum* in colonized plants has no effect on variables related to xylem ingestion showing an average duration of Xi of around 15 min in all treatments which, as described by Cornara et al. (20220) in olive, is enough time for bacterial acquisition in all cases. With respect to bacterial inoculation by *P. spumarius*, it has been associated with the so-called Xe waveform which likely represents fluid egestion regulated by

precibarial valve fluttering (Cornara et al., 2020). In this study Xe events were very occasional (lower than 1 event per probe) and not significantly different between treatments. Furthermore, the first Xe waveform appeared at a minimum of 40 mins after the beginning of the probe in all cases, not coinciding with the times observed for *X. fastidiosa* transmission by *P. spumarius* (ca. 2-7 min after the onset of the probe) (Cornara et al., 2020). The behaviour associated with *X. fastidiosa* inoculation could be triggered by accumulation of bacteria in the foregut which interferes with sap uptake, and also by unfavourable conditions of the host plant for the insect leading to a lack of phagostimulation and subsequent egestion (Cornara et al., 2020). Our results show that *M. brunneum* colonization of plants does not alter the Xe variables for *P. spumarius* between treatments, suggesting that egestion and inoculation with the bacterium would not increase on *M. brunneum*-colonized plants.

Insects feeding on colonised *S. oleraceus* leaves also reached xylem tissues more rapidly, showing shorter intervals between non-probing (Np), pathway phase (C), and first xylem contact (Xc). This may indicate that fungal colonisation facilitates stylet progression towards xylem vessels, potentially through histological modifications caused by fungal growth within intercellular spaces, as previously described in melon leaves colonised by *M. brunneum* (Garrido-Jurado et al., 2017).

Despite these alterations, xylem ingestion duration and the percentage of probing time spent in Xi remained similar between treated and control plants, indicating that *M. brunneum* colonisation in *S. oleraceus* did not reduce the ability of *P. spumarius* to successfully access and ingest xylem sap.

With regard to the feeding behaviour of *P. spumarius* in olive plants colonised by *M. brunneum*, the present study demonstrates that *M. brunneum* colonization interferes with several feeding behavioural patterns of *P. spumarius*, some of which are potentially involved in *X. fastidiosa* transmission (acquisition/inoculation) (Cornara et al. 2020). Spittlebugs feeding on *M. brunneum*-sprayed leaves exhibited a greater number of unsuccessful probes (i.e., probes in which the insect failed to reach xylem tissues) than insects feeding on sprayed control plants. They also showed an increased number and total duration of np events (i.e., insects with stylets outside the plant tissues) compared to the controls. In addition, individuals feeding on *M. brunneum*-treated leaves showed reduced xylem contact time (Xc) and a delayed onset of sustained xylem ingestion (Xi) relative to both control treatments, whereas a delay in non-sustained Xi was only detected when compared with non-sprayed controls.

The epiphytic presence and endophytic establishment of *M. brunneum* in olive plants may restrict the insect's ability to access xylem vessels and efficiently ingest xylem sap, thereby potentially lowering the probability of vector-pathogen contact required for *X. fastidiosa* transmission. In addition, spittlebugs feeding on both sprayed leaves and non-sprayed leaves from *M. brunneum*-treated plants showed fewer and shorter N events compared with

controls. Although the function of the non-pathway interruption (N) waveform is still not fully understood, it has been proposed that it may reflect valve-like activity or interruptions during xylem feeding, and could be associated with bacterial adhesion processes in the vector foregut, which are important for *X. fastidiosa* acquisition and retention (Backus and Shih 2020; Cornara et al. 2018b, 2020, 2021). Accordingly, acquisition may occur not only during continuous Xi but also during short interruptions embedded within feeding events (Cornara et al. 2018b; 2020). Therefore, the reduction in N activity observed in *M. brunneum*-colonized plants may indicate a disruption of mechanisms involved in *X. fastidiosa* retention, potentially leading to lower transmission.

However, xylem egestion (Xe) remains the only feeding waveform clearly associated with *X. fastidiosa* transmission (Cornara et al. 2024a). In this study, no Xe events were recorded when insects fed on *M. brunneum*-treated plants, while only two occurrences were observed in total, both in control plants. It should be noted that Xe is generally a rare behaviour, particularly in non-infective *P. spumarius*, such as the individuals used here (Cornara et al. 2020; Markheiser et al. 2023). Consequently, additional experiments using infective vectors will be necessary to determine whether fungal colonization has a direct effect on transmission efficiency.

Overall, *M. brunneum* significantly altered the probing and feeding behaviour of *P. spumarius* on olive plants, with stronger effects on sprayed leaves and weaker but still detectable effects on non-sprayed tissues, suggesting both epiphytic action and possible systemic plant responses. The occurrence of behavioural changes in untreated leaves points to a potential systemic effect, a rare finding in woody plants with practical relevance for incomplete canopy coverage. These results are consistent with previous studies showing that entomopathogenic agents can modify insect vector feeding behaviour (González-Mas et al., 2019a).

