

Direct and indirect effects of two endophytic entomopathogenic fungi on survival and feeding behaviour of meadow spittlebug *Philaenus spumarius*

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HIGHLIGHTS

- Direct exposure of *Philaenus* to *Metarhizium brunneum* reduces its survivorship.
- *M. brunneum* successfully colonises the host plant and reduces survival of *Philaenus*.
- *Philaenus* reaches the xylem faster on *M. brunneum*-colonised plants.
- Xylem sap ingestion by *Philaenus* is similar on non-colonised and colonised plants.
- Contact with *M. brunneum* does not alter the egestion pattern of *Philaenus*.

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ABSTRACT

The most effective way to contain spread of the plant pathogen *Xylella fastidiosa* in Europe is to manage populations of its main insect vector, *Philaenus spumarius* L. (Hemiptera: Aphrophoridae). The action of some entomopathogenic endophytic fungi (EEF) on insect pests, directly through contact or indirectly by internal colonization of host plant tissues, may offer an excellent control option within integrated pest management (IPM) programmes. This study evaluated the impact of two EEF strains as control agents of *P. spumarius* (*Metarhizium brunneum* strain EAMa 01/58-Su and *Beauveria bassiana* strain EABb 01/33-Su [Hypocreales: Clavicipitaceae and Cordycipitaceae, respectively]); both strains were known to be excellent control agents of many other serious chewing and sap-sucking agricultural pests. First, the pathogenicity of strains applied directly to *P. spumarius* adults was evaluated, resulting in 50.0% and 32.0% mortality and a mean survival time of 4.7 and 6.2 days for *M. brunneum* and *B. bassiana*, respectively. Furthermore, colonization of *Sonchus oleraceus* by EEFs was observed after both soil and foliar applications. Greatest colonization (91.0%) was observed in plants treated with foliar applications of *M. brunneum*. Based on these results, we evaluated the feeding behaviour of *P. spumarius* on *S. oleraceus* plants previously colonized by *M. brunneum* using the 'Electrical Penetration Graph' technique. *P. spumarius* fed in a similar way on EEF-colonized and non-colonized *S. oleraceus* plants, with the exception that insects feeding on colonized plants reached xylem vessels faster than insects feeding on non-colonized plants. In addition, significantly higher mortality of *P. spumarius* was observed when feeding on plants treated by *M. brunneum* compared with the control. The duration of xylem ingestion events indicates that *P. spumarius* preferred to feed on basal leaves of the plant compared with apical leaves. Our results indicate strong potential for strain EAMa 01/58-Su of *M. brunneum* to be used as part of an *X. fastidiosa* IPM programme.

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1. Introduction

The plant pathogen *Xylella fastidiosa* (Xanthomonadales: Xanthomonadaceae) has been listed recently as a priority pest for the European Union (EU). This is due to its severe economic, social and environmental impacts as indicated in the new Regulation (EU) 2016/2031, which became effective in December 2019. This phytopathogenic bacterium is highly destructive worldwide and can seriously damage many crops, particularly almond, grape, olive, citrus and many ornamental plants amongst others. A total of 638 plant species, 289 genera and 87 families are host plants of *X. fastidiosa* (Delbianco et al., 2022). *Xylella fastidiosa* is a xylem-limited bacterium, dependent on specialized xylem-feeding insects for spread (Houston et al., 1947; Frazier, 1965). The meadow spittlebug *P. spumarius* L. (Hemiptera: Aphrophoridae) has been identified as the key vector of *X. fastidiosa* in olive in Southern Italy causing Olive Quick Decline Syndrome (OQDS) (Cornara et al. (2017a), Cornara et al. (2017b)). It is actually considered to be the most important vector of *X. fastidiosa* in the European continent (Cornara et al. (2017a), Cornara et al. (2017b), 2019; Cruaud et al., 2018; Cavalieri et al., 2019).

Bactericides cannot penetrate xylem vessels. Therefore, the use of agrochemicals to prevent bacterial growth in the plant are not an effective strategy for dealing with diseases caused by *X. fastidiosa* (Scortichini et al., 2018). The development of successful methods against the bacterium is currently in progress (Scortichini et al., 2021; Morelli et al., 2021; Baccari et al., 2019; Antelmi et al., 2019)..

The European Plant Protection Organization (EPPO) recommends other control strategies targeting the vector or alternative host plants, such as removal of ground cover in crops susceptible to *X. fastidiosa* in conjunction with selective use of agrochemicals (EPPO 2020). Some studies have demonstrated that nymphs and adults of *P. spumarius* are susceptible to chemical insecticides (Dongiovanni et al., 2018; Dáder et al., 2019; Lago et al., 2022). Nevertheless, to comply with European legislation, which promotes the use of alternatives to synthetic insecticides for integrated pest management (IPM) (Directive 2009/128/EC and Regulation [EU] 2016/2031,) use of chemical insecticides should be very targeted and complement other more sustainable practices. Some agronomical measures such as soil tillage for weed elimination have also been suggested as ways to reduce *P. spumarius* populations (Sanna et al., 2021). However, such measures are currently not sufficient and have several environmental impacts on the agroecosystem, such as increased runoff or decreased populations of beneficial arthropods (pollinators and natural enemies) (Gómez et al., 2011, 2018; Paredes et al., 2013). Therefore, other alternative sustainable control methods are being proposed such as cover crop management that reduces *P. spumarius* populations (Morente et al., 2022).

