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Recovery of *Pseudomonas savastanoi* pv. *savastanoi* from symptomless shoots of naturally infected olive trees

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Summary. Seasonal dynamics of *Pseudomonas savastanoi* pv. *savastanoi* (Psv) on stems and leaves from symptomless shoots of naturally infected olive trees was monitored in Spanish olive orchards. Data inferred from the comparison between washing of leaves and dilution-plating versus leaf printing of individual leaves suggested that Psv population sizes varied by over several orders of magnitude, among leaves sampled concurrently from the same shoot. We did not find significant differences between leaves and stems, in respect to the number of samples where Psv was isolated or detected by PCR, showing that Psv colonizes both leaves and stems. The frequencies of Psv isolation and average populations were highly variable among field plots. No correlation between Psv populations and those of non-Psv bacteria in any plant material or field plot was observed. However, where both Psv and yellow *Pantoea agglomerans* colonies were isolated a positive correlation was found. In a selected field plot, dynamics of Psv over three years showed significant differences between summer and the rest of seasons. The highest Psv population occurred in warm, rainy months, while low numbers were generally found in hot and dry months. [**Int Microbiol** 2007; 10(2):77-84]

Key words: *Pseudomonas savastanoi* pv. *savastanoi* · *Olea europaea* L. · plant epidemiology · phylloplane · phyllosphere · leaf printing

Introduction

Pseudomonas savastanoi pv. *savastanoi* (Psv [38]) is the causal agent of olive knot disease [12], which is characterized by outgrowths on the trunk and branches, and less frequently on leaves and fruits of olive trees (*Olea europaea* L.). The development of knots depends on bacterial production of the phytohormones indole-3-acetic acid (IAA) and cytokinins [5,31,33]. It has been reported that olive knot disease is also *hrp/hrc* gene dependent [30]. The disease is widespread in most of the Mediterranean countries where olive crops have been grown for centuries.

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Besides from knots, Psv has been isolated from leaves and from the surfaces of stems and olive fruits [8,19]. However, few studies are available on the epidemiology of olive knot disease and most of the data on epiphytic populations of Psv on the phyllosphere of olive trees come only from southeastern Italy [8–10]. There, Psv had a resident phase colonizing the phyllosphere of healthy leaves, where it dominated bacterial communities throughout most of the year [8]. Psv populations have also been shown to display a seasonal distribution, reaching high levels on healthy leaves during spring and fall in such region, although statistical analyses were not applied [8]. The size of Psv populations in single leaves correlated with the age of the leaf, the season at which this leaf was formed and the time when the sample was taken [10]. The bacterial community composition found on olive tree leaves was more strongly influenced by the season than by the leaf age [10].

There are neither data available regarding isolation and molecular detection by polymerase chain reaction (PCR) of Psv, in a study lasting several years, nor any comparative analysis between the presence of Psv in stem and leaf surfaces. As Psv populations growing on any part of the plants may serve as an inoculum's reservoir for infection, monitoring bacterial populations could be a valuable means to forecast the development of olive knots and to prevent it with control-treatment applications. To know the distribution of Psv population among different leaves from the same shoot, the average number of Psv populations per leaf (using bulked samples) and the Psv distribution on individual leaves by leaf printing assays were compared. We also report the isolation and PCR detection of Psv on stem and leaf surfaces from symptomless shoots of naturally infected olive trees conducted for five field plots monitored up to three consecutive years in two of those plots. Moreover, relationships between Psv populations and climatic variables, and between Psv populations and other epiphytic bacteria were also studied.

