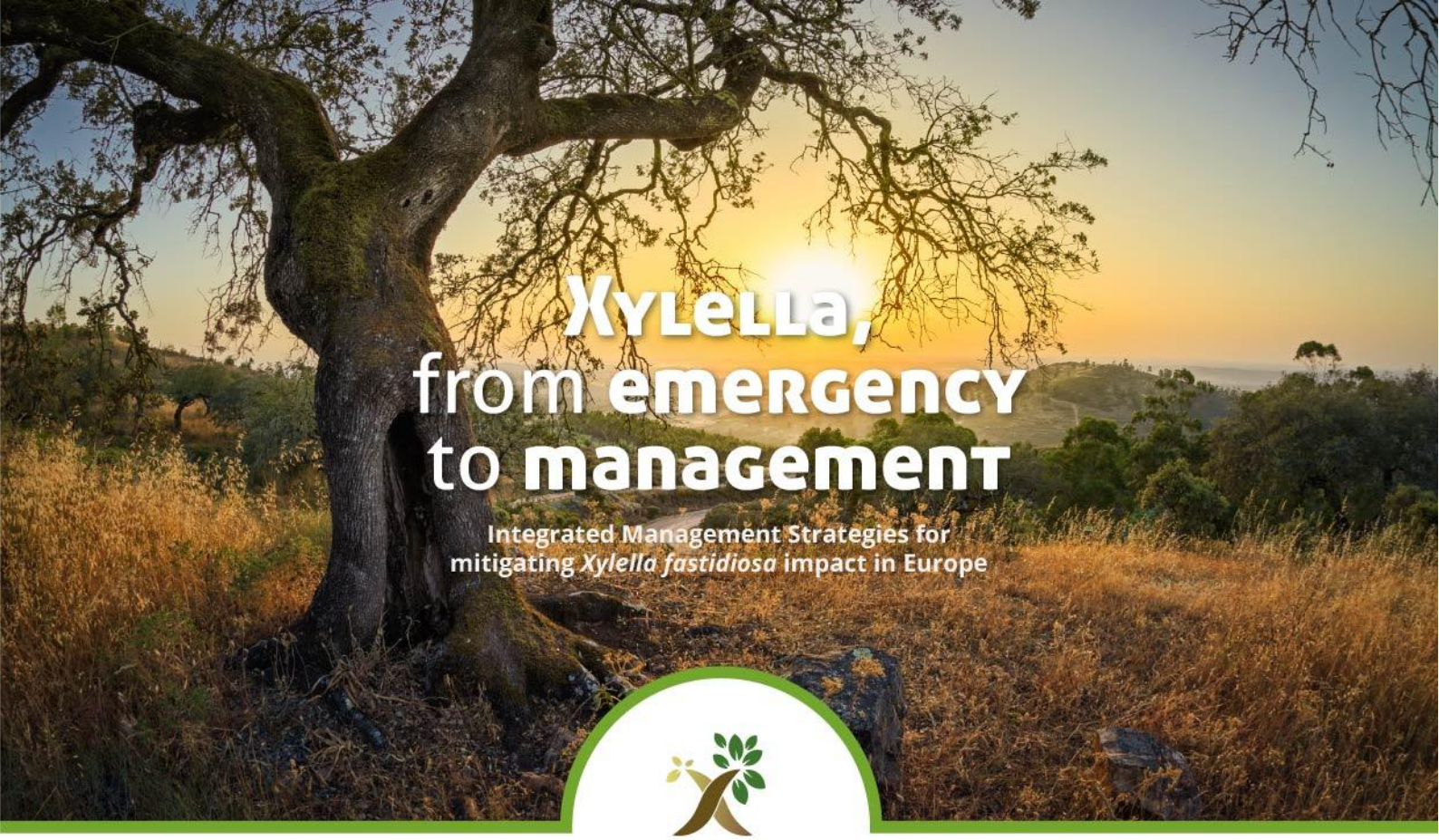



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# Development of Synthetic communities (SynComs) of xylem-inhabiting bacteria with key traits against *Xylella fastidiosa*

Deliverable 6.1

31 May 2025

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# ABSTRACT

This deliverable D6.1, entitled **"Development of Synthetic Communities (SynComs) of xylem-inhabiting bacteria with key traits against *Xylella fastidiosa* (*Xf*)"**, is part of the BeXyl project (Grant Agreement 101060593) and describes the design, selection, and preliminary testing of microbial consortia aimed at enhancing the resilience of woody crops against infections caused by *X. fastidiosa*. As no effective field treatments currently exist for this pathogen, and considering the European Union's increasing emphasis on sustainable agriculture through initiatives like the Green Deal and the Farm to Fork Strategy, this work explores the potential of using endophytic bacteria as eco-friendly biocontrol agents.

A diverse collection of 373 bacterial isolates belonging to 49 genera obtained from the xylem sap of olive, grapevine, and almond trees was used. These strains were subjected to comprehensive phenotypic screening for traits associated with direct antagonism (e.g., antimicrobial metabolite production, hydrolytic enzymes, siderophores) and indirect plant-beneficial mechanisms (e.g., nitrogen fixation, phosphate solubilization, indole acetic acid production). Each strain's ability to assimilate diverse carbon sources and tolerate biochemical stressors was evaluated using Biolog Gen III assays. In addition, inter-strain compatibility was tested through co-cultivation to identify combinations capable of stable co-existence in synthetic communities. Strains were further assessed for culturability and bioprocessability, two essential criteria for practical implementation. Selected strains were evaluated for their ability to grow in standardized and modified media under high-cell-density conditions. Parameters such as biomass yield, storage stability, colony morphology, and fermentation behaviour were measured to determine their scalability for large-scale production. This work enabled the identification of robust strains with high culturability and process reliability, facilitating future steps toward commercial formulation and field application.

From this extensive screening process, two SynComs—SC1 and SC2—were assembled using strains that demonstrated antagonistic activity against *X. fastidiosa*, ecological compatibility, and traits that promote plant growth and systemic resistance. For instance, *Bacillus safensis* XOL35 exhibited significant in vitro inhibition of *X. fastidiosa*, while other selected strains contributed additional functions and compatibility within the communities.

Preliminary in planta assays conducted on tobacco and olive plants suggest that both SC1 and SC2 reduce bacterial populations and may mitigate disease symptoms caused by *X. fastidiosa*. Notably, SC1 showed a marked ability to promote plant growth even under infection conditions, suggesting a dual role in disease suppression and plant health enhancement. Although these experiments are ongoing, initial results are promising and support the continued development of SynCom-based biocontrol strategies.

In summary, this deliverable highlights the potential of synthetic endophytic bacterial communities as a novel and sustainable tool in the fight against *X. fastidiosa*. The methodologies and findings presented offer a solid foundation for future application in integrated pest management programs aimed at reducing chemical inputs and enhancing plant resilience in Mediterranean and other susceptible agricultural systems.



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# 1. INTRODUCTION

Despite extensive research efforts, currently there is no effective cure for *X. fastidiosa*-associated diseases under field conditions (EFSA PLH Panel, 2019; Burbank, 2022). Most of the management strategies in place focus on containment measures, including intensive mandatory surveillance, the removal of infected plants and establishment of buffer zones, and vector control through insecticides (EFSA PLH Panel, 2018; Kyrkou et al., 2018). Moreover, the European Union is increasingly shifting towards more sustainable agricultural practices through policy frameworks such as the European Green Deal and the Farm to Fork Strategy. These initiatives promote a substantial reduction in the use of chemical pesticides and emphasize the adoption of integrated pest management (IPM) approaches (Tataridas et al., 2022). Therefore, there is a need to find new alternatives that are eco-friendly, comply with the EU regulations and that are able to manage the diseases caused by *X. fastidiosa*.

A promising strategy under investigation is the use of endophytic bacteria as biocontrol agents. These microorganisms reside within plant tissues without causing harm and form an integral part of the plant microbiome (Eljounaidi et al., 2016). They can provide a range of benefits to their host plants by either directly suppressing pathogens or indirectly enhancing plant fitness, thereby reducing disease development. Known mechanisms include the production of antimicrobial compounds, competition with pathogens for nutrients and ecological niches, secretion of lytic enzymes that degrade key structural components of pathogen cells, and the synthesis of siderophores that sequester essential micronutrients such as iron (Compant et al., 2005; Ali et al., 2024). Additionally, endophytes can activate induced systemic resistance (ISR), boosting the plant's immune response, and produce phytohormones that promote plant growth (PGP), thereby increasing overall plant fitness. An example would be *Paraburkholderia phytofirmans* strain PsJN which has demonstrated the ability to reduce *X. fastidiosa* colonization and disease symptoms in grapevines by inducing systemic resistance mechanisms (Baccari et al., 2019; Lindow et al., 2024).

Considering the potential beneficial traits of endophytic bacteria, there is growing interest in harnessing them not only as individual biocontrol agents, but also in the form of synthetic microbial communities (SynComs). SynComs are carefully designed consortia of compatible bacterial strains selected for their complementary functions and ecological compatibility (Martins et al., 2023). The goal of creating and applying a SynCom is to mimic or enhance the plant's natural microbiome, strengthening its protective and growth-promoting capabilities in a controlled and reproducible way. To design effective SynComs, it is essential to first identify suitable endophytic bacteria from the host plant of interest (Wang et al., 2023). In this regard, projects such as XF-ACTORS have contributed significantly by characterizing the microbiomes of *X. fastidiosa* hosts, including almond and olive, and by isolating their endophytic bacterial strains (Anguita-Maeso et al., 2020, 2022). Moreover, some of these studies have compared the microbiomes of infected and uninfected plants, highlighting potential genera that may serve as biocontrol agents against *X. fastidiosa*.

Based on these premises, one of the objectives of WP6, specifically Task 6.1, was to develop synthetic xylem bacterial communities (SynComs) with key traits to enhance



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the resilience of woody crops against *X. fastidiosa* infection. Initially, selected bacterial strains were characterized for their biocontrol-associated mechanisms, which included both direct actions against the pathogen and indirect mechanisms aimed at enhancing host plant fitness. Additionally, each strain was evaluated for carbon source assimilation and tolerance to biochemical stresses. Subsequently, the inhibitory activity of the selected strains against *X. fastidiosa* was assessed in vitro. Based on these findings, the compatibility among promising strains was evaluated, and potential SynComs were established. Currently, *in planta* experiments are being conducted to assess the protective effect of the assembled SynComs on plants infected with *X. fastidiosa*.

# 2. MATERIALS AND METHODS

## 2.1. Bacterial Strains and culture conditions

A collection of 373 bacterial strains (BXOL) used in the conducted experiments was obtained from previous research studies in which the xylem microbiome of olive trees was characterized using high-throughput sequencing and culture-dependent techniques (Anguita-Maeso et al., 2020; Anguita-Maeso, 2023) or were isolated de novo during the first year of BeXyl project. The initial collection consisted of 302 bacterial strains isolated from the xylem of different olive tree varieties, 31 from almond tree xylem sap and 40 from grapevine xylem sap. All bacterial isolates were identified through Sanger sequencing of the 16S ribosomal RNA region. In total these strains belong to 49 genera, with *Sphingomonas* spp., *Hymenobacter* spp., and *Methylobacterium* spp. being the most abundant (Anguita-Maeso et al., 2020), from which 46, 9 and 4 genera are present among the olive, grapevine and almond collection.

All strains were preserved at -80 °C in Reasoner's 2A (R2A) broth (50%) (Reasoner and Geldreich, 1985) supplemented with 25% of glycerol, and to start an active growth they were cultured in R2A solid medium. Plates were kept at 28 °C for 5 days. Afterward, colonies were scrapped and cultured in R2A medium for 5 additional days before being used in any of the experiments. When bacterial suspensions were required for the different tests, R2A broth was used.

## 2.2. In vitro inhibition assays against *Xylella fastidiosa*

The bacterial antagonism of the strains against *X. fastidiosa* (*Xf*) was assessed using dual in vitro cultures. These assays were conducted in the Biosafety Level 2 laboratory at the Institute for Sustainable Agriculture (IAS-CSIC) following the current legislation. The *Xf* strains used belonged to the subspecies *fastidiosa* (strains Temecula, TemeculaL, and IVIA5770) and *multiplex* (strains AlmaEm3 and ESVL). Active cultures of these strains for the experiments were obtained in PD3 solid medium (Davis et al., 1981) from glycerol stocks at -80 °C in PD3 broth supplemented in 25% of glycerol. Plates were kept at 28 °C for 7 days. Afterwards, a bacterial suspension of the strains was obtained in PD3 broth and was used to inoculate new PD3 plates using sterilized glass beads to obtain an even lawn culture. Plates were incubated at 28ºC for 5 days before starting the experiment



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due to the slow growth rate and low biomass yield of *X. fastidiosa* compared to BXOL strains.

Regarding the BXOL strains, 3 days after inoculation of *X. fastidiosa* using the sterilized glass beads, a single colony of each bacterial strain was inoculated into 5 mL of R2A broth which were incubated 28 °C for 3 days under continuous agitation (120 rpm). Then, 4 µL of the bacterial suspensions were inoculated on the *X. fastidiosa* inoculated plates and were incubated for 7 days at 28 °C as shown in Figure 1. Growth status and potential antagonistic effects were evaluated daily by monitoring the formation of inhibition halos. Three replicates were performed for each experimental combination.

Inhibitory activity was assessed using a semiquantitative scale from 0 to 3, where 0 indicated the absence of an inhibition halo (no antagonism) around the bacterial colonies; 1 (+) indicated a slight inhibition halo or a reduced *Xf* density around the bacterial perimeter; 2 (++) indicated an irregular inhibition halo and/or a clear decrease in *Xf* density, and 3 (+++) indicated a well-defined and prominent inhibition halo.

