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"PHYTOBACTERIOLOGICAL STUDIES RELATED TO THE NATIVE (*Olea cuspidata* Wall.) AND INTRODUCED (*Olea europaea* L.) OLIVE SPECIES IN NEPAL."

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Thesis
Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy
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by the authority of the Rector Magnificus
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To my wife Claudia and my son Himal

CONTENTS

GENERAL ABSTRACT

Two olive species, one introduced from Europe (*Olea europaea* L.) and cultivated in different Districts and the other one the native species of Nepal (*Olea cuspidata* Wall.), were studied regarding phytobacteriological aspects. Among the two olive species, the first was widely studied while no study is available on *O. cuspidata*. In particular, the presence and/or appearance of olive knot disease, the quali-quantitative composition of phylloplane bacterial populations of these species from different Districts, the epiphytic survival of *Pseudomonas savastanoi* pv. *savastanoi* (Psav) (the causal agent of olive knot disease) both on *O. europaea* and *O. cuspidata*, the susceptibility of these species to Psav and the antagonistic activity of the phylloplane bacteria against Psav *in vitro* have been studied. Different strains of Psav isolated from different geographic origins were used.

No presence of olive knot disease was found on *Olea* spp. surveyed during the study in Bajura, Dolpa and Kathmandu Districts. Whereas the disease was found on *O. europaea*, introduced two decades ago, in a commercial orchard of Makwanpur District. No presence of Psav on *Olea* phylloplane was found. The size of bacterial epiphytic populations ranged from very low (2.39×10^0 CFU/cm^2) to very high (1.09×10^5 CFU/cm^2). No quantitative differences statistically significant were found on *Olea* phylloplane sampled in the three Districts. Fifteen bacterial species were identified as colonizers of *Olea* phylloplane in Nepal which are so far different than those found in south-east Italy. Both the olive species were dominated by *Pantoea agglomerans* (former *Erwinia herbicola*). No qualitative differences statistically significant were seen on *Olea* phylloplane sampled in three Districts mentioned above.

Olive species showed a significant influence on bacterial epiphytic survival since Psav better multiplies on *O. cuspidata* compared to *O. europaea*. Leaf age and bacterial strains did not influence significantly the epiphytic survival of the pathogen. *O. cuspidata* showed higher susceptibility to Psav strains than *O. europaea* to artificial inoculations although no difference statistically significant was found. Plant age did not influence significantly host-pathogen interaction. The bacterial strains showed significant differences among them in terms of symptom appearance, knot size and bacterial populations into the knots. None of the phylloplane bacteria inhibited *in vitro* the growth of *P. savastanoi* pv. *savastanoi*.

ABBREVIATIONS

ASL	Above sea level
ANOVA	Analysis of Variance
CFU	Colony forming Unit
FAO	Food and Agriculture Organization of the United Nations
hrp genes	components of type III secretion systems, regulatory proteins
IAA	Indole-3-acetic acid
KOH	Potassium hydroxide
MOAC	Ministry of Agriculture and cooperatives
NA	Nutrient agar
NAG	Nutrient agar containing 1% glycerol
NAS	Nutrient agar supplemented by 5% sucrose
Psav	*Pseudomonas savastanoi* pv. *savastanoi*
rDNA	Ribosomal Deoxyribonucleic acid
RH	Relative humidity
RPM	Revolutions per minute
SDW	Sterile distilled water

CHAPTER 1.

GENERAL PART:

State of art and importance of olive cultivation in Nepal

1.1 State of art

The northwestern part of Nepal, where the altitude ranges from 1400 to 2500m asl, is known for the presence of big natural forests belong to two *Olea* species identified as as O. *cuspidata* and *O. glandulifera.* Secular plants of these species are present mostly in the District of Bajura, Mugu, Humla and Dolpa Districts (Lamichhane *et al*., 2010a). Thirty five forest types of *O. cuspidata* were identified and recorded from Nepal under Trans-Himalaya High Alpine Vegetation. The presence of these plants with vigorous growth and their healthy aspects indicate that *Olea* species find a suitable environmental conditions for growth, development and even for olive production since *O. cuspidtata* alone produces a remarkable quantity of olive fruit in these areas. Despite this great potentiality, no initiative was taken in the past to study and utilize these species which can give a significant contribution being a main source of income to the local people of these remote areas. Taking into account these factors, an effort was made for olive plantation through a project in collaboration between Italian Ministry of foreign affairs and Nepalese ministry of agriculture under FAO, from 2005.

For the plantation of European olive (*Olea europaea* L.), two different valleys have been selected, one in Bajura District (Kolti, 1400m asl where the project is in progress) and the other in Dolpa District (Juphal, 2500m where experimental fields are present). Twenty eight Italian olive cultivars (20 plants per variety) have been planted at Kolti in an experimental field together with local species. While at Juphal just a dozen of varieties were introduced. Moreover, a mother orchard has been established at Kirtipur (1300 m asl), in the valley of Kathmandu District.

European olive had already been introduced in 1994 by some private olive growers in Nepal (Chitlang valley, Makwanpur District) for commercial purpose. Olive production in this District was initiated since 2000 (Lamichhane *et al*., 2010a). This olive farm provides olive oil on the local market although in very small quantities. Since the beneficial properties of olive oil is well-known, its cultivation has continuously been a great concern all over the world.

1.2 Importance of olive cultivation in Nepal

In recent years, Nepal witnessed like most of the south Asian countries a constant growth in demand for edible oils which has essentially been met by imported products. The extent of the demand has induced the authorities, in particular the Government, to look for different alternatives which allow the increasing of the national output of edible oils. Furthermore, the government is committed to launch different projects attempting to introduce new species in

order to phase down imports in the coming years. In this regard, olive cultivation for the production of extra virgin olive oil at local level has been launched in collaboration with Italian Ministry of Foreign Affairs and Nepalese Ministry of Agriculture and cooperatives (MOAC) since 2006 and the project is entitled as “Promotion of olive production and Consumption in Nepal (GCP/NEP/056/ITA)”.

Most of the landscapes in Nepal is represented by mountains and hills with almost all terracement and slopes often not good for the cultivation of other plant crops because of low soil fertility and difficulties in practicing crops cultivation. These marginal soils are ideal for olive cultivation since this crop does not require high soil fertility and can give a satisfactory yield also without irrigation since a small quantity of rainfall is enough to obtain a good level of production. Moreover, since olive requires an annual pruning, the residues obtained by pruning, the herbaceous and the woody parts can be used respectively as fodder for animals and firewood at local levels in remote villages. Furthermore, since income source of local farmers is very limited, olive farming can really be a valid economic resource for their livelihood. A fundamental importance of olive cultivation, oil extraction and its use at local level is given by the benefits of olive oil for human health (De La Lastra *et al*., 2001; Owen *et al*., 2000). All these factors can contribute with strong and wide impacts on the agronomic, social and economic sectors of the remote and hilly areas of Nepal. Projects on olive cultivation can play a vital role through different way to improve the living and economic standards of farmers and mostly provide opportunities of work to local communities.

CHAPTER 2.

BACTERIAL PATHOGEN OF OLIVE

2.1 Introduction and overview

Like all plant species, also olive is subjected to the attacks of several pathogens. In particular, bacterium known as *Pseudomonas savastanoi* pv. *savastanoi* (Psav), the causal agent of olive knot disease, is considered an important problem for olive crops because of its effect on vegetative growth (Quesada *et al*., 2010., Wilson, 1935), olive yield (Schroth *et al*., 1973) and even on olive oil quality (Schroth *et al*., 1968). The disease can cause severe damages in olive groves, mainly when weather conditions favour the survival of epiphytic populations of the pathogen and their entry into the bark.

Olive knot is an endemic disease which occurs in almost all regions of the world where olive is grown. The bacterium produces tumorous outgrowths (knot) occurring on young twigs, branches and stems but occasionally on the leaves and fruits as well (Surico, 1986). For many years, knot development is thought to be the main cause due to the phytohormones indole-3-acetic acid (IAA) and cytokinins produced by the bacterium (Comai and Kosuge 1980; Rodrı´guez-Moreno *et al*., 2008; Smidt and Kosuge 1978; Surico *et al*. 1985; Surico and Iacobellis, 1992). Afterward, Psav mutants unable to produce IAA and/or cytokinins multiplied in the host tissues as well as the parental strain confirmed that the phytohormones alone are not responsible of the olive knot disease (Iacobellis *et al*., 1994). Some authors also proved that the virulence of Psav is due to the capacity of this bacterium to produce *hrp* genes like that of many other plant pathogenic bacteria (Lindgren, 1997; Penyalver *et al*. 2000; Sisto *et al*., 2004). More recently other auxin-producing bacterial species were found to be actively involved in association with Psav in knot development (Cimmino *et al*., 2006; Marchi *et al*., 2006; Ouzari *et al*., 2008).

2.2 Presence and risk of olive knot in Nepal

Although European olive has been introduced in Nepal about two decades ago and cultivated in different parts of the country even in small numbers, no attentions were paid for several years to this species. Recently, phytobacteriological surveys were carried out in different parts of the country both on European and local olive species. The presence of olive knots were not found neither on local olives present in Bajura, Dolpa, Mugu, Humla and Bhajang nor on European olive present in these Districts including the valley of Kathmandu (Lamichhane *et al*., 2010a). Nevertheless, the presence of olive knot disease were found to be widespread in a private European olive orchard situated in Makwanpur District (Balestra *et al*., 2009).

The presence of the bacterial pathogen in Nepal might be considered seriously and its finding in some Districts but not in others should be a matter of concern to avoid the diffusion of bacterial pathogen from one District to others. In particular, since the pathogen is absent in the Districts where the most widespread natural forests of wild olive species, in particular of *O. cuspidata*, are present and since this species was found to be highly susceptible to Psav (Lamichhane *et al*., 2010b), strong control measures must be adopted for the control of this disease.

2.3 Pathogen survival and multiplication

Besides from knots, Psav has been isolated from the phyllosphere of diseased and healthy olive leaves and stems where the pathogen has a resident epiphytic phase (Ercolani, 1978, 1985, 1991). Population sizes were influenced by seasons, reaching a higher level in spring and fall, when wet weather conditions occur. Tissues can be infected through leaf scars, wounds and fissures on stems and twigs, caused by meteorological phenomena and insect miners, as well as by harvest and pruning practices.

Psav can multiply in its saprophytic phase on olive tree phylloplane (Ercolani, 1978; Quesada *et al*., 2007) and its spread at short distances is caused by splashing rain, windblown aerosols, insects and cultural practices. Wounds caused by harvest and pruning, as well as by hail, frost and leaf scars create niches through which infection occurs (Wilson, 1935). The pathogen could also migrate within the host and develop secondary tumours in new wounds as suggested in oleander (*Nerium oleander*) (Wilson&Magie, 1964) and olive plants (Penyalver *et al*., 2006; Marchi *et al*., 2009). The bacterium can also survive inside knots from one season to the next and if the humidity and Psav populations are high enough, exudates containing the bacteria might be produced providing another source of inoculum (Wilson, 1935).

2.4 Possibility of control

The control measures of Psav is exclusively based on the prevention of the infection since no effective measures exist for an efficient control of this pathogen. Nevertheless, in the recent years, some positive results were obtained by using microbial antagonists able to perform growth inhibition *in vitro* (Lavermicocca *et al*., 2001., Rokni Zadeh *et al*., 2008). Regarding the prevention, the use of resistant cultivars is considered one of the most appropriate methods of control although very little information is available in this regard (Benjama, 1994; Di Rienzo *et al*., 2002; Marcelo *et al*., 1999; Varvaro and Surico, 1978). Particular attention must be paid during the

propagation of plant materials, pruning and manipulation of infected parts of the plants. In addition, elimination of infected tissues from the plants and their incineration must be done. Disinfection of plants with copper compounds are the essential practices.

The practices recommended for the control of olive knot disease are the following:

- Providing good growing conditions since healthy trees are less susceptible.
- Avoiding excessive fertiliser application and maintain nutrient balance to ensure that trees only produce the leaves necessary for development.
- Avoiding trees wounding.
- Practicing good hygiene by disinfecting tools after pruning suspect trees.
- Pruning of healthy plants before infected ones, not to prune in wet weather or just before wet weather, to prune out shoots and twigs damaged during harvesting since wounds remain susceptible for up to 10 days, to burn pruned branches immediately on site.

Moreover, plants such as Oleander, Ligustrum (Privet), Forsythia, Fraxinus (Ash), Jasmine, Phillyrea and any other plants of the Oleaceae family should not be planted near olives as they are potential hosts of *Pseudomonas savastanoi* pathovars. Finally, copper sprays should be applied in autumn and spring, before rain and after pruning and leaf fall to protect wound sites.

Psav is included in the list of transmissible agents of olive diseases, and its absence in propagating material is advisable for the certification of olive mother plants.

CHAPTER 3.

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CHAPTER 4.

AIM OF THE DOCTORAL THESIS

1. To verify the presence and/or appearance of *Pseudomonas savastanoi* pv*. savastanoi* both on native (*Olea cuspidata* Wall.) and introduced olive (*O. europaea* L.) species from different geographic regions in Nepal;

2. To study and compare quali-quantitative composition of bacterial epiphytes on those olive species;

3. To evaluate epiphytic survival of Psav on *O. europaea* and *O. cuspidata* by artificially contaminated olive phylloplane;

4. To assess susceptibility of two olive species by inoculating artificially with the Psav strains of different geographic origins;

5. To select possible biocontrol agents of Psav by performing *in vitro* antagonism.

CHAPTER 5.

EXPERIMENTAL PART

5.1 Preliminary tests

Calculation to find out the sample size (n) required for a test (processing of olive leaves to determine the quali-quantitative composition of the phylloplane)

Sample-size determination is an important step in planning a statistical study and it is usually rather complex matter. Among the important hurdles to be surpassed, an estimate of one or more error variances is of fundamental importance for successful and meaningful sample-size determination and specify an effect size of importance.

Sample size is important not only for statistical but also for economic reasons: an under-sized study can be a waste of resources for not having the capability to produce useful results, while an over-sized one uses more resources than are necessary.

The experiment has been carried out in laboratory using 10 replications of cultivar Maurino and the relative data on bacterial recovery has been worked out. Using the two tailed t-table, we found that five is the number of replications necessary to process the olive samples.

The appropriate formula to calculate the number of replication needed to detect is

$$n \geq 2(\sigma/\delta)^2 \{t_\alpha[v] + t_2(1\text{-}P)[v]\}^2$$

where, σ = true standard deviation

δ = the smallest true difference that is desired to detect (it is necessary to know only the ratio of σ/δ, not their actual values), we suppose 20%

v = degrees of freedom of the sample standard deviation with a groups and n replications per group

α = significance level, we suppose 5%

P = desired probability that a difference will be found to be significant (as small as δ)

$t_\alpha[v]$ and $t_2(1\text{-}P)[v]$ = values from a two-tailed t-table with v degrees of freedom and corresponding to probabilities of α and 2(1-P), respectively. (if P = ½ then $t_1 = 0$).

Preliminary analysis to determine the value of true standard deviation:

No. of replications 10

No. leaves/replicate 10

SDW 15 ml.

