

Chitosan-Coated Fosetyl-Al Nanocrystals' Efficacy on *Nicotiana tabacum* Colonized by *Xylella fastidiosa*

Giuseppe Tatulli,^{1,†} Francesca Baldassarre,^{2,3} Daniele Schiavi,⁴ Stefano Tacconi,⁵ Flavio Cognigni,⁶ Francesca Costantini,^{1,7} Giorgio Mariano Balestra,^{4,8} Luciana Dini,⁹ Nicoletta Pucci,¹ Marco Rossi,^{6,10} Valeria Scala,¹ Giuseppe Ciccarella,^{2,3} and Stefania Loreti¹

¹ Council for Agricultural Research and Economics, Research Centre for Plant Protection and Certification of Rome, 00156 Rome, Italy

² Department of Biological and Environmental Sciences, UdR INSTM of Lecce University of Salento, Via Monteroni, 73100 Lecce, Italy

³ Institute of Nanotechnology, CNR NANOTEC, Consiglio Nazionale delle Ricerche, Via Monteroni, 73100 Lecce, Italy

⁴ Department of Agriculture and Forest Sciences (DAFNE), University of Tuscia, Via S. Camillo de Lellis, Snc, 01100 Viterbo, Italy

⁵ CarMeN Laboratory, INSERM 1060-INRAE 1397, Department of Human Nutrition, Lyon Sud Hospital, University of Lyon, Lyon, France

⁶ Department of Basic and Applied Sciences for Engineering (SBAl), Sapienza University of Rome, Rome, Italy

⁷ Department of Environmental Biology, Sapienza University of Rome, p.le A. Moro 5, 00185, Rome, Italy

⁸ Phytoparasites Diagnostics (PhyDia) s.r.l. Via S. Camillo Delellis Snc 01100 Viterbo, Italy

⁹ Department of Biology and Biotechnology "C. Darwin", Sapienza University of Rome, Rome, Italy

¹⁰ Research Center on Nanotechnology Applied to Engineering of Sapienza (CNIS), Sapienza University of Rome, Rome, Italy

Accepted for publication 29 April 2024.

Abstract

Xylella fastidiosa (Xf) is a quarantine plant pathogen capable of colonizing the xylem of a wide range of hosts. Currently, there is no cure able to eliminate the pathogen from a diseased plant, but several integrated strategies have been implemented for containing the spread of Xf. Nanotechnology represents an innovative strategy based on the possibility of maximizing the potential antibacterial activity by increasing the surface-to-volume ratio of nanoscale formulations. Nanoparticles based on chitosan and/or fosetyl-Al have shown different in vitro antibacterial efficacy against Xf subsp. *fastidiosa* (Xff) and *pauca* (Xfp). This work demonstrated the uptake of chitosan-coated fosetyl-Al nanocrystals (CH-nanoFos) by roots and their localization in the stems and leaves of *Olea europaea* plants. Additionally, the antibacterial activity of fosetyl-Al, nano-fosetyl, nano-chitosan, and CH-nanoFos was tested on *Nicotiana*

tabacum cultivar SR1 (Petite Havana) inoculated with Xff, Xfp, or Xf subsp. *multiplex* (Xfm). The bacterial load was evaluated with qPCR, and the results showed that CH-nanoFos was the only treatment able to reduce the colonization of Xff, Xfm, and Xfp in tobacco plants. Additionally, the area under the disease progress curve, used to assess symptom development in tobacco plants inoculated with Xff, Xfm, and Xfp and treated with CH-nanoFos, showed a reduction in symptom development. Furthermore, the twitching assay and bacterial growth under microfluidic conditions confirmed the antibacterial activity of CH-nanoFos.

Keywords: disease control, nanotechnologies, *Nicotiana tabacum*, *Xylella fastidiosa*

Xylella fastidiosa (Xf) is a gram-negative bacterium of the family *Lysobacteraceae* (earlier described as *Xanthomonadaceae*). Xf colonizes the xylem of 690 host plant species distributed across the Americas, Europe, the Middle East, and Asia (European Food Safety Authority et al. 2023). In the United States, Brazil, and Costa Rica, Xf is the causal agent of several diseases, such as Pierce's disease in *Vitis* spp. (Hopkins 1989), citrus variegated chlorosis in *Citrus* spp. (Chang et al. 1993), and coffee leaf scorch in *Coffea arabica* (Rodriguez et al. 2001). Europe was pest free until the first detection of Xf subsp. *pauca* (Xfp) on *Olea europaea* in the Apulia region of Southern Italy (Saponari et al. 2013). New outbreaks occurred periodically in Europe (Denancé et al. 2017; Olmo et al.

2017), including the latest one involving Xf subsp. *fastidiosa* (Xff) ST1 in the Apulia region, Italy (EPPO Global Database 2024).

This emergency is managed through mandatory eradication in the presence of new outbreaks, but eradication efforts in the infected area of the Apulia region have been abandoned, with more than 6.5 million olive trees infected (Scholten et al. 2019). The European Food Safety Authority (EFSA) reports that there is no cure for Xf infections, with a cure defined as "eliminating the pathogen from plant tissues" (EFSA Panel on Plant Health 2016). Consequently, management in this area focuses on monitoring plants to detect new outbreaks in the "containment" and "buffer" areas proposed by Commission Implementing Regulation (EU) 2020/1201.

Substantial research investments from governments and private companies are founded with the purpose of individuating strategies able to control pest progression (Lindow 2019). The use of antibiotics in the treatment of plant diseases represents one of the potential causes of the spread of antibiotic resistance in the environment. Currently, the highest reports of antibiotic-resistant bacterial strains come from the United States, South America, and, to a lesser extent, Asia. In Europe, data are lacking, and further studies are needed (EFSA Panel on Plant Health et al. 2019; Verhaegen et al. 2024).

Several innovative approaches have emerged as alternatives to conventional agrochemicals for treating plant diseases, including the identification of resistant or tolerant olive germplasm

†Corresponding author: G. Tatulli; giuseppe.tatulli@crea.gov.it

G. Tatulli and F. Baldassarre contributed equally.

Funding: Support was provided by the Ministero delle Politiche Agricole Alimentari e Forestali (OLIVicoltura e Difesa da *Xylella fastidiosa* e da Insetti vettori in Italia; Oli.Di.X.I.It D.M. 23773).

e-Xtra: Supplementary material is available online.

The author(s) declare no conflict of interest.

(Giampetruzzi et al. 2016; Pavan et al. 2021; Vergine et al. 2020), management of the insect vectors (Dáder et al. 2019; Liccardo et al. 2020; Morente et al. 2022; Sanna et al. 2021), biological control strategies (Araújo et al. 2002; Baccari et al. 2019; Zicca et al. 2020), and the use of plant and microbial derived compounds (e.g., olive mill wastewaters, *Trichoderma* spp. crude culture extracts, phenolic compounds) (Bleve et al. 2018; Maddox et al. 2010), as well as physical techniques such as microwave irradiation or cold treatment (Hilton et al. 2021; Lieth et al. 2011).

The chemical approach is highly debated due to its detrimental effects on the environment, human health, and animal health (Annamalai and Namasivayam 2015; Marchand 2023; Savary et al. 2019). However, the possibility of using conventional agrochemicals or ions (e.g., copper, zinc, and citric acid) alone or in new formulations (Ge et al. 2020; Scortichini et al. 2018, 2021) or integrating them into nanotechnologies (An et al. 2022; Kumaraswamy et al. 2018; Orfei et al. 2023; Su et al. 2024; Trela-Makowej et al. 2024) holds promise.

The development of nanotechnologies in agriculture has accelerated in recent years, resulting in products that reduce the quantity of agrochemicals dispersed in the environment and subsequently their pollutant impact.

Chitosan is widely accepted as a plant defense inducer (El Hadrami et al. 2010), with direct antimicrobial properties (Li and Zhuang 2020; Sudarshan et al. 1992). Bulk chitosan is insoluble in water, which limits its use in agriculture, whereas chitosan nanoparticles are more bioavailable, ensuring its antimicrobial and plant biostimulatory properties (Wang et al. 2020).

In previous work, fosetyl-Al nano-formulation (nano-Fos) and nano-fosetyl coated with chitosan (CH-nanoFos) were developed, and their antibacterial efficacy in vitro against *Xff* and *Xfp* was assessed (Baldassarre et al. 2020). The bacteriostatic effect of CH-nanoFos on the planktonic and biofilm state of *Xff* and *Xfp* was demonstrated, whereas fosetyl-Al (Fos-Al) and nano-Fos showed no antibacterial activity against *Xff* and *Xfp*.

To assess the in planta efficacy of CH-nanoFos, its ability to move from the roots along the stem to the leaves was studied. *Olea europaea* plants were treated with CH-nanoFos (1 mg/ml), and X-ray tomography and transmission electron microscopy (TEM) images revealed higher localization of CH-nanoFos in the roots and stem than in leaves.

The antibacterial activity of CH-nanoFos in planta was tested with *Nicotiana tabacum* as a plant model (Francis et al. 2008; Oliver et al. 2014). CH-nanoFos, Fos-Al, nano-Fos, and nano-chitosan (nano-CH) were applied to tobacco plants inoculated with *Xff*, *Xf* subsp. *multiplex* (*Xfm*), and *Xfp*. Only CH-nanoFos showed a decrease of *Xf* colonization in tobacco plants and a reduction in symptom progression. Furthermore, the effect of CH-nanoFos on *Xf* adhesion and motility was evaluated using the twitching (Galvani et al. 2007) and microfluidic channel assays (Kandel et al. 2016), revealing the inhibitory efficacy of CH-nanoFos. Overall, the results confirmed the ability of CH-nanoFos to control *Xf* growth in vitro and colonization of tobacco plants.

Materials and Methods

Preparation of nano-formulations

NanoFos and CH-nanoFos were produced using the sonication-assisted method previously described (Baldassarre et al. 2020). The chitosan coating was obtained by adding chitosan medium MW solution (0.5% wt/wt in 0.1% glacial acetic acid) into the formulation during sonication. Nano-CH particles were synthesized as a positive control. Two solutions were prepared: (i) chitosan medium MW solution (1% wt/wt in 0.5% glacial acetic acid; it was kept stirring for 24 h) and (ii) sodium tripolyphosphate (TPP; 1 mg/ml) in distilled water. Sodium tripolyphosphate was used as a crosslinker. The CH solution was added dropwise to the sodium tripolyphosphate

solution under agitation. The resulting mixture was immediately processed using the Mini Spray Dryer (B-290) from BUCHI (New Castle, DE, U.S.A.). The atomization parameters were as follows: inlet temperature of 130°C, pump rate of 9.5 ml/min, 100% aspiration, and gas spray flow 50 mmHg. Finally, the resulting powder was collected and washed in water (centrifugation at 7,700 RCF for 30 min) for further tests. Nano-CH morphology was analyzed by TEM using a JEOL JEM1400Plus microscope (Peabody, MA, U.S.A.) equipped with a Gatan Orius SC600 bottom view camera (Pleasanton, CA, U.S.A.) and a LaB6 filament source (Supplementary Fig. S1). One drop (10 µl) of each sample solution was placed on a standard carbon-coated TEM Cu grid and allowed to dry at room temperature. The analysis was performed at an acceleration voltage of 80 kV.

CH-nanoFos roots uptake—plants and treatments

Single-stemmed 1-year-old *Olea europaea* tree (cultivar Ogliarola salentina) seedlings were obtained by cuttings from a local nursery sited in Sammichele di Bari, Apulia, Italy. Ten seedlings per treatment were placed in perlite and acclimatized in a grow chamber with a day/night cycle of 25/16°C for temperature and 16/8 h for light and air relative humidity of 65%. Plants were treated at the root level through a localized irrigation system for 7 days, using a CH-nanoFos water-based suspension of 1 mg/ml. Deionized water was used as the control. Different plant portions (leaves, stem, and roots) were collected and immediately prepared for the observation by chemical fixing, counterstaining, dehydrating, and embedding the samples in epoxy resin blocks (Schiavi et al. 2022).

X-ray microscopy (XRM)—image acquisition and dataset reconstruction

XRM was performed using a ZEISS Xradia Versa 610, available at the Research Center on Nanotechnology Applied to Engineering, Sapienza University of Rome, Italy. This instrument overcomes the limitations of traditional X-ray computed tomography with a two-stage magnification architecture (geometric magnification + optics) and a high-flux X-ray source to produce sub-micron-resolution images with enhanced contrast. XRM uses the same X-ray computed tomography approach to collect a series of 2D projections (radiographs) of the sample at different viewing angles, taken by rotating the sample and exposing it to the X-ray beam. The resulting projections are then reconstructed using a reconstruction algorithm to produce a 3D dataset. Spurr resin-embedded samples (see above) were investigated using the scan parameters reported in Table 1. XRM datasets were reconstructed using ZEISS Reconstructor Scout-and-Scan (v.16.1.14271; Carl Zeiss, Oberkochen, Germany). The software employs the Feldkamp-Davis-Kress algorithm.

The reconstructed datasets were visualized and analyzed using the software Dragonfly Pro (v.2022.1 Build 1259; Object Research Systems, Montreal, Quebec, Canada). The grayscale values of the 2D XRM images were inverted so that higher absorption regions appeared darker and lower absorption regions appeared lighter. The regions of interest were labeled using a histogram-based thresholding method.