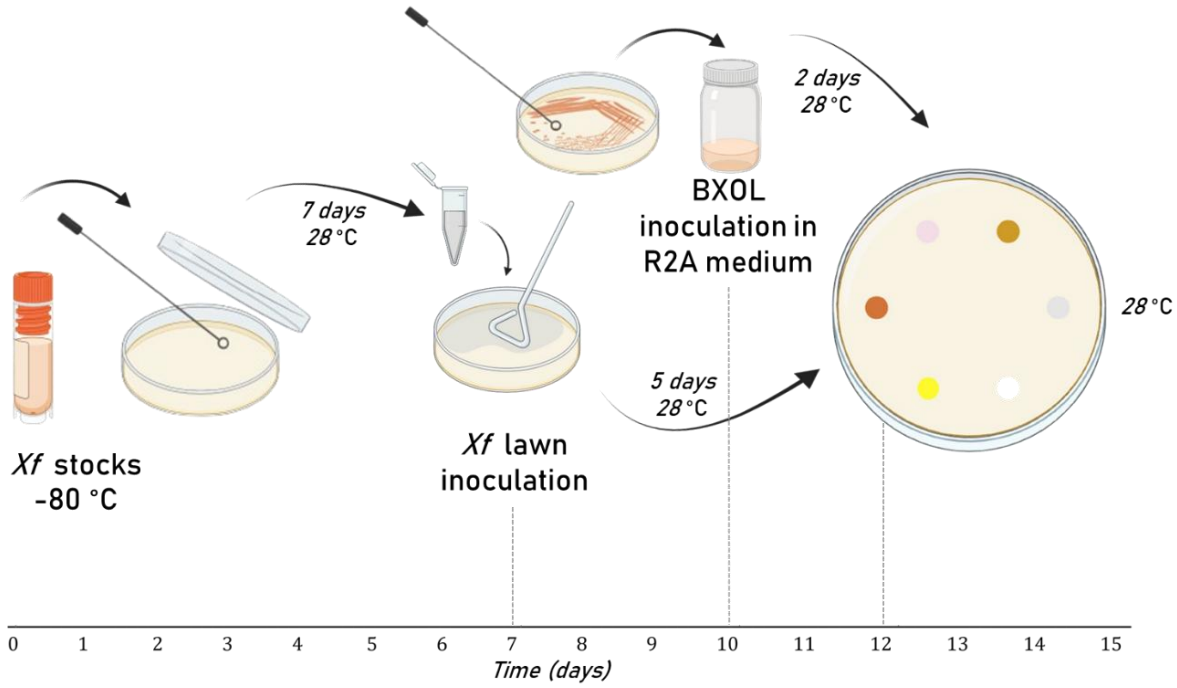



**Figure 1:** Workflow of the experimental procedure for evaluating *in vitro* inhibition against *Xylella fastidiosa*.

## 2.3. Evaluation of biocontrol-associated mechanisms

A total of 242 strains were phenotypically characterized by assessing various traits related to indirect and direct mechanisms of biocontrol (Table 1). Indirect mechanisms correspond to those that can influence the plant immune system and modulate hormone levels while direct mechanisms are related to the production of antimicrobial metabolites, hydrolytic enzymes, interference on quorum sensing systems, competition



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for nutrients or space between microorganisms, and siderophore production for iron acquisition (Legein et al., 2020).

**Table 1.** Summary of the culture media and criteria for the identification of positive bacteria for each test and reference.

| Test | Culture medium | Positive result | Reference |
|---|---|---|---|
| **Direct mechanisms: PGP** | | | |
| **Nitrogen fixation** | Nitrogen Fixating Bacteria medium | Growth | Döbereiner and Day, 1976 |
| **Phosphate Solubilization** | NBRIP | Transparent halo | Nautiyal, 1999 |
| **Potassium Solubilization** | Alexandrov medium | Transparent halo | Etesami et al., 2017 |
| **Siderophores** | CAS assay | Transparent halo | Schwyn and Neilands, 1987 |
| **IAA** | NB + tryptophan | Red color | Naik et al., 2008 |
| **Indirect mechanisms** | | | |
| **DNAse** | DNAse agar | Transparent halo | Scharlau, Spain |
| **Amylase** | Modified starch agar | Transparent halo | Tindall et al., 2007 |
| **Catalase** | - | Bubble formation | Holt et al., 1994 |
| **Oxidase** | - | Blue color after 10-15" | Kovács, 1956 |
| **Cellulase** | CMC solid medium | Transparent halo | Elbeltagy et al., 2000 |
| **Lipase** | Tween 80 solid medium | Precipitated halo | Prescott, 2002 |
| **Pectinase** | AMA medium | Transparent halo | McKay, 1988 |
| **Protease** | Casein agar | Transparent halo | Naik et al., 2008 |
| **Chitinase** | Minimal medium + colloidal chitin | Transparent halo | Murthy and Bleakley, 2012 |
| **Xylanase** | Xylan source medium | Transparent halo | Wood et al.,1988; Gong et al., 2012 |
| **Hydrogen cyanide (HCN)** | King's B medium + glycine | Reddish brown filter paper | Bano and Musarrat, 200; Lorck, 1948 |
| **2,3-Butanodiol** | MRVP Broth | Red color | Britania Lab, Argentina |



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### 2.3.1. General methods

Most of the experiments were carried out using solid media in 86 mm × 100 mm rectangular plates (Thermo Fisher Scientific, USA), each filled with 40 mL of the respective culture medium according to the test requirements (Table 1). Plates were inoculated with 4 µL of a bacterial suspension of each BXOL strain in TSB medium (Tryptic Soy Broth, Biolife, Milan, Italy). Specifically, each plate was inoculated with 10 bacterial strains resulting in a total of 26 plates for each test.

For each test, both negative and positive controls were conducted. TSB medium was used as the negative control in each test, while a bacterial strain known to demonstrate the analysed activity served as the positive control. Specifically, *Pseudomonas* sp. strain N8 was used for the nitrogen fixation, potassium solubilization, phosphate solubilization, siderophore production, cellulase, protease and DNAase activity tests. The strain *Pseudomonas* sp. strain JN4 was used to determine the production of IAA. Both were obtained from the laboratory of Rodríguez-Llorente (Dept. of Microbiology and Parasitology, University of Seville). The strain *Bacillus safensis* XOL35 was used as a positive control for lipase, amylase and xylanase activities and *Curtobacterium flaccumfaciens* strain BXOL74 to determine the chitinase activity since both showed their corresponding activities in initial experiments.

A binomial scale was used to evaluate catalase, oxidase, and nitrogen fixation activity tests, where 0 indicates no activity and 1 indicates activity. The rest of the activities (indole-3-acetic acid (IAA) production, lipase, DNase, amylase, cellulase, pectinase, protease, chitinase, xylanase, hydrocyanic acid, and siderophore production; phosphate and potassium solubilization; and the methyl red and Voges-Proskauer tests for determining 2,3-butanediol with MRVP medium) used a semiquantitative scale of five values: 0 for no activity, 1 for slight activity, 2 for moderate activity, 3 for high activity, and 4 for maximum activity.

### 2.3.2. Evaluation of indirect mechanisms associated with biocontrol

Four indirect mechanisms were evaluated: nitrogen fixation, phosphate and potassium solubilization, and indole-3-acetic acid (IAA) production. The first three assays determine the potential of the strains to increase the availability of essential macronutrients and micronutrients for plant development. The fourth assay involves a phytohormone from the auxin group, which promotes root elongation, root hair formation, and lateral root formation, thereby facilitating nutrient acquisition (Turan et al., 2016).

#### *2.3.2.1. Nitrogen Fixation*

To determine capacity to fixate nitrogen of the strains, solid Nitrogen Fixating Bacteria (NFB) medium (Döbereiner and Day, 1976) was used. This culture medium lacks combined nitrogen sources, so the bacteria must fix atmospheric nitrogen to grow. Plates were incubated at 28 °C for 7 days until results evaluation. A positive result (i.e., nitrogen fixation capacity) was considered when bacterial growth was observed on the medium.



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#### *2.3.2.2. Phosphate Solubilization*

To assess the ability to solubilise phosphate of the strains, the NBRIP (National British Research Institute Phosphate) solid medium (Nautiyal, 1999) was used. Plates were incubated at 28 °C for 7 days. Strains that developed a clear halo around their colonies were considered positive.

#### *2.3.2.3. Potassium Solubilization*

To evaluate the capacity of the strains to solubilize potassium, the Alexandrov medium described by Etesami et al. (2017) was used. Plates were incubated at 28 °C for 7 days. Strains surrounded by a clear halo were considered positive.

#### *2.3.2.4. Indole-3-acetic Acid (IAA) Production*

IAA production was assessed in tubes containing 3 mL of nutrient broth (NB, Biolab, Hungary) supplemented with tryptophan. Each tube was inoculated with 10 µL of the bacterial suspension and incubated for 72 hours at 28 °C under continuous shaking (120 rpm), including a negative control consisting of uninoculated medium. After incubation, the culture was centrifuged, and 1 mL of the supernatant was mixed with Salkowski's reagent (150 mL of concentrated $H_2SO_4$, 250 mL of distilled water, 7.5 mL of 0.5M $FeCl_3 \cdot 6H_2O$) (Gordon and Weber, 1951) and allowed to react for 20 min. The appearance of a red colour indicated a positive result for IAA production, which correlates with auxin production.

### 2.3.3. Evaluation of direct mechanisms associated with biocontrol

In this study, 13 enzymatic and/or antimicrobial activities were assessed as direct biocontrol mechanisms which include production of DNase, amylase, catalase, oxidase, cellulase, lipase, pectinase, protease, chitinase, xylanase, hydrogen cyanide (HCN), 2,3-butanediol, and siderophores.

#### *2.3.3.1. DNase Activity*

To determine the DNase activity of the strains solid DNAse agar (Scharlau, Spain) was used. Plates were incubated at 28 °C for 7 days. DNase activity was revealed by adding HCl 1N, which causes unhydrolyzed DNA to precipitate and darken the medium. Clear halos around bacterial colonies indicated DNA hydrolysis and a positive result.

#### *2.3.3.2. Amylase Activity*

To assess the amylase activity modified starch agar (Tindall et al., 2007) was used. Plates were incubated at 28 °C for 7 days. After incubation, Lugol's iodine solution (Sigma-Aldrich, Darmstadt, Germany) was added. The medium darkened, and clear halos around the colonies indicated amylase activity.

#### *2.3.3.3. Catalase Activity*

To evaluate the catalase activity, the Holt et al. (1994) protocol was followed. Individual bacterial colonies were placed on a sterile Petri dish lid using a sterile 10 µL pipette tip.



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A few drops of 3% hydrogen peroxide ($H_2O_2$) were added. Bubble formation indicated a positive catalase reaction.

#### *2.3.3.4. Oxidase Activity*

To determine the oxidase activity of the strains, colonies were placed on sterile filter paper using sterile 10 µL pipette tips. Then, tetramethyl-p-phenylenediamine (1%, Thermo Fisher Scientific Chemicals, Inc., MA, USA) was added (Kovács,1956). A blue color within 15 seconds indicated positive oxidase activity.

#### *2.3.3.5. Cellulase Activity*

Carboxy Methyl Cellulose (CMC) solid medium was used to determine the celullase activity of the strains (Elbeltagy et al., 2000). Plates were incubated at 28 °C for 7 days, then stained with 0.1% Congo red for 20 min, followed by decolorization with 1 M NaCl. Congo red binds to cellulose, darkening the medium. Clear halos after decolorization indicated cellulase activity.

#### *2.3.3.6. Lipase Activity*

Lipase activity was determined according to the method described by Prescott (2002), and Flores-Duarte et al. (2022). Tween 80 solid medium was used and plates were incubated at 28 °C for 7 days. Visual observations were performed with a EVOS M7000 fluorescence microscope (Thermo Fisher Scientific, USA). A positive result was indicated by a halo of precipitate formed from the reaction of $CaCl_2$ with fatty acids released by Tween 80 hydrolysis.

#### *2.3.3.7. Pectinase Activity*

Pectinase activity was determined as described by Elbeltagy et al. (2000). Ammonium Mineral Agar (AMA) medium was used and plates were incubated at 28 °C for 7 days, then treated with 2% cetyltrimethylammonium bromide (Merck KGaA, Darmstadt, Germany). This reagent precipitates non-hydrolyzed pectins, and clear halos indicated pectinase activity.

#### *2.3.3.8. Protease Activity*

Protease activity was assessed using modified casein agar described by Naik et al. (2008). Plates were incubated at 28 °C for 7 days and examined daily. Clear halos around colonies indicated protease activity.

#### *2.3.3.9. Chitinase Activity*

Chitinase activity was evaluated in minimal medium as described by Murthy and Bleakley (2012) supplemented with colloidal chitin. Plates were incubated at 28 °C for 7 days. Clear halos around the colonies indicated chitin degradation which corresponded to positive chitinase activity.



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### *2.3.3.10. Xylanase Activity*

The xylanase production was determined as described by Wood et al. (1988) and Gong et al. (2012). Corn xylan (Xylan from Corn Core, Tokyo Chemical Industry, Japan) was used as the source of xylan. The plates were incubated at 28 °C for 7 days, then revealed by adding commercial Lugol solution (Sigma-Aldrich, Darmstadt, Germany) to the plates. Strains that formed transparent halos around the colony were considered positive.

### *2.3.3.11. Hydrocyanic Acid or Hydrogen Cyanide (HCN) Production*

The evaluation of hydrocyanic acid or hydrogen cyanide (HCN) production was performed on solid King's B medium supplemented with 4.5 g of glycine (Bano and Musarrat, 2003; Lorck, 1948). Unlike the previous tests, this medium was dispensed into Petri dishes, inoculating one bacterial strain per plate. A sterile filter paper saturated with 1 mL of 2% sodium carbonate and 1 ml of 1% picric acid was placed on the lid of the plate. The plates were incubated at 28 °C for 7 days, and evaluations were performed on days four and seven. A positive result is indicated by a change in the colour of the filter paper from yellow to reddish brown, which indicates the presence of cyanogenic activity in the bacteria.

### *2.3.3.12. 2-3 Butanediol production*

Production of butanediol was evaluated using MRVP medium (Methyl Red-Voges Proskauer, Britanialab, Argentina). 10 µL of the bacterial suspension strain was inoculated into sterile test tubes containing 5 mL of MRVP broth and incubated at 33 °C for 72 h under continuous shaking (120 rpm). Following incubation, the medium was subjected to two biochemical assays: the Methyl Red (MR) test and the Voges–Proskauer (VP) test. For the VP test, 2.5 mL of the incubated culture was transferred to a new tube. Subsequently, 5% α-naphthol in absolute ethanol and 40% potassium hydroxide were added. The mixture was gently vortexed and allowed to stand for 15 min. The appearance of a red coloration was interpreted as a positive result, indicating the production of neutral end products such as acetoin. To the remaining 2.5 mL of the bacterial culture, 200 µL of methyl red indicator solution was added to perform the MR test. The development of a stable red colour indicated a positive reaction, confirming the accumulation of stable acidic end products resulting from glucose fermentation.

### *2.3.3.13. Siderophore production*

The strains' siderophore production was determined in modified solid medium of the universal CAS (Chrome Azurol S) assay (Schwyn and Neilands, 1987). Specifically, the buffer to PIPES (piperazine-N,N'-bis(2-ethanesulfonic acid), Thermo Fisher Scientific, USA) was used instead of MES and the incubation temperature was changed from 30 °C to 28 °C. The plates were incubated for 7 days and the results were evaluated. Bacteria that showcased a yellowish, orange, or purple halo on the periphery, which depended on the type of siderophore, were considered positive results (Pérez-Miranda et al., 2007).



