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RESEARCH PAPERS

A zinc, copper and citric acid biocomplex shows promise for control of *Xylella fastidiosa* subsp. *pauca* in olive trees in Apulia region (southern Italy)

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Summary. The bacterium *Xylella fastidiosa* subsp. *pauca* is associated with the "olive quick decline syndrome" in the Apulia region of southern Italy. To investigate control of this phytopathogen, a compound containing zinc and copper complexed with citric-acid hydracids (Dentamet®) was evaluated for *in vitro* and *in planta* bactericidal activity. Confocal laser scanning microscopy, fluorescent quantification and atomic emission spectroscopy were then used to determine if the compound reached the xylem networks of leaves, twigs and branches of olive, to release zinc and copper within the xylem. A 3-year field trial in an olive orchard containing mature Cellina di Nardò and Ogliarola salentina olive trees, and officially declared infected by *X. fastidiosa* subsp. *pauca*,was also carried out o to determine if the compound affected severity of the disease. Each year, from early April to October (excluding July and August), six spray treatments of 0.5% (v:v) Dentamet® were applied on the olive tree crowns. The compound reduced severity of symptoms in both cultivars. Most untreated trees died by the end of the trial, whereas all treated trees survived with good vegetative status as assessed by a normalized difference vegetation index. Quantitative real-time PCR was performed from June 2016 to September 2017, following the official procedures established by the European and Mediterranean Plant Protection Organization. The analysis revealed a statistically significant reduction of *X. fastidiosa* cell densities within the leaves of treated trees. These promising results suggest that integrated management to reduce severity of *X. fastidiosa* that includes regular pruning and soil harrowing with spring and summer spray treatments with Dentamet®, is likely to effectively control the disease.

Key words: Olive Quick Decline Syndrome, quarantine bacteria, endotherapy, confocal laser scanning microscopy, quantitative real-time PCR.

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Introduction

Xylella fastidiosa is a xylem-limited, γ-proteobacteria categorized as a quarantine plant pathogen by the European and Mediterranean Plant Protection Organization (EPPO) and the Committee of Plant Health of South America. In the United States *X. fastdiosa* subsp. *pauca*, the causal agent of citrus variegated chlorosis (CVC), is a regulated biological agent, and it is included in the Agricultural Bioterrorism Protection Act (Ancona *et al*., 2010). The European Union included *X. fastidiosa* in the Council Directive 2000/29/EC, All. I., Part. A, Sec. I and in the A1 list of EPPO quarantine pests and diseases. This Directive defines all the measures to be taken to avoid the introduction of the bacterium and its spread within all Member States, providing "legal obligation to abide by, once the organism is known to be present. Irrespective of the symptoms and subspecies concerned, all necessary measures to eradicate it, or if that is impossible, to inhibit its further spread, must be taken". Recently, the pathogen has been moved from the A1 to the A2 list (EPPO, 2017b) due to its establishment in southern Europe.

Strains of *X. fastidiosa* subsp. *pauca* were determined to be the main agent of the so called "olive quick decline syndrome" (OQDS) in the Salento peninsula (Apulia region, southern Italy) (Saponari *et al*., 2013; Martelli *et al*., 2015). The pathogen was presumably introduced from Central America (Marcelletti and Scortichini 2016; Giampetruzzi *et al*., 2017). Strains of the other two *X. fastidiosa* subspecies, subspp. *multiplex* and *fastidiosa*, were also recently found in southern France (Corsica and Maritime Alps) (EPPO, 2015a; 2015b), in southern Spain (Balearic Islands and Alicante province) (EPPO, 2016b; 2017a) and Germany (a single greenhouse in Saxony) (EPPO, 2016a).

The OQDS is a particularly severe disease that has spread through many hectares of Lecce province and, more recently, within the provinces of Brindisi and Taranto (Salento peninsula). Main symptoms include extensive leaf, twig and branch wilting, frequently accompanied by plant death. Consequently, upon implementation of the decision of the European Union 2015/2417/EU of 17th December 2015, the Italian Ministry of Agriculture, Food and Forestry released (18th February 2016), a new Ministerial Decree, updating a former decree titled "Emergency measures for the prevention and eradication of *X. fastidiosa* in the territory of Italian Republic". The decree delineated a series of mandatory technical and legislative measures aimed at preventing further disease spread.

Eradication (i.e., the complete removal and subsequent destruction of the infected field(s) and/or orchard(s)) is one pathogen control strategy applied after the involuntary introduction of a quarantine pest into a pest-free territory. However, to remove the initial disease focus the crop in question should be uprooted immediately upon identifying the pathogen. In some circumstances, when a new and unexpected introduction of a pest occurs, long periods are required for correct identification of causal agents. In these cases, the pathogens can become established on crops and in the environment, so that, for woody species in particular, effective eradication becomes very difficult. The OQDS symptoms in Apulia appeared suddenly towards the end of the 2000–2010 decade, in a restricted area of Gallipoli (Lecce province), but at the time of the etiology determination, in October 2013, the affected area was estimated at ca. 8,000 ha (Martelli, 2016).

Eradication of newly emerging plant pathogens is usually most effective with herbaceous crops that can be easily uprooted and substituted in fields or greenhouses, as seen for *Ralstonia solanacearum*-infected potato, eggplant, anthurium and geranium plants (Persson, 1998; Norman *et al*., 2009; EPPO, 2017c). In contrast, eradication efficacy with woody host species is usually transient.Indeed, repeated, robust, and large-scale attempts to eradicate *Xanthomonas axonopodis* pv. *citri*, the causal agent of citrus canker and another EPPO A1 quarantine pathogen, have failed in the long term in Florida and Brazil. Despite prolonged investment, eradication remains unsuccessful, and currently the only practical solution is to actively manage disease outbreaks (Schubert *et al*., 2001; Behlau *et al*., 2016). Similarly, *X. fastidiosa* elimination from Salento is not feasible due to the large area of infection determined from field monitoring (Almeida, 2016).

Xylella fastidiosa is a plant pathogenic bacterium that is not effectively and directly controllable using agrochemicals (Hopkins and Purcell, 2002; Cardinali *et al*., 2012; Martelli *et al*., 2015). This is mainly due to the inability of bactericides to penetrate host xylem vessels where the pathogen is located (Goodwin *et al*., 1988; Cobine *et al*., 2013). Additionally, the bacterium is spread in the environment through the feeding activities of insect vector(s), which are not easily controlled with insecticides (Hopkins and Purcell, 2002; Janse and Obradovic, 2010). Several alternative

strategies have been proposed to limit *X. fastidiosa*-induced damage, including the use of naturally or genetically engineered resistant or tolerant cultivars, cross-protection with avirulent or weakly-virulent *X. fastidiosa* strains, phage therapy and paratransgenic transformation of symbiotic bacteria (Hopkins and Purcell, 2002; Bruening *et al*., 2004; Arora *et al*., 2015; Das *et al*., 2015).

For Pierce's disease of *Vitis vinifera*, one of the main problems caused by *X. fastidiosa*in grapevine in the U.S.A., Kirkpatrick *et al.* (2003) stated: "if methods could be developed to effectively deliver prophylactic or therapeutic bactericides into grapevine, this could provide a comparatively straightforward solution to a very complex disease problem". Within the host xylem complex network, *X. fastidiosa* colonizes by forming biofilms and releasing cell wall-degrading enzymes to damage intervessel pit membranes (Perez-Donoso *et al*., 2010). Research on whether ions can influence *X. fastidiosa* growth, as biofilm and planktonic cells, revealed that at certain concentrations, copper (>200 μM) and zinc (0.25 mM) inhibited biofilm formation (Cobine *et al*., 2013). Other studies found that biofilm is less susceptible than planktonic cells to copper (Rodrigues*et al.*, 2008), while sublethal concentrations of zinc induce biofilm exopolysaccharide formation (Navarrete and De La Fuente, 2014). Zinc detoxification is required for full *X. fastidiosa* virulence, suggesting that the pathogen needs to remove zinc from host plant tissues before colonization can begin (Navarrete and De La Fuente, 2015). Related to these findings are the assumptions that the zinc concentration in the xylem represents a preformed defense that limits the growth of the bacterium, and that manipulation of plant mineral levels could be a potential disease management strategy (Navarrete and De La Fuente, 2015).

Based on these assumptions, we tested the field effectiveness of a zinc, copper and citric acid biocomplex **(**Dentamet®) for controlling *X. fastidiosa* on olive trees grown in the Salento area. The product is a water-soluble fertilizer comprised of zinc (4.0% w:w) and copper (2.0% w:w) complexed with hydracids of citric acid, obtained through natural fermentation processes using soil fungi. Patented in Israel, the biocomplex (specific weight = 1.280 gL^{-1}) has low environmental impact and is suitable for organic farming. Here, we focused on reduction of disease severity (Dowdle, 1998) for a quarantine phytopathogen. The goal was to decrease pathogen cell density in host plants, allowing for continued tree growth and yield, and thus avoiding uprooting. For this study we chose an orchard that had been ascertained to be infected by *X. fastidiosa* by the Phytosanitary Service of Apulia Region.

The specific aims of the study were as follows: first, we verified the *in vitro* and *in planta* bactericidal activity of Dentamet® towards *X. fastidiosa*. Next, we used spray treatments on the tree canopy of the affected olive orchard, along with endotherapy, to ascertain the fertilizer's ability to reach leaf, twig, and branch xylem tissues. These assessments were performed with confocal laser scanning microscopy, fluorescence quantification, and measuring zinc and copper content using inductively coupled plasma atomic emission spectroscopy. Third, we tested the effectiveness of Dentamet® for reducing disease symptoms over a 3-year period under field conditions. We used quantitative real-time PCR to determine *X. fastidiosa* density in olive leaves after spray and endotherapy treatments, and to assess the status of treated and non-treated olive trees through the normalized difference vegetation index. Zinc and copper content were also assessed in olive fruits collected at harvest. We then began to implement a *X. fastidiosa* control strategy for management of the pathogen in the infected area.

Materials and methods

Preliminary assays for inhibition efficacy of Dentamet® against *Xylella fastidiosa*

The zinc, copper and citric acid biocomplex to be tested, Dentamet® (Diagro srl, Italy), was assessed for bactericidal effects towards *X. fastidiosa* using two *in vitro* and two *in planta* assays.

For *In vitro* assay 1, a log-phase pure culture of *X. fastidiosa* subsp. *fastidiosa* strain M23 was used. This strain was originally isolated in California (U.S.A.) from almond (*Prunus amygdali*) trees showing symptoms of leaf scorching (Chen *et al*., 2005). Loopfuls of *Xylella* culture were streaked vertically and horizontally onto PW (Davis *et al*., 1983) plates. Sterile Whatman filter paper disks (8 mm diam.) were saturated with serial dilutions (1:20, 1:200, or 1:2,000) of Dentamet® in sterile water and placed on separate PW plate surfaces. The experiment was performed in triplicate, and the inhibition zones around the paper discs were checked 14 d later. *In vitro* assay 2 assessed the capability of Dentamet® to inhibit growth of

strain M23 in PW broth. Tubes containing PW broth (10 ml) were prepared with serial ten-fold dilutions (from 1:10 to 1:100,000) of Dentamet®. Each tube was then inoculated with 100 µl of *X. f.* subsp. *fastidiosa* M23 culture photometrically determined to be in log phase of multiplication. The tubes were placed in incubator at 28°C, under shaking. Bacterial growth was checked 6 and 15 d after the inoculation by SYBR Green real-time PCR (1 µL of 1:10 dilution of culture) using a primer set (Teme150fc = 5′-tctaccttatcgtgggg-gac-3′; Teme454rg = 5′-aacaactaggtattaaccaattgcc-3′) described by Chen *et al*. (2005). A dilution series using known concentrations of *X. fastidiosa* DNA for the standard curve was included in the PCR. Moreover, 15.0 µL of the *X. fastidiosa* M13 culture in each Dentamet® dilution were placed onto PW plates to assess bacterial viability as shown by colony formation. Bacterial growth was monitored after 7 d incubation.

In planta assay 1 was carried out in October 2016. Almond leaves showing symptoms of leaf scorching caused by *X. fastidiosa* were collected from an orchard in California known to be infected by *X. fastidiosa* (Supplementary Figure 1). In parallel, dilution series (1:10, 1:100, 1:200, 1: 1,000 or 1:10,000) of Dentamet® in water were prepared and 100 µL of each dilution were placed into each well of a 96 sterile microplate (200 µL capacity per well). A single symptomatic olive leaf was placed in each well overnight with the petiole portion of each leaf in each solution. For each dilution, four leaves were tested. Control leaves were placed in cells containing sterile water. One day later isolations were made from the leaves for recovery of *X. fastidiosa*. These were carried out on PW medium according to standardized procedures (Chen *et al*., 2005). The PW plates (i.e., four per Dentamet® dilution) were checked for *X. fastidiosa* growth 14–28 d after inoculation.

For *in planta* assay 2, almond leaves showing leaf scorching symptoms were sampled in October 2016, and were then each soaked for 15 min in sterile 50 ml tubes containing serial dilutions (1:20, 1:200 or 1:2,000) of Dentamet® in water. The leaves were then drained and air-dried overnight. For each dilution, four leaves were tested. Control leaves were soaked with sterile water. One day after the treatments, isolations from the leaves for recovering *X. fastidiosa* were carried out on PW medium according to standardized procedures (Chen *et al*., 2005). The PW isolation plates were checked for *X. fastidiosa* growth at 14 and 28 d after inoculation.