Average value of bacteria (log UFC/cm^2) obtained from the experiments, log X = 3.96

Standard deviation (of 3,96), σ = 0.24

True σ = 0.24/3.96 = 6%

We suppose that the difference of the average mean based on the discrimination capacity is of 20% (detection), hence, If we consider n= 10 and ν = n-1 = 9 from the values of two/tailed table we will obtain

$n \geq 2(\sigma/\delta)^2 \{t_\alpha[v] + t_2(1\text{-}p)[v]\}^2$

$n \geq 2\ (6\%/20\%)^2 \{1.8331+2.2622\}^2$

$N \geq 2.\ 6\%/20\%\ .(16,77)$

$N \geq 2.\ (0,3)^2.\ 16,77 = N \geq 2.\ 0,09.\ 16,77 = N \geq 0,18.\ 16,77$

$N \geq 3,01$

$N \geq 3$ (we considered this is too low)

If instead we consider n= 3 and ν = 3-1 = 2, from the values of two/tailed table we will obtain

$(2,92+4,30)^2 = 52,12$

$N \geq 2.\ 6\%/20\%\ .(52,12)$

$N \geq 2.\ (0,3)^2.\ 52,12 = N \geq 2.\ 0,09.\ 52,12 = N \geq 0,18.\ 52,12$

$N \geq 9,39$ (we considered this is too high)

If instead we consider n= 4 and ν = 4-1 = 3, from the values of two/tailed table we will obtain

$(2,35+3,18)^2 = 30,58$

$N \geq 2.\ 6\%/20\%\ .(30,58)$

$N \geq 2.\ (0,3)^2.\ 30,58 = N \geq 2.\ 0,09.\ 30,58 = N \geq 0,18.\ 30,58$

$N \geq 5,50$ (we considered that this is the right no. of replication to be followed)

So, with σ = 6% and δ = 20%, $N \geq 5$ (No. of final replications to be used)

CHAPTER 6.

Investigation on the efficiency of methods for sampling, recovery and enumeration of bacteria applied to the phylloplane of *Olea* spp.

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ABSTRACT

The development and application of precise and practical methods of assessment and enumeration of harmful bacteria, whether they are plant pathogenic or ice nucleation active bacteria, present on the plants phylloplane, require thorough studies able to provide detailed information. In this regard, olive has continuously been one of the most concerned species although none of the authors considered the aspects concerning the effectiveness of methods used for recovery and enumeration of bacteria. Different experiments were carried out to examine the quantitative recovery methods which are commonly used. In this experiments, *Pseudomonas savastanoi* pv. *savastanoi*, causal agent of olive knot disease, was applied to two different olive species. Among the most important features considered, type of sampling indicated that bulk leaf sampling allows a higher bacterial recovery from leaves respect to the single leaf sampling. Recovery of bacteria from leaves showed to be significantly better with lab blender followed by stirrer, stomacher blender and sonicator. The recovery efficiency of lab blender resulted to be constant among the proportions of different contaminated leaves. Storage of samples in a freezer for delay processing demonstrated that short storage of leaves sample without addition of buffer can be practiced but without delaying longer since the survival and recovery of bacteria tend to decline with longer storage. Among the two plating techniques, no difference significantly valid was found between sprayed and drop plating.

INTRODUCTION

Microorganisms of plant phylloplane are widely studied by many researchers for several reasons (Beattie, 2002; Colin *et al*., 1983; Knudsen *et al*., 1988; Lindow *et al*., 1988; Zagory *et al*., 1983). Aspects regarding survival and recovery of epiphytic microorganisms, in particular of plant pathogenic bacteria, their antagonist and ice nucleation active bacteria have largely been studied in the last years (Lindow *et al*., 1978; Lindow and Brandl, 2003; Morris et al., 1998). An efficient and accurate detecting method of sampling and recovery of harmful microorganisms is of fundamental importance, since an under-estimation can lead us to the remarkable economic losses, or an over-estimation may translate into several interventions of control and treatments with high cost of production (Donegan *et al*., 1991).

The supervising of microorganisms of our interest, whether they are genetically engineered microorganisms released into the environment or naturally present in the plant phylloplane, seeks accurate and efficient techniques for sampling, conservation of the samples, recovery and

enumeration. Very few researches have been carried out to study applied microorganisms on plant phylloplane (Donegan *et al*., 1991). Among the most important factors to be taken into account, way of sampling and interval of time from sampling to processing must be considered in developing a sample scheme for recovery of the microorganisms.

European olive (*Olea europaea* L.) has widely been studied from different reaserchers, mostly in regard to the epiphytic survival of olive knot pathogen (Ercolani, 1971, 1978 and 1979; Quesada *et al*., 2007; Varvaro and Ferrulli, 1983). No study is available on development and comparison to evaluate the methods for sampling, recovery and enumeration of bacteria. The objective of the study was to compare four commonly used processing methods (washing, blending, stomacher blending and sonication) for the recovery of microorganisms from olive leaves. Besides the influence of processing methods, also the influences of type of sampling, freezed and fresh samples, and plating techniques on bacterial recovery were studied.

Different aspects and methods were compared to address the aforementioned concerns in experiments following application of *Pseudomonas savastanoi* pv. *savastanoi* (hereafter Psav) to widely studied European olive (*O. europaea* L.) and a wild species of olive (*O. cuspidata* Wall.) which has not been the concern of study up to now.

Among the considered aspects, difference between the single and bulk samples was evaluated to examine sampling methodology. The concentration of microorganisms in the samples, the time between collection and processing of samples and the mechanical tools used for processing were considered in measuring the recovery of microorganisms from samples. In addition, two most widespread methods of plating (drop and spread) were compared to determine the most efficient, accurate and speeding method of plating.

MATERIALS AND METHODS

Olive plants: In case of *O. cuspidata*, plants were obtained by directly sowing the seeds. Before sowing, the seeds were put into a wide basin containing vermiculite, wetted and maintained at 4°C for 10 days since this species needs some chill hours for germination because of the seed dormancy. After 10 days of chilling, the basin was kept in a greenhouse and maintained at 20°C for 2 months. Afterwards, the plantlets were transplanted into the 2 litre plastic pots (15 cm wide and 30 cm deep) containing soil, pit and sand (ratio 1:1:1). Whereas for *O. europaea*, two years old plants (cv. Leccino) obtained from cuttings (vegetative propagation) were used. The plants were

kept into the same size plastic pots. All the plants were maintained in greenhouse at 25±1 °C with the relative humidity of 60-80%.

Bacterial strain and culture condition: *P. savastanoi* pv. *savastanoi*, (PseNE 107) obtained in Nepal (Balestra *et. al*., 2008) was used. It was maintained on nutrient agar or deep frozen in culture medium containing 15% glycerol. It was grown on nutrient agar (NA) medium. After 24 hours, fresh bacterial culture was re-streaked on the same medium to form a dense bacterial culture. Bacterial suspension was prepared taking the bacterial cultures and putting them inside a beaker containing phosphate buffer. The suspension was centrifuged at 15,000 x *g* for 20 min. The pellet was suspended to obtain an 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).

Contamination of plants: two year old olive plants of both species were sprayed (40 plants/species, total 80 plants) with the bacterial suspension which was distributed homogeneously on leaf surface by using an atomizer. The contamination by spraying was made until both the upper as well as lower leaf surfaces were fully wet. From 2 hours before till 2 hours after the contamination, the relative humidity was maintained at around 90% to slow down the inoculum evaporation. The experiments were carried out in April of 2009.

Statistical analysis: Data from each experiment were separately analyzed for *O. europaea* and *O. cuspidata* by analysis of variance (ANOVA). Duncan's multiple range test was used to calculate the difference within and among the cultivars.

Sampling experiments: single versus bulk leaf samples and sample size: plants grown as described above are sprayed with a high concentration of bacterial suspension (10^8 CFU/ml) by aforementioned method. For single leaf sample, four sets of 25 leaves/species were aseptically collected in sterile lab bags and processed in groups of 5 leaves (total 100 leaves) in 10 ml of phosphate buffer since olive leaves are particularly small to be processed singly. For bulk samples, 5 samples, each consisting of 20 leaves (total 100 leaves), were similarly collected and processed individually in 40 ml of phosphate buffer. Bulk and single leaf samples were collected from both olive species and immediately processed after 3 hours post-spray. All samples were blended for 5 min in a Lab blender. Samples were then serially diluted in phosphate buffer, spread plated in duplicate on nutrient agar medium supplemented by 5% sucrose (NAS) and amended by 800 µl of cycloheximide per liter of NAS. Colony counts of *P. savastanoi* pv. *savastanoi* for each single

sample were made after plating and incubation at 26±1°C for 48-72 h. APS assess software was used to measure leaf surfaces.

Recovery experiments: comparison of processing methods: five samples per species, each consisting of 20 leaves, per processing method were aseptically collected after 3 hours post-spray on the same day. Samples were processed by all the four techniques. For each technique, three sets of 20 leaves per sample, were used. (i) In washing (Lab therm shaker), the 20 leaves were aseptically placed in a sterile 250-ml conical flask with 40 ml of 0.01 M Tris buffer (pH 7.5), and shaken for 2h on a stirrer. (ii) In blending (Lab blender 80, PBI), the 20 leaves were aseptically placed in a sterile lab bag with 40 ml of 0.01 M Tris buffer, and blended at high speed for 5 min. (iii) In Stomacher blending (Stomacher® 400 Circulator, PBI), the sample was placed in sterile lab bags with 40 ml of 0.01 M Tris buffer, and processed for 5 min. (iv) For sonication, the sample was placed in a sterile 250-ml conical flask with 40 ml of 0.01 M Tris buffer, and processed in a sonicator (Sonic vibra cell) at amplitude 60 and pulser 0 for 7 min since these parameters resulted to be optimal from preliminary laboratory tests.

Samples from all processing methods were then serially diluted in phosphate buffer, spread plated in duplicate plate. Colony counts of *P. savastanoi* pv. *savastanoi* from duplicate plates for each replicate were made after plating and incubation at 26±1°C for 48-72 h. Data from each experiment were averaged, analyzed and converted to CFU/cm^2.

Effect of bacterial concentration in sample on recovery efficiency: plants grown as described above were sprayed with a low concentration of bacterial suspension (10^4 CFU/ml) and a set of five control plants per species was used where only SDW was sprayed. After 2 hours all leaves were removed and placed in sterile lab bags. Leaves contaminated with bacteria were mixed with leaves sprayed only with phosphate buffer in proportions so that the final sample contained 2g of leaf material and consisted of 5, 10, 25, 50 and 100% leaves contaminated with bacteria. Samples for each proportions were prepared in quadruplicate in sterile lab bags, processed in 40 ml of phosphate buffer in a lab blender for 5 min and serially diluted in phosphate buffer as described above. Dilutions were spread plated in duplicate onto NAS medium. Colony counts of *P. savastanoi* pv. *savastanoi* from duplicate plates for each replicate were made after plating and incubation at 26±1°C for 48-72 h. Data from each experiment were averaged, analyzed and converted to CFU/cm^2.

Effect of freezing samples on bacterial recovery: plants grown as described above are sprayed with a high concentration of bacteria suspended in phosphate buffer (10^8 CFU/ml) as mentioned previously. Twenty-four samples per species, each represented by 20 leaves, were aseptically collected and put in sterile lab bags immediately after three hours post-spray. Samples were divided in two groups for each species and freezed at -20°C with and without the addition of 40 ml of phosphate buffer before freezing. After 1, 5, 10, 15, 20 and 30 days, four frozen samples for each species were thawed for 30 min at room temperature and 40 ml of phosphate buffer was added to those sample frozen without buffer. Samples were then processed, plated and colony counts were averaged as described previously.

Comparison between two plating techniques: three sets of eight concentrations, ranging from 10^1 to 10^8 CFU/ml, were prepared by serially diluting a 10^8 CFU/ml concentration of bacterial suspensions of *P. savastanoi* pv. *savastanoi* for each strain after adjusting turbidimetrically as described by Varvaro and Surico (1987). Each "drop plate" contained five 10 µl drops of four concentrations, for a total of 20 drops per plate. Whereas, each "spread plate" consisted of 100 µl of each concentration spread onto NAS medium. Platings were made in duplicate for each techniques and incubated at 26±1°C for 48-72 h.

Colony counts were made from both plating techniques in Petri dishes with 30 to 300 colonies (Meynell and Meynell, 1965) and averaged for duplicate plates by adjusting the dilution factor. Data from each experiment were averaged, analyzed and converted to CFU/cm^2.

Sampling experiments: single towards bulk leaf sample

Tab. 1. Mean log CFU/cm^2 values of *Pseudomonas savastanoi* pv. *savastanoi* recovered from single and bulk leaf samples from two olive species

Species		**Log CFU/cm^2**	
Olea europaea	bulk	4.47±0.23*	a
	Single	3.92±0.03	b
Olea cuspidata	bulk	4.69±0.20	a
	Single	4.33±0.06	b

Means followed by the same letter are not significantly different at P = 0.05

* Standard error

In both experiments, bacterial counts from single leaf and bulk leaf sample were significantly higher for bulk samples.

Recovery experiments: comparison between the processing methods

Tab. 2. Comparison on efficiency of processing methods for recovery of *Pseudomonas savastanoi* pv. *savastanoi*

Technique	**n**	***Olea europaea***	
Lab blender	5	4.29±0.40*	a
Stirrer	5	4.09±0.16	b
Stomacher blender	5	3.88±0.18	c
Sonicator	5	3.57±0.03	d
Technique	**n**	***Olea cuspidata***	
Lab blender	5	4.52±0.18	a
Stirrer	5	4.38±0.11	b
Stomacher blender	5	4.18±0.21	c
Sonicator	5	4.02±0.01	d

Means followed by the same letter are not significantly different at P = 0.05

* Standard error

[n] Number of replicate

The ANOVA of the log CFU/cm^2 values from the four tested processing techniques indicated a significant differences on their efficiency on recovering *P. savastanoi* pv. *savastanoi* from leaf surfaces of two assayed olive species (Tab. 2). The bacterial recovery capacity appeared to be different from one method to another between the two assayed *Olea* species. All methods differ significantly from one another ranking from the most efficient to less efficient as Lab blender, stirrer, stomacher blender and sonicator.

Effect of bacterial concentration in sample on recovery efficiency

Plotting of the CFU/cm^2 values produced a straight line with high coefficient of determination value (r^2), In both experiments, by putting in relation different proportion of contaminated leaves (Fig. 1 and 2). The linearity of the data and the high value of r^2 demonstrate the efficiency, constancy and accuracy of the lab blender in recovering bacteria from leaf samples.

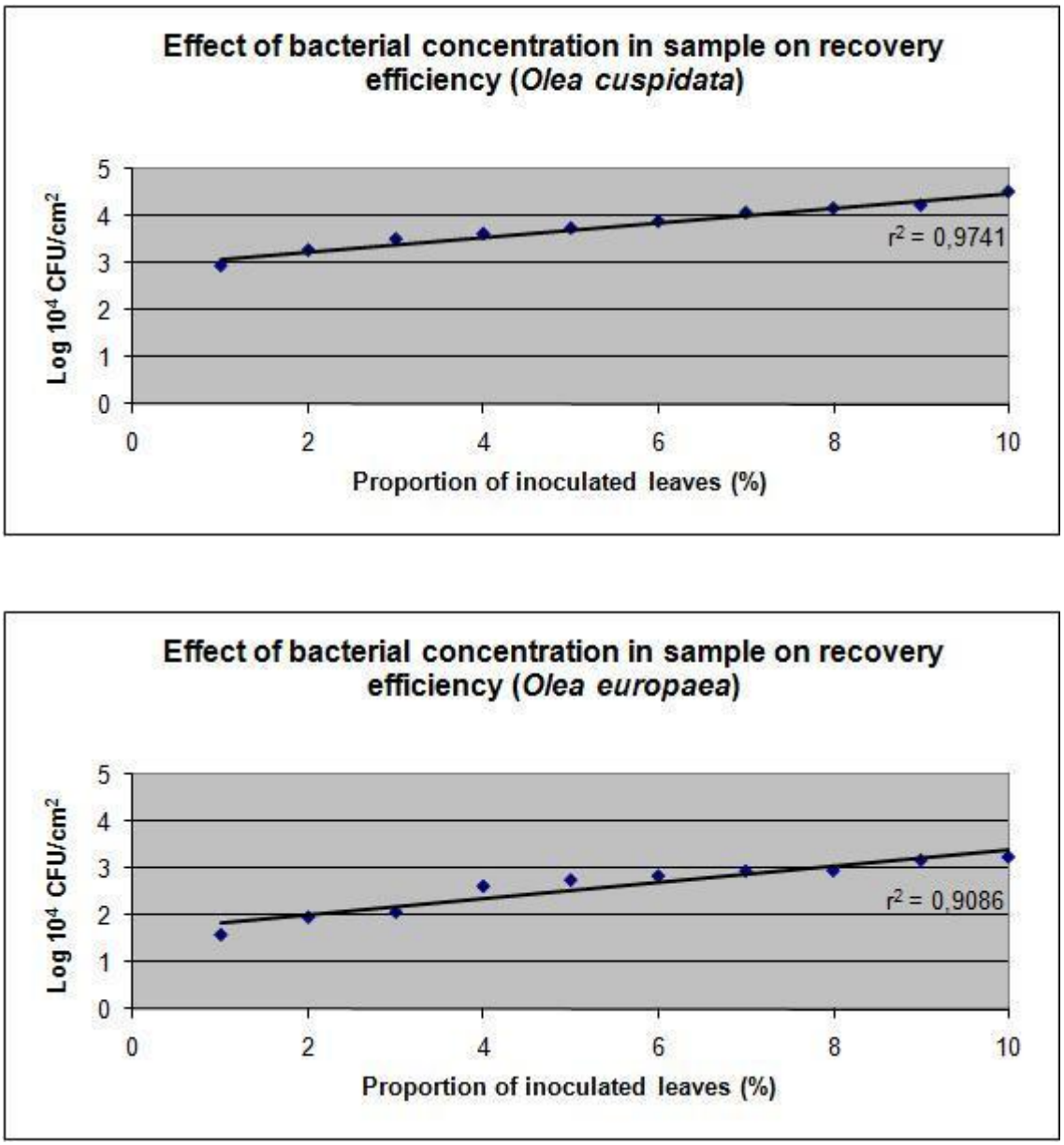

Fig. 1. Effect of bacterial concentration in sample of two *Olea* spp. on recovery efficiency of *Pseudomonas savastanoi* pv. *savastanoi*.

