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**Pest Organisms Threatening Europe (POnTE)**

# DELIVERABLE 2.1

# Knowledge of the pathogenicity and virulence of *Xylella fastidiosa* subsp. *pauca* ST53 on susceptible hosts

| | |
|---|---|
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| **Task number** | 2a. Isolation, biology and pathogenicity of *Xylella fastidiosa* |
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**Note:** Because at the time when the project was prepared, the only bacterial outbreak was the one reported in southern Italy, the research program proposed in POnTE on *X. fastidiosa* is mainly focused on the bacterial isolates associated to the severe infections on olive trees.based on the initial characterization the isolates associated to the infections were identified as "CoDiRO". However, in the framework of the project genetic studies allowed to identify a reference strain, which was fully sequenced and used as reference strain for all artificial inoculation and pathogenicity studies under controlled conditions. This strain was named "De Donno" and was deposited at the International Center for Microbial Resources-CFBP collection under the code CFBP 8402. Thus although in the initial version of the project, the Apulian strain is referred as "CoDiRO" strain, the results herein described refer to the strain De Donno.

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## 1. **OBJECTIVES IN WORK PACKAGE 2 AND TASK 2a**

The general objective of the research tasks planned in WP2 is the assessment of the biological properties of strains of the target pathogens (host range, pathogenicity). Studies include (i) field surveys to determine the occurrence, the natural host(s) and the symptoms induced under field infection conditions; (ii) artificial inoculations under controlled conditions to determine the pathogenicity of the target strain(s) on different host(s) or cultivar/varieties. In relation to the investigations on *Xylella fastidiosa* the main efforts have been devoted to investigate the biology and the pathogenicity of the bacterial isolates discovered in the region of Apulia (southern Italy), where the bacterium was discovered in 2013, associated to a novel severe disease decimating olive trees in this area. Genetic characterization of bacterial isolates carried out in the framework of WP3, indicated that infections on olives and other natural susceptible hosts were associated to *X. fastisiosa* subsp. *pauca*, genotype ST53. However, investigations have been extended to isolates recovered in Corsica and mainland France, where outbreaks have been discovered during the project lifespan.

The relevant part of the biological studies has been conducted by the Partners P1 (CNR) and P2 (UNIBA) working at the forefront of the epidemic in southern Italy, nevertheless experiments have been also carried out by the French partners, P3-INRA and P4-ANSES, but are still ongoing and hence too preliminary to be included in the present report, upon receiving the cultured reference strain De Donno from P1 (CNR). Both research Institutions have laboratories and greenhouses authorized to manipulate the quarantine bacterium (in vitro and in planta).

Experiments conducted by P1 and P2 were based on: i) surveys in the infected area (i.e. southern part of the Apulia region, under high pressure of inoculum) of natural susceptible hosts species supporting infections by *X. fastidiosa*, subsp. *pauca*, ST53, and (ii) artificial co-inoculations of X. fastidiosa and different fungal species to determine the role and the pathogenicity of the strain on different susceptible hosts. Because biological studies are in general long-term experiments, the activities of this research task took advantage from the activities started in early 2015 in the framework of the pilot project funded by EFSA (NP.EFSA.ALPHA.2014.07), whose duration was too limited to collect conclusive biological data, and thus field and greenhouse experiments were continued in the framework of "task 2a" of the project POnTE.

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## 2. DESCRIPTION OF DELIVERABLE 2.1

This deliverable summarizes the outcomes of experiments aiming at assessing the pathogenicity of isolates of *X. fastidiosa* subsp. *pauca*, ST53, infecting olives in Apulia, and to disclose the role of the bacterial infections in the etiology of the severe disease affecting olives in this area, the so called Olive Quick Decline Syndrome (OQDS).

OQDS was recognized as a complex disease, with olive trees found to be co-infected by *X. fastidiosa* and a number of sapwood fungal species. Thus, the main question driving the experiments was to assess the role of *X. fastidiosa* as causal agent of OQDS. To this, end, initial evidences that *X. fastidiosa* had major role in the disease were collected in the framework of the EFSA pilot project (NP.EFSA.ALPHA.2014.07). Whereas, in the framework of the project POnTE, the research teams of partners P1 and P2 performed co-inoculations under controlled conditions of *X. fastidiosa* and different fungal species, and conducted extensive field surveys in the OQDS-affected olive groves (in the demarcated infected area of the Apulia region) to establish rates of correlation between bacterial infections and disease symptoms. In addition, vector-mediated transmission and grafting were also used to assess if bacterial infections could be graft-transmitted and symptoms of OQDS reproduced by grafting infected cuttings or upon vector transmission.

## 3. MATERIALS AND METHODS

### 3.1 Artificial co-inoculations (P1-CNR, P2-UNIBA)

Inoculations were made on 2-year-old grafted plants of the cultivar Cellina di Nardò, one of the most common OQDS-affected cultivar under field conditions in the demarcated infected area of Apulia (southern Italy).

The selected reference strain De Donno (CFBP 8402) was used for infecting the plants in May 2015, by needle inoculations. Briefly, a bacterial suspension (>10^8 CFU/ml) was prepared by scraping and resuspending 8-10 day old colonies from BCYE medium, in 1-2 ml of PBS buffer. The bacterial suspension was then immediately used to inoculate 3-4 shoots for each plants. On each shoot, according to the length and hardness, 2-3 points of inoculations (5-10cm distant from each other) were performed generally in the basal portion. The point of inoculations and the shoots that received the inoculum were marked.

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One month after the bacterial inoculation the following fungal strains where inoculated on the main trunk of the plants, at 50-60cm from the soil. More specifically, inoculations were performed using *Phaeoacremonium aleophilum* B1a, *Ph. rubrigenum* N20, *Pseudophaeomoniella oleae* Fv84, *Ps. oleicola* M24 and M51. For fungal inoculation, the trunk of the plants was injured by removing approximately 5mm of bark and placing the mycelial plugs (4 mm in diameter) obtained from the edge of 14-day-old cultures of each isolate grown on PDA. Sterile non-colonized PDA plugs were used to inoculate control plants. The points of inoculation were then wrapped with sterile wet cotton and parafilm to avoid dehydration (Figure 1). Treatments consisted of 8 replicates for each combination of inoculation and 6 replicates for each control (Table 1).

**Figure 1.** Plants of cv Cellina di Nardò (2-year old) inoculated on the trunk with the different selected fungal isolates.

Plants were then periodically inspected for symptoms, tested for *X. fastidiosa* and 1.5 year later subjected to isolation for assessing the fungal colonization in the trunk (destructive test). Assays for *X. fastidiosa* were performed by sampling and testing separately leaves

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from the inoculated and non-inoculated shoots, to confirm that needle inoculations resulted in systemic infections. Diagnostic tests were performed by quantitative PCR using the primers designed by Harper et al. (2010) and following the procedure recommended in the EPPO standard 7/24 (3). Fungal colonization pattern was assessed by performing isolation at 7 representative points, below and above the inoculation point: -25 cm, -5 cm, +3 cm, +25 cm, +50 cm, +75 cm.