Systemic movement of Dentamet® within olive trees

To verify the capability of Dentamet® to reach the xylem of leaves, petioles, peduncles, twigs and branches of adult olive trees, an *ad hoc* investigation was carried out by coupling field and laboratory investigations. In the field study, 20-year-old olive trees (cv. Ogliarola salentina) were used, that were grown in the province of Taranto at a site where *X. fastidiosa* had not been detected (confirmed by the official monitoring surveys carried out by the Phytosanitary Service of Apulia Region). During the previous 4 years, the trees did not receive any pruning, and, at the time of investigation, did not show any visual symptoms of diseases. Trees each with at least three branches, were used for monitoring the systemic movement of Dentamet®. In the main trunk of each tree, an injection point (1.5 cm depth) was made using a drill, at 20 cm above the soil. This was filled with an ArborBioKaps® valve (8.0 mm diam.), patented for tree endotherapy. The trunk wound was protected from infection by microorganisms allowing the entering of pump beak. Safranin-O was used to elucidate the water-conducting pathways in trees (Orians *et al*., 2004; Umebayashi *et al*., 2007; Kim *et al*., 2014). This dye adheres to negatively charged plant cell wall polysaccharides (Sano *et al*., 2005). Safranin-O (Sigma-Aldrich), at 0.1% w:v, was mixed in deionized water with Dentamet®, at 0.5% v:v. An elastomeric pump (100 mL capacity) for continuous release of liquid (2.0 mL h^{-1}) was used for the trial. The pump was filled with the safranin-O/Dentamet® mixture and fitted into the endotherapy valve. The mobility of safranin-O alone was also assessed in the same way on six trees, as experimental controls. The experiments were repeated in two years. Data (see below) were subjected to ANOVA. Mean values were compared by Fisher's LSD test at (P<0,05) using SPSS software package, version 20.0 (SPSS Inc.). In addition, safranin-O and the safranin-O/Dentamet® mixture were also applied using the same system to some branches for further analyses.

The experiments were performed at the end of May 2015 and 2017, starting at 9.30 a.m. on the respective assessment days. Weather conditions were sunny days, with temperatures of 20 to 27°C and humidity of 35% in 2015, and 20 to 29°C and humidity of 35% in 2017. The mobility of the tracking dye was monitored at 4h, 24h and 48h after pump admission of the mixture into the injection points, by performing longitudinal sections along the trunk and the branches of each treated tree, and by measuring the length from

the injection point to the most distant point reached by the tracking dye.

In addition, migration of the dye mixture within each tree was also checked by inserting a disposable pharmaceutical syringe (10 mL capacity) without the needle into an injection point in the main trunk of the tree, at 20 cm above soil level. Ten mL of the safranin-O/Dentamet® mixture (0.1% safranin-O and 0.5% Dentamet®) were put into the syringe. Movement within the tree of the mixture was assessed as described for the elastomeric pump experiment. This experiment took place at the end of May 2017 at the same location, and six additional olive cv. Ogliarola salentina trees were used for each treatment, including safranin-O only experimental controls.

For olive leaves, the corresponding petioles, fruit peduncles, 2- and 5-year-old twigs, assessments were carried out either on samples sprayed with safranin-O at 0.1 % w:v or on samples sprayed with a mixture of safranin-O (0.1% w:v) and Dentamet® (0.5% v:v). Different cv. Ogliarola salentina trees growing in the same orchard were used for the trial. The trees were sprayed in the morning using an atomizer, applying 15 L of spray mixture to each tree. Samples were collected 48h afterwards. Samples from these trials were also used to assess diffusion of Dentamet® within treated trees using confocal laser scanning microscopy and for determining the Zn^{++} and Cu^{++} ions content in leaves and woody tissues(see below).

Assessment of systemic movement of Dentamet® within olive trees through confocal laser scanning microscopy and fluorescence quantification

To further ascertain and possibly quantify the systemic movement of Dentamet® within tissues of olive trees, a parallel investigation was performed using confocal laser scanning microscopy (CLSM) with a Carl Zeiss LSM710 microscope. After 48h from the canopy spraying samples of twigs were removed with from the tree and immediately transferred to the laboratory for further processing. Free hand sections of central veins of individual leaves were cut with a razor blade, and the corresponding petioles, fruit peduncles and 2 and 5-year-old twigs were separately collected.

Concerning endotherapy, after 48h of perfusion, branches (about 10 years old, 1 m length, 23-26 mm width) were removed from treated trees using a saw and transferred to the laboratory. Starting from the point of infusion (0 cm) and upward at 40 cm and 80 cm heights, razor blade free-hand sections of conducting xylem were observed and quantified using CLSM. In parallel, samples (300 mg each) of transverse and longitudinal tangential sections of branches were obtained and used for quantification of Cu^{++} and Zn^{++} ions characterizing the cyclic structure of Dentamet®, as described above. The assessments were performed on three replicates per each sample. The sections were each mounted in a drop of water and were observed using the confocal microscope. Safranin-O fluorescence emission was recorded with a 569–594 nm filter set, excited by a 488-nm argon ion laser line, while the autofluorescence of plant material was excited by a 543-nm He–Ne laser line and recorded with a 656–735 nm filter set and, then, eliminated. The specific setting for CLSM on the emission spectrum of safranin-O was fundamental for the comparison of the images and the quantified graphs among the different treatments. For fluorescence quantification, the Profile Tool of the ZEN2012 program of the LSM 710 confocal microscope was used. By using standardized settings, the ratio between green and red fluorescence pixels was used as a fluorescence index. For each of three images/experiments, nine emission peaks were measured to produce the quantification analysis, expressed as histograms. Because of the presence of the two factors (distance from injection point and Dentamet® treatment) in the experiments, the fluorescence index values were analyzed by two-way ANOVA (SigmaStat software) to determine if each factor and their interaction had significant effects ($P<0.05$) on the fluorescence indices (Press *et al*., 1992). The power of the laser line, the gain, and the offset were set on control samples to eliminate the autofluorescence of plant material in the safranin-O window.

Zinc and copper contents in tissues after safranin-O and safranin-O/Dentamet® treatments

Zn^{++} and Cu^{++} ion concentrations in sections of central veins of intact leaves plus the corresponding petioles, fruit peduncles, and 2- and 5-year-old twigs and branches, obtained from olive trees treated with safranin-O or safranin-O/Dentamet® mixture were analyzed using the Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). The analyses were performed on the same samples assessed by CLSM. Samples (300 mg each) were each mixed with 4.0 mL of H_2O_2 and 6.0 mL of superpure HNO_3 69%,

then treated at 180°C for 10 min. using a microwave digestion system (Milestone START D). The samples were then cooled, diluted with superpure water to a final volume of 20.0 mL, filtered through syringe filters (pore size 0.45 μm), and then measured for element content using an ICP-AES (Thermo Scientific, iCap 6000 Series). The spectrometer was previously calibrated for quantitative analysis with five standard solutions containing known concentrations of the elements, of 0.001, 0.01, 0.1, 0.5, and 1.0 mg L^{-1}. The calibration lines showed correlation coefficients (r)greater than 0.999 for both Cu^{++} and Zn^{++}. The results were expressed as the average (± standard deviation) of three different measurements on three different samples, and the element concentrations were expressed as ppm (mg kg^{-1} of sample weight). Data were subjected to ANOVA. Mean values were compared by Fisher's LSD test at (P<0,05) using the SPSS software package, version 20.0 (SPSS Inc.). The same techniques were used to assess the Zn^{++} and Cu^{++}contents in leaves of cv. Ogliarola salentina and Cellina di Nardò that served as treated and control trees for the quantitative real-time PCR assessment of *X. fastidiosa* cell densities.

Field effectiveness of Dentamet® for reducing *Xylella fastidiosa* symptoms through spray treatment to tree canopies

The field effectiveness of Dentamet® for reducing the density of *X. fastidiosa* within tree canopies was assessed in an adult olive orchard (Lat: 40°20′50″31N; Long: 17°54′24″55E), located at Veglie (Lecce province, Salento peninsula), where the presence of *X fastidiosa* was officially ascertained by the Phytosanitary Service of Apulia Region before beginning the trial. The farm was chosen for the homogeneity of the soil characteristics, for the uniform canopy volume, and as representative of the cultivation techniques currently performed on olive trees in the region. The experimental 70-year-old orchard contained 40 trees of the cv. Ogliarola salentina and Cellina di Nardò, spaced at 12 × 12 m and managed according to the cultivation techniques of the area (no irrigation, nor regular pruning, soil fertilization, control of main pests and diseases, herbicide treatments).Soil physicochemical characteristics of the trial plot are shown in Supplementary Table 1.

The effectiveness of Dentamet® was tested for three consecutive years (2015, 2016 and 2017). The experimental plot included two facing blocks of 20 trees each (20 treated and 20 untreated trees). Each block contained ten Ogliarola salentina and ten Cellina di Nardò trees, in randomized positions along each row. At the beginning of the trial, the mean incidence and severity of disease did not differ between the two blocks (i.e., 20% of trees showed symptoms with 10% severity). Dentamet® was sprayed on the canopy of the trees using an atomizer, with the rate of the compound being equivalent to 3.9 L ha^{-1} (280 mL in 100 hL of water). At each treatment, each tree received at least 20 L of spray solution. Six spray treatments were applied in each year, commencing in early April and ceasing in October, but avoiding July and August (Supplementary Table 2). No other treatments to control pests or pathogens were supplied to the trees during the trials. Control trees did not receive any Dentamet® treatments during the three consecutive years. However, compounds routinely applied for controlling the main diseases and pests (olive peacock spot, olive knot, olive fruit fly) were applied to the control plants. Assessments of disease through spring, summer and autumn were made by counting, at each survey and for each tree of each cultivar, the total number of wilted twigs and branches through the whole tree canopy. This method allowed precise estimates of disease progression during the seasons by revealing also the possible twig re-sprouting upon application of treatments. Data were subjected to ANOVA. Mean values were compared by Fisher's LSD test at (P<0,05). Two-way analyses of variance (ANOVA) were also performed on mean monthly values to assess effects of cultivar and the cultivar × treatment interaction. Statistical analyses were performed using SPSS software package, version 20.0 (SPSS Inc.). Climate data of the area were obtained from the Rete Agrometeorologica Regionale of Apulia Region-Assocodipuglia, Salice salentino (LE) station (Lat: 40°23′26″N; 17°52′ 31″E), checked by ACCREDIA.

Zinc and copper contents in olive drupes after Dentamet® spray treatments

At the end of October 2017, olive drupes were collected from treated and untreated cv. Ogliarola salentina and Cellina di Nardò trees to assess their zinc and copper contents. In each block, about 3.0 kg of olives from each tree were collected, from three trees of each cultivar. Each tree sample was mixed, and 2.0 kg was used for the analyses. These were performed accord-

ing to UNI EN (2002) and UNI EN 15763 (2010) 13805 Foodstuffs determination of trace elements standard methods.

Assessments of *Xylella fastidiosa* populations within olive leaves by quantitative real-time PCR

To further corroborate the reductions of disease symptoms after Dentamet® treatments, and to verify whether the *X. fastidiosa* populations within the leaves decreased following the Dentamet® spray treatments. Quantitative real-time PCR (Harper *et al.*, 2010) was applied from the end June 2016 to the end September 2017 on four trees (two each of cv. Ogliarola salentina and Cellina di Nordò) located in the Veglie experimental orchard. Samplings were carried out during the last week of each month. The trees were officially notified for the presence of *X. fastidiosa* by the Phytosanitary Service of Apulia Region before commencing the trial even though the pathogen populations within the trees was unknown. Two of the trees (one Ogliarola salentina and one Cellina di Nardò) received the Dentamet® treatments as described above, while the other two were included in the control plot and did not receive the treatments. At the beginning of the assessment (June 2016),the treated Ogliarola salentina and Cellina di Nardò trees showed the similar severity of disease (i.e., about 10% of wilted twigs per tree). Samples of young twigs were obtained as described by Valentini *et al*. (2017). At each survey, 12 samples of woody twigs, of 15–20 cm length with mature leaves, were taken from each tree. Each sample consisted in eight to ten twigs. The 12 samples represent three different heights of the canopy (i.e., low, medium, high) and the four canopy cardinal points. At each sampling, scissors were disinfected with a sodium hypochlorite solution. The samples were each place in a refrigerated bag and processed *in loco* for the DNA extraction within 30–45 min from their removal from the trees. The laboratory samples consisted in a minimum of 25 mature leaves from which the petioles, midribs and basal parts (0.7 g) were used directly for the preparation of plant extracts. From the end May to the end September 2017, leaf samples were also taken from ten trees that had received the trunk injections in Galatone (see below). For each tree, three young sprouts were collected and processed in the same way as described for the trees at Veglie. Olive trees grown in the same orchard, showing some twig and branch wilting were used as experimental controls for assessing *X. fastidiosa* cell numbers.

Leaf extracts were obtained by crushing the vegetal tissues of each sample in a plastic bag (Bioreba) containing 5.0 mL of Food Lysis Buffer of the DNeasy® mericon® Food Kit (50) (Qiagen), to obtain an homogeneous sap. The samples were processed as reported in the EPPO Standard PM 7/24(2) *Xylella fastidiosa* (Normes OEPP/EPPO Standards, 2016), and following the kit manufacturer's instructions. The entire DNA extraction procedures were performed in a dedicated *in situ* portable workstation located close to the Veglie orchard. The DNA concentrations of samples were assessed by NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific), and each sample was diluted to a final concentration of 20 ngμL^{-1}. Aliquots of 1.0 μL of DNA were assessed in triplicate by real-time PCR (Harper *et al*.,2010) in 11.0 μL of final reaction volume. Positive DNA controls of *X. fastidiosa* subsp. *pauca* CoDiRO strain ST53 were used; they were provided by Maria Saponari (CNR-ISPS, Bari, Italy). The specificity of PCR products was checked using either DNA extracted from previously assessed healthy olive trees or water. For quantification purposes, a standard curve was generated for each experiment using 10-fold serial dilutions of bacterial gDNA (10.0 ng to 10.0 fg per PCR reaction). The bacterial concentration was detected in each unknown sample expressed as ng per sample or colony forming units (CFUs) per sample, by following the procedures of Modesti *et al*. (2017). Data obtained by real-time PCR were analyzed independently for each cultivar and were subjected to ANOVA. Mean values were compared by Fisher's LSD test ($P<0.05$) using SPSS software package, version 20.0 (SPSS Inc.).

