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Report on solutions for the management of vector populations and *Xylella fastidiosa* in the host plant

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SUMMARY

This deliverable reports the activities and main achievements of BeXyl Task 7.3, *Testing solutions for the management of vector populations*, and Task 7.4, *Network of plots for monitoring and demonstration of innovative tools for the management of Xylella fastidiosa*. Together, these tasks focused on the field validation of sustainable management solutions targeting the insect vectors responsible for pathogen transmission and the management of *Xylella fastidiosa* infections under realistic European agricultural conditions. The activities assessed the performance, feasibility and applicability of the most promising solutions developed in previous work packages, providing evidence to support their integration into sustainable disease management strategies.

Task 7.3 evaluated biological and agronomic strategies for controlling juvenile populations of the vectors *Philaenus spumarius* and *Neophilaenus campestris*. Four field trials were conducted in olive orchards in Apulia during 2025 and 2026, comparing one or two applications of the entomopathogenic fungi *Metarhizium anisopliae* BP1q and *Beauveria bassiana* with mulching and untreated controls. Mulching provided the highest and most consistent reduction in vector abundance, decreasing *P. spumarius* populations by approximately 73–94% and *N. campestris* populations by 67–93% at the final assessments. Fungal treatments also reduced *P. spumarius* abundance, although their efficacy varied among sites, years and application strategies. These findings support their use as complementary, low-impact components of integrated vector management.

Task 7.4 established a network of field trials in naturally infected areas of Mallorca and Apulia to evaluate innovative tools for the management of *X. fastidiosa*. The network covers olive, almond and grapevine production systems and includes preventive and curative applications of synthetic microbial communities (SynComs), the defence-eliciting peptide PpPep1 and lytic bacteriophages. Treatments have been successfully implemented, and their performance is being monitored through symptom assessments and molecular detection of the pathogen. Regarding the SynComs, strain-specific qPCR assays have been developed and optimized. These tools will be implemented to monitor the population dynamics and persistence of the SynCom strains throughout the field trials.

Overall, this deliverable provides a robust framework for the field validation of sustainable management strategies targeting both insect vectors and *X. fastidiosa*. Although continued monitoring is required, particularly to assess the long-term effects of treatments in perennial hosts, the established experimental network provides a solid basis for identifying effective and operationally feasible solutions for future integration into European Integrated Pest Management (IPM) strategies.

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1 INTRODUCTION

Despite the considerable progress achieved in recent years, there is still no effective curative treatment for *Xylella fastidiosa* under field conditions. Current management strategies in infected and demarcated areas therefore rely primarily on containment measures, including mandatory surveillance, removal of infected plants, establishment of demarcated areas, vector control, and agronomic practices aimed at reducing pathogen spread. Although several innovative biological approaches have shown promising results under laboratory and greenhouse conditions, their practical implementation requires validation under representative field conditions.

Within the BeXyl project, complementary strategies have been developed to target the two key components of the *X. fastidiosa* disease cycle. WP5 focused on identifying environmentally sustainable solutions for the management of insect vectors and the reduction of pathogen transmission, including entomopathogenic fungi, agronomic practices and other biological approaches. Based on the results obtained, the most promising solutions, field applications of *Metarhizium anisopliae*, *Beauveria bassiana*, and weed management through mulching, were selected for validation under natural outbreak conditions. In parallel, WP6 developed innovative tools targeting *X. fastidiosa* within infected host plants, including synthetic microbial communities (SynComs), bacteriophages and defence-eliciting peptides, which were subsequently advanced to field evaluation.

Building on these developments, WP7 represents the field-validation phase of the project. Its objective is to evaluate the applicability, operational feasibility and performance of the most promising solutions under representative epidemiological conditions in European outbreak areas. To achieve this objective, a network of experimental field trials was established in naturally infected regions of Spain and Italy, covering different host crops, epidemiological contexts and management approaches. This network provides a common framework for assessing innovative management strategies under natural infection conditions and generating evidence to support their future implementation.

Specifically, Task 7.3 (*Testing solutions for the management of vector populations*) focuses on validating field-scale strategies based on biological control agents and agronomic practices for reducing vector populations. Task 7.4 (*Network of plots for monitoring and demonstration of innovative tools for the management of Xylella fastidiosa*) evaluates innovative tools targeting the pathogen within infected host plants under both preventive and curative application scenarios. Owing to the perennial nature of the host crops and the slow progression of *X. fastidiosa* infections, the experimental network has been designed to support long-term monitoring of disease development and treatment performance beyond the duration of the project, providing the scientific basis for the future integration of effective solutions into Integrated Pest Management (IPM) strategies.

This deliverable summarises the activities carried out within Tasks 7.3 and 7.4, describing the experimental field trials established, the management solutions evaluated and the current status of their assessment. Where available, preliminary results are presented; however, the long-term evaluation of treatments targeting *X. fastidiosa* will continue beyond the end of the project to fully assess their effectiveness under field conditions.

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2 MATERIAL AND METHODS

2.1 Field trials based on entomopathogenic fungi and agronomic measures for the management of *Philaenus spumarius* and *Neophilaenus campestris* vectors of *X. fastidiosa* (Task 7.3)

2.1.1 Experimental field sites

To evaluate the field performance of the entomopathogenic fungus *Metarhizium anisopliae* BP1q, four large-scale field trials were established in olive orchards characterized by high and homogeneous populations of juvenile *Philaenus spumarius*. Two trials were conducted in 2025 and two in 2026 in the municipalities of Cisternino (Province of Brindisi) and Crispiano (Province of Taranto), within the demarcated area of Apulia (Italy).

The efficacy of *M. anisopliae* BP1q (2.0×10^9 conidia g^{-1}), applied at 24 kg ha^{-1} (equivalent to 2×10^8 conidia m^{-2}), was compared with that of a commercial formulation of *Beauveria bassiana* strain ATCC 74040 (Naturalis®, 2.3×10^7 CFU mL^{-1}) applied at 2 kg ha^{-1}. Weed management through mulching was included as a reference agronomic strategy.

In 2025, six treatments were evaluated in both experimental sites: (i) one application of *M. anisopliae* BP1q (Ma1); (ii) two applications of *M. anisopliae* BP1q (Ma2); (iii) one application of *B. bassiana* (Na1); (iv) two applications of *B. bassiana* (Na2); (v) mulching; and (vi) an untreated control. The first fungal application was performed when most *P. spumarius* individuals had reached the third-to-fourth nymphal instars, whereas the second application was carried out 5–7 days later. Mulching was performed simultaneously with the second fungal application.

In 2026, the experimental design was expanded to investigate the effect of application timing. Besides the standard treatment timing corresponding to the peak of the third-to-fourth nymphal instars, an earlier application strategy was introduced at the peak of the first-to-second instars, based on the hypothesis that prolonged exposure of juvenile vectors to the entomopathogenic fungi could enhance treatment efficacy. The following treatments were therefore evaluated: (i) one application of *M. anisopliae* BP1q starting at the first-to-second instar peak (Ma1); (ii) two applications starting at the first-to-second instar peak (Ma2); (iii) one application starting at the third-to-fourth instar peak (Ma1-3); (iv) two applications starting at the third-to-fourth instar peak (Ma2-3); (v) one application of *B. bassiana* (Na1); (vi) two applications of *B. bassiana* (Na2); (vii) mulching; and (viii) an untreated control.

The experimental design consisted of a randomized complete block with four replicates per treatment. Each experimental plot covered an area of 20 m^2.

Treatment efficacy was assessed through three sampling campaigns: immediately before the first application, immediately before the second application, and 5–7 days after the second application. Juvenile populations of *P. spumarius* and *Neophilaenus campestris* were quantified using a 0.25 m^2 sampling frame placed at six randomly selected positions within each plot. For each sampling unit, the number of spittle masses, the number of nymphs per spittle mass, and the developmental instar of each individual were recorded.

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2.1.2. Statistical analysis

All statistical analyses were performed in **R** version 4.5.2 (R Core Team, 2026). The effects of the different treatments on the abundance of *P. spumarius* and *N. campestris* were analysed using quasi-Poisson generalized linear models (GLMs), fitted separately for each vector species, experimental site, and sampling date. Treatment and block were included as explanatory variables to account for the randomized block design.