Effect of freezing samples on bacterial recovery

Bacterial numbers decrease more significantly the longer the samples were frozen. In particular, bacterial numbers declined drastically in samples frozen with the addition of phosphate buffer. As shown in Tab. 3, in case of *O. europaea* the number of bacteria recovered from sample frozen for only 1, 5 or 10 days, without addition of buffer, did not significantly differ. Afterwards, the bacterial numbers tend to decline with longer freezing differing significantly between 10 and 15 days of freezing, with further significant decline after 20 days. Nevertheless, no significant difference was seen on the recovered bacterial number between 20 and 30 days. Whereas for the samples frozen with addition of buffer, the recovered bacterial populations for only 1 and five

days did not differ significantly, but subsequently bacterial numbers differ significantly among the samples frozen for 5, 10, 15 days. The populations recovery after the freezing of sample for 15 and 20 days was rather similar with a light decline. No bacterial populations were recovered from the samples frozen with addition of buffer for 30 days.

Bacterial recovery was slightly different in case of *O. cuspidata*, since the number of bacteria recovered from sample frozen for only 1 and 5 days, without addition of buffer, was not significantly different. Later, the bacterial numbers tend to decline with longer freezing with some similarities among them in their significance from samples processed after 10, 15, 20 and 30 days of freezing without addition of buffer. While for the samples frozen with addition of buffer, the number of bacteria recovered from sample frozen with addition of buffer for 1 day was not significantly different for those frozen for 5 and 10 days but differed significantly to those frozen for 15 and 20 days. No bacteria were recovered from the sample frozen for 30 days with addition of buffer in case of *O. cuspidata* as well.

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

Days	buffer	*Olea europaea* (log CFU/cm^2)		*Olea cuspidata* (log CFU/cm^2)	
1	+	2.24±0.67*	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		0.00±0.00	
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

Means followed by the same letter are not significantly different at P = 0.05

* Standard error

Plating experiments: comparison of drop *versus* spread plating

The average value expressed as CFU/cm^2 was higher from those obtained from spread plating respect to drop plating although no significant difference was found among them.

Tab. 4. Difference between the spread and drop plating methods

Plating technique	n	Mean log CFU/cm^2	
Spread plating	6	2.61±0.59*	a
Drop plating	6	2.49±0.77	a

Means followed by the same letter are not significantly different at P = 0.05

* Standard error

[n] Number of replicate

DISCUSSION

The results obtained from the sampling, recovery and enumeration of bacteria show considerable variation among the investigated features. Furthermore, the use of two different species belong to *Olea* showed that the parameters considered during the study differ from one species to another.

Concerning the type of sampling, generally, bulk leaf sampling is more quicker and easier at field level and thus it is frequently used respect to single leaf sampling. The latter, often, results unfeasible since it is laborious and time consuming. Furthermore, single leaf sampling, usually is imprecise since single sample could not be representative for quantifying microbial populations respect to the bulk leaf sample collected randomly. Nevertheless, results showed that the recovery of bacterial populations from leaf samples is higher from bulk sample rather than those from single samples from both olive species examined in the study. These results are in agreement with Crosse (1959) since larger bulk samples result in more accurate estimates of bacterial populations.

The recovery of bacterial populations by using the widely used four processing methods and the significant differences among them is of noteworthy importance. Nevertheless, our results suggest that the recovery of microorganisms from leaf surface varies with varying the plant species or at least between herbaceous and woody species. This hypothesis is concordant to the results obtained by Donegan *et al*., (1991) since in their study stomacher blending allowed the greatest recovery of microorganisms from the leaf surface of oat and bean plants. In our studies, lab blender allowed the greatest recovery of bacteria from leaf surfaces of both olive species. This is probably due to the fact that olive leaves are very particular with waxy and tough leaf surfaces

having numerous star hairs on the lower surface of the leaves (Surico, 1993). This finding is of considerable importance since microbial populations of olive is widely studied from several researchers and the advantages of lab blending like quick processing time (5 min. instead of 2h of stirrer and 7 min of sonicator), possibility of sample collection directly in the sterile lab bags suitable for lab blender, time-saving since the bags can be easily disposed without the need of cleaning and autoclaving of containers, like in case of sonication and stirrer, which is very laborious and time-consuming task mostly in experiments with numerous samples. Furthermore, the disadvantage of releasing plant cell contents, caused by beating action of paddles which apply pressure to the bagged sample, do not take place since olive leaves are very resistant. The capacity of lab blender in providing constant recovery confirms its effectiveness which keep our results far from any possible errors.

With regard to sample storage, since sample collections and immediate processing often results unfeasible especially in large scale experiments, an efficient way of storage, which allows on the one hand to ensure the conservation of leaf samples without any alteration and on the other hind the survival of microorganisms of our concern, would be advantageous. Our results show that the freezing of collected samples, with or without the addition of buffer, may negatively influence survival or recovery of microorganisms in the samples. The numbers of bacteria recovered from samples frozen for 1 and 5 days with addition of buffer and 1, 5, and 10 days without addition of buffer did not differ significantly in case of *O. europaea* and for 1, 5 10 and 15 days with addition of buffer and 1 and 5 days without addition of buffer for *O. cuspidata*, they indicate that a delay of a few days in sample processing is possible but longer storage should be avoided since the values tend to decline with time with significant differences.

The adverse effect of pre-freezing and the difference between the sample frozen with and without the addition buffer could be because of the bacterial injury caused from freezing and thawing of the buffer (Donegan *et al*., 1991) and can vary . Probably, also the type of leaf surface may influence since *O. cuspidata* has tender and less tough leaf surface respect to *O. europaea* leaves characterized by waxy and tough surface. In fact, different trend of decline in bacterial populations was observed among the two olive species.

No difference significantly valid was seen among the two plating techniques although the colony counts from spread plating were numerically higher than drop plating. Nevertheless, the lack of significant differences suggest several advantageous of drop plating since this technique is more convenient both economically, since the number of plates required is drastically lower given the

possibility of plating several concentrations per plate, and also from the point of physical time-saving given the easiness and quickness of the technique and the time saving also during the colony counts, since the quantity of bacterial suspension used for this technique is 10 fold less than the possible 100µl used for spread plates.

As conclusion, it is likely that a continuous effort is necessary for improvement and standardization of these techniques. In fact, the recovery of microorganisms probably depends not only from plant species but also from type of bacteria, whether they are plant pathogenic, antagonists or bacteria having ice nucleation activities. This is important in order to make right estimation and to intervene with proper control measures.

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CHAPTER 7.

Bacteria colonizing phylloplane of *Olea* spp. in the fields of Nepal

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ABSTRACT

Different bacterial species were isolated from olive leaves sampled in three different districts of Nepal (Bajura, Dolpa and Kathmandu). Almost all of them were aerobic, heterotrophic and mesophilic which were present in plant phylloplane of all the districts although in different percentages. Both of the olive species were dominated by *Pantoea agglomerans* (former *Erwinia herbicola*) and *Xanthomonas campestris* group in all the three districts. The size of the bacterial epiphytic populations ranged from very low (0.38 log CFU/cm^2 for cultivar Moraiolo sampled from Bajura district) to very high (5.03 log CFU/cm^2 for cv. Marina sampled from Kathmandu district). The isolates were characterized by morphological, physiological, biochemical and molecular assays and they were identified as, *Bacillus asahii*, *Bacillus cereus, Bacillus circulans*, *Bacillus megaterium*, *Bacillus pumilus, Bacillus silvestris*, *Bacillus simplex*, *Bacillus subtilis*, *Brenneria serratia*, *Microbacterium testaceum*, *Micrococcus luteus*, *Pantoea agglomerans*, *Pseudomonas fluorescens*, *Pseudomonas putida* and *Xanthomonas campestris* group. No presence of *Pseudomonas savastanoi* pv. *savastanoi* was found on olive leaves. No significant quantitative differences were found while significant differences in qualitative composition of epiphytic bacterial populations were observed among the three districts and among the two *Olea* species.

INTRODUCTION

Bacteria are common residents of leaves and there are many bacterial species which colonize plants leaf surfaces that provide physical environments suitable for growth and reproduction of epiphytic bacteria (Ercolani, 1978; Thomson *et al*., 1976). Almost all of these epiphytic bacteria play a different role which can influence the health of host plants under different conditions suitable for their activities, such as inciting disease in case of plant pathogenic bacteria (PPB) (Beattie and Lindow 1994a; Beattie and Lindow 1994b; Beattie and Lindow 1995; Hirano and Upper 1983; Mew and Kennedy, 1982), causing frost injury in case of ice nucleation active bacteria (INA) (Cambours, 2004; Cambours *et al*., 2005; Hirano *et al*., 1981; Lindow *et al*., 1978b; Lindow, 1986; Nejad *et al*., 2002, 2004, 2005; Pearce, 2001; Wilson and Lindow, 1994a) and altering plant growth in case of bacteria that produce plant hormons (Brandl and Lindow, 1998; Fett, 1987; Glickmann *et al*., 1998; Hirano and Upper, 2000; Lindow *et al*., 1998). In addition, there are also many saprophytes, which are epiphytic inhabitants naturally present on aerial parts of plants, with several beneficial effects to the plants such as growth inhibition of PPB acting as their antagonist with continuous worldwide interest, to attain sustainability in agriculture which is

essential to reduce or eliminate the use of chemical pesticides for plant disease control (Andrews, 1992; Blakeman and Fokeman, 1982; Gross, 1991; Ji *et al*., 1996; Kishore *et al*., 2005; Thomson *et al*., 1976). Thus, the knowledge of the bacterial species present on plant phylloplane and their accurate estimates are of essential importance to safely predict phenomena related to them, including disease or frost damage.

Although bacterial phylloplane communities on annual or perennials bearing deciduous leaves have largely been studied by several authors (Hirano and Upper, 1991; Ishimaru *et al*., 1991; Jacques *et al*., 1995; Leben and Daft, 1967; Lindemann *et al*., 1984; Lindow and Andersen, 1996; Malvick and Moore, 1988; Morris and Kinkel, 2002), only few is known of those occurring on the long living leaves of evergreen plants (de Jager *et al*., 2001; Karamanoli *et al.,* 2000; Périssol *et al*., 1993; Yadav *et al*., 2004), including european olive (*Olea europaea* L.) (Ercolani, 1978, 1991; Lavermicocca *et al.*, 1987). Whereas, no information is available on this issue on African olive (*Olea cuspidata* Wall.) which is a widespread olive species present mostly in African, Asian and Oceanian continents (Daniel and Boher, 1978; Spennemann and Allen, 2000). It is naturally present in several Himalayan regions of Nepal as well (Lamichhane *et al*., 2010a), particularly in Dopla, Bajura, Mugu and Humla districts at altitudes ranging from 1300 to 2500m above sea level (asl). Twenty eight Italian olive cultivars belonging to *O. europaea* are also present in the Himalayan regions of Bajura, Dolpa and Kathmandu districts of Nepal respectively at 1400, 1500 and 2500m (asl).

The aim of this study was to evaluate quali-quantitative composition of the bacterial populations present on olive phylloplane of two different olive species and to ensure whether there was the presence of *Pseudomonas savastanoi* pv. *savastanoi* (Psav) on epiphytic surfaces of asymptomatic olive plants present in three different districts of Nepal.

MATERIALS AND METHODS

FIELD

Three fields of european olive were concerned of our study. The fields were situated at Kolti, in the district of Bajura, at 1500m asl, at Juphal, in the district of Dolpa, at 2500m asl and at Kirtipur, Kathmandu district, at 1300m asl. Furthermore, wild olive belonging to *O. cuspidata* present in Bajura and Dolpa districts as natural forests were studied.

OLIVE CULTIVARS

For *O. europaea*, twenty eight Italian olive cultivars present in three different districts were used. The cultivars were, Anghiari, Ascolana, Bosana, Bourbon, Canino, Carboncella, Carolea, Cassanese, Cipressino, Coratina, Corona, Femminella, Frantoio, Frattese, Itrana, Leccino, Leoncino, Marina, Maurino, Moraiolo, Nocellara del Belice, Pendolino, Piantone, Rajo, Rasara, Rosciola, Taggiasca and Valle Corsana. Whereas, for *O. cuspidata*, plants naturally present in those area, were used.

SAMPLING OF OLIVE LEAVES

Leaf samples were collected randomly from all the asymptomatic plants during a 3-year period (April 2007- April 2009) in Bajura and only for one year period (April 2009) in Dolpa and Kathmandu districts. Sampling was made early in the morning. In case of *O. europaea*, leaf samples were randomly collected from 28 Italian cultivars by taking both young and old leaves. 40 leaves from each plant and five plants for each variety for a total of 200 leaves per variety were aseptically collected in sterile lab bags, bulked and put in an icebox. In case of *O. cuspidata*, the same procedure was used by collecting 10 samples each represented by 200 leaves. Samples were sent to Italy by air express, by air mail or in some cases, hand carried. Climatic data obtained from the meteorological stations installed to our study sites were used.

FREEZING OF THE SAMPLES

Since the volumes of the samples were too large, immediate processing of all the leaves was impossible. Donegan *et al*., (1991) showed that a delay of a few days in processing is possible since freezing of samples for a short period of time do not influence negatively bacterial recovery. For this reason samples were frozen at -20°C and processed within one week. Lindow *et al*. (1978a) worked with samples collected from distant locations and sent to laboratory with time between collecting and plating being 1 to 3 days in most cases, without any significant differences with those collected locally, although the effects of that delay have not been determined.

PROCESSING OF LEAF SAMPLES

Each bulk sample was aseptically divided in 10 groups (20 leaves per replicate) and placed in a sterile 250 ml conical flask with 40 ml of phosphate buffer and shaken in the dark by using an orbital stirrer at 26±1 °C and 150 rpm for 2h. Previous studies indicated that over 90% of the

phylloplane bacteria passed into suspension and that there was no error due to bacteria multiplying in the washing fluid (Crosse, 1959; Ercolani, 1978). These results were obtained by previous authors by plating 10-fold serial dilutions of homogenates of washed and unwashed leaves. Furthermore, Ercolani (1976) showed that virtually no bacteria passed into suspension from insides the leaves which allow us to make recovery of only phylloplane bacteria.