Infrared assessment of vegetation status using Normalized Difference Vegetation Index (NDVI)

On October 9, 2017, an estimation of the overall physiological status of the two blocks of trees (i.e., treated and untreated) was carried out using Normalized Difference Vegetation Index (NDVI). This value provides a vegetation indices that can be correlated with photosynthetic activity of individual trees (Rouse *et al*., 1974). The index is based on the relationship between absorption of visible light and reflectance of near-infrared light by chlorophyll in vegetation (Viña *et al*., 2011; Nouri *et al*., 2014). The structure of plant leaves reflects the near-infrared light, so that the more leaves present on a plant the greater is the NDVI value. The index can be applied to the live veg-

etation. NDVI values close to 1.0 indicate the greatest possible densities of green leaves; for temperate tree species greatest values range from 0.60 to 0.80. For the analyses, a drone camera sensor Sequoia 4.9, 4608 × 3456 (RGB) was used, flying at 100 m height and using an average ground sampling distance (GSD) of 2.73 cm.Data were subjected to ANOVA. Mean values were compared by Fisher's LSD test at (P<0,05) using SPSS software package, version 20.0 (SPSS Inc.).

Trunk injection of very severely infected olive trees

In April 2017, ten 70-year-old olive cv. Ogliarola salentina trees, located in Galatone (Lecce province, Salento peninsula) that were very severely affected by *X. fastidiosa* (i.e., wilting ranging from 75 to 90% of the canopies) were chosen for testing the possibility of recovery from the disease through trunk injection of Dentamet®. Four injection points located at the base of the trunk of each tree were established as described above. A disposable plastic pharmaceutical syringe was filled with 5.0 mL of Dentamet® (Supplementary Figure 2). The trees continued to receive doses of the diluted compound according to the following ratios of Dentamet®:water: 1:2 (in May); 1:5 (in June); and 1:5 (in July). Control trees received distilled water. In parallel, five other trees were treated in the same way, starting in July. During the trial, no irrigation or fertilization was provided to the trees. The occurrence of *X. fastidiosa* within the trees was previously tested at the end of winter by sampling twigs according to the procedures described above for the Veglie orchard. The occurrence of *X. fastidiosa* within the leaves of the new shoots sprouting from the main branches after trunk injection treatments were checked at the end of May, the end of July and end of September 2017. Three shoots per tree were removed from each branch, stored in a refrigerated plastic bag and processed for DNA extraction within 45 min from the sampling, as described above for the Veglie orchard. The occurrence of the pathogen was similarly monitored in olive trees grown a few meters from the treated trees, but did not receive any treatment.

Implementation of an integrated control strategy for *Xylella fastidiosa*

After observations that the olive trees in the experimental orchards of Veglie showed reductions of *X. fastidiosa* symptoms,despite receiving no Dentamet® treatments during July and August, it was decided to apply an integrated disease management strategy that included some agronomic techniques that could help to reduce the effects of *X. fastidiosa* in olive orchards. Such a control strategy could utilize tree pruning, removal of weeds which are likely to harbour the *X. fastidiosa* vector *Philaenus spumarius*, and spray applications of Dentamet® also in July and August each growing season. In order to select a suitable pruning strategy, a preliminary assessment was carried out in 2015 and 2016 to determine appropriate pruning methods for trees severely affected by OQDS. This was carried out in two olive orchards located in Galatina and Galatone (Lecce province, Salento peninsula, *X. fastidiosa* infected zone) (Scortichini, 2017). Two olive farms, located at Oria (Brindisi province, Salento peninsula, *X. fastidiosa* infected zone) and Copertino (Lecce province, Salento peninsula, *X. fastidiosa* infected zone) were chosen in which 90 to 100-year-old cv. Cellina di Nardò trees were present. on both farms, the occurrence of *X. fastidiosa* was ascertained using the methods described above. In the Oria farm,on March 2017, ten infected trees were uprooted according to the first European Union eradication programme. The trees (27 at Oria, and 16 at Copertino) received Dentamet® spray treatments once per month, from the end of April to mid October, 2017, at the dose rate used at Veglie (see above). Control plants (five per farm) did not receive any treatment. In addition, the soil at the sites was harrowed during March-July to remove weeds likely to harbour *P. spumarius*, and the trees were pruned twice per year (at the end winter and the end summer) to remove wilted twigs affected by *X. fastidiosa*. Fertilization and pest control were carried out using routine practices. Assessments of *X. fastidiosa* symptoms through the seasons was carried out as described above.

Results

Preliminary *in vivo* and *in planta* assays for testing the effectiveness of Dentamet® for inhibition of *Xylella fastidiosa* growth

For *in vitro* assay 1, clear inhibition zones were measured of 11 mm for Dentamet® at 1:20 dilution, and 10 mm for 1:200 dilution. No inhibition zones occurred where the 1:2,000 dilution was used. For *In vitro* assay 2, real-time PCR analyses indicated complete inhibition of *X. fastidiosa* subsp. *fastidiosa* M23 growth

in PW medium resulting from Dentamet® diluted at 1:100, both after 6 or 15 d from inoculation. These results were confirmed by the absence of *X. fastidiosa* M23 growth on PW plates as recorded 7 d after inoculation. Furthermore, in *In planta* assay 1, there was clear indication that Dentamet® inhibited growth of *X. fastidiosa* from almond leaves showing symptoms of "almond leaf scorch" after overnight soaking inten-fold serial dilutions of the compound. No growth was observed after 14 and 28 d incubation on PW plates inoculated with loops from tubes containing Dentamet® diluted from 1:10 to 1:1,000. Growth was observed only on PW plates from the 1:10,000 dilution. Isolations performed on PW from control leaves allowed developed colonies of *X. fastidiosa*. Similarly, *In planta* assay 2 revealed the absence of *X. fastidiosa* growth on PW plates after isolations from almond leaves, both after 10 and 28 d incubation after inoculations from culture medium containing Dentamet® diluted from 1:20 to 1:2,000. All tests indicated capability of this compound to cause bactericidal effects on *X. fastidiosa* subsp. *fastidiosa* strain M23, both in the in *in vitro* and *in planta* assays performed with almond leaves with symptoms of leaf scorching.

Systemic movement of Dentamet® in olive tree tissues

The visual assessments and measurements of dye transmission in olive trees were performed at 4h, 24h and 48h post-endotherapy (elastomeric pump fitted to tree trunks), as observed in longitudinal trunk or branch sections. At all these timepoints, safranin-O/Dentamet® mixture reached further in woody tissues compared with safranin-O alone. Significantly different values between the two treatments were observed 24h and 48h after endotherapy. Moreover, the safranin-O treatment gave very pale staining, whereas the safranin-O/Dentamet® combination always gave intensered stained woody tissue (i.e. RAL: 3024; HEX: F 80.000).Similarly, safranin-O/Dentamet® ascended further than did safranin-O alone in the treatment where plastic syringes were fitted into the tree trunks. Significantly different values between the two treatments were observed 48h after endotherapy The average ascent distances from the injection points for the two endotherapy treatments are shown in the Supplementary Figures 3a and 3b, whereas Supplementary Figure 4 depicts the capability of safranin-O/Dentamet® infusion to reach internal tissues of olive twigs.

Assessment of systemic movement of Dentamet® within tissues of olive trees using CLSM, fluorescence quantification and concentrations of zinc and copper

The CLSM images of samples from safranin-O (Supplementary Figure 5) and safranin-O/Dentamet® (Figure 1) treatments showed green fluorescence which clearly defined the different histological elements of leaves (Supplementary Figures 5A, and 1A), petioles (Supplementary Figures 5B and 1B), fruit peduncles (Supplementary Figures 5C and 1C), and 2 and 5-year-old twigs (respectively, Supplementary Figures 5D and 1D, and Supplementary Figures 5E and 6E). The images of all sections from control plant tissues showed no green autofluorescence (emission recorded with 569-594 nm filter set excited by a 488-nm argon ion laser), whereas red autofluorescence (emission excited by 543-nm He-Ne laser line and recorded with the 656–735 nm filter set) was observed mainly in heavily lignified, cutinized, cuticularized and suberized cell walls of all cell types (Supplementary Figure 6). The CLSM images obtained from olive trees sprayed with safranin-O/Dentamet® mixtures were similar to those obtained from the safranin-O treatment (Figures 1A-F and Supplementary Figure 5). This was not surprising because Dentamet® does not emit fluorescence. Safranin-O moved horizontally direction probably by capillary action and diffusion before reaching the sap of the xylem vessels (Figures 1A-F).

The transverse sections of primary and secondary xylem of 2- and 5-year-old twigs, and the longitudinal tangential sections of 5-year-old twigs showed numerous axial unicellular xylem rays of parenchyma cells (Figures 1A–F). The nuclei of ray and axial parenchyma cells, visible as round dots, showed strong green fluorescence due to the affinity of safranin-O to nucleic acids (Figure 1G). Further, this fluorescent mark showed the continuum of extracellular cell walls and intracellular spaces and the apoplastic to symplastic movement of safranin-O at cellular level. The morphology of transversal sections of secondary xylem of mature branches observed at different distance from the perfusion point was similar same for both treatments (Figures 2A–F). However, considerable differences between the two treatments were observed in the fluorescence intensity assessments as confirmed by the relative fluorescence quantification measurements. Statistically significant ($P<0.05$) decreases of fluorescence index were observed as the distance from the safranin-O injection points increased, regardless of the presence of Dentamet® and

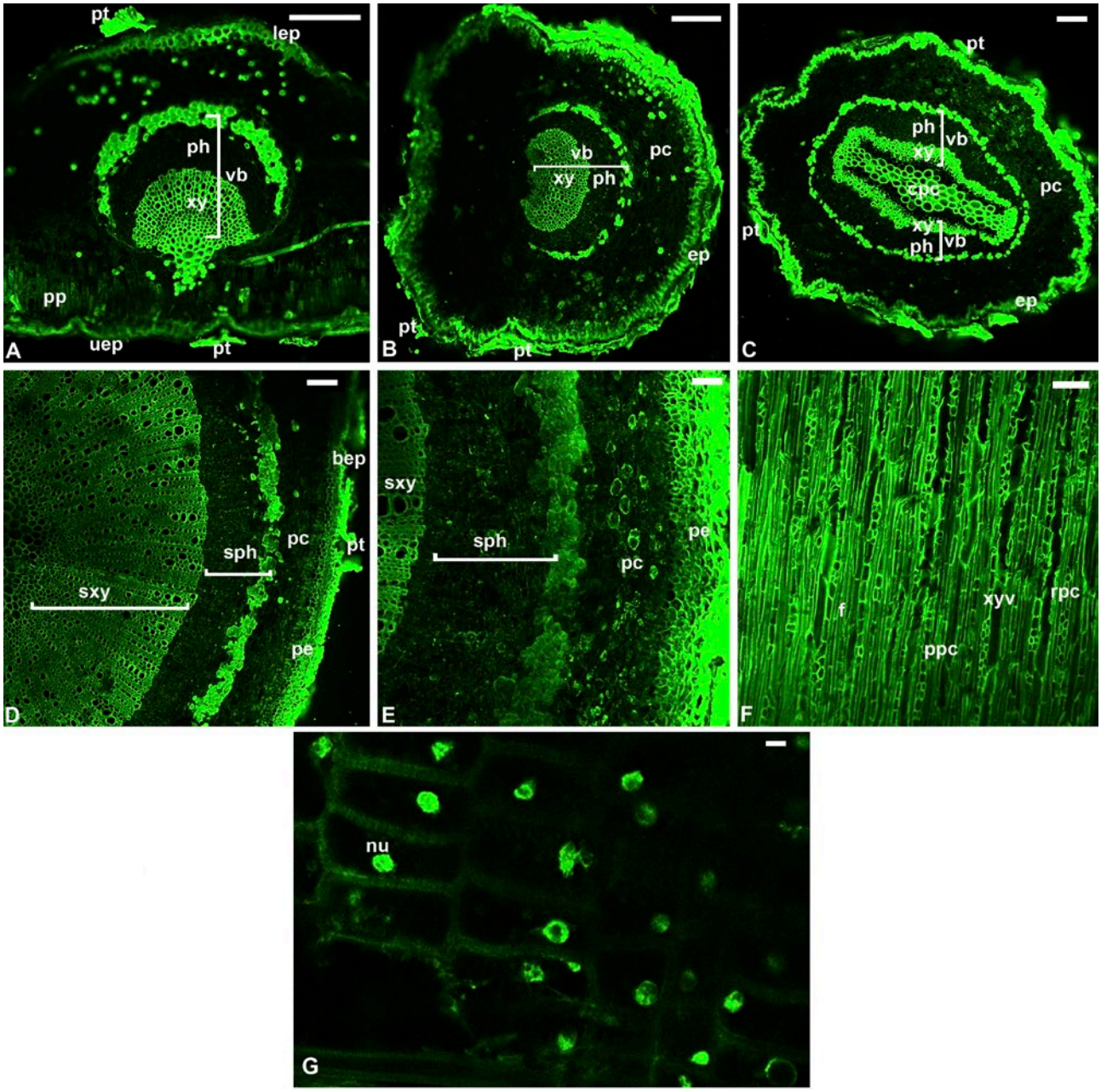

Figure 1. Confocal microscope images of transverse sections of leaf (A), petiole (B), peduncle of the fruit (C), two-year-old twig (D), five-year-old twig (E), and longitudinal tangential section of 5-year-old twig (F) excised from a healthy olive cv. Ogliarola salentina tree sprayed with a safranin-O/Dentamet® mixture. High magnification of the ray parenchyma cells (G). Upper epidermis (uep), peltate trichome (pt), palisade parenchyma (pp), vascular bundle (vb) with xylem (xy) and phloem (ph), lower epidermis (lep), epidermis (ep), parenchyma cells (pc), central parenchyma cells (cpc), secondary xylem (sxy), secondary phloem (sph), periderm (pe), broken epidermis (bep), xylem vessel (xyv), paratracheal parenchyma cells (ppc), fibers (f), ray parenchyma cells (rpc), nucleus (nu). Scale bars = 100 μm (A-F) and 5 μm (G).

within each treatment (presence or absence of Dentamet®), as well as, independently from safranin-O injection distance, within each treatment (Figure 2G). In particular, there was no significant fluorescence variation between the two treatments at the injection point, while a significantly greater amount of fluorescence was recorded for the safranin-O/Dentamet® treated samples, in comparison to those treated only with safranin-O, both at 40 cm and 80 cm from the injection points (Figure 2G). All these features results indicate major diffusion and/or fluidity of safranin-O in the presence of the cyclic compound Dentamet®.