The significance of treatment effects was assessed using F-tests. Model adequacy was evaluated by means of half-normal plots with simulated envelopes generated using the **`hnp`** package (Moral et al., 2017).

When significant treatment effects were detected, estimated marginal means were calculated using the **`emmeans`** package, and pairwise comparisons among treatments were performed with appropriate adjustment for multiple comparisons. Treatment efficacy was expressed as the percentage reduction in the model-predicted abundance relative to the untreated control and was calculated from the ratios between the estimated marginal means of each treatment and the control.

2.2 Establishment of a network of plots for monitoring and demonstration of innovative solutions for the management of *X. fastidiosa* (Task 7.4)

2.2.1 Experimental field sites

A network of eight field trials was established in *X. fastidiosa*-infected areas of Mallorca (Spain) and Apulia (Italy) to evaluate the most promising biological tools developed within WP6 under natural field conditions.

The trials were designed to represent different host crops and epidemiological contexts and included olive, almond and grapevine production systems. All sites were located in areas with confirmed natural disease pressure, where the presence of *X. fastidiosa* had previously been verified, thereby providing suitable conditions for the field evaluation of the proposed management solutions.

In Mallorca, the trials were established in areas naturally infected by different *X. fastidiosa* subspecies and sequence types, namely *X. fastidiosa* subsp. *fastidiosa* ST1, subsp. *multiplex* ST81, and subsp. *pauca* ST53, according to the host crop, trial objective and geographical location. In contrast, all field trials established in Apulia were located within the outbreak area of *X. fastidiosa* subsp. *pauca* ST53.

The geographical location, host crop, and corresponding *X. fastidiosa* subspecies and sequence type of each experimental site are summarised in Table 1 and illustrated in Figures 1 and 2.

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Table 1. Location, host plant and coordinates of the experimental field plots established in Mallorca (Spain) and Apulia (Italy).

Plot ID	Country	Town / area	Host	GPS coordinates
ES-P1	Spain	Mallorca	Almond	39°26'13.6"N, 2°56'38.1"E
ES-P4–P5	Spain	Felanitx, Mallorca	Grapevine	39°30’16”N, 3°09’33”E
ES-P7	Spain	Palma, Mallorca	Almond	39°36’49”N, 2°39’04”E
ES-P6	Spain	Mallorca	Grapevine	39°40'14"N, 3°14'17"E
ES-P8	Spain	Sencelles, Mallorca	Olive	39°39’14.9”N, 2°54’32.7”E
ITA-Ugento	Italy	Ugento, Lecce	Almond	39°58’36.8”N, 18°10’32.3”E
ITA-Squinzano-1	Italy	Squinzano, Lecce	Olive	40°27’00.14”N, 18°031’11.63”E
ITA-Squinzano-2	Italy	Squinzano, Lecce	Olive	40°27’03.86”N, 18°03’19.47”E

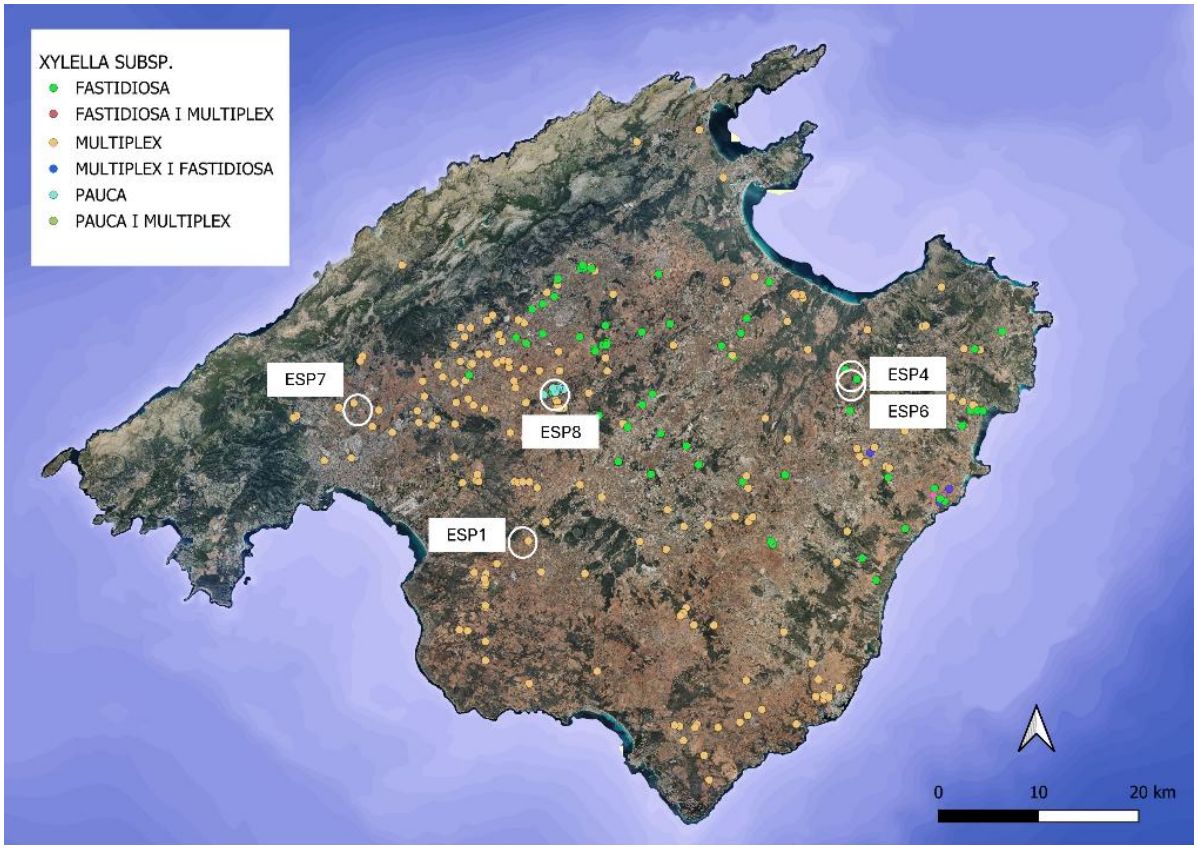


Figure 1. Location of the experimental field plots established in Mallorca (Balearic Islands, Spain).

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Figure 2. Location of the experimental field plots established in Apulia (Italy).

2.2.2. Biological solutions evaluated

The biological solutions evaluated in the field trials were selected based on their promising performance under controlled experimental conditions in WP6 and subsequently transferred to WP7 for validation under natural *X. fastidiosa* outbreak conditions. The selected tools comprised two synthetic bacterial communities (SynComs), one defence-eliciting peptide and three lytic bacteriophages.

The two SynCom formulations were assembled from bacterial endophytes previously isolated from naturally tolerant olive trees. SynCom 1 (SC1) consisted of *Bacillus safensis* XOL35, *Novosphingobium fluoreni* BXOL39 and *Variovorax* sp. BXOL162, whereas SynCom 2 (SC2) comprised *Rhizobium pusense*/*Agrobacterium pusense* XOL28, *Methylobacterium radiotolerans* XOL43 and *Novosphingobium fluoreni* BXOL39. These microbial consortia were designed to promote plant health and interfere with the establishment and multiplication of *X. fastidiosa* within host tissues.

The peptide evaluated was PpPep1, an endogenous defence-eliciting peptide derived from *Prunus persica*. PpPep1 was selected for its ability to activate plant immune responses and enhance host resistance against *X. fastidiosa*.

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The three lytic bacteriophages selected for evaluation in grapevine were Vise_2, a member of the genus *Bosavirus* (family *Mesyanzhinovviridae*); Vise_6, belonging to the genus *Pradovirus* (family *Autographiviridae*); and P19, an *Alphatectivirus* (family *Tectiviridae*). Vise_2 and Vise_6 were evaluated as a combined phage cocktail, whereas P19 was tested as a single-phage treatment. The discovery and initial development of P19 predated the BeXyl project through previous collaborative activities, and the corresponding field trial established in Mallorca was subsequently incorporated into WP7.