TEM analysis

For ultrastructural analysis, small pieces of leaves, roots, and stems from the different experimental groups were chemically fixed on ice with 2.5% glutaraldehyde in 0.1 mol/liter cacodylate buffer (pH = 7.4) for 1 h. Samples were post-fixed for 2 h with 1% OsO₄ in the same buffer and then dehydrated with ethanol (25, 50, 70, 90, and 100%). After embedding the samples in Spurr Resin Kit TK 4221D-1 (TAAB Laboratories Equipment, Berks, England), and ultramicrotome sectioning, 60-nm ultrathin sections were placed on grids and observed under a Zeiss Auriga transmission electron microscope (Zeiss) equipped with a STEM module, operating at 20 kV.

Bacterial strains and growth condition

Xff strain Temecula1 NCPPB 4605, *Xfm* strain CFBP 8416, and *Xfp* strain De Donno CFBP 8402 were grown on PD2 agar medium (Davis et al. 1980) for 15 to 20 days at 28°C. The colonies were then scraped off and resuspended in phosphate-buffered saline 1 × buffer. Each bacterial suspension was spectrophotometrically adjusted to OD_{600nm} = 0.1 and spotted on PD2 agar plates for further growth. The bacterial load was quantified, and the experiments were repeated three times with three technical replicates (*n* = 9 for each *Xf* subspecies). All strains were stored in PD2 broth with 20% glycerol at –80°C.

In planta bioassay—inoculation protocols and bacterial colonization evaluation

Two different *N. tabacum* cultivar Petit Havana SR 1 inoculation protocols were performed following Francis et al. 2008 (Protocol 1) and Oliver et al. 2014 (Protocol 2) with modifications.

Protocol 1. The schematic representation of inoculation Protocol 1 is illustrated in Figure 1A. Four-week-old *N. tabacum* plants cultivar SR1 (*n* = 30) were cut (10 to 12 cm tall) and pinprick-inoculated on two petioles, near the axils, with 20 µl of *Xfm* or *Xfp* bacterial suspension (OD_{600nm} = 0.1) for each petiole. Control plants (*n* = 10) were pinprick-inoculated with 20 µl of phosphate-buffered saline 1 × buffer. Thirty days after bacterial inoculation, tobacco plants were randomly divided for the treatments, as reported in Table 2. Four treatments (50 ml per treatment) were dispensed to the soil three times, 1 week apart (T0, T1, T2, and T3). Three days after the last treatment, the experiment was concluded (E4). Nebulization of leaves was excluded due to observed phytotoxicity (data not shown). The effect of inoculation/treatment on tobacco plant growth was assessed by measuring plant height at times T0, T1, T2,

T3, and E4. No symptoms described by Francis et al. (2008) (leaf scorch, interveinal chlorosis, leaf darkening and greening) were observed. The experiment was repeated two times with different inoculations. The bacterial load in all experimental conditions was evaluated at the end of the experiment (E4) as follows: All leaves were removed, and each stem was segmented into approximately 5- to 6-cm fragments, above the uppermost inoculation point, denoted from “a”, near the inoculation point, to “g”, at the apex of the plant (Fig. 1C). Genomic DNA was extracted using the CTAB method (EPPO 7/24 [5]), and qPCR was performed according to Francis et al. (2008) following EPPO (2023). The SYBR Green real-time PCR was used to verify the absence of nonspecific melting curves. *N. tabacum* actin primers were designed with Primer Blast software (forward: CCGGGCATTGCTGATAGGAT, reverse: CCTGCTGGAATGTGCTGAGA) and used as an internal control. The efficiency of actin primers was verified by the standard curve of *N. tabacum* DNA (*E* = 98.5%; *r*² = 0.997; slope = –3.359; *y*-int = 26.397). Standard curves generated by spiked samples (plant

TABLE 2. Treatments with Protocol 1 performed on *Nicotiana tabacum* plants

Treatments without <i>Xf</i> inoculation ^a	Treatments with <i>Xf</i> inoculation ^a
H ₂ O	<i>Xfp</i> - H ₂ O ^b
Nano-chitosan (1 mg/ml)	<i>Xfp</i> - nano-chitosan (1 mg/ml) ^b
CH-nanoFos (1 mg/ml) ^c	<i>Xfp</i> - CH-nanoFos (1 mg/ml) ^{b,c}
H ₂ O	<i>Xfm</i> - H ₂ O ^d
Fosetyl-Al (1 mg/ml)	<i>Xfm</i> - fosetyl-Al (1 mg/ml) ^d
Nano-fosetyl-Al (1 mg/ml)	<i>Xfm</i> - nano-fosetyl-Al (1 mg/ml) ^d

^a *Xf* = *Xylella fastidiosa*.

^b *Xfp* = *Xf* subsp. *pauca*.

^c CH-nanoFos = nano-fosetyl coated with chitosan.

^d *Xfm* = *Xf* subsp. *multiplex*.

TABLE 1. X-ray microscopy scan parameters

Sample	Objective	Pixel size (µm)	Voltage (kV)	Power (W)	Filter	Exposure time (s)	Number of projections
Roots CTR ^a	4×	1.43	30	2	LE1	25	1,601
Roots CH-nanoFos ^b	4×	1.43	30	2	LE1	25	1,601
Stems CTR ^a	4×	2.54	30	2	LE1	25	1,601
Stems CH-nanoFos ^b	4×	2.44	30	2	LE1	25	1,601
Leaves CTR ^a	4×	2.53	30	2	LE1	25	1,601
Leaves CH-nanoFos ^b	4×	2.74	30	2	LE1	25	1,601

^a CTR = control.

^b CH-nanoFos = nano-fosetyl coated with chitosan.

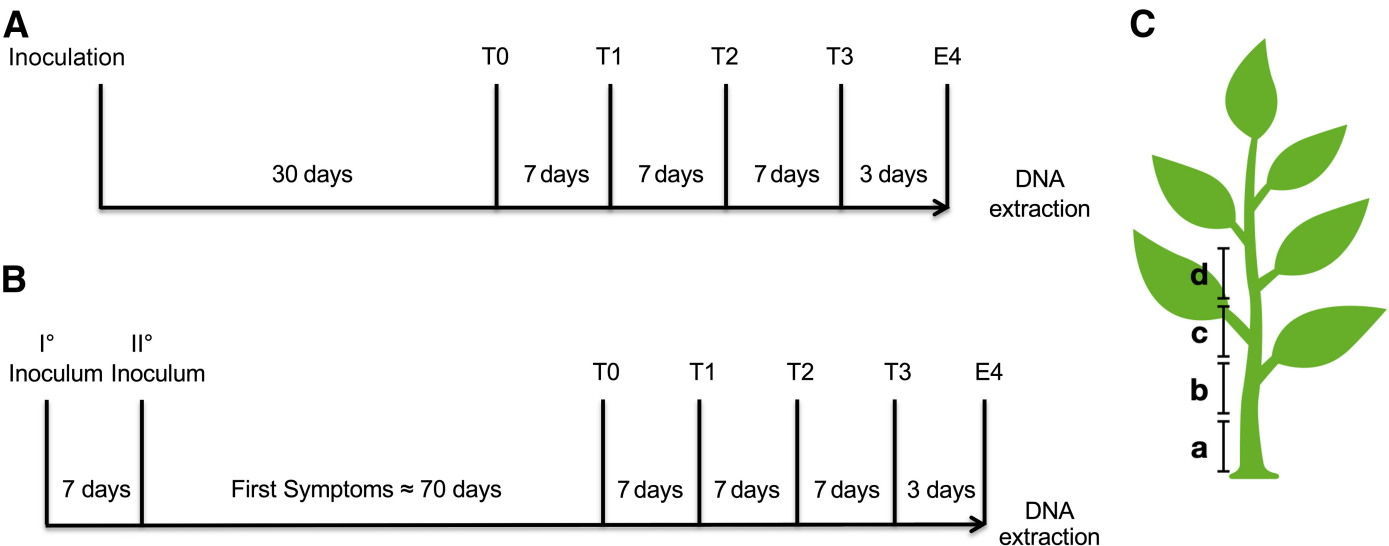


Fig. 1. **A**, Experimental time course of inoculation Protocol 1. **B**, Experimental time course of inoculation Protocol 2. **C**, Schematic representation of stem fragmentation.

extract added with 10-fold diluted bacterial suspensions at known concentrations, from 10^7 to 10^1 CFU/ml) were used for bacterial CFU quantification (Supplementary Fig. S2). The standard curves of spiked samples were compared with standard curves of bacterial suspensions at known concentrations (from 10^7 to 10^1 CFU/ml) to verify the absence of a possible inhibitory effect of the tobacco plant matrix on qPCR (Supplementary Fig. S2). The DNA was extracted with Puregene Yeast/Bact. Kit B (Qiagen, Hilden, Germany) for bacterial suspensions and with a CTAB-based protocol (PM 7/24 [5]) for spiked samples. Each experiment was carried out two times with a different inoculum (15 plants for each experimental group, $n = 30$).

Protocol 2. The schematic representation of inoculation Protocol 2 is illustrated in Figure 1B. Four-week-old *N. tabacum* plants cultivar SR1 ($n = 30$) were cut (10 to 12 cm tall) and pinprick-inoculated on two petioles, near the axils, with 20 μ l of *Xff*, *Xfm*, and *Xfp* bacterial suspension ($OD_{600nm} = 0.1$) for each petiole. Two inoculations were performed, 1 week apart. Control plants ($n = 10$) were pinprick-inoculated with 20 μ l of phosphate-buffered saline $1\times$ buffer. Symptoms first appeared on the tobacco plants approximately 70 days after inoculation for all *Xf* subspecies, after which they were randomly divided for treatment as follows: (i) noninoculated/ H_2O treated; (ii) noninoculated/CH-nanoFos treated; (iii) *Xff*, *Xfm*, or *Xfp* inoculated/ H_2O treated; and (iv) *Xff*, *Xfm*, or *Xfp* inoculated/CH-nanoFos treated. Each experiment was carried out twice for each *Xf* subspecies with different inoculations. The bacterial load in all experimental conditions was evaluated at the end of the experiment (E4). All leaves were removed, and each stem was segmented into approximately 5- to 6-cm fragments above the uppermost inoculation point (from a to g) (Fig. 1C). Genomic DNA was extracted with the CTAB method (PM 7/24 [5]), and qPCR was performed according to Harper et al.

(2010) following EPPO PM7/24 (5). Bacterial quantification was performed using the standard curve obtained from spiked samples (plant extract added with tenfold diluted bacterial suspensions at known concentrations, from 10^7 to 10^1 CFU/ml) (Supplementary Fig. S3). The standard curves obtained from spiked samples were compared with standard curves obtained from bacterial suspensions at known concentrations (from 10^7 to 10^1 CFU/ml) to verify the absence of a possible inhibitory effect of the tobacco plant matrix on qPCR (Supplementary Fig. S3). The DNA was extracted from bacterial suspensions and spiked samples as described for Protocol 1. Each experiment was carried out twice with a different inoculum (15 plants for each experimental group, $n = 30$).

N. tabacum symptom evaluation

The disease progression was followed weekly, and the percentage of symptoms incidence was calculated for each plant as $[(\text{symptomatic leaves}/\text{total leaves}) \times 100]$. The area under the disease progress curve (AUDPC) is the quantitative parameter used to evaluate the percentage of incidence of symptoms over time with respect to time 0. The AUDPC was calculated based on the midpoint rule method (Simko and Piepho 2012), $AUDPC = \sum_{i=1}^{n-1} [(y_i + y_{i+1})/2](t_{i+1} - t_i)$, where n is the number of times the symptoms assessment was performed, y is the score of incidence or severity for each plant, and t is the time of assessment (Simko and Piepho 2012).