After washing, the solution was serially diluted in phosphate buffer and spread plated in duplicate onto nutrient agar medium supplemented by 5% (v/v) of sucrose (NAS), amended by 800 µl /l of cycloheximide (previously prepared as stock solution by diluting 1g in 10 ml of ethanol) to prevent fungal contamination. One hundred µl of diluted solution was used for plating. The plates were then incubated at 26±1°C for 48-72 h. APS assess software was used to measure leaf surfaces.

COLONY COUNTS AND THEIR CHARACTERIZATION

Bacterial colonies were first counted with a stereomicroscope to determine the total bacterial population from NAS plates. The colonies were then purified by streaking on the same medium and incubating the plates at 26±1°C. Isolates obtained during the first, second and third year of work from all the districts were screened to determine their reaction towards the different assays. Three hundred and thirty isolates from Bajura district, 170 from Dolpa district and other 140 from Kathmandu district, for a total of 640 isolates collected during the three years of experiments, were tested. Morphological characteristics of the isolates were determined by streaking onto NSA medium followed by the microscopic observation. Gram test was performed by using KOH solution (3%). Levan production was observed after 3 days of incubation on NSA. Cowan and Steel's (1965) methods were used to detect catalase activity (method 1), citrate utilization (method 1), gelatin hydrolysis (method 1), indole production (method 2), urea hydrolysis (method 1), oxidation or fermentation of glucose, oxidase activity, nitrate reduction and starch hydrolysis. Arginine dihydrolase, lysine and ornithine decarboxylase activity (Taylor, 1961) and growth in MacConkey agar (Oxoid CM 7) were all recorded after 5 days of incubation.

IDENTIFICATION OF THE ISOLATES

Isolates were identified to the genus level by using the diagnostic tables of Cowan and Steel (1965) by which they were divided in groups to obtain only those effectively different since bacteria colonizing olive phylloplane are frequently represented by only few species (Ercolani,

1978). At the last, the isolates were identified to the species level by molecular analysis. Molecular identification was achieved by sequencing the 16S rDNA region of each representative isolate for each bacterial species. A 1500-bp region of the 16S rDNA was amplified by using the non specific primers NOC 1F and NOC 3R (Lamichhane *et al*., 2010b) and sequenced. A BlastN search was performed for comparing their similarity with the strains present in the database (GenBank, EMBL and DDBJ).

Table 1. Average Meteorological data recorded for one week before sampling at sampling sites during the year 2007-2009.

District	year	month	average air temp (°C)			rainfall (mm)	RH (%)
			min.	max.	mean		
Bajura	2007	may 23	6	23	14.5	0.7	21
Bajura	2008	may 19	8	24	16.0	0.3	26
Bajura	2009	may 26	6	24	15.0	0.0	25
Dolpa	2009	may 29	-1	16	7.5	0.4	30
Kathmandu	2009	June 3	5	26	15.5	7.4	68

STATISTICAL ANALYSIS

After colony counts, data from each experiment were separately analyzed and averaged to express the bacterial epiphytic populations colony forming unit/cm^2 (CFU/cm^2) by analysis of variance (ANOVA). Duncan's multiple range test was used to calculate the difference within and among the cultivars.

RESULTS

Bacterial populations of the phyllosphere of two olive species examined was highly variable among the samples collected in three different districts. Nevertheless, no significant quantitative differences were observed among the bacterial epiphytic populations of the different olive cultivars belong to the same species and between the two different species present in the same zone (Tab. 2). The size of the bacterial epiphytic populations ranged from very low (0.38 log CFU/cm^2 for cultivar Moraiolo, 0.54 log CFU/cm^2 for cv. Rasara and 1.15 log CFU/cm^2 for cv. Carboncella) to very high (2.99 log CFU/cm^2 for wild olive present near the orchard, 3.83 log CFU/cm^2 for cv. Frattese and 5.03 log CFU/cm^2 for cv. Marina) respectively for the samples collected in Bajura, Dolpa and Kathmandu districts.

Table 2. Bacterial epiphytic populations of olive leaves collected from three different districts in the year 2009

Cultivars	log (CFU/cm^2)		
	Bajura	Dolpa	Kathmandu
Cipressino	1.03±0.30*	2.50±0.22	3.60±0.12
Cassanese	2.56±0.25	2.87±0.19	3.46±0.16
Marina	0.75±0.74	2.78±0.30	5.03±0.03
Nocellara	2.11±0.19	2.52±0.15	2.94±0.41
Rosciola	1.51±0.32	2.19±0.15	2.90±0.14
Femminella	1.65±0.15	2.54±0.30	3.54±0.17
Leccino	1.02±0.52	2.67±0.21	3.32±0.23
Frantoio	2.24±0.39	3.07±0.43	3.64±0.17
Taggiasca	1.77±0.22	3.13±0.39	3.42±0.14
Moraiolo	0.38±0.33	3.11±0.51	4.02±0.08
Coratina	1.86±0.18	2.11±0.44	2.47±0.22
Ascolana	1.33±0.40	2.83±0.15	3.65±0.57
Bourbon	2.02±0.43	3.30±0.37	3.38±0.28
Rasara	0.54±0.44	1.62±0.21	2.33±0.51
Pendolino	2.09±0.39	2.93±0.36	3.80±0.20
Valle Corsana	2.77±0.11	3.59±0.35	2.90±0.39
Leoncino	2.73±0.37	3.30±0.26	2.25±0.76
Frattese	2.34±0.64	3.83±0.11	4.20±0.07
Itrana	1.80±0.34	2.01±0.33	2.52±0.48
Carboncella	1.15±0.63	2.21±0.41	1.52±0.76
Maurino	2.42±0.26	3.18±0.14	3.73±0.19
Piantone	1.70±0.14	3.29±0.16	3.33±0.14
Rajo	0.80±0.47	2.85±0.47	2.59±0.44
Canino	2.67±0.24	2.18±0.41	2.39±0.41
Anghiari	2.66±0.21	3.21±0.27	3.08±0.37
Carolea	0.49±0.47	1.67±0.11	2.00±0.28
Bosana	1.75±0.56	3.60±0.59	2.21±0.23
Corona	0.87±0.20	3.31±0.36	2.64±0.39
Wild from orchard	0.89±0.71	2.18±0.16	3.33±0.16
Wild near orchard	2.99±0.37	3.12±0.19	-
Wild far orchard	2.36±0.39	3.12±0.21	-
AVERAGE ± C. L.	**1.72±0.37**	**2.80±0.29**	**3.11±0.29**

(*C. L.) Confidence limit

Quantitatively, among the three districts, single and average values of epiphytic bacteria were lower for samples collected in Bajura followed by Dolpa and Kathmandu districts. No leaf samples with non-detectable bacterial populations were present since the leaf samples were collected in spring, season during which plant phyllosphere is often highly colonized by bacteria due to the favourable environmental conditions. There was not far larger variability in terms of bacterial quantitative populations neither among species nor within-species. Nevertheless, bacterial populations of olive phylloplane of wild species resulted to be the highest together with those of some European olive cultivars phylloplane. In particular, the values of phyllosphere bacterial populations of *O. cuspidata* resulted to be higher than the average values in all the districts (Tab. 2).

The average size of the phyllosphere bacterial populations of two olive species, present in Bajura district, during the three consecutive years resulted particularly different (Tab. 3). The highest average value was recorded in year 2007 with 3.83 log CFU/cm^2 followed by the year 2008 with 3.07 log CFU/cm^2; whereas the bacterial populations of olive phyllosphere were particularly low in the year 2009 with an average value of 1.72 log CFU/cm^2. On the other hand, also the single values in terms of bacterial epiphytic populations showed the same trend except for some cultivars.

It is likely that climatic conditions, in particular the average temperature and rainfall recorded for one week before sampling at sampling sites, influenced particularly the population of phyllosphere bacteria (Tab. 4). The average temperatures in Bajura during the sampling time were mild in the three consecutive years but the rainfalls were widely different from one year to another. Taking into consideration Bajura district, an increasing in rainfall directly influenced the number of epiphytic bacterial of olive with consequent increase in bacterial epiphytic populations. In particular, the dry climate of the year 2009 influenced negatively bacterial epiphytic populations. Whereas, a light rainfall in Dolpa could probably favoured the bacterial multiplication although the average temperature during sampling time was low. Regarding to the bacterial populations of phylloplane in Kathmandu district, the average values were the highest in the year 2009 among the three districts, since both the values of average rainfall and temperature were optimal for the bacterial growth and multiplication.

Table 3. Average values of bacterial epiphytic populations of olive leaves collected from Bajura district during the three years 2007-2009

Cultivars	Bajura, log (UFC/cm^2)			
	2007	2008	2009	Average
Cipressino	4.18±0.42*	4.08±0.15	1.03±0.30	3.10±0.29
Cassanese	4.00±0.30	3.02±0.17	2.56±0.25	3.19±0.24
Marina	3.70±0.38	3.12±0.30	0.75±0.74	2.52±0.47
Nocellara	3.95±0.22	2.69±0.39	2.11±0.19	2.91±0.27
Rosciola	2.86±0.02	2.80±0.24	1.51±0.32	2.39±0.19
Femminella	3.71±0.41	2.35±0.53	1.65±0.15	2.57±0.36
Leccino	3.94±0.21	2.46±0.28	1.02±0.52	2.47±0.34
Frantoio	3.92±0.13	4.06±0.19	2.24±0.39	3.41±0.24
Taggiasca	3.49±0.25	2.83±0.44	1.77±0.22	2.70±0.30
Moraiolo	3.02±0.05	2.69±0.11	0.38±0.33	2.03±0.16
Coratina	3.99±0.31	2.96±0.28	1.86±0.18	2.94±0.25
Ascolana	3.41±0.18	3.60±0.17	1.33±0.40	2.78±0.25
Bourbon	3.69±0.17	2.57±0.27	2.02±0.43	2.76±0.29
Rasara	2.99±0.01	3.05±0.24	0.54±0.44	2.19±0.23
Pendolino	3.06±0.25	2.88±0.27	2.09±0.39	2.68±0.30
Valle Corsana	3.97±0.17	2.34±0.15	2.77±0.11	3.02±0.14
Leoncino	2.94±0.01	3.41±0.46	2.73±0.37	3.03±0.28
Frattese	4.06±0.40	2.31±0.37	2.34±0.64	2.90±0.47
Itrana	3.86±0.13	3.34±0.43	1.80±0.34	3.00±0.30
Carboncella	2.72±0.01	2.96±0.29	1.15±0.63	2.28±0.31
Maurino	4.31±0.11	2.98±0.34	2.42±0.26	3.24±0.23
Piantone	3.16±0.13	2.64±0.31	1.70±0.14	2.50±0.19
Rajo	4.44±0.11	2.98±0.10	0.80±0.47	2.74±0.23
Canino	3.81±0.23	3.72±0.29	2.67±0.24	3.40±0.25
Anghiari	4.22±0.21	3.51±0.18	2.66±0.21	3.46±0.20
Carolea	4.28±0.29	3.30±0.16	0.49±0.47	2.69±0.30
Bosana	3.01±0.12	3.04±0.28	1.75±0.56	2.60±0.32
Corona	4.03±0.11	2.68±0.59	0.87±0.20	2.53±0.30
W orchard	5.23±0.06	2.14±0.14	0.62±0.20	2.66±0.14
W near orchard	5.44±0.08	4.46±0.35	2.99±0.37	3.73±0.36
W far Orchard	5.33±0.12	4.41±0.23	2.36±0.39	3.38±0.31
AVERAGE ± C. L.	**3.83±0.18**	**3.07±0.28**	**1.72±0.37**	**2.83±0.28**

(*C. L.) Confidence limit

Table 4. Relation between climatic conditions and average values of bacterial epiphytic populations of olive leaves

District	Year	Average temp. (°C)	Average rainfall (mm)	log CFU/cm^2
Bajura	2007	14.5	0.7	3.83
Bajura	2008	16.0	0.3	3.07
Bajura	2009	15.0	0.0	1.72
Dolpa	2009	7.5	0.4	2.80
Kathmandu	2009	15.5	7.4	3.11

The qualitative characterization of the bacterial epiphytic populations obtained from all the three districts showed that bacteria colonizing *O. europaea* and *O. cuspidata* are essentially the same but their number varies widely among the two *Olea* species and also among the three districts (Tab. 5). In general, the most abundant species on leaves were *Pantoea agglomerans* on both *Olea* species with 26.07% and 38.92% in Bajura district, 19.38% and 48.62% in Dolpa district and 20.03% and 0.00% in Kathmandu district respectively for *O. cuspidata* and *O. europaea* followed by *Xanthomonas campestris* group. Whereas, other bacterial species vary largely in their number from one species to another and mostly from one district to another. In particular, *Bacillus asahii*, *Micrococcus luteus* and *B. megaterium* were present consistently representing respectively third, fourth and fifth position on *O. cuspidata* and *O. europaea* sampled from Bajura district. On the other hand, numerically, third, fourth and fifth position on leaves of *O. cuspidata* sampled from Dolpa district were represented by *Microbacterium testaceum, Bacillus megaterium and B. circulans* but those position on *O. europaea* were represented by *Micrococcus luteus, Bacillus megaterium* and *Pseudomonas fluorescens.* The results were different on leaves sampled from Kathmandu district since those positions were held by *B. circulans*, *B. simplex* and *Microbacterium testaceum*.

Almost all the species colonizing olive phylloplane were found to be the same since they were successfully isolated from the leaf samples collected from both olive species in the three district except *Brenneria serratia* which was not found on *O. cuspidata* and *O. europaea* leaves collected respectively from Dolpa and Kathmandu districts and *B. subtilis* not present on *O. europaea* on leaves sampled from Bajura and Dolpa district.

Table 5. Isolated bacterial species and their percentage on two different species of olive leaves sampled in three different districts

Species	Bajura (%)		Dolpa (%)		Kathmandu (%)
	O. cuspidata	*O. europaea*	*O. cuspidata*	*O. europaea*	*O. europaea*
Bacillus asahii	11.73	3.20	5.01	1.08	5.22
Bacillus cereus	4.53	0.39	6.78	0.19	2.90
Bacillus circulans	4.89	0.89	8.32	1.23	11.53
Bacillus megaterium	7.66	5.38	8.45	5.68	4.56
Bacillus pumilus	0.17	0.31	3.23	0.11	4.37
Brenneria serratia	1.11	0.88	0.00	1.08	0.00
Bacillus silvestris	3.76	0.42	7.59	0.22	3.39
Bacillus simplex	3.49	0.58	4.38	0.38	9.00
Bacillus subtilis	5.37	0.00	4.32	0.00	4.11
Micrococcus luteus	8.63	7.65	8.76	9.65	6.29
Microbacterium testaceum	4.32	0.99	9.21	1.39	7.01
Pantoea agglomerans	26.07	38.92	19.38	48.62	20.03
Pseudomonas fluorescens	2.33	3.84	1.79	2.23	5.06
Pseudomonas putida	1.09	3.61	0.87	1.57	3.45
X. campestris group	14.85	32.94	11.91	26.57	16.32

DISCUSSION

Besides few studies relative to the bacterial ecology of European olives phylloplane present in the Mediterranean ecosystems (Ercolani, 1978, 1991; Lavermicocca *et al.*, 1987), no studies were made in other regions of the world neither on cultivated olive nor on other species belonging to *Olea*. The present study has shown the difference among the bacterial ecology of the phylloplane of both cultivated and naturally present olive species in several Himalayan regions of Nepal characterized by different climatic and geographic conditions.