2.2.3. Experimental design and treatment application

SynCom trials

Four field trials were established to assess the performance of the two SynComs selected (SC1 and SC2) under different epidemiological scenarios. In these trials, the preventive and curative application of both SynComs was evaluated. A preventive trial was established in Sencelles (ES-P8) using young plants of *Olea europaea* cv. 'Arbequina'. Additionally, a preventive and curative trial was conducted in naturally infected adult almond trees (ES-P1). In Italy, a preventive trial was conducted in young *O. europaea* cv Ogliarola trees. In addition, a curative application of both SynComs was evaluated in five-year-old *O. europaea* cv Favolosa under natural field conditions in Squinzano (Br) (ITA-Squinzano). The treatment rates were adjusted according to plant size (Table 2).

Inoculum for all strains that comprise the SynComs was produced and provided by ACIES-BIO in a lyophilized format. Each strain was rehydrated using R2A broth and was adjusted to a stock concentration of 10^9 CFU/mL. Finally, the strains were diluted and mixed, to create the corresponding SynComs (SC1 and SC2) in a 1:1:1 ratio, as detailed in D6.1. SynComs were applied by endotherapy to the trees either by direct application using a syringe for young trees or an endotherapy device provided by Fertinyect S. L. (Córdoba, Spain) for adult trees.

In the preventive trial established in Mallorca (ES-P8), nursery-produced 'Arbequina' olive plants were pre-inoculated with either SC1 or SC2 under controlled conditions during spring 2025 before field establishment. Untreated plants were included as controls. The pre-treated plants were transplanted to the experimental field in early autumn 2025, where the potential long-term protective effect of both SynComs is being assessed under natural exposure to *X. fastidiosa*. The field trial comprised a subset of 72 *Arbequina* plants arranged in a randomized complete block design with four blocks. Each block contained six plants treated with SC1, six plants treated with SC2 and six untreated control plants, resulting in 24 replicates per treatment. This subset was established within a larger olive cultivar collection maintained for complementary studies on cultivar response to *X. fastidiosa*. In the preventive trial established in Italy (ITA-Squinzano 2), 60 1-year-old *X. fastidiosa*-free 'Ogliarola' olive trees were planted in the field. The trees were subsequently treated with either SC1 or SC2, while untreated trees served as controls. The experiment was arranged in five randomized blocks, with four trees per treatment in each block, resulting in 20 replicates per treatment.

In contrast, the almond (ES-P1) and olive (ITA-Squinzano 1) trials followed both a curative and preventive approach, in which the two SynCom formulations were applied by endotherapy to naturally infected, or not infected yet field-grown trees. Treatments were carried out in Mallorca and in Apulia. In the Italian trial, 72 mature 'Favolosa' olive trees were selected, with 24 trees assigned to each treatment (SC1, SC2 and untreated control). Plants were arranged

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in a randomized block design with 4 blocks per treatment and each block was comprised of six trees. Untreated trees were maintained as controls throughout the experiments, and treatment performance is currently being monitored through long-term visual assessments and molecular analyses.

Table 2. Experimental field plots established for testing SynComs (SC1, SC2) solutions against *Xylella fastidiosa*.

Plot	Host	Cultivar	No. Plants	Treatment approach	Treatment conditions	Rate (per plant)
ES-P8	Olive	Arbequina	72	Preventive	pre-inoculated plants	30 µl
ES-P1	Almond	Marta, Marinada	96	Preventive/ Curative	field treatment	100 ml
ITA-Squinzano-1	Olive	Favolosa	72	Curative	field treatment	30 ml
ITA-Squinzano-2	Olive	Ogliarola	60	Preventive	field treatment	500 µl

Peptide trials

The plant endogenous peptide PpPep1, is being evaluated in two naturally infected almond orchards: plot ES-P7 in Mallorca, using cv. 'Masbovera', and plot ITA-Ugento in Apulia, using cv. 'Genco'. In both trials. The peptide was diluted in ultrapure water to a final concentration of 0.1 µM and applied once by foliar spraying during the active vegetative growth period. Five young leaves per plant were selected, and two to three sprays were applied to both the adaxial and abaxial surfaces of each leaf. Untreated plants were maintained as controls. Treatments were applied in Mallorca on 4 June 2025 and in Apulia on 11 June 2025.

Table 3. Experimental field plots established for testing Peptide PpPep1 tools against *Xylella fastidiosa*.

Plot	Species	Cultivar	No. Plants	Tool tested
ES-P7	Almond	Masbovera	48	PpPep1
ITA-Ugento	Almond	Genco	18	PpPep1

In Mallorca (ES-P7), the trial comprised 48 two-year-old healthy almond trees of cv. 'Masbovera', which had been specifically planted for the trial in an area with natural *X. fastidiosa* inoculum pressure. The experimental design consists on 24 treated trees and 24 untreated trees as controls, arranged in a randomized block design of 6 blocks with 6 replicates per block (Figure 3).

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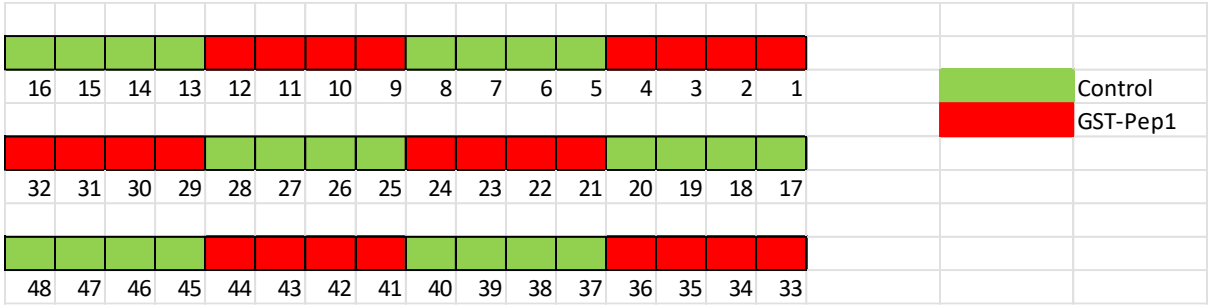


Figure 3. Scheme showing the randomized distribution of almond cv 'Masbovera' across three blocks in the trial plot for testing peptide located in Mallorca, Spain.

In Apulia (ITA-Ugento), the trial included 18 one- year-old almond trees cv 'Genco', specifically planted for the trial on 20 May 2025, arranged in a randomized block design consisting of three blocks. Each block contained three peptide-treated trees and three untreated control trees, resulting in nine plants per treatment (Figure 4).

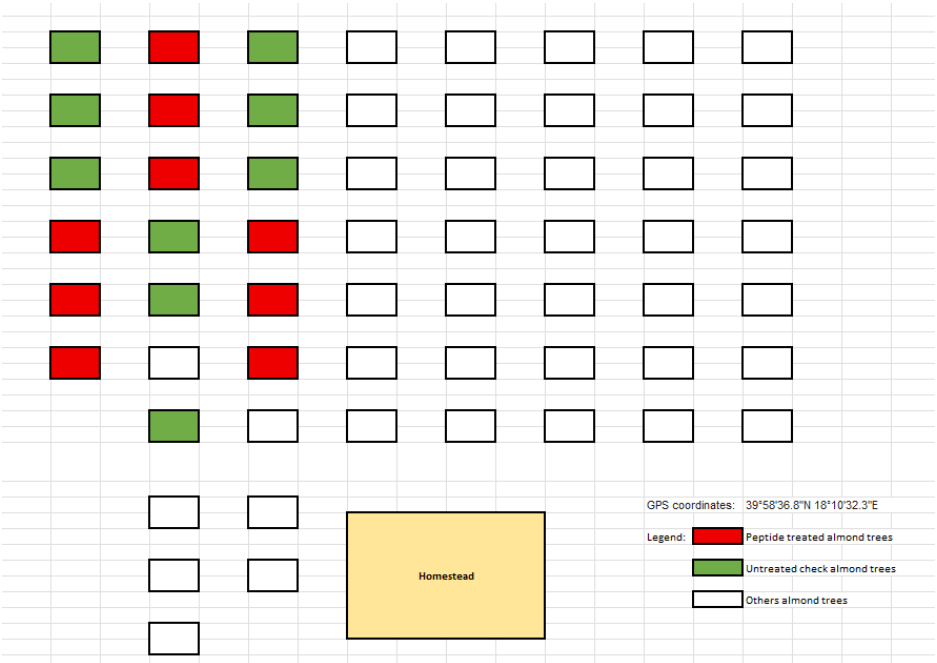


Figure 4 - Scheme showing the randomized distribution of almond cv 'Genco' across three blocks in the trial plot for testing peptide located in Apulia, Italy.