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## 2.4. Evaluation of carbon sources assimilation and biochemical sensitivity

The carbon source utilization and biochemical sensitivity for a selection of strains were carried out using the Gen III Microplate plates from Biolog (Hayward, CA, USA). These plates include 71 wells for carbon source utilization profiling and 23 wells for assessment of sensitivity to chemical compounds such as sodium chloride at different concentrations, acid pH and antibiotics. The substrates and biochemical indicators are lyophilized in each of the 96 wells, along with tetrazolium, a colorimetric redox indicator. Each plate was inoculated with a suspension of the corresponding bacterial strain prepared in one of the buffers provided in the kit (IFA, IFB, IFC1, or IFC2), selected according to the bacterial genus and following the manufacturer's instructions.

The plates were then incubated at 28 °C for 7 days, with visual inspections carried out every 2 days to observe the appearance of a purple colour indicating growth and assimilation of the tested compound. Cellular respiration increases in proportion to the utilization of the carbon source or the growth of the bacterial colony. Increased respiration caused a reduction of the tetrazolium redox dye, producing a violet colour in the well. Well A1 served as a negative control for carbon source utilization, while well A10 was used as a positive control in the biochemical sensitivity assays.

## 2.5. Compatibility assays among BXOL strains

Twelve bacterial strains were selected from the total analysed based on all the assessed characteristics. Their compatibility to assemble a synthetic community (SynCom) was evaluated qualitatively using a co-cultivation assay on R2A agar plates. For each pair, one strain was uniformly spread onto the surface of the R2A agar plate using a sterile Digralsky spreader. The plates were allowed to dry for 15-30 min at room temperature. Then, 4 µL of the second strain was spotted on the plate by triplicate. Plates were incubated at 28 °C for 7 days. Interactions were assessed visually, and compatibility was determined based on the absence of inhibition halos and the preservation of colony morphology. Strains were considered compatible if the spotted culture exhibited normal growth without visible inhibition or interference on the strain spread on the R2A plate.

## 2.6. Selection and characterization of the SynComs

The bacterial strains to establish two SynCom were selected based on phenotypic and metabolic traits determined in previous experiments, taking into account their potential as biocontrol agents through indirect and direct mechanisms and their genus diversity.

After the selection, compatibility among the strains of the two SynComs in pairs and as triplet and their ability to inhibit *X. fastidiosa* subsp. *pauca* (strain CoDiRO) were determined as described in section 2.5 and section 2.2, respectively.



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## 2.7. Selection of strains for SynComs based on culturability and bioprocessability

### 2.7.1. Development and optimisation of culture media and growth conditions for selected strains from xylem sap

Activities were conducted to develop and optimise growth media and fermentation procedures for the production of selected bacterial strains to be used as members of the SynCom. The main aim was to develop a culture medium to achieve high-cell density to enable supply of viable material for in planta studies in WP7 and to test potential for large-scale production of co-cultures. Following the optimization of growth conditions and media for high-cell-density fermentation, various methods, including storage of cultivation broth and lyophilization, were tested to ensure sufficient material production.

Initially, a literature review was conducted to explore potential growth media for SynComs bacteria. The consortium partner P1-CSIC selected and transferred 10 strains isolated from olive trees' xylem sap to P22-ACIES facilities. Initially, 2 strains (*Curtobacterium flaccumfaciens* and *Pseudomonas stutzeri*) were eliminated due to biosafety hazard. The 10 strains were revitalised on solid on R2A medium.

Microscopic examination was conducted to assess cell morphology, and growth on various standard microbiological media (2TY, YEPD, PDA) was assessed to rule out potential contamination. Master cell banks were prepared by streaking the strains on the selected medium, cultivating for 1-2 days, and storing in 20% glycerol.

### 2.7.2. Selection of strains for SynComs based on bioprocessability

Several parameters were used as indicators of good scalability and bioprocessability potential to select the strains for further media development. Biomass yield in modified media, storage stability, cell cluster formation during fermentation, and spore formation in bacillus strains were assessed in all 10 strains. Some strains had strikingly low biomass yield at all of the tested media variations, which increases a risk and poses potential challenges for upscaling. Next, we assessed storage stability tests and conducted survival tests to identify strains with at least $10^8$ CFU/mL and a good stability at 4°C for an extended period.



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## 2.8. *In planta* effects of SynComs against *Xylella fastidiosa* infection

Although it was outside the scope of this deliverable the protective effect of the designed SynComs (SC1 and SC2) in infected plants with *X. fastidiosa* is being evaluated in planta at the moment. Two in planta experiments are being carried out using different hosts. Specifically, one of them is being performed tobacco plants (*Nicotiana benthamiana*) and the other one in olive plants.

### 2.8.1. Plants and greenhouse conditions

For experiments using tobacco plants, three-week-old seedlings were transplanted into 11.5 cm square pots containing a sterile peat-based substrate specifically formulated for small pots. The substrate consisted of a mixture of acidic peat, neutral peat, wetting agent, and a supplemented compound mineral fertilizer (NPK) (Flora gard, Oldenburg, Germany).

Tobacco plants were initially maintained in an environmentally controlled quarantine greenhouse at 25 ± 2 °C with a 14 h/10 h light/dark photoperiod until reaching the 6–8 leaf stage. Subsequently, plants were transferred to a glasshouse and grown under natural sunlight at 20 to 25 °C.

For experiments using olive plants (*Olea europaea*), nine-month-olive plantlets of the cultivar Arbequina, with a minimum height of 50 cm, supplied by Plantas Continental, S.A. nursery (Posadas, Córdoba, Spain) were used. Plantlets had been propagated at the nursery following standard semi-woody stacking procedure. The same peat-based substrate described for tobacco plants was used for olive plantlets. Plants were maintained in an environmentally controlled quarantine greenhouse under optimal growth conditions, at 25-28°C with a natural photoperiod.

For both tobacco and olive plants, irrigation was applied on demand, according to the specific needs of each plant, determined through substrate moisture levels and visual evaluation of plant condition. All plants were fertilized weekly with a balanced, water-soluble NPK formulation to support uniform growth under greenhouse conditions.

### 2.8.2. Inoculation of SynComs and *X. fastidiosa*

Both SynComs were applied through endotherapy as shown in Figure 2 three days before *X. fastidiosa* inoculation. Each SynCom suspension was prepared in R2A broth as described in section 2.1. and three injections of 10 µL were performed. Not-treated plants were included using R2A instead of the SynCom. The inoculation of *Xf* was performed with three injections of 10 µL of a suspension of *Xf* subsp. *pauca* strain CoDiRo at approximately $10^8$ CFU/mL ($OD_{600}$=1) in PD3 as described in Baró et al. (2021). The SynCom and *Xf* bacterial suspension concentrations were confirmed by plate counting. Not-inoculated controls were included by injecting PD3 instead of the bacterial suspensions. Additionally, plants only inoculated with each SynCom were included.



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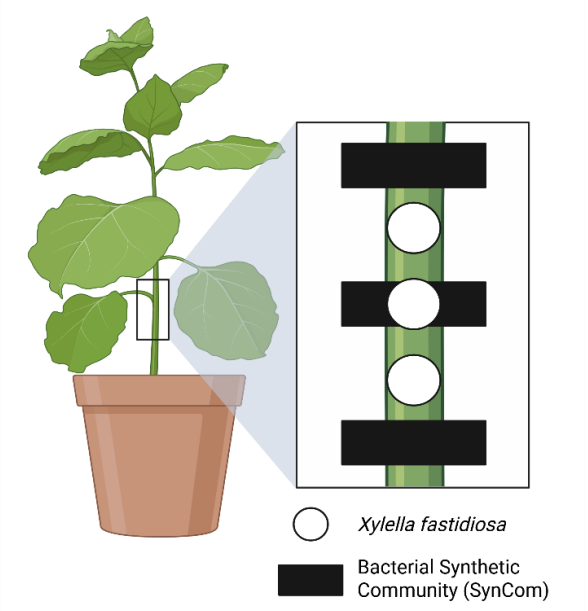



**Figure 2:** Diagram of SynCom and *Xf in planta* inoculation by endotherapy.

Both in tobacco and olive, the treatments being evaluated include: a non-inoculated and not-treated control (NTC), plants treated with SynCom 1 (SC1), plants treated with SynCom 2 (SC2), plants inoculated with *X. fastidiosa* (*Xf*), plants treated with SC1 and inoculated with *X. fastidiosa* (SC1+*Xf*) and plants treated with SC2 and inoculated with *X. fastidiosa* (SC2+*Xf*).

In the case of tobacco plants, the experimental design will consist of three replicates of three plants per each treatment and sampling time (15, 30, 60 days post-inoculation (dpi)) resulting in a total of 54 tabaco plants. In the case of olive plants, the experimental design will consist of four plants per each treatment and sampling time (30, 90, 150, 210 dpi) resulting in 96 olive plants.

### 2.8.3. Effect of treatment on populations of *X. fastidiosa*, disease severity, and plant physiological parameters in tobacco and olive plants

To determine the effect of the treatments on the populations of *X. fastidiosa* and symptoms development, at each sampling time, stem samples were collected, and qPCR analysis were performed as described in Harper et al. (2010) and Ouyang et al. (2013).

Symptoms severity is being assessed following the severity scale described by Baró et al. (2021). Leaf physiological parameters (such as chlorophyll content) are being determined using Dualex leaf clip sensor (METOS Iberia, Seville, Spain) and Polypen (Photon Systems Instruments, Drásov, Czech Republic). Measurements are being taken on six leaves per plant, above the highest point of inoculation between the centre and the margin of the leaves. Other plant physiological parameters such as stem length and fresh weight will also be assessed.



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# 3. RESULTS

## 3.1. In vitro inhibition assays against *Xylella fastidiosa*

The growth inhibition activity against several subspecies of *X. fastidiosa* was assessed for BXOL strains. Halo formation was measured on the 4$^{th}$ and 8$^{th}$ day after inoculation. In all cases the strain *Bacillus* sp. XOL35 was the one that presented the strongest growth inhibition effect showing a sharp inhibition halo (+++) against *X. fastidiosa* subsp. *multiplex* (AlmaEm3 and ESVL) and subsp. *fastidiosa* (TemeculaL). Additionally, it also showed a blurrier halo (++) against *X. fastidiosa* subsp. *fastidiosa* (IVIA5770).

Other strains that showed relevant activity against *X. fastidiosa* included *Bacillus safensis*/*Priestia megaterium* BXOL182, which caused a decrease in *X. fastidiosa* colony density (++), and *Nocardioides* sp. BXOL257, *Rhizobium pusense* XOL28 and *Bacillus* sp. BXOL215, which resulted in a slight inhibition (+). The remaining BXOL bacterial strains that were tested showed no inhibitory effect against *X. fastidiosa*.

## 3.2. Phenotypical characterization: Evaluation of biocontrol-associated mechanisms

Results of phenotypical characterization of biocontrol-associated mechanisms underscored that the results were strain-dependent rather than strictly linked to taxonomic identity at the genus level. This intra-species variation highlights the metabolic diversity that can exist even among closely related bacterial isolates and the possible influence of host environment on bacterial functional traits, reinforcing the importance of strain-level characterization in functional screening assays. Additionally, this functional variability highlights the diverse metabolic strategies among the isolated strains and can guide their selection for SynCom development based on ecological compatibility and functional traits.

### 3.2.1. Evaluation of indirect mechanisms associated with biocontrol

#### *3.2.1.1. Nitrogen Fixation*

Nitrogen Fixation was determined by evaluating the ability of bacterial strains to grow in nitrogen-free NFB medium. Strains were classified based on their activity using a semiquantitative scale (0 to 4). Most of them, 97%, were positive for nitrogen fixation activity. The strains that did not show nitrogen fixating activity were *Sphingomonas* spp. (BXOL77), *Micrococcus* sp. (BXOL6) and *Curtobacterium* sp. (BXOL24-8). It is interesting to note that other strains belonging to the same genera did result positive for this assay indicating that this activity was not genus dependent.

#### *3.2.1.2. Phosphate Solubilization*

Phosphate solubilization activity was assessed in NBRIP medium and strains were classified based on their activity using semiquantitative scale (0 to 4). From all the evaluated strains, 32% were positive for phosphate solubilization. The ones that showed the highest activity (level 3) were *Pseudomonas* spp. BXVI24-26, BXVI24- 27, BXVI24-28



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and BXVI24-29, which were isolated from grape vine xylem. Most of the other strains that resulted positive for phosphate solubilization presented a low level of solubilization (level 1).

#### *3.2.1.3. Potassium Solubilization*

Potassium solubilization activity was determined using Alexandrov medium and strains were classified based on their activity using semiquantitative scale (0 to 4). A total of 22% of the evaluated strains were positive for potassium solubilization. The strain that showed the highest solubilizing capability was *Bacillus pumilus* XOL18A with an intensity level of 1.5. The rest of the positive strains showed levels of solubilization that oscillated between 0.5 and 1.

#### *3.2.1.4. Indole-3-acetic Acid (IAA) Production*

IAA production was evaluated in tubes containing nutrient broth supplemented with tryptophan and strains were classified based on their activity using semiquantitative scale (0 to 4). Overall, 38% of the tested strains were positive for IAA production. Among these, the average intensity score was 1,26. The strains that exhibited the maximum levels of IAA production (level 4) were *Micrococcus luteus* BXOL6, *Sinomonas* sp. BXVI24-19.1 and *Arthrobacter* sp. BXVI24-19.2.

### 3.2.2. Evaluation of direct mechanisms associated with biocontrol

#### *3.2.2.1. DNase Activity*

DNase activity was assessed in DNAse agar (Scharlau, Spain) and strains were classified based on their activity using a semiquantitative scale (0 to 4). Over 20% of the strains resulted positive for DNAse activity. The strains that presented the highest DNAse activity were *Bacillus salipaludis* BXOL108A and *Micrococcus luteus* BXOL6 with an intensity level of 3.

#### *3.2.2.2. Amylase Activity*

Amylase activity was determined in modified starch agar which was later revealed with Lugol solution. Strains were classified based on their activity using a semiquantitative scale (0 to 4). For all of the evaluated strains, 88% of them presented amylase activity. Specifically, most of them exhibited high amylase activity showcasing a level 3 of activity. Interestingly, variability was observed not only across genera but also between strains of the same genus. For instance, while *Bacillus* spp. XOL35 (*B. safensis*) and XOL18A (*B. pumilus*) showed no amylase activity, other strains from the same genus, *B. salipaludis* XOL108A and *B. halotolerans* BXOL262 exhibited scores of 2.5 and 4, indicating moderate and maximum activity respectively. A similar pattern was observed within the genus *Brachybacterium* spp., where *B. ginsengisoli* BXOL279 showed no activity, whereas *Brachybacterium* sp. BXOL267 exhibited moderate amylase production.