These data were confirmed by the concentration levels of Zn^{++} and Cu++ ions in the leaves (i.e., central veins), as well as in petioles, fruit peduncles, and in 2- and 5-year-old twigs and branch samples, as revealed by the ICP-AES and ANOVA analyses. Significantly greater values were recorded in the samples treated with safranin-O/Dentamet® (Figure 3). The amounts of zinc and copper within the main leaf vein plus petiole in the samples treated with safranin-O/Dentamet® was of 20 times greater for Zn^{++} (mean = 144.3 ppm) and six times greater for Cu^{++} (mean = 90.5 ppm), than for the samples receiving safranin-O (Zn^{++}, 7.2 ppm; Cu^{++}, 14.6 ppm). Similarly, the zinc and copper amounts in the fruit peduncles treated with safranin-O/Dentamet® were 17-fold greater for Zn^{++} (mean =72.4 ppm) and 1.5-fold greater for Cu^{++}(mean = 38.8 ppm) than those obtained for the samples treated only with safranin-O. For the analyses carried out on branch woody tissue receiving endotherapy, the zinc and copper amounts in the samples treated with safranin-O/Dentamet® were significantly greater than measured for samples treated with safranin-O. For zinc ions, the transverse sections of branches, taken at 0, 40 or 80 cm from the injection points, gave values of, respectively, 8.9 (mean = 217.0 ppm), 8.7 (13.1 ppm) and 8.0 (8.6 ppm) greater than those observed for the safranin-O-treated branches. In the same samples, the copper contents in tissues treated with safranin-O/Dentamet® was, respectively, 44.0 (146.0 ppm), 3.7 (7.1 ppm), and 1.25 (2.0 ppm) times greater than in for samples treated with safranin-O (Figure 3).

Field effectiveness of Dentamet® for reducing *X. fastidiosa* symptoms through tree canopy spray treatments

Figure 4 shows the effectiveness of Dentamet® spray treatments during the 2015 to 2017 growing seasons for reducing *X. fastidiosa* symptoms in the Veglie olive orchard. This orchard experienced adverse climatic events during the experimental period (Supplementary Figure 7). For more than 40 consecutive days in the 2015 summer the maximum temperature was 1–4°C greater than historical records for the region. In addition, drought occurred throughout the 2017 summer 2017, when total rainfall was 7.8 mm from 1 June to 30 August. During January 2017, the orchard experienced unusual frost accompanied by heavy snow, and sub-zero temperatures were recorded for 11 d in the Salento peninsula. The weather station near the experimental orchard recorded daily minimum temperatures of -0.2°C to -5.1°C from the 6 to 12 of January. In the orchard, snow remained on the trees for one week. During the following spring, variable leaf fall related to the frost event was observed in the experimental orchard. Furthermore, the orchard soil is characterized by very low zinc and boron contents (Supplementary Table 1).

In general, symptoms progressed in the orchard during the duration of the experiment. The total number of wilted twigs increased during spring and summer (until September). To a lesser extent, disease symptoms also increased in winter and early spring, especially during 2015–2016. This pattern occurred for both treated and untreated trees, but with clear significant differences in severity between the treatments. Increases in symptom severity were less evident in spring 2017, possibly due to the reduction of *X. fastidiosa* populations density in the tree canopies during the prolonged mid-January frost (see also the real-time PCR data). At the end of the second year and during 2017, mortality of untreated trees increased. During the first two years, cv. Cellina di Nardò trees exhibited slightly less disease progression than cv. Ogliarola Salentina, but by the end of the experiment, untreated trees of both cultivars experienced the same death rates (in total, six dead trees per cultivar). From June 2015 onwards, Dentamet® spraying induced statistically significant decreases in numbers of wilted twigs for both cultivars compared with untreated trees. Furthermore, all Dentamet®-treated trees survived during the 3-year experiment.

The absence of treatments in July and August increased the numbers of wilted twigs during those months, and Dentamet® treatment in September then either reduced or resulted in only a slight increase in wilted twigs during October. By the end of the trial, treated Cellina di Nardò and Ogliarola salentina trees

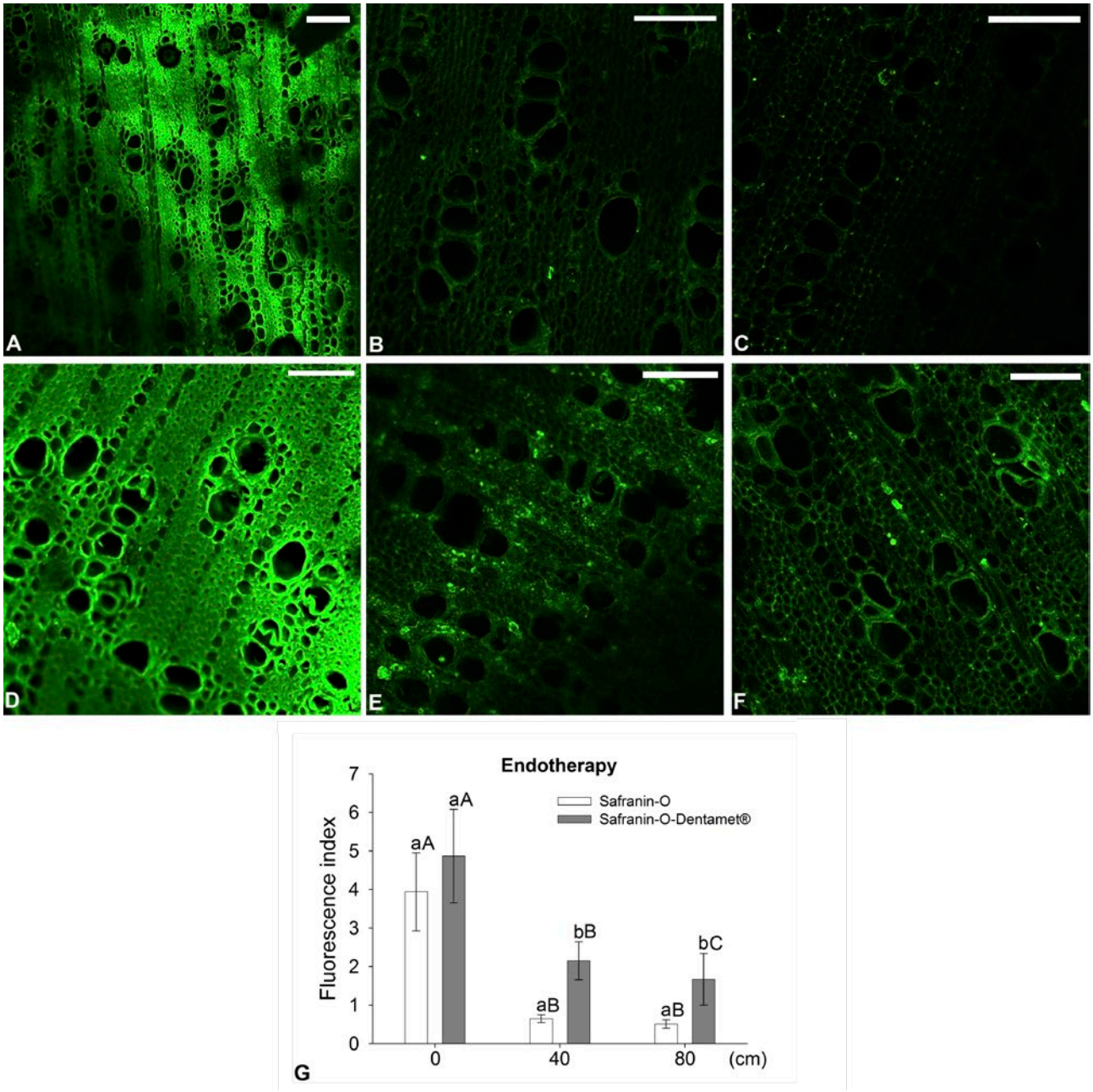

Figure 2. Confocal microscope images of transverse sections of olive cv. Ogliarola salentina healthy branches (approx. 10-year-old, 1 m length, 23-26 mm width) at 0 cm (A, D), 40 cm (B, E) or 80 cm (C, F) from the injection point of endotherapy treatment with safranin-O (A-C),or with the mixture safranin-O/Dentamet® (D-F).Scale bars = 100 μm (A-F). (G) Fluorescence index of safranin-O quantified from confocal images of transverse sections of olive branch at 0 cm, 40 cm or 80 cm from the injection point of endotherapy treatment with safranin-O,or with the mixture safranin-O/Dentamet®. Data are means ± standard deviations of nine emission peaks per each endotherapy point. Different lowercase letters indicate significant differences between treatments for each distance from the injection points, uppercase letters indicate significant differences among the different distances within each treatment ($P<0.05$).

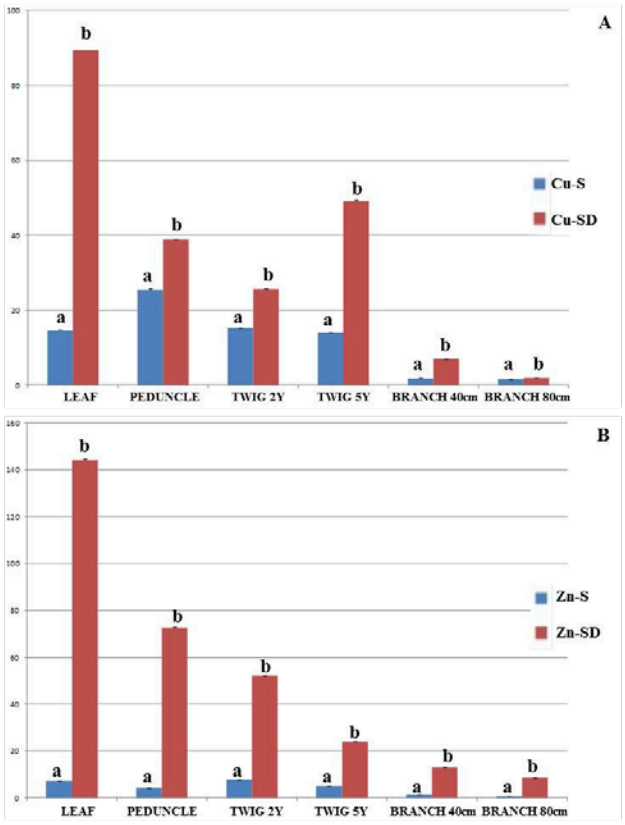

Figure 3. (A) Mean amounts of copper (Cu^{++}) and (B) of zinc (Zn^{++}) (ppm) within olive tree leaves, petioles, fruit pedunclesor stems of two-year-old twigs, or five-year-old twigs, and branches at 40 cm and at 80 cm from endotherapy injection points (see also Figures 1 and 2). Data were obtained using the Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) technique, and are means± standard deviations. Cu: copper; Zn: zinc; S. safranin-O; SD: safranin-O/Dentamet® mixture. Bars represent standard errors. Different letters indicate statistical significance at $P<0.001$.

had, respectively, 50 and 60 wilted twigs (including branches), whereas the untreated trees (four per cultivar) had means, respectively of 164 and 167 wilted twigs. These trees were also close to death (Figure 5). The two-way ANOVA table shows a highly significant effect of treatment and no significant effect of cultivar ($P = 0.294$) or treatment × cultivar interaction ($P= 0.748$) (Table 1). At the end of the trial, the mean Zn^{++} and Cu^{++} ion contents in treated leaves were significantly greater than in the untreated leaves (cv. Cellina di Nardò: mean Zn^{++} = 29.16 ppm (treated) and 4.29 ppm (untreated); mean Cu^{++} = 28.87 ppm (treated) and 3.02 ppm (untreated)). Treated cv. Ogliarola trees also had large amounts of Zn^{++} (mean = 47.70 ppm) and Cu^{++} (21.81 ppm),but the values for the untreated control tree could not be determined because it died during December 2016. These data confirm that Dentamet® was effective in reaching, and remaining within, the olive tree leaves without causing any phytotoxic effects.