Previous studies showed that average population densities of epiphytic bacteria cover a huge range, from 5.0 log CFU/cm^2 to 9.0 log CFU/cm^2 (Morris and Kinkel, 2002). They are often encountered in sizes averaging 7.0 log CFU/cm^2 to 8.0 log CFU/cm^2 of leaf (Beattie and Lindow, 1995; Andrews and Harris, 2000; Hirano and Upper, 2000). Such high numbers were not found on the leaves of the species that we examined since our maximum values of some individual species only reached this minimum range (5.23, 5.44 and 5.33 log CFU/cm^2 in the year 2007 from leaves of *O. cuspidata* and 5.03 log CFU/cm^2 in the year 2009 from leaves of *O. europaea* cv. Marina

sampled from Kathmandu) found by these authors with average values lower than these levels. Ercolani (1991) found a minimum of 4.23 log CFU/cm^2 and maximum of 5.53 log CFU/cm^2 on the leaves of cultivar Coratina (*O. europaea* L.) with an average value of 4.88 log CFU/cm^2. These average values are much higher than those of our two olive species that we examined. There are two possible reasons explaining this lower colonization of olive leaves: a) our study was carried out in an environment which is new for european olive which has recently been introduced in these regions, so colonization of a new species probably takes longer time respect to that of the Mediterranean ecosystem, which is native of *Olea* species with thousand years of interactions with bacterial phylloplane community; b) drought stress could have influenced negatively the growth and multiplication of epiphytic bacteria (Bosabalidis and Kofidis, 2002), given that average values of rainfall are very low in these districts during the spring since almost all rainfall occurs during the monsoon season between July and August.

Studies on the ecology of plants leaf surface showed that epiphytic bacterial community dynamics are influenced by external factors such as temperature, relative humidity, wind speed, radiation, and crop management (Andrews *et al*., 1980; Andrews and Kenerley, 1978, 1980; Balestra and Varvaro, 1997; Blakeman, 1985; Dickinson, 1973., Hirano and Upper, 2000; Jacques *et al*., 1995; Ji *et al.*, 1996; Lindow and Andersen, 1996). Furthermore, high variability in space and time is a typical feature of epiphytic bacterial populations, the size of which can vary widely even among adjacent, visually identical leaves of the same plants (Andrews, 1996). Since climatic and geographic conditions of the aforementioned three districts are widely different, this can be possible reason of the different colonization of olive phyllosphere. Moreover, high altitudes and the presence of constant afternoon strong wind blows, which characterize Bajura and Dolpa districts, may have negatively effect on bacterial epiphytic survival reducing the moisture of leaf surfaces and relative humidity which favour epiphytic bacterial multiplication as demonstrated by Burrage (1976). The wind can even transport bacteria from this area to the lower parts of that region as bacterial immigration of consistent levels has been demonstrated already which probably is favoured also through wind (Lindow and Andersen, 1996). Moreover, lower bacterial epiphytic populations can be explained since when the prevailing high temperature can enhance bacterial growth. Often, water is scarce in Bajura and Dolpa districts unlike Kathmandu district and when water is available, temperatures are not always favourable.

Regarding bacterial communities of *Olea* phylloplane, the dominant bacterial colonizers were *P. agglomerans* and *X. campestris* group followed by different endospore-forming *Bacillus*

spp. The latter, because of its resistant structures, is frequently present and isolated from plants phylloplane also from difficult environments. The abundance of *P. agglomerans* can be explained easily since this species is considered to be ubiquitous in the phylloplane of various crop plants, as other pseudomonads (Eicker, 1976; Ercolani, 1991; Hirano and Upper 1991; Mansvelt and Hattingh, 1987).

Many bacterial species identified from olive phylloplane in Nepal are completely different than those found by Ercolani (1978) from south-east Italy. The latter found *P. savastanoi* pv. *savastanoi* as the most predominant, with more than 65% of isolates obtained from the phylloplane, whereas no Psav was isolated from *Olea* phylloplane in Nepal. Moreover, among the isolates identified from our *Olea* phylloplane, only six species (*Pantoaea agglomerans, Bacillus megaterium, Micrococcus luteus, Pseudomonas fluorescens, Bacillus subtilis and Xanthomonas campestris* group) were found by Ercolani (1978). Besides Psav, bacterial phylloplane colonizers such as *Arthrobacter globiformis, Lactobacillus plantarum, Klebsiella pneumoniae, Serratia marcescens, Acetobacter aceti, Leuconostoc dextranicum* and *Pseudomonas delafieldii,* found by Ercolani (1978), were not found from *Olea* phylloplane in Nepal. In addition, new bacterial species such as *Bacillus asahii, Bacillus cereus, Bacillus circulans, Bacillus pumilus, Bacillus silvestris, Bacillus simplex*, *Brenneria serratia, Microbacterium testaceum* and *Pseudomonas putida* were identified from *Olea* phylloplane from our areas of study. The presence of new bacterial species and the absence of those identified from Mediterranean areas can be associated to the different climatic conditions among the two areas of study.

Previous studies demonstrated that bacterial pathogen of European olive plants is easily detectable also from asymptomatic olive plants including leaves (Bertolini *et al*., 2003; Ercolani, 1978; Quesada *et al*., 2007) but during our study no isolates of *P. savastanoi* pv. *savastanoi* were detected. Our study demonstrated that, Psav is not present yet in these Himalayan districts (Bajura, Dolpa and Kathmandu) although the presence of this pathogen has already been reported from Makwanpur district of Nepal where European olive was brought from some private olive growers (Balestra *et al*., 2009). The absence of the pathogen in these districts is probably because of the introduction of strictly controlled European olive plants from Italy. Furthermore, a large numbers of bulk leaf samples collected in spring, period of time when phyllosphere bacterial populations were recorded highest for most plant species as shown by previous studies (Daniel and Boher, 1978; Lindow, 1986), were used during the three years of study, hypothesis of non

delectableness can not be associated to some errors given the efficiency of this type of sampling (Crosse, 1959).

Since numbers of cultivars and volumes of leaf samples taken during our study were very big, with consequent high number of bacterial isolates, we were unable to consider other characteristics such as seasonal effect, percentage of INA bacteria present on the phylloplane of two *Olea* species. Thorough studies on these regards would be desirable in the next future to obtain detailed information.

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CHAPTER 8.

Survival of *Pseudomonas savastanoi* pv. *savastanoi* on phylloplane of two olive species

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ABSTRACT

Two olive species (*Olea europaea* and *O. cuspidata*) were used to study the epiphytic survival of *Pseudomonas savastanoi* pv. *savastanoi*, the causal agent of olive knot disease. Bacterial strains of different origin were used. Epiphytic survival of bacterial populations was studied applying artificially a bacterial suspension on leaf surfaces and observing plant reaction over the time. The influence of olive species, bacterial strains and leaf age were considered as factors influencing bacterial epiphytic survival. Leaf age and bacterial strains did not influence significantly the bacterial epiphytic survival but plant species showed a significant influence. Wild olive belong to *O. cuspidata* was found to be more suitable for the multiplication of the bacterial pathogen respect to European olive belong to *O. europaea*.

Keywords: *Olea europaea*, *Olea cuspidata*, epiphytic survival, olive knot pathogen, Nepal

INTRODUCTION

Pseudomonas savastanoi pv. *savastanoi* (Gardan *et al*., 1992) (hereafter Psav) causes the endemic disease of olive known as olive knot or tubercle. Besides olive (*O. europaea* L.), the pathogen and other pathovars of the same species (pv. *nerii* and pv. *fraxinii*) cause a similar disease on oleander (*Nerium oleander* L.), ash (*Fraxinus* spp.), jasmine (*Jasminum* spp.), privet *(Ligustrum* spp.), *Forsythia* spp. and *Phillyrea* spp. (Bradbury, 1986). Recently the disease is reported on myrtle (*Myrtus communis* L.) and buckthorn (*Rhamnus alaternus* L.) (Saad and Hanna, 2002; Saad and Melkonian, 1992). Psav has a resident phase on olive phylloplane and can multiply in its saprophytic phase on olive tree phylloplane (Dye *et. al*., 1980; Ercolani, 1978; Quesada *et al*., 2007). Furthermore, the bacterium can spread within the short period of time through several vectors such as splashing rain, insects and windblown aerosols. Once there are suitable conditions, mostly represented by wounds caused on parts of plants by cultural practices (pruning, harvest) and meteorological phenomena (frost and hail), which is an entranceway of the pathogen, infection occurs. Psav population on leaf surface varies widely in the space of a year reaching the maximum values in spring and autumn, periods when the cambium is highly active and the plant can be easily attacked by the pathogen (Armenise, 1950). The bacterial populations decrease strongly during summer and winter, since the climatic conditions are unfavorable for the survival and multiplication of the bacteria. Several studies on Psav have been made in the last years, mostly regarding its dissemination, multiplication, mechanism of attack and its bio-control

(Lavermicocca *et al*., 2002; Marchi *et al*., 2008; Parret *et al*., 2005; Rodriguez-Moreno *et al*., 2009; Quesada *et. al*., 2010; Young, 2004).

Olea cuspidata (Wall.), commonly known as African wild olive, is a widespread olive species present mostly in African, Asian and Oceania continents (Spennemann and Allen, 2000; Crossman, 2002). This species grows to 25 m tall and is widely distributed in drier forest and forest margins from 750 to 3000 m a.s.l. Once established, the tree is hardy and drought resistant (Noad and Birnie, 1989; Negash, 1995). The vegetative propagation of plants is an important tool for capturing genetic variation in tree improvement and multiplication programmes (Davis and Haissig, 1994; Howard, 1994). Ritchie (1994) has recommended vegetative propagation as a powerful mean of exploiting genetic gains through capture of both additive and non-additive genetic variance. *O. cuspidata* is naturally present in several Himalayan regions of Nepal (Bartolucci and Dhakal, 1999; Lamichhane *et al*., 2010), particularly in Dolpa, Bajura, Mugu and Humla districts. In the last two decades, European olive (*O. europaea* L.) has been introduced in different districts of Nepal including those areas where *O. cuspidata* is natively present.

In contrast to studies on the phytobacteriological characteristics of the European olive, African olive has received no research attention on this issue. The aims of our study were to examine the survival, multiplication and quantitative variation of Psav populations on the leaves of different age of *O. cuspidata* comparing with those of *O. europaea*.

MATERIALS AND METHODS

Experiments were carried out in a greenhouse of the 'Lupori' farm of the University of Tuscia in spring 2008 and repeated in the same period of 2009.

Olive plants: In case of *O. cuspidata*, plantlets were obtained by directly sowing the seeds. Before sowing, the seeds were put into a wide basin containing vermiculite, wetted and maintained at 4°C for 10 days since this species needs some chill hours for germination because of the seed dormancy. After 10 days of chilling, the basin was kept in a greenhouse and maintained at 20°C for 2 months. The plantlets were then 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*, two year old plants (Cv. Leccino) obtained from cuttings (vegetative propagation) were used. The plants were kept into the same size plastic pots. Cultivar Leccino was used since previous study showed its susceptiblity

towards Psav. All the plants were maintained in greenhouse at 25±1 °C with the relative humidity of 60-80%.

Bacterial culture: In the experiments, two bacterial isolates of Psav were used: one (PseNE 107) obtained in Nepal (Balestra *et. al*., 2008) and another (ITM 105) Italian isolate belong to Istituto Tossine e micotossine da parassiti vegetali, Bari (Marchi *et al*., 2009).

Preparation of bacterial suspension: Bacterial isolates preserved on NGA medium were streaked on NA medium. After 24 hours, fresh bacterial cultures were re-streaked on the same medium to form a dense bacterial culture. Each bacterial suspension was prepared taking the bacterial culture and putting them inside a beaker containing sterilized distilled water (SDW). The suspension was centrifuged at 15,000 x *g* for 20 min. The pellet was used to obtain an homogeneous bacterial suspension in SDW. The concentration of the bacterial suspension was adjusted turbidimetrically to about 10^6 CFU/ml by reference to a calibration curve (Varvaro and Surico, 1987).

Contamination of plants: two year old olive plants of both species were inoculated (20 plants/species/isolate, for a total of 80 plants) with the bacterial suspension which was distributed homogeneously on leaf surface using spray inoculation method. The contamination by spraying was made until both the upper as well as lower leaf surfaces were fully wet. A set of five control plants per cultivar was used, where only SDW was sprayed. From 2 hours before till 2 hours after the contamination, the relative humidity was maintained at around 90% to slow down the inoculum evaporation.

Calculation of the bacterial populations from leaves: four leaves from each plant, 2 younger and 2 older respectively from the upper and lower part of the plant, for a total of 80 leaves/species/isolate were aseptically collected after 1, 5, 14, 21, 28, 35 and 42 days. The bulk sample was divided in 2 groups, each consisting of 40 leaves. Each single group was put in sterile conical flasks and processed individually in 40 ml of phosphate buffer. The processing was made by using the stirrer at 180 revolution per minute (rpm) for 2 hours at 26±1 °C. Samples were then serially diluted in phosphate buffer, spread plated in duplicate on nutrient agar medium supplemented by 5% sucrose and 2,5 µg/l of crystal violet (Varvaro and Surico, 1978). From the washing of control leaves, very lower bacterial populations were recovered (less than 1 CFU/cm^2). Colony counts of Psav from duplicate plates for each replicate were made after plating and incubation at 26±1°C for 48-72 h. Data from each experiment from both years were averaged,

analyzed by analysis of variance (ANOVA) and converted to CFU/cm^2. Duncan's multiple range test was used to calculate the difference within and among the species.

RESULTS

In case of *O. europaea* cv. Leccino, as reported in figure 1, immediately after contamination, the number of bacterial populations began to decrease until 2.2 x 10^1 (1.35 log) and 1.4 x 10^1 (1.15 log) CFU per cm^2 for the strain ITM 105 and 1.2 x 10^1 (1.08 log) and 9.0 x 10^0 (0.96 log) CFU per cm^2 for the strain PseNE 107 within 5 days, respectively for old and new leaves. For both strains, on old leaves, the bacterial populations started to increase gradually from day 5 until the day 21. From day 21 on, the bacterial populations started again to decrease gradually. On new leaves, the bacterial populations for the strain ITM 105 had an irregular trend as they increased from day 5 to day 14 and reduced from day 14 to day 21 and increased until the day 35. Unlike the strain ITM 105, for PseNE 107, the bacterial populations had the same inclination as that on old leaves until the day 14. Afterwards, the populations increased gradually until the day 28 and resumed to decrease.

Survival of two different bacterial populations of different origin resulted not significantly different on leaves of the same age within the same species whereas the survival resulted to be influenced significantly between the leaves of different age (Figs. 1 and 2). Regarding the bacterial survival on leaves of two different species, data shown remarkable and significant differences.

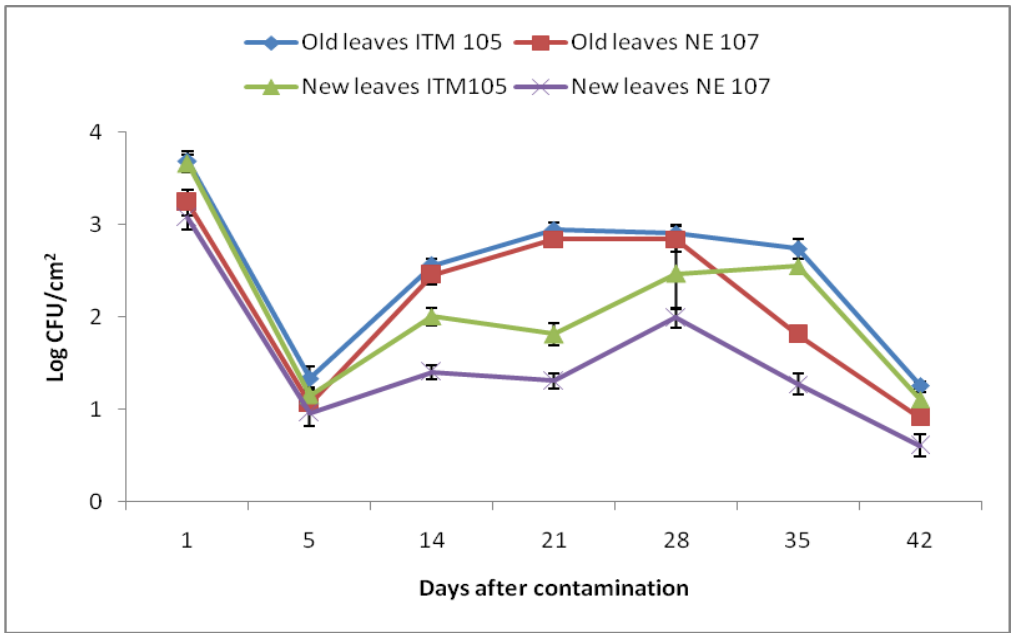

Fig. 1. Survival of two different isolates of *Pseudomonas savastanoi* pv. *savastanoi* on leaf surface of *Olea europaea* cv. Leccino.