**Table 1.** Combinations of the co-inoculations performed on potted olive plants

| N. of replicates and ID | Fungal isolate | *X. fastidiosa* | N. of replicates | Fungal isolate | *X. fastidiosa* |
|---|---|---|---|---|---|
| 8 (X1-X8) | No | Yes | 6 (X49-X54) | M24 | No |
| 8 (X9-X16) | B1a | Yes | 6 (X55-X60) | M51 | No |
| 8 (X17-X24bis) | N20 | Yes | 6 (X61-X66) | Fv84 | No |
| 8 (X25-X32) | Fv84 | Yes | 6 (X67-X72) | N20 | No |
| 8 (X33-X40) | M51 | Yes | 6 (X73-X78) | B1a | No |
| 8 (X41-X48) | M24 | Yes | 6 (X79-X84) | No | No |

### 3.2 Vector mediated transmission

Ten 1-year-old grafted plants (Figure 2) of cv Cellina di Nardò were caged with 15 adults of *Philaenus spumarius*, collected in September 2016 from the canopies of *Xylella*-infected olives severely affected by extensive dessications. The incidence of infected specimens, previously determined by testing in qPCR, was in the average range of 60%. Plants were maintained caged with the insects for approximately 15 days (i.e. till the majority of them died) to simulate field situation multiple re-infections occur on the trees.

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Plants were then tested 6 and 12 months after the transmission test, by randomly collecting leaves, and then re-tested 1 year after. Plants were also inspected for symptoms.

**Figure 2**. Plants of Cellina di Nardò used for vector transmission. On the left side panel a plant caged with adults of *Philaenus spumarius*.

### 3.3 Graft-transmission of *X. fastidiosa* to olives.

Cuttings of a size suitable for grafting (6-7 mm in diameter) were collected from green branches of a OQDS-affected tree of 'Ogliarola Salentina', checked by isolation and qPCR for the presence of *X. fastidiosa,* and top-grafted on a total of 48 potted 3-year-old olive seedlings. After graft-take, the bacterial population was monitored in the new sprouts pushed by the scions, and in the rootstocks, to determine whether the bacterium had moved into them.

### 3.4 Assessment of the pathogenicity of *X. fastidiosa* on olives under field conditions

This experiment is the follow up of the field trial started in April 2015 in the framework of the EFSA pilot project and in collaboration with the local olive producer organization "A.PR.O.L. Lecce". The experimental plot included 24 replicates for each selected cultivar planted in four randomized blocks of six plants each, indeed plants of *Polygala myrtifolia* and *Nerium oleander* were also planted in a single row surrounding the borders of the experimental plot (Table 2). To increase the vector transmission rate from the surrounding environment, in July 2015 all plants were caged with naturally infected specimens of *P.*

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*spumarius* collected in the surrounding *Xylella*-affected olive groves. Plants of the olive selections Don Carlo and FS17 were not caged due to their small size, nor the plants of *P. myrtifolia* and oleander. Cages with insects were maintained on the plants for 4 weeks. This first trial was extended in the framework of the project POnTE with additional 19 cultivars planted in April 2016, using the same randomized scheme. Symptom inspection and sampling were conducted at least once a year (in general sampling was done between the end of the winter season and early spring, so that the infections occurred the year before between late spring and late summer could likely be already detectable). Samples consisted of 4-5 cuttings collected randomly from the canopy, from which 8-10 leaves were excised and used for ELISA (as first test) and DNA extraction and qPCR test (Harper *et al.,* 2010) for all ELISA-negative samples. None of the plants was pruned to avoid risks of removing shoots with initial stage of bacterial colonization.

### 3.5 Field surveys in olive groves located the demarcated infected area

#### 3.5.1. Isolation of the bacterium from OQDS symptomatic trees

Surveys were carried out between 2014 and 2017 in olive groves with typical OQDS symptoms. The minimum distance between the closest foci was 2 km. In each of the 58 foci, one diseased grove was selected, and in each grove, from 3 to 4 ancient trees of 'Ogliarola Salentina' or 'Cellina di Nardò' were sampled by collecting from each tree 8-10 twigs next to symptomatic branches. Prior to perform bacterial isolation, the presence of the bacterium in the samples was determined by quantitative real time PCR (qPCR) (Harper et al., 2010). Isolation was then immediately performed (within 3 days from sampling), using cuttings of 0.4-0.8 cm in diameter, i.e. 1 to 2-year-old, from at least one qPCR-positive tree from each selected grove. A total of 10-12 pieces of 5-6cm in length were recovered from the selected cuttings and abundantly washed under tap water with dish soap. Subsequently, they were surface sterilized by soaking the cuttings in 2% sodium hypochlorite solution for 2min, followed by 2min in ethanol 70%. After rinsing the cuttings in sterile water, they were dried, divided in half and squeezed with a plier to make imprints on BCYE agar plates, by gently pressing the fresh made cut on the agar surface. At least two plates were made for each tree.

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### 3.5.2 Large scale sampling in OQDS affected olive groves

In 2017, an extensive sampling was conducted in 16 different sites of the demarcated infected area. In 13 out of 16 olive groves, all trees were of cv. 'Ogliarola salentina' and 'Cellina di Nardò' showing severe OQDS symptoms (over 70% of the canopy collapsed). In the remaining 3 groves all trees were of cv 'Leccino', and showed milder or no evident symptoms. From 25 to 50 trees of 'Cellina di Nardò' and 'Ogliarola Salentina' were sampled in each site for a total of 500 trees, whereas 100 trees of cv 'Leccino' were sampled in total. Samples consisted of 20 hardwood cuttings (at least 1-year old) collected from the canopy (i.e. avoiding suckers). All samples were subjected to a double tests: serological test ELISA (at the laboratory of CRSFA- linked party P2) and molecular test qPCR (Harper et al., 2010) (at the jointed laboratory of P1-P2). An estimation of the differential bacterial population size detected in trees of 'Ogliarola salentina', 'Cellina di Nardò' and 'Leccino' was attempted by using a reference standard curve generated by testing a serial dilution of samples spiked with known bacterial concentration (from 10^7 to 10^2 CFU/ml). Indeed, a comparison of the OD values recovered in ELISA was also made to get a rough indication of the bacterial concentration in the positive trees.

