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A method for estimation of population densities of ice nucleating active *Pseudomonas syringae* in buds and leaves of mango

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F.M. CAZORLA, L. OLALLA, J.A. TORÉS, A. PÉREZ-GARCÍA, J.C. CODINA AND A. DE VICENTE. 1995. Active ice nucleation strains of *Pseudomonas syringae* pv. *syringae* have been associated with a necrotic disease in mango trees growing in Málaga (southern Spain). In this paper a simple multiple-tube test is described to estimate the number of active ice nucleation bacteria associated with plant tissues and, also from suspensions of isolated bacterial strains. This method is based on the most probable number technique developed for microbiological analysis of water. The tube test presented a higher detection sensitivity of active ice nuclei than the traditional drop-freezing test, because a larger amount of plant material could be analysed routinely. Both methods demonstrated a similar accuracy. A high correlation was obtained between the tube test-estimated number of ice nuclei and populations of *Ps. syringae*-like organisms enumerated on King's agar B.

INTRODUCTION

A bacterial infection has been observed in mango trees growing in Málaga (Spain), which contributes to development of necrotic lesions on buds, stems and leaves. *Pseudomonas syringae* pv. *syringae* has been associated with the appearance of this disease (Cazorla *et al.* 1992). The symptoms are greater during tree dormancy, in late autumn and winter. Disease development is favoured by cool and rainy weather, which suggests that ice nucleation activity (INA) of the bacteria may be involved (Montesinos and Vilardell 1991). INA at about $-2°C$ has been detected in bacterial suspensions; thus, certain epiphytic bacteria may serve as the ice nuclei that prevent supercooling of plants (Lindow *et al.* 1982). It is generally accepted that certain bacteria associated with plants can contribute to frost damage by serving as nuclei for ice formation. Several bacterial species have been reported to be INA organisms, *Ps. syr.* pv. *syringae* appears as one of the most common and active bacteria associated with many plant species (Lindow *et al.* 1978a; Endert and Ritchie 1984; López 1989; Montesinos and Vilardell 1991; Morris *et al.* 1992). Frost damage to plants is related to both the number of INA bacteria present in plant tissues and the nucleation frequency of those bacterial cells (Lindow *et al.* 1982; Hirano *et al.* 1985).

Rapid and accurate diagnosis of INA bacteria is essential to control the development of diseases, but the INA detection by the traditional drop-freezing methods (Lindow *et al.* 1978b; Govindarajan and Lindow 1988; Olive and McCarter 1988; Strong-Gunderson *et al.* 1992) is too laborious for routine assays and not very sensitive for low INA bacterial populations. Ice nucleation tube tests have been proposed to detect INA from bacterial isolates (Hirano *et al.* 1985; Cody *et al.* 1987; Lindow 1993) but they were not able to quantify the number of ice nuclei. Montesinos and Vilardell (1991) described a tube assay to determine the number of ice nuclei on the basis of the reciprocal of the highest dilution factor that froze.

In this report a multiple-tube nucleation test is described to estimate the number of ice nuclei from bacterial suspensions of isolated strains and, especially, to quantify the INA populations associated with plant parts. This method is applied to suspensions of *Ps. syr.* pv. *syringae* strains and buds and leaves of mango trees, and it is compared with the

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classical drop-freezing method. Also, the number of active ice nuclei is related to the *Ps. syringae* population levels in plant tissues.

MATERIALS AND METHODS

Plants sampling and enumeration of *Ps. syringae* populations

Buds and leaves of mangoes (*Mangifera indica* L.) were obtained from a tree-growing area in the Experimental Station 'La Mayora' belonging to Consejo Superior de Investigaciones Científicas (CSIC) and located in Algarrobo, near Málaga (Spain). Samples of 3–5 leaves or buds (total sample about 5 g) were collected in sterile polythene bags, stored at 4°C and processed the same day of sampling. Each sample was homogenized in 50 ml of 0·1 mol l^{-1} phosphate buffer, pH 7·0 by stomaching (Colworth Stomacher-400, Seward Ltd, London) for 3 min. Tenfold serial dilutions of this leaf or bud homogenate were prepared in phosphate buffer and then, plated onto King's B agar (KMB) (King *et al.* 1954) amended with cycloheximide (100 mg l^{-1}) to inhibit fungal growth (Olive and McCarter 1988). These plates were incubated for 2–3 d at 22°C. Fluorescent colonies being negative for oxidase and arginine dihydrolase (ADH) were enumerated as *Pseudomonas syringae*-like (Hildebrand *et al.* 1988; Lindow 1993).