Materials and methods

Field plots, sampling and enumeration of bacterial population of bulked samples. Five olive orchards located in two olive-growing regions in Spain (Valencian Community and Andalusia) were selected. Plant samples were taken from summer 1999 to spring 2002 in plots 1 and 2 from Valencia, and from spring 2001 to winter 2002 in plots 3, 4 and 5 from Jaén (Andalusia). Five naturally infected olive trees with the same amount of knots (ca. 400) were selected in each plot for sampling. The olive tree cultivars were 'Blanqueta' in plot 1, 'Cornicabra' in plot 2, 'Picual' in plot 3 and 4, and 'Manzanilla de Jaén' in plot 5. Three main branches surrounding the trunk were marked in each selected tree and a bulked sample from marked branches was randomly collected each season from symptomless shoots as described below. Plant samples were kept in plastic bags at 4ºC and processed 24 h afterwards as follows. The youngest part of about ten centimetres of each shoot was removed before analysis to process two-year-old leaves. Stems were defoliated and cut in ca. 3.5 cm per piece. Of each bulked sample, 20 g of stem (95 to 120 cut pieces) and leaves (105 to 156 leaves) were processed. The areas of detached leaves of each sample (phylloplane) were measured on an area meter apparatus (Model 3100 Area Meter, Li-Cor), with a total area of 763 to 1178 cm^2 per sample. Stem and leaves of each bulked sample were placed in 100 ml of Ringer ¼ solution [29], adding 0.05% Tween 20, and shaken for 90 min at 200 rpm at room temperature. Washing solutions, obtained after running the shaking procedure, were 10-fold diluted in phosphate buffer saline (PBS); an aliquot (50 µl) was plated onto King's medium B [17] and another one onto the semi-selective PVF-1 medium [34], supplemented with cycloheximide at 250 and 125 mg/l, respectively. Plates were incubated for five days at 26ºC. Psv-like colonies were counted in both media and a proportion of putative Psv colonies were confirmed by PCR according to Penyalver et al. [27]. Total bacterial colonies and the number of yellow *Pantoea agglomerans* (*Erwinia herbicola*)-like colonies were also counted in King's medium B. Identification of a proportion of putative *P. agglomerans* colonies was confirmed by biochemical tests according to Ercolani et al. [8]. Bacterial populations were expressed as colony forming units (CFU) per gram of stem, per gram and cm^2 of leaf and $\log_{10}$-transformed before populations were calculated [14]. Climatic variables were recorded from the field plot 1 in an automatic weather station located 5 km from the orchard: cumulative rainfall, average temperature and average relative humidity were calculated from a given sampling date to the next one. Psv populations were contrasted to the amount of total non-Psv remaining bacteria and to the populations of *P. agglomerans*.

Leaf printing assay. Averages of Psv populations per leaf from five bulked samples were estimated in each sample by the washing followed by dilution-plating as described previously. In parallel, 50 upper and lower surfaces of individual leaves from shoots of each bulked sample from marked branches of the same tree were pressed firmly for 20 s against the surface of solid PVF-1 medium [34] supplemented with cycloheximide (125 mg/l). Leaves were left on the medium for 4 h, to ensure bacterial transfer and then removed. Plates were incubated for five days at 26º C. Psv-like colonies were counted and a proportion of the presumptive Psv colonies were confirmed by PCR according to Penyalver et al. [27].

Molecular detection of Psv. Psv detection was carried out by bacterial enrichment in liquid PVF-1 medium and subsequent nested-PCR in a single closed-tube and performing dot-blot hybridization and colorimetric detection of amplicons as described by Bertolini et al. [2].

Statistical methods. Data on Psv isolation and PCR detection were statistically analyzed yearly using the generalized linear model assuming binomial distribution [26] with field plot, season and vegetal material as fixed main factors, and tree within field plot as random factor. Psv populations were analyzed using a similar mixed linear model. Means for each season were compared using contrast and applying the Bonferroni's correction with global significance of 5%. Linear regression analysis performed with Excel 2000 (Microsoft) was used to study the correlation between Psv and non-Psv bacteria densities and between Psv and *P. agglomerans* densities. Means of Psv populations were analysed by a triple factorial model (year, season and vegetal material) for the seasonal dynamics in field plot 1. Means of each season were separated by using Di Rienzo, Guzmán and Casanoves (DGC) test [7]. Covariance analysis using year as factor and cumulative rainfall, average temperature and average relative humidity as covariates, was used to asses the influence of climatic parameters on Psv populations in field plot 1.