Zinc and copper contents in olive drupes after Dentamet® spray treatments

The analyses carried out on olive drupes showed that similar and not statistically different contents of zinc and copper in olives from treated and untreated cv. Ogliarola salentina and cv. Cellina di Nardò trees (Supplementary Table 3). This indicates that Dentamet® treatments did not cause increases in the contents of these ions within the drupes before harvest.

Quantitative real-time PCR assessment of *Xylella fastidiosa* density within olive leaves

Figures 6 and 7 show the quantitative real-time PCR assessments of *X. fastidiosa* cell numbers in treated and untreated trees from June 2016 to September 2017. The two control trees exhibited heavy pathogen damage during the trial. As stated above, the cv. Ogliarola Salentina control tree died in December 2016, and this tree showed partial sucker resprouting during early summer of 2017, and it was not possible to completely refer present results from this tree in Figures 6 and 7. The cv. Cellina di Nardò control almost collapsed in April 2017, but samples could be collected from suckers inside the tree even although it was not possible to account for three canopy height samples during the samplings. When the average bacterial concentration found during the 13 months of assessment, expressed in ng of DNA then converted to colony forming unit (CFU) equivalents (Modesti *et al.*, 2017), the treated trees were shown to contain low CFU equivalents (approx. 10^2 CFU equivalents) in comparison with the untreated cv. Cellina di Nardò tree (10^4 to 10^5 CFU equivalents) (Figure 6). Both the treated cultivars showed similar trends of decreasing/increasing of bacterial concentrations during the 13 month sampling period, and these trends were different from that for the control tree (Supplementary Figure 8). This indicates a direct

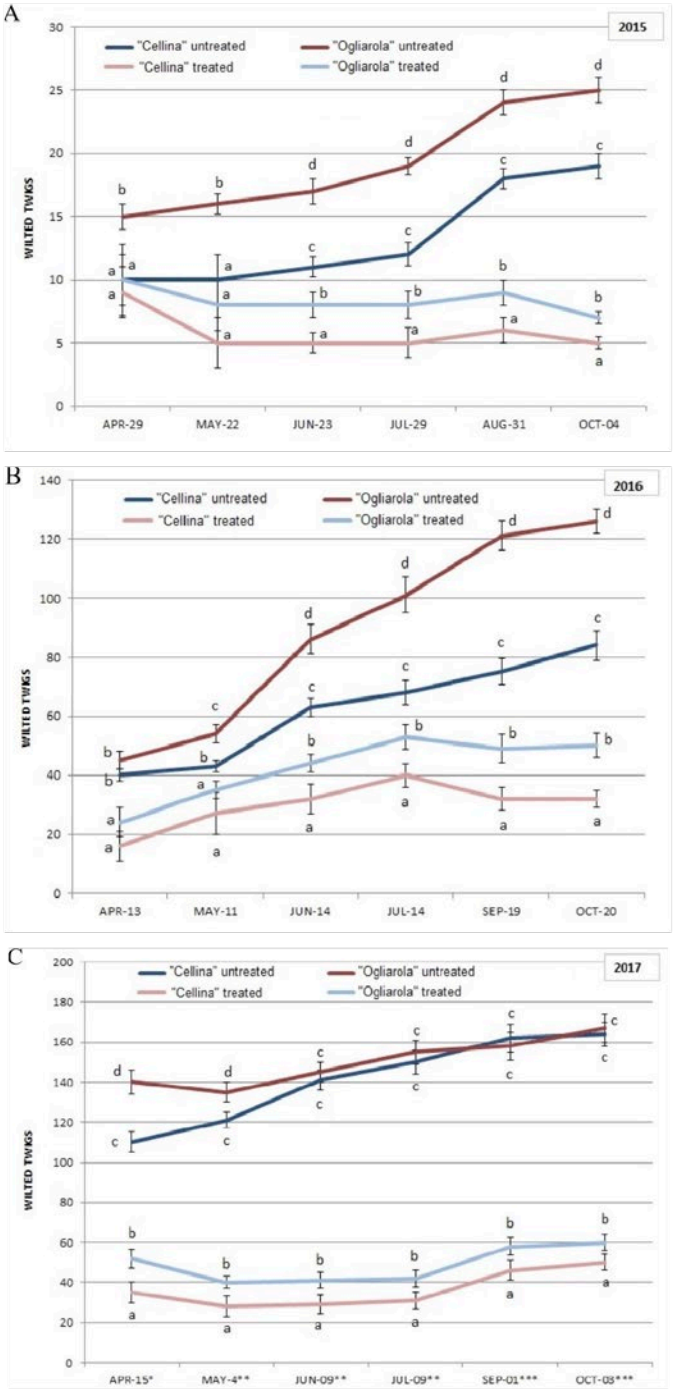

Figure 4. Mean numbers of wilted twigs on olive trees treated with Dentamet® during 2015 (A), 2016 (B) and 2017 (C), in an olive orchard located in Veglie (Lecce province) planted with cv. Ogliarola salentina and Cellina di Nardò and infected by *Xylella fastidiosa* subsp. *pauca*. Means were compared $P<0.05$. Bars represent standard deviations. Different letters indicate statistically significant differences. In the control block, trees began to die in December 2016. *: for control trees, data were for eight cv. Ogliarola salentina and ten cv. Cellina di Nardò trees. **: for control trees, data were for seven cv. Ogliarola salentina and seven cv. Cellina di Nardò trees. ***: for control trees, data were for cv. Ogliarola salentina and four cv. Cellina di Nardò trees.

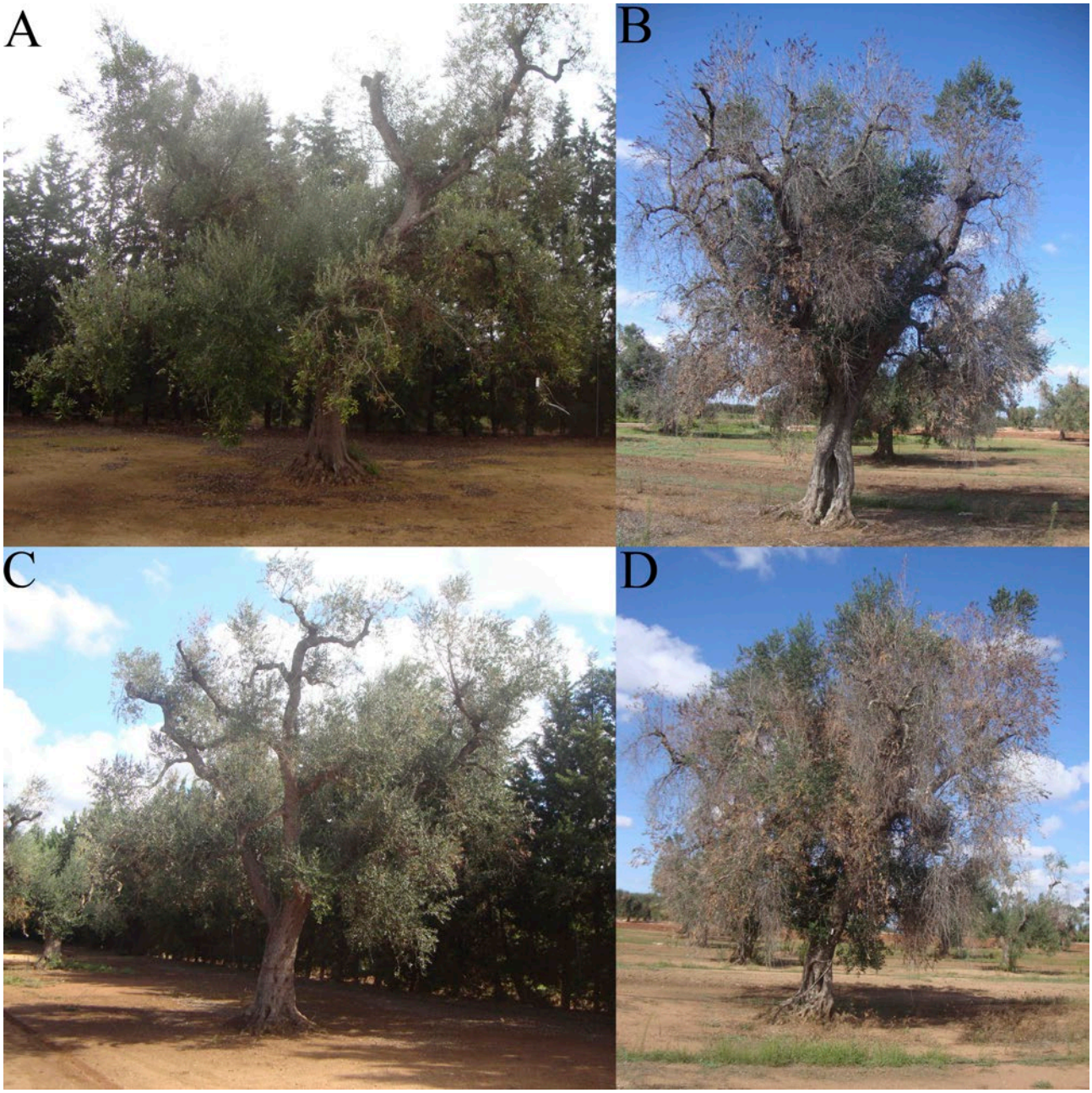

Figure 5. Olive trees photographed at the beginning of October 2017 in Veglie orchard (Lecce province). A: cv. Ogliarola salentina treated with Dentamet®. B: cv. Ogliarola salentina untreated control. C: cv. Cellina di Nardò treated with Dentamet®. D: cv. Cellina di Nardò untreated control. The treated cv. Ogliarola salentina and cv. Cellina di Nardò trees would have been uprooted on April 2015, according to the prescribed quarantine eradication programme.

effect of the Dentamet® treatments, reducing bacterial populations within olive tree canopies of the two cultivars.The ANOVA results showed that Dentamet® treatments significantly reduced pathogen populations in both cultivars from initial-assessment levels during June 2016 (Figure 7). After the extensive wilting of the cv. Cellina di Nardò control tree during spring 2017, only a small part of the canopy

Table 1. Two-way ANOVA results considering two factors (treatment and cultivar, and their interactions) for monthly mean values as obtained from the experimental blocks in Veglie (LE). The analysis shows a very significant effect of treatment and a not significant effect of cultivar and interaction treatment × cultivar.

Source	Sum of squares	df	Mean square	F	*P*-value
Corrected model	48299.167	3	16099.722	8.992	**< 0.0001**
Treatment	46106.722	1	46106.722	25.752	**< 0.0001**
Cultivars	2005.556	1	2005.556	1.12	0.294
Treatment × Cultivar	186.889	1	186.889	0.104	0.748
Error	121749.444	68	1790.433		
Corrected total	170048.611	71			

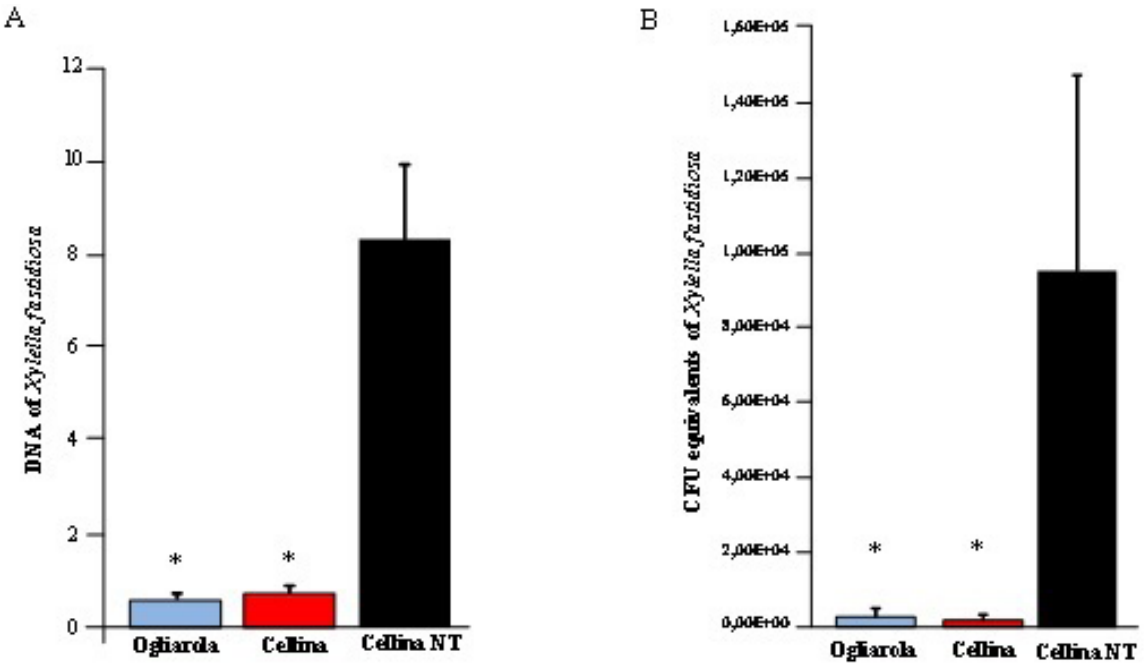

Figure 6. Relationships between the mean total *X. fastidiosa* subsp. *pauca* DNA population (A) and colony forming unit (CFU) equivalents (B), calculated following Modesti *et al*. (2017), determined for Dentamet®-treated (cv. Ogliarola salentina and Cellina di Nardò) and untreated control (cv. Cellina di Nardò) olive trees. NT: not treated. Data refer to the trees still alive at the end of the trial, where a cv. Ogliarola salentina control tree was dead on December 2016. *: statistically significant value compared with the untreated tree ($P<0.05$). Bars represent standard errors of means.