Bacterial twitching

The twitching motility of *Xff* and *Xfm* was evaluated by measuring the colony fringe width on PD2 agar supplemented or not with CH-nanoFos (100 μ g/ml) (Galvani et al. 2007). The agar plates were incubated at 28°C for 8 days, and colony fringe width was measured using ImageJ software. Four replications were performed

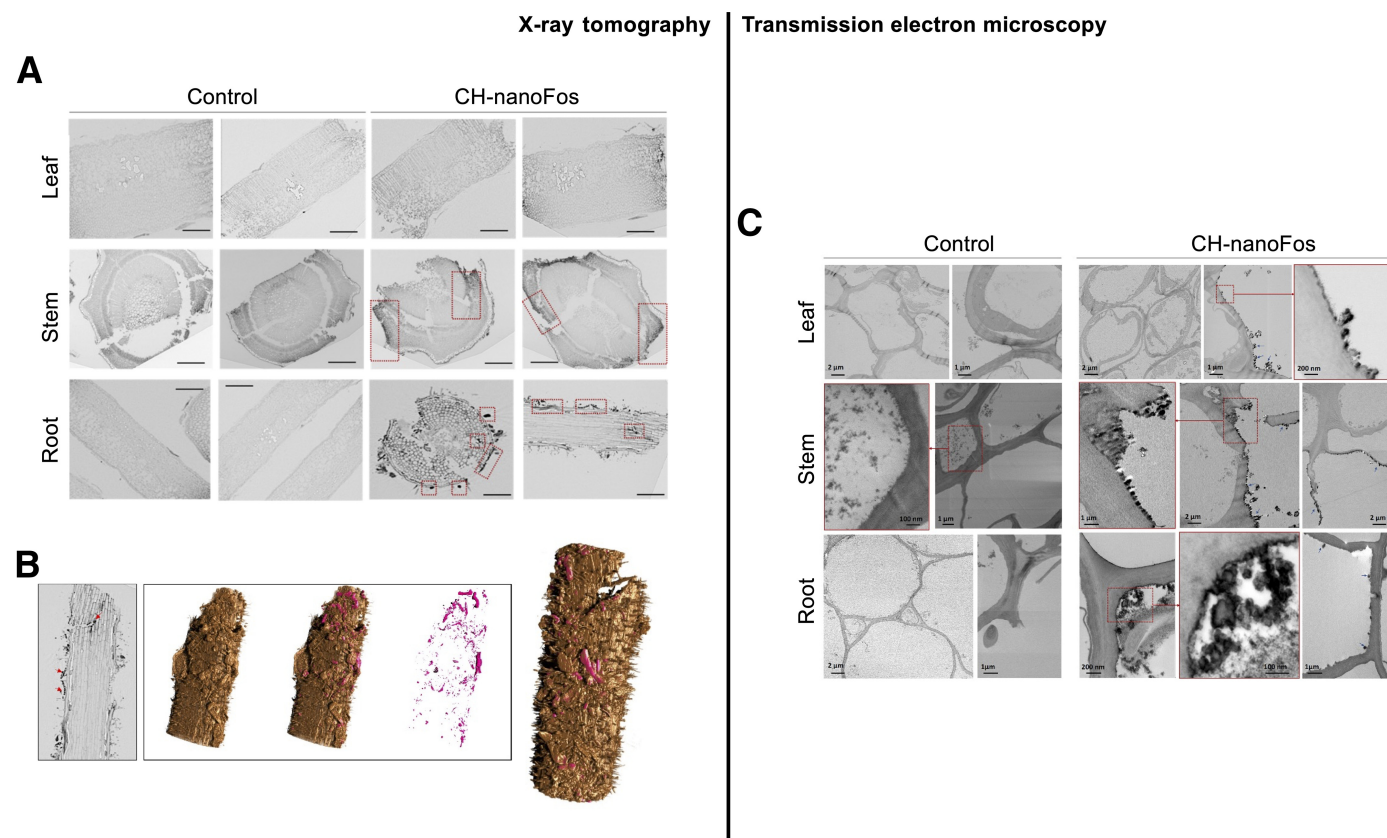


Fig. 2. X-ray tomography and transmission electron microscopy (TEM) analysis. **A**, Tomographic analysis was performed on sections of leaves, stems, and roots from both control and nano-fosetyl coated with chitosan (CH-nanoFos)-treated plants. Scale bar = 1 mm. **B**, 3D reconstruction of roots after treatment with CH-nanoFos. **C**, TEM images showing samples of leaves, stems, and roots from control and CH-nanoFos-treated plants. CH-nanoFos appears as black dots and clusters.

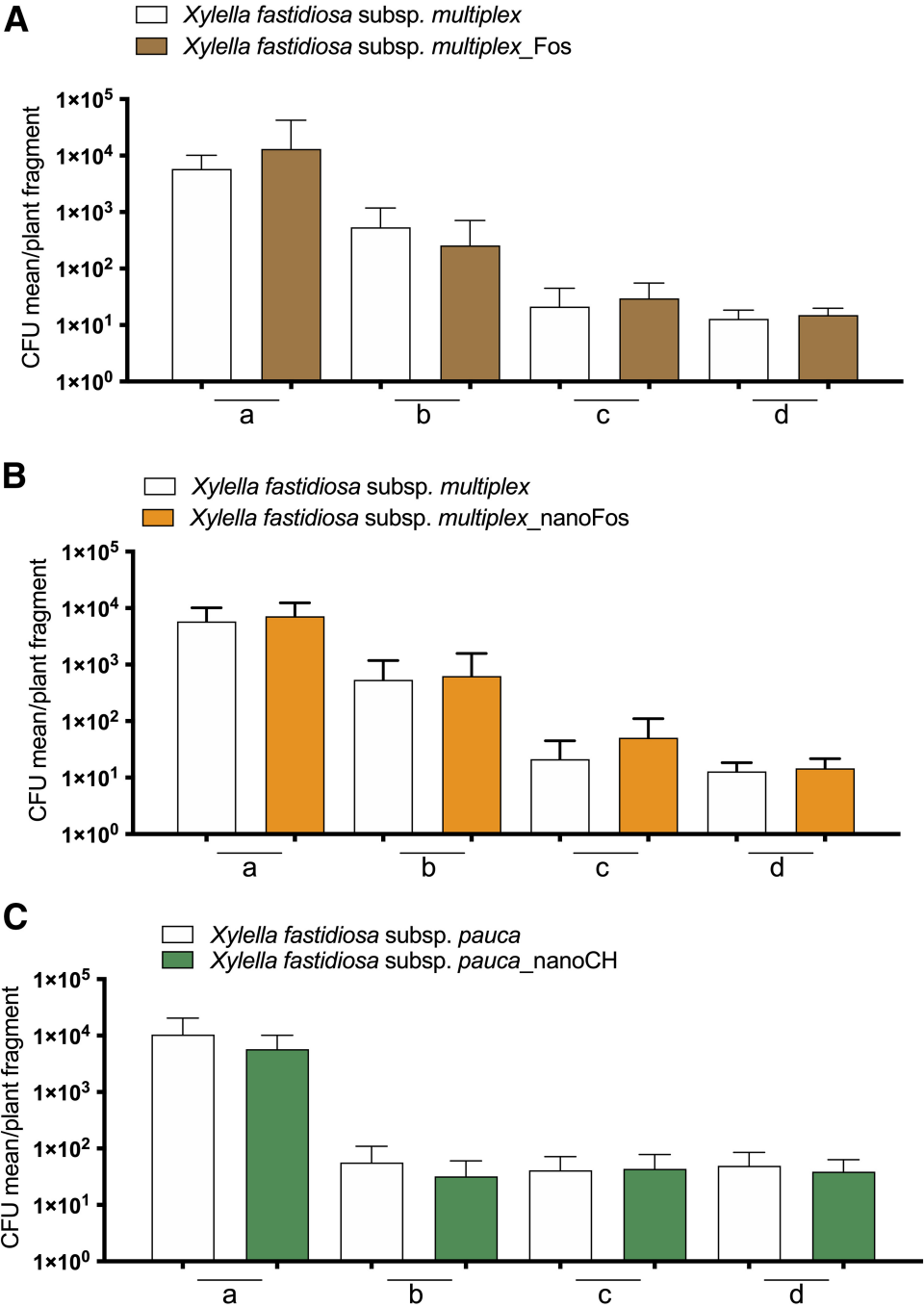
for each dilution, and each experiment was carried out two times with different inoculum ($n = 8$). Colonies with a peripheral fringe were designated as twitch-positive. All images were captured by using a stereomicroscope (Wild Heerbrugg, Heerbrugg, Switzerland) connected to a digital camera (Leica IC80 HD) in the same conditions of brightfield illumination. Twenty fringe-positive colonies were used for measurement; four measurements of peripheral fringe width were carried out for each colony, after correcting image contrast and brightness under the same conditions, by using ImageJ software 1.52v ($n = 20$).

Microfluidic device

The microfluidic network utilized in this study comprised a glass slide (2.4×6 cm) with a thickness of 0.2 mm bonded to a polydimethylsiloxane (PDMS) slab. The PDMS slab featured three channels with separate inlets and outlets, each measuring 3 cm in

length, 1 mm in width, and 0.05 mm in depth (Supplementary Fig. S4). The PDMS slab was fabricated following a previously reported procedure and bonded to the glass slide (Costantini et al. 2016; Mirasoli et al. 2018). Inlet and outlet connections were established by inserting stainless steel pins (0.013" ID $\times$ 0.025" OD $\times$ 0.5" long) purchased from New England Small Tube Corporation (Litchfield, NH, U.S.A.) in the inlet/outlet hole previously formed. Solutions were flowed in/out of the microfluidic channels by connecting Tygon tubing (0.020" ID $\times$ 0.060" OD) purchased from General Control Spa (Milan, Italy) to the pins inserted in the device. Sample solutions were driven by a PHD 2000 series syringe pump (Harvard Apparatus, Crisel Instruments S.r.l., Rome, Italy) equipped with six sterile plastic syringes of 1 ml volume (Thermo Scientific, Waltham, MA, U.S.A.), each fitted with needles measuring 0.8×38 mm. Prior to use, the device was exposed to UV light for 10 min and rinsed for 1 h with ethanol (70%) at a flow rate of 0.1 μ l/min.

Fig. 3. Inoculation Protocol 1; effect of Fosetyl-Al (Fos-Al), nano-fosetyl (nanoFos), and nano-chitosan (nanoCH) on *Nicotiana tabacum* plants inoculated or not with *Xylella fastidiosa* subsp. *multiplex* (*Xfm*) and *X. fastidiosa* subsp. *pauca* (*Xfp*). Graphical representation of **A**, Fos-Al and **B**, nanoFos efficacy on *Xfm* bacterial colonization of *N. tabacum* plants, and **C**, nanoCH efficacy on *Xfp* bacterial colonization of *N. tabacum* plants. *N. tabacum* stem fragments indicated as a, b, c, and d. Data are presented as colony forming units (CFU) mean $\pm$ standard deviation ($n = 22$, experiment repeated two times with different inocula).



Xff was grown on PD2 agar for 4 to 5 days at 28°C; afterward, the bacterial cells were scraped off and resuspended in PD2 broth. The microfluidic channels were individually allocated as (i) PD2, (ii) PD2-*Xff*, (iii) PD2-CH-nanoFos, and (iv) PD2-*Xff*-CH-nanoFos and filled with bacterial suspension ($OD_{600nm} = 0.8$) or PD2 broth at a flow rate of 0.1 $\mu\text{l}/\text{min}$. Two and a half hours after the injection commenced, the flow was halted for 30 min. Subsequently, syringes and inlets used for bacterial injection were replaced, and all channels were rinsed with PD2 broth at 0.01 $\mu\text{l}/\text{min}$ overnight to remove bacteria not bound to the glass-PDMS channel internal surface. Following this rinsing step, the channels were examined using an optical microscope. At this stage, PD2 broth supplemented or not with CH-nanoFos (100 $\mu\text{g}/\text{ml}$ final concentration) was flowed at 0.01 $\mu\text{l}/\text{min}$ in the channels with or without *Xff*. Planktonic bacterial growth was assessed after 3 and 7 days using an optical microscope. Biofilm growth was evaluated at the end of the experiment (day 7) with a crystal violet solution (0.1% wt/vol). The staining solution was introduced into the channel at a flow rate of 0.1 $\mu\text{l}/\text{min}$ for 10 min and subsequently rinsed with distilled water overnight at a flow rate of 0.01 $\mu\text{l}/\text{min}$. The channels were then observed using an optical microscope.

Microfluidic optical image acquisition and analysis

Time-lapse images were captured using an optical microscope equipped with a 40 $\times$ objective lens (Zeiss Axioskop 2 Phase Contrast Brightfield Microscope, Nikon digital sight DS-F1). All images underwent processing using ImageJ free software. Initially, the images were converted to 8-bit type, the background was subtracted, and auto-contrast was applied to brightfield images to enhance the definition of cell edges. The brightfield images were then utilized to measure cell area using the “analyze particle” tool in ImageJ. The micrometer scale was calibrated using the scale bar provided in the microscope image.

Statistical analyses

In planta data were analyzed by performing one-way analysis of variance at the 95% confidence level. Bacterial twitching statistical significance was determined by the Student's *t* test for pairwise comparison of means. All statistical analyses were carried out using GraphPad Prism 5 Software (GraphPad Software, San Diego, CA, U.S.A.). *P* values less than 0.05 were considered significant. Data are presented as mean plus or minus standard deviation.