Philaenus spumarius depends on herbaceous ground cover for most of its life cycle. Eggs are laid in vegetation debris in autumn, nymphs feed on fresh grasses in spring, as do young adults before leaving and entering crops as the vegetation cover dries out (Morente et al., 2018b; Antonatos et al., 2020; Bodino et al., 2020). Although nymphs concentrate on ground vegetation, in the Iberian Peninsula *P. spumarius* is almost absent in the olive canopy (Morente et al., 2018b). Consequently, management of ground cover that reduces vector populations may offer a good opportunity to control *X. fastidiosa* diseases. With the same aim, several studies have explored the potential of bacterial and fungal endophytes for biological control of the main insect vectors of *X. fastidiosa*. The use of entomopathogenic endophytic mitosporic ascomycetes (EEMAs) such as *Metarhizium brunneum* Petch. and *Beauveria bassiana* (Balsamo) Vuill. has acquired unprecedented interest as an important component of modern IPM programmes. This is due to promising results against chewing and sap-sucking insect pests (Barelli et al., 2016; Resquín-Romero et al., 2016; Natalia González-Mas et al., 2019). EEMAs applied either as biological mycoinsecticides with contact action, or as endophytes that reach the internal tissues of host plants, can negatively affect the survival and development of cryptic pests (Quesada-Moraga et al., 2020). EEMAs also provide multifunctional benefits as they are

rhizosphere competent; induce systematic resistance; improve plant nutritional status and responses to biotic and abiotic factors (Sánchez-Rodríguez et al., 2016; Raya-Díaz et al., 2017; Branine et al., 2019; Quesada Moraga, 2020). González-Mas et al. (2019a), González-Mas et al. (2019b) found that endophytic colonization of melon plants by the EEMA *B. bassiana* EABb strain 01/33-Su decreased transmission of persistent and non-persistent aphid-transmitted viruses. Also, the endophytic behaviour of *M. brunneum* strain EAMa 01/58-Su caused additional mortality to sap-sucking and chewing target pests (Resquín-Romero et al., 2016; Garrido-Jurado et al., 2017). These findings have encouraged our search for similar solutions to be found for *X. fastidiosa*, i.e. by targeting its vectors at field level. In our previous studies, we made soil applications of *M. brunneum* strain EAMa 01/58-Su as a sustainable economically friendly control method for olive fruit fly that targeted third instar larvae in the soil beneath the tree canopy (Yousef et al., 2017, 2018). The abovementioned strain is also endophytic and rhizosphere competent providing additional benefits such as improved plant nutrition and mediating multitrophic interactions (Sánchez-Rodríguez et al., 2016; Miranda-Fuentes et al., 2020b, 2021; Gonzalez-Guzman et al., 2021; Quesada-Moraga et al., 2022). In addition, *M. brunneum* strain EAMa 01/58-Su is antagonistic to important phytopathogenic fungi including *Verticillium dahliae*, *Phytophthora inunctata* and *Cadophora helianthi* (Lozano-Tovar et al., 2013; Miranda-Fuentes et al., 2020a).

Hence, the main goal of this study was to: assess the direct effects of *M. brunneum* and *B. bassiana* strains EAMa 01/58-Su and EABb 01/33-Su on adult *P. spumarius*; and the indirect effects of their possible endophytic colonization of the common olive orchard weed, *Sonchus oleraceus* L. on vector survival and feeding behaviour events related to transmission of *X. fastidiosa*.

2. Material and methods

2.1. Plant material

Sowthistle (*Sonchus oleraceus* L.), a broadleaf annual weed commonly present in the olive agroecosystem was used as the host plant for *P. spumarius*. This plant has been effectively used for continuous indoor rearing of *P. spumarius* (Morente et al., 2018a, 2021). *Sonchus oleraceus* plants used to rear *P. spumarius* were grown from seeds sown in pots filled with a 1:2 mixture of vermiculite (Asfaltex S.A., Barcelona, Spain) and soil substrate (Jiffy substrate, Jiffy Products International, BV, Moerdijk, The Netherlands). For inoculation experiments, *S. oleraceus* seeds were pre-germinated to ensure viability and then transferred into plastic pots of 7x7 cm diameter with a 1:1 mixture of sterilized vermiculite (No. 3, Asfaltex S.A., Barcelona, Spain) and soil (Floragard, Oldenburg, Germany) and maintained in an environmental chamber under controlled conditions: 25 ± 2 °C, photoperiod 16: 8 h (light/dark). The substrate in which they were grown was sterilized twice in an autoclave for 20 min at 121 °C with a 24 h interval between each sterilization process. Plants were watered three times a week and a nutrient complex of 20: 20: 20 (N: P: K) Nutrichem 60 fertilizer (Miller Chemical & Fertilizer Corp., Hanover, PA, USA) was added to the irrigation water at a rate of 1 g/L.

2.2. Collection and rearing of *Philaenus spumarius*

Philaenus spumarius individuals used in the study were collected at a recreational area located in Villanueva de la Cañada, Madrid (40°27.15'15.9", 4°00'21.1"W) where *Quercus ilex* L., *Eryngium campestre* L. and Asteraceae were the dominant vegetation. Late-instar nymphs were collected mainly from *Eryngium campestre* L. Leaves colonized by nymphs were removed from the plant and placed inside plastic bags for transportation to the laboratory. Nymphs were gently transferred using the tip of a fine paintbrush and caged on five-leaf stage *S. oleraceus* plants. A total of ten nymphs were released onto each plant

and reared in insect-proof cages until adulthood under ambient semi-field conditions (T max: 23.5 ± 0.82 °C, T med: 17.87 ± 0.67 °C, T min: 12.24 ± 0.57 °C) in a greenhouse at Instituto de Ciencias Agrarias (ICA-CSIC) (Madrid, Spain). *Sonchus oleraceus* plants were changed every two weeks to ensure optimal rearing conditions.

2.3. Fungal strains and inoculum preparation

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).

For inoculum preparation, strains were grown aseptically on potato dextrose agar (PDA) in Petri dishes for 15 days at 25 °C in darkness. Conidial suspensions were prepared by scraping conidia from the dishes into an aqueous sterile solution of 0.1% Tween 80. The resulting conidial suspension was filtered through several layers of sterile cheesecloth to remove mycelia and sonicated for 5 min to homogenize the inoculum. Conidial concentrations were determined using a haemocytometer and appropriate dilutions made in 0.1% Tween 80 to achieve a concentration of 1×10^8 conidia mL⁻¹ for experiments. Prior to experimentation conidial viability was determined on liquid Czapek-Dox broth plus 1% (w/v) yeast extract medium (Yousef et al., 2014). Only suspensions with > 97.0% germination after 24 h were used.