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#### *3.2.2.3. Catalase Activity*

Catalase activity was evaluated by exposing bacterial biomass to hydrogen peroxide. Strains were classified as positive or negative. Most of the strains, specifically 97%, were catalase positive. Although bubble size formation or speed was not taken into account in this study, it is relevant to note that variations in reaction intensity were observed. Specifically, some strains quickly generated large and vigorous bubbles while others showed smaller or slower-forming ones.

#### *3.2.2.4. Oxidase Activity*

Oxidase activity was determined applicating tetramethyl-p-phenylenediamine to the bacterial biomass and strains were classified as positive or negative. Half of the evaluated strains were positive for oxidase activity. Positive reactions were typically characterized by the rapid development of a dark blue coloration, while negative strains showed no visible change. The assay does not provide quantitative data, and thus, variations in colour intensity were not interpreted as differences in enzyme levels.

#### *3.2.2.5. Cellulase Activity*

Cellulase activity of the bacterial strains was assessed in carboxy methyl cellulose solid medium and strains were classified based on their activity using a semiquantitative scale (0 to 4). In this case, 33% of the evaluated strains were positive for cellulase activity. The strains that showed the highest cellulase activity (level 4) were *Curtobacterium flaccumfacciens* BXVI24-37, *C. flaccumfacciens* BXVI24-34, *C. ammoniigenes* BXVI24-30 and *C. flaccumfacciens* BXVI24-13, which were isolated from grapevine. The positive strains presented an overall average intensity level of 2.19. Interestingly, variability in cellulase activity was observed not only across genera but also among strains belonging to the same species but isolated from different host plants. For example, the strains *C. flaccumfacciens* BXVI24-37, BXOL74, and BXAL24-26, which were isolated from grapevine, olive and almond tree, respectively, only BXVI24-37 showed cellulase activity.

#### *3.2.2.6. Lipase Activity*

Lipase activity was determined in Tween 80 solid medium and strains were classified based on their activity using a semiquantitative scale (0 to 4). Considering both the initial visual assessment and the microscopic evaluation, a total of 67% of the tested strains showed positive lipase activity. In fact, several strains that were initially scored as 0 (no visible precipitation of $CaCl_2$) showed microscopic evidence of precipitate formation, indicating very low but present enzymatic activity. A wide range of activity levels was observed across the strain collection ranging from 0 to 4, and the average intensity was 2. The strains that exhibited the maximum activity belonged to diverse genera, such as *Bacillus* sp., *Variovorax* sp., *Bosea* sp. and *Pseudomonas* sp., which highlights the broad taxonomic distribution of this trait.

#### *3.2.2.7. Pectinase Activity*

Pectinase activity was determined in AMA medium and strains were classified based on their activity using a semiquantitative scale (0 to 4). From the evaluated strains, 11% presented pectinase activity. The strain that presented the highest activity was *Bacillus*



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sp. BXOL262 which showcased a moderate activity level (level 2.5). The other strains that were positive exhibited very low to low pectinase activity (level between 0.5 and 1).

### *3.2.2.8. Protease Activity*

Protease activity was evaluated in Casein agar and strains were classified based on their activity using a semiquantitative scale (0 to 4). A total of 44% of the tested strains exhibited positive protease activity in this assay. Among these, the average score was 2.83 out of 4, indicating moderate to high enzymatic production. The highest activity levels were observed in strains belonging to the genera *Bacillus* and *Curtobacterium*. Specifically, *B. safensis* XOL35, *Bacillus* sp. BXOL182, *B. altitudinis* BXOL228, *Curtobacterium* spp. BXOL186, BXOL68 and *C. flaccumfaciens* BXAL24-18, BXOL24-8 and BXVI24-13. In contrast, all evaluated strains from the genera *Methylobacterium* (BXOL170, BXOL175, BXOL70, BXOL125, among others) and *Pseudomonas* showed no detectable activity (level 0), indicating genus-level trends in enzyme production within the tested collection. In addition, variability was observed in the intensity of the response of all the positive strains, reflected by differences in halo diameter and clarity, suggesting strain-specific enzymatic diffusion or activity levels.

### *3.2.2.9. Chitinase Activity*

Chitinase activity was determined in minimal medium supplemented with colloidal chitin and strains were classified based on their activity using a semiquantitative scale (0 to 4). Among the evaluated strains, those that tested positive which corresponded to 15% of the total, showed an average activity level of 1.61 out of 4, indicating a generally low to moderate level of enzyme production.

The strains that showed the highest activity levels belonged to the genera *Stenotrophomonas* (e.g, BXOLR24-7) and *Saccharopolyspora* (XOL8) (score 3), followed by strains from the genera *Curtobacterium* (e.g., BXAL24-18, score 2 and BXOL209, score 1.5), *Bacillus* (e.g., XOL18A, score 1), and *Methylobacterium* (BXOL140, score 1). The strains from other genera showed no detectable activity suggesting an absence or very low expression of the target enzymatic function under the tested conditions.

### *3.2.2.10. Xylanase Activity*

Xylanase activity was evaluated in xylan source medium and strains were classified based on their activity using a semiquantitative scale (0 to 4). A total of 55% of the tested strains exhibited positive xylanase activity. Among these, the average score was 1.46, indicating low to moderate enzymatic production. A wide range of activity levels was observed across the strain collection, covering the entire scale from no activity (0) to maximum activity (4). Strains exhibiting high activity included representatives from *Methylobacterium* sp. (BXOL24-4) and *Agrococcus* (XOL1). Strains like *Novosphingobium* sp. BXOL39 or BXOL215 showed moderate activity.

### *3.2.2.11. Hydrocyanic Acid or Hydrogen Cyanide (HCN) Production*

Hydrocyanic acid production was evaluated in King's B medium supplemented with glycine. Colour changes were observed either in the filter paper indicator or directly in



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the bacterial colonies, allowing for a semi-quantitative assessment of HCN production. A total of 18% of the evaluated strains tested positive for the HCN production. In some strains, the filter paper turned a reddish-brown colour, indicating a positive reaction. In other cases, the colony itself developed a red pigmentation, which was considered indicative of very low activity and scored as 0.5. These observations reflect variability in the intensity and localization of HCN production among the tested strains.

#### *3.2.2.12. 2-3 Butanediol production*

Production of this compound was evaluated by incubating bacteria in MRVP medium (Methyl Red-Voges Proskauer, Britanialab, Argentina) and subsequently performing Methyl Red (MR) test and Voges-Proskauer test (VP). Strains were classified based on their activity using a semiquantitative scale (0 to 4). In the VP test, the most prominent positive reactions were observed among *Bacillus* spp. (XOL35, BXOL228), with some strains exhibiting strong reddish coloration indicative of robust 2,3-butanediol production. This suggests that certain *Bacillus* strains preferentially utilize the butanediol fermentation pathway, resulting in neutral metabolic byproducts. Conversely, the MR test identified strains producing stable acidic end-products, particularly among genera such as *Cellulomonas* (BXOL220), *Bacillus* (XOL18A) and *Paenibacillus* (XOL15). These strains triggered a distinct red coloration upon addition of the methyl red indicator, confirming the maintenance of low pH conditions due to acid accumulation. Overall, the results indicate clear metabolic divergence among the evaluated taxa. Some strains favours acidogenic pathways (MR-positive), while others demonstrate a preference for neutral end-product formation (VP-positive).

#### *3.2.2.13. Siderophore production*

Siderophore production was evaluated in the solid medium of the universal CAS (Chrome Azurol S) assay (Schwyn and Neilands, 1987). Strains were classified based on their activity using a semiquantitative scale (0 to 4). A total of 12% of the evaluated strains resulted positive for siderophore production. Among these, the average intensity score was 1,57 out of 4, indicating low to moderate siderophore production. The strains that exhibited the highest levels of activity belonged to the genus *Pseudomonas* spp. (BXVI24-26, BXVI24-27 and BXVI24-28), followed by *Methylobacterium* sp. XOL47, *Rhizobium pusense* XOL28 and *Stenotrophomonas* spp. BXOLR24-7 and BXOLR24-8.

### 3.2.3. Multivariate analysis for direct and indirect mechanisms associated with biocontrol of xylem-inhabiting bacterial strains

Results of Ward's Cluster analysis of 105 xylem-inhabiting olive strains, 40 grapevine strains and 31 almond strains are shown in Figures 3, 4 and 5, respectively. The Ward's Cluster analysis showed in Figure 3 presents the phenotypic activity profiles of the olive-isolated bacterial strains based on the results of 18 biocontrol-associated assays. A green colour scale was used to visually represent the intensity of activity for each trait, where darker shades indicate higher activity levels (maximum score of 4), and lighter shades correspond to lower intensities, with white denoting no detectable activity (score 0). Activity scores were assigned after the appropriate incubation period and subsequent



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revealing agent addition, when applicable. No clear clustering patterns were observed either among the tested traits or the bacterial strains, suggesting a high degree of functional variability. Strain *Bacillus halotolerans* BXOL262 exhibited the highest aggregated activity score across all evaluated mechanisms. In contrast, some strains such as *Gottfriedia solisilvae* XOL39 showed a broad activity spectrum but with mostly low-intensity responses, while others like strain *Novosphingobium fluoreni* BXOL39 was positive in fewer assays but displayed higher intensity levels. Overall, the results reflect a heterogeneous distribution of biocontrol-related traits among the strains.

The Ward's Cluster analysis showed in Figure 4 presents the phenotypic activity profiles of the almond-isolated bacterial strains, based on the results of 18 biocontrol-associated assays. Strain *Curtobacterium flaccumfaciens* BXAL24-18 exhibited the highest aggregated activity score across all evaluated mechanisms and the highest number of positive assays. In contrast, some strains such as *Curtobacterium ammoniigenes* BXAL24-20 showed a moderately wide positive activity but displayed higher intensity levels, while others like strain *Sphingomonas ginsenosidivorax* BXAL24-2 was positive in fewer assays, with mostly low-intensity responses.

A third Ward's Clustering analysis (Figure 5) was performed with the strains isolated from grapevine, following the same scoring criteria and colour scale described above. Strain BXVI24-20 exhibited the highest aggregated activity score across all evaluated mechanisms and the highest number of positive assays. *Arthrobacter* sp. BXAL24-19.2 showed a moderate-broad positive overall activity but displayed higher intensity levels in 3 of the 9 positive assays. Strain *Moraxella osloensis* BXVI24-6.1 also showed a moderate-broad positive overall activity, producing high lipase activity and generally low activities in the rest of the positive assays. As observed in the olive-derived isolates, no clear grouping patterns were detected among strains or activities, reinforcing the high phenotypic variability across the evaluated traits. Again, certain strains showed broad yet low-intensity activity profiles, while others were highly active but in fewer tests.



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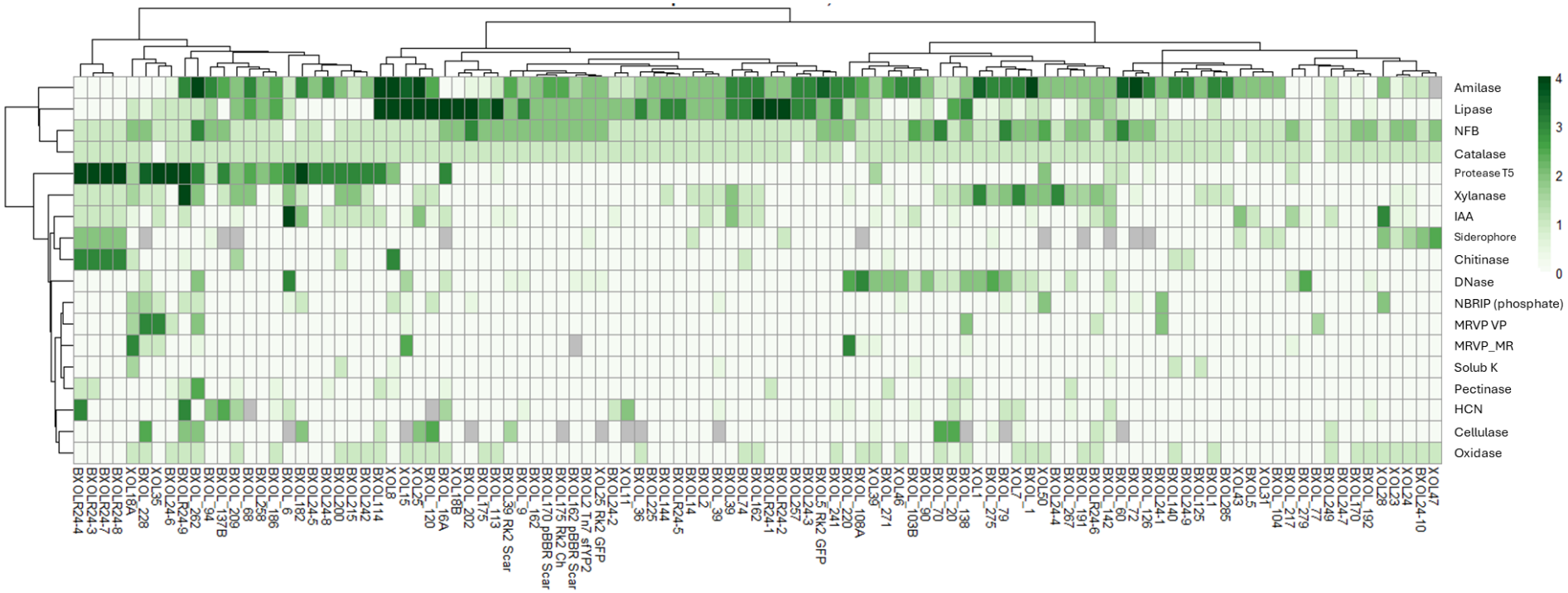



**Figure 3:** Ward's Cluster analysis of 19 biocontrol-associated mechanisms evaluated in this work for the olive xylem-inhabiting strains. The colour gradient indicates activity levels from 0 (no detectable activity, white) to 4 (maximum observed activity, dark green), based on the intensity of each functional activity