remained alive for sampling, and a significant difference from the treated tree was not found for May and July. However, the *X. fastidiosa* populations cell densities decreased significantly both in the treated and untreated trees, immediately after the mid-January frost events of 2017. Subsequently, pathogen inoculum increased in the untreated trees and, to a lesser extent, in treated trees as well. Pathogen cell numbers were low (0.15 ng DNA) in both treated trees by experiment end. This reflected the vegetative indices of both trees (see also Figure 8). Taking into account the different heights of the canopies for

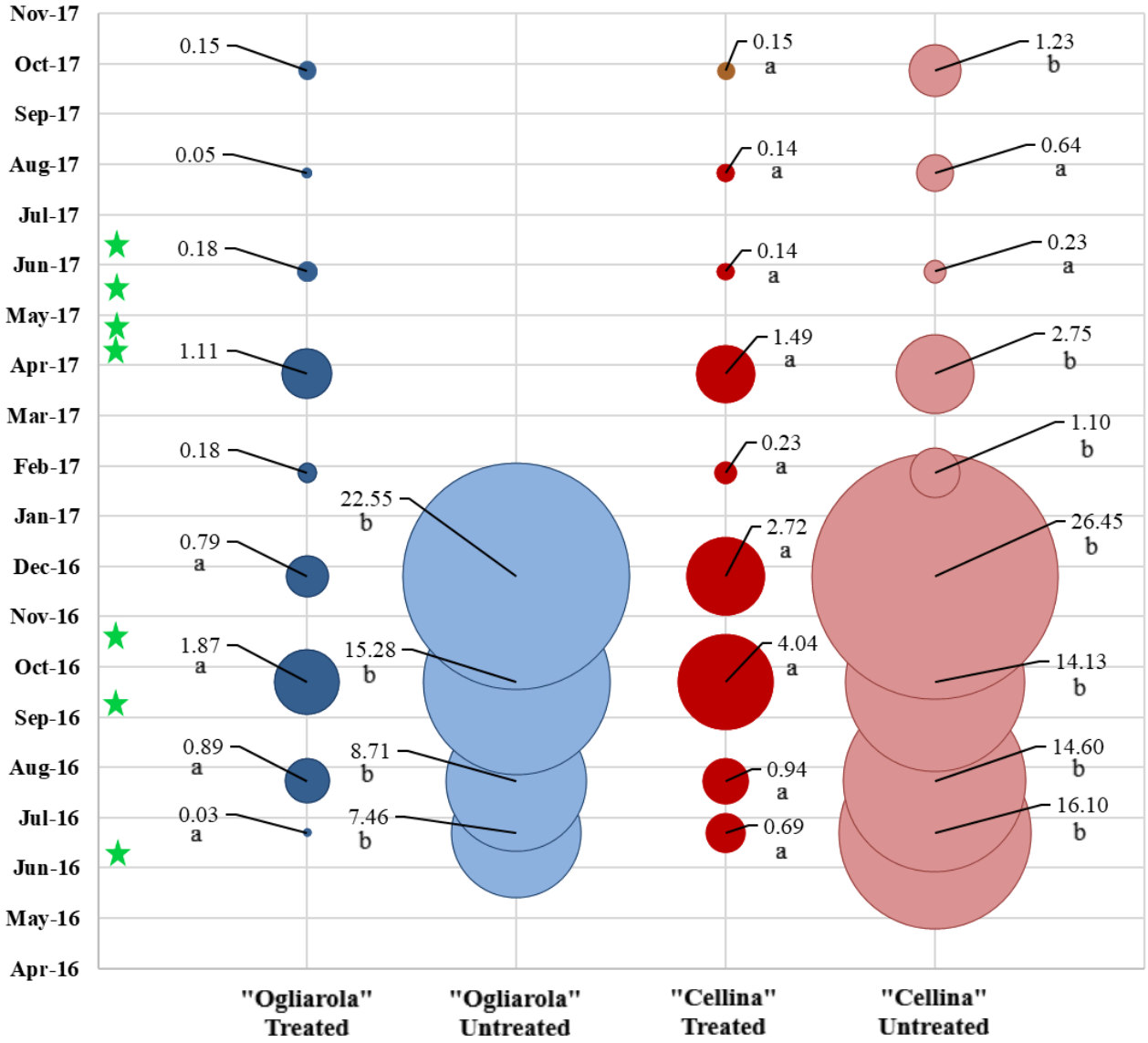

Figure 7. *Xylella fastidiosa* subsp. *pauca* DNA concentration detected in olive trees at each sampling, and expressed as mean ng DNA per tree, determined using the methods of Modesti *et al*. (2017), and obtained from June 2016 to September 2017 in the olive orchard of Veglie (Lecce province) planted with cv. Ogliarola salentina and Cellina di Nardò. Statistical significance was evaluated at $P<0.05$. Different letters indicate significance of the treatment. Asterisk refers to the day of Dentamet® treatment in the treated block. Note the reduction of *X. fastidiosa* DNA after the frost events of January 2017. The cv. Ogliarola salentina untreated control tree died in December 2016, whereas the cv. Cellina di Nardò untreated control tree became almost completely withered on April 2017, so that samples were taken from the remaining live canopy without respecting sampling heights.

the samplings (low, medium and high) performed in the non-treated cv. Cellina di Nardò control tree (36 samples per plant at each of the 10 samplings) until March 2017, there were slightly greater bacterial concentrations for the upper canopy in comparison with the lower canopy, and no statistical differences were detected between the medium and low parts of the canopies (data not shown).

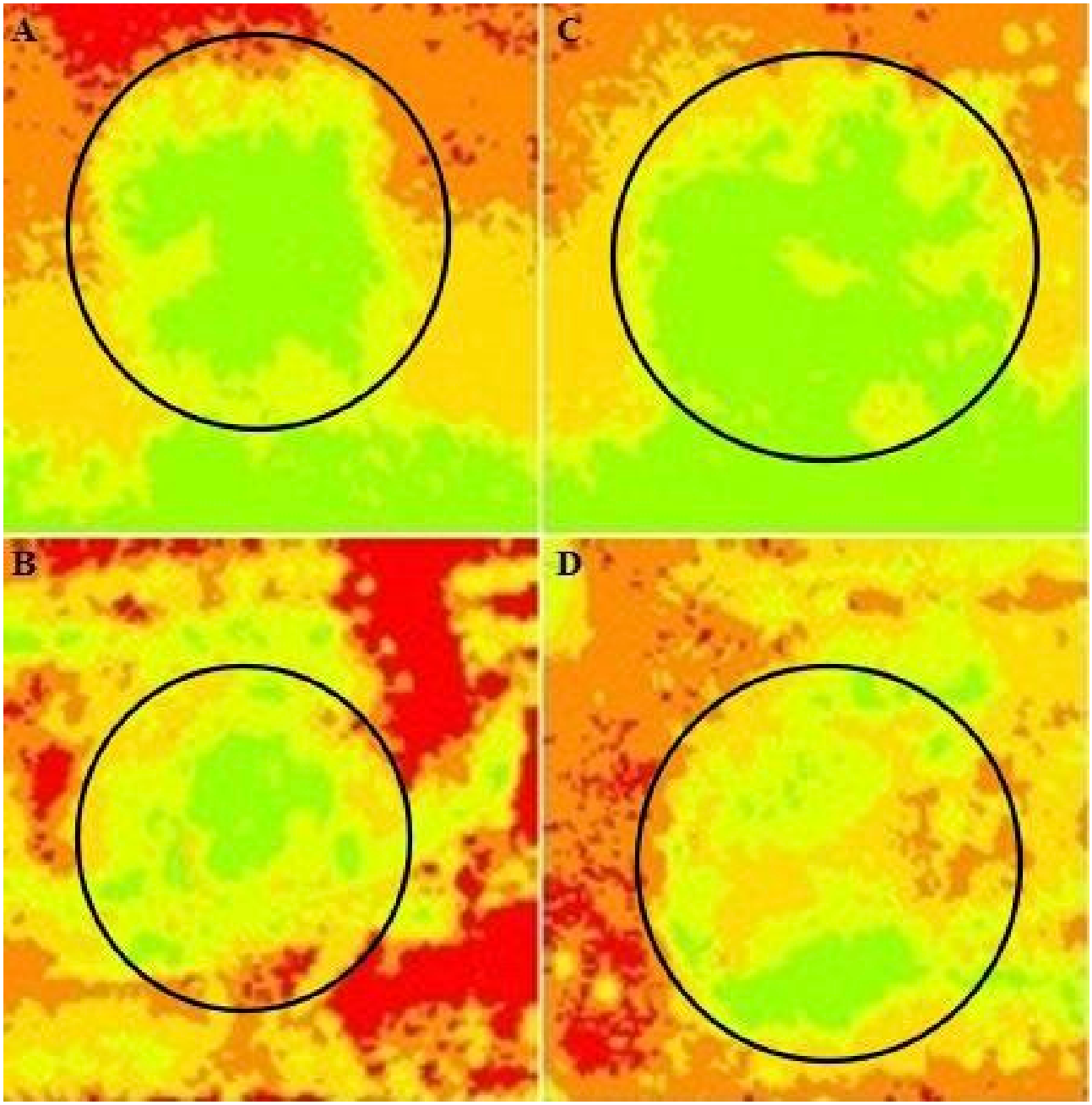

Figure 8. Olive tree patterns (within circle) obtained through the normalized difference vegetation (NDVI) index assessed on 9 October, 2017, at the end of the trial performed in the orchard of Veglie (Lecce province). A: cv. Ogliarola salentina tree treated with Dentamet®. B: cv. Ogliarola salentina untreated control tree. C: cv. Cellina di Nardò tree treated with Dentamet®. D: cv. Cellina di Nardò untreated control tree. Green: indicates the vegetation as revealed by the drone measurement; red: is the soil surface. Note that for A and C patterns (treated trees), the intensity of the green colour (NDVI) of the olive canopies is similar to that of *Cupressus arizonica* var. *stricta* trees growing closeby.The same olive trees were assessed using quantitative real-time PCR analyses to determine *Xylella fastidiosa* populations.

Figure 9. Recovery of a cv. Ogliarola salentina olive tree severely damaged (more than 80% of the canopy dead) by *Xylella fastidiosa* subsp. *pauca* grown in Galatone (Lecce province) after trunk injection with Dentamet® from April 2017 to July 2017 (see text for details). A: the tree before treatment. B: initial re-sprouting in May of shoots from the main tree branches as observed 20 d after treatment. C: new shoots continued to grow during June. D: at the end of July, part of the canopy was developed. E: despite the drought occurring during summer 2017, the tree showed in September a number of suckers as well as part of the canopy.

Infrared assessment of the vegetation status through the Normalized Difference Vegetation Index (NDVI)

At the end of the trial, the NDVI measurements revealed significant differences of the vegetative status between the two blocks of trees (treated and untreated). Regardless of cultivar, the treated trees showed a mean NDVI index per tree always greater than 0.60, with maximum relative peaks in parts of the tree canopies of 0.85, found both in cv. Cellina di Nardò and cv. Ogliarola salentina trees. In contrast, the untreated trees still with vegetation showed a mean NDVI index of 0.45. These statistically significant data are further indication of the effectiveness of Dentamet® in enhancing the vegetative status of olive trees. Representative patterns of the NDVI of treated and untreated trees are shown in Figure 8.

Endotherapy of severely affected olive trees

The trunk bases of cv. Ogliarola salentina trees that were severely affected by were injected with pure Dentamet® in spring of 2017. Assessment of disease severity once per month from May to the end of September revealed that the treatment induced extensive shoot re-sprouting along the main branches (Figure 9). Re-sprouting was observed 15–20 d after the first trunk injection. In September 2017, treated trees had numerous young shoots along the main branches and suckers at collar level, indicating that severely diseased trees can recover from the disease. In some cases, a third of the crowns had been regenerated by the end of summer. Control trees receiving water injections did not show any new vegetation. Dentamet® should be diluted to 1:5 solution after the first treatment application in April, because trees receiving 1:1 Dentamet® in May subsequently exhibited new shoots with extensive necrosis, possibly due to the high concentration of the product. Quantitative real-time PCR analyses performed after re-sprouting demonstrated that *X. fastidiosa* was absent from the leaves of young shoots in May. However, the pathogen was detected in the shoots of one tree during July and in six trees during September. Control trees nearby also contained the pathogen. An initial re-sprouting occurred in the five trees injected during July, followed by subsequent shoot wilting.

Implementation of integrated *Xylella fastidiosa* control strategies

Preliminary trials at Galatone and Galatina during 2015–2016 revealed that heavy pruning to remove all visibly infected branches increased *X. fastidiosa* symptoms and often caused death of trees. Many trees that were heavily pruned in April collapsed in the following months after partial re-sprouting. Consequently, starting from April 2017, the olive orchards of Oria and Copertino were subjected to an integrated control plan. Accompanied by routine fertilization and pest control, the strategy included weed removal with soil harrowing during spring to summer, regular light pruning twice per year, and Dentamet® spray treatments (0.5% v:v) to tree canopies from the end of April to beginning of October (including also July and August). This protocol maintained tree health despite the occurrence of *X. fastidiosa* inoculum in the surrounding area as revealed by the monitoring carried out by Regione Puglia in the Oria area. In both orchards, during mid-October, Dentamet®-treated trees had 45% fewer wilted twigs than control trees.