Comparing these results with those obtained when the insect feeds on a *S. oleraceus* plant colonised by *M. brunneum*, several differences can be observed that are likely linked to the suitability of the host plant, as *P. spumarius* shows a clear preference for herbaceous hosts such as *S. oleraceus*, whereas the olive tree acts primarily as a seasonal or secondary host during periods of sparse vegetation (Bodino et al. 2019; Morente et al. 2018b). On the most suitable hosts, the insects may be less affected by fungal stress, whilst less preferred hosts, such as the olive tree, may intensify behavioural disruption.

Endophytes are known to induce plant defence mechanisms and metabolic reprogramming that vary depending on the specific plant-fungus interaction (García-Espinoza et al. 2023, 2024; Rasool et al. 2021). These changes can include modifications in sap composition and nutritional quality, including xylem sap traits that are crucial for host selection in xylem-feeding insects (Andersen et al. 1992; Brodbeck et al. 1990; Cornara et al. 2024b; Horsfield 1977). Further research is needed to clarify how these plant-mediated changes contribute to behavioural alterations across different *X. fastidiosa* host plants.

The behavioural alterations observed in *P. spumarius* may be explained by both physical and chemical interference caused by *M. brunneum* colonization. Epiphytic fungal structures on leaf surfaces may hinder stylet insertion and disrupt stylet insertion, similarly to mineral oils or kaolin particles in other sap-feeding insects, increasing probing activity and delaying access to feeding tissues (Hamouche et al. 2024; Norris et al. 2023; Powell et al. 1998). In addition, fungal structures within plant tissues and around xylem vessels may further interfere with stylet movement and feeding efficiency (Quesada-Moraga et al. 2006; Vega 2018).

It is also known that entomopathogenic fungi induce the activation of the plant's defence system, thereby enhancing resistance to both herbivores and pathogens; consequently, given the limited detection of the fungus on untreated leaves, it may be that the observed changes are driven by the plant's systemic response (García-Espinoza et al. 2023; Quesada-Moraga et al. 2023). It will be essential to conduct future studies integrating gene expression and metabolomic analyses to determine whether *M. brunneum* activates defence pathways in the olive tree that contribute to the disruption of *P. spumarius*'s feeding.

Finally, regarding the feeding behaviour of directly treated insects, significant alterations in feeding activity were also observed. As in previous cases, these effects varied depending on the host plant, even though the insects were feeding on healthy rather than colonised plants. As previously discussed, these host-related differences may be linked to the insect's natural host preference, independently of fungal presence.

In the analysis using *S. oleraceus* as the host plant, the main changes detected in the non-sequential variables involved the duration of Xc and R waveforms. The Xc waveform is typically very brief in these insects, since once the xylem has been located, *P. spumarius* rapidly initiates xylem ingestion and therefore does not exhibit a prolonged xylem contact phase. Although there is no established average duration for this contact phase, several studies in the literature have shown that *P. spumarius* is able to initiate xylem ingestion relatively quickly (Malone et al., 1999). Treated individuals exhibited a longer xylem contact phase than control insects. Another variable significantly affected by *M. brunneum* treatment was the R waveform. Resting time was significantly greater in treated individuals compared with controls. This effect could be associated with a sublethal impact of the fungus, as reduced mobility has previously been reported in insects treated with entomopathogenic fungi (Pelizza et al., 2013; de Oliveira et al., 2026). These behavioural changes suggest that entomopathogenic fungi impair insect's ability to initiate and sustain effective feeding, as described by de Oliveira et al. (2026). One hypothesis explaining these findings is that fungal infection compromises neuromuscular coordination, possibly through the production of mycotoxins such as destruxins (associated with *Metarhizium* spp.), which have been shown to interfere with the physiological functions of insects (Barelli et al., 2022). Another aspect evaluated during the feeding behaviour assays was insect escape during recording. A higher proportion of escaped individuals was observed in the control group, which could also be explained by the sublethal effects of the fungus on insect mobility and activity.

Regarding the sequential variables, namely the time elapsed between non-probing (np) or pathway (C) activities and xylem contact (Xc) or xylem ingestion (Xi), as well as the time required to initiate sustained Xi events, significant differences were observed between treated and control insects. Treated individuals reached xylem tissues and initiated sustained ingestion more rapidly than control insects. One possible explanation is that fungal treatment may induce early sublethal effects that alter both the energetic balance and feeding behaviour of the insects. Insects subjected to physiological stress or pathogen challenge have been reported to modify nutrient intake and feeding dynamics in order to compensate for infection-associated physiological costs (Shikano and Cory, 2016). More generally, stress-related changes in feeding activity and resource acquisition have been widely described in insects under adverse physiological conditions (Pompon et al., 2011).

Although these compensatory responses have been mainly described in non-hemipteran insects (Shilakano and Cory, 2016), similar alterations in probing behaviour and vascular tissue access have also been reported in Hemiptera. For example, Valenzuela et al. (2020) observed that infected individuals of the psyllid *Bactericera cockerelli* reached vascular tissues more rapidly than non-infected controls, suggesting that physiological alterations within the insect may modify probing dynamics independently of plant-mediated effects. In xylem-feeding hemipterans, feeding itself represents a considerable energetic challenge due to the negative pressure and low nutritional quality of xylem sap (Andersen et al. 1992; Bergman et al. 2021). Therefore, it is plausible that fungal-induced stress could alter probing decisions and promote a more rapid access to xylem tissues in treated insects.

