ECOPRINT **17:** 47-52, 2010 ISSN 1024-8668
Ecological Society (ECOS), Nepal
www.nepjol.info/index.php/eco; www.ecosnepal.com

ESTIMATING BACTERIAL POPULATION ON THE PHYLLOSPHERE BY SERIAL DILUTION PLATING AND LEAF IMPRINT METHODS

Ram Kailash P. Yadav[1]*, Katerina Karamanoli[2] and **Despoina Vokou[3]**
[1]Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal
[2]Laboratory of Agricultural Chemistry, School of Agriculture, Aristotle University, Gr-541 24 Thessaloniki, Greece
[3]Department of Ecology, School of Biology, Aristotle University, Gr-541 24 Thessaloniki, Greece
*E-mail: rkp.yadav@cdbtu.edu.np

ABSTRACT

The plant phyllosphere constitute a habitat for variety of microorganisms; bacteria are the most common ones. In this study, we assessed the ability of serial dilution plating and leaf imprint methods to elucidate population abundance on the phyllosphere of eight plant species co-occurring in the same area in mediterranean ecosystem (northern Greece). The serial dilution plating method outweighed the leaf imprint method in determining the total epiphytic bacterial population and the estimates were significantly higher. However, the ability to detect presence of bacterial colony on leaf surface is higher for the leaf imprints, suggesting specific use of this method for practical purposes.

Key words: Phyllosphere, bacterial population, serial dilution plating, leaf imprint.

INTRODUCTION

Phyllosphere bacteria have agricultural and environmental importance as they can affect plant growth and can also suppress and stimulate the colonization and infection of tissues by plant pathogens (Lindow and Brandl 2003, Rasche *et al*. 2006). Bacterial populations on the phyllosphere are very diverse and vary in size both among and within species, and also over short time scales (Kinkel *et al*. 1995, Yadav *et al*. 2004, Lambais *et al*. 2006). Furthermore, interactions in the phyllosphere determine the extent to which human pathogens are able to colonize and survive on plant tissues, such as fresh salad, fruit and vegetable produce (Whipps *et al*. 2008, Berger *et al*. 2010).

Culture methods to enumerate bacterial population have revealed high numbers of bacterial strains inhabiting leaf surfaces. For instance, 37 genera and 78 species of bacteria were isolated from wheat and 20 genera from mango leaves (Legard *et al*. 1994, de Jager *et al*. 2001). Culture-independent methods have shown that the phyllosphere bacterial communities are more diverse than previously detected (Yang *et al*. 2001). Studies involving leaf imprints have shown that bacteria do not occur in a uniform pattern across leaf surfaces (Weller and Saettler 1980, Leben 1988).

Inability to grow most bacteria in the laboratory and the difficulties associated with bacterial

identification set limitations in the description of microbial communities (Torsvik *et al*. 2002). In recent times, therefore, characterization of microbial communities by culture dependent methods have been under much scrutiny and voices are being raised in favour of culture independent methods such as molecular methods (McCaig *et al*. 1999), and characterization of community level physiological profile (Garland *et al*. 2001, Preston-Mafhan *et al*. 2002, Yadav *et al*. 2008). However, culture methods (e.g., serial dilution plating, liquid broth culture, leaf imprints, etc.) are still thought appropriate and being widely used to characterize microbial populations from different habitat (Ritz 2007). In the present study, we attempt to compare the ability of the two culture methods (serial dilution plating and leaf imprint) in characterizing the bacterial abundance on the phyllosphere.

MATERIALS AND METHODS

We studied eight plant species (woody and non-woody perennials) naturally occurring and co-existing in the same area in the Mediterranean ecosystem in Halkidiki (northern Greece). The woody species are the evergreen-sclerophyllous shrubs, *Arbutus unedo* L., *Quercus coccifera* L., *Pistacia lentiscus* L. and *Myrtus communis* L. and the seasonal dimorphic shrubs, *Lavandula stoechas* L. and *Cistus incanus* L. They are all common components of the mediterranean-type ecosystems of the country. The non-woody perennials are *Calamintha nepeta* (L.) Savi and *Melissa officinalis* L. Sampling was done in the morning and leaf samples were placed in sterile plastic bags, were transported to the laboratory in an icebox, and were analyzed within 24 h. The serial dilution plating method (Lindow *et al*. 1978) was used. Each sample was weighed and immersed in 25 ml sterile phosphate buffer (0.01 M, pH 7.3) supplemented with 0.1% bactopeptone, in a 100 mL Erlenmeyer flask. Flasks were sonicated in an ultrasonic cleaner for 10 min. Portions (100 µL) from the original wash and appropriate dilutions thereof prepared in 0.01 M phosphate buffer (pH 7.3) were plated onto nutrient agar (NAG) medium, supplemented with 2.5% (v/v) glycerol, and amended with 30 µg mL^{-1} natamycin to prevent fungal contamination.