Discussion

This study has demonstrated that Dentamet® exhibits *in vitro* bactericidal activity towards *X. fastidiosa*, as shown by the inhibition of the test strain M23 of *X. fastidiosa* subsp. *fastidiosa*, isolated from almond. *In planta* assays also showed that soaking in Dentamet® solution caused infected almond leaves to rapidly (15 min) absorb the biocomplex, resulting in subsequent inhibition of the M23 strain. The Dentamet® concentrations of zinc (0.4 g L^{-1}) and copper (0.02 g L^{-1}) are greater than that reported by Cobine *et al.* (2013) regarding the capability of these ions to inhibit *X. fastidiosa* biofilm formation in growth medium (0.032 g L^{-1} for zinc and 0.012 g L^{-1} for copper). However, when complexed with citric acid, the two elements actively reduced *X. fastidiosa* growth, both in agar medium and in leaf assays, at a 1:10 dilution (corresponding to 34 μM for zinc and 17 μM for copper). In comparison, catechol is the most active phenol inhibitor of *X. fastidiosa*, but this compound is active at 100 μM (Maddox *et al*., 2010).

In the present study, several experiments, involving direct and indirect approaches, showed that Dentamet® reaches the xylem vessel networks of leaves, peduncles, twigs and branches of olive trees. Indirect evidence of rapid and effective potential to permeate leaves was shown by the absence of *X. fastidiosa* growth on PW medium after repeated isolations from almond leaves showing symptoms of "leaf scorch". The leaves were previously soaked into different con-

centrations of Dentamet® for 15 min or overnight immersion. Secondly, field studies using endotherapy and spray applications along with subsequent laboratory analyses, provide direct evidence of systemic Dentamet® uptake in plants.

The endotherapy experiments demonstrated the ascending movement of safranin-O and safranin-O/Dentamet® mixtures within olive woody tissue. At 48 h post-injection, safranin-O/Dentamet® reached higher points in tree stems than safranin-O alone. Field data were confirmed using relative fluorescence quantification measurements as well as ICP-AES analyses of zinc and copper contents. Samples containing Dentamet® had greater fluorescence indices and elevated levels of both copper and zinc ions. In general, CLSM analyses indicated that in sprayed and endotherapy samples, zinc and copper moved together to reach xylem complex networks. Like safranin-O, Dentamet® may also remain trapped in the free space between the primary and secondary walls of all living and dead cells, including fibres and xylem vessel elements. Dead xylem cells are continuously provided with molecules or ions from the surrounding living cells (e.g., epidermal, cortex, phloem, axial, and ray parenchyma cells). Thus, the transverse and longitudinal conduction systems of plants tend to be in equilibrium. Pit membranes that occur between pit pairs are specialized cell wall structures that connect vessel elements to their neighbours facilitating element conduction (Esau, 1977; Evert, 2006). When Dentamet® is trapped inside olive cell walls and on cell surfaces, it may decrease the secretion of cell wall-degrading enzymes by *X. fastidiosa*, thus limiting the breakdown and enlargement of pores in intervessel pit membranes. The pathogen would then be unable to spread through the xylem vessel systems (Pérez-Donoso *et al*., 2010; Sun *et al*., 2011), and/or promote the synthesis of xanthan and tylose, compounds that contribute to occlusion of vessel elements (Sun *et al*., 2013; De Benedictis *et al*., 2017).

The 0.5% (v:v) Dentamet® spray treatments consistently reduced field *X. fastidiosa* symptoms, during the three-year field experiment. At the end of the experiment, none of the treated trees died. This was despite experiencing frost and heavy snow in January 2017, followed by high temperatures and drought during summer 2017 that probably provided additional stress to the trees (Choi *et al*., 2013). Such adverse climatic events can damage olive tree metabolism (Sanzani *et al*., 2012). However, the NDVI measurements confirmed that treated trees reached a satisfactory vegetative index only a few months after treatment. In contrast, six cv. Ogliarola Salentina and six cv. Cellina di Nardò trees died in the untreated block. These results confirmed that the effectiveness of sprayed Dentamet® relies on its bactericidal activity towards *X. fastidiosa* and on its ability to reach the xylem complex networks of diseased plants.

At the end of the experiment, olive leaves contained very elevated amounts of zinc and copper, providing indirect evidence of Dentamet® absorption. High zinc levels in the xylem strongly limited *X. fastidiosa* multiplication, thus interfering with its virulence (Navarrete and De La Fuente, 2015), while the bactericidal activity of copper is well established. Indeed, copper and zinc concentrations in treated cv. Cellina di Nardò leaves were, respectively, about 10 and 7 times greater than in the untreated leaves. These results reinforce previous assumptions made by Navarrete and De La Fuente (2015) regarding the important role played by the mineral status of the host plant infected by *X. fastidiosa*. that could be manipulated as a disease management strategy. For the present study, zinc content in the field soil was very low (0.5 ppm) (Neilsen *et al*., 1986; Adhikari and Rattan, 2007). Therefore, Dentamet® spray treatments were likely to be the primary sources of high zinc concentration in treated olive trees, and the supply of zinc from the Dentamet® spray treatment probably reduced pathogen levels.

Pathogen cell population assessments with quantitative real-time PCR confirmed field results showing reduced disease severity after Dentamet® treatments. Treated trees exhibited lower *X. fastidiosa* cell numbers than untreated trees. Frost events, occurring in mid-January 2017 over 1 week, reduced the cell populations of the bacterium both in the Dentamet®-treated and the untreated trees. Pathogen densities then increased during post-frost months. This confirms the assumption that prolonged cold poses a disadvantage to *X. fastidiosa*, but not to the point of completely curing infected host plants (Purcell, 1977; 1980). Previous research found that *X. fastidiosa* can survive for 4 d at -5°C in buffers, culture media, and grapevine xylem sap (Meyer and Kirkpatrick, 2008). Additionally, temperatures below -6°C across multiple days are more effective in killing the pathogen (Lieth *et al*., 2011). In the area of the experimental field used in the present study, the minimum temperature on 12 January 2017, during the frost, was of -5.1°C.

The post-frost survival of the *X. fastidiosa* subsp. *pauca* CoDiRO strain in temperate Mediterranean climates was postulated by Marcelletti and Scortichini (2016), who found the presence of the Csp1 cold-shock protein in the strain genome, putatively enabling the pathogen to adapt to cold (Burbank and Stenger, 2016). Previous studies on *X. fastidiosa*-infected grapevine revealed correlations between elevated abscisic acid (ABA) and cold exposure (Kirkpatrick and Meyer, 2005). ABA is involved in the synthesis of cold-shock proteins (Bravo *et al*., 1998; Thomashow, 1998), so future studies should examine whether ABA levels increase in olive trees that experienced frost events.

Although we did not have data on the initial levels of pathogen inoculum, we noted clear links between Dentamet® spray treatments and subsequent decreases in *X. fastidiosa* cell densities, as well as pathogen increases after July and August when spray treatments ceased. In the two untreated trees, the opposite trend was observed. The untreated cv. Ogliarola Salentina tree exhibited a generally linear disease progression during summer and autumn, whereas the untreated cv. Cellina di Nardò tree exhibited a slight decrease in pathogen cell density from June to September, followed by an increase in November. Infected and healthy trees of both cultivars differ in phenolic-compound and sugar levels (Girelli *et al*., 2017; Luvisi *et al*., 2017a). Thus, further assays performed using known initial doses of *X. fastidiosa* inoculum, coupled with age- and environment-controlled potted plants, could reveal putative cultivar-specific responses to the pathogen.

The present study indicated that endotherapy (Dentamet® release through plastic syringes) resulted in extensive re-sprouting of new shoots from heavily infected olive trees. All ten trees receiving endotherapy recovered from *X. fastidiosa* during the following months, and by autumn, large parts of the tree canopies were restored. Control trees supplemented with water did not show any new vegetation during the growth seasons. However, further research is necessary to understand the re-occurrence of *X. fastidiosa* in new shoots, which occurred during July and September in this study. We hypothesize that the pathogen may have moved from branch xylem vessels to the new shoots. Alternatively or simultaneously, insect vectors may have caused re-infection. Regardless, endotherapy is a promising technique for recovering severely infected trees for subsequent management using routine agronomic disease management methods and spray treatments. Whether Dentamet® can stimulate adventitious buds along tree branches during spring should be further investigated. We stress that commencement of endotherapy during summer is inadvisable because new shoots could potentially wilt after their initial growth.

The two olive orchards of Oria and Copertino are within the *X. fastidiosa* infected area and are surrounded by sources of pathogen inoculum. As a result, trees in both orchards experienced previous wilting and were treated with the integrated *X. fastidiosa* management strategy, including regular pruning, soil harrowing in spring and summer, and canopy spray treatments from April to October. Tree health in both orchards improved, even after the first seven months of Dentamet® applications, with -treated trees exhibiting fewer wilted twigs than control trees.Disease severity was much greater in nearby olive orchards that did not follow this disease management strategy. Other sites in Lecce province also have olive orchards applying the integrated strategy, and they sometimes appear as green 'oases' surrounded by dead trees.

This study has demonstrated that field control of *X. fastidiosa* infection in olive trees could be possible in the Apulia region of Italy. Dentamet® was effective in permeating olive xylem networks with zinc and copper ions without affecting their contents in the drupes collected before harvest, and effectively reducing pathogen populations over time. We recommend the use of this biocomplex containing zinc, copper and citric acid, such as the formulation used in this study, as an eco-friendly tool that can be included as part of *X. fastidiosa* management. Similar to the procedures used in this study, such a strategy should incorporate agronomic techniques, including regular light pruning of olive trees and soil harrowing during spring and summer to remove weeds, along with traditional soil fertilization and pest control. This integrated approach should be routinely applied each growing season. It is also a practical approach for areas where *X. fastidiosa* is less severe close to southern Salento, or as a preventive approach in the disease-free regions. Plant uprooting may be unnecessary where integrated management is used, because this approach reduces pathogen inoculum (Stacey *et al*, 2004; Cunniffe and Gilligan, 2010). Extensive eradication programmes similar to that proposed for the *X. fastidiosa* in Apulia failed to completely eliminate bacterial phytopathogens in the long term. For *Xanthomonas axonopodis* pv. *citri* in Brazil, the pathogen

rapidly re-infected the region after ceasing tree uprooting (Behlau *et al*., 2016).

Other host plants of *X. fastidiosa* subsp. *pauca* are found in Apulia, including many wild species (EFSA, 2016). Uprooting olive trees would not completely eliminate the pathogen from the region. It has been postulated that the risk of pathogen expansion to uninfected sites would increase if all the alternative host plants in OQDS-affected areas are not effectively controlled (White *et al*., 2017). Ultimately, the integrated strategy for the control of *X. fastidiosa* subsp. *pauca* would reflect the requirements for a more inclusive governance and management of a quarantine plant pathogen infecting a vast area (Mills *et al*., 2011; Luvisi *et al*., 2017b).

Conflicts of interest

The authors declare that they have no relevant conflicts of interest.

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Literature cited

Adhikari T. and R.K. Rattan, 2007. Distribution of zinc fractions in some major soils of India and the impact on nutrition of rice. *Communications in Soil Science and Plant Analysis* 38, 2779–2798.

Almeida R.P.P., 2016. Can Apulia's olive trees be saved ? *Science* 353, 346–348.

Ancona V., D.N. Appel and P. De Figuereido, 2010. *Xylella fastidiosa*: a model for analyzing agricultural biosecurity.*Biosecurity and Bioterrorism: Biodefense Strategy, Practice, and Science* 8, 171–182.

Arora A.K., A. Forshaw, T.A. Miller and R. Durvasula, 2015. A delivery system for field application of paratransgenic control. *BMC Biotechnology* 15, 59.

Behlau F., A.E. Fonseca and J. Belasque jr, 2016. A comprehensive analysis of the Asiatic citrus canker eradication programme in São Paulo state, Brazil, from 1999 to 2009. *Plant Pathology* 65, 1390–1399.

Bravo L.A., G.E. Zuñiga, M. Alberdi and L.J. Corcuera, 1998. The role of ABA in freezing tolerance and cold acclimation in barley. *Physiologia Plantarum* 103, 17–23.

Bruening G., B.C. Kirkpatrick, T. Esser and R.K. Webster, 2004. Cooperative efforts contained spread of Pierce's disease and found genetic resistance. *California Agriculture* 68, 134–141.

Burbank L.P. and D.C. Stenger, 2016. A temperature-dependent cold-shock protein homolog acts as a virulence factor in *Xylella fastidiosa. Molecular Plant-Microbe Interactions* 29, 335–344.

Cardinali M.C.B., P.R. Villas-Boas,D.M.B.P. Milori, E.J. Ferreira, M.F.E. Silva, M.A. Machado, B.S. Bellete and M.F.D.F. Da Silva, 2012. Infrared spectroscopy: a potential tool in Huanglongbing and citrus variegated chlorosis diagnosis. *Talanta* 91, 1–6.

Chen J., R. Groves, E.L. Civerolo, M. Viveros, M. freeman and Y. Zheng, 2005. Two *Xylella fastidiosa* genotypes associated with almond leaf scorch disease on the same location in California. *Phytopathology* 95, 708–714.

Choi H-K., A. Iandolino, F. Goes da Silva and D.R. Cook, 2013. Water deficit modulates the response of *Vitis vinifera* to the Pierce's disease pathogen *Xylella fastidiosa*. *Molecular Plant-Microbe Interactions*, 26, 643–657.

Cobine P.A., L.F. Cruz,F. Navarrete, D. Duncan, M. Tygart and L. De La Fuente, 2013. *Xylella fastidiosa* differentially accumulates mineral elements in biofilm and planktonic cells. *PLoS ONE* 8, e54936.

Cunniffe N.J. and C.A. Gilligan, 2010. Invasion, persistence and control in epidemic models for plant pathogens: the effect of host demography. *Journal of the Royal Society Interface* 7, 439–451.