Bacterial strains

Eleven strains of *Pseudomonas syringae* pv. *syringae* were isolated as epiphytic bacteria from healthy leaves and buds of mango trees, and also 43 strains of *Ps. syr.* pv. *syringae* were isolated from typical necrotic lesions in mango tissues. Buds and leaves were superficially disinfected in a solution of mercury chloride (1 g l^{-1}) for 2 min, washed twice in sterile distilled water and dried in aseptic conditions. Then, they were cut into 1–2 cm pieces and placed on KMB plates, and incubated at 22°C for 48–72 h. All strains were isolated on KMB agar containing cycloheximide, and identified following standard diagnostic tests (Hildebrand *et al.* 1988; Cazorla *et al.* 1993).

For INA assays, cells from 48 h pure cultures grown on KMB agar were resuspended (10^7 cells ml^{-1}) in phosphate buffer (0·1 mol l^{-1}, pH 7·0).

Determination of ice nuclei number

Multiple-tube nucleation test. Test tubes (diam. 18 mm) containing 9 ml of sterile phosphate buffer were prechecked at −10°C for 30 min, then they were shaken vigorously and all tubes in which the buffer had frozen were discarded. The valid tubes were then warmed to over 5°C.

Triplicate 10-fold serial dilutions of the homogenized samples or bacterial suspensions were carried out and subsequently the tubes were reimmersed for 10 min in a circulating water–ethanol bath maintained at a temperature in the range −3°C to −9°C. The cumulative number of tubes which had frozen at a given temperature was recorded for each dilution and the number of ice nuclei per g fresh weight of sample was estimated, in terms of the most probable number (MPN) from the table of MPN and 95% confidence limits given in Standard Methods for the Examination of Water and Wastewater (Anon. 1975).

Drop-freezing method. This is basically the conventional technique described by Lindow *et al.* (1978b). For each bacterial suspension or homogenized sample, 20 droplets of 10 μl of each serial dilution were placed on the test surface. The number of droplets which froze within 30 s was recorded. The number of ice nuclei active at a given temperature was calculated following the method of Govindarajan and Lindow (1988).

Statistical method

Estimates of bacterial population size and active ice nuclei number from individual samples were log transformed to achieve normality. The χ-square statistical test was used to test the null hypothesis that the number of ice nuclei determined for both compared methods were equal. Linear regression analysis was performed to test the relationship between population levels of *Ps. syringae* and number of active ice nuclei.

RESULTS

Tube assay optimization

A simple tube test to estimate the number of bacterial ice nuclei associated with individual plant parts was compared with the classical drop-freezing technique. This method estimates the number of bacterial ice nuclei using a multiple-tube technique and a table of most probable numbers (Anon. 1975). The first experiments were designed to determine the optimal assay conditions.

The cumulative number of frozen tubes at −5°C in a given time, for different densities of active ice nuclei is represented in Fig. 1. When the number of ice nuclei per tube was higher than 10, an assay time of 5 min gave clear detection of INA; however, when the number of ice nuclei per tube was lower than 10, an assay time of approximately 10 min was required. From these results, a standard assay time of 10 min was selected.

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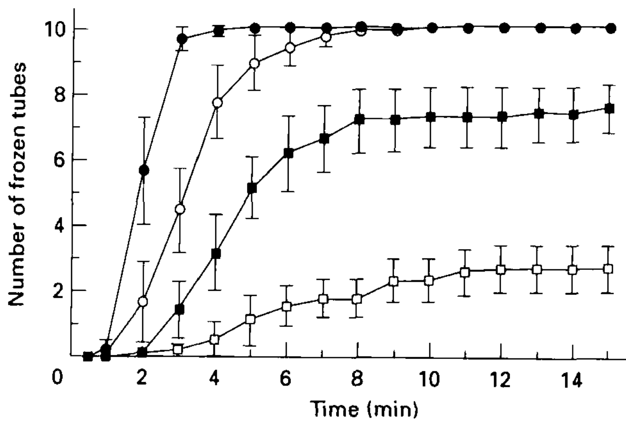

