www.czasopisma.pan.pl PAN POLSKA AKADEMIA NAUK www.journals.pan.pl

JOURNAL OF PLANT PROTECTION RESEARCH Vol. 54, No. 1 (2014)

DOI: 10.2478/jppr-2014-0004

Comparison of methods used in the recovery of Phylloplane bacteria: a case study of *Pseudomonas savastanoi* pv. *savastanoi* applied to the Phylloplane of *Olea europaea* sub-species

Jay Ram Lamichhane[1, 2]*, *Leonardo Varvaro*[1]

[1] Department of Science and Technology for Agriculture, Forestry, Nature and Energy (DAFNE), Tuscia University, Via San Camillo de Lellis, 01100 Viterbo, Italy

[2] Fruit Development Directorate (FDD), Kirtipur, Kathmandu, Nepal

Received: April 20, 2013
Accepted: January 14, 2014

Abstract: An efficient and accurate method of sampling, recovery, and enumeration of epiphytic bacterial populations are of fundamental importance for their precise estimation. In this study, effectiveness and reliability of processing methods, sampling type, sample storage, and plating techniques, for the recovery of the epiphytic bacterial populations, were evaluated. *Pseudomonas savastanoi* pv. *savastanoi*, the causal agent of olive knot disease, and two olive sub-species were used. Bulk-leaf sampling allowed for a higher number of the bacterial recovery. The use of a lab blender was the most effective and reliable method among the four commonly used processing methods. A short storage of leaf samples was possible through freezing. Bacterial survival was not influenced as long as the samples were processed within 5 days and frozen without a buffer. No difference was observed among the drop and spread platings, suggesting that one of them can be used and the choice depends on the cost and time required.

Key words: *Olea europaea* subsp. *cuspidata*, *Olea europaea* subsp. *europaea*, sampling and processing methods

Introduction

So far, epiphytic bacterial populations (EBP) have been studied by many researchers who deal with plant pathogenic bacteria (PPB), ice nucleation active (INA) bacteria, and plant growth promoting leaf-associated (PGPL) bacteria (Lindow *et al*. 1978; Hirano *et al*. 1982; Colin and McCarter 1983; Zagory *et al*. 1983; Knudsen *et al*. 1988; Lindow *et al*. 1988; Beattie 2002). The use of an efficient and accurate method of sampling, recovery, and enumeration of the EBP is of primary importance for precise estimations. This aspect is even more important, in the case of harmful bacteria, to predict the risk phenomena related to the presence of bacteria in association with the plant surfaces. An adequate addressing of these methodological considerations can make sure that the risks associated with an underestimation as well as an overestimation are avoided.

European olive (*Olea europaea* L. subsp. *europaea*, hereafter *O. europaea*) is one of the most economically important species worldwide. Although the EBP of this sub-species has been studied by different researchers (Ercolani 1971, 1978, 1979; Varvaro and Surico 1987; Ercolani 1991; Quesada *et al*. 2007), no report is available to date, on the accurate methods of sampling, processing, and quantification. It is reported that, *Pseudomonas savastanoi* pv. *savastanoi* (Psav), the causal agent of olive knot disease, and the group of INA bacteria, represent respectively, 67% and 10% of the EBP of European olive (Ercolani 1978, 1991). Hence, the consistent presence of these species represents a serious risk and requires periodic supervision, in order to contain their inoculum density, through preventive control measures (Quesada *et al*. 2010). Besides the European olive, another naturally growing olive sub-species in the Himalayas, known as *O. europaea* L. subsp. *cuspidata* (hereafter *O. cuspidata*) (Lamichhane *et al*. 2010a; Lamichhane 2011), was tested to confirm the validity of the results.

The objective of this study was to compare the effectiveness of four commonly used processing methods. In addition, the influences of sampling type, sample freezing, and plating techniques were investigated.