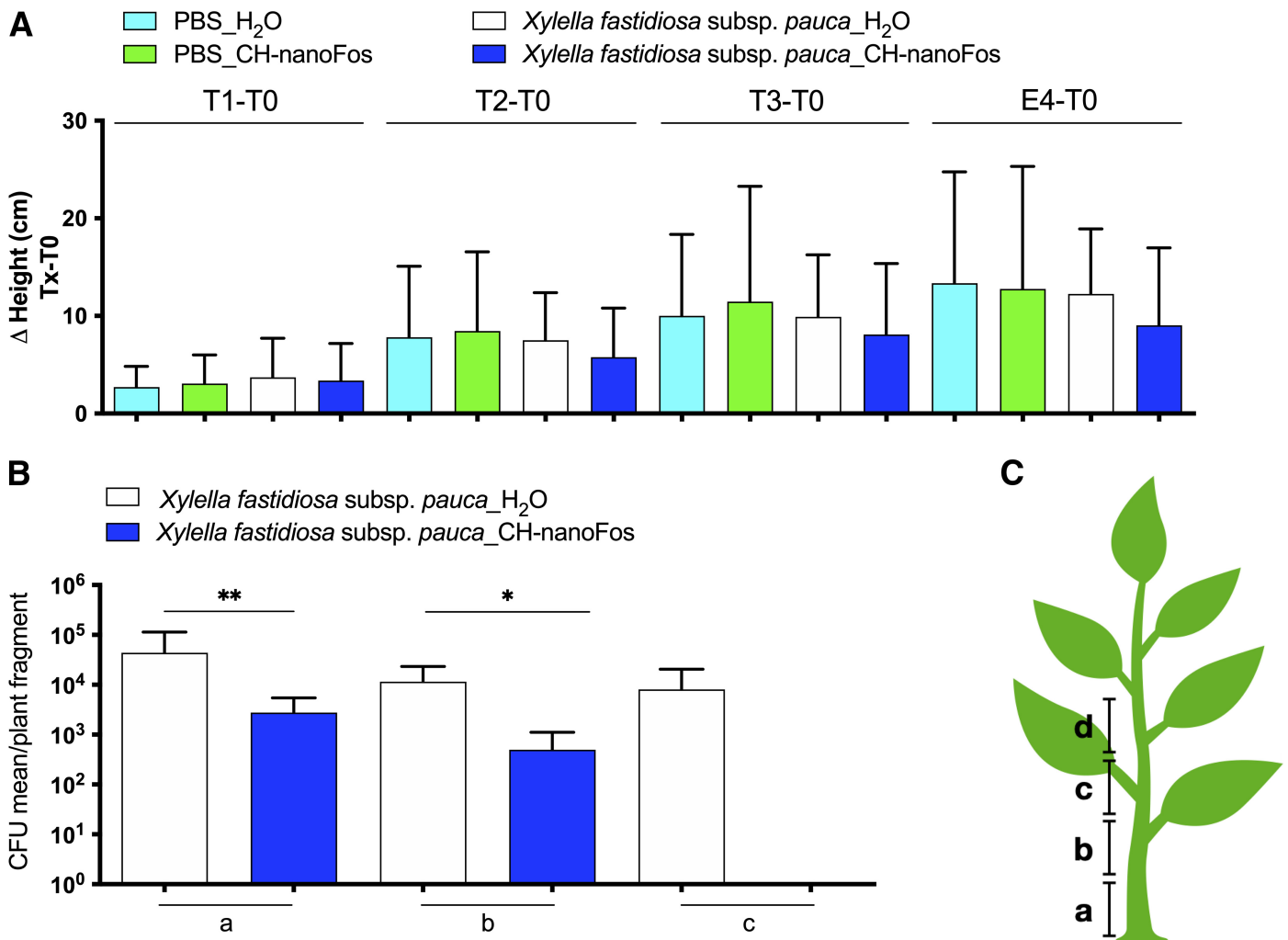


Fig. 4. Inoculation Protocol 1; effect of nano-fosetyl coated with chitosan (CH-nanoFos) (1 mg/ml) on *Nicotiana tabacum* plants inoculated or not with *Xylella fastidiosa* subsp. *pauca* (*Xff*). **A**, Graphical representation of *N. tabacum* plant height during the treatment time course of Protocol 1 presented as ΔHeight (difference between plant height measured at time T1, T2, T3, and E4 and first-day treatment [T0]) (T1-T0, T2-T0, T3-T0, and E4-T0). Data presented as mean values $\pm$ standard deviation (SD); experiment repeated two times with different inocula ($n = 30$). **B**, Graphical representation of CH-nanoFos efficacy on *Xff* bacterial colonization of *N. tabacum* plants. Data are presented as colony forming units (CFU) mean $\pm$ SD ($n = 30$, experiment repeated two times with different inocula; * $P < 0.05$, ** $P \leq 0.005$ versus control). **C**, Graphical representation of the *N. tabacum* stem fragments collected for the evaluation of *X. fastidiosa* in planta (a, b, c...).

Results

CH-nanoFos uptake by *Olea europaea* plants

Previously, the bacteriostatic efficacy of CH-nanoFos on *Xff* and *Xfp* planktonic and biofilm growth was demonstrated (Baldassarre et al. 2020). However, the in planta efficacy of CH-nanoFos depends on its ability to penetrate through the roots and translocate along the xylematic apparatus. To assess the movement of CH-nanoFos, *O. europaea* plants (cultivar Ogliarola salentina) were treated or not with CH-nanoFos (1 mg/ml) for 7 days. X-ray tomography and TEM analysis were performed on leaf, stem, and root samples (Fig. 2). The images obtained by X-ray tomography were analyzed using inverse contrast to better determine the presence of CH-nanoFos aggregates in the samples under investigation (Fig. 2A). Aluminum, a constituent of the nano-formulation, appeared as dark aggregates that tended to be black and were easily distinguishable from the structures in the plant sample under investigation. As shown in Figure 2A, a significant amount of CH-nanoFos was detected mainly in the outer root parts of the treated plants. In contrast, the amount of these aggregates was reduced in the stem, and only a few aggregates were observed in the leaves (Fig. 2A). Additionally, the 3D structure of the root samples was reconstructed from the obtained tomographic scans through meticulous image analysis and reprocessing using Dragon Fly software. Taking advantage of the different X-ray absorption indices, it was possible to highlight the presence of CH-nanoFos aggregates and mark them with a specific color (violet, Fig. 2B). Subsequently, the presence of nanoparticles in the different test samples was characterized by TEM, considering the instrumental limitations in terms of micro- and nanoscopic resolution. In agreement with the tomographic analysis, the TEM micrographs showed

that CH-nanoFos particles were more abundant in the roots of the treated plants, much less in the stem, and almost absent in the leaves (Fig. 2C).

Treated *N. tabacum* plants' response to *Xf* subspecies inoculation

The uptake of CH-nanoFos by olive roots prompted us to evaluate the antibacterial efficacy of chitosan and fosetyl-Al nano-formulations in planta. *N. tabacum* plants were chosen as the in planta model (Lopes et al. 2000; Pereira et al. 2020). Inoculations were performed with *Xfm* and *Xfp* following Francis et al. (2008; inoculation Protocol 1). Tobacco plant growth (height in centimeters) and bacterial load (CFU/plant fragment quantification) were the parameters used to assess the efficacy of treatments. Fos-Al, nano-Fos, and nano-CH treatments did not influence the tobacco growth (data not shown) or the bacterial load (Fig. 3A to C). As for other treatments, CH-nanoFos did not influence tobacco plant growth (Fig. 4A). The reduction of bacterial load in tobacco plants treated with CH-nanoFos was statistically significant in fragments a and b and undetectable in fragment c (Fig. 4B). Bulk chitosan is insoluble in water (Qin et al. 2006). It was therefore solubilized in acetic acid solution (0.1% vol/vol), which, however, showed a phytotoxic effect in the plant (data not shown). No symptoms were observed in *N. tabacum* plants artificially inoculated with *Xfp* and *Xfm* (data not shown).

N. tabacum plants inoculation with *Xf* subspecies—symptom progression

The inoculation protocol described in Figure 1A did not induce symptom progression and was subsequently modified as described in Figure 1B (inoculation Protocol 2). Tobacco plants were inoculated with *Xff*, *Xfm*, and *Xfp*, and the first symptoms were

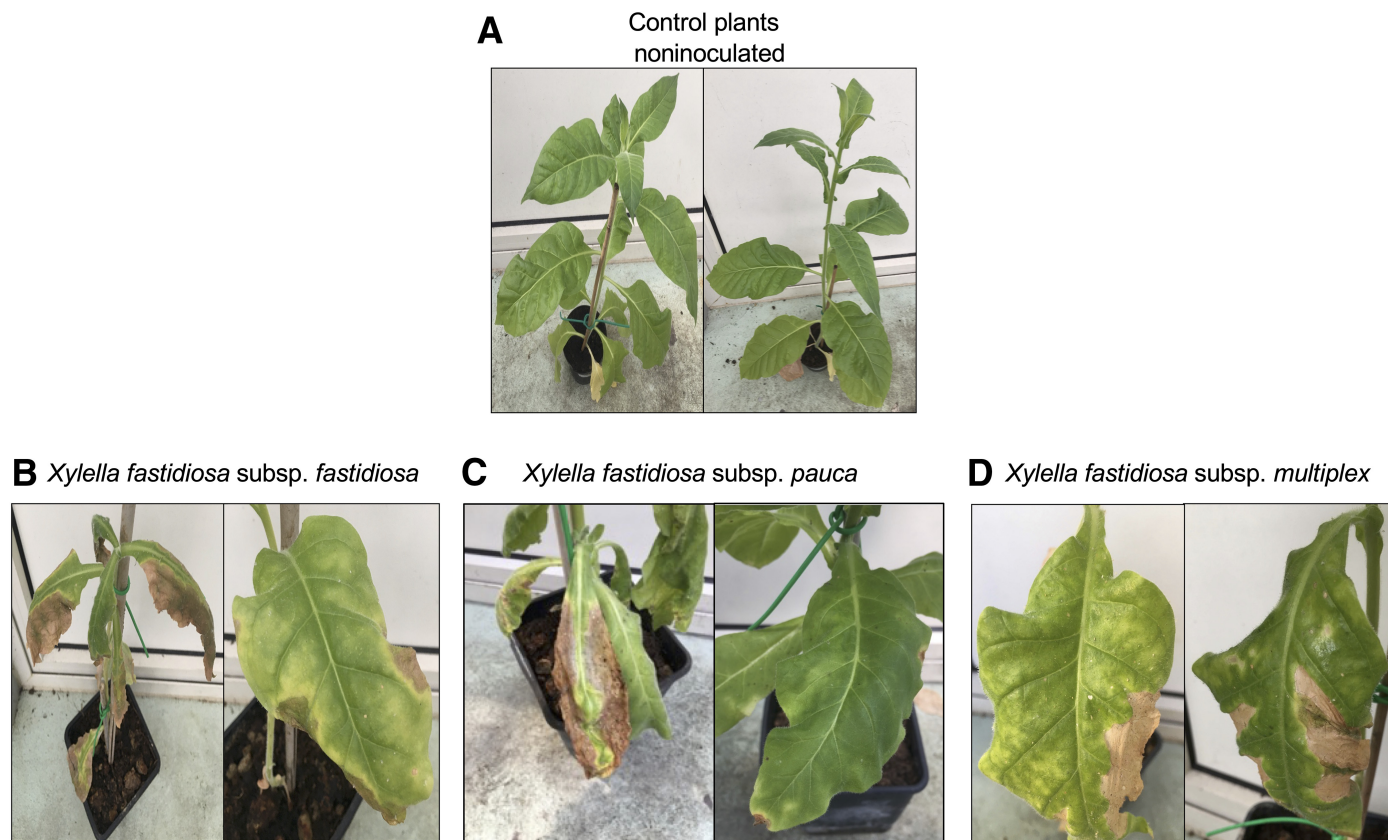


Fig. 5. Inoculation Protocol 2; symptom development of *Nicotiana tabacum* plants inoculated with *Xylella fastidiosa* subsp. *fastidiosa* (*Xff*), *X. fastidiosa* subsp. *multiplex* (*Xfm*), and *X. fastidiosa* subsp. *pauca* (*Xfp*). **A**, Noninoculated control of *N. tabacum* plant. **B**, *N. tabacum* plants inoculated with *Xff*. **C**, *N. tabacum* plants inoculated with *Xfp*. **D**, *N. tabacum* plants inoculated with *Xfm*. The photos were taken approximately 100 days after the first inoculation.

observed approximately 70 days after the second inoculation, when CH-nanoFos treatments were started.

The characteristic symptoms developed on *N. tabacum* plants (leaf scorch, interveinal chlorosis, leaf darkening and greening) are shown in Figure 5B to D, according to Oliver et al. (2014).

Xff, *Xfm*, and *Xfp* inoculations, as well as CH-nanoFos treatments, did not influence plant growth (Supplementary Fig. S5A to C), as observed in the previous experimental conditions with *Xfm* and *Xfp* (Protocol 1). Flowering occurred randomly in all ex-

perimental conditions (data not shown). Symptom development of *N. tabacum* plants, assessed by the AUDPC, showed a statistically significant reduction only in CH-nanoFos-treated plants inoculated with *Xfm* (Fig. 7C). Treated plants inoculated with *Xfp* and *Xff* (Figs. 6A and 7E) showed an overall trend in symptom reduction, which was evident at the end of the trial, particularly for *Xfp*. Representative images of symptoms developed on *N. tabacum* plants inoculated with *Xff*, *Xfm*, and *Xfp* are presented in Figure 6B, D, and F.