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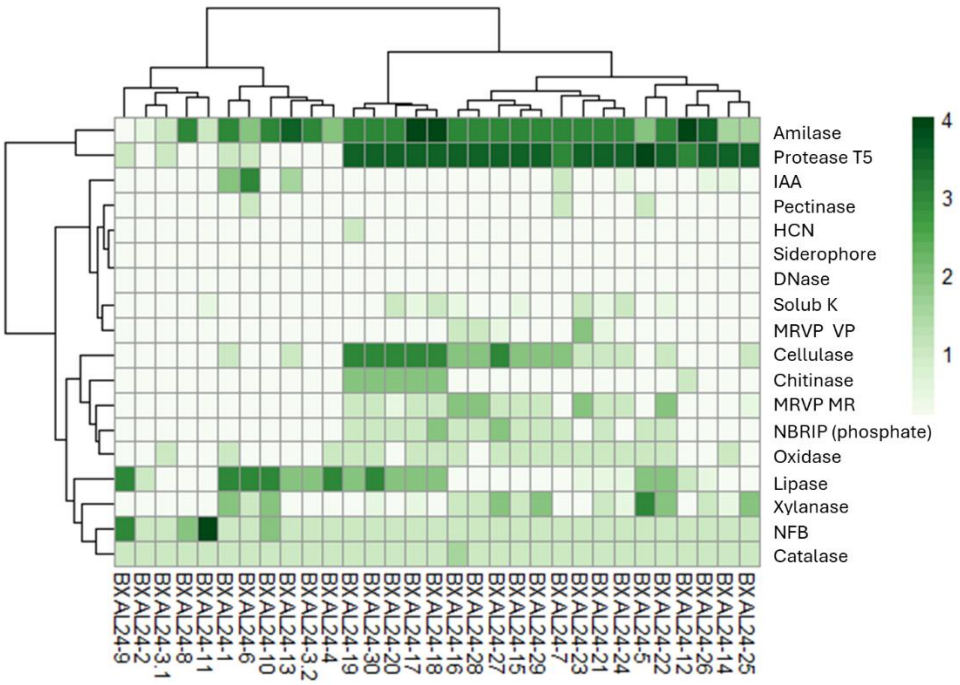



**Figure 4:** Ward's Cluster analysis of 19 biocontrol-associated mechanisms evaluated in this work for the almond xylem-inhabiting strains. The color gradient indicates activity levels from 0 (no detectable activity, white) to 4 (maximum observed activity, dark green), based on the intensity of each functional activity

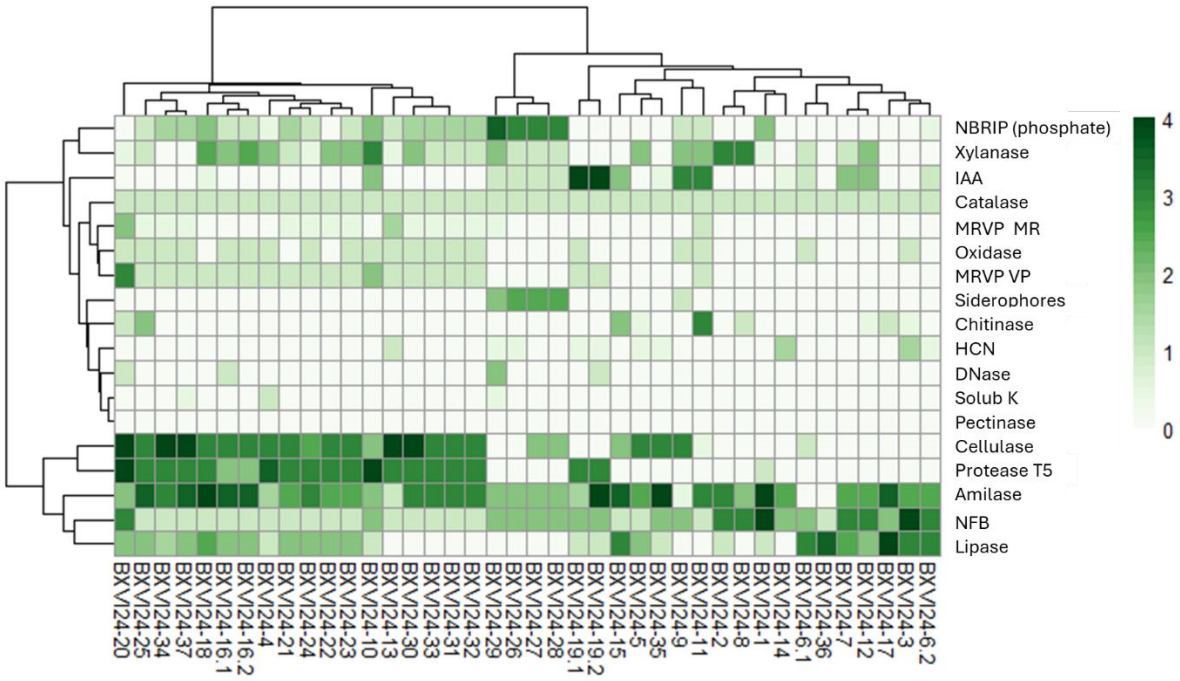



**Figure 5:** Ward's Cluster analysis of 19 biocontrol-associated mechanisms evaluated in this work for the grapevine xylem-inhabiting strains. The color gradient indicates activity levels from 0 (no detectable activity, white) to 4 (maximum observed activity, dark green), based on the intensity of each functional activity



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## 3.3. Phenotypical characterization: Evaluation of carbon sources assimilation and biochemical sensitivity

The Biolog Gen III Microplate test (Hayward, CA, USA) analyses a microorganism through 94 biochemical tests: 71 carbon source utilization assays and 23 chemical sensitivity assays, for instance, sodium chloride at different concentrations, acidic pH, and antibiotics. These experiments were carried out on a selection of 23 bacterial isolates. Buffers A, B, C1 or C2 were selected for each strain based on manufacturer specifications. In some strains, a false positive appeared in A-1 well, so experiments were repeated using a different buffer to find a more suitable one. *Microbacterium* spp. BXOL225 and BXOL285 showed utilization of a wide range of carbon sources and resistance to most sensitivity tests. *Bacillus safensis* XOL35, *Methylobacterium* sp. 17Sr1-43 XOL43 and BXOL140 and *Novosphingobium* sp. BXOL39 showed growth in a small range of carbon sources and no growth in some sensitivity tests. *Pseudomonas stutzeri* XOL25 showed growth in a small range of carbon sources and resistance to all sensitivity tests. *Methylobacterium* sp. C1 BXOL125 and *Bacillus firmus* BXOL249 showed minimal proliferation in less than 20 out of 72 carbon sources, and sensitivity to most biochemical tests.

## 3.4. In vitro compatibility assays

To assess potential antagonistic interactions among bacterial isolates candidates to guide in the design of SynComs, compatibility assays were conducted. Each strain was tested both as a lawn and as a spot-inoculated drop on solid media. Controls of the bacterial spot-inoculated without bacterial lawn were included, as well as background controls with bacterial lawn and no drop tests (Table 2).

Globally, 59.7% of the total interactions exhibited mutual tolerance or growth promotion of one of the strains, without compromising the other one's growth. Strain BXOL215 (*Bacillus* sp.) consistently inhibited the growth of nearly 70% of other strains when used as a lawn. Despite this, BXOL215 did not show significant sensitivity when spotted onto the lawns of most of the other strains, suggesting a resistance to reciprocal antagonism. BXOL170 (*Methylobacterium* sp.) exhibited poor growth when inoculated as a spot onto several lawns, indicating its sensitivity to competition or inhibitory effects from other strains. Notably, the lawn from *Methylobacterium* spp. strains (XOL43, BXOL170, BXOL175) demonstrated the highest overall compatibility, allowing the growth of all other isolates but XOL35 (*Bacillus safensis*) and BXOL170. This high level of tolerance made them strong candidates for inclusion in synthetic communities. XOL28 (*Pseudomonas stutzeri*) grew successfully when inoculated as a spot onto all evaluated lawns (Table 2).



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**Figure 6:** Ward's Cluster analysis using the assimilated carbon sources and the sensitivity compounds from the Biolog Gen III test. Purple color indicates positive growth.



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**Table 2:** Compatibility matrix between 12 strains initially selected as candidates for synthetic community assembly

| | Strain (tested) ⇒ | BXOL 2 | BXOL 39 | BXOL 162 | BXOL 200 | BXOL 215 | BXOL 249 | XOL 25 | XOL 28 | XOL 35 | XOL 43 | BXOL 170 | BXOL 175 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Strain (lawn) ⇓** | **Species** | ***Sphingomonas aerolata*** | ***Novosphingobium fluoreni*** | ***Variovorax* sp.** | ***Curtobacterium flaccumfaciens*** | ***Bacillus* sp.** | ***Bacillus firmus*** | ***Pseudomonas stutzeri*** | ***Rhizobium pusense*** | ***Bacillus safensis*** | ***Methylobacterium radiotolerans*** | ***Methylobacterium* sp.** | ***Methylobacterium* sp.** |
| **BXOL 2** | ***Sphingomonas aerolata*** | + | - | + | + | + | + | + | + | + | + | - | + |
| **BXOL 39** | ***Novosphingobium fluoreni*** | + | + | ++ | ± | + | ± | ± | ++ | ± | ++ | - | + |
| **BXOL 162** | ***Variovorax* sp.** | + | + | + | + | + | + | + | + | - | + | - | - |
| **BXOL 200** | ***Curtobacterium flaccumfaciens*** | ++ | + | + | + | + | - | + | ++ | ± | ± | -- | + |
| **BXOL 215** | ***Bacillus* sp.** | -- | -- | -- | -- | + | -- | + | -- | - | -- | -- | - |
| **BXOL 249** | ***Bacillus firmus*** | + | - | ± | ± | + | + | ++ | + | - | ± | - | + |
| **XOL 25** | ***Pseudomonas stutzeri*** | ± | + | + | + | + | - | + | + | + | + | - | - |
| **XOL 28** | ***Rhizobium pusense*/ *Agrobacterium pusense*** | + | ± | ± | - | -- | ± | + | + | - | - | - | - |
| **XOL 35** | ***Bacillus safensis*** | - | ± | + | ± | - | ± | + | ± | + | - | -- | - |
| **XOL 43** | ***Methylobacterium radiotolerans*** | + | + | + | + | - | + | + | + | -- | + | -- | + |
| **BXOL 170** | ***Methylobacterium* sp.** | + | + | + | + | + | + | + | + | ± | + | + | + |
| **BXOL 175** | ***Methylobacterium* sp.** | ++ | ++ | + | + | + | + | + | + | -- | + | - | + |

Each strain was tested both as a lawn (rows) and a drop test (columns). Interactions were evaluated visually based on the presence of inhibition zones. Results represent the average outcome of three biological replicates and at least two experiments following the same procedure. Colour codes: + green: compatible interactions; ++ dark green: compatible interactions with equal or higher growth than the control (background strain alone), - orange: incompatible interactions with less growth that the control, -- red: incompatible interactions with high growth inhibition of the tested strain, ± yellow: ambiguous result between comparing experiments.



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## 3.5. Selection and characterization of the SynComs

Based on the results obtained from in vitro inhibition assays, evaluation of biocontrol-associated mechanisms, assessment of carbon source assimilation and biochemical sensitivity, strain compatibility tests, as well as culturability and bioprocessability (section 3.6) a selection of bacterial strains was made for the construction of two synthetic communities (SynCom).

The selection process aimed to ensure that the chosen strains exhibited complementary biocontrol-associated traits, thereby increasing the likelihood of synergistic interactions and broader functionality when applied together. Strains capable of producing different types of lytic enzymes, siderophores, or volatile compounds were combined to enhance the spectrum of antagonistic activity against phytopathogens. Additionally, priority was given to strains displaying plant growth-promoting (PGPR) characteristics, such as nitrogen fixation, indole acetic acid (IAA) production, phosphate solubilization, to support plant health and resilience, and their culturability, bioprocessability and survival after storage at 4ºC or dry formulation. The selected strains, based on their phenotypic characterization and antagonistic activity against *Xf*, as well as the total added values of intensity results and the number of positive assays per strain, are presented in Table 3. Importantly, at least one of the selected strains demonstrated significant in vitro inhibition of *Xf*.

**Table 3:** Results of phenotypic characterization of selected bacterial strains for SynCom development and their in vitro inhibition against *Xylella fastidiosa*

| Assay → Strain↓ | Phosphate Solubilization | Siderophores | IAA | Nitrogen fixation | Potassium Solubilization | DNAse | Amylase | Cellulase | Pectinase | Protease | Chitinase | Lipase | Xylanase | Hydrogen cyanide (HCN) | Catalase | Oxidase | MRVP - MR | MRVP - VP | Total | Assays > 0 | *Xf* Inhibition |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **BXOL 39** | 0 | 0 | 2 | 1 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 3 | 2 | 0 | 1 | 0 | 0 | 0 | 11 | 6 | - |
| **BXOL 162** | 0 | 0 | 1 | 1 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 4 | 1 | 0 | 1 | 1 | 0 | 0 | 10 | 7 | + |
| **XOL 28** | 2 | 2 | 3 | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 10 | 7 | + |
| **XOL 35** | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 3 | 12 | 8 | ++ |
| **XOL 43** | 0 | 1 | 2 | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 4 | - |

Colour codes: 0 (white) = no activity, 1 (light grey) = low activity, 2 (medium grey) = moderate activity, 3 (dark grey) = high activity, 4 (darkest grey) = maximum activity. For the "Total" and "Assays>0" columns, a colour gradient was applied, ranging from red (lowest values), through yellow (intermediate), to green (highest values), to facilitate visual comparison of overall performance. Codes in *Xf* inhibition assay: -, no inhibition; +, slight inhibition; ++, an irregular inhibition halo and/or a clear decrease in *Xf* density.



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Compatibility among strains was also a key selection criterion, ensuring stable coexistence within the strains within the SynCom and minimizing antagonism among their members. Results of the compatibility assay performed between SynCom candidates and their combinations are presented in Table 4.

**Table 4.** Compatibility matrix between SynCom strains

| Test strain⇒ Background strain ⇓ | T1 XOL35 | T2 BXOL39 | T3 BXOL162 | T1+T2 | T2+T3 | T1+T3 | T1+T2+T3 |
|---|---|---|---|---|---|---|---|
| **XOL35** | + | + , ↓ ø2 | + , ↓[3] | + | + | + , ↓[3] | + |
| **BXOL39** | + , ↓ ø1 | + | + | + | + | + | + |
| **BXOL162** | + | + | + | + | + | + | + by intersection |

| Test strain⇒ Background strain ⇓ | T4 XOL28 | T5 XOL43 | T6 BXOL39 | T4+T5 | T5+T6 | T4+T6 | T4+T5+T6 |
|---|---|---|---|---|---|---|---|
| **XOL28** | + | - | + | + | + | + | + by intersection |
| **XOL43** | ++ | ++ | ++ | + | + | + | + |
| **BXOL39** | + | ++ | + | + | ++ | + | + by intersection |

Results represent the average outcome of three biological replicates. Colour codes: + green: compatible interactions; ++ dark green: compatible interactions with equal or higher growth than the control (background strain alone), - orange: incompatible interactions. Growth reductions are indicated with ↓ and accompanied by observations (e.g., ↓ ø: reduction on colony diameter, reduction on). "+ by intersection" indicates compatibility inferred from shared behaviour across multiple combinations.