Bacteriophage trials

Field trials were established in vineyards located in Mallorca (ES-P4, ES-P5, ES-P6, Table 4) to evaluate the efficacy of lytic bacteriophages against *X. fastidiosa* subsp. *fastidiosa* ST1 under natural field conditions in collaboration with Evolving Therapeutics S.L.. The trials involved the application of two bacteriophages, Vise_2 and Vise_6, as a combined treatment on 6 August 2025, and the application of bacteriophage P19, which was initially applied on August 2023 and subsequently reapplied on August 2024.

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In plot ES-P5 (Aubocassa), a total of 25 mature vines of the cultivar 'Prensal' and 24 mature vines of the cultivar 'Cabernet Sauvignon' were treated. The plants were selected using a semi-random sampling approach from among vines considered likely to develop symptoms associated with *X. fastidiosa* infection.

In contrast, the trial in plot ES-P4 (Son Nadal) was established using 22 newly planted young vines cv. 'Tempranillo'. These vines received the bacteriophage treatment as a preventive measure prior to symptom development; consequently, no untreated control vines were included in this trial.

Additionally, an ongoing field trial in plot ES-P6 (Sos Ferres), originally established through previous collaborations with partners who later became involved in the BeXyl project, was incorporated into the project's field trial network. The trial had been initiated before the BeXyl field trials described above and evaluated the efficacy of bacteriophage P19 in 30 mature vines, comprising 15 plants of cv. 'Parellada' and 15 plants of cv. 'Callet'. Two plants of Parellada and three of Callet were established as negative controls. Therefore, the experimental design allowed the treatment to be assessed primarily in vines already infected with *X. fastidiosa* and, to a more limited extent, as a preventive intervention in initially uninfected vines.

Table 4. Experimental field plots established for testing Phages against *Xylella fastidiosa*.

Plot	Species	Cultivar	Phages Tested	No. Plants	Treatment approach
ES-P4	Grapevine	'Tempranillo'	Vise_2 + Vise_6	22	Preventive
ES-P5	Grapevine	'Pensal' 'Cabenrnet Sauvignon'	Vise_2 + Vise_6	49	Preventive /Curative
ES-P-6	Grapevine	'Callet' 'Parrellada'	P19	30	Preventive /Curative

All bacteriophage treatments were applied by trunk endotherapy at a dose of 2 mL of phage suspension per plant using a trunk injection device (All bacteriophage treatments were applied by trunk endotherapy at a dose of 2 mL of phage suspension per plant using a Wedgle Direct-Inject® trunk injection device (ArborSystems, Omaha, NE, USA) (Figure 5). For the combined treatment, the final suspension contained Vise_2 and Vise_6 in a 1:1 ratio, with a total phage concentration of approximately $1x10^8$ PFU/mL. The phage suspensions were prepared before application by propagating the phages in LB medium and were then delivered directly into the vine vascular system following the protocol developed in WP6. Briefly, a hole was drilled into the trunk, and the phage suspension was administered in two consecutive 1 mL flushes. The injection site was subsequently sealed with a plug to minimize tissue damage and protect the wound.

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Figure 5. Application of bacteriophage treatments by trunk injection.

2.3.4. Monitoring and assessment of treatment performance

The performance of all biological tools is being assessed through long-term field monitoring combining visual symptom evaluation and periodic molecular analyses. Disease development is being monitored by regular inspections of treated and control plants, recording symptom appearance and progression throughout the duration of the trials.

In addition, plant samples are periodically collected for laboratory analysis to determine the presence of *X. fastidiosa* by quantitative real-time PCR (qPCR), following the diagnostic protocols routinely used by the participating institutions. Sampling is carried out during the periods considered optimal for reliable pathogen detection in each host species, taking into account the seasonal dynamics of bacterial populations and local field conditions.

The frequency and timing of assessments may vary among trials according to host species, environmental conditions and logistical constraints. All trials are currently being monitored, and additional sampling campaigns will be performed throughout the duration of WP7 to evaluate treatment performance over time.

2.3.5. Monitoring of SynCom strains population levels

In order to monitor the population levels of the strains of the SynComs that are being tested on field, strain specific qPCRs have developed and optimized for each strain of SC1 AND SC2.

As indicated in the technical report, the genome of several bacterial isolates, including the strains used to assemble the SynComs, were sequenced using a hybrid approach using Oxford nanopore Technologies (ONT, long reads) and Illumina (short reads). Genome assembly was performed using the BacFlux+ v1.1.2. pipeline (Antonielli et al. 2024; Velasco-Amo et al., 2024). Primer pairs were designed using the primerForge v1.5.3 workflow (https://github.com/dr-joe-wirth/primerForge). TaqMan probes were designed for each primer pair using Genious v2024.0.7. Up to two primer-probe sets were selected for each strain when they fit the established criteria, including positioning the probes away from both amplicon ends and avoiding a guanine (G) residue at 5' end of the probe (Arya et al. 2005). Finally, potential self-complementarity and cross-complementarity among all the designed primers and probes was evaluated using Primer3 v2.6.1 (https://github.com/primer3-org/primer3).

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DNA from the SynCom strains was extracted from bacterial suspensions using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA). The quality and quantity of the extracted DNA was assessed by Qubit 4 Fluorometer (ThermoFisher Scientific, MA, USA).

Initially, primer specificity at different annealing temperatures was assessed by PCR. PCR reactions were carried out in a T100™ Thermal Cycler (Bio-Rad, USA) using a temperature gradient ranging from 53 to 61°C. All reactions were repeated at least twice and included a negative control (no DNA). Amplification products were separated by electrophoresis in 1.5% agarose gels and visualized with the GelDoc GO Imaging System (BIORAD, CA, USA). Once the optimal annealing temperature had been determined, PCR assays were performed to identify the primer pair with the highest specificity for each strain. The resulting amplicons were then sequenced to confirm that they corresponded to the expected target sequences.

DNA concentration was normalized to 10^7 genome copies/µL. DNA standard curves for each strain were obtained from 10-fold dilutions ranging from 10^7 to 1 copy. Each DNA standard curve was tested using its corresponding primer–probe set in singleplex format, as well as in a triplex assay combining the three primer–probe sets corresponding to the strains comprising each SynCom. The amplification efficiency and limit of quantification (LOQ) were determined for all qPCR assays.

Finally, DNA was normalized to 3 ng/µL for all strains, and two types of samples were prepared. First, the DNA of each strain was diluted 1:2 in water. Second, DNA from the three strains comprising each SynCom was mixed in a 1:1:1 ratio. These samples were then analyzed using the optimized triplex assay.

3 RESULTS

3.1 Testing vector control strategies (Task 7.3)

At the pre-treatment sampling, the mean abundance of *P. spumarius* and *N. campestris* did not differ significantly among treatments ($p > 0.05$), indicating homogeneous initial population densities across plots. In both sites and years, *P. spumarius* was more abundant than *N. campestris* (Figures 6–9).

In 2025, the abundance of *P. spumarius* nymphs and early adults on ground-cover vegetation was significantly reduced in plots treated with mulching, two applications of *M. anisopliae* Bp1q (Ma2), and two applications of *B. bassiana* Naturalis® (Na2), compared with untreated control plots at Cisternino (Figure 6). At Crispiano, significant reductions in *P. spumarius* abundance were detected for all treatments at the final post-treatment evaluation (Figure 7). For *N. campestris* in 2025, in the field trial carried out at Cisternino a significant treatment effects were detected only for mulching. At Crispiano, all treatments except Na2 significantly reduced *N. campestris* abundance at the first post-treatment evaluation, whereas no significant differences among treatments were observed at the second evaluation.

