# Relationships Among Population Levels of *Pseudomonas syringae*, Amount of Ice Nuclei, and Incidence of Blast of Dormant Flower Buds in Commercial Pear Orchards in Catalunya, Spain

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## ABSTRACT

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Flower bud necrosis of commercial pear trees during winter dormancy is the most destructive phase of bacterial blast of pear caused by *Pseudomonas syringae* in the fruit tree-growing area of Catalunya, Spain. Forty percent of strains of *P. syringae* isolated from buds were ice nucleation active (INA) and 70% were phytopathogenic to pear. Thirty-nine percent were both INA and phytopathogenic. They were capable of developing blast of dormant buds in the absence of cold depending on the inoculum dose. However, cold temperatures increased disease incidence in inoculated flower buds from cut branches forced to bloom. Winter populations of *P. syringae* in dormant flower buds ranged from less than $10^3$ to $10^8$ colony-forming units (cfu) per gram fresh weight. Ice nuclei (i.n.) active at −5 C were associated with populations of *P. syringae* during 1988 and 1989 and ranged from undetectable to about $10^5$ i.n./g fresh wt. Mean nucleation frequency was $3.2 \times 10^3$ cfu/ice nucleus. Mean nucleation temperatures ($NT_{50}$) of detached flower buds during tree dormancy ranged from −2.7 to −6 C and depended upon population levels of *P. syringae* and amount of ice nuclei. A significant relationship was found among the population levels of *P. syringae*, the amount of ice nuclei in dormant flower buds at the start of winter, and subsequent disease incidence measured as bud failure assessed in early March after several winter frosts. The threshold level producing a 5% disease incidence in six orchards and three cultivar types was $9.3 \times 10^2$ cfu/bud or 1 i.n./bud, and the slope of the log dose-log response curve was 0.2.

Bacterial blast of pear is a widespread disease that results in significant economic losses in fruit tree-growing areas around the world. The disease has been reported from England (8), Italy (15), Greece (51), France (37), New Zealand (13), South Africa (38), Chile (10), Canada (39), and the United States (14). Symptoms include blossom blast, leaf or tip necrosis, young fruit spotting, papyraceous cankers of bark, and blast of buds before budbreak or prebloom.

*Pseudomonas syringae* pv. *syringae* has been implicated as a causal agent of bacterial blast of pear. Its pathogenic properties and the mechanisms of invasion and damage to host tissues are partially known through laboratory studies. Hydrolytic enzymes, toxins, ice-nucleation-active (INA) proteins, and possibly plant hormones are involved in the disease process (18). However, their relative importance remains unknown. Much attention has been given to the role of ice nucleation activity in the development of plant damage because many diseases induced by *P. syringae* require or are favored by ice formation before disease development (34).

Flower bud necrosis of pear trees is a problem of economic importance in some deciduous fruit tree-growing areas of Catalunya, Spain. The most destructive phase occurs during tree dormancy. Symptoms start in late November and reach maximum development during March (40). The fact that they appear predominantly after frost periods suggests that INA bacteria may be implicated and that blast of dormant buds is an early stage of the disease process commonly known as bacterial blast of pear.

It is essential to develop threshold actions for decision making. To do so, the pathogen *P. syringae*, or its associated pathogenic factors, should be detected before disease symptoms occur, and different population levels should be quantitatively related to disease incidence. This may be accomplished by selecting sites at which naturally occurring pathogen populations differ or by means of combinations of inoculation and chemical control procedures (28,48,50,54). Population size and ice nucleation temperature have been found to affect frost injury in different kinds of plants and organs that have been artificially inoculated (22,23,33). However, dose-effect responses for disease epidemics of bacterial blast of dormant buds of pear trees were not included in any previous studies.

The objective of this work was to study the role of *P. syringae* as a causal agent of dormant bud necrosis of pear trees. This paper reports the characteristics of strains isolated from the gemmisphere, the effect of cold in combination with its pathogenic properties on disease development, and the quantitative relationship between the population size of the pathogen and subsequent disease incidence in commercial orchards of Catalunya, Spain.