Materials and Methods

Olive plants

Olea cuspidata plants were obtained by directly sowing the seeds. Before sowing, the seeds were put into a wide basin containing vermiculite. Then, wet seeds were maintained at 4°C for 10 days, since this sub-species needs some hours of chill temperatures for germination because of the seed dormancy (Lamichhane and Varvaro 2012). After 10 days of chilling, the basin was kept in a greenhouse and maintained at 20°C for 2 months. Subsequently, the

*Corresponding address:
jayram.lamichhane@gmail.com

plantlets were transplanted into 2-litre plastic pots (15 cm wide and 30 cm deep) containing soil, pit, and sand (ratio 1 : 1 : 1). Whereas for *O. europaea*, plants obtained from cuttings of cv. Leccino were used. The plants were kept in the same size plastic pots. All the plants were maintained in a greenhouse at 25±1°C with a relative humidity of 60–80%. In all the experiments, two year old plants of both olive sub-species were used.

Bacterial strain and culture condition

Pseudomonas savastanoi pv. *savastanoi* (PseNE 107) isolated in a Himalayan region of Nepal was used (Balestra *et al*. 2009). Bacterial strain preserved on nutrient agar supplemented by 2% of glycerol (NAG) at 4°C was grown on nutrient agar (NA) medium. After 24 h, fresh bacterial culture was re-streaked on the same medium to form a dense bacterial culture. Bacterial suspension was prepared by putting the culture inside a beaker containing phosphate buffer. The suspension was centrifuged at 15,000 × g for 20 min (Lamichhane *et al*. 2010b). The pellet was suspended to obtain a homogeneous bacterial suspension in phosphate buffer. The concentration of the bacterial suspensions was adjusted turbidimetrically to about 10^4 and 10^8 CFU/ml by reference to a calibration curve (Varvaro and Surico 1987).

Inoculation of plants

Plants (40 plants/sub-species) of both sub-species were sprayed with the bacterial suspension which was distributed homogeneously on the leaf surface by using an atomizer (Lamichhane *et al*. 2010b). Inoculation by spraying was made until both the upper as well as lower leaf surfaces were fully wet. From 2 h before till 2 h after the inoculation, the relative humidity was maintained at around 90% to slow down the inoculum evaporation. The experiments were carried out twice on both olive sub-species in April of 2009 and April of 2010.

Sampling experiments: single *vs.* bulk leaf samples and sample size

Plants were sprayed with a high concentration of bacterial suspension (10^8 CFU/ml). For the single leaf sample, four sets of 25 leaves/sub-species were aseptically collected in sterile lab bags and processed in groups of 5 leaves (given its very small size) in 10 ml of phosphate buffer. For bulk samples, 5 samples, each consisting of 20 leaves, were similarly collected and processed individually, in 40 ml of phosphate buffer. Leaves were collected and processed 3 h post-spray. All samples were blended for 5 min in a lab blender (optimal time as shown by preliminary tests). Suspensions were then serially diluted in phosphate buffer and spread plated in duplicate on NA medium. The medium was amended by 150 ppm of cycloheximide to avoid fungal growth. Colony counts for each single sample were made after plating and incubation at 26±1°C for 72 h. To measure leaf surfaces, APS assess software was used.

Recovery experiments: comparison of processing methods

Two sets of samples/sub-species (each 20 leaves/processing method), previously inoculated with a 10^8 CFU/ml bacterial suspension, were aseptically collected on days 0 (3 h post-spray), 1, 3, and 5 and processed separately with 40 ml of 0.01 M Tris buffer (pH 7.5). The procedure was: (i) washing (Lab therm shaker) done in a sterile 250-ml conical flask, for 2 h at 150 revolutions per minute (rpm) on a rotary shaker, (ii) blending (Lab blender 80, PBI) at high speed and (iii) stomacher blending (Stomacher® 400 Circulator, PBI) for 5 min, (iv) sonication (Sonic vibra cell) was performed, in a sterile 250-ml conical flask (amplitude 60, pulser 0) for 7 min. The parameters used for each processing method were chosen according to the optimal results from our preliminary tests (data not shown). Suspension dilution and plating, colony counts and data analysis, for each method and for each sub-species, were made as described above.