## 4. RESULTS AND DISCUSSION

### 4.1 Co-inoculations of fungal species and *X. fastidiosa*

Successful host colonization was obtained upon inoculations of the different fungal isolates and *X. fastidiosa* strain De Donno. The results collected six months post inoculation when leaf samples (from the inoculated an non-inoculated shoots) were tested for *X. fastidiosa*, showed that the bacterium was successfully inoculated in all replicates and infections were established. Similarly, as shown in figure 3 fungal isolation confirmed successful colonization, with *Ps. oleicola* M24 showing the larger colonization pattern and the highest isolation frequency, compared to the other isolates tested. In particular, this strain was found both 75cm above and 25cm below the inoculation point, with an isolation frequency around 30-60%. Conversely, the strains *Pseudophaeomoniella* sp. M51 and *Ph. aleophilum* B1a showed the lowest colonization pattern, remaining confined around the inoculation point and reaching 25cm upward at very low isolation frequency. *Ps. oleae* Fv84 showed the most intense and large

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discoloration (Fig. 4).

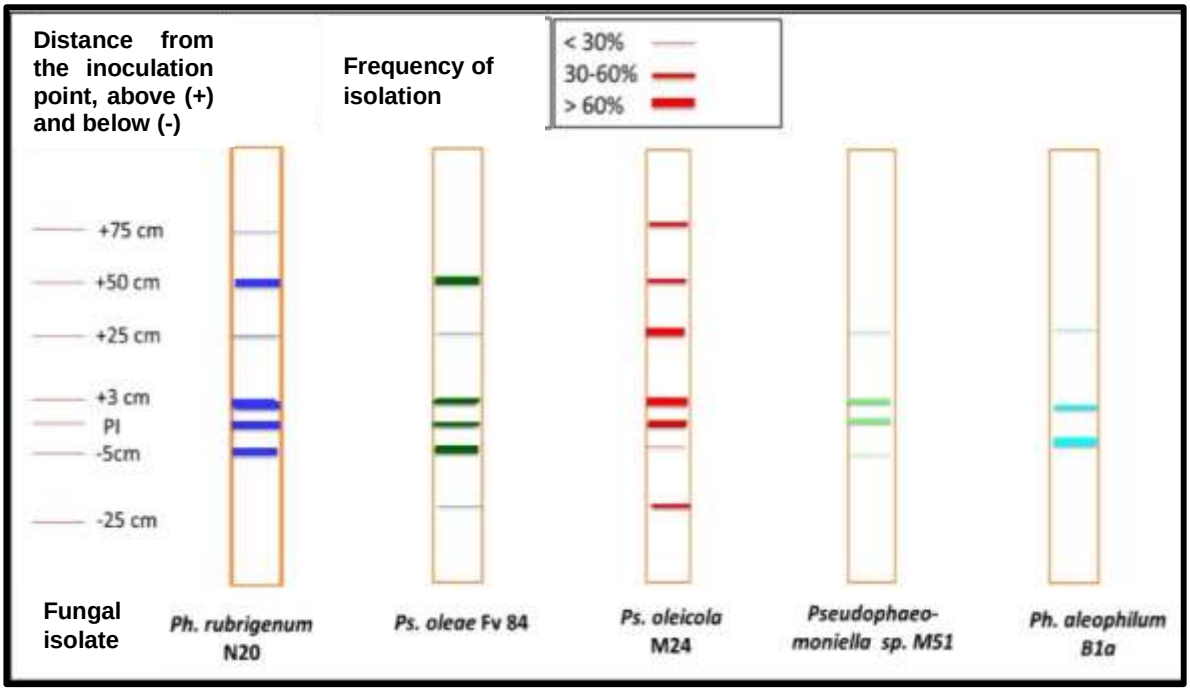


**Figure 3**. Schematic representation of the host colonization and frequency of the isolation of the five fungal species inoculated in olive plants.


**Figure 4**. Wood discoloration detected at 75cm above the inoculation points in the plants inoculated with the isolate Fv84 of *Ps. oleae.*

With regard to the symptoms scored on the inoculated plants, results showed clear distinct desiccation phenomena: (i) plants inoculated only with the fungal isolates (regardless the species/isolates) showed mild wilting symptoms, limited to few (2-5) basal twigs per plants, always close to the inoculum point on the main trunk (Fig. 5-7); (ii) plants

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inoculated with *X. fastidiosa* started to show symptoms of dieback and desiccation, 8 months after the inoculations, regardless the presence or not of the co-inoculated selected fungal species; (iii) no differences were recorded in the severity of the desiccation (number of desiccated shoots) among plants that harbored only *X. fastidiosa* strain De Donno or those where the different fungal isolates were also added. However, the desiccation phenomena recorded on the plants infected with *X. fastidiosa* and those recorded on the plants where only the fungal isolates were inoculated were clearly distinguishable (Fig. 5-7), with those associated to *X. fastidiosa* being more severe and causing the dead of the entire canopies of the plants.

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**Figure 5.** Plants of Cellina di Nardò 14 months post inoculation. On the right panel one of the plant co-inoculated with *Xylella fastidiosa* strain De Donno and isolate Fv84 of Ps oleae. The co-infected plant clearly shows severe desiccations on the upper part of the canopy. On the rigth side a plant inoculated only with isolate Fv84 of *Ps. oleae*, showing wilting and desiccations on few shoots at bottom of the canopy (i.e. close the inoculation point of the fungal isolate).

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**Figure 6**. Plants of Cellina di Nardò 14 months post inoculation. On the right panel one of the plant inoculated with the isolate Ps. oleicola M24 and with isolate Fv84 of Ps oleae on the left panel. Both plants show typical wilting and desiccation of the basal shoots.

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**Figure 7.** Overview of the plants of Cellina di Nardò 14 months post inoculation. On the left side the plants co-inoculated with *X. fastidiosa*; on the right side the plants inoculated only with the different fungal species.

### 4.2 Host colonization and symptoms upon vector-transmission of *X. fastidiosa*.

Six months after the vector-transmission experiments, 7 out of 10 plants resulted systemically infected by *X. fastidiosa*. Quantitative PCR results indicated quantitation cycles varying from 23.54 to 31.51. None of the three negative plants tested positive when they were re-sampled 12 months post transmission. Symptoms of wilting appeared as soon as 12 months post transmission, on 1 of the 7 infected plants, later on symptoms were recorded on all infected plants, starting with chlorosis, wilting and progressing in the typical dieback and desiccation of the shoots (Table 3, Figure 8).