## MATERIALS AND METHODS

**Study area and meteorological conditions.** This work was conducted in deciduous fruit tree-growing areas of Catalunya in the northeastern part of Spain. Commercial orchards were located near the Mediterranean coast (Emporda County) and in a 25-km land valley (Girones and La Selva counties). Weather parameters were monitored at the meteorological station of the Mas Badía Agricultural Experiment Station. Conditions were exceptionally mild; average temperature was 9 C from November 1988 to March 1989. However, starting in the middle of November 1988, several winter frosts occurred with air temperatures ranging between −2 and −4 C at 2 m from the surface level and between −5 and −9 C at soil surface.

**Measurement of bacterial populations and ice nuclei on flower buds.** During 1986–87, 35 orchards were surveyed two times in winter and spring. During 1988–89, six orchards and three cultivar

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types per orchard were sampled on 13 December, 3 January, 26 January, 22 February, and 13 March. A bulked sample of 10–20 g of buds (100–200 buds) was collected from 50 randomly selected trees for each cultivar type and orchard at each sampling date. Samples were placed in plastic bags, transported to the laboratory, and processed the same day of sampling. Ordinary surveying of population levels and ice nuclei (i.n.) was performed on two subsamples of approximately 5 g of buds. On December 1988, eight orchards of cultivar General Leclerc were sampled, and for each of them, 25 buds were processed individually to determine population levels per bud and ice nucleation temperatures.

Buds were cut across the longitudinal axis into four pieces to increase extraction efficiency of epiphytic bacteria located in inner parts. The pieces were placed in sterile 0.1% peptone water and vigorously shaken on a rotary shaker for 30 min. Approximately 10 ml of the wash solution was added per gram of plant material. The supernatant was used for 10-fold serial dilutions in sterile Ringer solution, which was used for determination of bacterial populations and numbers of ice nuclei. Bacterial populations were assessed by plating duplicate 50-μl samples onto King's B agar (25). Cycloheximide (100 mg/L) was added to all media to prevent fungal growth. Plates were incubated at 25 C for 2–3 days. Fluorescent colonies under ultraviolet light were tested for presence of oxidase. We noticed that up to 86% of the colonies which were fluorescent and oxidase negative pertained to *P. syringae*. Representative colonies of fluorescent *Pseudomonas* were selected and purified on King's B agar for further analysis.

To determine number of ice nuclei, triplicate serial dilution tubes were preincubated at 0 C in a refrigerator and subsequently incubated for 15 min in a refrigerated water-alcohol bath at −5 C. The reciprocal of the highest dilution factor of the tubes that froze was recorded, and results were expressed as the concentration of ice nuclei per gram fresh weight of bud tissue (52).

**Characterization and preservation of bacterial strains.** Fluorescent strains of *Pseudomonas* were tested for oxidase reaction, levan production, arginine dihydrolase, ice nucleation activity, and phytopathogenicity (52). All bacterial isolates were tested on the API 20E and 20 System tests (API System, S. A., Vercieu, France). Strains were preserved in a glycerol-mineral salts buffer and stored at −20 C (19).

**Determination of ice nucleation temperatures of detached flower buds.** Nucleation temperatures were determined by the tube nucleation test (23). The method consisted of exposing individual buds to decreasing temperatures. Each sample consisted of 25 flower buds. Each bud was cut into four pieces across the longitudinal axis and immersed in a test tube containing 10 ml of phosphate buffer equilibrated to −0.5 C. Temperature then was lowered successively by decrements of 0.5 C until −10 C. Tubes were maintained for 30 min at each equilibrated temperature, and the number of frozen tubes was recorded. The temperature at which 50% of flower buds froze ($NT_{50}$) was calculated from the cumulative percentage of frozen buds at each temperature.