Effect of bacterial concentration in the sample, on recovery efficiency

Plants were sprayed with a low concentration of bacterial suspension (10^4 CFU/ml). A set of 5 plants per sub-species were sprayed with only sterile distilled water (SDW) and used as the control. After 2 h, the leaves were removed and placed in sterile lab bags. Leaves inoculated with bacteria were mixed with leaves sprayed only with phosphate buffer so that the final sample contained 2 g of leaf material and consisted of 5, 10, 25, 50, and 100% of leaves inoculated with bacteria. Samples for each proportion were prepared in quadruplicate in sterile lab bags, and separately processed in 40 ml of phosphate buffer by blending. Suspension dilution and plating, colony counts, and data analysis, for each method and for each sub-species were done as described above. Data were used in the SPSS regression analysis, with CFU/cm^2 as the dependent variable and the proportion of leaves inoculated with bacteria as the independent variable.

Effect of sample freezing on bacterial recovery

Twenty-four samples per sub-species, each represented by 20 leaves, were aseptically collected and immediately put in sterile lab bags 3 h post-spray (10^8 CFU/ml). Samples were divided in 2 groups, for each sub-species, and frozen at –20°C with and without the addition of 40 ml phosphate buffer. After 1, 5, 10, 15, 20, and 30 days, 4 frozen samples for each sub-species were thawed for 30 min at room temperature and 40 ml of phosphate buffer was added to those samples frozen without buffer and processed in a lab blender. Another group of samples (2 samples/sub-species, each represented by 20 leaves) were immediately processed in a lab blender 3 h post-spray, in 40 ml of buffer. Samples were then processed and plated. Colony counts were averaged as described previously.

Comparison between two plating techniques

Three sets of bacterial suspensions with different concentrations (10^3–10^8 CFU/ml) were prepared by serially diluting a 10^8 CFU/ml. Each "drop plate" contained five 10 µl drops per plate. Regardless of the spread plate, each plate contained 100 µl of the bacterial suspension which were spread onto the NA medium. After 60 h of incubation, colony counts were made from the plates that contained between 30 to 300 colonies (Meynell and Meynell 1965). Finally, the data were analyzed.

Statistical analysis

Data from each experiment were analyzed by using the SPSS software and subjected to analysis of variance (ANOVA). Duncan's multiple range test was used to calculate the differences among the methods and treatments.

Results

Sampling experiments: single and bulk leaf samples

In both the experiments, bacterial counts were done from single leaf and bulk leaf samples. Bulk samples had significantly higher bacterial counts (Table 1). For both the olive subspecies, bulk leaf sampling allowed for a higher recovery compared to single leaf sampling (4.47 and 4.69 log CFU/cm^2 *vs*. 3.92 and 4.33 log CFU/cm^2) for *O. europaea* and *O. cuspidata*, respectively.

Table 1. Mean log CFU/cm^2 values of *P. savastanoi* pv. *savastanoi* recovered from the single and bulk leaf samples of two olive sub-species

Sample	*O. europaea*		*O. cuspidata*	
Bulk	4.47[a]±0.23[b]	a[c]	4.69±0.20	a
Single	3.92±0.03	b	4.33±0.06	b

[a] values are the mean of five replicates
[b] standard error
[c] means followed by the same letter are not significantly different at p = 0.05

Recovery experiments: comparison between the processing methods

The ANOVA of the log CFU/cm^2 values from the 4 processing techniques, indicated significant differences concerning the efficiency of the techniques for recovering *P. savastanoi* pv. *savastanoi* from the leaf surfaces of the two olive sub-species (Table 2). The lab blender was most effective when it came to the recovery of epiphytic bacteria with values of 4.29 and 4.52 log CFU/cm^2 from *O. europaea* and *O cuspidata*, respectively. Similarly, the stirrer recovered 4.09 and 4.38 CFU/cm^2 of bacteria from *O. europaea* and *O. cuspidata*, respectively. The stomacher blender recovered 3.88 and 4.18 CFU/cm^2 and in last place, the sonicator recovered 3.57 and 4.02 CFU/cm^2. The number of bacteria recovered from two olive sub-species did not differ significantly. However, significant differences were observed among the processing methods.