Results

Psv distribution among leaves. A comparative analysis of washing followed by dilution-plating and leaf printing assay of 5 bulked samples is shown in Table 1. The number of Psv colonies isolated by individual leaf printing ranged from 1 to 112 CFU [data not shown]. However, in 13 out of 15 leaf surfaces where Psv was isolated, the number of Psv colonies was lower than 10 CFU. Psv colonies were distributed all over the leaf surface. When contrasting both techniques, Psv was isolated by leaf replica printing in a very low number of leaf surfaces in the 5 analyzed samples (15 out of 500), showing that Psv did not colonize all leaves from the same shoots in a similar way.

Psv colonization of stems and leaves. Independently of the season and year, average of Psv populations were low and quite similar on stems and leaves in all evaluated field plots (Table 2) due to the fact that Psv was not isolated in 73% of the analyzed samples (Fig. 1A). Psv populations in

Table 1. Average of *Pseudomonas savastanoi* pv. *savastanoi* populations (Psv) per leaf assessed by two methods: dilution-plating of bulked samples and individual leaf printing

	Washing of leaves and dilution-plating of bulked samples		Leaf printing assay using individual leaves from the same bulked samples	
Sample No.[a]	Number of leaves[b]	Psv population size per leaf (log CFU/leaf)[c]	Psv isolation from upper surface[d]	Psv isolation from lower surface[e]
1	100	1.48	3	6
2	80	1.57	1	1
3	124	NI[f]	0	1
4	152	2.11	0	0
5	40	3.90	2	1

[a]Bulked samples were taken from shoots of marked branches of a selected tree of plot 1 in spring 2004.
[b]Number of leaves analyzed in the bulked sample by washing of leaves followed by dilution-plating procedure.
[c]Average of Psv population size per leaf calculated in each bulked sample.
[d]Number of upper leaf surfaces where Psv was isolated by the leaf printing assay from 50 analyzed leaves.
[e]Number of lower leaf surfaces where Psv was isolated by the leaf printing assay from 50 analyzed leaves.
[f]NI = Psv was not isolated.

samples where Psv was isolated ranged from 2 to 6 log CFU/g, showing a typical log distribution (Fig. 1B).

In the two plots analyzed over the three-year study (Plot 1 and 2) the average Psv populations were higher in plot 1 than in 2 (Table 2). There were no significant differences between either plant materials in any year in the overall study (*P*-values were 0.257, 0.705 and 0.168 for the first, second and third year, respectively). The number of samples where