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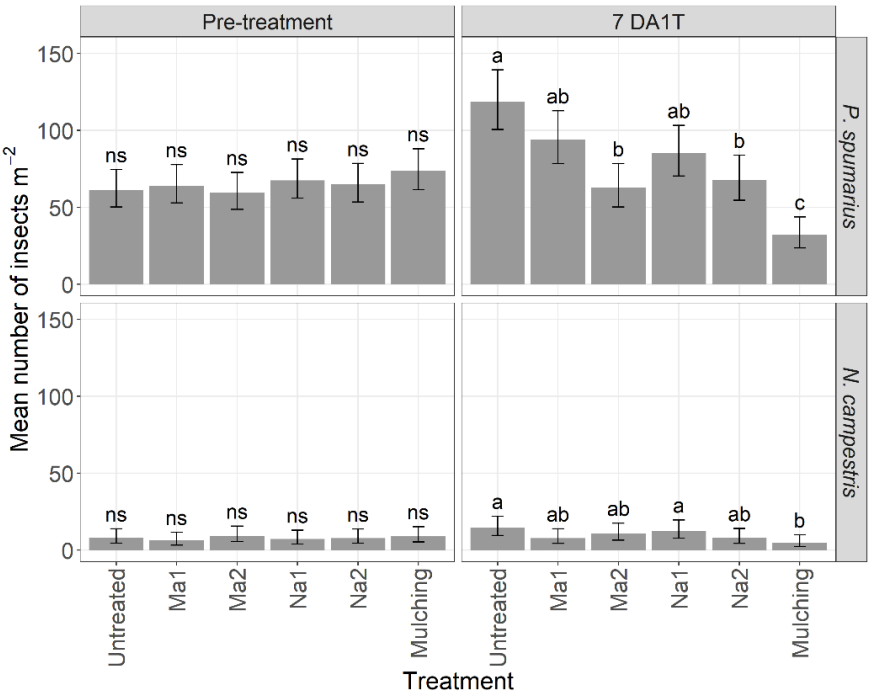


Figure 6. Mean number (± confidence interval) of nymphs and early adults of *Philaenus spumarius* and *Neophilaenus campestris* per m² in plots subjected to different treatments at Cisternino, Apulia, Italy, in 2025, at pre-treatment and 7 days after the first treatment application (7 DA1T). Bars followed by different letters differ significantly ($p < 0.05$). Ma1: one application of *Metarhizium anisopliae* Bp1q; Ma2: two applications of *M. anisopliae* Bp1q; Na1: one application of *Beauveria bassiana* Naturalis®; Na2: two applications of *B. bassiana* Naturalis®.

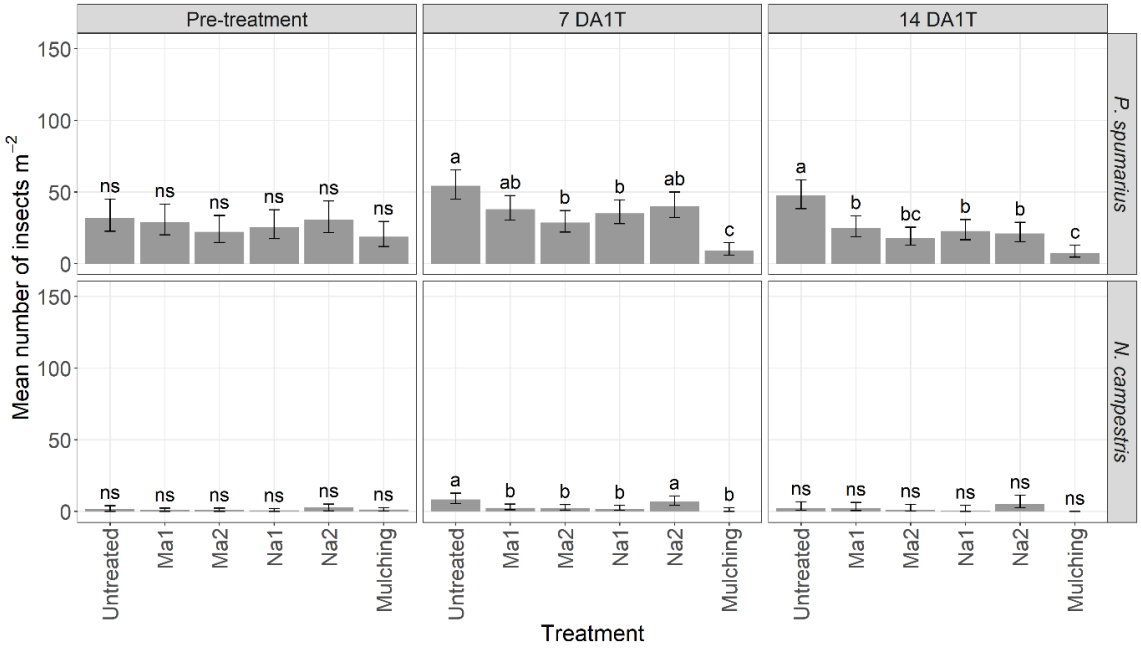


Figure 7. Mean number (± confidence interval) of nymphs and early adults of *Philaenus spumarius* and *Neophilaenus campestris* per m² in plots subjected to different treatments at Crispiano, Apulia, Italy, in 2025, at pre-treatment, 7 days after the first treatment application (7 DA1T), and 14 days after the first treatment application (14 DA1T). Bars followed by different letters differ significantly ($p < 0.05$). Ma1: one application of *Metarhizium anisopliae* Bp1q; Ma2: two applications of *M. anisopliae* Bp1q; Na1: one application of *Beauveria bassiana* Naturalis®; Na2: two applications of *B. bassiana* Naturalis®.

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In 2026, significant reductions in the abundance of *P. spumarius* on ground-cover vegetation were detected only at 14 days after the first treatment application (14 DA1T) at both experimental sites (Figures 8 and 9). At Cisternino, all entomopathogenic fungi treatments significantly reduced *P. spumarius* abundance compared with the untreated control. At Crispiano, although lower abundances were observed in all treated plots, significant reductions relative to the untreated control were detected only for the treatment with two applications of *M. anisopliae* Bp1q initiated at the N3–N4 nymphal stages, and for both *B. bassiana* Naturalis® treatments (Na1 and Na2). At both sites, mulching resulted in the lowest *P. spumarius* abundance among all treatments at the final evaluation date. For *N. campestris*, a significant treatment effect in 2026 was detected only for mulching at Cisternino at the final evaluation date, whereas no significant differences among fungal treatments were observed at either site.