Table 2. Comparison of the efficiency of the processing methods for recovery of *P. savastanoi* pv. *savastanoi*

Technique	*O. europaea*		*O. cuspidata*	
Lab blender	4.29[a]±0.40[b]	a[c]	4.52±0.18	a
Stirrer	4.09±0.16	b	4.38±0.11	b
Stomacher blender	3.88±0.18	c	4.18±0.21	c
Sonicator	3.57±0.03	d	4.02±0.01	d

[a] values are the mean of eight replicates, processed on days 0, 1, 3, and 5 post-spray
[b] standard error
[c] means followed by the same letter are not significantly different at p = 0.05

Effect of bacterial concentration in samples, on recovery efficiency

Plotting of the CFU/cm^2 values produced a straight line with a high coefficient of determination value (r^2), in both the experiments. Such a plotting result was achieved by placing different proportions of inoculated leaves as they related to each other (Fig. 1). The linearity of the data and the high value of r^2 demonstrated the efficiency, constancy, and accuracy of the lab blender in recovering bacteria from leaf samples.

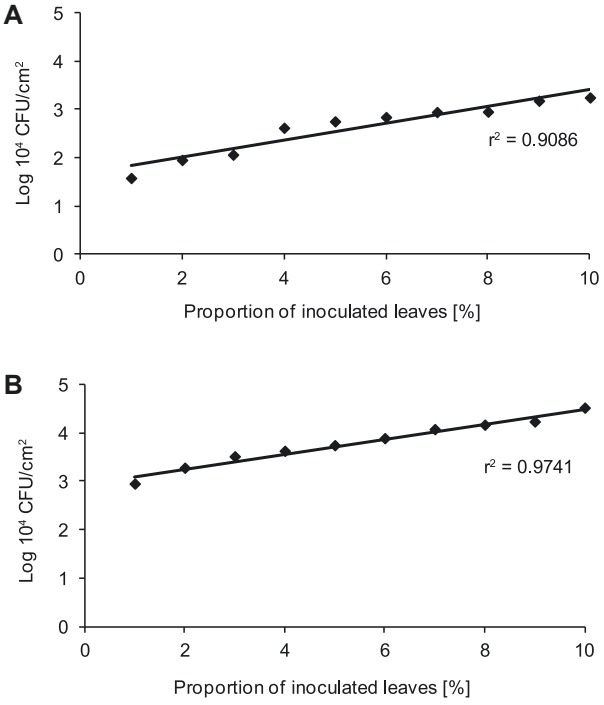

Fig. 1. Effect of bacterial concentration in the samples of *O. europaea* (A) and *O. cuspidata* (B) on recovery efficiency of *P. savastanoi* pv. *savastanoi*

Table 3. Comparison of the immediate and delayed processing of samples collected 3 h post-spray, on recovery of *P. savastanoi* pv. *savastanoi* from two olive sub-species

Days	Buffer	*O. europaea*		*O. cuspidata*	
		log CFU/cm^2		log CFU/cm^2	
1	+	2.24[a]±0.67[b]	c[c]	2.72±0.16	e
5	+	1.78±0.24	c	2.46±0.28	cde
10	+	0.99±0.02	b	1.83±0.27	bcde
15	+	0.29±0.29	ab	1.52±0.47	b
20	+	0.19±0.19	a	0.31±0.31	a
30	+	0.00±0.00	a	0.00±0.00	a
0	–	5.13±0.07	e	5.19±0.05	f
1	–	4.94±0.02	e	4.16±0.08	f
5	–	4.91±0.00	e	3.97±0.21	f
10	–	4.80±0.05	e	2.58±0.58	de
15	–	3.95±0.04	d	1.74±0.04	bcd
20	–	2.25±0.24	c	1.60±0.10	bc
30	–	2.10±0.18	c	1.83±0.11	bcde

[a] values are the mean of five replicates

[b] standard error

[c] means followed by the same letter are not significantly different at p = 0.05