In order to estimate the bacterial population on the adaxial and abaxial leaf surfaces, leaf imprints were made on agar media prepared as in dilution plating method. An intact individual leaf was placed onto agar plate and was pressed with the smooth end of a sterile glass rod until a clear imprint of the entire leaf was obtained on the agar surface (Aneja 2003). Different leaves were used for imprinting the adaxial and abaxial surfaces to avoid disturbances in surface community distribution. After bacterial extraction, each leaf was used to measure its area with leaf area meter (Ejkelkamp, Agrisearch Equipment, Netherland).

Plates with serial dilution plating and leaf imprints were incubated at 24^{o}C for 2-5 days. Bacterial populations were enumerated from the plates and expressed as log CFU (colony forming units) per square centimeter of leaf area. The reason for transforming CFUs into log CFUs is described in Yadav *et al*. (2004). CFU on adaxial and abaxial leaf surfaces were added to represent the bacterial population on leaf surface by imprint method. In case of dilution plating, leaf weights for each species were translated into leaf area (adaxial and abaxial) using leaf weight-leaf area relationship (linear regression model) with 30 samples. The highest degree of relationship was found in *Myrtus communis* (R^2 = 0.986, p < 0.0001) and the lowest was found in *Quercus coccifera* (R^2 = 0.739, p < 0.0001). Comparison of bacterial population estimates were done with one-way ANOVA and t-test by using SPSS for Windows (11.0.1, SPSS Inc., USA).

RESULTS AND DISCUSSION

The size of bacterial population on the phyllosphere of eight species estimated by serial dilution plating and leaf imprint methods is presented in Fig. 1. There was significant variation ($p < 0.0001$) in population size between the groups and within the groups (Table 1). The average epiphytic bacterial population on the phyllosphere ranged from as low as 0.94 log CFU cm^{-2} in case of *Q. coccifera* to as high as 3.92 log CFU cm^{-2} in *C. nepeta* as estimated by the serial dilution plating method. Results of leaf imprint method showed the highest population on the leaves of *C. incanus* (1.37 log CFU cm^{-2}) and the lowest population in case of *A. unedo* (0.59 log CFU cm^{-2}). Overall, *A. unedo* and *Q. coccifera* (evergreen sclerophyllous) are the least populated species based on either of the population estimation methods, while *C. incanus* (seasonal dimorphic), *C. nepeta* and *M. officinalis* (non-woody perennials and aromatic) were the most populated. This pattern is consistent with the population pattern found in these species over seasons (Yadav *et al*. 2004, 2005).

In this study, greater variation in population size in *C. incanus*, *C. nepeta* and *M. officinalis* determined by both methods can be attributed to the hairy and rough leaf surfaces they bear (Yadav *et al*. 2004a). However, leaf imprint method appeared as better one in terms of extent of detection of microbes on the leaf surface is concerned. This was evident by the fact that only one out of forty eight leaf samples had no detectable bacteria against nine in as many samples estimated by serial dilution plating method.

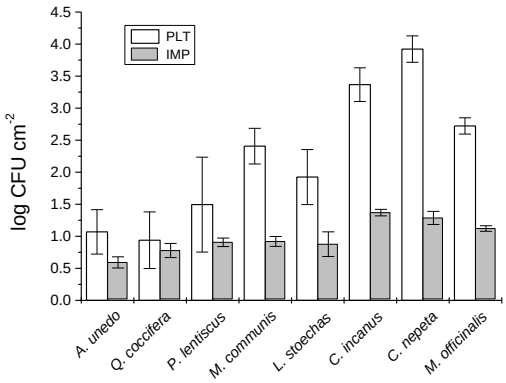

Fig. 1. Bacterial population of the phyllosphere of mediterranean species estimated by serial dilution plating method (PLT) and leaf imprint method (IMP). Bars represent mean values of log CFU cm^{-2} and error bars represent standard errors of the means (n = 6).