2.4. Direct lethal effects of *M. brunneum* and *B. bassiana* on *P. spumarius* adults

To assess pathogenicity of the fungal strains against *P. spumarius*, adults were sprayed with fungal suspensions (1×10^8 conidia mL⁻¹) of one or other strain; 5 mL of fungal suspension were applied per replicate using an aerograph 27,085 (piston compressor of 23 l/min, 15–50 PSI and a 0.3 mm nozzle diameter, China). Control insects were sprayed with the same volume of sterile aqueous 0.1% Tween 80. For each fungal strain and control, five replicates of five *P. spumarius* adults were used. After spraying, each group of five adults was transferred to a *S. oleraceus* plant. Each plant with the five *P. spumarius* adults was confined in a cage and maintained under the following conditions: 26 ± 2 °C, $70 \pm 5\%$ RH and a photoperiod of 16:8 (L:D) h. Mortality was recorded daily for one week. To determine whether mortality was due to the entomopathogenic fungus, dead *P. spumarius* adults were removed daily, immediately surface sterilized during 1 min in 1% sodium hypochlorite, rinsed three times in sterile distilled water (1 min each), placed on sterile wet filter paper in sterile Petri plates, sealed with laboratory film, incubated at 25 °C in darkness and inspected for fungal outgrowth (Quesada-Moraga et al., 2006).

2.5. Endophytic colonization of *S. oleraceus* by *M. brunneum* and *B. bassiana*

The endophytic capacity of both fungal strains was assessed following two different inoculation methods: soil treatment and leaf spraying. For each fungal strain, four groups of *S. oleraceus* plants (five plants each) at the four-leaf stage were prepared. Leaves of the first group were sprayed with 2 mL per plant of conidial suspension using the

abovementioned aerograph. In the second group, leaves were sprayed with sterile aqueous 0.1% Tween 80 (foliar spray control). In the third group the substrate in which the plants were growing was inoculated with 2 mL of conidial suspension per plant using a pipette. In the fourth group the substrate in which the plants were growing was treated in the same way but only with sterile aqueous 0.1% Tween 80 (soil drench control). The substrate of plants subjected to foliar treatments was carefully covered with aluminium foil to prevent them from being contaminated by run-off. After inoculation, treated and control plants were covered with transparent plastic shielding bags and maintained in a growth chamber at 25 °C and a photoperiod of 16:8 (Light/Dark) for 48 h 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 48 h after treatment and endophytic colonization was determined (Resquín-Romero et al. 2016; Garrido-Jurado et al. 2017). Specifically, 2 leaves were removed from each treatment and control replicate in each group and surface-sterilized by washing for 2 min in 1% sodium hypochlorite followed by two rinses (2 min) in sterile deionized water. Surface sterilized leaves were then air dried under sterile air flow and ten leaf fragments (1 cm²) excised from each leaf of each plant using a sterile scalpel (in total, 50 fragments per treatment), and plated onto selective culture medium SDA (Sabouraud Dextrose Agar) supplemented with 0.5 g l⁻¹ chloramphenicol. The scalpel was changed for another sterile one between plants. Plates were sealed and incubated in darkness at 25 °C for 7 days. Colonization was expressed as the percentage of fragments from each leaf presenting visible growth of *B. bassiana* or *M. brunneum* respectively, which was observed using a light microscope (Nikon SMZ800 Model CP-S, Tokyo, Japan) and identified based on morphological features. To confirm that surface sterilization was effective, 40-µl aliquots from the last rinse water were plated on SDAC and incubated under the same conditions. The same procedure was followed with stems and roots.

Based on the results of the endophytic colonization experiment above, a second experiment was established to measure the progression of endophytic colonization by strain *M. brunneum* EAMa 01/58-Su after foliar application. For that, ten *S. oleraceus* four-leaf stage plants were used. Adaxial and abaxial leaf surfaces of the two basal leaves were sprayed with conidial suspension using the aerograph (2 mL per plant). The two upper leaves of plants and the soil were carefully covered with plastic shielding bags and aluminium foil, respectively, to prevent them from being inoculated. Control plants were sprayed similarly with a sterile aqueous solution of 0.1% Tween 80. After treatment, plants were maintained under the same conditions as described above. After fungal inoculation, two plants in the treatment and control were selected for destructive sampling at each of five time point: 2, 7, 14, 21 and 28-days post-inoculation (dpi). On each occasion, one treated and one untreated leaf were sampled from each plant and each leaf preserved individually in an auto-sealing plastic bag; ten fragments per leaf were evaluated for endophytic colonization as described above. The same procedure was followed with stems and roots.

Table 1
Fungal isolates used in this study.

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

2.6. Effect of endophytic colonization of *S. oleraceus* plants by *M. brunneum* on the feeding behaviour and mortality of *P. spumarius* adults

Eight-week-old *P. spumarius* adults and *S. oleraceus* plants (5 to 6-leaf stage) were used for all EPG experiments. Prior to behavioural observation, adult *P. spumarius* were starved for one hour inside 1.5 mL aerated tubes, slightly anesthetized by exposure to CO₂ for ca. 5 s, immobilized with a cased diaphragm pump (Eyela Aspirator A3S; Rikakikai, Tokyo, Japan) and then tethered on experimental plants according to the protocol described by Cornara et al. (2018a), Cornara et al. (2018b). Briefly, the tip of an 18 µm gold wire (3 cm long) was bent to create a loop that was placed on the insect pronotum and glued in place on the leaf with a double layer of water-based silver conductive paint (EPG Systems, Wageningen, The Netherlands).