Fig. 1 Cumulative number of frozen tubes (average of *n* experiments ± S.E./2) at −5°C as a function of exposure time. Each experiment was carried out with 10 tubes containing homogenized samples of buds or leaves of mango with an estimated number of: 100–1000 ice nuclei active at −5°C per tube (●), $n = 9$; 1–10 ice nuclei active at −5°C per tube (○), $n = 15$; 0·6–0·9 ice nuclei active at −5°C per tube (■), $n = 11$; <0·5 ice nuclei active at −5°C per tube (□), $n = 9$

Comparison with droplet test

A comparative study of the sensitivity of the two assayed INA methods was carried out from homogenized buds and leaves of mango (Table 1). In general, the tube test was sensitive and detected active ice nuclei from a higher percentage of samples than the drop-freezing test. The detection of INA was also studied from bacterial suspensions (3–5 10^7 cfu ml^{-1}) of *Ps. syr.* pv. *syringae* strains ($n = 31$). A slight improvement of the sensitivity was observed by the tube test (48·4% of strains were INA at −3°C and 96·8% at −5°C and at −8°C) with respect to the droplet test (45·2% and 83·9%).

The number of samples with INA detected by the droplet test compared to the number of ice nuclei estimated by the tube test in the same samples showed a general sensitivity of both methods when the samples contain a high number of ice nuclei (>1000 per g of fresh weight). However, if the number of ice nuclei was lower than 1000 per g, the sensitivity of the tube test was higher than the drop test (Table 2).

The tube test and the classical droplet test were compared with respect to the efficiency of both methods for quantitative estimation of the number of bacterial ice nuclei. These experiments were conducted employing samples in which INA was detected by both methods. The results of experiments carried out with bacterial suspensions at −5°C and −8°C showed no significant differences ($P > 0·99$) between the methods (Fig. 2a). Results obtained from similar experiments carried out with natural samples of mango buds and leaves colonized by cells of *Ps. syr.* pv. *syringae* also showed no significant difference ($P > 0·99$) between the number of ice nuclei estimated by both methods, at −5°C (Fig. 2b) and at −9°C (Fig. 2c).

Ps. syringae counts and INA

The relationship between numbers of ice nuclei at −5°C (Fig. 3a) and −9°C (Fig. 3b) and population levels of *Ps.*

Table 1 Detection of ice nucleation activity by multiple-tube nucleation test and drop-freezing method at different temperatures in 10 min, from homogenized buds and leaves of mangoes

Temperature (°C)	Number of samples	Multiple-tube test		Drop-freezing method	
		n	%	*n*	%
−5	114	62	54·4	28	24·6
−7	113	75	66·4	41	36·3
−9	111	102	91·9	62	55·9

n, Number of samples with ice nucleation activity at each temperature.
%, Percentages of samples with ice nucleation activity at each temperature.

Table 2 Detection of ice nucleation activity by the drop-freezing method at different temperatures in 10 min, from homogenized buds and leaves of mangoes, in relation to the estimated number of active ice nuclei obtained by the multiple-tube test.

Temperature (°C)	Number of ice nuclei detected by the tube test					
	No detection		<1000 g^{-1}		>1000 g^{-1}	
	n	droplet +	*n*	droplet +	*n*	droplet +
−5	52	0	39	5	23	23
−7	38	0	45	11	30	30
−9	9	1	70	29	32	32

n, Number of assayed samples.
droplet +, Number of samples with ice nucleation activity detected by the drop-freezing method.