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**Table 3**. Results of the diagnostic tests and symptoms evaluation on the olive plants infected by vector-mediated transmission

| ID of the plants | % of positive insects caged on each plant | Cq | Results of the qPCR test performed 6 months post transmission (March 2017) | Symptoms of desiccation recorded 18 months after the transmission |
|---|---|---|---|---|
| PA16-360 | Not done | 23.72 | Positive | Present |
| PA16-361 | 35.71 | 31.51 | Positive | Present |
| PA16-362 | 64.28 | 24.22 | Positive | Present |
| PA16-363 | 66.66 | 0 | Negative | Absent |
| PA16-364 | Not done | 25.45 | Positive | Present |
| PA16-365 | 75 | 30.32 | Positive | Present |
| PA16-366 | 50 | 0 | Negative | Absent |
| PA16-367 | 33.33 | 0 | Negative | Absent |
| PA16-368 | 75 | 23.54 | Positive | Present |
| PA16-369 | 57.14 | 26.23 | Positive | Present |
| PA-HC | Negative control | | | Absent |
| PA-HC | Negative control | | | Absent |
| PA-HC | Negative control | | | Absent |

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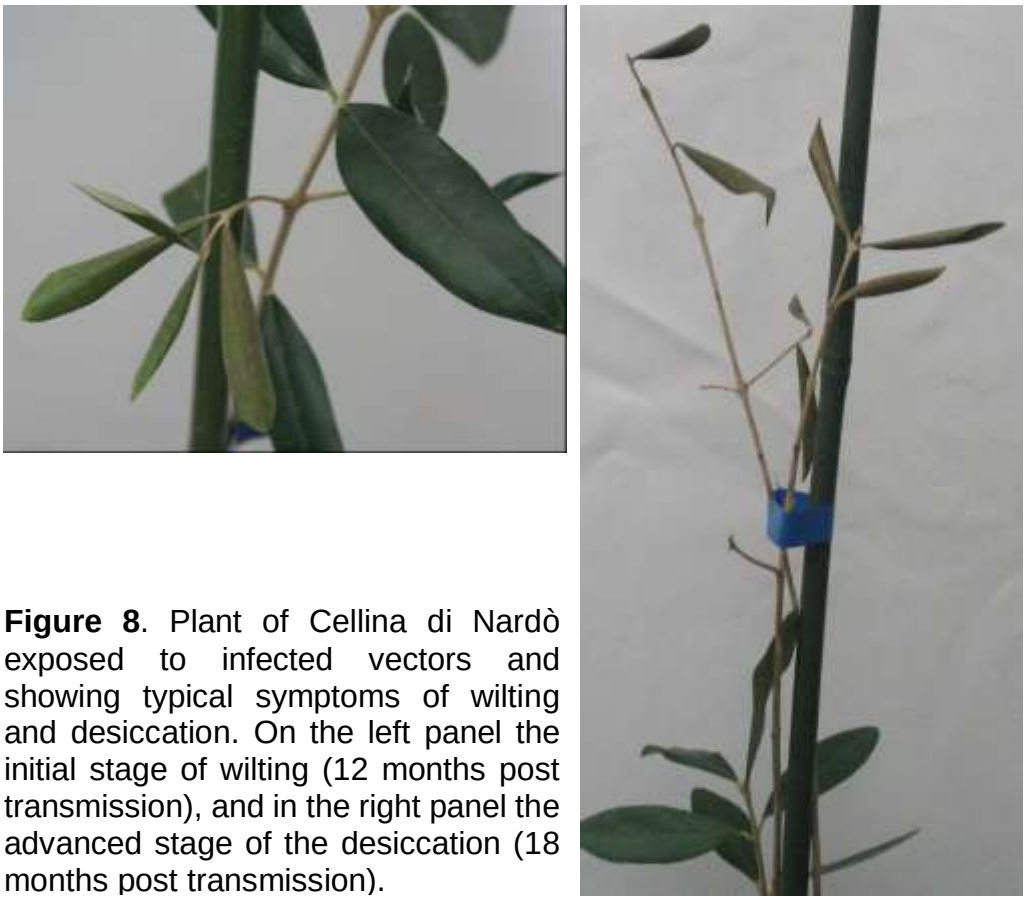

**Figure 8**. Plant of Cellina di Nardò exposed to infected vectors and showing typical symptoms of wilting and desiccation. On the left panel the initial stage of wilting (12 months post transmission), and in the right panel the advanced stage of the desiccation (18 months post transmission).

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### 4.3 Graft-transmission of *X. fastidiosa* to olives

Graft take was approximately 30% (15 grafted plants out of 48). Five of these plants originated from qPCR-negative cuttings and 10 from positive cuttings. Two and six months after grafting, consistent negative responses by qPCR came from the five plants derived from healthy cuttings (qPCR negative) whereas at 2 and 6 months after grafting, 6 and 10 positives plants were detected among the plants originated from the infected cuttings. Twelve months after grafting rootstocks, roots were also checked for bacterial presence, yielding positive results consistent with the presence of typical OQDS symptoms. None of scions of these infected and symptomatic plants survived 16 months post-grafting, although sprouts from the rootstocks continued to grow (symptomless or with some leaf-scorch symptoms). Whereas plants from the qPCR-negative cuttings continued to grow and remained symptomless (Fig. 9)

**Figure 9.** Symptoms of desiccation on a plant originated by grafting an infected cutting onto a healthy olive seedling.

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### 4.4 Assessment of the pathogenicity of *X. fastidiosa* on olives under field conditions

The data so far collected refer mainly to the rates of infected olive plants for the different cultivars, since upon 3 and 4 years from planting no clear-cut symptoms could be recorded under field conditions on the trees of different cultivars, except some suspicious symptoms recorded on few plants (6 out 24) of Cellina di Nardò recorded 1,5 year after planting (i.e. in July 2016). Indeed, the symptoms did not progress rapidly as observed under greenhouse conditions, in fact after 4 years none of the infected plants showed severe desiccation compromising the survival of the infected plants.

The data reported in the following tables 4 and 5 and in figure 12 include the cumulative results of ELISA and qPCR assays.

**Table 4.** Progression of the infections under field conditions in the plot planted in April 2015

| **Olive cultivars** | **Results of the diagnostic assays: % of positive plants** | | | |
|---|---|---|---|---|
| | **2015** | **2016** | **2017** | **2018** |
| CORATINA | 44 (8/18) | 69.6 (16/23) | 58.33(14/24) | 70.83(17/24) |
| LECCINO | 8.3(2/24) | 33.3 (7/21) | 45.83(11/24) | 45.83(11/24) |
| ARBOSANA | 58.3 (14/24) | 85.7 (18/21) | 84(21/25) | 84 (21/25) |
| ARBEQUINA | 69.5 (16/23) | 85 (17/20) | 79.17(19/24) | 79.17(19/24) |
| KORONEIKI | 75 (18/24) | 90 (19/21) | 82.61(19/23) | 86.96(20/23) |
| CELLINA DI NARDÒ | 62.5 (15/24) | 95 (20/21) | 83.33(20/24) | 95.83(23/24) |
| CIMA DI MELFI | 50 (12/24) | 75 (18/24) | 75(18/24) | 75(18/24) |
| FRANTOIO | 45.8 (11/24) | 61.9 (13/21) | 54.17(13/24) | 54.17(13/24) |
| **Other susceptible hosts** | | | | |
| *Nerium oleander* | 10 (2/20) | 50.00 (9/18) | | 92.31(12/13) |
| *Polygala myrtifolia* | 81.82 (9/11) | 85.71 (6/7) | | 88.89(8/9) |

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**Figure 10**. Olive tree of the cultivar Cellina di Nardò planted in the infected area in April 2015 and showing shoot dieback in July 2016.