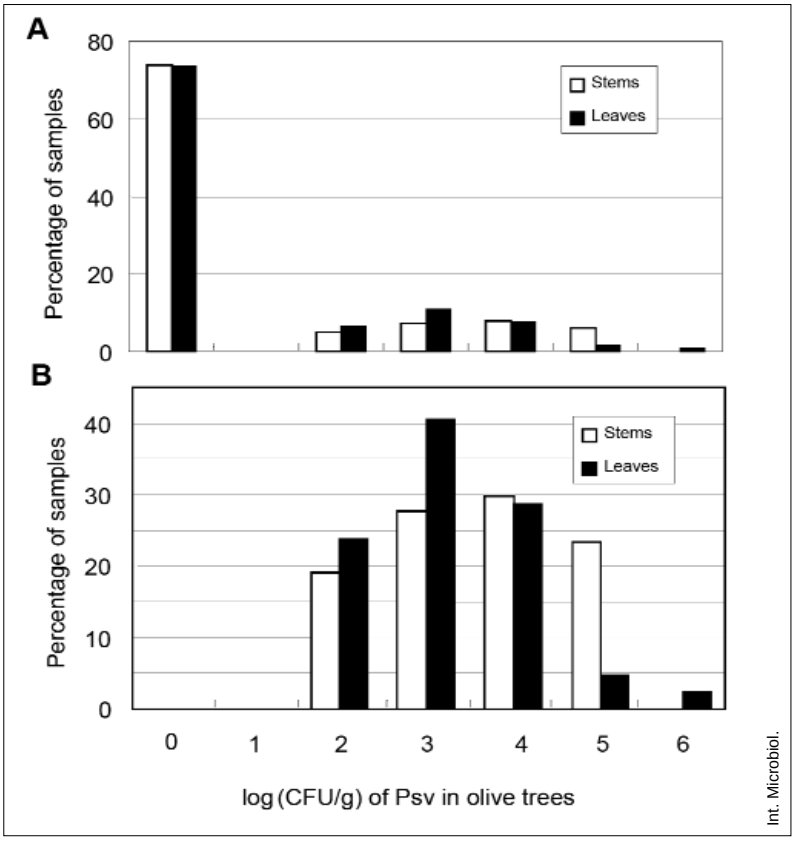

Fig. 1. (**A**) Frequency of *Pseudomonas savastanoi* pv. *savastanoi* (Psv) populations on stems and leaves from symptomless shoots of naturally infected olive trees from five field plots evaluated (number of stem and leaf analysed samples were 179 and 159, respectively). (**B**) Distribution of Psv populations among stem and leaf surfaces in samples where the bacterium was isolated (number of stem and leaf samples were 47 and 42, respectively).

Table 2. Isolation, detection and enumeration of *Pseudomonas savastanoi* pv. *savastanoi* (Psv) populations on symptomless stem and leaf surfaces from shoots of naturally infected olive trees

Field plot	Plant material analysed	Isolation of Psv[a]	Average of Psv populations (log CFU/g)	Molecular detection of Psv[b]
1	Stems	30 (60)	2.01 ± 0.28[c]	27 (60)
	Leaves	25 (50)	1.80 ± 0.27	32 (50)
2	Stems	6 (60)	0.44 ± 0.18	8 (60)
	Leaves	7 (50)	0.37 ± 0.14	9 (50)
3	Stems	3 (20)	0.48 ± 0.29	4 (20)
	Leaves	4 (20)	0.86 ± 0.43	5 (20)
4	Stems	4 (20)	0.61 ± 0.30	4 (20)
	Leaves	3 (20)	0.57 ± 0.32	4 (20)
5	Stems	4 (19)	0.94 ± 0.44	4 (19)
	Leaves	3 (19)	0.57 ± 0.33	5 (19)
Total	Stems	47 (179)	1.04 ± 0.14	47 (179)
	Leaves	42 (159)	0.93 ± 0.13	55 (159)

[a]Number of samples in which Psv was isolated by washing of stems or leaves followed by dilution-plating (analyzed samples).
[b]Number of samples in which Psv was detected on stems or leaves by bacterial enrichment and subsequent nested-PCR in a single closed tube (analyzed samples).
[c]Standard error of the mean.

Psv was detected by the molecular technique was also similar on stems and on leaves without significant differences in any year in the overall study (P = 0.317, 0.275 and 0.739). Significant differences were observed between the two plots analyzed over the three-year study on Psv isolation and detection being higher in plot 1 than in 2. The frequency of Psv isolation and detection in the field plots from Jaén (plots 3, 4 and 5) was very low during the first year of study and for this reason they were not analyzed in the following years.

Correlation of Psv and other bacteria population densities. The correlation coefficient r value relating the density of Psv to the density of non-Psv bacteria on 73 individual samples from all field plots (74% of contrasted data come from plot 1) was very low (r = 0.2) (Fig. 2A). The same