Overall, all SynCom strains were found to be compatible, except for T5 (*Methylobacterium* sp. XOL43) when tested on background strain B4 (*Rhizobium pusens*e XOL28), where inhibition was observed. However, no compatibility issues were detected for T5 on other backgrounds, nor on plates containing SynCom mixtures, suggesting that the interaction may not affect the overall community performance.

SynCom1 and SynCom2 were designed considering the mentioned criteria, resulting in the following strain combinations:

| SynCom1 | Isolate code | Species |
|---|---|---|
| | **XOL35** | *Bacillus safensis* |
| | **BXOL39** | *Novosphingobium fluoreni* |
| | **BXOL162** | *Variovorax* sp. |
| **SynCom2** | **Isolate code** | **Species** |
| | **XOL28** | *Rhizobium pusense/Agrobacterium pusense* |
| | **XOL43** | *Methylobacterium radiotolerans* |
| | **BXOL39** | *Novosphingobium fluoreni* |



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## 3.6. Selection of strains for SynComs based on culturability and bioprocessability

### 3.6.1. Development and optimisation of culture media and growth conditions for selected strains from xylem sap

The growth of strains was tested on a selection of different media identified from a literature review to identify medium that would provide good growth of the strains (Table 5). The optimal cultivation medium for 10 of the 12 strains selected in Table 2 was identified as R2A solid media at 28°C (Figure 7).

**Table 5:** Results of test growth on different media

| Strain ID | Species | XF26 | SXM | XVM2 | PW | PD3 | R2A | M9 | 2TY | LBS + glucose |
|---|---|---|---|---|---|---|---|---|---|---|
| **BXOL2** | *Sphingomonas aerolata* | ++ | + | ++ | +++ | ++ | ++ | ++ | ++ | ++ |
| **BXOL39** | *Novosphingobium fluoreni* | ++ | + | ++ | +++ | +++ | ++ | + | +++ | ++ |
| **BXOL162** | *Variovorax sp.* | + | + | + | + | +++ | ++ | + | + | ++ |
| **BXOL175** | *Methylobacterium sp.* | + | ++ | + | ++ | ++ | ++ | + | + | + |
| **BXOL170** | *Methylobacterium sp.* | + | + | + | ++ | ++ | ++ | + | + | + |
| **BXOL215** | *Bacillus sp.* | + | + | + | + | ++ | ++ | + | +++ | + |
| **BXOL249** | *Bacillus firmus* | + | + | + | + | + | ++ | + | + | + |
| **XOL28** | *Rhizobium pusense* | ++ | ++ | ++ | +++ | ++ | ++ | ++ | +++ | ++ |
| **XOL35** | *Bacillus safensis* | ++ | ++ | ++ | ++ | ++ | ++ | + | +++ | ++ |
| **XOL43** | *Methylobacterium radiotolerans* | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |

Based on the results of this screening, R2A medium supported good growth for all strains and it was selected for further optimisation with the goal to achieve better biomass yield. Initially, the strains were cultivated in liquid R2A medium using previously identified growth conditions. Growth was evaluated spectrophotometrically as well as visually, since most of the strains showed tendencies to form aggregates and cell clusters in liquid R2A medium.

Systematic identification of important nutrients supporting better growth was performed sequentially for all strains. Carbon and nitrogen sources were modified initially. Addition of C source (glucose) resulted in higher biomass only for *Sphingomonas aerolata* BXOL2 and *Novosphingobium fluoreni* BXOL39. However, addition of carbon and nitrogen source improved growth of all strains except *Bacillus* sp. BXOL215. For *Methylobacterium* spp. strains, methanol was evaluated as a potential carbon source; however, it did not result in increased biomass production.



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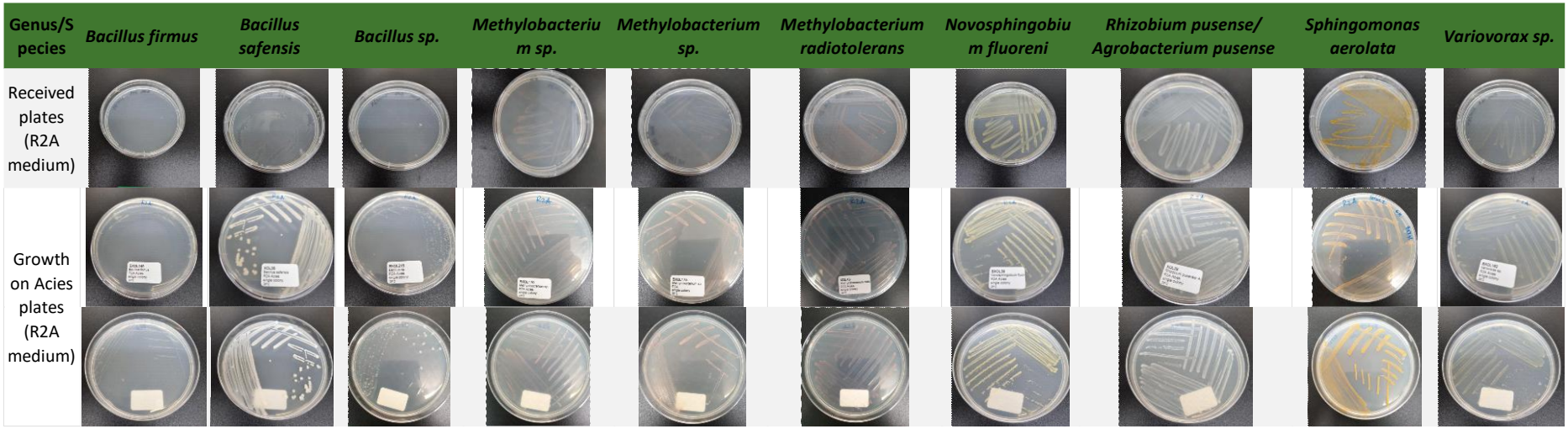



**Figure 7:** Strains received at ACIES-BIO facilities from P1-CSIC growing on R2A solid medium.

Microorganisms were grouped into three groups based on their growth profiles:

- **Fast-growing strains**, with no cluster formation (*Bacillus safensis* XOL35)
- **Slow-growing strains forming cell clusters** (*Novosphingobium fluoreni* BXOL39 and *Sphingomnas aerolata* BXOL2- very viscous, *Rhizobium pusense* XOL28, *Methylobacterium radiotolerans* XOL43, *Methylobacterium* sp. BXOL175, *Variovorax* sp. BXOL162, *Bacillus firmus* BXOL249, *Bacillus* sp. BXOL215- forms small clusters)
- **Slow-growing strains with no clusters** observed in liquid media (*Methylobacterium* sp. BXOL175)



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For some strains, very high pH levels (8–9) were observed during cultivation, which have negative effects on growth. Further medium development addressed pH stabilisation by several buffering systems. Phosphate buffer was identified as the optimal buffering system.

Next round of medium development included fine-tuning of mineral and vitamin contents. Vitamins improved growth of *Sphingomonas aerolata* BXOL2*, Bacillus* sp. BXOL215*, Bacillus safensis* XOL35*, Methylobacterium* sp. BXOL170*, Methylobacterium* sp. BXOL175*, Methylobacterium radiotolerans* XOL43 and *Novosphingobium fluoreni* BXOL39*.* Addition of minerals had effect on faster growth of *Bacillus safensis* XOL35 and *Bacillus firmus* BXOL249 and slightly higher final $OD_{600}$ of *Methylobacterium radiotolerans* XOL43 and *Novosphingobiuzm fluoreni* BXOL39 but were not beneficial for other strains (even harmful to *Sphingomonas aerolata* BXOL2 and *Rhizobium pusense* XOL28). Additionally, higher concentration of vitamins was tested and have proven to be beneficial for growth of *Variovorax* sp. BXOL162, *Rhizobium pusense* XOL28*, Methylobacterium radiotolerans* XOL43 and *Bacillus safensis* XOL35*.*

Based on the results of growth in various amended media, the composition was adjusted for limiting nutrients. The optimal medium composition for the majority of the strains was identified by adding the missing nutrients yeast extract, glucose, a vitamin mix, and the medium BEXm_7 was developed. For some of the strains, such as *Variovorax* sp. BXOL162*, Rhizobium pusense* XOL28, *Methylobacterium radiotolerans* XOL32 and *Bacillus safensis* XOL35, a higher concentration of vitamins was used.

### 3.6.2. Selection of strains for SynComs based on bioprocessability

Biomass yield in modified media, storage stability, and cell cluster formation during fermentation were assessed in the 10 selected strains. Additionally, spore formation was determined in *Bacillus* strains. Based on results, 5 strains were the most promising for the development of SynComs based on biomass yield and stability (Table 6). These are *Bacillus safensis* XOL35*, Methylobacterium radiotolerans* XOL43*, Novosphingobium fluoreni* BXOL39*, Rhizobium pusense* XOL28 and *Variovorax* sp. BXOL162.

All strains achieved at least $10^8$ CFU/mL after 48 hours of cultivation, with nearly all exhibiting a survival rate above 84% after 10 days at 4°C. The exception was *Bacillus safensis* XOL35 which, despite a high initial CFU/mL, showed significantly reduced viability after storage. Efforts to enhance sporulation were considered but were not successful.

Medium development for these strains resulted in good biomass. Each of these strains was re-tested in shake flasks in three newly developed media, and their growth was evaluated (Figures 8 – 12) to select the optimal culture medium for production of biomass for the trials on plants.



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**Table 6:** Viability and storage stability of the 10 selected strains

| Strain | Colony forming unit (CFU)/mL | | Survival rate (%) |
|---|---|---|---|
| | 48h cultivation | 10 days at 4 °C | |
| ***Bacillus firmus* BXOL249** | 2.2E+04 | 0.0E+00 | 0% |
| ***Bacillus safensis* XOL35** | 1.3E+09 | 1.9E+05 | 0% |
| ***Bacillus* sp. BXOL215** | 5.8E+04 | 2.8E+04 | 48% |
| ***Methylobacterium radiotolerans* XOL43** | 8.7E+08 | 7.3E+08 | 84% |
| ***Methylobacterium* sp. BXOL170** | 1.5E+07 | 1.2E+07 | 82% |
| ***Methylobacterium* sp. BXOL175** | 7.9E+05 | 7.8E+05 | 99% |
| ***Novosphingobium fluoreni* BXOL39** | 5.6E+08 | 4.8E+08 | 86% |
| ***Rhizobium pusense* XOL28** | 1.9E+10 | 1.8E+10 | 92% |
| ***Sphingomonas aerolata* BXOL2** | 1.1E+08 | 2.4E+07 | 22% |
| ***Variovorax sp.* BXOL162** | 1.8E+09 | 1.7E+09 | 94% |

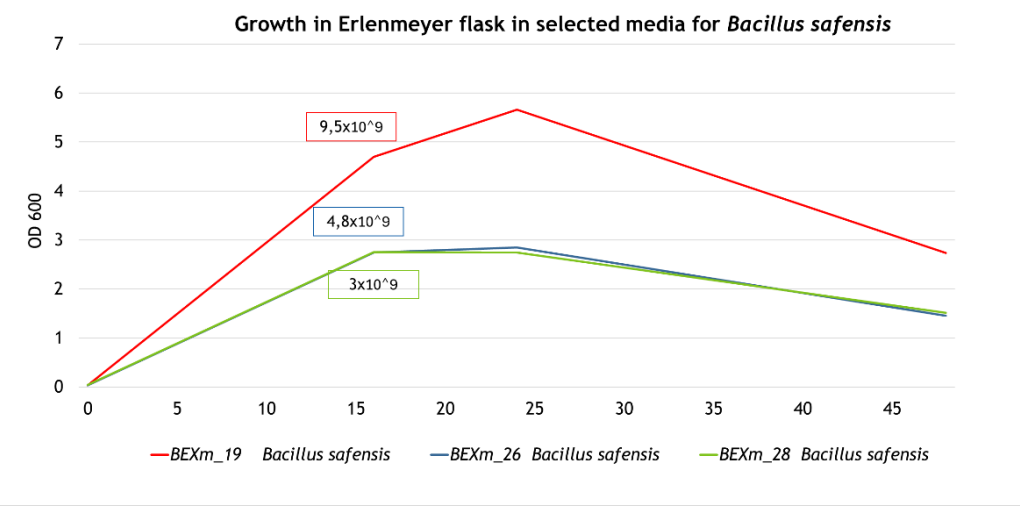



**Figure 8:** Growth of *Bacillus safensis* XOL35 cultivated in 3 best media



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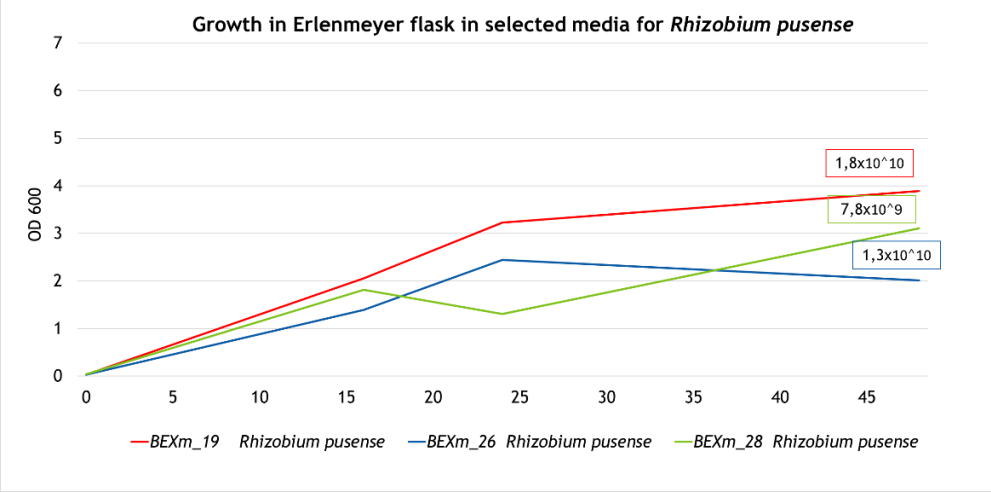