**Figure 11.** Overview of the plants 3 years after planting (picture taken in 2018). Although as shown in table 4 the incidence of infections is relatively high, none of the plants show severe desiccation phenomena.

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**Table 5**. Progression of the infections under field conditions in the plot planted in April 2016

| Olive cultivars | Results of the diagnostic assays: % of positive plants | | |
|---|---|---|---|
| | 2016 | 2017 | 2018 |
| TOSCANINA | 0.00 | 4.35 | 5.00 |
| PERANZANA | 4.17 | 8.33 | 17.39 |
| PHICOLINE | 4.17 | 13.04 | 18.18 |
| LECCINO* | 4.17 | 4.17 | 8.33 |
| PENDOLINO | 20.83 | 20.83 | 45.83 |
| ITRANA | 0.00 | 9.09 | 31.82 |
| SIMONE | 4.17 | 4.35 | 8.70 |
| CELLINA DI NARDO'** | 4.17 | 12.50 | 34.78 |
| NOCELLARA MESSINESE | 8.33 | 16.67 | 21.74 |
| ASCOLANA TENERA | 4.17 | 8.33 | 17.39 |
| BELLA DI SPAGNA | 4.17 | 12.50 | 30.43 |
| TERMITE DI BITETTO | 4.17 | 4.17 | 12.50 |
| MAIATICA | 4.17 | 4.55 | 9.09 |
| DOLCE DI CASSANO | 20.83 | 27.27 | 45.45 |
| NOCIARA | 8.33 | 17.39 | 21.74 |
| OGLIAROLA Barese | 4.17 | 12.50 | 37.50 |
| CIPRESSINO | 4.17 | 8.33 | 16.67 |
| OLIASTRO | 8.33 | 12.50 | 30.43 |
| NOCELLARA ETNEA | 12.50 | 25.00 | 25.00 |

* cultivar used as reference control for resistance; ** cultivar used as reference control for high susceptibility.

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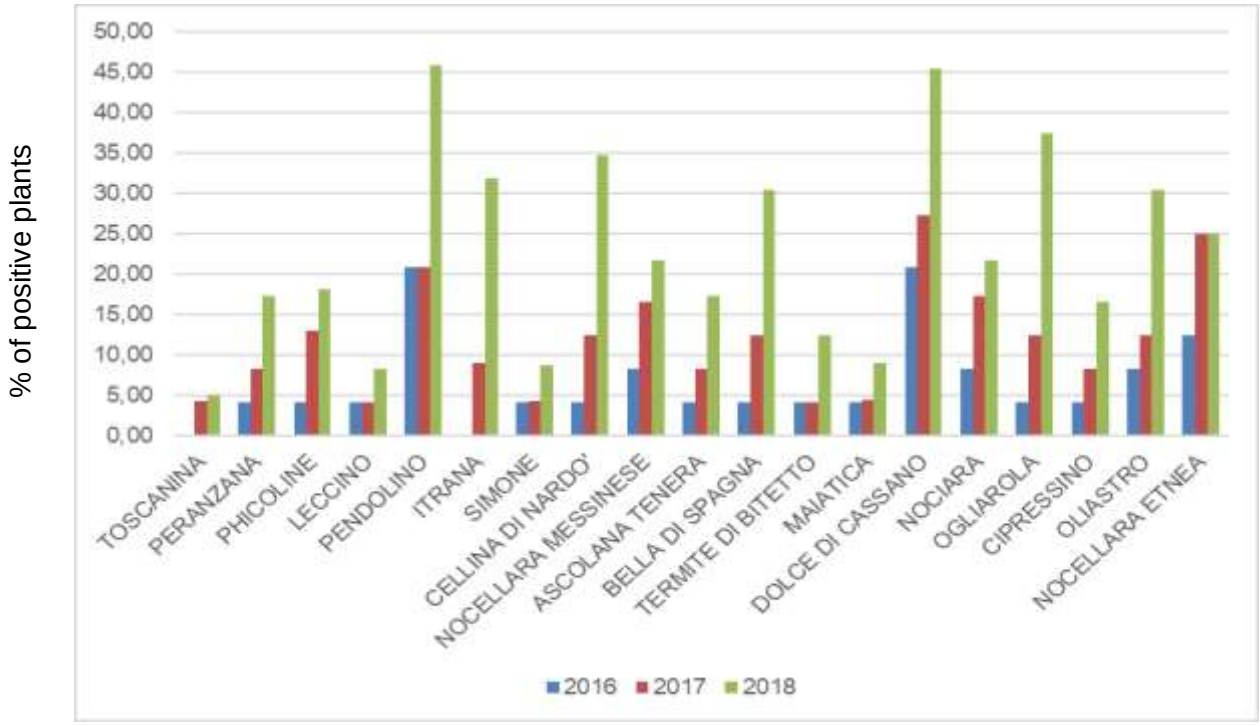


**Figure 12**. Infection rates (%) on 19 olive cultivars exposed to natural infections from 2016 to 2018.

While in the plot realized in April 2015, infections were "boosted" by caging insects on the plants (Table 4); the data reported in table 5 and figure 12 are indicative of the natural spread and progression of the infections in the area under high pressure of inoculum. As shown, after three years (i.e. three seasons of exposure to infected vectors) the incidence of infections can reach values higher of 30% for some cultivars. Interestingly, based on the data so far collected on the incidence of infections, cultivars could be grouped in three clusters: one including the reference resistance cultivar Leccino and those showing low percentage of infections (i.e. lower or close to 10%) (Toscanina, Simone, Termite di Bitetto, Matiatica), a second cluster grouping those showing high incidence of infections likewise the susceptible cultivar Cellina di Nardò (i.e. higher than 35%) (Ogliarola Barese, Dolce di Cassano, Pendolino, Bella di Spagna, Oliastro), and a third group including those with intermediate values.

Although not all positive olive trees detected during one year were re-tested during the subsequent year, the data collected for the positives that were repeatedly tested showed consistent results, i.e. agreement between the results obtained in two different years.

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## 4.5 Field surveys

### 4.5.1 Isolation of *X. fastidiosa* from symptomatic olive trees

*X. fastidiosa* was detected by qPCR in all the symptomatic trees sampled all fifty-eight OQDS outbreaks of different age, from the oldest (2013) to the more recent (2016). Actively growing *X. fastidiosa* colonies were successfully obtained from 51 of the 58 olive samples, collected in distinct OQDS foci (Fig.13, Table 6). Colonies grew relatively slowly, requiring 7 to 15 days to become visible and developed in several spotted imprints of each sample, an indication of the wide distribution of the bacterium in symptomatic trees. The highest number of spots per tree with actively growing colonies (i.e. 10-30 spots/tree) was obtained in May-June, whereas isolations made in August and January yielded colonies in fewer spots (i.e. 3-4 spots/tree). All cultured isolates were confirmed as *X. fastidiosa* by qPCR and were triple cloned prior to being stored in glycerol at -80°C. Thus, strong correlation was found between the occurrence of *Xylella*-infections and OQDS symptoms.