Table 1. Comparison of bacterial populations estimated by plate dilution (PLT) and leaf imprint (IMP) methods using one-way ANOVA.

Variables		Sum of squares	d.f.	Mean square	F-value	Sig.
PLT	Between groups	48.46	7	6.92	7.37	0.000
	Within groups	37.60	40	0.94		
	Total	86.07	47			
IMP	Between groups	2.86	7	0.41	6.624	0.000
	Within groups	2.47	40	0.06		
	Total	5.32	47			

Table 2. Comparison of phyllosphere bacterial populations in all species estimated by serial dilution plating (PLT) and leaf imprint (IMP) methods.

Methods	log CFU cm^{-2}	t-test
PLT	2.23 ± 0.19 (n = 48)	t = 7.09 $p < 0.0001$
IMP	0.98 ± 0.05 (n = 48)	

In case of pair wise comparisons of two methods, bacterial population size varied significantly in most of the species (Fig. 1). The estimate by dilution plating outweighed that by the leaf imprint. When population data of all eight species were pooled for each method and compared (Table 2), the estimate by serial dilution plating method was found significantly higher ($p < 0.0001$). The leaf imprint method underestimated the size of population of epiphytic bacteria compared to the dilution plating method, as has also been reported by other researchers (Jacques *et al*. 1995). Furthermore, the common sites of bacterial colonization on leaf surface are base of trichomes, stomata, cell wall junctions, vein endings and even beneath the cuticles (Mansvelt and Hattings 1987, Mariano and McCarter 1993, Knief *et al*. 2010). It is apparent that bacterial colonies in such sites are less likely to be recovered in a leaf imprint made on the nutrient agar. Leaf washings, on the other hand, can possibly remove cells from the colonies situated even under cuticle through pores and cracks (Corpe and Rheem 1989).

In conclusion, it is obvious that serial dilution plating is better than leaf imprint method regarding characterization of microbial abundance on the phyllosphere. Leaf imprint method only presents an underestimate of the population size but its ability to detect bacterial colony on leaf surface is higher. Moreover, these culture methods are still being used as an alternative to other methods in contemporary microbial ecology.

ACKNOWLEDGEMENT

R.K.P. Yadav was supported by a grant from the State Scholarships Foundation (IKY), Greece. He acknowledges Tribhuvan University, Kathmandu for granting him study leave. This project was also supported by the General Secretariat for Research and Technology, Ministry of Development, Greece (01 ED 317).

REFERENCES

Aneja, K.R. 2003. *Experiments in Microbiology, Plant Pathology and Biotechnology*. New Age International (P) Ltd., New Delhi.

Berger, C.N., S.V. Sodha, R.K. Shaw, P.M. Griffin, D. Pink, P. Hand and G. Frankel. 2010. Fresh fruit and vegetables as vehicles for the transmission of human pathogens. *Environ. Microbiol.* **12(9):**2385-2397.

Corpe, W.A. and S. Rheem. 1989. Ecology of the methylotrophic bacteria on leaving leaf surfaces. *FEMS Microbiol. Ecol.* **62:**243-250.

de Jager, E.S., F.C. Wehner and L. Korsten. 2001. Microbial ecology of the mango phylloplane. *Microb. Ecol.* **42:**201-207.

Garland, J.L., A.L. Mills and J.S. Young. 2001. Relative effectiveness of kinetic analysis vs single point readings for classifying environmental samples based on community-level physiological profiles (CLPP). *Soil Biol. Biochem.* **33:**1059-1066.

Jacques, M.-A., L. Kinkel and C.E. Morris. 1995. Population sizes immigration and growth of epiphytic bacteria on leaves of different ages and position of field-grown endive (*Cichorium endivia* var. *latifolia*). *Appl. Environ. Microbiol.* **61:**899-906.

Kinkel, L., M. Wilson and S.E. Lindow. 1995. Effects of scale on estimates of epiphytic

bacterial populations. *Microb. Ecol.* **29:**282-297

Knief, C., A. Ramette, L. Frances, Alonso-Blanco C. and J.A. Vorholt. 2010. Site and plant species are important determinants of the *Methylobacterium* community composition in the plant phyllosphere. *The ISME Jour.* **4:**719-728.