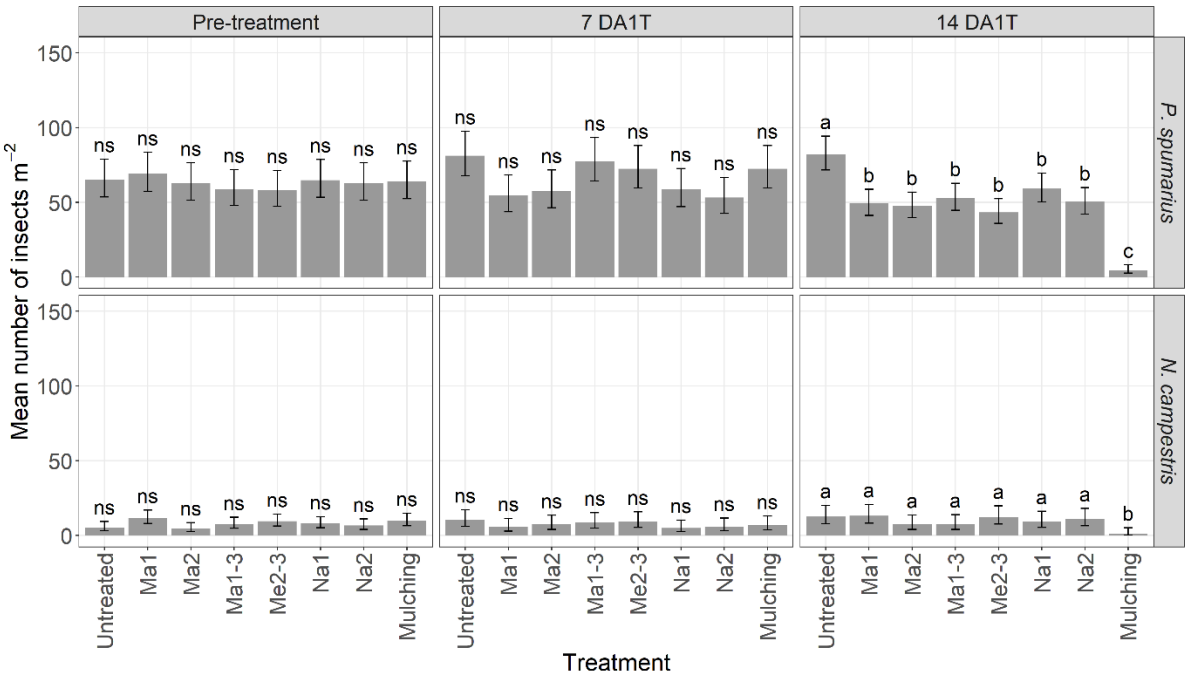


Figure 8. Mean number (± confidence interval) of nymphs and early adults of *Philaenus spumarius* and *Neophilaenus campestris* per m² in plots subjected to different treatments at Cisternino, Apulia, Italy, in 2026, at pre-treatment, 7 days after the first treatment application (7 DA1T), and 14 days after the first treatment application (14 DA1T). Bars followed by different letters differ significantly ($p < 0.05$). Ma1: one application of *Metarhizium anisopliae* Bp1q, starting at 1-2 *P. spumarius* peak stages; Ma2: two applications of *M. anisopliae* Bp1q, starting at 1-2 *P. spumarius* peak stages; Ma1-3: one application of *Metarhizium anisopliae* Bp1q, starting at 3-4 *P. spumarius* peak stages; Ma2-3: two applications of *M. anisopliae* Bp1q, starting at 3-4 *P. spumarius* peak stages; Na1: one application of *Beauveria bassiana* Naturalis®; Na2: two applications of *B. bassiana* Naturalis®.

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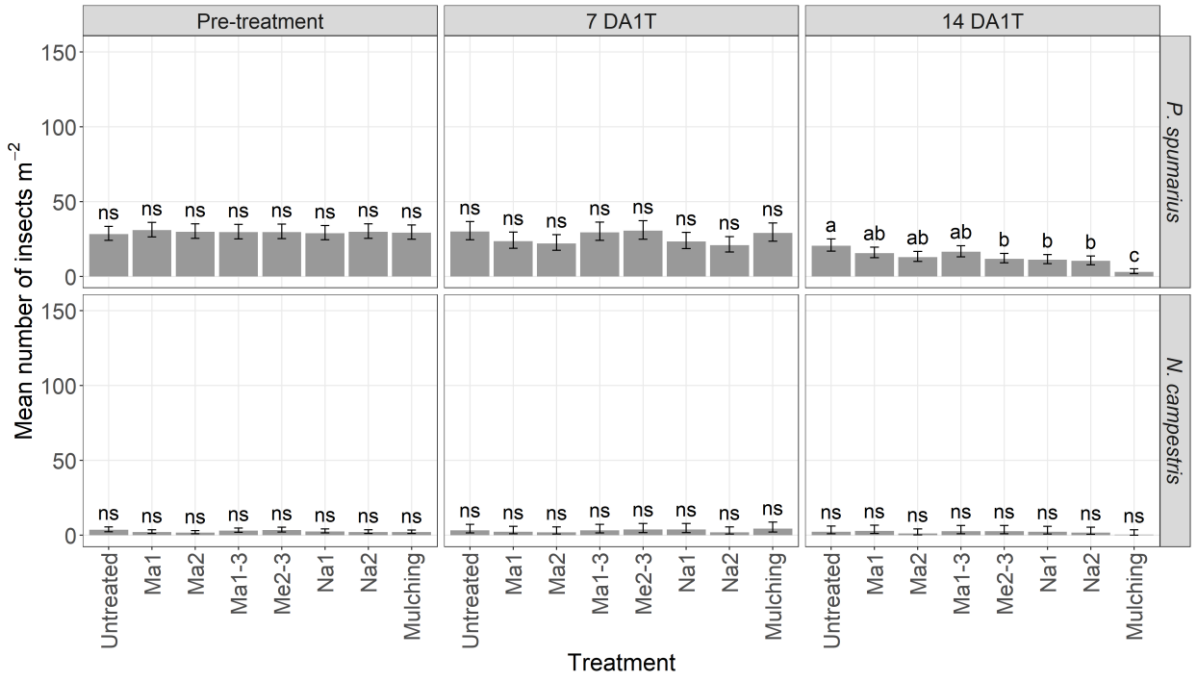


Figure 9. Mean number (± confidence interval) of nymphs and early adults of *Philaenus spumarius* and *Neophilaenus campestris* per m² in plots subjected to different treatments at Crispiano, Apulia, Italy, in 2026, at pre-treatment, 7 days after the first treatment application (7 DA1T), and 14 days after the first treatment application (14 DA1T). Bars followed by different letters differ significantly ($p < 0.05$). Ma1: one application of *Metarhizium anisopliae* Bp1q, starting at 1-2 *P. spumarius* peak stages; Ma2: two applications of *M. anisopliae* Bp1q, starting at 1-2 *P. spumarius* peak stages; Ma1-3: one application of *Metarhizium anisopliae* Bp1q, starting at 3-4 *P. spumarius* peak stages; Ma2: two applications of *M. anisopliae* Bp1q, starting at 3-4 *P. spumarius* peak stages; Na1: one application of *Beauveria bassiana* Naturalis®; Na2: two applications of *B. bassiana* Naturalis®.

Mulching was particularly effective in reducing spittlebug nymph abundance in both experimental olive orchards and in both experimental years. At the final evaluation dates, mulching reduced the estimated abundance of *P. spumarius* on ground-cover vegetation by 72.84–94.40% relative to the untreated control, and that of *N. campestris* by 67.03–92.50% (Table 5). In plots treated with entomopathogenic fungi, the reduction in *P. spumarius* abundance was less pronounced than in mulching-treated plots, ranging from 20.00% to 61.72% relative to the untreated control. Treatments involving two fungal applications tended to show slightly higher efficacy against *P. spumarius* than those involving a single application, although no significant differences in population abundance were detected among the fungal treatments. At the final evaluation date, no significant reduction in *N. campestris* abundance was detected in plots treated with any entomopathogenic fungi treatments.

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Table 5. Estimated treatment efficacy in reducing nymph abundance of *Philaenus spumarius* and *Neophilaenus campestris* in the Cisternino and Crispiano sites in 2025 and 2026. Efficacy values represent the percentage reduction in model-predicted insect abundance relative to the untreated control at the final evaluation date. Positive values indicate reduced abundance compared with the untreated control, whereas negative values indicate higher abundance than in the untreated control. Significance levels refer to pairwise comparisons with the untreated control: ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

SITE	Treatment	2025		2026	
		P. spumarius	*N. campestris*	*P. spumarius*	*N. campestris*
CISTERNINO	Ma1	20,68% ^{ns}	46,15% ^{ns}	40,20% ***	-3,75% ^{ns}
	Ma2	47,03% ***	26,37% ^{ns}	42,20% ***	41,25% ^{ns}
	Ma1 – 3			35,60% ***	40,00% ^{ns}
	Ma2 – 3			47,20% ***	2,50% ^{ns}
	Na1	27,97% *	14,29% ^{ns}	28,00% *	26,25% ^{ns}
	Na2	42,84% ***	45,05% ^{ns}	38,80% ***	13,75% ^{ns}
	Mulching	72,84% ***	67,03% **	94,40% ***	92,50% ***
CRISPIANO	Ma1	47,24% **	11,11% ^{ns}	24,00% ^{ns}	-17,65% ^{ns}
	Ma2	61,72% ***	50,00% ^{ns}	36,80% *	52,94% ^{ns}
	Ma1 – 3			20,00% ^{ns}	-11,76% ^{ns}
	Ma2 – 3			42,40% **	-11,76% ^{ns}
	Na1	52,07% ***	77,78% ^{ns}	45,60% **	5,88% ^{ns}
	Na2	55,52% ***	-127,78% ^{ns}	49,60% ***	23,53% ^{ns}
	Mulching	83,79% ***	-	84,80% ***	82,35% ^{ns}

3.2. Field evaluation of innovative biological tools targeting *Xylella fastidiosa* in host plants (Task 7.4)

As described above, the field trials included in WP7 are designed as long-term evaluations under natural epidemiological conditions. Because the biological tools developed in WP6 were transferred to WP7 only after their successful development and validation, most field trials could only be established during the later stages of the project. Consequently, the results presented in this section represent the current status of the ongoing evaluations rather than final outcomes. Long-term monitoring will continue beyond the end of the BeXyl project to assess the efficacy and persistence of the different biological tools under field conditions.