Before tethering the insects, adaxial and abaxial leaf surfaces of two basal leaves were sprayed with the conidial suspension of the strain EAMa 01/58-Su while the upper leaves were protected from inoculation (as described in 2.5). After 48 h of incubation (as in 2.5), two *P. spumarius* adults were tethered on each plant (as described above), one on the abaxial side of the directly sprayed leaf (basal leaf) and the second on the abaxial side of the uninoculated but endophytically colonized leaf (apical leaf). The same procedure was undertaken on control plants (sprayed with sterile aqueous 0.1% Tween 80). Feeding behaviour was recorded under laboratory conditions (22–24 °C) with a Giga-8 direct current (DC) EPG device (EPG-systems, Wageningen, The Netherlands) at 1 Giga Ohm input resistance. Output from the EPG at 100x gain was digitalized at a rate of 100 samples per s per channel, and recorded using Stylet + software (EPG systems, Wageningen, The Netherlands). Substrate voltage was adjusted following the calibration instructions of the DC-EPG equipment so that EPG output signals fitted the +5 V to –5V window provided by the software.

Each plant was used for EPG recording only once. A total of 28 *S. oleraceus* plants were used each time: 14 colonized by the fungus (basal leaves directly inoculated; apical leaves endophytically colonized) and 14 control plants (basal leaves sprayed with 0.1% Tween 80; apical leaves un-colonized). Four-hour EPG recordings were made for each insect/plant combination inside a Faraday cage to prevent electrical noise. The inoculation protocol was the same as described previously and the procedure was repeated on three consecutive days with new plants, fungal suspension and *P. spumarius* adults (total number of plants per treatment was 42).

We considered the following six main EPG waveforms as described by Cornara et al. (2018a), Cornara et al. (2018b), 2020): C (corresponding to stylet penetration activities during the pathway phase); Xc (xylem contact/pre-ingestion); Xi (active xylem sap ingestion); R (a resting phase alternating with xylem ingestion); N (interruption during the xylem phase); Xe (an occasional behaviour potentially related to egestion). Waveforms were manually marked and registered by EPG Stylet + software (EPG systems, Wageningen, The Netherlands). Failed recordings (showing unusual feeding behaviours or when insects escaped during the recording) were identified and removed before statistical analysis.

Once the EPG ended, insects were divided by treatment and replicate, and caged on five-leave stage *S. oleraceus* plants (not treated) under semi-natural conditions. Mortality was recorded daily for 7 days. Both dead and living adults were removed at the end, stored individually in 1.5 mL Eppendorf tubes and kept at –80 °C for endophytic colonization study. Insects were inspected for fungal outgrowth as an indication of fungal-induced mortality (as in 2.3). Moreover, apical and basal leaves on which insects had fed were collected and endophytic fungal colonization evaluated to verify that contamination had not occurred in control plants (as in 2.4). Leaves were cut with sterile scissors and stored in sealable plastic bags at –80 °C until analysis of fungal outgrowth.

2.7. Statistical analyses

Endophytic colonization data of the first experiment were subjected to ANOVA and analyzed per application method. Means from different treatments (three treatments: two fungal strains and control in each application method) were compared using a Tukey's test ($\alpha = 0.05$); Statistix®10 (Analytical Software, Tallahassee, USA) was used for these data analyses.

EPG variables describing *P. spumarius* feeding were calculated using the CSIC-UAL v.3.0 EPG workbook developed by Garzo et al., (in preparation). Non-sequential and sequential variables were calculated. Non-sequential variables were: 1) the number of waveform events per insect (min); 2) the total waveform duration per insect (min); 3) the mean duration of waveform events per insect (min). Sequential variables were: 1) the time from non-probing (Np)(min) to the first xylem contact (Xc), to the first xylem ingestion (Xi), to the first sustained xylem ingestion (Xi > 10 min), and to the first fluid egestion (Xe); 2) the time from the first pathway (C) (min) to the first Xc, the first Xi, the first Xe; 3) the time to the first probe with Xi (min) and the first probe with sustained Xi (min). Moreover, the percentage of the total probe in Xi was calculated. A total of 36 EPG variables were obtained (Supplementary Material 1). All EPG raw data were checked for normality and homogeneity of variance using the Shapiro-Wilk and Levene test; data were log transformed as needed to reduce heteroscedasticity.

First, changes in the feeding behaviour of *P. spumarius* related to the position of the leaf (basal or apical) were evaluated. To identify potential changes in EPG variables data between basal and apical leaves in control plants and in treated plants separately, pairwise comparisons were made using a Mann–Whitney *U* test for non-gaussian variables, or a Student's *t*-test for gaussian variables. Statistical analyses were done in R (R Core Team 2021). The effect of inoculation of *S. oleraceus* with *M. brunneum* on the feeding behaviour of *P. spumarius* was assessed by Linear Models (LMs) or Generalized Linear Models (GLMs) (quasipoisson distribution of errors and link logit) (Zuur et al., 2009) as appropriate using the R packages lme4 and nlme respectively. In cases where significant differences were found, a *post-hoc* least square pairwise test was done using the lsmeans package in R (Lenth, 2016).

Mortality data for *P. spumarius* adults were analyzed 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).

3. Results

3.1. Direct lethal effects of *M. brunneum* and *B. bassiana* against adult *P. spumarius*

Direct spraying of *P. spumarius* adults with *M. brunneum* strain EAMa 01/58-Su or *B. bassiana* strain EABb 01/33-Su had a significant effect on mortality ($\chi^2 = 9.20$ $P < 0.001$), which reached ca. 50% for *M. brunneum* and 32% for *B. bassiana* (Table 2). Fungal outgrowth was observed in some of the dead insects. However, a large percentage of dead adults treated with *B. bassiana* strain EABb 01/33-Su did not show any fungal outgrowth after incubation under optimal conditions (Table 2).