**Pathogenicity tests.** Strains isolated from orchards were tested for phytopathogenicity by inoculating immature pear fruits. Immature pear fruits of the cultivar Passa Crassana (approximately 6 wk after fruit set) were surface disinfected by soaking them in a 0.5% Na-hypochlorite-Triton ×100 solution and then rinsing in distilled water according to Gross et al (20). Each pear fruit was injected four times with 10 μl of a bacterial suspension of $10^8$ cfu/ml using a microsyringe. Controls were injected with sterile potassium phosphate buffer. Fruits were incubated at 25 C for 3–5 days in plastic containers lined with moist paper towels.

**Infectivity titrations on dormant flower buds.** Pathogenicity tests on dormant flower buds were performed on detached branches forced to bloom in an environmental chamber. Branches with flower buds were taken from an orchard where *P. syringae* was undetected (less than $10^3$ cfu/g fresh wt. of bud). Branches, aged 2 yr, of 40- to 60-cm length, each containing 15–25 flower buds, were cut in February at stage of development A (dormant buds) described by Fleckinger (17). The cut ends were immersed immediately in water, transported to the laboratory, and stored at 2 C in the dark for about 4 wk. Before use, branches were surface disinfected, their base end pruned diagonally while submersed in water to avoid occlusion of vascular system by air, and placed in small containers with 100 ml of a sterilized 1% sucrose solution. The method used to force blooming was a modification of the method of Szkolnik and Hickey (53). Branches were placed in an environmental chamber at 22–25 C with a 12-hr photoperiod at 3,000 lx and 70–80% relative humidity. Periodically, the base end was pruned and the sucrose solution was replaced to avoid yeast growth and occlusion of the vascular system. Under these conditions, full bloom occurred in about 3 wk. Inoculations were performed by microinjection of 5 μl of a phosphate-buffered bacterial suspension of strain EUPG94 of *P. syringae* previously shown to be phytopathogenic and INA. The needle was carefully introduced into the bud through the distal end until half of its longitudinal axis was reached. Three branches and 40–60 buds per treatment were inoculated. A nested factorial design was used to determine the incidence of injured forced-to-bloom flower buds in relation to inoculation of *P. syringae* EUPG94 ($5 \times 10^6$ cfu/bud, buffer inoculated and uninoculated), cold temperature shocks of 12 hr (not applied, −2 and −4 C), and at two stages of development (dormant buds and swollen buds). In another experiment done on dormant buds, a nested design was used for the effect of dose of inoculum ($10^3$, $10^6$, and $10^7$ cfu/bud), and cold-temperature shock (not applied and −2 C). Disease incidence was estimated as the proportion of blasted or undeveloped buds per branch 10 and 25 days after inoculation.

**Assessment of disease incidence in orchards.** Disease incidence in commercial orchards from December 1988 until March 1989 was determined in situ as the proportion of diseased flower buds per tree. Generally, diseased buds appeared with the scales opened, dry, and with necrosis affecting the internal bractea and primordial flowers. Confirmation of damage occasionally required microscopic examination. A total of six orchards 5–7 yr old and of three cultivar types (Conference, Doyenne du Comice, and General Leclerc) were surveyed. For each cultivar type within a given orchard, 50 trees and two branches per tree (25–50 buds per branch) were selected at random, and all flower buds were examined.

## RESULTS

**Bacteria associated with dormant buds.** The epiphytic bacterial flora of buds was composed mainly of *Flavobacterium* spp., *Erwinia herbicola*, *Pseudomonas* spp., *Micrococcus* spp., and *Bacillus* spp. Fluorescent *Pseudomonas* spp. accounted for less than 0.01% to more than 90% of the total heterotrophic bacteria. Up to 100 strains of fluorescent *Pseudomonas* spp. were isolated at random from samples taken from several cultivars and 30 pear orchards during 1987. After species determination, they were characterized for ice nucleation activity and pathogenicity to pear. Among the fluorescent strains of *Pseudomonas*, 39% were *P. syringae*, 38% were *P. fluorescens*, 8% were *P. viridiflava*, and the remaining were unidentifiable. Among the oxidase-negative strains, 86% were *P. syringae*. Among the phytopathogenic strains of *Pseudomonas*, 78% were *P. syringae*, 4% were *P. viridiflava*, and the remaining were unidentified forms. Among INA strains, 95% were *P. syringae* and 5% were *P. viridiflava*. Seventy percent of the strains of *P. syringae* were phytopathogenic and 40% were INA. Only 39% of strains of *P. syringae* were found to be both INA and phytopathogenic to pear.