3.2.1. SynComs trials

Following treatment application, the experimental plots have been monitored through periodic visual inspections and qPCR analyses to assess treatment performance under natural field conditions.

At ES-P8 (Mallorca, Spain), with young olive trees, the first qPCR analyses were performed in June 2026. All sampled trees remained qPCR-negative and symptomless, and no differences among treatments could therefore be detected at this point. Additional sampling campaigns are planned to assess the long-term performance of the SynCom applications.

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At ES-P1 (Mallorca, Spain), with young almond trees, the trial was established shortly before the preparation of this deliverable and treatment performance has not yet been assessed. Visual and molecular evaluations will begin during the forthcoming sampling campaigns and will continue as part of the long-term follow-up.

A comparable situation applies to the Squinzano site (Apulia, Italy), where the trials with young 'Ogliarola' and mature 'Favolosa' olive trees are not yet sufficiently advanced to identify differences among treatments. Continued observations and periodic analyses will be required to determine their performance over time.

Regarding the development and optimization of qPCRs for the monitoring of the SynCom strains, Primers and TaqMan probes were designed using primerForge v1.5.3 and Geneious v2024.0.7. Following *in silico* evaluation, primer–probe sets meeting the predefined design criteria were selected (**Table 6**).

Table 6. Sequences and characteristics of the primer–probe sets designed for the SynCom strains.

Name	Forward/Probe (5' - 3')	Reverse (5' - 3')	Primer/probe size	Amplicon size	Tm (ºC)	GC content (%)
BsXOL35-P1	CCGAAGAAGCCGTCAAAG	TCGATCAGGGTACGAAGC	18	114	56.1	55.6
BsXOL35-P1_probe	ATCCCGTAGCTATGACGCCC	-	20	-	61.4	60
BsXOL35-P2	CTCTGTCGCCGTAAATTTCGTC	GCGAAGGTTAAGTCGATTTCGG	22	102	60	50
BsXOL35-P2_probe	AGTGCGACGTTCTGAAGGTC	-	20	-	59.3	55
VaBXOL162-P1	TGAGGGACGGCATGGAAGTC	TCCGTGATCTGGCTTGACCC	20	101	61.9	60
VaBXOL162-P1_probe	TTGTAAGTACAAAGTACTCT	-	20	-	49.1	30
VaBXOL162-P2	ACCGCTGTCGAACAAGGAG	TCACGAGACGCAAACCCTC	19	101	60	57.9
VaBXOL162-P2_probe	AGAAGGGCTTCGTGCACAAG	-	20	-	59.3	55
MrXOL43-P1	GCGTCCGCTTTCAAGATGC	ATTCGTCGGACTTCGCTCG	19	105	60.2	57.9
MrXOL43-P1_probe	CGGCGGTAATGGGCGCAAAG	-	20	-	63.4	65
MrXOL43-P2	TCACGCGTCATGATCACGGC	ATGAAGCCGTCTGCGTACGC	20	119	63.5	60
MrXOL43-P2_probe	ATGCCTGCTTCGGCCGGTAG	-	20	-	63.4	65
NfBXOL39-P1	CAGAACGCGGAGGCAATCAG	CCGCGCTCAAGACCATCAAG	20	106	61.7	60
NfBXOL39-P1_probe	ACAGATCGATCAGCAACCGC	-	20	-	59.3	55
NfBXOL39-P2	ACTGCTGGCTTGCGAGGAAC	AAGTGCAAGGCGCTCCAGTC	20	114	63.3	60
NfBXOL39-P2_probe	TCCGGCACTCCTGCCAACAA	-	20	-	61.4	60
RhXOL28-P1	CAGGGCAAAGACATCACAC	CCATTCGACGATCTGCTTG	19	111	56.5	52.6
RhXOL28-P1_probe	CGCTACGGTCCGATGACCGC	-	20	-	65.5	70
RhXOL28-P2	TGAATGAGCTCGTTGCCCG	AATCAAGACGCGGTCCAGC	19	119	60.7	57.9
RhXOL28-P2_probe	ATTGATCCTGAAGGCGGCCG	-	20	-	61.4	60

Using the designed primers, a temperature-gradient PCR was performed to verify primer performance and determine the optimal annealing temperature. An annealing temperature of 61 °C yielded the highest specificity across all primer pairs and was therefore selected for subsequent analyses. Each primer pair was then tested against DNA from the non-target strains to evaluate its specificity. Some primer pairs produced non-specific amplification with one or more non-target strains. The primer pairs exhibiting the highest specificity were selected for qPCR assays being BsXOL35-P2, VaBXOL162-P2, NfBXOL39-P2, RhXOL28-P1, and MrXOL43-P1 the final candidates (**Figure 10**). Finally, all PCR amplicons were sequenced to confirm that they matched the expected target sequences.

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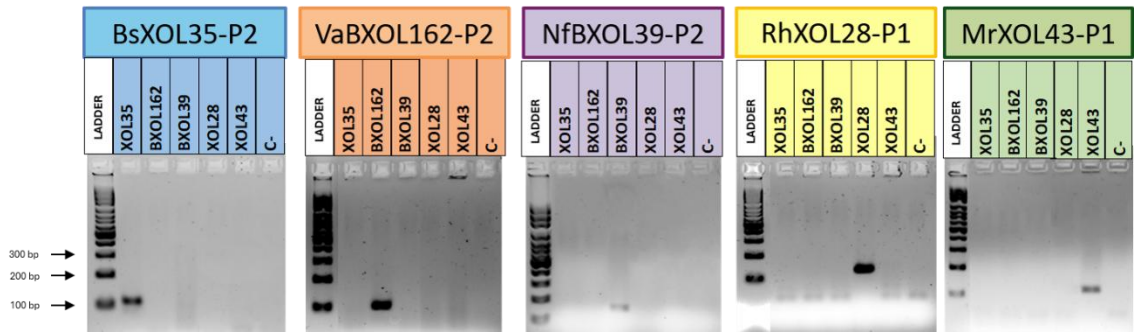


Figure 10. Agarose gel electrophoresis of PCR products amplified using the primer pairs selected for their high specificity. A molecular weight ladder is shown on the left side of each gel. The three lowest bands of the ladder correspond to 300, 200, and 100 bp (from top to bottom).

Standard regression lines were generated for each DNA standard curve using the selected primer-probe sets and DNA concentrations ranging from 10^7 to 1 genome copy/µL. Each standard curve was evaluated in both singleplex and triplex formats. The qPCR assays showed high performance, with amplification efficiencies above 80% (**Table 7**). Furthermore, all standard curves exhibited excellent linearity across the tested dynamic range, with coefficients of determination (R^2) ranging from 0.98 to 0.99. In addition, minimal differences in amplification efficiency were observed between the singleplex assays and their corresponding triplex assays in most cases.