**Figure 9:** Growth of *Rhizobium pusense* XOL28 cultivated in 3 best media

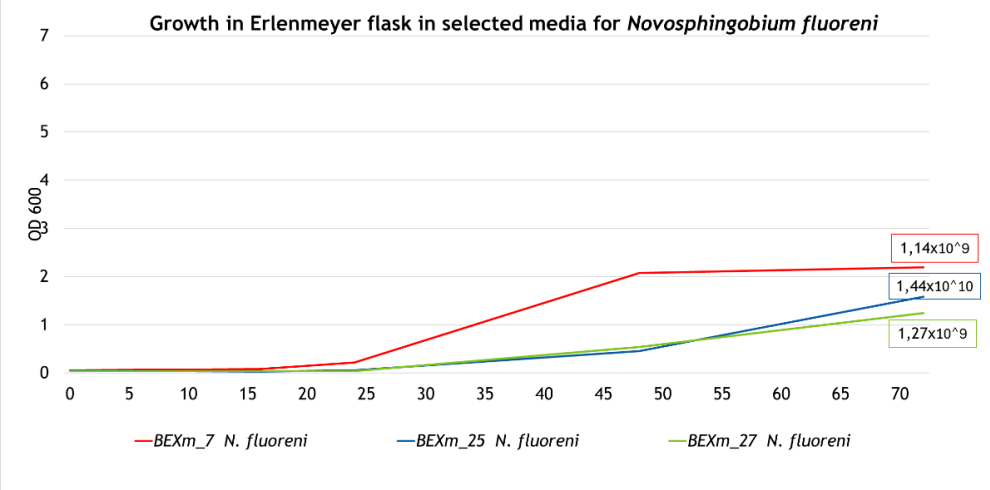



**Figure 10:** Growth of *Novosphingobium fluoreni* BXOL39 cultivated in 3 best media

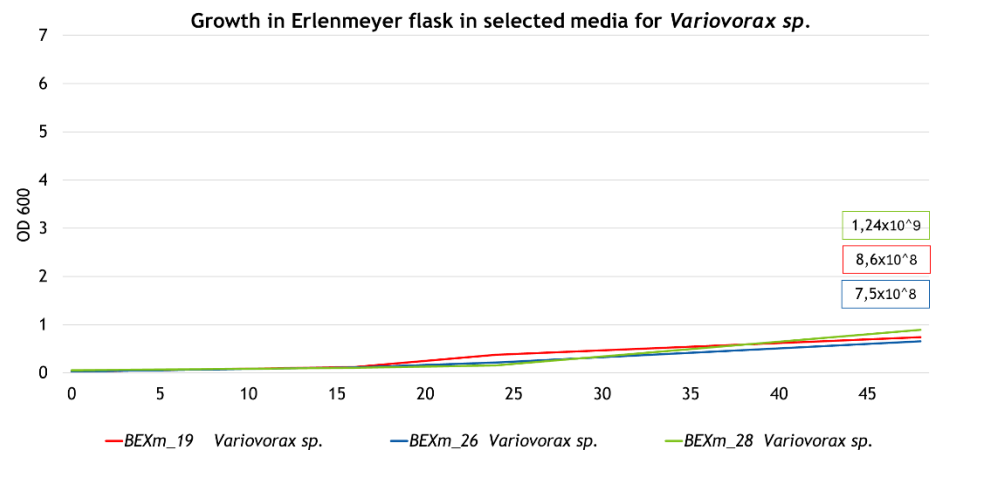



**Figure 11:** Growth of *Variovorax* sp. BXOL162 cultivated in 3 best media



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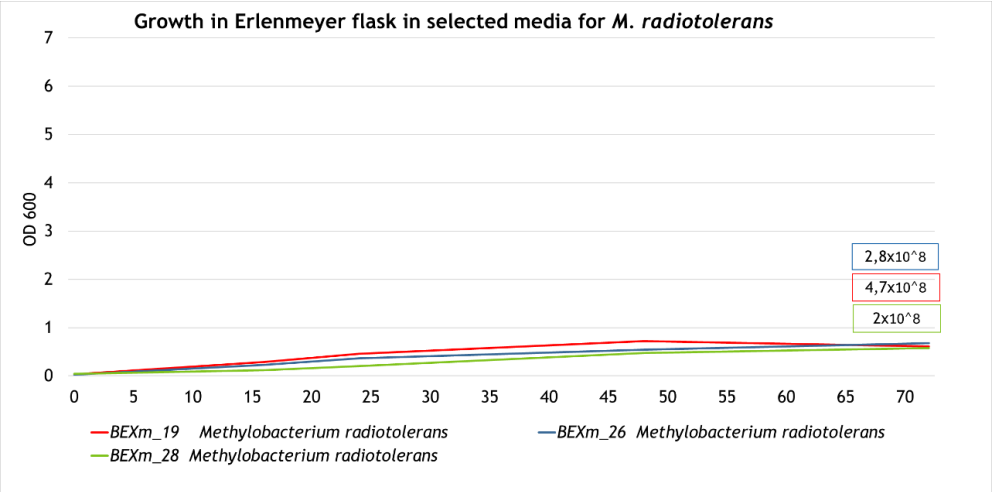



**Figure 12:** Growth of *Methylobacterium radiotolerans* XOL43 cultivated in 3 best media

The developed media ensure sufficient cell density for production of the test materials for the trials.

The survival of bacteria after the end of fermentation and until the application to plants is critical for the efficacy of the developed SynComs. Therefore, storage stability after production was tested at 4°C (Figure 13). *Bacillus safensis* XOL35 had the lowest survival rates. Attempts to enhance its sporulation to increase survival were not successful.

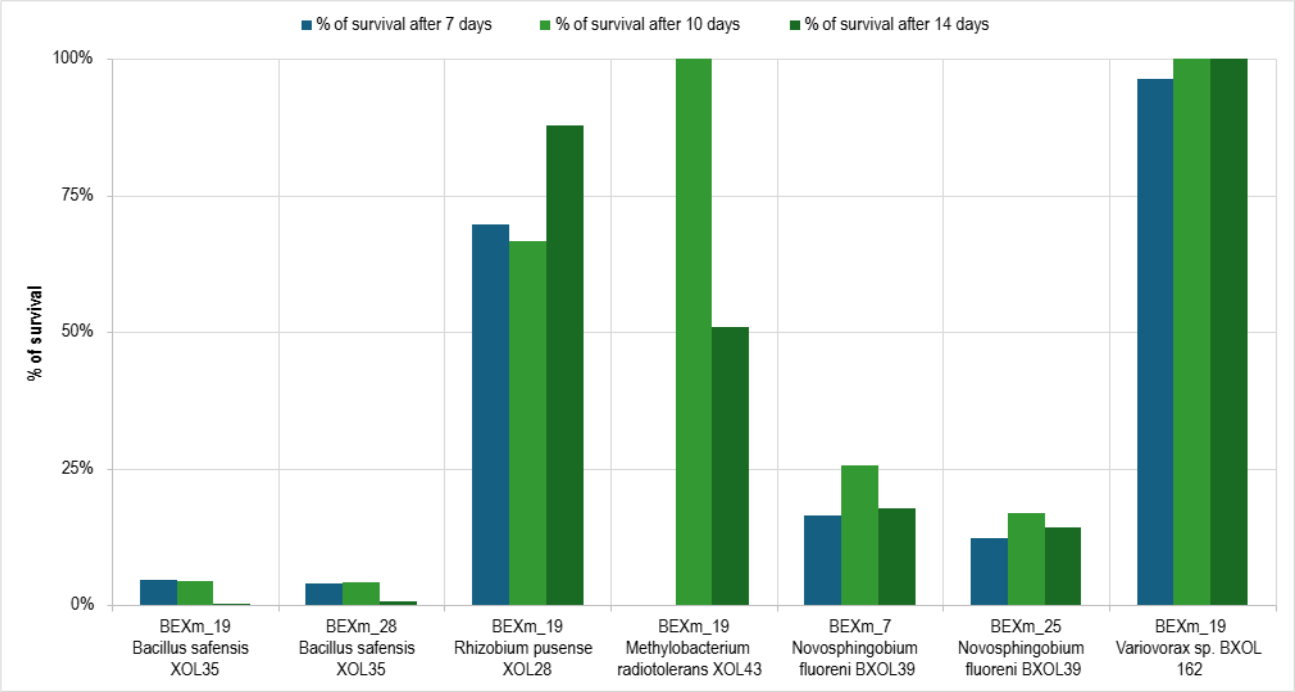



**Figure 13:** Viability of five selected strains after different storage time and growing media at 4 °C.



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*Rhizobium pusense* XOL28 maintained approximately 75% cell viability during storage. For *Methylobacterium radiotolerans* XOL43, a decline in cell viability to 51% was observed after 14 days. *Variovorax* sp. BXOL162 demonstrated the highest storage stability. An efficient microbial formulation should enable high viability and longevity of bacteria for consistent efficacy (Figure 13).

Dry formulation provides a longer shelf life and the flexibility of use; it can be dissolved in water to be used for spraying or irrigation. Therefore, a test of lyophilisation was performed with selected strains (Figure 14) to evaluate the survival following lyophilization, as this method could potentially provide sufficient material for in planta studies for more sensitive strains, such as *Bacillus safensis* XOL35.

Most of the bacterial strains have a cell survival >70%, with the exception of *Novosphingobium fluoreni* BXOL39 (<50%) and *Bacillus safensis* XOL35 (<30%) (Figure 14).

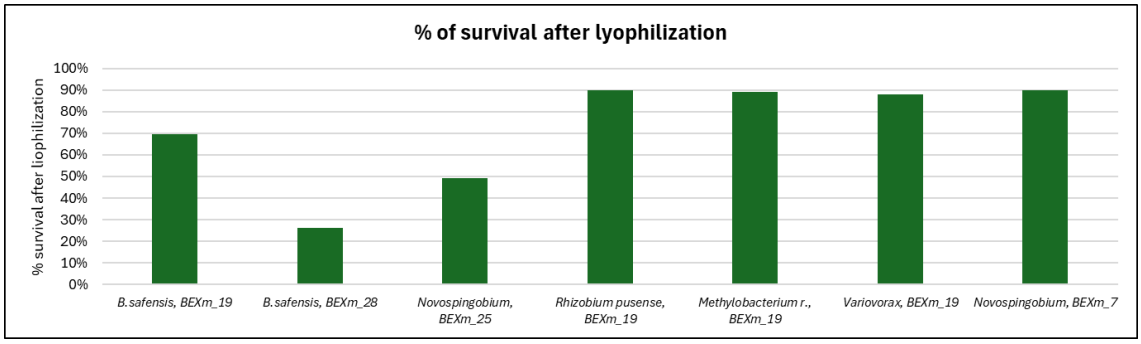



**Figure 14:** Percentage of cell survival after lyophilization

## 3.7. *In planta* effects of assembled SynComs against *Xylella fastidiosa* infection

The protective effect of the assembled SynComs (SC1 and SC2) on tobacco and olive plants infected with *X. fastidiosa* is currently being evaluated, as disease symptoms caused by this fastidious pathogen take time to develop. The impact of SC1 and SC2 on disease severity, leaf physiological parameters (e.g., chlorophyll content), and plant growth-related traits (e.g., stem length) is being assessed in both pathosystems.

### 3.7.1. Protective effect of assembled SynComs in infected tobacco plants

In the infected tobacco plants, two of the three scheduled samplings were completed at 15- and 30-days post-inoculation (dpi) with *X. fastidiosa*. The effects of SC1 and SC2 on bacterial population levels are shown in Figure 15. The preventive application of SC1 led to a significant reduction in *X. fastidiosa* populations at both 15 and 30 dpi in all of the sampled zones, as



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indicated by higher $C_T$ values compared to the control group inoculated only with *X. fastidiosa*. In the case of SC2, a significant reduction in bacterial population was observed at 30 dpi. The observed reduction in *X. fastidiosa* population levels in plants treated with SC1 and SC2 seems to increase over time (Figure 15).

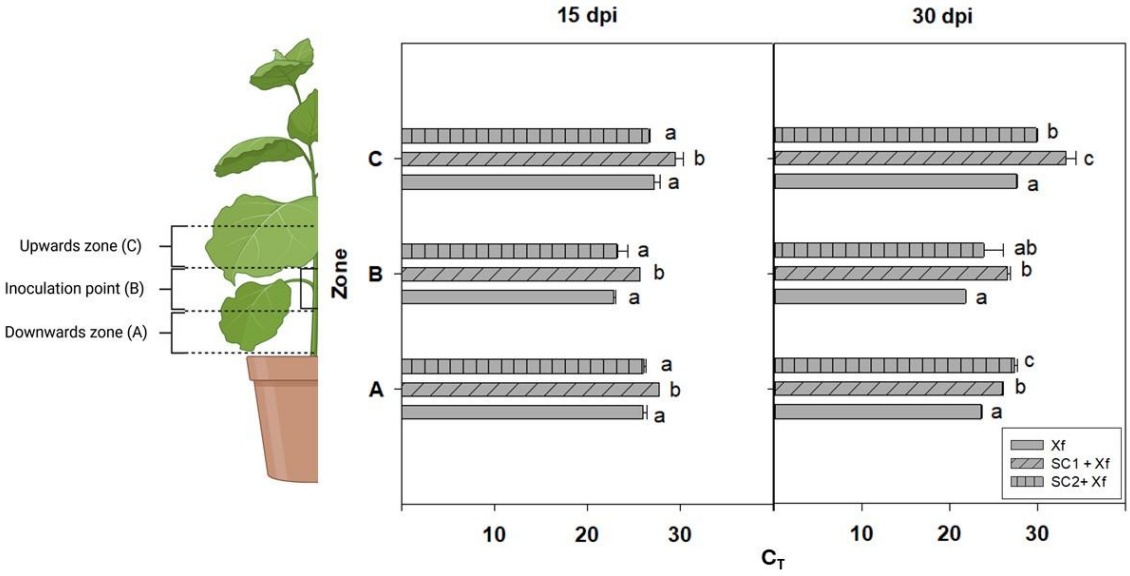



**Figure 15:** Effect of the SC1 and SC2 on *X. fastidiosa* population levels in infected tabaco plants at 15- and 30-days post-inoculation (dpi) in the three sections that were sampled (downwards zone (A), inoculation point (B), and upwards zone (C)). Values are the means of three plants and error bars represent the confidence interval (α=0.05). Different letters between treatments at each sampling time and zone indicate significant differences between treatments for each analyzed section according to Tukey's test ($P < 0.05$).

The effects of SC1 and SC2 treatments on disease severity and physiological parameters were assessed at 15- and 30-days post-inoculation (dpi) and compared to plants treated with the corresponding SynCom or R2A medium but not inoculated with *X. fastidiosa* (SC1, SC2, and NTC) (Figure 16). Disease symptoms begun to appear in plants inoculated with *X. fastidiosa* at 30 dpi. Although the plants inoculated with *X. fastidiosa* alone show the highest disease severity, no significant differences between treatments have been observed so far.