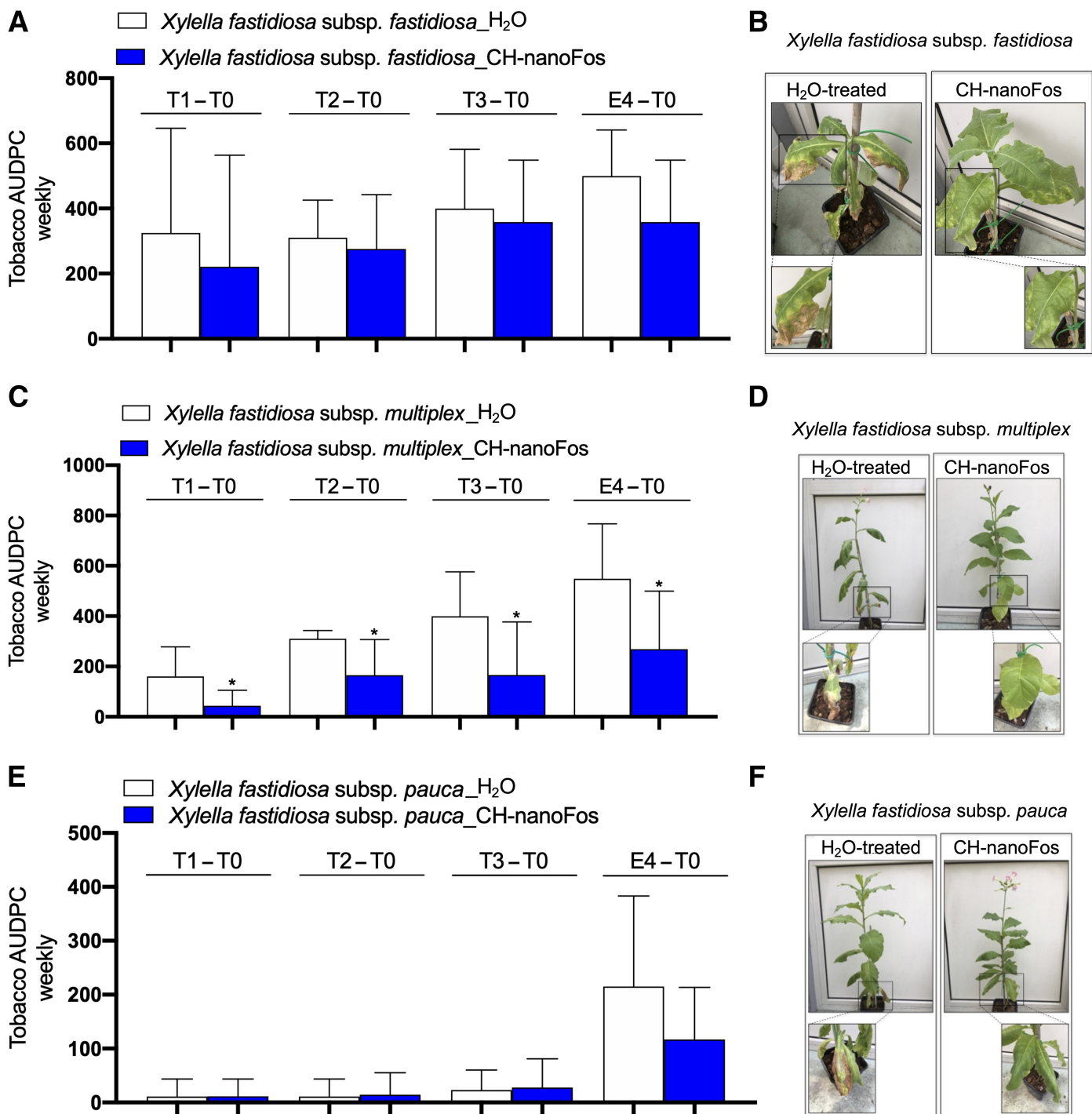


Fig. 6. Inoculation Protocol 2; symptom development of *Nicotiana tabacum* plants inoculated with **A and B**, *Xylella fastidiosa* subsp. *fastidiosa* (*Xff*); **C and D**, *X. fastidiosa* subsp. *multiplex* (*Xfm*); and **E and F**, *X. fastidiosa* subsp. *pauca* (*Xfp*). **B, D, and F**, Representative images of symptoms on *N. tabacum* plants at the end of the experimental Protocol 2. Area under disease progress curve (AUDPC) for evaluation of symptom development in *N. tabacum* plants inoculated with **A**, *Xff*; **C**, *Xfm*; and **E**, *Xfp*. Data were collected every week and presented as mean values $\pm$ standard deviation; the experiment was repeated two times with different inocula ($n = 30$; * $P < 0.05$ versus control).

Xf quantification in response to CH-nanoFos treatment

The relationship between symptom development and *Xf* subspecies plant colonization was assessed by bacterial CFU per plant fragment quantification. All *Xf* subspecies exhibited a high colonization capability (Fig. 7, white bars). CH-nanoFos treatment

resulted in a statistically significant reduction of bacterial load in tobacco plants, particularly evident from fragments b to g (Fig. 7A to C), with the exceptions of *Xfm*-infected plants, where a significant reduction started from fragment a, and of *Xfp*-infected plants, where the reduction was significant up to fragment e.

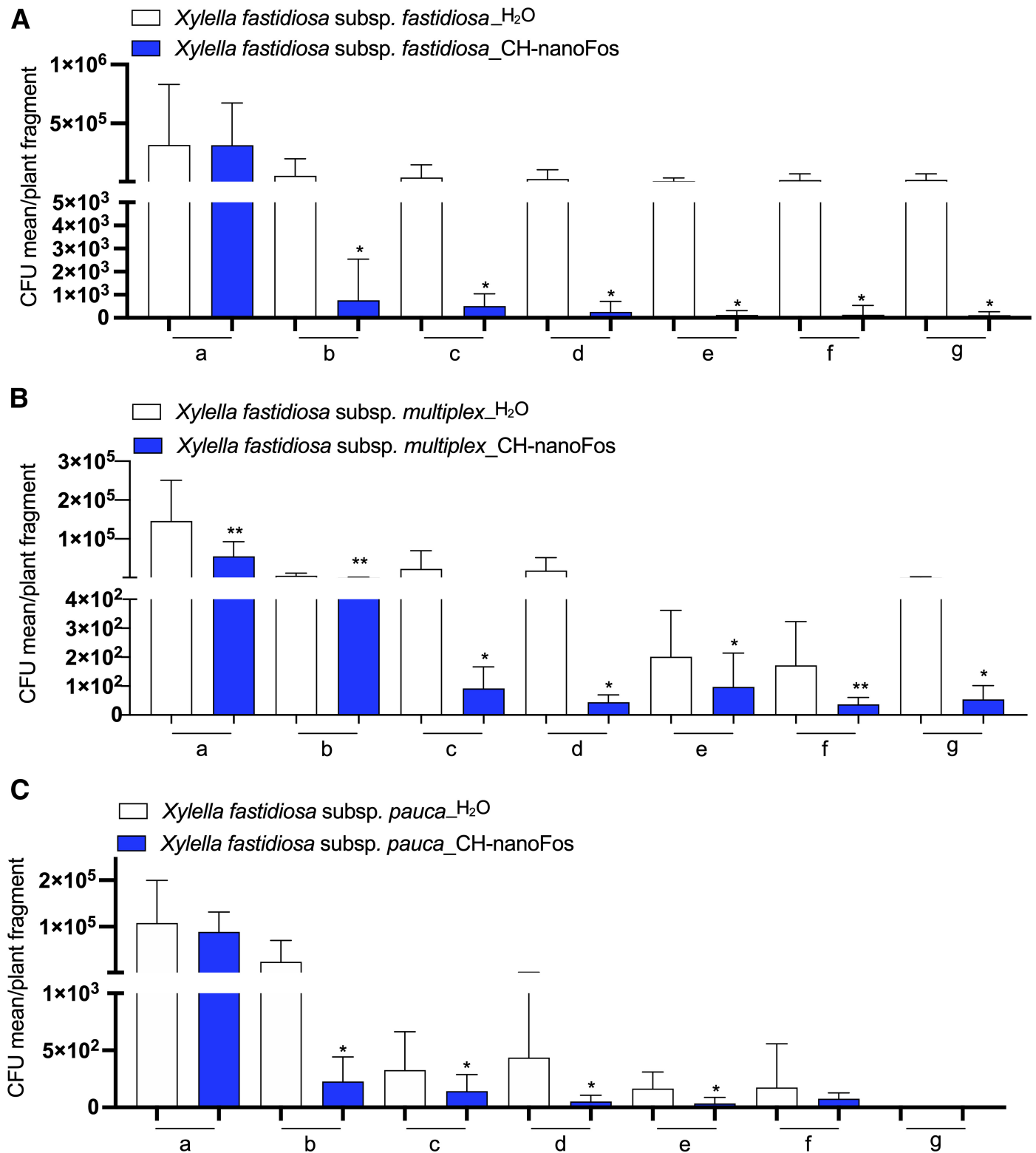


Fig. 7. Inoculation Protocol 2; nano-fosetyl coated with chitosan (CH-nanoFos) effect on *Nicotiana tabacum* plant colonization by **A**, *Xylella fastidiosa* subsp. *fastidiosa* (*Xff*); **B**, *X. fastidiosa* subsp. *multiplex* (*Xfm*); and **C**, *X. fastidiosa* subsp. *pauca* (*Xfp*) presented as colony forming unit (CFU)/plant fragment. Each plant stem was segmented in about 4- to 5-cm fragments starting 5 cm above the uppermost inoculation point (from a to g). Data are presented as CFU mean $\pm$ standard deviation ($n = 30$, experiment repeated two times with different inocula; * $P < 0.05$, ** $P \leq 0.005$ versus control).

Bacterial twitching and microfluidic growth assay

The assessment of the antibacterial effect of CH-nanoFos on *Xf* growth was investigated using two methods: the twitching motility assay and the evaluation of planktonic and biofilm growth in a microfluidic device. The twitching assay is based on the motility of *Xf*, which is characterized by a typical morphology with a peripheral fringe around the colonies. This morphology is observed in *Xff* and *Xfm*, whereas it is absent for *Xfp* (D'Attoma et al. 2020). Microscopic evaluation of the fringed margin revealed the complete absence of the typical fringe of *Xff* and *Xfm* grown on CH-nanoFos-supplemented media, unlike bacterial colonies grown on control media (Fig. 8A and C). The mean measurements of the fringe width are shown in Figure 8B and D. This evidence showed that CH-nanoFos inhibits the bacterial motility of *Xff* and *Xfm*. The microfluidic device was used to simulate the bacterial growth in a xylematic flow. The results showed that when flowing PD2 supplemented with CH-nanoFos, the area occupied by *Xff* colonies at days 3 and 7 is similar to that at day 0, unlike in control conditions (only PD2 flow), where there is a progressive significant increase in the area occupied by bacterial colonies (Fig. 9B). As with planktonic growth, the biofilm of *Xff* is significantly reduced in CH-nanoFos-enriched PD2 flow (Fig. 9D). The overall results highlight the inhibitory efficacy of CH-nanoFos toward *Xff* and *Xfm*.

Discussion

Xff, *Xfm*, and *Xfp* are widely accepted by the scientific community as the main groupings (Hopkins 1989; Johnson et al. 2022; Sicard et al. 2018) due to their extensive host variety and significant economic value (EFSA Panel on Plant Health et al. 2019). Moreover, based on comparative genomic analyses, *Xff*, *Xfm*, and *Xfp* have been proposed, with the subsp. *fastidiosa* including the subspecies *sandyi* and *morus* (Denancé et al. 2017; Marcelletti and Scortichini 2016).

Historically, known occurrences of *Xf* were confined to the Americas until 2013 when *Xfp* was associated with olive quick decline syndrome in the Apulia region of Southern Italy (Saponari et al. 2013). Since then, the spread of the pest in Italy has had a significant impact on the economy, estimated to be between 1.9 to 5.2 billion euros over 50 years (Strona et al. 2017), and on the landscape of Salento, which hosts hundreds of centuries-old olive trees. The *Xfp* strain De Donno affected approximately 6.5 million olive trees (Martelli 2016; Scholten et al. 2019), and 10,000 ha of the Salento territory were classified as infected (Martelli 2016), with the unfortunate impossibility of eradicating the infected plants. Several years later, it was reported that killed or damaged plants amounted to 54,000 ha of olive orchards (Frem et al. 2021). It is worth noting that environmental conditions favor the proliferation of the vector and transmission of *Xf*. In particular, the extensive cultivation of the two indigenous cultivars sensitive to *Xf* (Cellina di Nardò and Ogliarola salentina) has facilitated the spread of *Xfp* in the area (Martelli 2016).