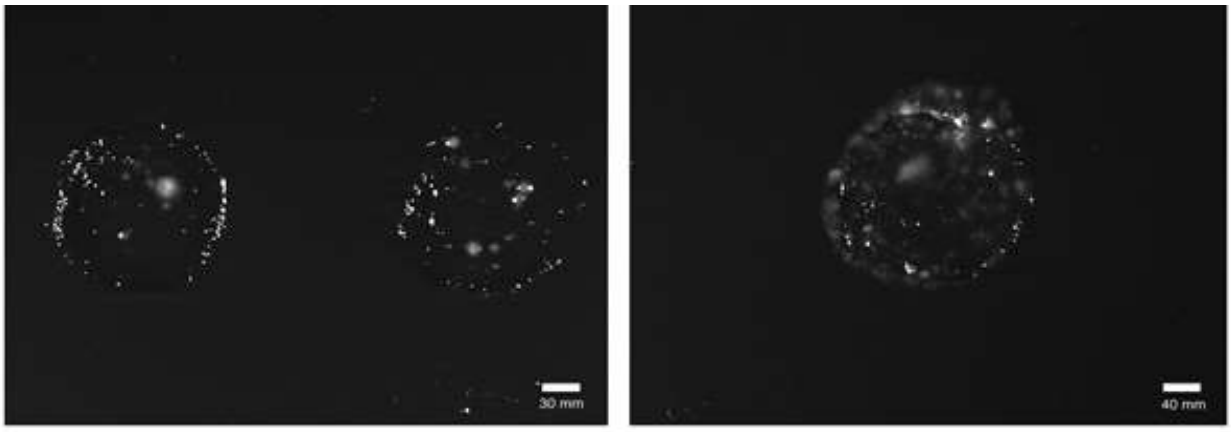


**Figure 13**. Imprints with actively growing *Xylella fastidiosa* colonies. Two spots with a low density of colonies, compared with a stem-imprint full of colonies (right).

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**Table 6.** List of the olive groves showing symptoms of Olive quick decline syndrome (OQDS) and representing the different foci used to collect the olive samples for the identification of *Xylella fastidiosa* by quantitative PCR (qPCR) (Harper et al., 2010) and isolation in axenic culture. In each grove, three symptomatic trees were selected and sampled.

| Foci OQDS | Code of the samples | Municipality (Province) | Date of sampling | Results of the qPCR for *X. fastidiosa*[a] | Cultured isolate |
|---|---|---|---|---|---|
| APL-1 | WPT 1129 | Minervino di Lecce (Lecce) | May, 2016 | Positive | YES |
| APL-2 | WPT 1130 | Uggiano la Chiesa (Lecce) | May, 2016 | Positive | YES |
| APL-3 | WPT 1133 | Cursi (Lecce) | May, 2016 | Positive | YES |
| APL-4 | WPT 1134 | Supersano (Lecce) | May, 2016 | Positive | YES |
| APL-5 | WPT 1135 | Maglie (Lecce) | May, 2016 | Positive | YES |
| APL-6 | WPT 1140 | Muro Leccese (Lecce) | June, 2016 | Positive | YES |
| APL-7 | WPT 1141 | Palmariggi (Lecce) | June, 2016 | Positive | YES |
| APL-8 | WPT 1144 | Spongano (Lecce) | June, 2016 | Positive | YES |
| APL-9 | WPT 1145 | Andrano (Lecce) | June, 2016 | Positive | YES |
| APL-10 | WPT 1148 | Tricase (Lecce) | June, 2016 | Positive | YES |
| APL-11 | AS | Cutrofiano (Lecce) | June, 2016 | Positive | YES |
| APL-12 | AV | Avetrana (Taranto) | June, 2016 | Positive | YES |
| APL-13 | CS | Campi Salentina (Lecce) | June, 2016 | Positive | YES |
| APL-14 | CIST | Alliste (Lecce) | May, 2016 | Positive | YES |
| APL-15 | CUT | Cutrofiano (Lecce) | June, 2016 | Positive | YES |
| APL-16 | FP | Presicce (Lecce) | June, 2016 | Positive | YES |
| APL-17 | GC | Gagliano del capo (Lecce) | June, 2016 | Positive | YES |
| APL-18 | GD | Gagliano del capo (Lecce) | June, 2016 | Positive | YES |
| APL-19 | Gigante | Alliste (Lecce) | May, 2016 | Positive | YES |
| APL-20 | Giug | Giuggianello (Lecce) | May, 2016 | Positive | YES |

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| | | | | | |
|---|---|---|---|---|---|
| APL-21 | La Castellana | Matino (Lecce) | June, 2016 | Positive | YES |
| APL-22 | San CAS | San Cassiano (Lecce) | May, 2016 | Positive | YES |
| APL-23 | TK | Nociglia (Lecce) | May, 2016 | Positive | YES |
| APL-24 | SP1 | Morciano di leuca (Lecce) | June, 2016 | Positive | YES |
| APL-25 | SP3 | Salve (Lecce) | June, 2016 | Positive | YES |
| APL-26 | SP4 | Presicce (Lecce) | June, 2016 | Positive | YES |
| APL-27 | SP7 | Specchia (Lecce) | June, 2016 | Positive | YES |
| APL-28 | Dedonno (CFBP 8402) | Gallipoli (Lecce) | June, 2014 | Positive | YES |
| APL29 | SZ | Squinzano (Lecce) | May, 2016 | Positive | YES |
| APL-30 | TR | Alliste (Lecce) | May, 2016 | Positive | YES |
| APL-31 | UG | Ugento (Lecce) | May, 2016 | Positive | YES |
| APL-32 | ORIA | Oria (Brindisi) | June, 2016 | Positive | YES |
| APL-33 | VEG | Veglie (Lecce) | June, 2016 | Positive | YES |
| APL-34 | CU | Cutrofiano (Lecce) | June, 2016 | Positive | YES |
| APL-35 | FO | Taviano (Lecce) | June, 2016 | Positive | YES |
| APL-36 | VN | Gallipoli (Lecce) | June, 2016 | Positive | YES |
| APL-37 | ST | Sternatia (Lecce) | June, 2016 | Positive | YES |
| APL-38 | GA | Gagliano del capo (Lecce) | June, 2016 | Positive | YES |
| APL-39 | MELC A | Ugento (Lecce) | August, 2014 | Positive | YES |
| APL-40 | COP | Copertino (Lecce) | August, 2014 | Positive | YES |
| APL-41 | CUR | Cursi (Lecce) | August, 2014 | Positive | YES |
| APL-42 | SC | Presicce (Lecce) | August, 2014 | Positive | YES |
| APL-43 | LI SAULI | Gallipoli (Lecce) | June, 2016 | Positive | YES |
| APL-44 | WPT 1137 | Salve (Lecce) | May, 2016 | Positive | YES |
| APL-45 | WPT 1139 | Specchia (Lecce) | May, 2016 | Positive | NO |