Table 7. Standard regression equations from seven-point 10-fold serial dilutions of DNA of the strains assembled in the SynComs ranging from 107 to 1 genome copies/µL. Coefficient of determination (R2), efficiency of qPCR reactions and limit of quantification (LOQ) are also indicated.

qPCR mix	Primer set	Intercept	Slope	R^2	Efficiency (%)	LOQ (Ct)
Singleplex	BsXOL35-P2	36.51	-3.08	0.99	111.19	36.02
	VaBXOL162-P2	34.94	-3.33	0.99	99.66	34.45
	MrXOL43-P2	41.55	-2.92	0.98	120.02	35.67
	NfBXOL39-P2	37.31	-3.06	0.99	112.23	34.28
	RhXOL28-P1	38.70	-3.16	0.99	107.23	32.98
Triplex SC1	BsXOL35-P2	38.73	-2.86	0.99	123.69	35.33
	VaBXOL162-P2	37.85	-3.29	0.99	101.35	34.45
	NfBXOL39-P2	37.99	-3.34	0.99	99.25	34.73
Triplex SC2	MrXOL43-P2	46.13	-3.36	0.98	98.44	37.56
	NfBXOL39-P2	34.55	-2.87	0.99	123.07	29.71
	RhXOL28-P1	38.72	-3.15	0.99	107.71	32.95

Finally, the DNA of each strain was normalized to a final concentration of 1 ng of genomic DNA/µL, either individually or in a mixture with DNA from the other strains belonging to the corresponding SynCom. All samples were analyzed using their respective triplex qPCR assays. The results showed minimal interference in target detection and quantification caused by the

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presence of DNA from the other strains, with ΔCq values below 0.8 (**Figure X**). These findings confirm the specificity of the designed triplex qPCR assays.

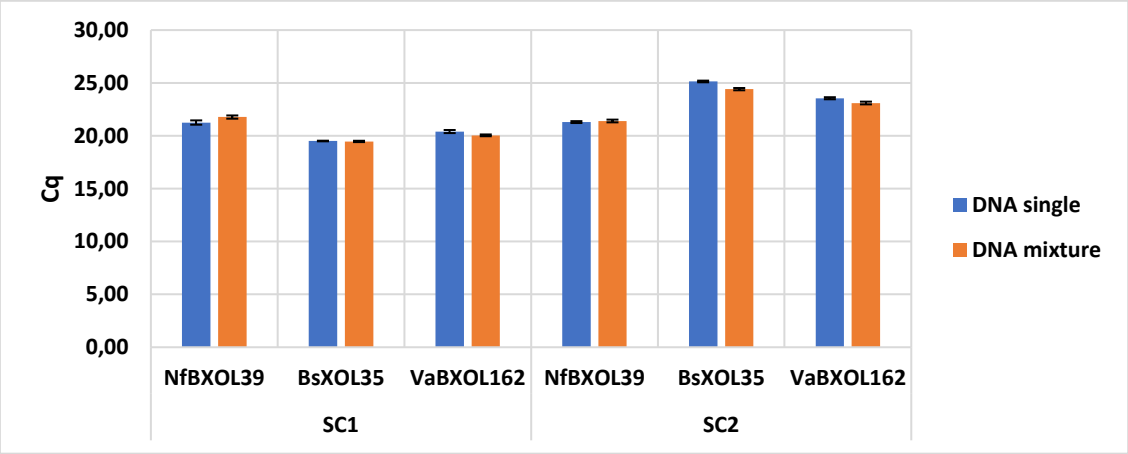


Figure 2. Comparison of Cq values obtained from individual and mixed genomic DNA samples for each strain within the corresponding SynCom (SC1 and SC2).

Future work will include evaluating the potential interference of plant-derived components during DNA extraction and qPCR analysis. Subsequently, the population dynamics of the SynCom strains will be monitored over time in plants from the field experiments. Same DNA already used to check for X. fastidiosa infection will be also assessed ensuring the monitoring of the presence of SynCom strains across experimental time.

3.2.2. Peptide trials

At the time of preparation of this deliverable, the peptide field trials established with almond trees in Spain and Italy had not yet progressed sufficiently to allow treatment performance to be determined. Further visual and molecular assessments will therefore be required before conclusions can be drawn.

3.2.3. Bacteriophages trials

The vineyard trials established in Mallorca with the Vise_2 + Vise_6 bacteriophage combined treatment have only recently entered the evaluation phase, and differences among treatments cannot yet be determined. By contrast, the previously established ES-P6 trial, which was incorporated into the BeXyl project while already ongoing, has yielded encouraging preliminary evidence of the potential of bacteriophage-based treatments under natural field conditions, including a progressive reduction in disease severity and bacterial populations following treatment. Nevertheless, all trials remain subject to long-term follow-up, and further assessments will be needed before definitive conclusions can be reached.

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4 SUMMARY OF RESULTS AND CONCLUSIONS

4.1 Testing vector control strategies (Task 7.3)

Across the field trials, the efficacy of entomopathogenic fungi against spittlebug vectors was variable and strongly dependent on the experimental site, year, target species, and application strategy. *M. anisopliae* BP1q and the *B. bassiana*-based product Naturalis® generally reduced *P. spumarius* abundance compared with the untreated control, although the statistical significance of these reductions was not consistent across trials. Efficacy against *N. campestris* was more variable and generally less pronounced, with no consistent significant reductions associated with fungal treatments at the final evaluation. Repeated applications tended to provide greater reductions in *P. spumarius* abundance than single applications, although differences among fungal treatments were not consistently significant. The results obtained in 2026 did not provide clear evidence that advancing the first application to the first-to-second instar stages improved the efficacy of *M. anisopliae* compared with applications initiated at the third-to-fourth instar stages.

Taken together, these findings indicate that entomopathogenic fungi may contribute to the management of spittlebug vectors under field conditions, although their performance is influenced by multiple environmental, biological, and operational factors. Notably, *M. anisopliae* BP1q was applied as a simple aqueous conidial suspension, without an optimized formulation, yet achieved approximately 40% efficacy against the juvenile stages of *P. spumarius* following two applications. This result suggests that further improvements in formulation, application timing, and treatment frequency could enhance the performance of this approach. Multi-year studies conducted under different environmental and agronomic conditions are therefore needed to assess its consistency and determine how entomopathogenic fungi can best be integrated with mulching and other vector-management practices.

The relatively greater and more consistent efficacy of mulching indicates that agronomic management of ground vegetation should remain a key component of integrated strategies aimed at reducing populations of *P. spumarius* and *N. campestris* and, consequently, limiting the abundance of vectors associated with *X. fastidiosa*. Low-impact products, including entomopathogenic fungi, should therefore be considered complementary tools rather than standalone solutions. Their combination with conservative soil-management practices, or their use to support agronomic measures such as mowing, may improve overall control while reducing reliance on intensive plowing, a well-established and widely recommended agronomic practice whose repeated use may negatively affect soil structure. This approach is also consistent with the broader direction of European agricultural policies towards reducing intensive soil disturbance and promoting more sustainable soil-management practices.

4.2 Field evaluation of innovative biological tools targeting *Xylella fastidiosa* in host plants (Task 7.4)

A comprehensive network of field trials was successfully established in Spain and Italy providing a unique framework for the long-term validation of biological control strategies under European outbreak conditions.

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The experimental network includes different host crops (olive, almond and grapevine), geographical regions and epidemiological scenarios, allowing both preventive and curative approaches to be assessed under realistic field conditions.

Although most trials are still at an early stage of evaluation because of the slow development of *X. fastidiosa* infections and the recent establishment of several experiments, the field infrastructure required for long-term assessment is now fully operational. Preliminary observations obtained from the previously established bacteriophage trial incorporated into BeXyl further support the potential of this approach, although continued monitoring will be required before definitive conclusions can be drawn. Overall, the experimental network established within WP7 provides a robust platform for the long-term validation of innovative biological tools and their future integration into sustainable *X. fastidiosa* management strategies.

4.3. Main achievements

- A coordinated network of field trials was established in Spain and Italy to validate innovative management tools for *Xylella fastidiosa* under natural outbreak conditions.
- The experimental network covers olive, almond and grapevine production systems and includes both preventive and curative treatment approaches.
- The most promising tools developed in WP6, including synthetic microbial communities (SynComs), the defence-eliciting peptide PpPep1 and bacteriophage-based treatments, were successfully implemented under field conditions.
- Harmonised experimental protocols and monitoring procedures were established to enable robust and comparable assessment of treatment performance across sites.
- The established network provides a long-term platform for evaluating treatment efficacy and generating the evidence required to support the future integration of effective solutions into sustainable *X. fastidiosa* management strategies.

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