New outbreaks of different *Xf* subspecies have been reported in new areas both in Italian regions (Marchi et al. 2018) and across Europe (EPPO Global Database 2018, 2019a, b; Trkulja et al. 2022). The high rate of genetic recombination facilitates the identification of variants, allowing *Xf* to jump to different host species, expand its host range, increase virulence, and adapt to new ecological niches (Landa et al. 2022; Vanhove et al. 2019). These factors pose significant challenges to control strategies (Quetglas et al. 2022; Strona et al. 2017). Currently, efforts to address the progression of the infection include identifying resistant cultivars, reducing vector populations, implementing proper orchard management, and developing new products or formulations able to control the bacterium and/or inhibit bacterial virulence (Kyrkou et al. 2018; Pavan et al. 2021; Scortichini et al. 2018, 2021). However, the uncontrolled use of pesticides and fertilizers has led to increased pathogen and pest resistance, soil biodiversity depletion, and pesticide accumulation in

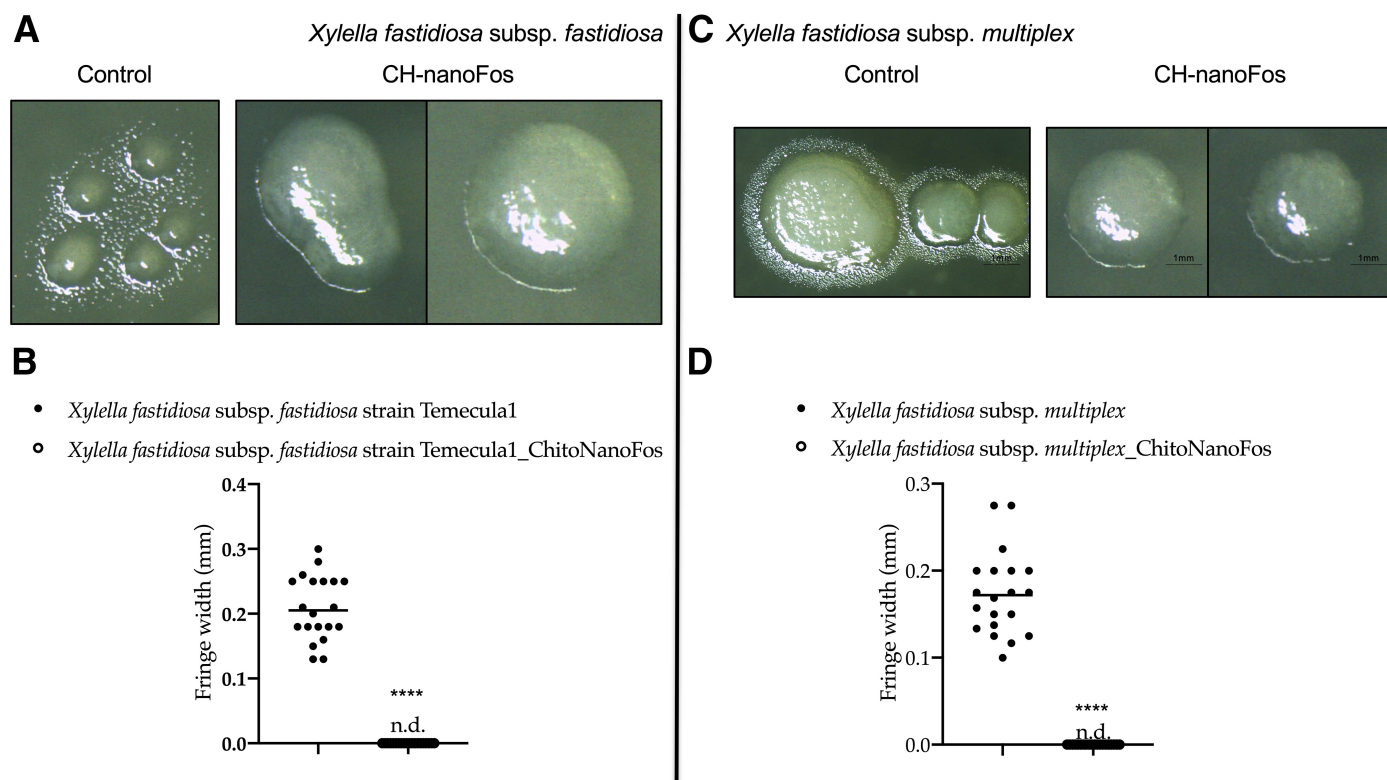


Fig. 8. Bacterial twitching assay. *Xylella fastidiosa* subsp. *fastidiosa* (*Xff*; left panel) and *X. fastidiosa* subsp. *multiplex* (*Xfm*; right panel) were grown on PD2 agar supplemented or not with nano-fosetyl coated with chitosan (CH-nanoFos). **A and C**, Fringes around colonies due to bacterial motility. **B and D**, Fringe width quantified with ImageJ software ($n = 20$; **** $P \leq 0.0001$ versus control). n.d., not determined.

soil and water (Tilman et al. 2002). Consequently, the use of chemicals in agriculture is strictly regulated. In the United States, unlike in European Union (EFSA Panel on Plant Health et al. 2019), the use of antibiotics (e.g., oxytetracycline) in agricultural practice is permitted but only results in a reduction in symptom development, with the infection persisting (Kyrkou et al. 2018). The endophytic lifestyle of *Xf* presents a significant obstacle to the use of effective pesticides in open fields. Chemicals applied in open fields often reach target sites in crops at very low concentrations due to degradation by photolysis, hydrolysis, and microbial degradation (Nair et al. 2010). This limitation makes it challenging to identify substances that can reach the xylem vessels without undergoing structural alterations while maintaining their bioactive effects. Consequently, repeated applications are often necessary, leading to soil and water pollution (Nair et al. 2010). To address these challenges, conventional agricultural practices and agrochemicals are being combined with technological innovations to produce nano-compounds (He et al. 2019; Zhang and Goss 2022).

Nanoparticles, ranging in size from about 1 to 100 nm, have various applications in agriculture, including controlled release of pesticides, herbicides, and plant growth regulators, which can improve crop production (Mitrano et al. 2015; Soenen et al. 2015; Stark et al. 2015). Furthermore, nanocarrier exploitation enables the controlled release of bioactive molecules, protecting them from environmental stress and degradation (Chhipa and Joshi 2016).

As part of this innovative process to find highly efficient and environmentally friendly solutions, two substances commonly used in agricultural practice were investigated: chitosan and fosetyl-Al. Chitosan is the second most abundant polymer after cellulose (Elgadir et al. 2015). It has significant direct antimicrobial potential against a wide range of bacteria, fungi, and viruses and indirect potential in inducing resistance (El Hadrami et al. 2010; Riseh et al. 2022). Moreover, chitosan is biodegradable and nontoxic, and all these characteristics favor its use in agricultural practice over synthetic pesticides and fertilizers. It is also known for its ability to promote biodiversity in the rhizosphere at the expense of potential pathogenic microorganisms (Park and Chang 2012). The versatility of chitosan is also highlighted in its use as a nanocarrier, improving the stability and bioavailability of bioactive substances. Fosetyl-Al, despite being a synthetic fungicide, has a high degree of soil degradability and no impact on the microbiome (Fournier et al. 2020).

Considering the potential of these two substances, Baldassarre et al. (2020) described the synthesis of CH-nanoFos using an ultrasonication process. Unlike Fos-Al and nano-Fos, CH-nanoFos exhibited *in vitro* bacteriostatic effects on planktonic growth and inhibited biofilm in *Xff* and *Xfp* (Baldassarre et al. 2020).

In view of the possible efficacy of fosetyl-Al and chitosan as plant immune response inducers (Brunetti et al. 2020), these molecules were investigated in several formulations (Fos-Al, nano-Fos,

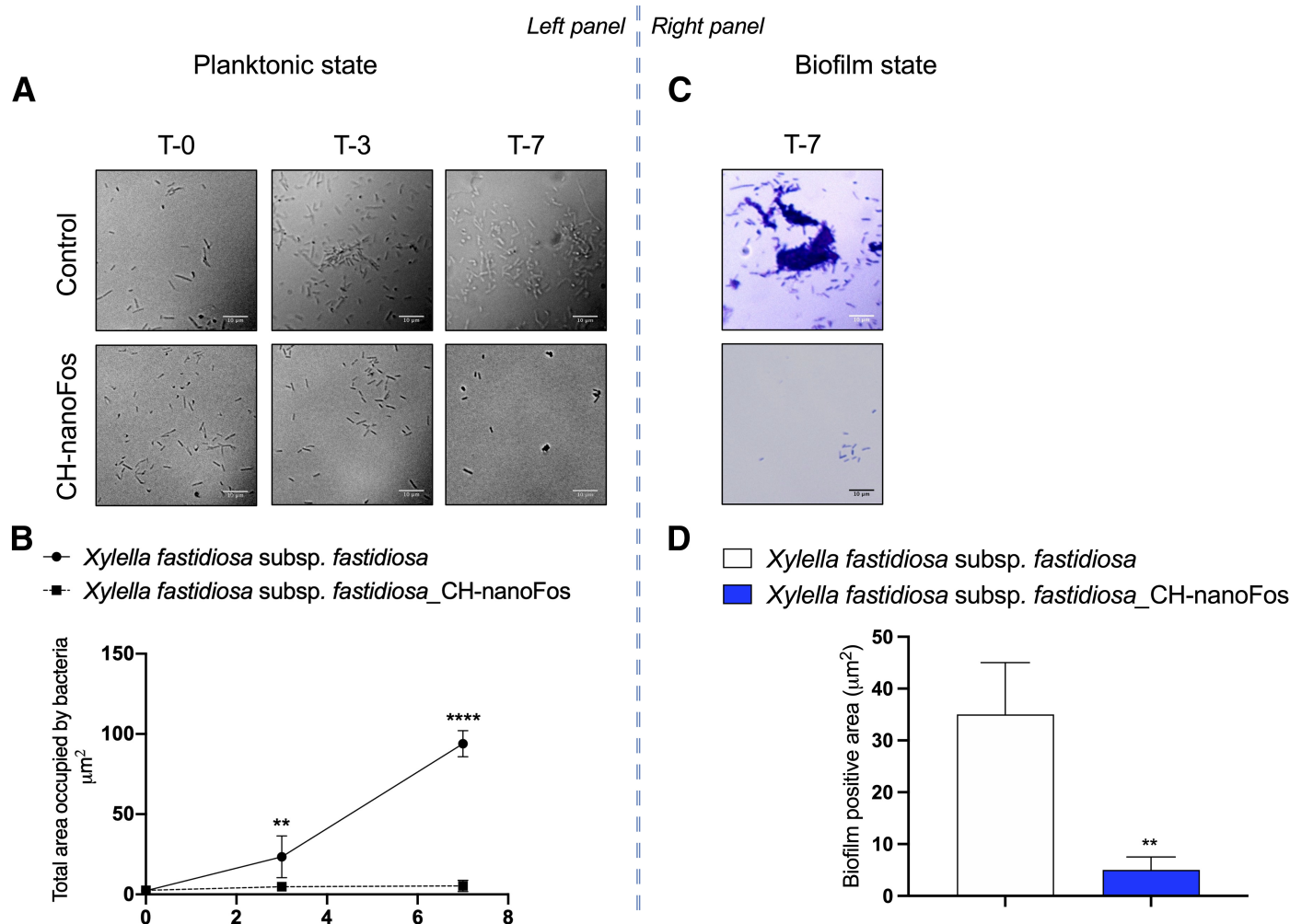


Fig. 9. *Xylella fastidiosa* subsp. *fastidiosa* (*Xff*) microfluidic growth. Planktonic (left panel) and biofilm (right panel) growth of *Xff* in microfluidic channels with PD2 supplemented or not with nano-fosetyl coated with chitosan (CH-nanoFos; 100 $\mu\text{g}/\text{ml}$). **A and C,** Representative images of bacterial growth and biofilm assay. **B and D,** Graphs show total area occupied by *Xff* colonies and biofilm, quantified with ImageJ software ($n = 8$; ** $P < 0.01$, **** $P \leq 0.0001$ versus control).

nano-CH, and CH-nanoFos) in the model system *N. tabacum* cultivar Petit Havana SR 1. For this purpose, the bacterial plant colonization and the symptom development were evaluated as reliable indicators of *Xf* infection (Gómez et al. 2020; Lopes et al. 2000). Interestingly, we did not observe leaf scorch symptoms until 60 to 70 days after *Xf* inoculation, nor inhibition of plant growth and flowering, contrary to previous reports (Francis et al. 2008; Pereira et al. 2017). Moreover, among the treatments, CH-nanoFos was the only effective one to contain symptom development and colonization of all *Xf* subspecies in the in planta *N. tabacum* model, compared with fosetyl-Al and nano-formulated fosetyl-Al and chitosan. Real-time PCR analyses confirmed a significant reduction in bacterial CFU per plant fragment with all *Xf* subspecies, supporting the efficacy of CH-nanoFos in reducing plant colonization. Although CH-nanoFos showed a tendency to reduce symptom severity, this was significant only in *Xfm*-inoculated plants.

Twitching motility, mediated by type IV pili, is a critical virulence trait for *Xf* (Cursino et al. 2011), facilitating its transition to the planktonic phase in xylem vessels (Meng et al. 2005). In vitro experiments revealed a complete inhibition of twitching motility of *Xff* and *Xfm* treated with CH-nanoFos, likely due to interference with type IV pilus-mediated twitching (Li et al. 2007). Similarly, CH-nanoFos treatment in a nano-microfluidic flow device resulted in the loss of *Xff* ability to form typical biofilm aggregates.

These results support previous in vitro findings (Baldassarre et al. 2020) and demonstrate the efficacy of CH-nanoFos in reducing *Xf* colonization and symptom development in planta. The lower antibacterial activity of nanoFos observed previously (Baldassarre et al. 2020) was also confirmed in planta. The synergistic action of Fos-Al and CH, maximized by nano-downsizing, likely contributes to the efficacy of CH-nanoFos.

Finally, the possible movement of CH-nanoFos within the xylem vessels of the host plant was assessed by XRM and TEM images. The images demonstrated the ability of CH-nanoFos to penetrate the root system and, to a lesser extent, reach the stem and leaves. It is worth noting that Baldassarre et al. (2020) showed that CH-nanoFos nanoparticles were less toxic than conventional formulations on human keratinocyte cells, an in vitro model to test the toxicity of pesticides, proving it to be a promising product for eco-sustainable use.

The results of this study together with the previous in vitro evidence on this nano-formulation highlight that CH-nanoFos is promising for *Xf* control. These conclusions, together with a future evaluation of the feasibility of producing large volumes of CH-nanoFos and the relative cost per treated plant, can be considered a good starting point to be able to test its efficacy in open fields on naturally infected plants. The proven bacteriostatic effect of CH-nanoFos could allow the susceptible Ogliarola salentina and Cellina di Nardò cultivars to develop a viable and productive canopy in the infected areas, although they may represent a reservoir of *Xf*.

Furthermore, the evidence on the reduction of the bacterial CFU in the treated model plant could support the open field treatments to reduce the availability of *Xf* load that can be transmitted by the insect, slowing down the progression of the infection. The effectiveness of CH-nanoFos on the three subspecies, *multiplex*, *pauca*, and *fastidiosa*, is of strategic importance also in consideration of the recent discoveries of *Xff* and *Xfm* in the province of Bari, a short distance from the *Xfp* demarcated area.