Das M., T.S. Bhowmick, S.J. Ahern, R. Young and C.F. Gonzalez, 2015. Control of Pierce's disease by phage. *PLoS ONE* 10, e0128902.

Davis M.J., W.J. French and N.W. Schaad, 1983. Axenic culture of the bacteria associated with phony peach and plum leaf scald. *Current Microbiology* 6, 309–314.

De Benedictis M., M. De Caroli, I. Baccelli,G. Marchi,G. Bleve,A. Gallo,F. Ranaldi,V. Falco,V. Pasquali,G. Piro,G. Mita and G.P. Di Sansebastiano, 2017. Vessel occlusion in three cultivars of *Olea europaea* naturally exposed to *Xylella fastidiosa* in open field. *Journal of Phytopathology* 165, 589–594.

Dowdle W.R., 1998. The principles of disease elimination and eradication. *Bulletin of the World Health Organization* 76, Suppl. 2, 22–25.

EFSA (European Food Safety Authority), 2016. Scientific report on the update of a database of host plants of *Xylella fastidiosa*: 20 November 2015. *EFSA Journal* 14, 4378, 40 pp.

EPPO 2015a. First report of *Xylella fastidiosa* in France. *EPPO Reporting Service* 144.

EPPO 2015b. *Xylella fastidiosa* detected in Alpes-Maritimes, mainland France. *EPPO Reporting Service* 180.

EPPO 2016a. First report of *Xylella fastidiosa* subsp. *fastidiosa* on *Nerium oleander* in Germany. *EPPO* 133.

EPPO 2016b. First report of *Xylella fastidiosa* in Spain. *EPPO Reporting Service* 213.

EPPO 2017a. First report of *Xylella fastidiosa* in mainland Spain. *EPPO Reporting Service* 133.

EPPO 2017b. New additions to the A1 to A2 list. *EPPO Reporting Service* 158.

EPPO 2017c. *Solanum melongena* plants for fruit production: a successful eradication. *EPPO Reporting Service* 017.

Esau K., 1977. *Anatomy of Seed Plants*, 2nd ed., Wiley, New York, 524 pp.

Evert R.F., 2006. *Esau's Plant Anatomy*. Meristems, cells, and tissues of the plant body: their structure, function, and development. 3rd ed., John Wiley & Sons Inc., New York, 624 pp.

Giampetruzzi A., M. Saponari, G. Loconsole, D. Boscia, V.N. Savino, R.P.P. Almeida, S. Zicca, B.B. Landa, C. Chacon-Diaz and P. Saldarelli, 2017. Genome-wide analysis provides evidence on the genetic relatedness of the emergent *Xylella fastidiosa* genotype in Italy to isolates from Central America. *Phytopathology* 107, 816–827.

Girelli C.R., L. Del Coco, M. Scortichini, M. Petriccione, L. Zampella, F. Mastrobuoni, G. Cesari, A. Bertaccini, G. D'Amico, N. Contaldo, D. Migoni and F.P. Fanizzi, 2017. *Xylella fastidiosa* and olive quick decline syndrome (CoDiRO) in Salento (southern Italy): a chemometric 1H-NMR-based preliminary study on Ogliarola salentina and Cellina di Nardò. *Chemical and Biological Technologies in Agricolture* 4, 25.

Goodwin P.H., J.E. De Vay and C.P. Meredith, 1988. Roles of water stress and phytotoxins in the development of Pierce's disease of the grapevine. *Physiological and Molecular Plant Pathology* 32, 1–16.

Harper S.J., L. Wardand G.R.G. Clover, 2010. Development of LAMP and qPCR methods for the rapid detection of *Xylella fastidiosa* for quarantine and field applications. *Phytopathology* 100, 1282–1288.

Hopkins D.L. and A.H. Purcell, 2002. *Xylella fastidiosa*: cause of Pierce's disease of grapevine and other emergent disease. *Plant Disease* 86, 1056–1066.

Janse J.D. and A. Obradovic, 2010. *Xylella fastidiosa*: its biology, diagnosis, control and risks. *Journal of Plant Pathology* 92, 35–48.

Kailis S. and D.J. Harris, 2007. *Producing Table Olives*. Landlinks Press, Collingwood, Australia, 328 pp.

Kim H.K., J. Park and I. Hwang, 2014. Investigating water transport through the xylem network in vascular plants. *Journal of Experimental Botany* 65, 1895–1904.

Kirkpatrick B., P. Anderson, E. Civerolo, D.-D. Jones, A.H. Purcell, R. Smith, C. Vargas and E. Weber, 2003. Evaluation of bactericides and modes of delivery for managing Pierce's disease. In: *Pierce's Disease Research Symposium*101-103.

Kirkpatrick B. and M. Meyer, 2005. Identification of mechanisms mediating cold therapy of *Xylella fastidiosa*-infected grapevines. In: *Proceedings of the Pierce's Disease Research Symposium*, California Department of Food and Agriculture, 242–246.

Lieth J.H., M.M. Meyer, K.H. Yeo and B.C. Kirkpatrick, 2011. Modeling cold curing of Pierce's disease in *Vitis vinifera* "Pinot Noir" and "Cabernet Sauvignon" grapevines in California. *Phytopathology* 101, 1492–1500.

Luvisi A., A. Aprile, E. Sabella, M. Vergine, F. Nicolì, E. Nutricati, A. Miceli, C. Negro and L. De Bellis, 2017a. *Xylella fastidiosa* subsp. *pauca* (CoDiRO strain) infection in four olive (*Olea europea* L.) cultivars: profile of phenolic compounds in leaves and progression of leaf scorch symptom. *Phytopathologia Mediterranea* 56, 259–273.

Luvisi A., Y. Ampatzidis and L. De Bellis, 2017b. Plant pathology and information technology: opportunity for management of disease outbreak and applications in regulation frameworks. *Sustainability* 8, 831.

Maddox C.E., L.M. Laur and L. Tian, 2010. Antibacterial activity of phenolic compounds against the phytopathogen *Xylella fastidiosa*. *Current Microbiology* 60, 53–58.

Marcelletti S. and M. Scortichini, 2016. *Xylella fastidiosa* CoDiRO strain associated with the olive quick decline syndrome in southern Italy belongs to a clonal complex of the subspecies *pauca* that evolved in Central America. *Microbiology* 162, 2087–2098.

Martelli G.P., D. Boscia, F. Porcelli and M. Saponari, 2015. The olive quick decline syndrome in south-east Italy: a threatening phytosanitary emergence. *European Journal of Plant Pathology* 144, 235–243.

Martelli G.P., 2016. The current status of the quick decline syndrome of olive in southern Italy. *Phytoparasitica* 44, 1–10.

Meyer M.M. and B.C. Kirkpatrick, 2008. Examining the effects of cold therapy on Pierce's disease-infected grapevines and on the viability of *Xylella fastidiosa* cells *in vitro*.In: *Proceedings of the 2nd Annual National Viticulture Research Conference*, University of California, Davis, 56–57.

Mills P., K. Dehnen-Schmutz, B. Ilbery, M. Jeger, G. Jones, R. Little, A. MacLeod, S. Parker, M. Pautasso, S. Pietravalle and D. Maye, 2011. Integrating natural and social science perspectives on plant disease risk, management and policy formulation. *Philosophical Transactions of the Royal Society B* 366, 2035–2044.

Modesti V., N. Pucci, S. Lucchesi, L. Campus and S. Loreti, 2017. Experience of the Latium region (central Italy) as a pest-free area for monitoring of *Xylella fastidiosa*: distinctive features of molecular diagnostic methods. *European Journal of Plant Pathology* 148, 557–566.

Navarrete F. and L. De La Fuente, 2014. Response of *Xylella fastidiosa* to zinc: decreased culturability, increased exopolysaccharide production, and formation of resilient biofilm under flow conditions. *Applied and Environmental Microbiology* 80, 1097–1107.

Navarrete F. and L. De La Fuente, 2015. Zinc detoxification is required for full virulence and modification of the host leaf ionome by *Xylella fastidiosa*. *Molecular Plant-Microbe Interactions* 28, 497–507.

Neilsen D., P.B. Hoyt and A.F. MacKenzie, 1986. Distribution of soil Zn fractions in British Columbia interior orchard soils. *Canadian Journal Soil Science* 66, 445–454.

Norman D.J., M. Zapata, D.W. Gabriel, Y.P. Duan, J.M.F. Yuen, A. Mangravita-Novo and R.S. Donahoo, 2009. Genetic diversity and host range variation of *Ralstonia solanacearum* strains entering North America. *Phytopathology* 99,1070–1077.

Normes OEPP/EPPO Standards, 2016. PM 7/24 (2) *Xylella fastidiosa*. *Bulletin OEPP/EPPO Bulletin* 46, 463–500.

Nouri H., S. Beecham, S. Anderson and P. Nagler, 2014. High spatial resolution worldview-2 imagery for mapping NDVI and its relationships to temporal urban landscape evapotranspiration factors. *Remote Sensing* 6, 580–602.

Orians C.M., M.M. Van Vuuren, N.L. Harris, B.A. Babst and G.S. Ellmore, 2004. Differential sectoriality in a long dis-

tance transport in temperate tree species, evidence from dye flow, ^{15}N transport, and vessel element pitting. *Trees* 18, 501–509.

Pérez-Donoso A.G., Q. Sun, M.C. Roper, L.C. Greve, B. Kirkpatrick and J.M. Labavitch, 2010. Cell wall-degrading enzymes enlarge the pore size of intervessel pit membranes in healthy and *Xylella fastidiosa*-infected grapevines. *Plant Physiology* 152, 1748–1759.

Perrson P., 1998. Successful eradication of *Ralstonia solanacearum* from Sweden. *Bulletin OEPP/EPPO Bulletin* 28, 113–119.

Press W.H.,S.A. Teukolsky,W.T. Vetterling and B.P. Flannery, 1992. Numerical recipes in C++. *The art of scientific computing*. Cambridge University Press, Cambridge, UK, 616 pp.

Purcell A.H., 1977. Cold therapy of Pierce's disease of grapevines. *Plant Disease Reporter* 61, 514–518.

Purcell A.H., 1980. Environmental therapy for Pierce's disease of grapevines. *Phytopathology* 64, 388–390.

Rodrigues C.M., M.A. Takita, H.D. Coletta-Filho, J.C. Olivato, R. Caserta R, M.A. Machado and A.A. de Souza, 2008. Copper resistance of biofilm cells of the plant pathogen *Xylella fastidiosa*. *Applied Microbiology and Biotechnology* 77: 1145–1157.

Rouse J.W., R.H. Haas, J.A. Schell and D.W. Deering, 1974. Monitoring vegetation systems in the Great Plains with ERTS. In: *Proceedings of the Third Earth Resource Technology Satellite (ERTS) Symposium* Vol. 1, 48–62.

Sano Y., Y. Okamura and Y. Utsumi, 2005. Visualizing water-conduction pathways of living trees: selection of dyes and tissue preparation methods. *Tree Physiology* 25, 269–275.

Sanzani SM., L. Schena, F. Nigro, V. Sergeeva, A. Ippolito and M.G. Salerno, 2012. Abiotic diseases of olive.*Journal of Plant Pathology* 94, 469–491.

Saponari M., D. Boscia, F. Nigro and G.P. Martelli, 2013. Identification of DNA sequences related to *Xylella fastidiosa* in oleander, almond and olive trees exhibiting leaf scorch symptoms in Apulia (southern Italy). *Journal of Plant Pathology* 95, 659–668.

Schubert T.S., S.A. Rizbi, T.R. Gottwald, J.H. Graham and W.N. Dixon, 2001. Meeting the challenge of eradicating citrus canker in Florida—again. *Plant Disease* 85, 340–356.

Scortichini M., 2017. Preliminary results on field trial to control *Xylella fastidiosa* on olive trees in Puglia. *Options Méditerranéennes* 121, 77–78.

Stacey A.J., J.E. Truscott, M.J.C. Asher and C.A. Gilligan, 2004. A model for invasion and spread of rhizomania in the UK: implications for disease control strategies.*Phytopathology* 94, 209–215.

Sun Q., L.C. Greve and J.M. Labavitch, 2011. Polysaccharide composition of intervessel pit membranes contribute to Pierce's disease resistance of grapevines. *Plant Physiology* 155, 1976–1987.

Sun Q., Y. Sun, M.A. Walker and J.M. Labavitch, 2013. Vascular occlusions in grapevine with Pierce's disease make disease symptom development worse. *Plant Physiology* 161, 1529–1541.

Thomashow M.F., 1998. Role of cold responsive genes in plant freezing tolerance. *Plant Physiology* 118, 1-7.

Umebayashi T., Y. Utsumi, S. Koga, S. Inoue, Y. Shiiba, K. Arakawa, J. Matsumura and K. Oda, 2007. Optimal conditions for visualizing water-conducing pathways in a living tree by the dye injection method. *Tree Physiology* 27, 993–999.

Valentini F., G. Cavallo and A.A. D'Onghia, 2017. Sampling procedures of plant material for the survey of *Xylella fastidiosa* in Puglia Region.*Options Méditerranéennes* 121, 49–52.

Viña A., A.A. Gitelson, A.L. Nguy-Robertson and Y. Peng, 2011. Comparison of different vegetation indices for the remote assessment of green leaf area index of crops. *Remote Sensing and Environment* 115, 3468–3478.

White S.M., J.M. Bullock, D.A.P. Hooftman and D.S. Chapman, 2017. Modelling the spread and control of *Xylella fastidiosa* in the early stages of invasion in Apulia, Italy. *Biological Invasions* 19, 1825–1837.

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