**Disease development on inoculated dormant buds forced to bloom.** Flower buds inoculated with a high dose of strain EUPG94 of *P. syringae* ($5 \times 10^6$ cfu and 50 i.n. at −5 C per bud) developed after 10 days a significantly higher proportion of injury than uninoculated or buffer-inoculated buds (Table 1). Moreover, among buds inoculated with strain EUPG94, higher injury levels were observed after exposure to cold-shock temperatures of −4 C than during continuous incubation at 20 C. After 25 days, injury was observed on controls inoculated with buffer, presumably due to damage by the needle, and on controls of swollen buds submitted

to −4 C. Another experiment consisted of inoculations of the same strain with three different inoculum levels ($10^3$, $10^6$, and $10^7$ cfu/bud) on flower buds in a dormant stage of development (Table 2). Continuous incubation at 20 C did not produce significant injury after 25 days for the dose of $10^3$ cfu/bud, compared with the buffered checks. However, the exposure to cold shock at −2 C significantly increased injury levels for all doses of inoculum in comparison with continuous incubation at 20 C.

**Relationships among population levels of *P. syringae*, amount of ice nuclei, and nucleation temperatures of dormant flower buds in orchards.** A significant relationship between the log of pathogen populations and the log of numbers of ice nuclei active at −5 C in dormant buds was observed for the data collected during the winter and spring of 1988 and 1989 (Fig. 1). The analysis indicated that slope of the regression lines was about 1 (0.88 in 1988 and 1.04 in 1989) and that the intercept (when $\log_{10}$ i.n./g fresh wt. = 0) was approximately 3.5 (3.74 in 1988 and 2.92 in 1989). Because the intercepts correspond to the number of bacteria necessary to develop an ice nucleus, an average of $3.2 \times 10^3$ bacteria was needed to act as an ice nucleus at −5 C under field conditions.

Cumulative percentages of flower buds frozen at decreasing temperatures differed among orchards depending on population sizes (Fig. 2). On 12 December 1988 in orchard A, population levels and amount of ice nuclei were about 100,000-fold higher than in orchard F. Ninety percent of buds from orchard A contained populations above $10^4$ cfu/g fresh wt.; only 20% of buds from orchard F exceeded this value and ice nuclei were not detected at −5 C. The temperature at which 50% of dormant flower buds froze ($NT_{50}$) was −2.7 C for orchard A and −6 C for orchard F. Nucleation events started at about −2 C in orchard

TABLE 1. Incidence of injured forced-blossomed flower buds at two different stages of development on cut branches after exposure to *Pseudomonas syringae* and cold temperature

| | Treatment | | | |
|---|---|---|---|---|
| | Cold shock[d] | | Disease incidence[a,b] | |
| Inoculation[c] | Temperature (C) | Stage[e] | 10 | 25 |
| UI | na[f] | A | 0.02 c | 0.04 e |
| UI | −2 | A | 0.06 c | 0.03 e |
| UI | −2 | C | 0.04 c | 0.07 e |
| UI | −4 | A | 0.05 c | 0.12 d |
| UI | −4 | C | 0.01 c | 0.85 b |
| BI | na | A | 0.07 c | 0.19 e |
| BI | −4 | A | 0.06 c | 0.27 d |
| I | na | A | 0.87 b | 0.86 b |
| I | −2 | A | 0.80 b | 0.78 c |
| I | −2 | C | 0.71 b | 0.73 c |
| I | −4 | A | 1.00 a | 1.00 a |
| I | −4 | C | 1.00 a | 1.00 a |