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| | | | | | |
|---|---|---|---|---|---|
| APL-46 | WPT 1142 | Otranto (Lecce) | June, 2016 | Positive | YES |
| APL-47 | WPT 1143 | San Cassiano (Lecce) | June, 2016 | Positive | YES |
| APL-48 | WPT 1146 | Specchia (Lecce) | June, 2016 | Positive | NO |
| APL-49 | WPT 1147 | Alessano (Lecce) | June, 2016 | Positive | NO |
| APL-50 | SP2 | Salve (Lecce) | June, 2016 | Positive | NO |
| APL-51 | SP5 | Miggiano (Lecce) | June, 2016 | Positive | NO |
| APL-52 | SP6 | Montesano Salentino (Lecce) | June, 2016 | Positive | NO |
| APL-53 | SP8 | Specchia (Lecce) | June, 2016 | Positive | NO |
| APL-54 | FP | Presicce (Lecce) | June, 2016 | Positive | YES |
| APL-55 | RAC | Racale (Lecce) | June, 2016 | Positive | YES |
| APL-56 | TR | Trepuzzi (Lecce) | June, 2016 | Positive | YES |
| APL-57 | SQ1 | San Vito dei Normanni (Brindisi) | January, 2017 | Positive | YES |
| APL-58 | SQ2 | Carovigno (Brindisi) | January, 2017 | Positive | YES |

[a] Samples were assessed as "Positive" when qPCR reactions produced quantitative cycle (Cq)>0 and <32; "Negative" when no fluorescence was detected in the reaction, Cq = 0. None of the samples tested as "doubtful" (Cq>32)

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### 4.5.2 Occurrence of *X. fastidiosa* infections in commercial olive trees in the infected area

All 500 symptomatic trees (severely affected, with over 70% of the canopy collapsed) belonging to cv Ogliarola salentina and Cellina di Nardò yielded positive reactions when tested by qPCR, few of them (n. 14) yielded negative results when tested by ELISA (Table 7). Whereas, when 100 trees of the cv Leccino, mostly symptomless, were tested using both methods, the bacterium was detected in 35% of the trees by qPCR and in 15% of the trees by ELISA. Quantitative analysis of the results recovered by ELISA (OD values) and qPCR (Cq values) clearly showed that infections supported by trees of the cultivar Cellina di Nardò, Ogliarola salentina and Leccino were associated to significant different bacterial population size in the infected trees (Table 8). Infected trees of the cultivar Leccino harbored lower bacterial populations (approx. 100 times lower) than the trees of the other 2 cultivars. Such difference was also evident when comparing the OD values recorded in the Elisa tests (Table 8).

**Table 7.** Results of ELISA and qPCR assays on susceptible olive cultivars selected in 13 different sites in the contaminated area.

| **Ogliarola salentina and Cellina di Nardò** (500 samples) | | | |
|---|---|---|---|
| **ELISA** | | **qPCR** | |
| **Positive** | 486 | **Positive** | 497 |
| **Negative** | 14 | **Negative** | 3* |
| **% of infected plants** | **97.2%** | **% of infected plants** | **99.2%** |
| **Leccino (100 samples)** | | | |
| **ELISA** | | **qPCR** | |
| **Positive** | **15** | **Positive** | **35** |
| **Negative** | **85** | **Negative** | **65** |
| **% of infected plants** | **15%** | **% of infected plants** | **35%** |

* After a second round of sampling these 3 samples tested positives

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**Table 8**. Estimation of the population size (CFU/ml) in the positive samples of the different cultivars. As shown, the data obtained are in agreement with the OD values recovered in the ELISA assays.

| CULTIVAR | CFU/ml | Mean of O.D. values (405 nm) of the positive samples |
|---|---|---|
| **Leccino** | 29.800 | 1.06 |
| **Cellina di Nardò** | 1.060.000 | 2.04 |
| **Ogliarola Salentina** | 1.320.000 | 2.23 |

The overall data collected in the field confirm: (i) the strict association of the bacterial infections and OQDS symptoms in susceptible trees; (ii) that differential response and susceptibility to *X. fastidiosa* exists under field conditions, as showed by the data collected on Cellina di Nardò and Ogliarola salentina *vs* Leccino; (iii) when testing olive trees of the susceptible varieties high agreement (>90%) is obtained between serological and molecular diagnostic assays; (iv) that with high pressure of inoculum, field infections in olive trees can reach level close to 100%, as the case of trees of susceptible cultivars.

## 5. CONCLUSIONS

The work and the results summarized in this deliverable refer mainly to the evaluation of the pathogenicity of *Xylella fastidiosa* subsp. *pauca*, ST53 on olive, the main susceptible host for this genotype. The overall research program herein described took advantage from the procedure standardized and the trials set up in 2015 in the framework of the EFSA pilot project (NP.EFSA.ALPHA.2014.07).

The results gathered in the project further extended the knowledge on the biology, virulence and latent period of the bacterial infections caused by *X. fastidiosa* subsp. *pauca*, ST53.

Main outcomes can be summarized as follows:

- Co-inoculations of different fungal species and *X. fastidiosa* on susceptible olive plants clearly showed that the typical symptoms of shoot dieback and desiccation could be reproduced only when *X. fastidiosa* was infecting these plants, either alone or in combination with fungal species causing vascular discolorations in olives. This is a relevant contribution that further support the role of *X. fastidiosa* in the etiology of the OQDS, confirming the aggressiveness of this *pauca* genotype on olives.