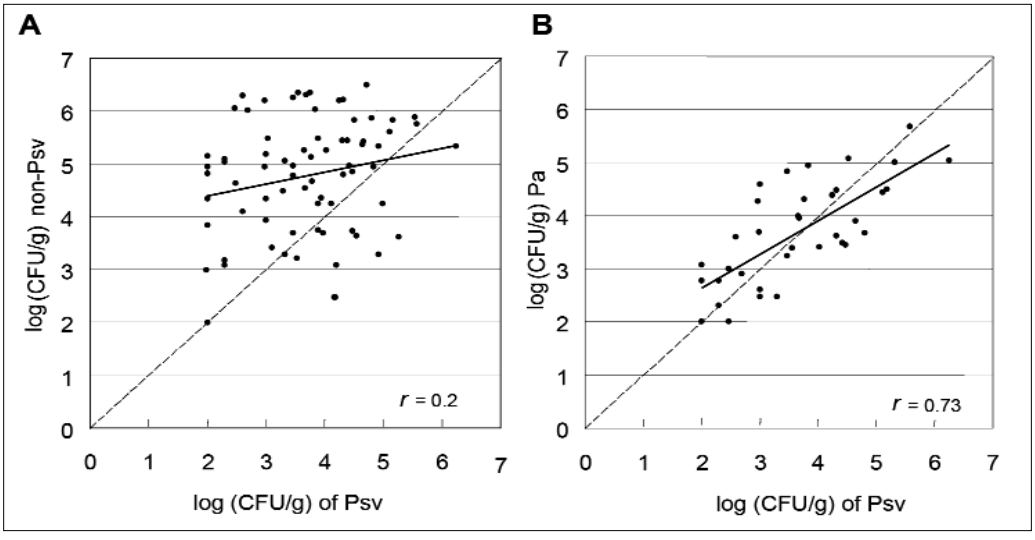

Fig. 2. Correlation of densities of *Pseudomonas savastanoi* pv. *savastanoi* (Psv) and **(A)** non-Psv bacteria on 73 individual samples from all field plots, and (**B**) *Pantoea agglomerans* (Pa) on 36 individual samples from all field plots. The solid line (correlation coefficient) describes the relationship between Psv populations and total non-Psv populations (A), or the relationship between Psv populations and the populations of Pa (B).

Table 3. Seasonal distribution of *Pseudomonas savastanoi* pv. *savastanoi* (Psv) in stem and leaf surfaces from symptomless shoots of naturally infected olive trees over the three-year study in field plot 1

Season	Isolation of Psv*	Average of Psv populations (log CFU/g)#
Summer	4 (25)	0.37a
Fall	12 (25)	1.51b
Winter	17 (30)	2.11b
Spring	22 (30)	2.92b

*Number of samples in which Psv was isolated by washing either on stems or leaves followed by dilution-plating (analysed samples).
#Values followed by the same letter do not significantly differ, based on the DGC test ($P < 0.05$).

correlation pattern was found when analyzing samples from stems or leaf surfaces separately, and for field plot 1 [data not shown]. Note that a high positive correlation ($r = 0.73$) was found between the density of Psv and that of *P. agglomerans* on 36 individual samples where both bacteria were isolated (61% of contrasted data come from plot 1) (Fig. 2B). The same correlation pattern was found when analyzing samples from stems or leaf surfaces separately and for the field plot 1 [data not shown].

Seasonal distribution of Psv populations. As Psv was isolated in significantly higher percentages in plot 1 than in plot 2 over the three-year study (50% of analyzed samples, see Table 2), we selected plot 1 to monitor seasonal differences of Psv populations and the influence of climatic variables (cumulative rainfall, average temperature and average relative humidity). In summer, Psv was isolated at significantly lower frequencies than in fall and spring, but only for the second and third year ($P = 0.042$ and 0.037, respectively). Significant differences were observed in the average Psv populations between summer and the other seasons (Table 3). Seasonal distributions of average Psv populations were quite similar in stem and leaf surfaces over the three-year study (Fig. 3A). On stem surfaces, Psv reached the highest population sizes mainly in spring, while the lowest population sizes were observed mainly in summer. Quite similar patterns were found on leaf surfaces. The highest Psv populations in either stems or leaf surfaces generally occurred when average temperature was moderate, from 10 to 20ºC. Low Psv popula-