[a]Disease incidence was assessed on triplicate branches containing 15 to 25 buds. It was determined 10 and 25 days after starting the experiment and expressed as the proportion of injured buds per branch.
[b]Means followed by the same letter do not differ significantly ($P = 0.05$) according to the Student-Kneuman-Keuls test. The $F$ number for the different treatments was 118 for disease assessment 10 days after inoculation and 47 for disease assessment 25 days after inoculation.
[c]UI = uninoculated; BI = inoculated with sterile buffer phosphate; I = inoculated with strain EUPG94 of *P. syringae*. Average inoculum concentration was $5 \times 10^6$ colony-forming units/bud and 50 ice nuclei at −5 C/bud.
[d]Cold temperature shock was applied after inoculation. Exposure time was 12 hr.
[e]A = dormant buds; C = swollen buds.
[f]na = cold shock not applied; branches maintained continuously at 20 C.

TABLE 2. Infectivity titration of *Pseudomonas syringae* and effect of a 12-hr exposure to cold shock at −2 C on dormant flower buds from cut branches of pear cultivar Conference forced to bloom

| | Treatment | | |
|---|---|---|---|
| Inoculation[a] | Cold shock temperature (C) | Dose (colony-forming units/bud) | Disease incidence[b,c] |
| BI | na[d] | ··· | 0.21 d |
| BI | −2 | ··· | 0.17 d |
| I | na | $10^3$ | 0.20 d |
| I | na | $10^6$ | 0.52 c |
| I | na | $10^7$ | 0.59 c |
| I | −2 | $10^3$ | 0.55 c |
| I | −2 | $10^6$ | 0.88 ab |
| I | −2 | $10^7$ | 0.91 a |

[a]BI = inoculated with sterile buffer phosphate; I = inoculated with strain EUPG94 of *P. syringae*.
[b]Disease incidence was assessed on triplicate branches containing 15 to 25 buds. It was determined 25 days after starting the experiment and expressed as the proportion of injured buds per branch.
[c]Means followed by the same letter do not differ significantly ($P = 0.05$) according to the Student-Kneuman-Keuls test. The $F$ number for the different treatments as 7.4.
[d]na = cold shock not applied; branches maintained continuously at 20 C.