For *O. cuspidata*, as reported in figure 2, immediately after contamination, the number of bacterial populations began to decrease until 9.1 x 10^1 (1.96 log) and 4.8 x 10^1 (1.69 log) CFU per cm^2 for the strain ITM 105 and 7.2 x 10^1 (1.86 log) and 4.1 x 10^1 (1.62 log) CFU cm^2 for the strain PseNE 107 within 5 days, respectively for old and new leaves. For both strains, in case of old leaves, the bacterial populations started to increase gradually from day 5 until the day 21. From day 21 on, the bacterial populations started again to decrease gradually. Whereas in case of new leaves, for the strain ITM 105, the bacterial populations had the same trend as of *O. europaea* as they increased from day 5 to day 14 and reduced again from day 14 to day 21 then increased again until the day 35. Unlike the strain ITM 105, for PseNE 107 the bacterial populations had the same trend as that on old leaves until the day 14. From day 14 on, the populations increased gradually until the day 28 and then resumed to decrease.

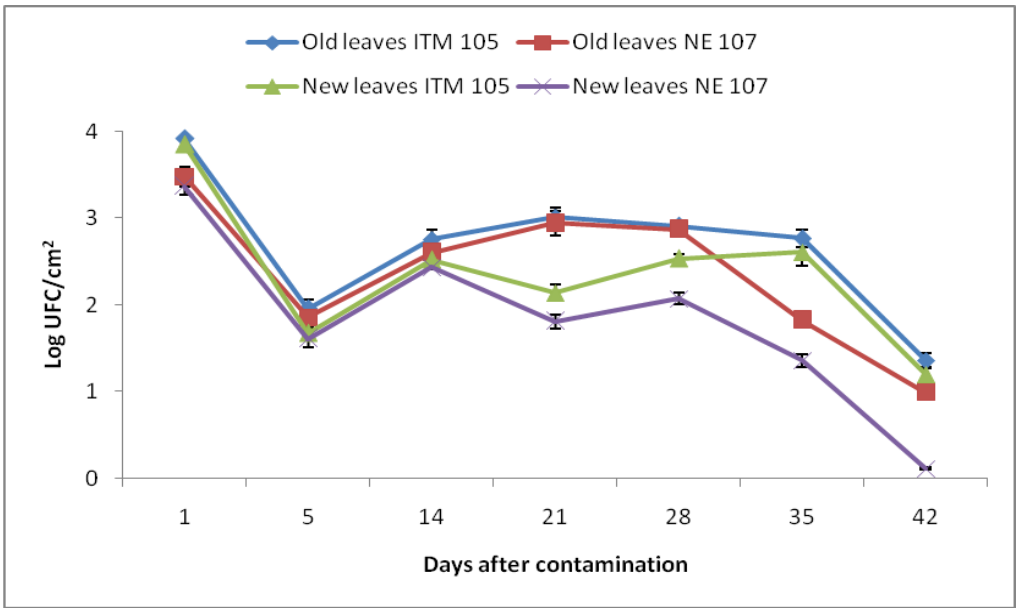

Fig. 2. Survival of two different isolates of *Pseudomonas savastanoi* pv. *savastanoi* on leaf surface of *Olea cuspidata*.

DISCUSSION

The leaf surface of a given plant represent a natural habitat of a large number of microorganisms and as the consequence they are naturally colonized by many species. Several factors play a vital role on epiphytic survival of phyllosphere bacteria. Among them the role of pigmentation, ultraviolet radiation and leaf colonization strategies are widely studied (Jacobs *et al*., 2004). Since olive represents a potential host of Psav and since this pathogen is very

consistently present on the olive phylloplane respect to other microorganisms, also on the asymptomatic plant phylloplane present in the open air, as demonstrated by Ercolani, 1978, we focused to evaluate the survival capacity of this pathogen in a controlled environment in relation to the different *Olea* species and leaf age.

Results obtained from both the experiments showed the ability of olive knot pathogen not only to survive but to higher multiply on *O. cuspidata* respect to *O. europaea*. Both the strains of Psav higher multiply on wild olive. In fact, beginning from very low CFU/cm^2 remained from the initial fall, the bacterium within 4 weeks reached a maximum peak. Nevertheless, bacterial survival showed a further fall afterwards which has not been studied since this can happen only in controlled environment while in the field other bacterial inocula can reach on the phylloplane from different source adding or substituting to those previous and allowing a constant and continue presence of the pathogen on the plant surface unlike to other pseudomonads (Ercolani, 1978).

The initial fall of bacterial population during the first five days could be probably due to the fact that bacteria coming from natural (from infected tissues such as tubercle) or artificial (substrate of culture) environments, suitable for their growth, do not posses a good capacity to survive as epiphytes (Varvaro and Ferrulli, 1978). Only a few bacterial cell, probably due to the modification caused by environment, are able to adjust and continue to live in a state of reduced metabolism known as hypobiosis (Leben, 1974).

Italian isolate of Psav better multiplied on both olive species, respect to the isolate obtained in Nepal with higher value expressed as CFU/cm^2. This is probably because this isolate possesses a high level of virulence attributable to the continue contact of the pathogen with the host from centuries since olive is native of the Mediterranean region unlike European olive plants recently introduced in Nepal.

The fact that the pathogen better multiplies on *O. cuspidata*, where the initial fall was significantly lower respect to that observed on *O. europaea*, can be opined because of the capacity of wild olive to release different solutes in leaf exudates and nutrients given their broad and tender leaves. In fact, higher nutrient availability in the leaf exudates is found to be positively correlated with the higher colonization of olive phylloplane by Psav (Balestra and Varvaro, 1997). Higher multiplication of Psav on *O. cuspidata* phylloplane is very important since the presence of this pathogen has not been reported in Nepal from areas where wild olive is present. For this

reason, strong control measures will be taken to avoid the diffusion of this pathogen from area where infected European olive was found.

Only few significant differences were observed regarding the influence of leaf age on multiplication of bacteria. Generally, higher multiplication was observed on old leaf surfaces than the young ones.

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CHAPTER 9.

Reaction of two olive species to different *Pseudomonas savastanoi* pv. *savastanoi* strains

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ABSTRACT

A native species of olive (*Olea cuspidata* Wall.) widespread in the Himalayan regions of Nepal was tested for the first time for its susceptibility to *Pseudomonas savastanoi* pv. *savastanoi*, the causal agent of olive knot disease. Five different strains of the bacterial pathogen isolated from different geographic areas were used. European olive (*O. europaea* L.) was used as control. Both *Olea* species were artificially inoculated by pricking plant stems of 3, 9 and 20 month of age. *O. europaea* showed less susceptibility to *P. savastanoi* pv. *savastanoi* compare to *O. cuspidata* although no difference statistically significant was found. Plant age did not influence significantly the host-pathogen interaction. The bacterial strains showed differences statistically significant among them in terms of bacterial populations inside the knot.

Key words: *Olea europaea*, *Olea cuspidata*, artificial inoculation, olive knot disease, Nepal

INTRODUCTION

Pseudomonas savastanoi pv. *savastanoi* (Gardan *et al*., 1992) (hereafter Psav) causes the endemic disease of European olive (*Olea europaea* L.) known as olive knot or tubercle which occurs in almost all the regions of the world where olive is grown, including the regions where this crop has recently been introduced like Australia and Nepal (Balestra *et al*., 2009; Hall *et al*., 2004). The bacterium survives epiphytically on asymptomatic olive plants (Bertolini *et al*., 2003; Ercolani, 1978; Marchi *et al*., 2009; Quesada *et al*., 2007; Penyalver *et al*., 2006) and enters into the plant tissues once there are suitable conditions and multiplies rapidly affecting olive trees by producing tumorous outgrowths on trunks, branches and occasionally on the leaves and fruits (Wilson, 1935). The pathogen can cause severe damage in olive groves, mainly when weather conditions favour the survival of epiphytic populations of the bacterium and their entry into the bark. Psav has epiphytic resident populations on olive twigs, leaves and drupes. Population sizes were influenced by seasons, reaching a higher level in spring and fall, when wet weather conditions occur (Ercolani, 1978). Tissues can be infected through leaf scars, wounds and fissures on stems and twigs, caused by meteorological phenomena and insect miners, as well as by harvest and pruning practices. Both olive yield and quality can be reduced as a consequence of bacterial infections of the plant by the pathogen (Schroth, 1973).

The secreted phytohormones produced by Psav were thought to be the main factors responsible for the disease caused by this plant pathogen for many years (Surico *et al*., 1985; Surico and Iacobellis, 1992). Afterward, Psav mutants unable to produce IAA and/or cytokinins

multiplied in the host tissues as well as the parental strain confirmed that the phytohormones alone are not responsible of the olive knot disease (Iacobellis *et al*., 1994). Some authors also proved that the virulence of Psav is due to the capacity of this bacterium to produce *hrp* genes like that of many other plant pathogenic bacteria (Lindgren, 1997; Sisto *et al*., 2004). More recently other auxin-producing bacterial species were found to be actively involved in association with Psav in knot development (Cimmino *et al*., 2006; Marchi *et al*., 2006; Ouzari *et al*., 2008).

Although European olive and its cultivar behaviour has largely been studied towards Psav (Ercolani, 1978, 1991; Penyalver *et al*., 2006, Rodríguez-Moreno *et al*., 2008, Rodríguez-Moreno *et al*., 2009; Quesada *et al*., 2010; Varvaro and Surico, 1978a, 1978b), no information is yet available concerning the reaction of other olive species. In particular, we focused on African olive (*Olea cuspidata* Wall.), which is widespread mostly in African, Asian and Oceanic regions (Crossman, 2002; Spennemann and Allen, 2000), to several isolates of Psav. *O. cuspidata* is widespread in Himalayan regions of Nepal and represents an economically and ecologically very important crop particularly in Dolpa, Bajura, Mugu and Humla districts (Bartolucci and Dhakal, 1999; Lamichhane *et al*., 2010b). The objective of our study was to evaluate the susceptibility of *O. cuspidata* comparing with that of *O. europaea* to determine the difference in their susceptibility to different Psav isolates.

MATERIALS AND METHODS

Plant materials

In case of *O. cuspidata*, plants were obtained by directly sowing the seeds. Before sowing, the seeds were put into a wide basin containing vermiculite, wetted and maintained at 4°C for 10 days since this species needs some chill hours for germination because of the seed dormancy. After 10 days of chilling, the basin was kept in a greenhouse and maintained at 20°C for 2 months. Afterwards, the plantlets were transplanted into the 2 litre plastic pots (15 cm wide and 30 cm deep) containing soil, pit and sand (ratio 1:1:1). Whereas for *O. europaea*, two year old plants obtained from rooted cuttings (vegetative propagation) were used. The plants were kept into the same size plastic pots. Cultivar Leccino was used since this is well-known for its susceptibility to Psav.

Bacterial cultures

In the experiments, five bacterial strains of Psav were used: one (PseNE 107) isolated in Nepal (Balestra *et. al*., 2008), other Italian strains (ITM 105, strain 311) belong to Istituto Tossine e

micotossine da parassiti vegetali Bari (Marchi *et al*., 2009), two local isolates obtained in Viterbo (VT) and Montefiascone (MF). The isolates were first grown for 48 h at 27±1°C in nutrient agar containing 1% glycerol (NAG). Inoculum was prepared from 24 h cultures of Psav re-streaked on the same medium and standardized turbidimetrically to a concentration of 10^8 CFU per ml of sterile distilled water (SDW).

Inoculation techniques

Plants belonging both to *O. europaea* and *O. cuspidata* were inoculated at different stages of development (3, 9 and 20 months of age) by pricking the stems with a 5-10 µl aliquot of bacterial inoculums with a hypodermic needle containing bacterial suspension. Five plants per isolate (total 25 plants for each species) were inoculated at each stage of development. Each plant was inoculated at two sites. Another set of plants (2 plants per strain per species) was inoculated with 5-10 µl of sterile distilled water (SDW) as negative control. Inoculated plants were kept in a glasshouse where the temperature ranged from 20 to 24°C for the 120-day duration of the experiment.

Disease observation

Inoculated plants belonging to both olive species were periodically controlled for the observation of disease symptoms.

Statistical analysis

Data from each experiment were averaged, analyzed by analysis of variance (ANOVA) and converted to CFU/g of knot weight. The average number of bacteria present into the every single knot was determined.

RESULTS

Disease observation

Characteristic knot symptoms were observed on the inoculated plants of both olive species within 5 weeks after inoculation. The values averaged from first, second and third artificial inoculations on the disease appearance were reported in table 1. The symptoms were observed before on *O. cuspidata* within the range of time between 3 and 4 weeks, whereas the knots appeared lately on *O. europaea* (within 4 and 5 weeks) except those caused by Local Vt isolate.

Table 1. Appearance of olive knot symptoms on two olive species by strains of *Pseudomonas savastanoi* pv. *savastanoi*

	Week after inoculation	
Strains	*O. cuspidata*	*O. europaea*
ITM 105	3	4
311	4	5
PseNE 107	3	4
MF4	4	4
Local Vt	3	3

Sampling of knots and processing

After 6 months from the artificial inoculation the knots formed on the plants of both *Olea* species were aseptically cut and weighed separately. Every single knot was separately fragmented and homogenized by using a mixer (Ultra-Turrax T25), in presence of 20 ml of phosphate buffer 0.05 M, pH 6.6, for 2 min at 10,000 rpm. The suspensions obtained after mixing were then used for ten-fold serial dilutions in phosphate buffer. Duplicate plating was carried out transferring an aliquot of 0,1 ml in Petri dishes containing a specific medium PVF1 for *P. savastanoi* pv. *savastanoi* (Surico and Marchi, 2002). Colony counts were performed after incubation of the dishes at 26±1°C for 72 hours. The average number of bacteria present into every single knot was determined.

Calculation of bacterial populations present into the knots

The bacterial populations into each inoculation site (expressed as CFU/g of knot) obtained separately in three different periods of time were reported in table 2. No difference statistically significant was observed among the two olive species inoculated in the same period of time since both of them were susceptible to the strains of bacterial isolates. A clear difference with values statistically significant among the bacterial isolates were observed on plants inoculated at 3 months but not those inoculated at 9 and 20 months, except for the strain 311.