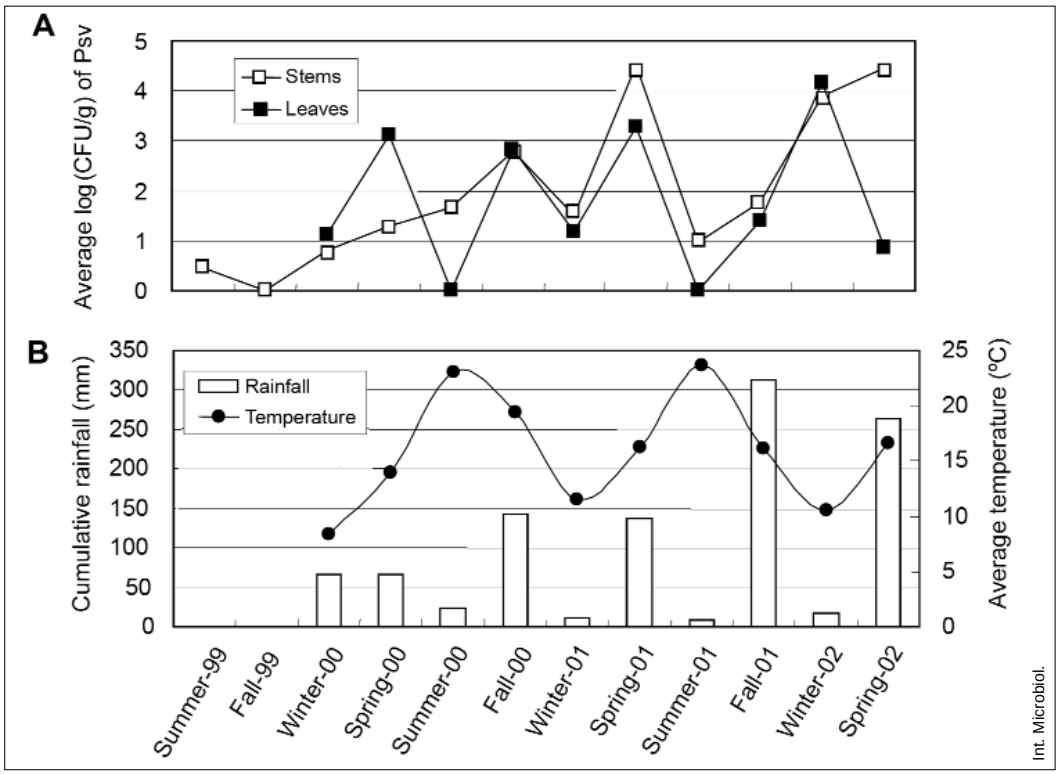

Fig. 3. Average of *Pseudomonas savastanoi* pv. *savastanoi* populations (Psv) on stem and leaf surfaces of naturally infected olive trees over the three-years study from field plot 1 (**A**), and cumulative rainfall and average temperature calculated from a given sampling date to the next one (**B**).

tions were generally observed when temperatures were higher, and cumulated rainfall values from the previous samplings were lower than 25 mm (Fig. 3B). Note that Psv population sizes in stems and leaf surfaces in this field plot correlated (with r^2 values of 0.7 and 0.43, respectively) with rainfall, temperature and relative humidity.

In the overall study, independently of the field plot, plant material and year, the samples with Psv were 33 out of 88 in spring, 27 out of 90 in winter, 16 out of 80 in fall, and 13 out of 80 in summer. No significant differences between seasons in any year were observed. Average of Psv populations per season were quite similar over the study. Nor significant differences were found with respect to the detection of Psv by PCR in any year [data not shown]. .

Discussion

Our study provides new data on the dynamics and plant distribution of Psv populations in non-irrigated olive orchards in dry Spanish areas. We decided to monitor Psv populations by analyzing bulked samples according to Jacques and Morris [16], after comparing washing followed by dilution-plating vs the Psv distribution on individual leaves by leaf print assays in the samples. As described for other epiphytic bacteria and hosts [18], Psv populations were highly variable among leaves from a bulked sample as inferred from the leaf printings assays, suggesting differences in several orders of magnitude among leaves. Due to the low detection level of leaf printing [16], such results also suggested that Psv should probably colonize low numbers of leaves with high population sizes in a bulked sample. So, when using bulked samples, we may have overestimated the population median [14], but we surely improved the efficiency of diagnosis.