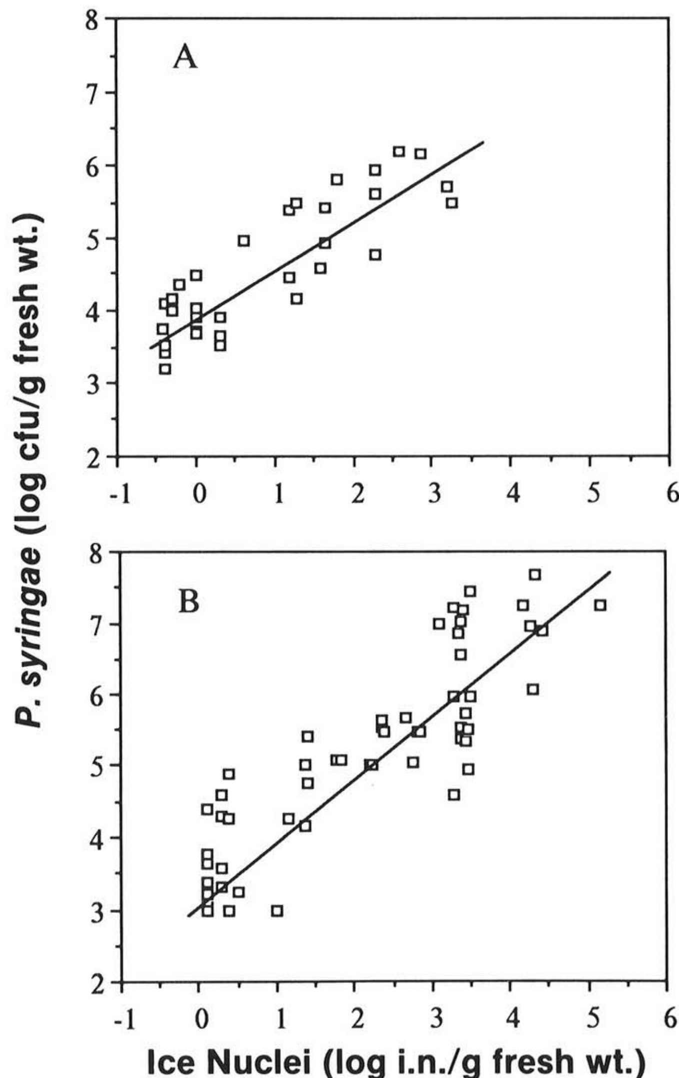


**Fig. 1.** Relationship between population levels of *Pseudomonas syringae* and numbers of ice nuclei (i.n.) active at −5 C on dormant flower buds of pear trees. The lines represent the regressions of $\log_{10}$ (colony-forming units [cfu] per gram fresh weight) against $\log_{10}$ (−5 C i.n./g fresh wt.). The data correspond to: **A**, samples from March 24, 1988, from diseased trees of an orchard of pear cultivar Passa Crassana (log cfu/g fresh wt. = 0.88 log i.n./g fresh wt. + 3.74, $r = 0.857$, $P < 0.01$) and **B**, samples taken during the period of December 1988 to March 1989 from eight commercial orchards, including cultivars General Leclerc, Conference, and Dovenne du Comice (log cfu/g fresh wt. = 1.04 log i.n./g fresh wt. + 2.92, $r = 0.966$, $P < 0.01$).

A, whereas a decrease to −4.5 was needed for a significant number of tubes to freeze in orchard F. $NT_{50}$ was inversely related with the mean population size of *P. syringae* for the eight commercial orchards surveyed at the end of December 1988 (Fig. 3).

**Disease incidence in orchards after several frosts in relation to winter populations of *P. syringae* and amount of ice nuclei.** Based on local information, 35 orchards were classified either as having repeated outbreaks of blast of dormant buds year after year or as never or only occasionally affected. Among orchards with a history of disease during December 1986 to April 1987, about 50% of samples exceeded $10^5$ cfu/g fresh wt., and the mean $\log_{10}$ population level of *P. syringae* was 4.49 ± 1.36 cfu/g fresh wt. In orchards that never or only occasionally had been affected, only 5% of samples surpassed $10^5$ cfu/g fresh wt., and the mean $\log_{10}$ population was 3.14 ± 0.91.

Winter population levels of *P. syringae* for December 1988 to April 1989 depended on the orchard (Fig. 4). Orchards A and B, which had problems in the past, had a higher number of ice nuclei and population levels of *P. syringae* than orchards C (occasionally affected) and F (not diseased). Disease symptoms appeared after the first November 1988 frosts and were severe during the first week of March 1989 in orchards that traditionally harbored high populations of *P. syringae*.

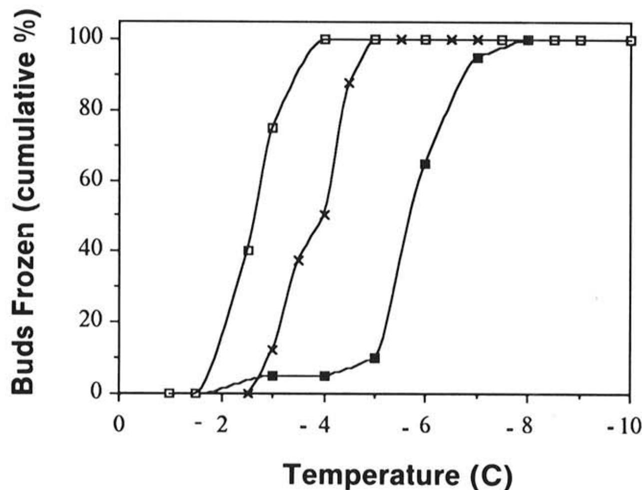


**Fig. 2.** Cumulative percent of frozen buds as a function of termperature during an exposure time of 30 min. Samples are from pear cultivar General Leclerc and were taken from different affected orchards. Orchards A (□) and B (x) were diseased in the past. Orchard F (■) was not diseased.