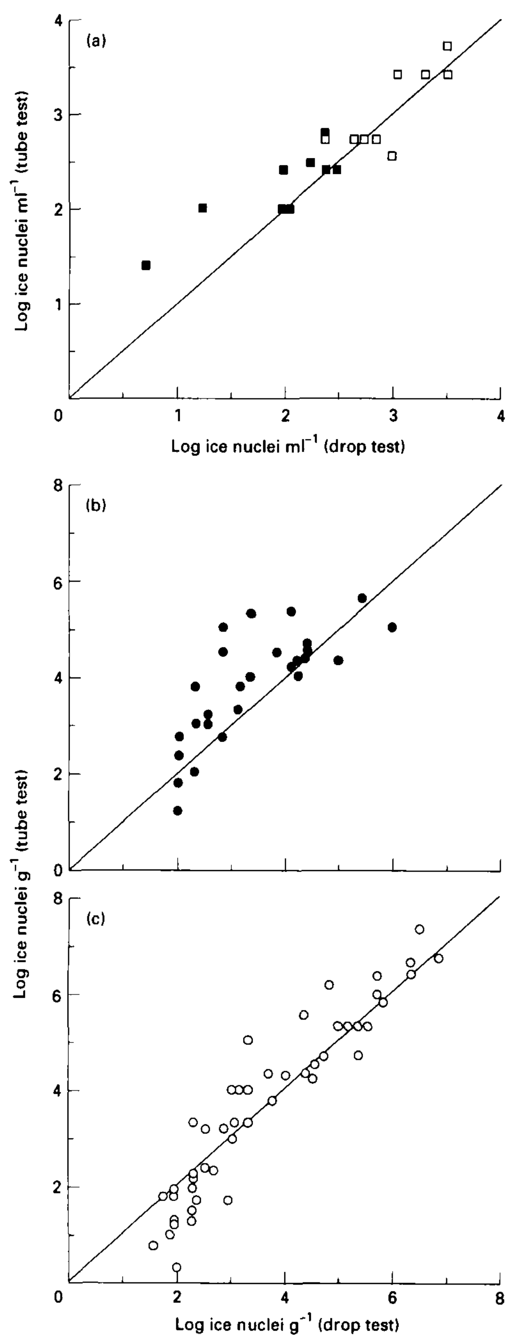

Fig. 2 Comparison between the number of active ice nuclei estimated by the tube nucleation test and by the drop-freezing method. (a) From suspensions of *Pseudomonas syringae* strains isolated from mango tissues (log ice nuclei ml^{-1}). The assay temperatures were -5°C (■, $n = 9$) and -8°C (□, $n = 9$). (b) From homogenized samples of buds or leaves of mango (log ice nuclei g^{-1} fresh weight), when the assay temperature was -5°C (●, $n = 28$). (c) From homogenized samples of buds or leaves of mango (log ice nuclei g^{-1} fresh weight), when the assay temperature was -9°C (○, $n = 59$). The estimated number of ice nuclei by both methods does not differ significantly ($P > 0·99$) according to the χ-square test

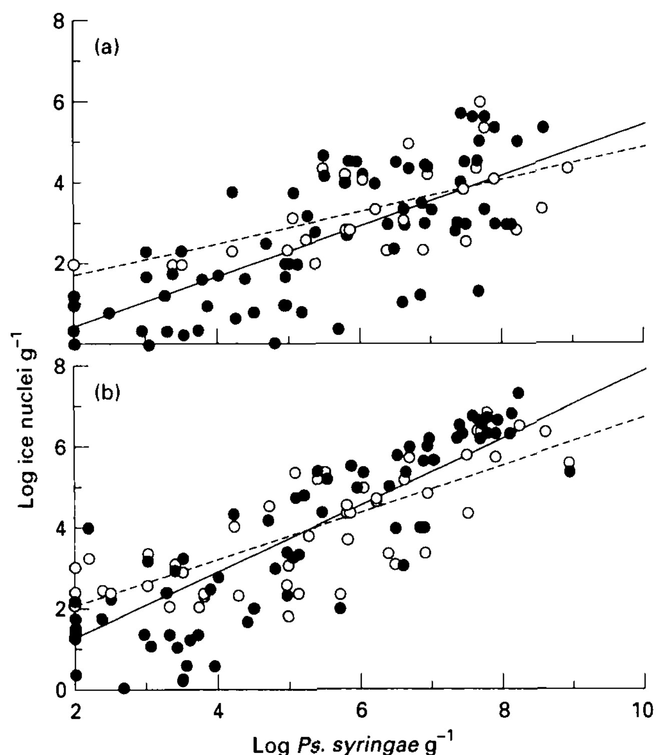

Fig. 3 Relationship between population levels of *Pseudomonas syringae* (log cfu g^{-1} fresh weight) and numbers of ice nuclei (log ice nuclei number g^{-1} fresh weight) in leaves and buds of mango trees. The numbers of ice nuclei were detected at -5°C (a) and at -9°C (b). Ice nucleation activity for each sample was determined by the multiple-tube nucleation test (— ● —) and by the drop-freezing method (- - - ○ - - -). The calculated linear regressions are: (a) -5°C: multiple-tube test, $y = -0·84 + 0·63x$ ($n = 83$, $r = 0·755$, $P < 0·001$); drop-freezing test, $y = 0·90 + 0·40x$ ($n = 28$, $r = 0·589$, $P < 0·001$); (b) -9°C: multiple-tube test, $y = -0·44 + 0·83x$ ($n = 108$, $r = 0·887$, $P < 0·001$); drop-freezing test, $y = 0·83 + 0·59x$ ($n = 57$, $r = 0·841$, $P < 0·001$)

syringae in buds and leaves of mango trees was studied in Fig. 3. From most samples, numerous (10^2–10^9 cfu g^{-1} fresh weight) typical colonies of *Ps. syringae* (fluorescent, oxidase-negative and ADH-negative) appeared after 2–3 d at 22°C. A direct correlation ($P < 0·001$) was observed between both parameters, regardless of the ice nucleation method used.