3.2. Endophytic colonization of the common olive orchard weed *S. oleraceus* by *M. brunneum* and *B. bassiana*

Statistically significant differences were found between strains for each application method: foliar ($F_{2,15} = 11.10$, $P \leq 0.001$) and soil ($F_{2,15} = 11.50$, $P \leq 0.001$). The strain *M. brunneum* EAMa 01/58-Su had a higher colonization capacity than *B. bassiana* strain EABb 01/33-Su in both foliar and soil treatments 48 h after fungal application; the average

Table 2

Susceptibility of *P. spumarius* adults to *B. bassiana* isolate EABb 01/33-Su and *M. brunneum* isolate EAMa 01/58-Su after spraying with a conidial suspension.

Treatment*	Mortality (%)		Kaplan-Meier survival analysis		
	Mean ± SE	Fungal outgrowth**	AST*** (±SE; days)	Upper limit	Lower limit
Control	4.00 ± 4.00 a	0.00 ± 0.00	6.84 ± 0.16 a	7.15	6.53
EABb 01/33-Su	32.00 ± 10.20b	12.50 ± 5.00	6.24 ± 0.31b	6.84	5.64
EAMa 01/58-Su	48.00 ± 10.20b	50.00 ± 5.30	4.72 ± 0.51b	5.72	3.72

* The spray treatment was done with a 1×10^8 conidia/mL suspension (LSD test ($p < 0.05$)).

** Percentages represent the proportion of dead insects that showed fungal outgrowth.

*** AST: average survival time; these were limited to 7 days. Within columns, means with the same lowercase letter were not significantly different to each other according to log rank test ($p < 0.05$). Significant differences are indicated with letters.

percent recovery of *M. brunneum* EAMa 01/58-Su from leaf fragments was 91.6% and 58.3%, respectively (Fig. 1).

When progress of systematic endophytic colonization of *S. oleraceus* plants by *M. brunneum* EAMa 01/58-Su was evaluated in both directly treated leaves and untreated ones, the fungus was reisolated from all the treated leaves at high percentage recovery rates 14 dpi. The percentage recovery decreased from day 2 (95%) to day 14 post-inoculation (41.2%) (Fig. 2A). The fungus also systematically colonized untreated leaves endophytically; percentage recovery ranged between 28.5% (2dpi) and 10% (7dpi) (Fig. 2B) but fell to 0% at 14 dpi. The fungus was not detected in stem and root tissues. It is worth mentioning that leaves taken 7 dpi onwards were new leaves.

3.3. Feeding behaviour of *P. spumarius* adults on endophytically colonized *S. oleraceus* plants

A total of 95 valid EPG recordings were obtained and analysed: 25 control leaves sprayed with aqueous Tween 0.1% (basal-control: BC), 19 control leaves not sprayed (apical-control: AC), 23 leaves sprayed with

and endophytically colonized by *M. brunneum* (basal-treated: BT) and 28 leaves that were not sprayed with *M. brunneum* but were endophytically colonized (apical-treated: AT).

The percentage of *M. brunneum* EAMa 01/58-Su directly sprayed leaves from which the fungus was reisolated ranged between 93% (repetition 1) and 20% (repetition 3). The fungus successfully colonized plants with sprayed leaves since it was also reisolated from untreated leaves with an average percentage recovery of over 50% in repetition 1 and 40% in repetition 2.

Due to differences in percentage fungal recovery from plated leaf fragments from plants in the three replicates, the results of the EPG variables obtained in replicates 1 and 2 were compared with the results of replicate 3 (by Linear Model or Generalised Linear Model according to the distribution of data residuals and data homoscedasticity). No significant differences were observed between the EPG variables in the different replicates. Therefore, we used the results obtained from all three replicates.

3.3.1. Effect of fungal colonization on the feeding behaviour of *P. spumarius*

Feeding behaviour of *P. spumarius* on *M. brunneum*-treated and on non-treated plants showed the same trends in non-sequential EPG variables associated with Np, C, Xc, R and Xe (Fig. 3). Regarding the xylem ingestion (Xi) phase, there was no effect overall of EPF treatment on the number or duration of Xi events. Nevertheless, considering the position of the leaf and the plant treatment, Xi events tended to be higher in *P. spumarius* feeding on basal treated leaves (BT) than on basal control leaves (BC) (t-ratio = -1.866, df = 91, $p = 0.06$) (Fig. 3).

Moreover, colonization of *M. brunneum* affected the time elapsed before contacting the xylem vessels. Thus, the time between Np and the first Xc ($F = 5.21$, df = 3, $p = 0.002$) and between the first C and the first Xc ($F = 5.23$, df = 3, $p = 0.002$) were shorter in insects feeding on treated plants than in insects feeding on control plants (Fig. 3, Supplementary Material 1). There were no differences in EPG variables related to the probe: total time of probing, time to the first probe and percentage of probes (Supplementary Material 1).

3.3.2. Effect of leaf position on feeding behaviour of *P. spumarius*

In control plants, the position of the leaf where *P. spumarius* was feeding had a significant impact on total number of Xi events, total and