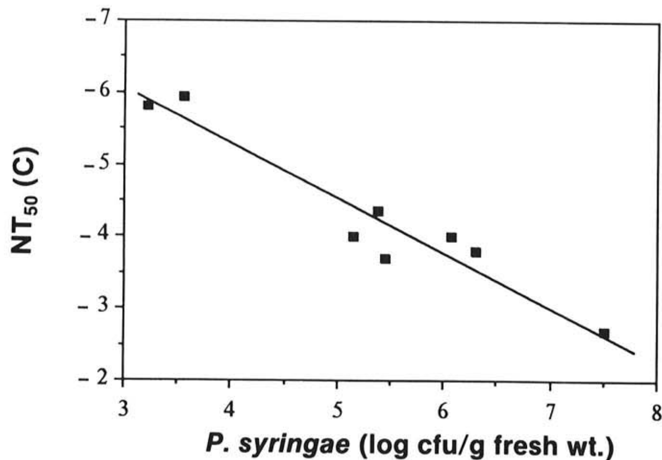


**Fig. 3.** Relationship between the mean nucleation temperature ($NT_{50}$) of detached dormant buds and the mean population size of *Pseudomonas syringae* in eight orchards containing pear cultivar General Leclerc. The data correspond to December 1988. Regression equation: $NT_{50} = 0.76$ $\log_{10}$ colony-forming units (cfu)/g fresh weight − 8.33, $r = 0.96$, $P < 0.01$.

A direct relationship was found between log colony-forming units per gram fresh weight or log ice nuclei per gram fresh weight assessed on December 1988 or January 1989 and the subsequent disease incidence observed in March 1989 for combined data of six orchards and three cultivars (Fig. 5). However, this relationship was not linear. The regression of the log of disease incidence on the log of population size ($r = 0.888$, $P < 0.01$) had a slope of 0.20. The regression of the log of disease incidence on the log of amount of ice nuclei ($r = 0.882$, $P < 0.01$) had a slope of 0.19. The threshold levels for a 5% disease incidence calculated from the regression lines were $9.8 \times 10^3$ cfu/g fresh wt. and 10 i.n./g fresh wt. of bud tissue.