Lambais, M.R., D.E. Crowley, J.C. Cury, R.C. Büll and R.R. Rodrigues. 2006. Bacterial diversity in tree canopies of Atlantic forest. *Science* **312:**1917.

Leben, C. 1988. Relative humidity and the survival of epiphytic bacteria with buds and leaves of cucumber plants. *Phytopathology* **78:**179-185.

Legard, D.E., M.P. McQuilken, J.M. Whipps, J.S. Fenlon, I.P. Fermor, I.P. Thompson, M.J. Bailey and J.M. Lynch. 1994. Studies of seasonal changes in the microbial populations on the phyllosphere of spring wheat as a prelude to the release of a genetically modified microorganism. *Agr. Ecosyst. Environ.* **50:**87-101.

Lindow, S.E., D.C. Arny and C.D. Upper. 1978. Distribution of ice nucleation-active bacteria on plants in nature. *Appl. Environ. Microbiol.* **36:**831-838.

Lindow, S.E. and M.T. Brandl. 2003. Microbiology of the phyllosphere. *Appl. Environ. Microbiol.* **69:**1875-1883.

Mansvelt, E.L. and M.J. Hattingh. 1987. Scanning electron microscopy of colonization of pear leaves by *Pseudomonas syringae* pv. *syringae*. *Can. J. Bot.* **65:**2517-2522.

Mariano, R.L.R. and S.M. McCarter. 1993. Epiphytic survival of *Pseudomonas viridiflava* on tomato and selected weed species. *Microb. Ecol.* **26:**47-58.

McCaig, A.E., E.A. Glover and J.I. Prosser. 1999. Molecular analysis of bacterial community structure and diversity in unimproved and improved grass pastures. *Appl. Environ. Microbiol.* **65:**1721-1730.

Preston-Mafham, J., L. Boddy and P.F. Randerson. 2002. Analysis of microbial community functional diversity using sole-carbon source utilization profiles–a critique. *FEMS Microbiol. Ecol.* **42:**1-14.

Rasche, F., E. Marco-Noales, H. Velvis, L.S. van Overbeek, M.M. Lopez, J.D. van Elsas and A. Sessitsch. 2006. Structural characteristics and plant-beneficial effects of bacteria colonizing the shoots of field grown conventional and genetically modified T4-lysozyme producing potatoes. *Plant Soil* **289:**123-140.

Ritz, C. 2007. The plate debate: cultivable communities have no utility in contemporary environmental microbiology. *FEMS Microbiol. Ecol.* **60:**358-362.

Torsvik, V., L. Ovreas and T.F. Thingstad. 2002. Prokaryotic diversity – magnitude, dynamics and controlling factors. *Science* **296:**1064-1065.

Weller, D.M. and A.W. Saettler. 1980. Colonization and distribution of *Xanthomonas phaseoli* and *X. phaseoli* var. *fuscans* in field-grown navy beans. *Phytopathology* **70:**500-506.

Whipps, J.M., P. Hand, D.A.C. Pink and G.D. Bending. 2008. Human pathogens and the phyllosphere. *Adv. Appl. Microbiol*. **64:**183-221.

Yadav, R.K.P., A.M. Bosabalidis and D. Vokou. 2004a. Leaf structural features of Mediterranean perennial species: plasticity and life form specificity. *J. Biol. Res.* **2:**21-34.

Yadav, R.K.P., E.M. Papatheodorou, K. Karamanoli, H.A. Constantinidou and D. Vokou. 2008. Abundance and diversity of the phyllosphere bacterial communities of Mediterranean perennial plants that differ in leaf chemistry. *Chemoecology* **18:**217-226.

Yadav, R.K.P., J.M. Halley, K. Karamanoli, H.-I. Constantinidou and D. Vokou. 2004. Bacterial populations on the leaves of Mediterranean plants: quantitative features and testing of distribution models. *Environ. Exp. Bot.* **52:**63-77.

Yadav, R.K.P., K. Karamanoli and D. Vokou. 2005. Bacterial colonization of the phyllosphere of Mediterranean perennial species as influenced by leaf structural and chemical features. *Microb. Ecol.* **50:**185-196.

Yang, C-H., D.E. Crowley, J. Morneman and N.T. Keen. 2001. Microbial phyllosphere populations are more complex than previously realized. *Proc. Nat. Acad. Sci. USA*, **98:**3889-3894.