Table 2. Bacterial populations present in each gram of knot (log CFU/g) caused by different strains of *Pseudomonas savastanoi* pv. *savastanoi* on two *Olea* species of different age

	3 months				9 months				20 months			
Strains	A		B		A		B		A		B	
ITM 105	7.47±0.17*	f	7.26±0.23	ef	7.16±0.31	b	7.14±0.26	b	6.45±0.16	bc	6.35±0.17	bc
311	4.95±0.08	a	5.03±0.14	a	5.29±0.26	a	5.35±0.29	a	4.89±0.20	a	4.69±0.11	a
NEP	6.69±0.13	cd	6.94±0.15	de	6.76±0.20	b	6.77±0.17	b	6.16±0.15	b	5.79±0.13	b
MF4	6.07±0.22	b	6.28±0.09	bc	6.78±0.77	b	6.69±0.27	b	6.40±0.47	bc	6.08±0.41	b
Local Vt	7.67±0.07	f	7.47±0.11	f	7.02±0.10	b	7.23±0.05	b	7.07±0.32	c	6.39±0.07	bc

A *Olea cuspidata* B *Olea europaea*

Means followed by the same letter are not significantly different at P = 0.05

* Standard error

On 3 month old plants, the bacterial populations of the local isolate Vt were the highest with 4.68 x 10^7 (7.68 log) and 2.96 x 10^7 (7.47 log) CFU/g of knot respectively on *O. cuspidata* and *O. europaea*. In terms of bacterial populations per gram of knot, the isolate Vt was followed by the strain ITM 105, NEP, MF and the strain 311. The latter was characterized by the lowest values of bacterial populations with 8.92 x 10^4 (4.95 log) and 1.08 x 10^5 (5.03 log) CFU/g of knot respectively on *O. cuspidata* and *O. europaea*. Same trend was observed also on the plants inoculated at 9 and 20 months where bacterial populations expressed as CFU/g remained unvaried (Tab. 2). Individual values of bacterial populations obtained on plants inoculated at 3, 9 and 20 months showed that there was no difference statistically significant since all the plants were infected and every bacterial strain multiplied similarly in the host tissues (Tab. 2).

The values averaged from the three individual experiments on *O. cuspidata* showed that there are difference statistically significant among the isolates in terms of bacterial populations per gram of knot weight on the same olive species. In particular the isolate Local Vt, MF4 and 311 differed significantly among them with respective values of 1.8 x 10^7, 2.6 x 10^6 and 1.1 x 10^5 (7.25 log, 6.42 log and 5.04 log) CFU/g of olive knot. Whereas no difference statistically significant was found between the isolate ITM 105 and NEP (Fig. 1). The values obtained on *O. europaea* confirmed the same trend since only isolate MF differed significantly with other four isolates (Fig. 2). Interestingly, rather than some difference statistically significant among the isolates on the

same species, no significant differences were found in bacterial populations of the same isolate between the two *Olea* species (Figs. 1 and 2).

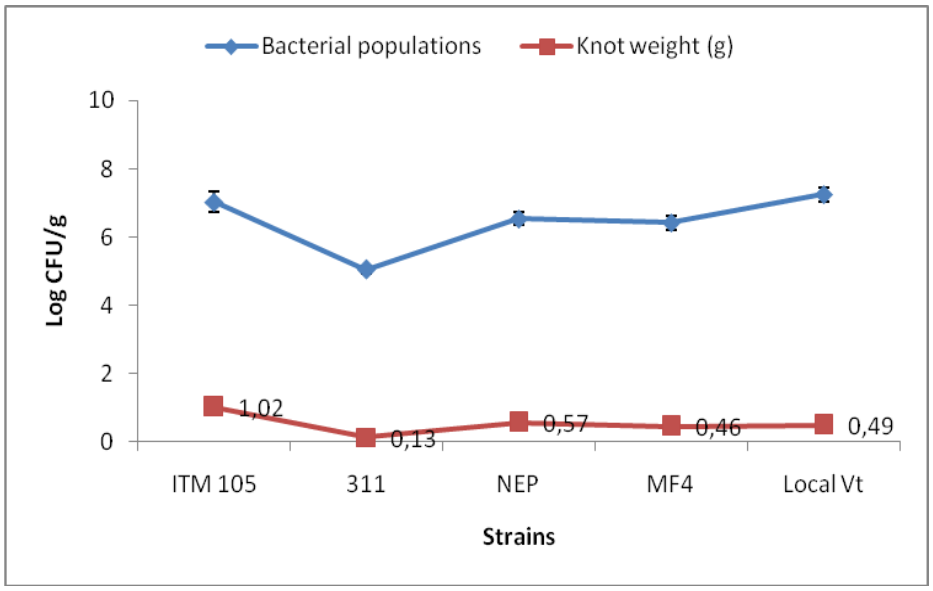

Fig. 1. Relation between average values of knot weight and bacterial populations inside the knots on *Olea cuspidata*

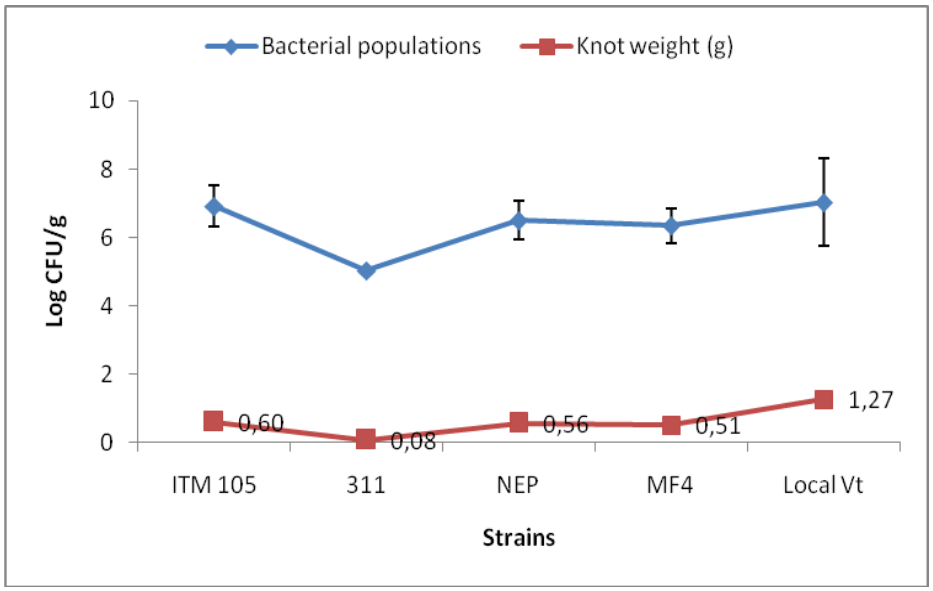

Fig. 2. Relation between average values of knot weight and bacterial populations inside the knots on *Olea europaea*

No correlation was found among the knot weight and their respected bacterial populations on both olive species since knots with lower weight showed higher contents of bacterial populations (Fig. 1). In fact, on *O. cuspidata* the local isolate Vt with average knot weight of 0.49 g

showed the highest bacterial populations of 1.8 x 10^7 (7.25 log) CFU/g compared to the isolate ITM 105 which rather than a higher average knot weight (1.02 g) had a lower value with 1.07 x 10^7 (7.02 log) CFU/g of bacterial populations (Fig. 1). The same trend was confirmed also on *O. europaea* where no proportional increase in bacterial populations was found with higher knot weight (Fig. 2).

DISCUSSION

Pseudomonas savastanoi pv. *savastanoi* strains inoculated artificially on two *Olea* species on different stage of development showed that *O. cuspidata* reacts positively as *O. europaea* to Psav, which is a new finding since this species native of Himalaya was unknown up to now for its reaction to Psav. Unlike many wild plant species which are generally resistant to plant pathogens and often used as source of resistance in plants breeding, *O. cuspidata* surprisingly gave positive response to Psav. Values averaged from the three individual experiments showed that the symptoms appearance was early on *O. cuspidata* by 1 week, except some isolates (MF4 and Local Vt) which gave the same result on both *Olea* species. Nevertheless, the average knot weight was higher on *O. cuspidata* for some strains (ITM 105, PseNE 107 and 311) and lower for other (MF4 and Local Vt). The average values of bacterial population expressed as CFU/g were higher for all strains on *O. cuspidata* (Figs. 1 and 2) than on *O. europaea* although with difference not statistically significant.

Preliminary study by using two different strains of Psav (ITM 105 and PseNE 107) on one year old plants of *O. europaea* and *O. cuspidata* showed that the latter is more susceptible to the bacterial pathogen both in terms of symptom appearance, knot weights and values of bacterial populations (Lamichhane *et al*., 2010a). Our results are in accordance with the previous study, since Psav strains previously used confirmed a clear difference both in terms of symptoms appearance, knot weight and bacterial populations per gram of knot. Nevertheless, the new strains of Psav used in this study did not give a clear difference between the two *Olea* species in terms of knot weight and symptoms appearance with higher values on *O. cuspidata*. Less susceptibility of *O. europaea* compare to that of *O. cuspidata* to Psav is probably due to the continuous contact of *P. savastanoi* pv. *savastanoi* from centuries with *O. europaea* that led this species to develop some kind of resistance compared to *O. cuspidata,* which had never been in contact with Psav. Plant age did not influence significantly host-pathogen interaction since the

strains caused knot symptoms similarly on plants of different age with similar Psav average populations.

The different time of symptom appearance and the different knot weight caused by the strains of Psav on two different species was probably due to the different reactivity showed by the two *Olea* species to artificial inoculations with Psav strains. The same hypothesis was previously confirmed by Varvaro and Surico (1978a) on three cultivars of *O. europaea*. The lack of correlation between the knot weight and their respective Psav populations confirm that knot weight is independent by the number of Psav populations inside the infected tissues but depend probably on the concentration of IAA produced by auxin-producing bacteria associated to Psav-induced olive knots as described by previous authors (Cimmino *et al*., 2006; Marchi *et al*., 2006; Ouzari *et al*., 2008). Hence, lower values of Psav population into the knot of bigger size might be because of the higher population of other auxin-producing bacteria which probably limit Psav multiplication by producing antibiotic compounds and competing for space and nutrients (Cimmino *et al*., 2006).

The overall findings reported in this study lead us to conclude that the absence of olive knot symptoms on *O. cuspidata* and the consequent lack of Psav colonies from the leaves processed during previous study (Lamichhane *et al*., 2010b) was not because of the resistance of *O. cuspidtata* but due to the fact that the causal agent of olive knot disease is not present in the Himalayan regions where *O. cuspidata* is widespread. Since Psav was already reported from Nepal on *O. europaea* (Balestra *et al*., 2009) and given a high susceptibility of *O. cuspidata* to Psav, an immediate implementation of control strategies based on the good agronomic and cultural practices are strongly recommended to avoid the contamination and consequent diffusion of the pathogen on *O. cuspidata,* given its wide economical and ecological importance in Nepal. In particular, the infected materials from *O. europaea* must be collected and destroyed to avoid the use of infected materials for vegetative propagation. If possible, it is to be hoped to spray copper compounds on olive plants.

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CHAPTER 10.

In vitro antagonistic action of the bacterial species from *Olea* spp. phylloplane against *Pseudomonas savastanoi* pv. *savastanoi*

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ABSTRACT

Over three growing seasons, more than 250 bacterial strains were isolated from the phylloplane of two asymptomatic species of olive plants in Nepal. Eighty six representative strains of them were screened for their antagonistic behavior against five different strains of *Pseudomonas savastanoi* pv. *savastanoi*, the causal agent of olive knot disease. None of the bacterial isolates exhibited *in vitro* antagonism against the tested phytopathogen.

Key words: Antagonism, phylloplane isolates, Nepal

INTRODUCTION

Aerial leaf surfaces are habitat for epiphytic bacteria, which are able to colonize plant leaf surfaces and survive for a long time even under difficult physical conditions (temperature, rainfall). Bacteria are generally the predominant initial inhabitants of the newly expanded phylloplane, while yeasts and filamentous fungi dominate after in the growing season (Kinkel *et al*., 1987). The common phylloplane resident bacteria including plant pathogens (PPB) live epiphytically on host and nonhost plant species (Beattie and Lindow, 1995; Blakeman and Fokkema, 1982). Several studies provided information regarding antagonism between the microorganisms on the leaf surface: in particular, the role of some saprophytic bacteria from plant phylloplane in controlling the growth and multiplication of pathogenic bacteria, known as biological control. Hence, the latter maybe an alternative to chemicals in the control of some pathogenic bacteria which provide many economic and ecologic advantages.

Bacterial knot of olive (*Olea europaea* L.) caused by *Pseudomonas savastanoi* pv. *savastanoi* (Psav) is an endemic disease of great economic importance which has been reported in almost all olive cultivated regions. Besides olive, the pathogen causes a similar disease on many other plant species (Bradbury 1986; Saad and Hanna, 2002). The pathogen causes outgrowths (tumorous growth or knots) on the stems, branches and occasionally on the leaves and fruits of host plants. Besides from knots, Psav has been isolated from the phylloplane of diseased and healthy olive leaves and stems where the pathogen has a resident epiphytic phase. Psav enters into the plant tissues and causes the disease only by means of wounds created by cultural practices and meteorological phenomena (Ercolani, 1991; Quesada et al., 2007).

Biological control of several plant pathogens have widely been studied in the last decades. Nevertheless, only little is known on biocontrol of *P. savastanoi* pv. *savastanoi* by using

fluorescent pseudomonas (Lavermicocca *et al*., 2002, Zadeh *et al*., 2008). Since the effectiveness of a bacterial strain as a biocontrol agent depend on the ecological similarity between the antagonist and the target pathogen in the phyllosphere of the host plant (Völksch and May, 2001) we focused to the saprophytic bacteria from olive plants in searching those antagonist against Psav. The objective of this study was to isolate the bacterial species from the phylloplane of field grown and naturally present olive species in Nepal and to assess their potential antagonistic activities against Psav by using *in vitro* screening methods.

MATERIALS AND METHODS

Plant material and field

Two species of olive plants, one field grown *(Olea euro*paea L.) and another native species of Nepal (*O. cuspidata* Wall.) were used in this study. Twenty eight olive cultivars belong to *O. europaea* from three different districts (Bajura, Dolpa and Kathmandu) situated respectively at 1500, 2500 and 1300m ASL and *O. cuspidata* from the same districts were used in this study. The cultivars belong to *O. europaea* were: Anghiari, Ascolana, Bosana, Bourbon, Canino, Carboncella, Carolea, Cassanese, Cipressino, Coratina, Corona, Femminella, Frantoio, Frattese, Itrana, Leccino, Leoncino, Marina, Maurino, Moraiolo, Nocellara del Belice, Pendolino, Piantone, Rajo, Rasara, Rosciola, Taggiasca and Valle Corsana.

Sampling of olive leaves

Leaf samples were randomly collected from all the asymptomatic plants during a 3-year period (April 2007- April 2009) in Bajura and only for one year period (April 2009) in Dolpa and Kathmandu districts. Sampling was made early in the morning. For *O. europaea*, 40 leaves (both young and old leaves) from each plant and five plants for each variety for a total of 200 leaves per variety were aseptically collected in sterile lab bags and bulked. For *O. cuspidata*, the same procedure was used by collecting 10 samples, each represented by 200 leaves.

Isolation of bacteria from plant material

Each bulk sample was aseptically divided in 10 groups (20 leaves per replicate), placed in a sterile 250ml conical flask with 40 ml of phosphate buffer (0.1M, pH 6.8) and shaken in the dark by using an orbital stirrer at 26±1 °C and 150 rpm for 2h. Previous studies indicated that over 90% of the phylloplane bacteria passed into suspension and that there was no error due to bacteria multiplying in the washing fluid (Crosse, 1959; Ercolani, 1978). These results were obtained by previous authors by plating 10-fold serial dilutions of homogenates of washed and unwashed

leaves. Furthermore, Ercolani (1976) showed that virtually no bacteria passed into suspension from insides the leaves which allow us to make recovery of only phylloplane bacteria. After washing, the solution was serially diluted in phosphate buffer and spread plated in duplicate onto nutrient agar medium supplemented by 5% (v/v) of sucrose (NAS), amended by 800 µl/l of cycloheximide (previously prepared as stock solution by diluting 1g in 10 ml of ethanol) to prevent fungal contamination. One hundred µl of diluted solution was used for plating. After 48-72 h of incubation at 26±1°C, single colonies were removed with a sterile needle and transferred to fresh NAS medium. Examination by means of a stereomicroscope of the developed bacterial colonies, Gram stain and fluorescent tests were carried out in order to classify the isolates into groups for further identification. For *in vitro* biocontrol assay 45, 23 and 18 representative isolates, including both non-pseudomonads and fluorescent pseudomonads, respectively from Bajura, Dolpa and Kathmandu, were used. Although fluorescent pseudomonads are known to be the most efficient antagonists (Kearns and Hale, 1996; Patil *et al*., 1998; Zadeh *et al*., 2008), we evaluated all phylloplane bacteria.