A molecular detection technique based on enrichment-PCR was applied to complement and improve the reliability of the information obtained with the isolation procedure based on washing and dilution plating [23]. We did not isolate neither did we detect Psv in most of the analysed samples during the study, either because Psv was not present or because the populations from naturally infected olive trees in symptomless shoots were below the sensitivity level of isolation (ca. 10^2 CFU/g) or the detection level of enrichment-PCR (ca. 1 CFU/g). Similar erratic detection has been described for other epiphytic bacteria in natural conditions [13,20,24,40]. Obviously, this situation was not found when evaluating inoculated bacteria instead of indigenous populations [18]. In our study, average Psv population sizes ranged from 2 to 6 log CFU/g. These values were quite similar to those found in other studies in naturally infected olive trees [8–10,19,34], but lower than those found for other epiphytic *Pseudomonas* in inoculated plants grown in greenhouses or in open fields [36]. Psv detection frequencies differed broadly among plants, within and from different field plots, as described for other epiphytic bacteria [18,22]. These differences may be due to the many factors affecting epiphytic bacterial populations in nature and their rapid fluctuations [1,6,9,15,18,21,35]. Monitoring methods based on the cultivation of bacteria may underestimate the actual population size because bacteria could enter in a viable but non-culturable state [4], as reported for *P. syringae* in bean leaves [39]. However, our study showed very small differences between positive Psv isolation and positive PCR detection, suggesting both that the methodology was appropriated, and that the pathogen was generally culturable.

We compared seasonal variations in Psv populations between stem and leaf surfaces. We did not find significant differences between both plant materials with respect to the number of analysed samples where Psv was isolated, detected by PCR or the average Psv populations. This suggests that Psv colonizes either stem and leaf surfaces, but in the conditions we used, growing at very low numbers. It has been suggested, however, that epiphytic bacteria might be better protected when they grow on leaves than on stem surfaces. In fact, when growing on leaves of olive trees, they stick to the depressions of the veins and in specific structures such as the shields of pectate hairs [32]. In contrast, Lavermicocca and Surico [19] reported larger Psv population sizes on stems than on leaves from naturally infected olive trees, but their study was performed for only one year and without statistical analyses. However, as we evaluated bacteria on stems after cutting stems into pieces, we could count also some endophytic Psv [28]. Furthermore, our results suggest that for monitoring Psv populations leaves and stems should be evaluated.

We found no correlation between Psv populations and those of non-Psv in any plant material or field plot. However, there was a clear positive correlation between Psv population sizes and those of yellow *P. agglomerans*, either on stems or leaf surfaces. Such colonies have been described in association with Psv in olive knots and in the olive phyllosphere elsewhere [8,25], but this is the first report showing a similar fluctuation of both bacterial populations on the same host. This is of interest because both bacteria produce IAA and its production has been demonstrated to contribute to the epiphytic fitness in olive for Psv and in pear (*Pyrus communis*) for *P. agglomerans* [3,37]. The correlation found could be based on this similar interaction with its hosts based on IAA production or even on a possible physical interaction between both bacteria in the phyllosphere community.

Futhermore, a synergistic effect of *P. agglomerans* on the development of olive knot symptoms caused by Psv has been also reported [11,25].

As the frequencies of Psv isolation and average populations were highly variable among field plots, the seasonal dynamics of Psv populations was evaluated only for field plot 1, where Psv was consistently isolated from leaf and stem surfaces over the three-year study. We found that seasonal fluctuations of Psv populations in this plot fell into a recognizable pattern. Higher populations were found in warm and rainy months and lower populations in hot and dry months, either on leaf or stem surfaces, with the exception of winter 2002. Previous data from Italy indicated that Psv populations on leaves were greater in spring and fall (ca. 10^4 cfu/cm^2) than in winter and summer (ca. 10^2–10^3 cfu/cm^2), although statistics were not applied [8]. In field plot 1, multiple regression analysis between Psv population sizes on stem and leaf surfaces and climatic variables (such as temperature, rainfall and relative humidity), showed that those variables exert a strong influence on the Psv population values.

In conclusion, these assays have provided comprehensive new information to understand the behavior of Psv on olive plants. As epiphytic Psv on trees might be a major source of available inoculum to develop knots, monitoring the populations of the pathogen under natural conditions provides the basis to manage the disease and to know the microbial ecology of olive plants.

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