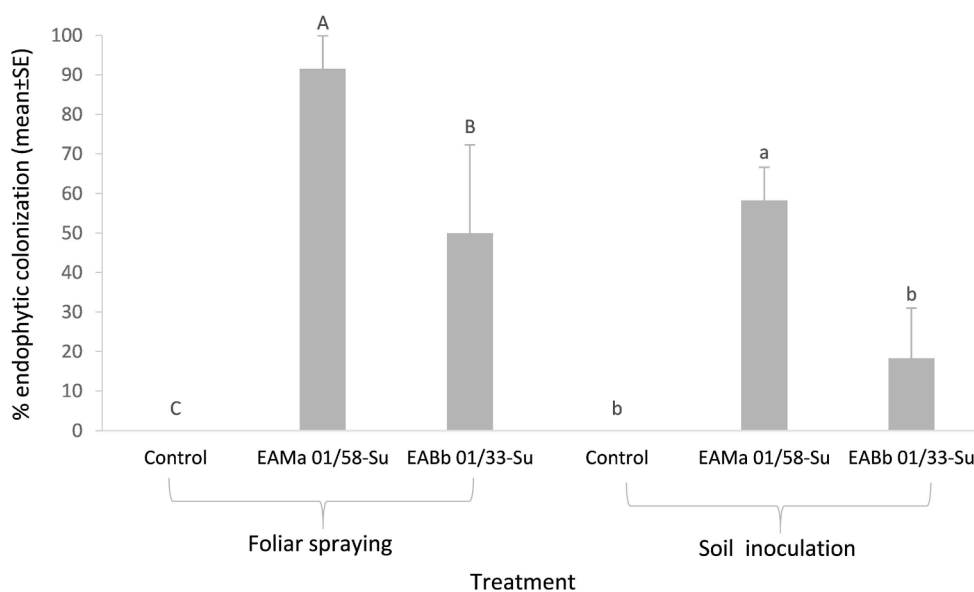


Fig. 1. Percentage of *S. oleraceus* leaves endophytically colonized by entomopathogenic fungi 48 h after either foliar spraying or soil inoculation with *M. brunneum* isolate EAMa 01/58-Su or *B. bassiana* isolate EABb 01/33-Su compared with control plants. Within treatments, bars with different upper (foliar) or lower (soil) case letters are significantly different to each other following ANOVA ($p < 0.05$) and Tukey test ($\alpha = 0.05$).

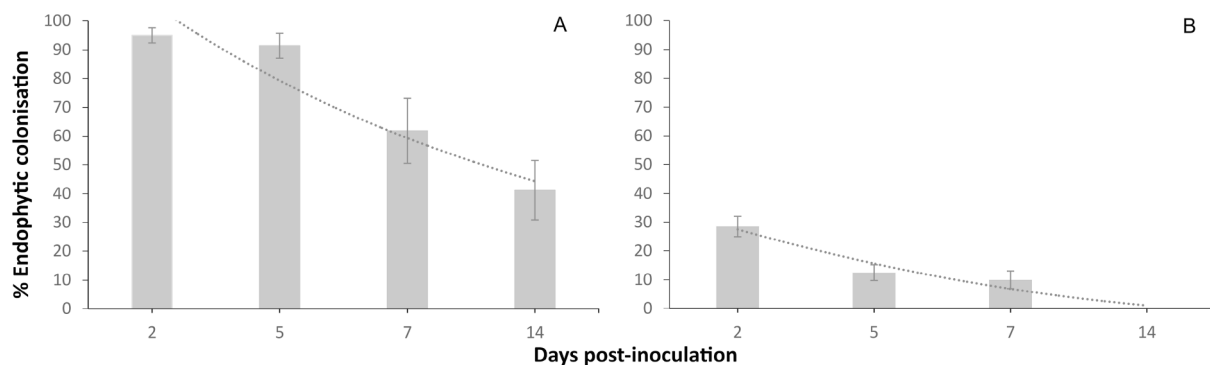


Fig. 2. Percentage of *S. oleraceus* plants endophytically colonized by *M. brunneum* isolate EAMa 01/58-Su 2, 5, 7 and 14 days after spraying the basal leaves with a conidial suspension. A: timeline of fungal colonization in directly sprayed leaves, B: timeline of fungal colonization in leaves not treated directly but receiving the fungus endophytically.

mean duration of Xi and the percentage of probing in Xi (BC and AC treatments). Specifically, insects feeding on AC showed a higher number of Xi events ($t = 2.12$, $df = 91$, $p\text{-value} = 0.04$) with a lower total and mean duration of Xi (total duration: $W = 149$, $p\text{-value} = 0.04$; mean duration: $t = -2.45$, $df = 37.60$, $p\text{-value} = 0.02$) and percentage of the probe in Xi ($W = 134$, $p\text{-value} = 0.01$) than those feeding on BC (Fig. 3). Moreover, the total duration of R was longer in AC than in BC ($W = 337$, $p = 0.02$) (Fig. 3). No differences were found in the remaining sequential and non-sequential EPG variables between basal and apical leaves in control plants and no effect of the position of the leaves were found in any of the EPG variables in treated plants (Supplementary Material 1).

3.4. Effect of presence of the endophytic *M. brunneum* on survival of *P. spumarius*

Philaenus spumarius adults feeding on *S. oleraceus* plants colonized by the fungus *M. brunneum* for four hours showed a significantly higher mortality than those feeding on the control plants. Specifically, adults feeding on directly treated leaves (BT) had a mortality rate of 53.8% compared with 13.6% on control leaves (BC; treated with Tween 0.1%); of note, adults feeding on untreated leaves endophytically colonized by the fungus (AT) had a mortality rate of 48.2% compared with 18.1% on control leaves (AC).

Interestingly, fungal outgrowth was only observed on some dead insects that have been fed on directly treated leaves (BT 18%) while a large percentage of dead adults did not show any fungal outgrowth after incubation under optimal conditions. Furthermore, none of the adults that died after feeding on untreated but endophytically colonized leaves showed fungal outgrowth.

4. Discussion

Entomopathogenic fungi represent a group of microorganisms with great potential for use in microbial control of insect pests due to the multiple attributes and benefits they provide and their complex modes of action (Quesada-Moraga et al., 2020, 2022). The present study evaluated the potential of two entomopathogenic fungal strains, *M. brunneum* EAMa 01/58-Su and *B. bassiana* EABb 01/33-Su as regulators of *P. spumarius* populations under direct and indirect control scenarios. Results showed that the *M. brunneum* strain was more pathogenic to *P. spumarius* when directly applied, and colonized the host plant *S. oleraceus* more efficiently, than the *B. bassiana* strain. *Beauveria bassiana* EABb 01/33-Su was infrequently isolated from dead treated adults, likely in accordance with the 'toxin strategy' proposed by Ker-shaw et al., (1999). In addition, survival of *P. spumarius* fed on host plants endophytically colonized by *M. brunneum* EAMa 01/58-Su was reduced.