Regarding the physiological parameters related to the growth-promoting activity of the assembled SynComs, no significant differences in stem height were observed between treatments (Figure 16). Nevertheless, significant differences were detected in shoot system fresh weight. Interestingly, the NTC, SC1, and *Xf* plants exhibited lower shoot fresh weight compared to SC2, SC1+*Xf*, and SC2+*Xf* plants, indicating the potential growth-promoting effect of the assembled SynComs (Figure 16). Finally, regarding the chlorophyll content index, plants inoculated with *X. fastidiosa* but left untreated (*Xf*) showed a significant decrease



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compared to the other treatments, suggesting that the application of SC1 and SC2 may help protect plant fitness (Figure 16).

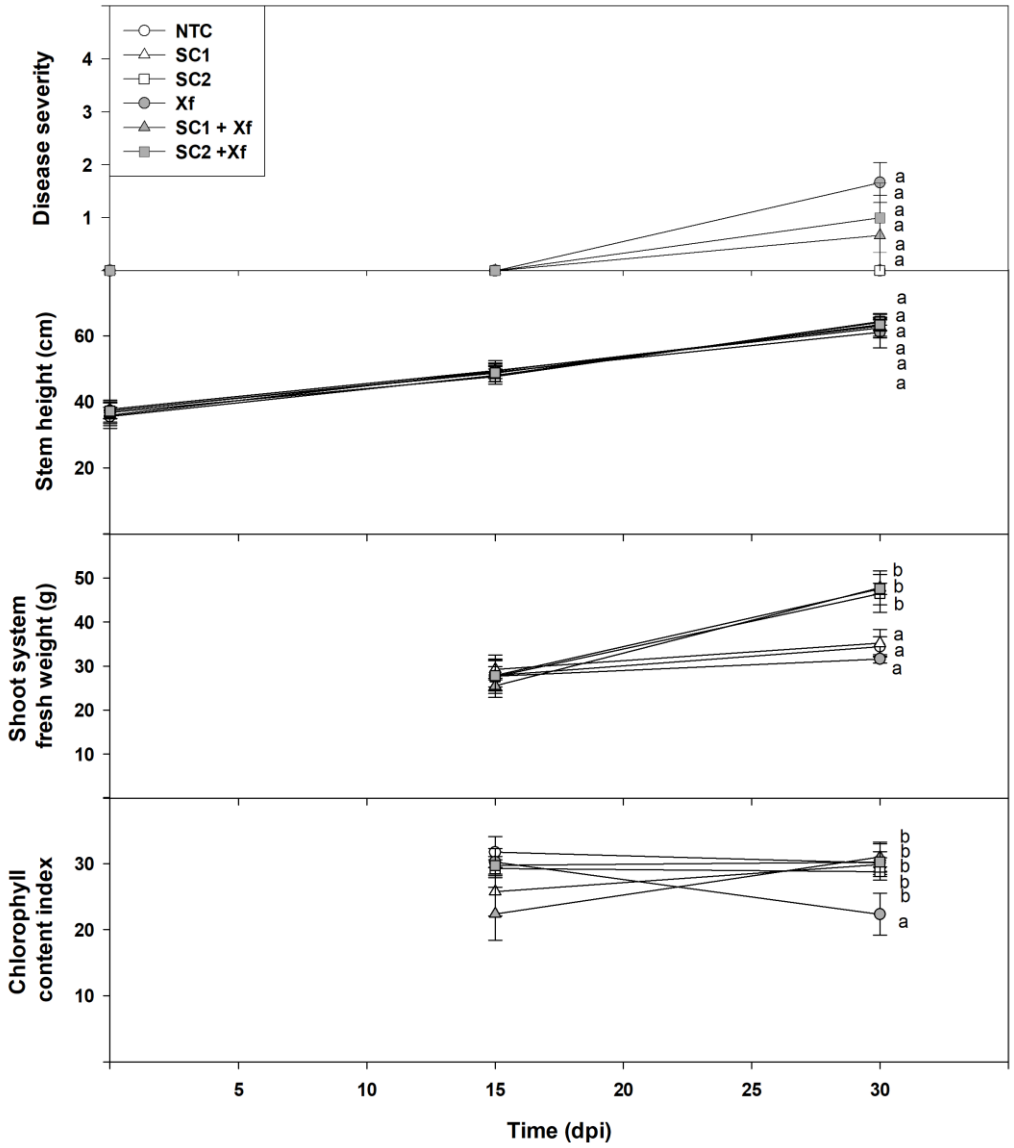



**Figure 16:** Disease severity, physiological parameters related to plant growth promotion (stem height and shoot fresh weight), and leaf physiological parameter (chlorophyll content index) of tobacco plants inoculated with *X. fastidiosa* and treated with SC1 and SC2 compared with a non-treated control (*Xf*). A non-inoculated and not treated control (NTC) was added for reference for all the physiological parameters. For disease severity, stem height and shoot system fresh weight, values are the mean of three plants. For the chlorophyl content index, values are the means of 4 leaves/plant for a total of three plants. Error bars represent the confidence interval (α=0.05). Different letters between treatments indicate significant overall differences between the treatments for each analyzed parameter according to Tukey's test ($P < 0.05$).



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### 3.7.2. Protective effect of assembled SynComs in infected olive plants

In the infected olive plants with *X. fastidiosa*, one of the four scheduled samplings was completed at 30 dpi. The effects of SC1 and SC2 on bacterial population levels are shown in Figure 17. The preventive application of SC1 resulted in a significant reduction of *X. fastidiosa* populations (higher $C_T$ values) in zones A and B at 30 dpi, while SC2 treatment led to a significant reduction only in zone A.

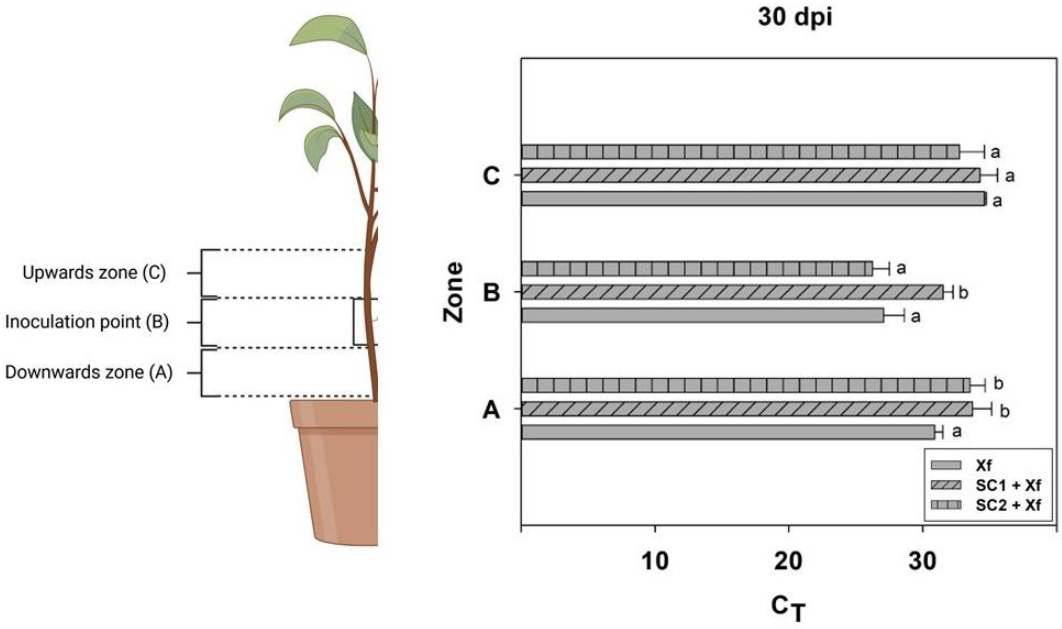



**Figure 17:** Effect of the SC1 and SC2 on *X. fastidiosa* population levels in infected olive plants at 30 days post-inoculation (dpi) in the three sections that were sampled (downwards zone (A), inoculation point (B), and upwards zone (C)). Values are the means of four plants and error bars represent the confidence interval (α=0.05). Different letters between treatments at each sampling time and zone indicate significant differences between treatments for each analyzed section according to Tukey's test ($P < 0.05$).

The effects of SC1 and SC2 treatments on disease severity, physiological parameters related to plant growth promotion and leaf physiology were assessed 30 dpi and compared to plants treated with the corresponding SynCom or R2A medium but not inoculated with *X. fastidiosa* (SC1, SC2, and NTC) (Figure 18). At 30 dpi, no disease symptoms associated with *X. fastidiosa* infection were observed in the olive plants, so they were not represented. Regarding the physiological parameters related to the growth-promoting activity of the assembled SynComs, no significant differences were detected in stem height. However, significant treatment effects were observed for shoot fresh weight, number of secondary branches, and total length of secondary branches. In all cases, the highest growth-promoting activity was observed in plants treated with SC2 but were not infected and in those treated with SC1 and inoculated with *X. fastidiosa* (Figure 10). These results are interesting, as SC1 appears to promote plant growth in the presence of *X. fastidiosa* infection. In contrast, while SC2 also enhances plant growth, the values for all measured parameters are lower when the treated



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plants are infected with *X. fastidiosa* and values obtained are similar to the NTC and *Xf* treatments.

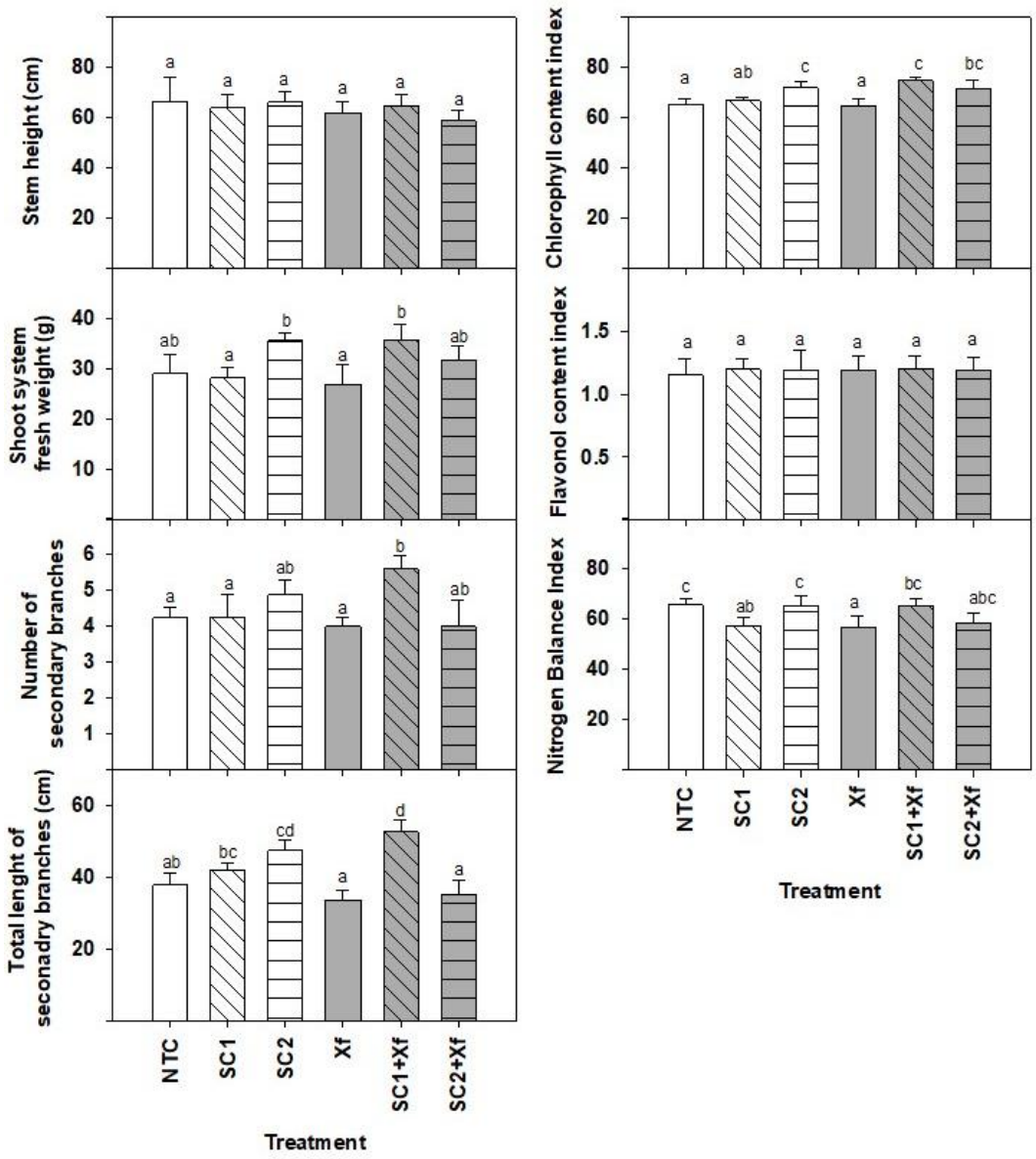



**Figure 18:** Disease severity, physiological parameters related to plant growth promotion (stem height, shoot system fresh weight, number of secondary branches, total length of secondary branches), and leaf physiological parameter (chlorophyll and flavonol content index and nitrogen balance index) of olive plants inoculated with *X. fastidiosa* and treated with SC1 and SC2 compared with a not-treated control (Xf). A not-inoculated and not treated control (NTC) was added for reference for all the physiological parameters. For physiological parameters related to plant growth promotion, values are the mean of four plants. For the leaf physiological parameters, values are the means of 6 leaves/plant for a total of four plants. Error bars represent the confidence interval (α=0.05). Different letters between treatments indicate significant differences between the treatments for each analyzed parameter according to Tukey's test ($P < 0.05$).



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Finally, significant differences in chlorophyll content index were observed depending on the treatment in olive plants. Specifically, olive plants treated with SC2 but not inoculated with *X. fastidiosa* (SC2), as well as those treated with SC1 and inoculated with *X. fastidiosa* (SC1+Xf), showed higher chlorophyll content index values compared to the NTC, suggesting a potential role in supporting plant fitness (Figure 18). Interestingly, flavonol content index values remained consistent across treatments. Since flavonol levels often increase in response to plant stress, this suggests that the analyzed plants were not experiencing significant stress at 30 dpi due to the *X fastidiosa* infection. Finally, the nitrogen balance index did not show statistically significant differences ($P \geq 0.05$) between treatments, likely due to high variability within the dataset.

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