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- An additional insight gathered from the above mentioned experiment is the timeframe for the symptom development. In this experiment, symptoms on infected plants started to appear earlier (8 months post-inoculation) than those recorded in a previous experiment when clear symptoms could be observed not early than 12 months post-inoculation. The plants used in this experiment were older than those previously inoculated, suggesting that older plants may develop quickly the symptoms.
- Large scale field survey identified strong correlations among the presence of OQDS symptoms, the detection of the *Xylella*-infections, and the isolation of the bacterium from the diseased plants.
- Vector-mediated transmission using naturally infected adults of *Philaenus spumarius*, reproduced similar results as those obtained by needle inoculations. Within 6 months, plants were successfully colonized and the bacterium reached detectable level (although the plants were still symptomless); 12 months after the transmission (i.e. the infection events) symptoms started to appear and then progressed rapidly.
- Graft-transmission of *X. fastidiosa* was successfully obtained after grafting field-infected cuttings on healthy rootstocks, confirming that the bacterium can move against the transpiration stream. Indeed, symptoms were recovered approximately 12 months after graft take.
- Field experiments provided some important insights: (i) in the case of olive young plants, under field conditions, the latency period in the infected olives can be longer than under controlled conditions and symptoms progress slower than under greenhouse conditions; (ii) differences in the incidence of the infections among olive cultivars may represent a promising feature that can support the differentiation of highly susceptible, tolerant and resistant cultivars; (iii) once infected trees are detected in a plot, infections spread rapidly as shown in the plots planted in the infected area, where the number of infected plants can double over a one year period; (iv) differently from cherry trees, for which a sort of recovery was observed on young trees (see Deliverable 2.2 for more details), in the case of young olive trees once a new infection occurs it most likely will persist and progress within the tree.

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## 6. DISSEMINATION AND COMMUNICATION ACTIVITIES RELATED TO THIS DELIVERABLE

**Peer-reviewed papers**

1. M. Saponari, D. Boscia, G. Altamura, G. Loconsole, S. Zicca, G. D'Attoma, M. Morelli, F. Palmisano, A. Saponari, D. Tavano, V. N. Savino, C. Dongiovanni, G. P. Martelli, 2017. Isolation and pathogenicity of *Xylella fastidiosa* associated to the olive quick decline syndrome in southern Italy. Scientific Reports 7, 17723, doi:10.1038/s41598-017-17957-z.

2. D. Boscia, G. Altamura, M. Saponari, D. Tavano, S. Zicca, P. Pollastro, M.R. Silletti, V.N. Savino, G.P. Martelli, A. Delle Donne, S. Mazzotta, P.P. Signore, M. Troisi, P. Drazza, P. Conte, V. D'Ostuni, S. Merico, G. Perrone, F. Specchia, A. Stanca, M. Tanieli, 2017. Incidenza di *Xylella* in oliveti con disseccamento rapido. L'Informatore Agrario 27, 47-51.

**Contributions presented in conference and meetings**

1. **Biology and pathogenicity of *Xylella fastidiosa* associated to olive quick decline syndrome.** Saponari M., Boscia D., Altamura G., Loconsole G., Zicca S., D'Attoma G., Morelli M., Palmisano F., Saponari A., Dongiovanni E., Cavalieri V., Savino V.N., Martelli G.P. Oral presentation. European conference on Xylella fastidiosa: finding answers to a global problem: Palma de Mallorca, 13-15 November 2017.

2. **Screening of olive germplasm for resistance to *Xylella fastidiosa* ST53: the state of the art.** Boscia D., Altamura G., Dongiovanni C., Giampetruzzi A., La Notte P., Loconsole G., Martelli G.P., Morelli M., Palmisano F., Potere O., Saldarelli P., Savino V., Susca L., Tavano D., Zicca S., Saponari M. Oral presentation. European conference on Xylella fastidiosa: finding answers to a global problem: Palma de Mallorca, 13-15 November 2017.

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3. **Detection of *Xylella fastidiosa* in susceptible and resistant field olive trees.** Boscia D, Silletti MR, Pollastro P, Zicca S, Altamura G, Tavano D, Loconsole G, Saponari. Poster. European conference on Xylella fastidiosa: finding answers to a global problem: Palma de Mallorca, 13-15 November 2017.
4. **Screening olive cultivars for resistance to *Xylella fastidiosa.*** M. Saponari. Oral presentation. POnTE - XF-ACTORS, 2nd Joint Annual Meeting: European Research on Emerging Plant Diseases. Instituto Valenciano de Investigaciones Agrarias (IVIA) (Valencia), Spain 23-26 October 2018.

**Book of abstracts**

1. **Boscia D., Silletti M.R., Pollastro P., Zicca S., Altamura G., Tavano D., Loconsole G., Saponari M., 2017.** Detection of Xylella fastidiosa in susceptible and resistant field olive trees. In Book of abstract: European conference on Xylella fastidiosa: finding answers to a global problem: Palma de Mallorca, 13-15 November 2017. https://www.efsa.europa.eu/sites/default/files/event/171113/171113_book-of-abstracts.pdf.

2. **Boscia D., Altamura G., Dongiovanni C., Giampetruzzi A., La Notte P., Loconsole G., Martelli G.P., Morelli M., Palmisano F., Potere O., Saldarelli P., Savino V., Susca L., Tavano D., Zicca S., Saponari M., 2017**. Screening of olive germplasm for resistance to Xylella fastidiosa ST53: the state of the art. In Book of abstract: European conference on Xylella fastidiosa: finding answers to a global problem", Palma de Mallorca, 13-15 novembre 2017.

3. **Biology and pathogenicity of Xylella fastidiosa associated to olive quick decline syndrome.** Saponari M., Boscia D., Altamura G., Loconsole G., Zicca S., D'Attoma G., Morelli M., Palmisano F., Saponari A., Dongiovanni E., Cavalieri V., Savino V.N., Martelli G.P. In Book of abstract: European conference on Xylella fastidiosa: finding answers to a global problem" , Palma de Mallorca, 13-15 novembre 2017.

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**Preparation of technical reports submitted to EFSA and phytosanitary authority**

1. Upon a specific request by EFSA, about scientific evidences collected/developed in the framework of the project POnTE for X. fastidiosa subsp. pauca, ST53, the consortium POnTE submitted in March 2017 a detailed report on host susceptibility. The data contained in the report, were evaluated by EFSA and used in their pubhlished Statement: "EFSA (European Food Safety Authority), Bau A, Delbianco A, Stancanelli G andTramontini S, 2017. Statement on susceptibility ofOlea europaeaL. varieties toXylella fastidiosasubsp.paucaST53: systematic literature search up to 24 March 2017. EFSA Journal 2017;15(4):4772,18 pp. doi:10.2903/j.efsa.2017.4772".

2. Upon a specific request received by P1 (CNR) from the Regional Phytosanitary Authority, a report was prepared concerning the evidence of differential susceptibility of olive cultivars and other crop species susceptible to X. fastidiosa subsp. pauca, ST53. A report describing the results collectively gathered in the framework of the project POnTE and XF-ACTORS was submitted and used as supporting material for the implementation of the legislative measures and in particular to lift the prohibition of planting olive trees in the demarcated infected area of Apulia, a measure of relevant impact to sustain the re-establishment of olive production in this area where olive orchards of the local cultivars were decimating by the infections.

This project has received funding from the European Union's Horizon 2020 research and innovation

## 7. REFERENCES


- Harper SJ, Ward LI and Clover GRG, 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.
- EPPO (European and Mediterranean Plant Protection Organization), 2018. PM 7/24 (3) *Xylella fastidiosa*. Bulletin OEPP/EPPO Bulletin. 48:175–218.