In directly treated insects feeding on olive plants during the EPG recordings, a similar pattern of escape events was observed, once again suggesting that the reduced activity in treated individuals may be associated with sublethal effects of the fungus (Barelli et al., 2022). Within feeding behaviour, only sequential variables were significantly affected. In particular, the duration of xylem contact (Xc) was significantly lower in treated individuals compared with controls, in contrast to the pattern observed in *S. oleraceus* recordings. Conversely, the duration of xylem ingestion (Xi) was significantly higher in treated insects than in control individuals. This increase in Xi duration may be associated with compensatory adjustments in xylem feeding driven by the insect's physiological state, as hemipteran xylem feeders are known to modulate the balance and duration of xylem ingestion in response to internal osmotic and nutritional demands (Pompon et al., 2011).

Across the different exposure scenarios evaluated in this study, *M. brunneum* showed a clear but context-dependent effect on *P. spumarius* survival, strongly influenced by the plant host and the mode of exposure.

When insects fed for 4 h on *S. oleraceus* colonised by *M. brunneum*, mortality was significantly higher than in controls. This outcome is consistent with the observed alterations in feeding behaviour, which included a faster access to xylem vessels while maintaining sustained

ingestion on colonised plants. Rather than reducing feeding activity or limiting exposure, this behavioural shift likely increases the probability of contact with fungal propagules, thereby enhancing infection risk and contributing to the increased mortality recorded. In this context, plant-fungus-insect interactions appear to facilitate pathogen exposure without necessarily impairing overall feeding performance, effectively turning sustained feeding into a pathway for increased fungal contact.

By contrast, no effect on mortality was detected when *P. spumarius* fed for 4 h on *M. brunneum*-colonised olive plants. This lack of lethality suggests that successful infection likely requires more prolonged and/or more efficient contact between the insect cuticle and viable fungal propagules, which may not have been achieved under the short exposure conditions of the EPG assays. Several factors may help explain this discrepancy: first, colonisation levels in olive were lower than those reported in *S. oleraceus* and may not have reached the threshold required to induce significant mortality, particularly given that olive is not a preferred feeding host for xylem-feeding vectors (Cuenca-Medina et al. 2025; Morente et al. 2018b). In addition, spittlebugs tend to exhibit more intense feeding activity on herbaceous hosts, especially when these are colonised by *M. brunneum*, which increases the likelihood of contact with fungal propagules and may partly account for the higher mortality observed in the first assay. Finally, plant-mediated physiological and metabolic responses induced by fungal colonisation may differ substantially among host species, potentially modulating insect susceptibility in a host-dependent manner (Meesters et al. 2023).

Direct application of *M. brunneum* resulted in significantly higher mortality compared with controls in all experimental conditions, both in assays performed on *S. oleraceus* and on olive. Notably, mortality was highest in insects whose feeding behaviour had been assessed on olive plants. Overall, these results indicate that *M. brunneum* pathogenicity against *P. spumarius* is strongly dependent on exposure pathway and host plant context, with direct contact representing the most effective route for inducing mortality, whereas plant-mediated effects are highly contingent on host suitability and colonisation efficiency.

With regard to *Xylella* transmission experiments, the results should be interpreted with caution, as additional temporal replicates are required to strengthen the robustness of the findings and to determine whether the observed absence of significant differences reflects a true lack of effect or is instead driven by experimental variability. The reason for this lack of effects could be related to the transmission mode of *X. fastidiosa* by *P. spumarius*. Transmission of *X. fastidiosa* to test plants is stochastic and can occur in a matter of a few minutes; EPF has been shown to reduce, but not hamper, vector feeding in the xylem. Future studies incorporating repeated temporal sampling, larger sample sizes, and, where appropriate, varying numbers of infective vectors per plant will be essential to more conclusively elucidate the role of entomopathogenic fungal colonization in modulating transmission efficiency under realistic conditions.

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SUPPLEMENTARY MATERIAL

Supplementary material 1. Effect of the inoculation of *Metarhizium brunneum* on Sonchus oleraceus plants on the feeding behaviour of *Philaenus spumarius*. Results from the LM and GLM tests and the pairwise post-hoc for the EPG variables of the study. Significant results are highlighted in bold numbers.

Supplementary material 2. Summary effect of the inoculation of *Metarhizium brunneum* on *Olea europaea* plants on the feeding behaviour of *Philaenus spumarius.*

Supplementary material 3. Raw EPG data of *Philaenus spumarius* probing and feeding behaviour on *Metarhizium brunneum* treated olive plants.

Supplementary material 4. Effect of Direct *Metarhizium brunneum* application on the feeding behaviour of *Philaenus spumarius* on *Sonchus oleraceous.*

Supplementary material 5. Effect of Direct *Metarhizium brunneum* application on the feeding behaviour of *Philaenus spumarius* on *Olea europaea.*