Feeding and development of *P. spumarius* is closely linked to the

presence of fresh vegetation (Wise et al., 2006). Therefore, the efficacy of strains colonizing host plants via the endophytic pathway is essential to ensure exposure of *P. spumarius* to EEMA in crops. Results show that *M. brunneum* EAMa 01/58-Su colonized *S. oleraceus* plants inoculated both by soil application and leaf spraying more efficiently than *B. bassiana* EABb 01/33-Su. Moreover, *M. brunneum* strain EAMa 01/58-Su had a high colonization rate after 14 days on directly sprayed leaves and the plant underwent progressive systemic endophytic colonization of unsprayed leaves. Recently, García-Espinoza et al., (2023) demonstrated by molecular techniques the capacity of this strain to activate defence reaction in primed plants with upregulation of genes that regulate Jasmonic, Salicylic acids and Ethelyn pathways that directly impact chewing insects' fitness. This is an important advantage for control strategies since endophytic EEMAs persist better in the plant tissue than on the plant surface (Vega et al., 2008; Quesada-Moraga et al., 2014); they may also confer systemic protection against insect pests due to chemical changes induced in the plant by the endophyte or the secondary metabolites secreted by the fungus (Gurulingappa et al., 2010; Akello and Sikora, 2012; Castillo Lopez et al., 2014; Ríos-Moreno et al., 2016). However, results showed that the fungus/plant association was temporary and neither new leaves grown after spraying nor the stem and roots showed fungal colonization, similar to our previous study that showed a temporary transient endophytic behaviour of this strain (Garrido-Jurado et al., 2017). Also, studies conducted with the EPF *B. bassiana* showed a similar temporary transient endophytic behaviour in leaf tissues (Landa et al., 2013; Ullrich et al., 2017; Koch et al., 2018). Considering that hatching of *P. spumarius* eggs and emergence of adults is asynchronous (Weaver and King, 1954), when assessing the efficacy of *M. brunneum* in the field, it would be necessary to synchronize spray applications with peaks of adult abundance in the ground cover vegetation.

Results discussed above on the mode of action of *M. brunneum* on *P. spumarius* and the host plant *S. oleraceus* show that this strain could be a potential control agent of *P. spumarius* populations in *X. fastidiosus*-susceptible crops. Indeed, soil application of this strain have been widely used in olive orchards for control of olive fruit fly *Bactrocera oleae* (Yousef et al., 2017, 2018). These applications may offer a good opportunity to target *P. spumarius* populations through direct spraying or endophytic colonization of the surrounding plant cover, which remains to be investigated.

Some effect of leaf position on feeding behaviour of *P. spumarius* was observed in control plants. *Philaenus spumarius* preferred to feed on basal leaves as indicated by longer xylem ingestion (Xi) events, less numerous Xi and higher percentage of the Xi probes in basal control leaves (BC) than in apical control leaves (AC). Moreover, the resting (R) duration was shorter in BC than in AC. The resting waveform (R) represents a resting phase during xylem ingestion when neither fluid excretion nor movements are made while the stylets remain within the xylem (Cornara