In conclusion, *Xf* infection represents a challenge for the scientific community to understand, on the one hand, how the environment tries to adapt to this pathogen and, on the other hand, to explore innovative solutions to reduce the spread of the infection and allow for the recovery of productivity in these severely affected areas. There is no single strategy to control the spread of the pathogen. However, integrated approaches based on agronomic practices and environmentally friendly products can lead to the vegetative recovery of affected olive trees, making them more resilient to this dangerous

disease. This is particularly important for the two typical Salento varieties, Ogliarola Salentina and Cellina di Nardò, whose susceptibility has seriously compromised the landscape and productivity of Salento olive cultivation.

Acknowledgments

We thank Alessia L'Aurora and Simone Lucchesi for their support in experimental procedures. *Xfp* strain De Donno (CFBP 8402) was provided by Maria Saponari, CNR-IPSP, Bari, Italy.

Literature Cited

- An, C., Sun, C., Li, N., Huang, B., Jiang, J., Shen, Y., Wang, C., Zhao, X., Cui, B., Wang, C., Li, X., Zhan, S., Gao, F., Zeng, Z., Cui, H., and Wang, Y. 2022. Nanomaterials and nanotechnology for the delivery of agrochemicals: Strategies towards sustainable agriculture. *J. Nanobiotechnol.* 20:11.
- Annamalai, J., and Namasivayam, V. 2015. Endocrine disrupting chemicals in the atmosphere: Their effects on humans and wildlife. *Environ. Int.* 76:78-97.
- Araújo, W. L., Marcon, J., Maccheroni, W., Jr., van Elsland, J. D., van Vuurde, J. W. L., and Azevedo, J. L. 2002. Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. *Appl. Environ. Microbiol.* 68:4906-4914.
- Baccari, C., Antonova, E., and Lindow, S. 2019. Biological control of Pierce's disease of grape by an endophytic bacterium. *Phytopathology* 109:248-256.
- Baldassarre, F., Tatulli, G., Vergaro, V., Mariano, S., Scala, V., Nobile, C., Pucci, N., Dini, L., Loreti, S., and Ciccarella, G. 2020. Sonication-assisted production of fosetyl-Al nanocrystals: Investigation of human toxicity and in vitro antibacterial efficacy against *Xylella fastidiosa*. *Nanomaterials* 10: 1174.
- Bleve, G., Gallo, A., Altomare, C., Vurro, M., Maiorano, G., Cardinali, A., D'Antuono, I., Marchi, G., and Mita, G. 2018. In vitro activity of antimicrobial compounds against *Xylella fastidiosa*, the causal agent of the olive quick decline syndrome in Apulia (Italy). *FEMS Microbiol. Lett.* 365:fnx281.
- Brunetti, A., Pucci, N., Modesti, V., Lumia, V., Latini, A., Loreti, S., and Pilotti, M. 2020. In vitro and in planta screening of compounds for the control of *Pseudomonas syringae* pv. *actinidiae* in *Actinidia chinensis* var. *chinensis*. *Eur. J. Plant Pathol.* 158:829-848.
- Chang, C. J., Garnier, M., Zreik, L., Rossetti, V., and Bové, J. M. 1993. Culture and serological detection of the xylem-limited bacterium causing citrus variegated chlorosis and its identification as a strain of *Xylella fastidiosa*. *Curr. Microbiol.* 27:137-142.
- Chhipa, H., and Joshi, P. 2016. Nanofertilisers, nanopesticides and nanosensors in agriculture. Pages 247-282 in: *Nanoscience in Food and Agriculture 1, Sustainable Agriculture Reviews*. S. Ranjan, N. Dasgupta, and E. Lichtfouse, eds. Springer International Publishing, Cham, Switzerland.
- Costantini, F., Sberna, C., Petrucci, G., Reverberi, M., Domenici, F., Fanelli, C., Manetti, C., de Cesare, G., DeRosa, M., Nascetti, A., and Caputo, D. 2016. Aptamer-based sandwich assay for on chip detection of Ochratoxin A by an array of amorphous silicon photosensors. *Sens. Actuators B Chem.* 230:31-39.
- Cursino, L., Galvani, C. D., Athinuwat, D., Zaini, P. A., Li, Y., De La Fuente, L., Hoch, H. C., Burr, T. J., and Mowery, P. 2011. Identification of an operon, Pil-Chp, that controls twitching motility and virulence in *Xylella fastidiosa*. *Mol. Plant-Microbe Interact.* 24:1198-1206.
- Dáder, B., Viñuela, E., Moreno, A., Plaza, M., Garzo, E., del Estal, P., and Fereres, A. 2019. Sulfoxaflor and natural pyrethrin with piperonyl butoxide are effective alternatives to neonicotinoids against juveniles of *Phlaenus spumarius*, the European vector of *Xylella fastidiosa*. *Insects* 10:225.
- D'Attoma, G., Morelli, M., De La Fuente, L., Cobine, P. A., Saponari, M., de Souza, A. A., De Stradis, A., and Saldarelli, P. 2020. Phenotypic characterization and transformation attempts reveal peculiar traits of *Xylella fastidiosa* subspecies *pauca* strain De Donno. *Microorganisms* 8:1832.
- Davis, M. J., Purcell, A. H., and Thomson, S. V. 1980. Isolation media for the Pierce's disease bacterium. *Phytopathology* 70:425-429.
- Denancé, N., Legendre, B., Briand, M., Olivier, V., de Boisseson, C., Poliakov, F., and Jacques, M.-A. 2017. Several subspecies and sequence types are associated with the emergence of *Xylella fastidiosa* in natural settings in France. *Plant Pathol.* 66:1054-1064.
- EFSA Panel on Plant Health (PLH). 2016. Treatment solutions to cure *Xylella fastidiosa* diseased plants. *EFSA J.* 14:e04456.
- EFSA Panel on Plant Health (EFSA PLH Panel), Bragard, C., Dehnen-Schmutz, K., Di Serio, F., Gonthier, P., Jacques, M.-A., Jacques Miret, J. A., Justesen, A. F., MacLeod, A., Magnusson, C. S., Milonas, P., Navas-Cortés, J. A., Potting, R., Reignault, P. L., Thulke, H.-H., Van der Werf, W., Vicent Civera, A., Yuen, J., Zappalà, L., Makowski, D., Delbianco, A., Maiorano, A., Muñoz Guajardo, I., Stancanelli, G., Guzzo, M., and Parnell, S. 2019. Effectiveness of in planta control measures for *Xylella fastidiosa*. *EFSA J.* 17:e05666.

- El Hadrami, A., Adam, L. R., El Hadrami, I., and Daayf, F. 2010. Chitosan in plant protection. *Mar. Drugs* 8:968-987.
- Elgadir, M. A., Uddin, M. S., Ferdosh, S., Adam, A., Chowdhury, A. J. K., and Sarker, M. Z. I. 2015. Impact of chitosan composites and chitosan nanoparticle composites on various drug delivery systems: A review. *J. Food Drug Anal.* 23:619-629.
- EPPO. 2023. PM 7/24 (5) *Xylella fastidiosa*. EPPO Bull. 53:205-276.
- EPPO Global Database. 2018. Eradication of the isolated infestation of *Xylella fastidiosa* in Germany. EPPO Reporting Service no. 07-2018.
- EPPO Global Database. 2019a. First report of *Xylella fastidiosa* in Israel. EPPO Reporting Service no. 06-2019. <https://gd.eppo.int/reporting/article-6551>
- EPPO Global Database. 2019b. First report of *Xylella fastidiosa* subsp. *multiplex* in Portugal. EPPO Reporting Service no. 01-2019.
- EPPO Global Database. 2024. *Xylella fastidiosa* subsp. *fastidiosa* detected on grapevine in Italy. EPPO Reporting Service no. 03-2024.
- European Food Safety Authority (EFSA), Giblin, D., Pasinato, L., and Delbianco, A. 2023. Update of the *Xylella* spp. host plant database – systematic literature search up to 31 December 2022. EFSA J. 21:e08061.
- Fournier, B., Pereira Dos Santos, S., Gustavsen, J. A., Imfeld, G., Lamy, F., Mitchell, E. A. D., Mota, M., Noll, D., Planchamp, C., and Heger, T. J. 2020. Impact of a synthetic fungicide (fosetyl-Al and propamocarb-hydrochloride) and a biopesticide (*Clonostachys rosea*) on soil bacterial, fungal, and protist communities. *Sci. Total Environ.* 738:139635.
- Francis, M., Civerolo, E. L., and Bruening, G. 2008. Improved bioassay of *Xylella fastidiosa* using *Nicotiana tabacum* cultivar SR1. *Plant Dis.* 92:14-20.
- Frem, M., Santeramo, F. G., Lamonaca, E., El Moujabber, M., Choueiri, E., La Notte, P., Nigro, F., Bozzo, F., and Fucilli, V. 2021. Landscape restoration due to *Xylella fastidiosa* invasion in Italy: Assessing the hypothetical public's preferences. *NeoBiota* 66:31-54.
- Galvani, C. D., Li, Y., Burr, T. J., and Hoch, H. C. 2007. Twitching motility among pathogenic *Xylella fastidiosa* isolates and the influence of bovine serum albumin on twitching-dependent colony fringe morphology. *FEMS Microbiol. Lett.* 268:202-208.
- Ge, Q., Cobine, P. A., and De La Fuente, L. 2020. Copper supplementation in watering solution reaches the xylem but does not protect tobacco plants against *Xylella fastidiosa* infection. *Plant Dis.* 104:724-730.
- Giampetruzzi, A., Morelli, M., Saponari, M., Loconsole, G., Chiumenti, M., Boscia, D., Savino, V. N., Martelli, G. P., and Saldarelli, P. 2016. Transcriptome profiling of two olive cultivars in response to infection by the CoDiRO strain of *Xylella fastidiosa* subsp. *pauca*. *BMC Genomics* 17:475.
- Gómez, L. M., Teixeira-Silva, N. S., Caserta, R., Takita, M. A., Marques, M. O. M., and de Souza, A. A. 2020. Overexpression of *Citrus reticulata* SAMT in *Nicotiana tabacum* increases MeSA volatilization and decreases *Xylella fastidiosa* symptoms. *Planta* 252:103.
- Harper, S. J., Ward, L. I., and Clover, G. R. G. 2010. Development of LAMP and real-time PCR methods for the rapid detection of *Xylella fastidiosa* for quarantine and field applications. *Phytopathology* 100:1282-1288.
- He, X., Deng, H., and Hwang, H.-m. 2019. The current application of nanotechnology in food and agriculture. *J. Food Drug Anal.* 27:1-21.
- Hilton, A., Jeong, M., Hsu, J.-H., Cao, F., Choi, W., Wang, X., Yu, C., and Jo, Y.-K. 2021. Thermal treatment using microwave irradiation for the phytosanitation of *Xylella fastidiosa* in pecan graftwood. *PLoS One* 16:e0244758.
- Hopkins, D. L. 1989. *Xylella fastidiosa*: Xylem-limited bacterial pathogen of plants. *Annu. Rev. Phytopathol.* 27:271-290.
- Johnson, K. A., Bock, C. H., Brannen, P. M., and Chen, J. 2022. A genome resource for *Xylella fastidiosa* subsp. *multiplex* strain P5A2 causing phony peach disease in the Southeastern United States. *Phytopathology* 112:2466-2470.
- Kandel, P. P., Lopez, S. M., Almeida, R. P. P., and De La Fuente, L. 2016. Natural competence of *Xylella fastidiosa* occurs at a high frequency inside microfluidic chambers mimicking the bacterium's natural habitats. *Appl. Environ. Microbiol.* 82:5269-5277.
- Kumaraswamy, R. V., Kumari, S., Choudhary, R. C., Pal, A., Raliya, R., Biswas, P., and Saharan, V. 2018. Engineered chitosan based nanomaterials: Bioactivities, mechanisms and perspectives in plant protection and growth. *Int. J. Biol. Macromol.* 113:494-506.
- Kyrkou, I., Pusa, T., Ellegaard-Jensen, L., Sagot, M.-F., and Hansen, L. H. 2018. Pierce's disease of grapevines: A review of control strategies and an outline of an epidemiological model. *Front. Microbiol.* 9:2141.
- Landa, B. B., Saponari, M., Feitosa-Junior, O. R., Giampetruzzi, A., Vieira, F. J. D., Mor, E., and Robatzek, S. 2022. *Xylella fastidiosa*'s relationships: The bacterium, the host plants, and the plant microbiome. *New Phytol.* 234:1598-1605.
- Li, J., and Zhuang, S. 2020. Antibacterial activity of chitosan and its derivatives and their interaction mechanism with bacteria: Current state and perspectives. *Eur. Polym. J.* 138:109984.
- Li, Y., Hao, G., Galvani, C. D., Meng, Y., Fuente, L. D. L., Hoch, H. C., and Burr, T. J. 2007. Type I and type IV pili of *Xylella fastidiosa* affect twitching motility, biofilm formation and cell-cell aggregation. *Microbiology* 153:719-726.
- Liccardo, A., Fierro, A., Garganese, F., Picciotti, U., and Porcelli, F. 2020. A biological control model to manage the vector and the infection of *Xylella fastidiosa* on olive trees. *PLoS One* 15:e0232363.
- Lieth, J. H., Meyer, M. M., Yeo, K.-H., and Kirkpatrick, B. C. 2011. Modeling cold curing of Pierce's disease in *Vitis vinifera* 'Pinot Noir' and 'Cabernet Sauvignon' grapevines in California. *Phytopathology* 101:1492-1500.
- Lindow, S. 2019. Money matters: Fueling rapid recent insight into *Xylella fastidiosa*—An important and expanding global pathogen. *Phytopathology* 109:210-212.
- Lopes, S. A., Ribeiro, D. M., Roberto, P. G., França, S. C., and Santos, J. M. 2000. *Nicotiana tabacum* as an experimental host for the study of plant–*Xylella fastidiosa* interactions. *Plant Dis.* 84:827-830.
- Maddox, C. E., Laur, L. M., and Tian, L. 2010. Antibacterial activity of phenolic compounds against the phytopathogen *Xylella fastidiosa*. *Curr. Microbiol.* 60:53-58.
- Marcelletti, S., and Scortichini, M. 2016. Genome-wide comparison and taxonomic relatedness of multiple *Xylella fastidiosa* strains reveal the occurrence of three subspecies and a new *Xylella* species. *Arch. Microbiol.* 198:803-812.
- Marchand, P. A. 2023. EU chemical plant protection products in 2023: Current state and perspectives. *Agrochemicals* 2:106-117.
- Marchi, G., Rizzo, D., Ranaldi, F., Ghelardini, L., Ricciolini, M., Scarpelli, I., Drosera, L., Goti, E., Capretti, P., and Surico, G. 2018. First detection of *Xylella fastidiosa* subsp. *multiplex* DNA in Tuscany (Italy). *Phytopathol. Mediterr.* 57:363-364.
- Martelli, G. P. 2016. The current status of the quick decline syndrome of olive in southern Italy. *Phytoparasitica* 44:1-10.
- Meng, Y., Li, Y., Galvani, C. D., Hao, G., Turner, J. N., Burr, T. J., and Hoch, H. C. 2005. Upstream migration of *Xylella fastidiosa* via pilus-driven twitching motility. *J. Bacteriol.* 187:5560-5567.
- Mirasoli, M., Bonvicini, F., Lovecchio, N., Petrucci, G., Zangheri, M., Calabria, D., Costantini, F., Roda, A., Gallinella, G., Caputo, D., de Cesare, G., and Nascetti, A. 2018. On-chip LAMP-BART reaction for viral DNA real-time bioluminescence detection. *Sens. Actuators B Chem.* 262:1024-1033.
- Mitrano, D. M., Motellier, S., Clavaguera, S., and Nowack, B. 2015. Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products. *Environ. Int.* 77:132-147.
- Morente, M., Ramírez, M., Lago, C., de las Heras-Bravo, D., Benito, A., Moreno, A., and Fereres, A. 2022. Habitat manipulation for sustainable management of *Philaenus spumarius*, the main vector of *Xylella fastidiosa* in Europe. *Pest Manag. Sci.* 78:4183-4194.
- Nair, R., Varghese, S. H., Nair, B. G., Maekawa, T., Yoshida, Y., and Kumar, D. S. 2010. Nanoparticulate material delivery to plants. *Plant Sci.* 179:154-163.
- Oliver, J. E., Sefick, S. A., Parker, J. K., Arnold, T., Cobine, P. A., and De La Fuente, L. 2014. Ionome changes in *Xylella fastidiosa*-infected *Nicotiana tabacum* correlate with virulence and discriminate between subspecies of bacterial isolates. *Mol. Plant-Microbe Interact.* 27:1048-1058.
- Olmo, D., Nieto, A., Adrover, F., Urbano, A., Beidas, O., Juan, A., Marco-Noales, E., López, M. M., Navarro, I., Monterde, A., Montes-Borrego, M., Navas-Cortés, J. A., and Landa, B. B. 2017. First detection of *Xylella fastidiosa* infecting cherry (*Prunus avium*) and *Polygala myrtifolia* plants, in Mallorca Island, Spain. *Plant Dis.* 101:1820.
- Orfei, B., Moretti, C., Loreti, S., Tatulli, G., Onofri, A., Scotti, L., Aceto, A., and Buonauro, R. 2023. Silver nanoclusters with Ag^{2+/3+} oxidative states are a new highly effective tool against phytopathogenic bacteria. *Appl. Microbiol. Biotechnol.* 107:4519-4531.
- Park, K.-C., and Chang, T.-H. 2012. Effect of chitosan on microbial community in soils planted with cucumber under protected cultivation. *Hortic. Sci. Technol.* 30:261-269.
- Pavan, S., Vergine, M., Nicoli, F., Sabella, E., Aprile, A., Negro, C., Fanelli, V., Savoia, M. A., Montilon, V., Susca, L., Delvento, C., Lotti, C., Nigro, F., Montemurro, C., Ricciardi, L., De Bellis, L., and Luvisi, A. 2021. Screening of olive biodiversity defines genotypes potentially resistant to *Xylella fastidiosa*. *Front. Plant Sci.* 12:723879.
- Pereira, W. E. L., Fischer, E., Souza-Neto, R. R., Saldarelli, P., Saponari, M., Coletta-Filho, H. D., and De Souza, A. A. 2017. Characterization of *Nicotiana tabacum* as an alternative host for *X. fastidiosa* subsp. *pauca*. Page 40 in: Proceedings of the European Conference on *Xylella fastidiosa*: Finding Answers to a Global Problem, Palma de Mallorca, Spain, 13-15 November 2017.
- Pereira, W. E. L., de Andrade, S. M. P., Del Ponte, E. M., Esteves, M. B., Canale, M. C., Takita, M. A., Coletta-Filho, H. D., and De Souza, A. A. 2020. Severity assessment in the *Nicotiana tabacum*–*Xylella fastidiosa* subsp. *pauca* pathosystem: Design and interlaboratory validation of a standard area diagram set. *Trop. Plant Pathol.* 45:710-722.