The effect of sample freezing on bacterial recovery

Bacteria decreased more significantly, the longer the samples were frozen. In particular, a drastic decline in bacteria was observed in samples frozen with the addition of a phosphate buffer. In the case of *O. europaea*, the number of bacteria recovered from the sample which had been frozen for only 1, 5 or 10 days, without the addition of a buffer, did not significantly differ with those processed immediately (4.94, 4.91 and 4.80 log CFU/cm^2, respectively) (Table 3). Afterwards, the bacterial numbers tended to decline with longer freezing. There was a significant difference between 10 and 15 days (4.80 and 3.95 log CFU/cm^2, respectively), with further significant decline after 20 days (2.25 log CFU/cm^2). No significant difference was observed between 20 and 30 days of freezing (2.25 and 2.10 log CFU/cm^2, respectively). Regarding the samples frozen between 1 and 5 days with the addition of a buffer, the bacterial populations did not differ significantly (2.24 and 1.78 log CFU/cm^2, respectively). But a significant decrease occurred after 5 days. The recovery after the freezing of samples for 15 and 20 days was rather similar with a slight decline (0.29 and 0.19 log CFU/cm^2, respectively). No bacteria were recovered from the samples frozen for 30 days with the addition of a buffer.

Bacterial recovery was somewhat different in the case of *O. cuspidata*. The number of bacteria recovered from the samples frozen for 1 and 5 days, without the addition of buffer, was not significantly different (2.72 and 2.46 log CFU/cm^2, respectively). Bacterial numbers from samples processed after 10, 15, 20, and 30 days of freezing without the addition of a buffer, tended to decline with longer freezing, and there were some similarities among the numbers (Table 3). For the samples frozen with the addition of buffer, the recovery after 1 day of freezing was not significantly different from those frozen for 5 and 10 days. The values differed significantly from those frozen for 15 and 20 days. Also, for *O. cuspidata*, no bacteria were recovered from the sample frozen for 30 days, with the addition of a buffer (Table 3).

Plating experiments: comparison of drop versus spread plating

No significant difference was found among the plating techniques. Spread and drop plating allowed the recovery of log 2.61 and 2.49 CFU/ml, respectively.

Discussion

The results showed a substantial difference among the methods examined, in the recovery of EBP. In addition, the type of sampling, the way, and the duration of sample freezing, significantly affected the number of EBP recovered. The high r^2 values showed the effectiveness of the processing methods confirming the reliability of the methods. The type of plating, however, did not show statistically significant differences. It is important to note, that the efficiency of a certain technique in the recovery of bacteria did not differ among the two sub-species.

The type of sampling significantly affected the recovery of EBP. Among the single and bulk leaf samplings, the latter was more effective. Our results are in agreement with Crosse (1959) since larger bulk samples result in more accurate estimates of bacterial populations and it is commonly used in field research. Moreover, bulk leaf sampling is considered to be fast, when compared to single leaf sampling (Donegan *et al*. 1991). Regarding the effectiveness of the processing methods in the recovery of EBP, the lab blender was the most reliable followed by the stirrer, then the stomacher blender, and in last place, the sonicator. Despite the application of the same bacterial

suspension, the numbers of bacterial colonies recovered differed numerically among the two olive sub-species, although the differences were not statistically significant. A possible explanation might be the influence of the host on the EBP, which so far, has been reported by Jacques (1996) and Lindow and Brandl (2003). The ability of an instrument in recovering the bacteria appears to be influenced by the type of the leaves. Donegan *et al*. 1991 recovered the highest number of the bacteria associated with bean leaves, using stomacher blending followed by the lab blender, sonication, and washing. In fact, unlike bean leaves, olive leaves are very particular with waxy and coriaceous surfaces. There are numerous star hairs on the lower surface of the leaves (Surico 1993).