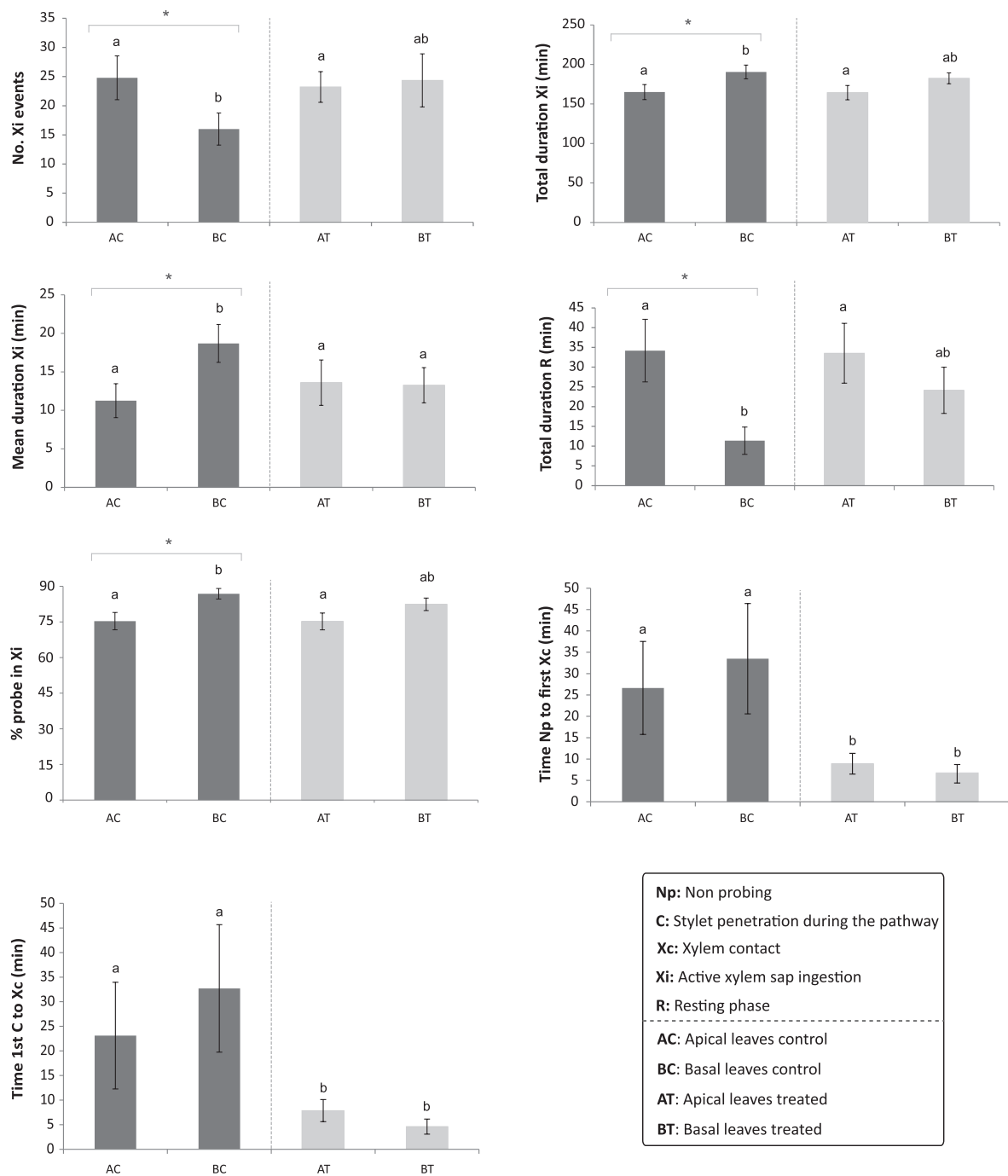


Fig. 3. Electrical Penetration Graph (EPG) variables (mean ± SE) affected by inoculation with *M. brunneum* (significant differences signalled by letters) (LM and GLM analysis; $p < 0.05$) and by the position of the leaves (apical or basal) in control and treated plants (significant differences signalled by asterisks) (Student's *T*-test and Mann-Whitney *U* test; $p < 0.05$). AT: Apical leaves treated, BT: Basal leaves treated, AC: Apical leaves control, BC: Basal leaves control.

et al. (2018a), Cornara et al. (2018b)). Therefore, a more prolonged resting, together with short xylem ingestion events may indicate that feeding on apical leaves in the control plant is more energy costly than in the basal leaves of *S. oleraceus*. In fact, negative pressure in xylem vessels increases from the roots to the apical parts of the plant (Zimmermann, 1978; Hacke and Sperry, 2001). Interestingly, colonization by *M. brunneum* minimizes leaf positional effects on feeding behaviour of *P. spumarius* and no differences were found between the apical and basal leaves of treated plants.

Despite some minor changes in some observed EPG variables, *P. spumarius* was able to feed well on *S. oleraceus* plants colonized by the

fungus. 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 (Cornara et al., 2020). 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. (2020) 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.

Nevertheless, we did find other significant differences that indicate that feeding behaviour of *P. spumarius* adults is affected by the presence of *M. brunneum* in the leaf. For example, insects feeding on endophytically colonized leaves exhibited a shorter time between non-probing (Np) and xylem contact (Xc) and between pathway phase (C) and the first xylem contact (Xc), which suggests that xylem vessels can be reached faster in plants colonized by the fungus. It is known that *P. spumarius* introduce their stylets directly into xylem vessels through the periderm, cortex and phloem cells (Cornara et al. (2018a), Cornara et al. (2018b)). Our findings suggest that the presence of *M. brunneum* may facilitate and speed up stylet contact with xylem vessels. This could be because *M. brunneum* causes histological changes in *S. oleraceus* leaves which has been described in melon plants where hyphal colonization by *M. brunneum* reaches the intercellular spaces of leaves (Garrido-Jurado et al., 2017).

However, despite xylem ingestion (Xi) events tending to be more numerous in treated plants, xylem ingestion time and percentage of the probe in Xi was the same in both the treatment and the control. These results suggest that endophytic colonization by *M. brunneum* in *S. oleraceus* plants does not interfere with *P. spumarius* xylem ingestion or other feeding processes that favour exposure to *M. brunneum* EAMa 01/58-Su strain. In fact, *M. brunneum* increases *P. spumarius* mortality rate, not only 7 dpi (as demonstrated in the pathogenicity experiment) but also after the 4-hour feeding period on treated plants that was monitored by EPG. Thus, insects fed on *M. brunneum*-colonized *S. oleraceus* plants suffered a significantly higher mortality rate than insects fed on control plants.

Host population-level development of diseases caused by *X. fastidiosa* depends on plant-to-plant transmission by insect vectors (Frazier and Freitag, 1946; Houston et al., 1947). They also depend on ecological interactions amongst plant, pathogen and vector, and the effect of external biotic and abiotic factors, including anthropogenic activities and policy decisions (Sicard et al., 2018). At present, *P. spumarius* is the only vector of *X. fastidiosa* with epidemiological importance in Europe (Cornara et al., 2019). The rate at which *P. spumarius* acquires *X. fastidiosa* is extremely low, however, transmission can occur within a few minutes (Cornara et al., 2020). Hence, population control of adult *P. spumarius* is essential to control spread of the bacterium in crops. Newly emerged adults are not infective until they feed on infected plants and acquire the bacterium (Cornara et al., 2016). Thus, foliar application of *M. brunneum* strain EAMa 01/58-Su to the ground cover around susceptible crops, at the same time as insect-vector emergence peaks in spring, could reduce insect-vector populations before they become infective, potentially minimising plant-to-plant transmission of the bacterium in the infected zone.

5. Conclusions

This study proposes that *M. brunneum* strain EAMa 01/58-Su is a candidate for use within sustainable control strategies targeting *P. spumarius* populations. *Metarhizium brunneum* successfully colonized the host plant *S. oleraceus* endophytically and had a lethal effect on *P. spumarius* both when sprayed directly on insects and when those insects had fed for 4 h on *M. brunneum*-colonized host plants. The meadow spittlebug, *P. spumarius*, feeds in a similar way on non-colonized and colonized plants favouring contact with the EEMA and thus, increasing its mortality rate. Therefore, application of *M. brunneum* EAMa-158-Su by spraying the ground cover around crops susceptible to *X. fastidiosa* may be a promising alternative to control its insect vectors instead of using agrochemicals and removal of the ground cover.

CRedit authorship contribution statement

Meelad Yousef-Yousef: Investigation, Formal analysis, Visualization, Writing – original draft. **Marina Morente:** Investigation, Formal analysis, Visualization, Writing – original draft. **Natalia González-Mas:** Investigation, Formal analysis, Visualization, Writing – original draft. **Alberto Fereres:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Enrique Quesada-Moraga:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Aranzazu Moreno:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biocontrol.2023.105348>.

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