- Qin, C., Li, H., Xiao, Q., Liu, Y., Zhu, J., and Du, Y. 2006. Water-solubility of chitosan and its antimicrobial activity. *Carbohydr. Polym.* 63:367-374.
- Quetglas, B., Olmo, D., Nieto, A., Borràs, D., Adrover, F., Pedrosa, A., Montesinos, M., de Dios García, J., López, M., Juan, A., and Moralejo, E. 2022. Evaluation of control strategies for *Xylella fastidiosa* in the Balearic Islands. *Microorganisms* 10:2393.
- Riseh, R. S., Hassanisaadi, M., Vatankhah, M., Babaki, S. A., and Barka, E. A. 2022. Chitosan as a potential natural compound to manage plant diseases. *Int. J. Biol. Macromol.* 220:998-1009.
- Rodriguez, C. M., Obando, J. J., Villalobos, W., Moreira, L., and Rivera, C. 2001. First report of *Xylella fastidiosa* infecting coffee in Costa Rica. *Plant Dis.* 85:1027.
- Sanna, F., Mori, N., Santoiemma, G., D'Ascenzo, D., Scottillo, M. A., and Marini, L. 2021. Ground cover management in olive groves reduces populations of *Philaenus spumarius* (Hemiptera: Aphrophoridae), vector of *Xylella fastidiosa*. *J. Econ. Entomol.* 114:1716-1721.
- Saponari, M., Boscia, D., Nigro, F., and Martelli, G. P. 2013. Identification of DNA sequences related to *Xylella fastidiosa* in oleander, almond and olive trees exhibiting leaf scorch symptoms in Apulia (southern Italy). *J. Plant Pathol.* 95:668.
- Savary, S., Willocquet, L., Pethybridge, S. J., Esker, P., McRoberts, N., and Nelson, A. 2019. The global burden of pathogens and pests on major food crops. *Nat. Ecol. Evol.* 3:430-439.
- Schiavi, D., Francesconi, S., Taddei, A. R., Fortunati, E., and Balestra, G. M. 2022. Exploring cellulose nanocrystals obtained from olive tree wastes as sustainable crop protection tool against bacterial diseases. *Sci. Rep.* 12:6149.
- Scholten, R., Sanchez, L. M., Hornero, A., Navas-Cortes, J. A., Zarco-Tejada, P. J., and Beck, P. S. A. 2019. Monitoring the impact of *Xylella* on Apulia's olive orchards using Sentinel-2 satellite data and aerial photographs. Second European Conference on *Xylella fastidiosa*, Ajaccio.
- Scortichini, M., Chen, J., De Caroli, M., Dalessandro, G., Pucci, N., Modesti, V., L'aurora, A., Petriccione, M., Zampella, L., Mastrobuoni, F., Migoni, D., Del Coco, L., Girelli, R. C., Piacente, F., Cristella, N., Marangi, P., Laddomada, F., Di Cesare, M., Cesari, G., Fanizzi, F. P., and Loreti, S. 2018. A zinc, copper and citric acid biocomplex shows promise for control of *Xylella fastidiosa* subsp. *pauca* in olive trees in Apulia region (southern Italy). *Phytopathol. Mediterr.* 57:48-72.
- Scortichini, M., Loreti, S., Pucci, N., Scala, V., Tatulli, G., Verweire, D., Oehl, M., Widmer, U., Codina, J. M., Hertl, P., Cesari, G., De Caroli, M., Angilè, F., Migoni, D., Del Coco, L., Girelli, C. R., Dalessandro, G., and Fanizzi, F. P. 2021. Progress towards sustainable control of *Xylella fastidiosa* subsp. *pauca* in olive groves of Salento (Apulia, Italy). *Pathogens* 10:668.
- Sicard, A., Zeilinger, A. R., Vanhove, M., Schartel, T. E., Beal, D. J., Daugherty, M. P., and Almeida, R. P. P. 2018. *Xylella fastidiosa*: Insights into an emerging plant pathogen. *Annu. Rev. Phytopathol.* 56:181-202.
- Simko, I., and Piepho, H.-P. 2012. The area under the disease progress stairs: Calculation, advantage, and application. *Phytopathology* 102:381-389.
- Soenen, S. J., Parak, W. J., Rejman, J., and Manshian, B. 2015. (Intra)Cellular stability of inorganic nanoparticles: Effects on cytotoxicity, particle functionality, and biomedical applications. *Chem. Rev.* 115:2109-2135.
- Stark, W. J., Stoessel, P. R., Wohlleben, W., and Hafner, A. 2015. Industrial applications of nanoparticles. *Chem. Soc. Rev.* 44:5793-5805.
- Strona, G., Carstens, C. J., and Beck, P. S. A. 2017. Network analysis reveals why *Xylella fastidiosa* will persist in Europe. *Sci. Rep.* 7:71.
- Su, X., Li, B., Chen, S., Wang, X., Song, H., Shen, B., Zheng, Q., Yang, M., and Yue, P. 2024. Pore engineering of micro/mesoporous nanomaterials for encapsulation, controlled release and variegated applications of essential oils. *J. Control. Release* 367:107-134.
- Sudarshan, N. R., Hoover, D. G., and Knorr, D. 1992. Antibacterial action of chitosan. *Food Biotechnol.* 6:257-272.
- Tilman, D., Cassman, K. G., Matson, P. A., Naylor, R., and Polasky, S. 2002. Agricultural sustainability and intensive production practices. *Nature* 418:671-677.
- Trela-Makowej, A., Orzechowska, A., and Szymańska, R. 2024. Less is more: The hormetic effect of titanium dioxide nanoparticles on plants. *Sci. Total Environ.* 910:168669.
- Trkulja, V., Tomić, A., Iličić, R., Nožinić, M., and Milovanović, T. P. 2022. *Xylella fastidiosa* in Europe: From the introduction to the current status. *Plant Pathol. J.* 38:551-571.
- Vanhove, M., Retchless, A. C., Sicard, A., Rieux, A., Coletta-Filho, H. D., De La Fuente, L., Stenger, D. C., and Almeida, R. P. P. 2019. Genomic diversity and recombination among *Xylella fastidiosa* subspecies. *Appl. Environ. Microbiol.* 85:e02972-18.
- Vergine, M., Meyer, J. B., Cardinale, M., Sabella, E., Hartmann, M., Cherubini, P., De Bellis, L., and Luvisi, A. 2020. The *Xylella fastidiosa*-resistant olive cultivar "Leccino" has stable endophytic microbiota during the olive quick decline syndrome (OQDS). *Pathogens* 9:35.
- Verhaegen, M., Mahillon, J., Caulier, S., Mingeot-Leclercq, M.-P., and Bragard, C. 2024. Data collection on antibiotics for control of plant pathogenic bacteria. EFSA Support. Publ. 21:8522E.
- Wang, W., Meng, Q., Li, Q., Liu, J., Zhou, M., Jin, Z., and Zhao, K. 2020. Chitosan derivatives and their application in biomedicine. *Int. J. Mol. Sci.* 21:487.
- Zhang, Y., and Goss, G. G. 2022. Nanotechnology in agriculture: Comparison of the toxicity between conventional and nano-based agrochemicals on non-target aquatic species. *J. Hazard. Mater.* 439:129559.
- Zicca, S., De Bellis, P., Masiello, M., Saponari, M., Saldarelli, P., Boscia, D., and Sisto, A. 2020. Antagonistic activity of olive endophytic bacteria and of *Bacillus* spp. strains against *Xylella fastidiosa*. *Microbiol. Res.* 236:126467.