Identification of bacterial isolates

Isolates were identified at genus level by using the diagnostic tables of Cowan and Steel (1965) by which they were divided in groups to obtain only those effectively different, since bacteria colonizing olive phylloplane are frequently represented by only few species (Ercolani, 1978). Later, the isolates were identified at species level by molecular analysis by sequencing the 16S rDNA region of each representative isolate for each bacterial species. A 1500-bp region of the 16S rDNA was amplified by using the non specific primers NOC 1F and NOC 3R and sequenced. A BlastN search was performed for comparing their similarity with the strains present in the database (GenBank, EMBL and DDBJ).

Strains of bacterial pathogen

Five strains of *P. savastanoi* pv. *savastanoi* with different geographical origins were used. PseNE 107 isolated from olive in Nepal (Balestra *et. al*., 2008), Italian strains ITM 105 and 311, belong to Istituto Tossine e micotossine da parassiti vegetali Bari (Marchi *et al*., 2009) and two local isolates obtained in Viterbo (VT) and Montefiascone (MF), were used in the tests.

Detection of antagonistic activity

Conventional deferred antagonism assays were performed as described by Parret *et al*. (2003): agar diffusion method relying on detection of halo formation in an overlay of indicator

cells due to growth inhibition by chloroform-killed producer cells. The growth inhibition was quantified by measuring IZR (inhibition zone radius) from the edge of the producer colony.

RESULTS

Molecular identification as described above showed that the phylloplane isolates obtained from olea species were *Bacillus asahii*, *Bacillus cereus, Bacillus circulans*, *Bacillus megaterium*, *Bacillus pumilus, Bacillus silvestris*, *Bacillus simplex*, *Bacillus subtilis*, *Brenneria serratia*, *Microbacterium testaceum*, *Micrococcus luteus*, *Pantoea agglomerans*, *Pseudomonas fluorescens*, *Pseudomonas putida* and *Xanthomonas campestris* group.

None of the bacterial isolates from phylloplane mentioned above inhibited growth of Psav strains *in vitro*.

DISCUSSION

Although related phytopathogenic and non-pathogenic *Pseudomonas* bacteria have been reported to inhibit *P. savastanoi* pv. *savastanoi* strains (Lavermicocca *et al*., 2002; Zadeh *et al*., 2008), in our case no growth inhibition of Psav was observed by the phylloplane bacteria isolated from olive plants. The absence of the possible bacterial antagonist of Psav on olive phylloplane is probably due to the fact that all the leaf samples have been collected from the asymptomatic olive plants where Psav was not present (Lamichhane *et al*., 2010). Previous study demonstrated that the presence and the effectiveness of a given antagonist from phyllosphere of the infected plants depend on the ecological similarity between the antagonist and the target pathogen (May *et al*., 1996; Völksch and May, 2001). Hence, the lack of olive knot pathogen on olive phylloplane could be related to the consequent absence of the antagonist. For this reason, the study of phylloplane bacteria of the infected plants together with those of healthy one will give a more precise idea on the possible presence of antagonist in relation to that of the pathogen and their effectiveness as biocontrol agents.

Antagonistic bacteria of *P. savastanoi* pv. *savastanoi* reported until now are only few and they all are isolates from plant rhizoplane (Zadeh *et al*., 2008). However, we attempted to isolate the antagonist of Psav for the first time but with no satisfactory result.

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CHAPTER 11.

GENERAL DISCUSSION

Olive knot disease was observed in Nepal only on the plants present in the private orchard of Makwanpur where *O. europaea* was introduced two decades ago unlike those introduced in Bajura, Dolpa and Kathmandu three years ago. This finding might be linked to the fact that *Pseudomonas savastanoi* pv. *savastanoi* arrived together with the plant materials shipped by private growers from other countries given that no necessary control measures were performed during plant's shipment.

Besides few studies relative to the bacterial ecology of European olive phylloplane present in the Mediterranean ecosystems, no studies were made in other regions of the world neither on cultivated olive nor on other species belonging to *Olea*. The present study has shown the difference among the bacterial ecology of the phylloplane of both cultivated and naturally present olive species in several Himalayan regions of Nepal characterized by different climatic and geographic conditions. The lower colonization of olive phylloplane respect to the values previously found by some authors can be explained by two possible reasons: a) our study was carried out in an environment which is new for european olive which has recently been introduced in these regions, so colonization of a new species probably takes longer time respect to that of the Mediterranean ecosystem which is native of *Olea* species with thousand years of interactions with bacterial phylloplane community; b) drought stress could have influenced negatively the growth and multiplication of epiphytic bacteria, given that average values of rainfall are very low in these Districts in the spring, since almost all rainfall occurs during the monsoon season between July and August. Widely different climatic and geographic conditions of the three Districts can be possible reason of the different colonization of olive phyllosphere. Moreover, high altitudes and the presence of constant afternoon strong wind blows which characterize Bajura and Dolpa Districts may have had negatively effect on bacterial epiphytic survival reducing the moisture of leaf surfaces and relative humidity which favours epiphytic bacterial multiplication. The wind can even transport bacteria from this area to the lower parts of that region, as bacterial immigration of consistent levels has been demonstrated already which probably is favoured also through wind. Moreover, lower bacterial epiphytic populations can be explained since when the prevailing high temperature can enhance bacterial growth, often water is scarce in Bajura and Dolpa Districts, unlike in Kathmandu District, and when water is available, temperatures are not always favourable. Regarding bacterial communities of *Olea* phylloplane, the dominant bacterial colonizers were *P. agglomerans* and *X. campestris* group followed by different endospore-forming *Bacillus* spp., the latter because of their resistant structures are frequently present and isolated

from plant phylloplane also from difficult environments. The abundance of *P. agglomerans* can be explained easily since this species are considered to be ubiquitous in the phylloplane of various crop plants together with *Pseudomonas*. No detection of *P. savastanoi* pv. *savastanoi* on *Olea* phylloplane is due to the non presence of this bacterial pathogen in these Himalayan Districts, except in Makwanpur where olive has been introduced before. Bacterial species identified from *Olea* phylloplane in Nepal are significantly different respect to those found in south-east Italy.

Our results showed the ability of olive knot pathogen not only to survive but to higher multiply on *O. cuspidata* respect to *O. europaea*. In fact, beginning from very low CFU/cm^2 the bacterium remained from the initial fall and within 4 weeks its population reached a maximum peak. The initial fall of bacterial population during the first five days is probably for the fact that bacteria coming from natural (from infected tissues such as tubercle) or artificial (substrate of culture) environments, suitable for their growth, do not posses a good capacity to survive as epiphytes. Italian isolate of Psav better multiplied on both olive species, respect to the isolate obtained in Nepal with higher value expressed as CFU/cm^2. This is probably because this isolate possesses a high level of virulence attributable to the higher capacity of the pathogen in producing auxin and cytokinins. Only few significant differences were observed regarding the influence of leaf age on multiplication of bacteria. Generally, higher multiplication was observed on old leaf surfaces than the young one, which probably be given by higher nutrient contents.

Pseudomonas savastanoi pv. *savastanoi* strains artificially inoculated on two *Olea* species on different stage of development showed that *O. cuspidata* reacts positively as *O. europaea* to Psav, which is a new finding since this species native of Himalaya was unknown up to now for its reaction to Psav. Less susceptibility of *O. europaea* compare to that of *O. cuspidata* to Psav is probably due to the continuous contact of *P. savastanoi* pv. *savastanoi* from centuries with *O. europaea* that led this species to develop some sort of resistance compared to *O. cuspidata,* which had never been in contact with Psav. The different time of symptom appearance and the different knot weight caused by the strains of Psav on two different species was due to the different reactivity showed by two *Olea* species to artificial inoculations. The lack of correlation between the knot weight and their respective bacterial populations confirms that knot weight is independent by the number of bacterial populations inside the infected tissues but depend on the concentration of growth hormone (IAA and Cytokinin) produced by the bacterial pathogen inside the infected tissues. Hence higher values of bacterial populations inside the knot, in particular those of the strain Local Vt and ITM 105, were due to the higher virulence of these strains given

probably by the higher concentration of plants hormones produced by them. In addition, plants age did not influence significantly host-pathogen interaction since all the strains reacted similarly on plants of different age with similar values.

Although related phytopathogenic and non-pathogenic Pseudomonas bacteria have been reported to inhibit *P. savastanoi* pv. *savastanoi* strains, no growth inhibition of Psav was observed by our olive phylloplane bacteria. The absence of the possible bacterial antagonist against Psav on olive phylloplane is probably due to the fact that all the leaf samples have been collected from the asymptomatic olive plants, where Psav was not present. Previous study demonstrated that the presence and the effectiveness of a given antagonist from phyllosphere of the infected plants depend on the ecological similarity between the antagonist and the target pathogen. Hence, the lack of olive knot pathogen on olive phylloplane could be related to the consequent absence of the antagonist. For this reason, the study of phylloplane bacteria of the infected plants together with those of healthy one will give a more precise idea on the possible presence of antagonist in relation to that of the pathogen and their effectiveness as biocontrol agents. In addition, antagonist bacteria of *P. savastanoi* pv. *savastanoi* reported until now are very few and they all have been isolated from plant rhizoplane. However, we attempted to isolate the antagonist of Psav for the first time but with no satisfactory result.

CHAPTER 12.

CONCLUSIONS

The finding of *Pseudomonas savastanoi* pv. *savastanoi* in Nepal must be considered seriously and seeks immediate implementation of control strategies since this pathogen is not present in other areas, except than in Makwanpur, where *Olea* spp. are present. Only the use of the adequate agronomic and cultural practices can be the effective way to avoid the contamination and consequent diffusion of Psav to the other areas where wild olive species, in particular *O. cuspidata,* are present since this species is found to be very susceptible to Psav.

Bacterial phylloplane colonizers of *O. europaea* and *O. cuspidata* were almost the same, although in different proportions. Only some of them are are similar as those described by some previous authors on *O. europaea* from the Mediterranean areas. Lower average bacterial phylloplane populations respect to those found in Mediterranean climate might probably be due to the less suitable climatic conditions in Nepal.

Olea cuspidata, an unknown olive species up to now for its reaction to Psav showed to be highly suitable for the survival and multiplication of Psav, which is a new finding. In fact, Psav better multiply on *O. cuspidata* respect to *O. europaea*. Higher bacterial multiplication on old leaves compare to that on new ones is associated to the higher nutrient contents of the old leaves.

Psav not only better survive and multiply epiphytically on *O. cuspidata* but also into the plant tissues since both the symptom appearance, average knot weight and the bacterial populations inside the knots are higher on this species respect to that of *O. europaea* during the artificial inoculations. Higher susceptibility of *O. cuspidata* to Psav is probably due to the fact that this olive species has never been in contact with Psav and the plant did not develop the resistance, unlike *O. europaea* which has continuously been in contact with Psav with consequent evolution from centuries.

In nature, the survival and multiplication of a pathogenic bacteria are often influenced by other saprophytic and possible antagonistic species, since ecological similarity between different bacterial species on phylloplane has been demonstrated. The lack in finding from *Olea* phylloplane of possible antagonists against Psav can be related to the absence of the bacterial pathogen on *Olea* phylloplane.

CHAPTER 13.

PHOTOGRAPHS

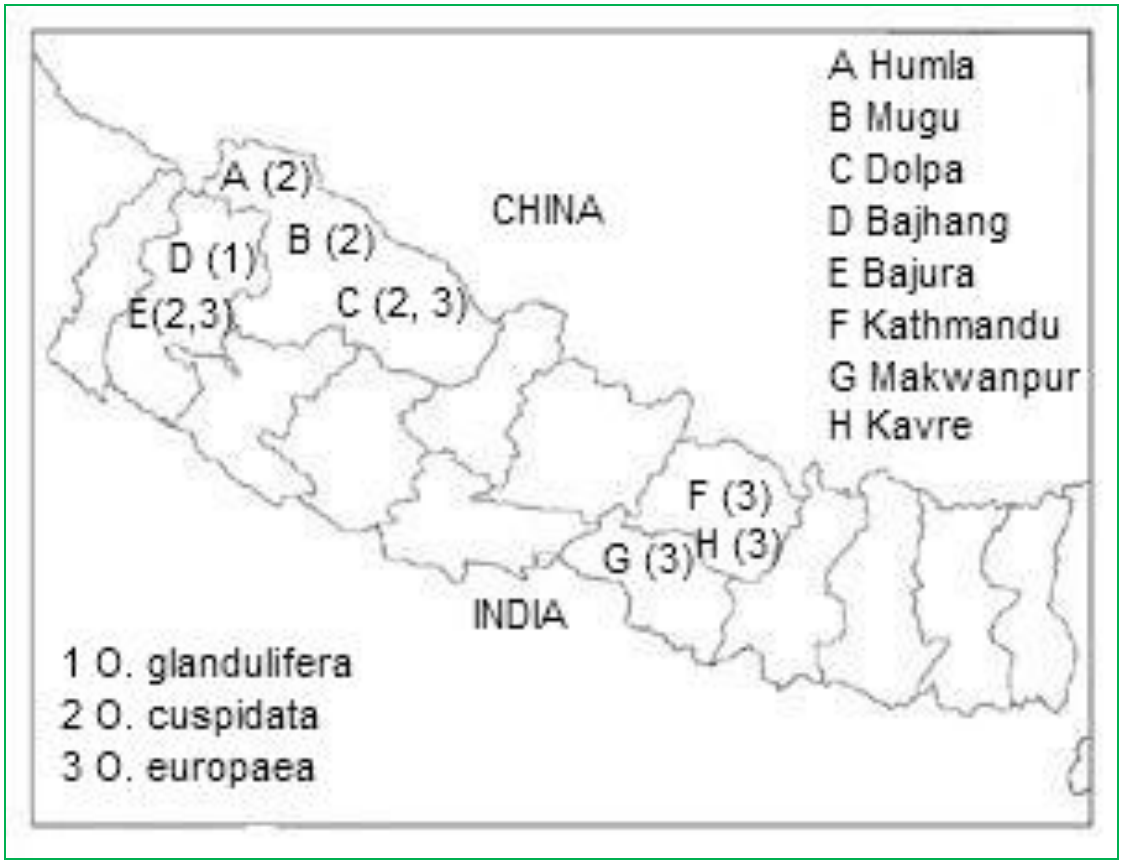

Fig. 1. Surveyed Districts and reported olive species in Nepal

Fig. 2. European olive planted within the FAO project in Kathmandu District

Fig. 3. European olive planted within the FAO project in Bajura District

Fig. 4. Olive knot symptoms on the main trunk of European olive (Cv. Frantoio) observed in a private orchard of Makwanpur District

Fig. 5. Natural forests of wild olive (*Olea cuspidata*) present in Bajura District (a)

Fig. 6. Natural forests of wild olive (*Olea cuspidata*) present in Bajura District (b)

Fig. 7. Secular plant of wild olive (*Olea cuspidata*) present in Dolpa District

Fig. 8. Olive fruits of *Olea cuspidata* (left) and *Olea europaea* (right)

Fig. 9. Production of wild olive (*Olea cuspidata*) by seed in the greenhouse

Fig. 10. Olive knot symptoms developed on 9 month old plantlets of artificially inoculated wild olive (*Olea cuspidata*)

Fig. 11. Olive knot symptoms developed on 20 month old plantlets of artificially inoculated wild olive (*Olea cuspidata*)

Fig. 12. Olive knot symptoms developed on 20 month old plantlets of artificially inoculated european olive (*Olea europaea* cv. Frantoio)

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