For the researchers working on the olive phylloplane bacteria, the higher effectiveness of the lab blender compare to the stirrer is a very encouraging result. In fact, compared to the stirrer, the lab blender has a quick processing time (5 min *vs.* the 2 h of the stirrer). The possibility of samples being collected directly into the sterile lab bags is practical when using the lab blender, since later the bags can be easily disposed of, without cleaning and autoclaving. When using the stirrer, all these steps are very laborious and time-consuming, especially in experiments with numerous samples. Furthermore, the disadvantage of releasing plant cell contents because of the beating action of the paddles applying pressure to the bagged sample, does not take place since olive leaves are very resistant. The ability of the lab blender in providing constant recovery, confirms its effectiveness which keeps our results far from error. There were different concentrations of inoculated leaf samples of both olive sub-species. The constant recovery of the bacteria with the lab blender confirms the reliability of this technique.

Regarding the effect of delayed processing on the recovery of the EBP, our results showed that the freezing of the samples, with or without the addition of a buffer, may negatively influence the survival or recovery of microorganisms in the samples over time. The lack of significant differences, among the quantitative recovery of bacteria from samples immediately processed and those frozen for 1 and 5 days, with and without the addition of a buffer, shows that a delay of up to 5 days in processing is possible. After a delay of more than 5 days, the decline in bacterial number is significant and processing cannot be done. These results are encouraging mostly in large-scale experiments where an immediate processing is not possible. It is noteworthy to report the differences statistically significant in quantitative recovery of the bacteria, among the samples frozen with and without the addition of a buffer. The addition of a buffer when pre-freezing might have a negative effect since bacteria can be injured from the freezing and thawing of the buffer (Donegan *et al*. 1991). The type of leaf surface may also be an influence since *O. cuspidata* has a delicate, less resistant leaf surface, whereas *O. europaea* is characterized by a waxy, tough surface. The difference in leaf surfaces might be the reason that bacterial numbers recovered from the immediate processing and those recovered from the frozen samples were lower for *O. cuspidata* compare to those of *O. europaea*.

No statistically significantly difference was observed among the two plating techniques. For this reason, we suggest using drop plating since this technique is more convenient economically. The number of plates required is significantly lower given the possibility of plating several concentrations per plate. In addition, this technique allows for considerable time-saving, both during the plating and colony counts, since the quantity of the bacterial suspension used for this technique is ten-fold less than that used for spread platings.

References

Balestra G.M., Lamichhane J.R., Kshetri M.B., Mazzaglia A., Varvaro L. 2009. First report of *Pseudomonas savastanoi* pv. *savastanoi* on olive in Nepal. Plant Pathol. 58 (2): 393–393.

Beattie G.A. 2002. Leaf surface waxes and the process of leaf colonization by microorganisms. p. 3–26. In: "Phyllosphere Microbiology" (S.E. Lindow, E.I. Hecht-Poinar, V.J. Elliott, eds.). APS Press, Minnesota, 408 pp.

Conlin K.C., McCarter S.M. 1983. Effectiveness of selected chemicals in inhibiting *Pseudomonas syringae* pv. *tomato in vitro* and in controlling bacterial speck. Plant Dis. 67 (6): 639–644.

Crosse J.E. 1959. Bacterial canker of stone fruits: IV. Investigation of a method for measuring the inoculums potential of cherry trees. Ann. Appl. Biol. 47 (2): 306–317.

Donegan K., Matyac C., Seidler R., Porteous A. 1991. Evaluation of methods for sampling, recovery and enumeration of bacteria applied to the phylloplane. Appl. Environ. Microbiol. 57 (1): 51–56.

Ercolani G.L. 1971. Presenza epifitica di *Pseudomonas savastanoi* (E.F. Smith) Stevens sull'Olivo, in Puglia. Phytopathol. Mediterr. 10: 130–132.

Ercolani G.L. 1978. *Pseudomonas savastanoi* and other bacteria colonizing the surface of olive in the field. J. Gen. Microbiol. 109 (2): 245–257.

Ercolani G.L. 1979. Distribuzione di *Pseudomonas savastanoi* sulle foglie dell'olivo. Phytopathol. Mediterr. 18: 85–88.

Ercolani G.L. 1991. Distribution of epiphytic bacteria on olive leaves and the influence of leaf age and sampling time. Microb. Ecol. 21 (1): 35–48.