DISCUSSION

A quantitative tube nucleation test to determine the number of bacterial ice nuclei present in mango tissues is described in this paper, that may be applicable to any plant material. Assay conditions that would maximize the efficiency of the tube nucleation test were studied. The assay temperature could be variable; however, at -5°C the

sensitivity was enough to detect INA from suspensions of bacterial cultures. If the assay was carried out at −9°C, the detection of INA from natural samples was clearly increased (Table 2), but this could be due to interferences of ice nuclei from plant tissues (Hirano *et al.* 1985; Baertlein *et al.* 1992). However, the presence of active ice nuclei in leaves and buds of mango were always correlated with detection of *Ps. syringae* in these samples. Other workers have reported that nucleation events in leaves with no detectable INA bacteria do not occur at −5°C (Lindow *et al.* 1978b; Hirano *et al.* 1985) and they have selected this temperature for routine determination of bacterial INA (Lindow *et al.* 1982; Hirano and Upper 1986; Olive and McCarter 1988). In this study the optimum exposure time for the tube nucleation test carried out at −5°C was 10 min (Fig. 1).

Several authors have proposed tube nucleation procedures to detect bacterial ice nuclei in plants (Hirano *et al.* 1985; Lindow 1993), but generally, these assays are not quantitative. Montesinos and Vilardell (1991) have used a tube assay to estimate the number of ice nuclei on the basis of the reciprocal of the highest dilution factor of the tubes that froze. The tube nucleation test to quantify bacterial ice nuclei associated with plants developed here is based on the multiple-tube technique or most probable number (MPN) method used for the determination of coliforms from water (Anon. 1975). This method increased the statistical precision of the reciprocal tube method described by Montesinos and Vilardell (1991). The MPN method assumes that one tube froze if it contained at least one ice nucleus or *Ps. syringae* cell, because few if any bacterial cells contain more than one ice nucleus (Govindarajan and Lindow 1988). The MPN determinations from only three replicate series are relatively inefficient, but the multiple-tube test described in this work can be made more precise by increasing the number of tubes and/or the number of the dilutions assayed and also by the use of a computer program to perform the calculations for MPN (Klee 1993). The use of homogenized samples produced in a lab blender allowed greater detection of bacterial cells present in plant tissues than the standard washing procedures, which are less efficient (Lindow *et al.* 1978b; Fry *et al.* 1985).

Current assays used to estimate concentrations of ice nuclei in bacterial suspensions are basically variations of the drop-freezing method (Lindow *et al.* 1982; Hirano and Upper 1986), a procedure described by Vali (1971) and Lindow *et al.* (1978b). This method has also been the general method to quantify ice nuclei associated with plant material. The detection of INA was higher by the multiple-tube method described here than by drop-freezing technique, irrespective of the temperature. Furthermore, the tube test was more sensitive when fewer than 1000 ice nuclei per g fresh weight were present in samples (Table 2). This may be explained by the total amount of sample analysed by each technique, 3·3 g (33·3 ml of homogenized samples) by tube test compared to 0·02 g (20 droplets of 10 μl) by the drop-freezing method.

INA values determined by tube and droplet methods were very similar and estimated numbers of ice nuclei from bacterial suspensions and from natural samples were not significantly different. This confirms the accuracy of the multiple-tube test relative to the drop-freezing technique. Also, the tube assay presents improvements with respect to the classical method, since it is simpler and more reliable, allows routine analyses of larger amounts of sample. Furthermore, it could be adapted easily to check the number of ice nuclei in other plant species. The high correlation between the number of estimated ice nuclei and the number of cells of *Ps. syringae* enumerated on King B agar may be considered a validation of the efficiency of the multiple-tube test. Results presented here support the role of the epiphytic populations of *Ps. syringae* in mango trees as INA observed in buds and leaves. This is in agreement with other authors (Hirano *et al.* 1985; Marshall 1988; Montesinos and Vilardell 1991; Lindow 1993) who have related the epiphytic populations of *Ps. syringae* and its INA with the frost damage in plants.

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