Hirano S.S., Nordheim E.V., Arny D.C., Upper C.D. 1982. Lognormal distribution of epiphytic bacterial populations on leaf surfaces. Appl. Environ. Microbiol. 44 (3): 695–700.

Jacques M.A. 1996. The effect of leaf sge and position on the dynamics of microbial populations on aerial plant surfaces. p. 233–248. In: "The Aerial Plant Surface Microbiology"(C.E. Morris, C. Nguyen, P.C. Nicot, eds.). NetLibrary, Incorporated, Netherlands, 307 pp.

Knudsen G.R., Walter M.V., Porteous L.A., Prince V.J., Armstrong J.L., Seidler R.J. 1988. Predictive model of conjugative plasmid transfer in the rhizosphere and phyllosphere. Appl. Environ. Microbiol. 54 (2): 343–347.

Lamichhane J.R. 2011. Phytobacteriological studies related to the introduced and native olive sub-species in Nepal. Ph.D. Thesis, Tuscia, University Viterbo, Italy, 99 pp.

Lamichhane J.R., Varvaro L. 2013. Epiphytic *Pseudomonas savastanoi* pv. *savastanoi* can infect and cause olive knot disease on *Olea europaea* subsp. *cuspidata*. Aust. Plant Pathol. 42 (2): 219–225.

Lamichhane J.R., Balestra G.M., Varvaro L. 2010a. Phytobacteriological investigation on *Olea* spp. in different districts of Nepal. Petria 20 (2): 147–148.

Lamichhane J.R., Balestra G.M., Varvaro L. 2010b. Responses of tomato cultivars largely cultivated in Nepal to *Pseudomonas syringae* pv. *tomato*. Phytopathol. Mediterr. 49: 406–413.

Lindow S.E., Brandl M.T. 2003. Microbiology of the phyllosphere. Appl. Environ. Microbiol. 69 (4): 1875–1883.

Lindow S.E., Arny D.C., Upper C.D. 1978. Distribution of ice nucleation active bacteria on plants in nature. Appl. Environ. Microbiol. 36 (6): 831–838.

Lindow S.E., Knudsen G.R., Seidler R.J., Walter M.V., Lambou V.W. 1988. Aerial dispersal and epiphytic survival of *Pseudomonas syringae* during a pretest for the release of genetically engineered strains into the environment. Appl. Environ. Microbiol. 54 (6): 1557–1563.

Meynell G.G., Meynell E. 1965. Theory and Practice in Experimental Bacteriology. Cambridge University Press, USA, 288 pp.

Surico G. 1993. Scanning electron microscopy of olive and oleander leaves colonized by *Pseudomonas syringae* subsp. *savastanoi*. J. Pytopathol. 138 (1): 31–40.

Quesada J.M., Garcia A., Bertolini E., Lopez M.M., Penyalver R. 2007. Recovery of *Pseudomonas savastanoi* pv. *savastanoi* from symptomless shoots of naturally infected olive trees. Int. Microbiol. 10 (2): 77–84.

Quesada J.M., Penyalver R., Perez-Panades J., Salcedo C.I., Carbonell E.A., Lopez M.M. 2010. Comparison of chemical treatments for reducing epiphytic *Pseudomonas savastanoi* pv. *savastanoi* populations and for improving subsequent control of olive knot disease. Crop Prot. 29 (12): 1413–1420.

Varvaro L., Surico G. 1987. Multiplication of wild types of *Pseudomonas Savastanoi* pv. *savastanoi* (Smith) Young *et al.* and their indolacetic deficient mutants in olive tissues. p. 556–565. In: "Plant Pathogenic Bacteria" (E.L. Civerolo, A. Collmer, R.E. Davis, A.G. Gillaspie, eds.). Springer, Dordrecht.

Zagory D., Lindow S.E., Parameter J.R. 1983. Toxicity of smoke to epiphytic ice nucleation active bacteria. Appl. Environ. Microbiol. 46 (1): 114–119.