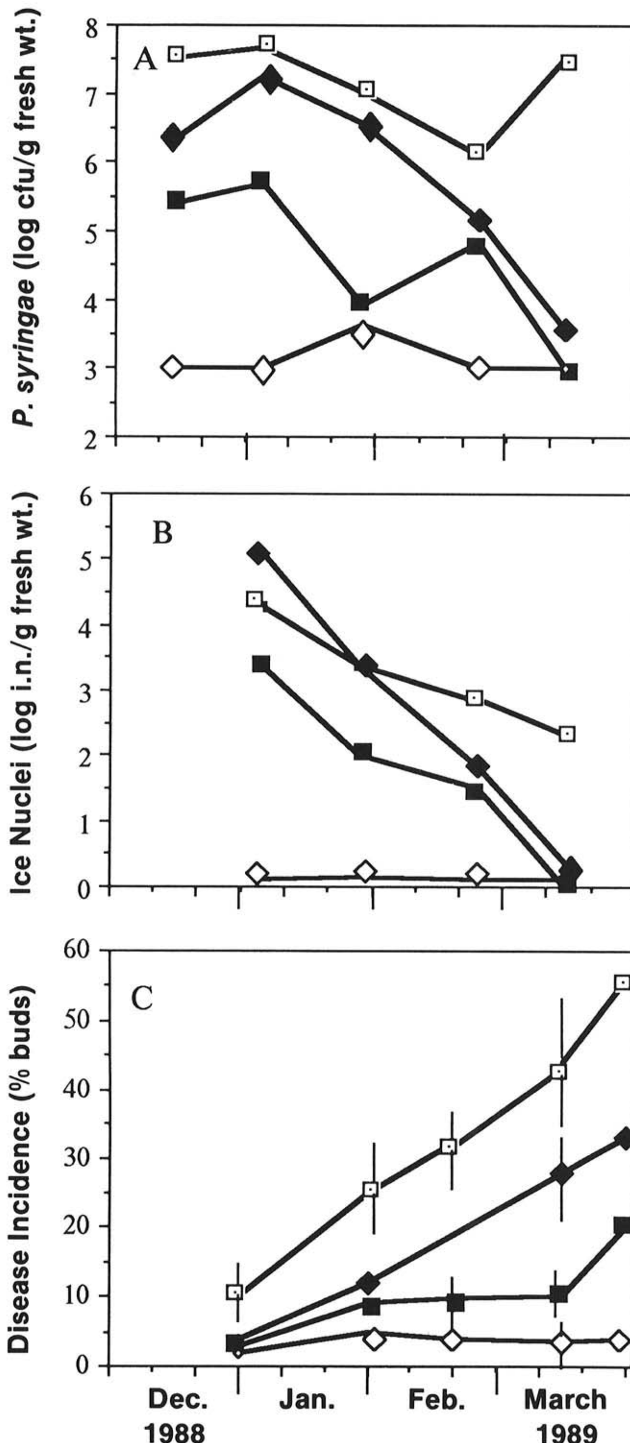


**Fig. 4.** Dynamics of: **A,** population levels of *Pseudomonas syringae*, **B,** amount of ice nuclei (i.n.), and **C,** disease incidence for pear cultivar General Leclerc in four representative orchards with varying histories of disease incidence. Orchards A (□), B (◆), and C (◆) were diseased in the past. Orchard F (□) was not diseased. cfu = colony-forming units.

## DISCUSSION

Bacterial blast of pear caused by infections of *P. s. syringae* has been described as producing usually blossom blast and tip, fruit, or leaf necrosis during spring (14,37,38). However, blast of dormant buds is the most destructive phase of the disease in certain Mediterranean countries (16,40).

Because the incidence of lesions in diseased orchards of Catalunya was observed mainly in dormant buds, composition and characteristics of the microflora associated with the gemmisphere environment during winter dormancy received highest priority. The microflora of gemmisphere of commercial pear trees in Catalunya was composed mainly of *Flavobacterium* spp., *E. herbicola*, *P. fluorescens*, and *P. syringae*. Similarly, the epiphytic strains of *Pseudomonas* isolated from woody plants (11), fruit trees (12,19), and cereals or vegetable plants (24) are strains of *P. fluorescens* and *P. syringae*. The fact that 40% of our isolates of *P. syringae* showed ice nucleation activity is in agreement with previously reported data because at least half of the pathovars of *P. syringae* from various origins have the capacity to act as ice nucleating agents (20,30,38,41). The observation that up to 39% of our isolates of *P. syringae* are both INA and phytopathogenic to pear suggests that some strains that incite frost injury may benefit from this action for subsequent tissue invasion.

The population levels of *P. syringae* and the amount of ice nuclei on dormant flower buds in the orchards we studied were considerably high. This finding contrasts with the work of Gross et al (19,22) in which extensive winter surveys of deciduous fruit trees did not detect resident INA bacteria (detection level 10 cfu/g fresh wt.). However, most of the winter populations of "epiphytic" *P. syringae* that we detected in buds resided in the inner scales (Montesinos and Vilardell, *unpublished data*). This finding agrees with previous observations of overwintering of *P. syringae* in apple and pear buds (9,16,49) and with other reports stressing the importance of buds as protected positions for many pathogenic and nonpathogenic epiphytic bacteria in plants (26,27). This may explain why, in the orchards that we studied, standard bactericide sprays applied during fall, winter, or spring (prebloom stages) have a low efficiency in control of incidence of internal bud necrosis and do not significantly decrease populations of the pathogen in dormant buds (Montesinos and Vilardell, *unpublished data*).

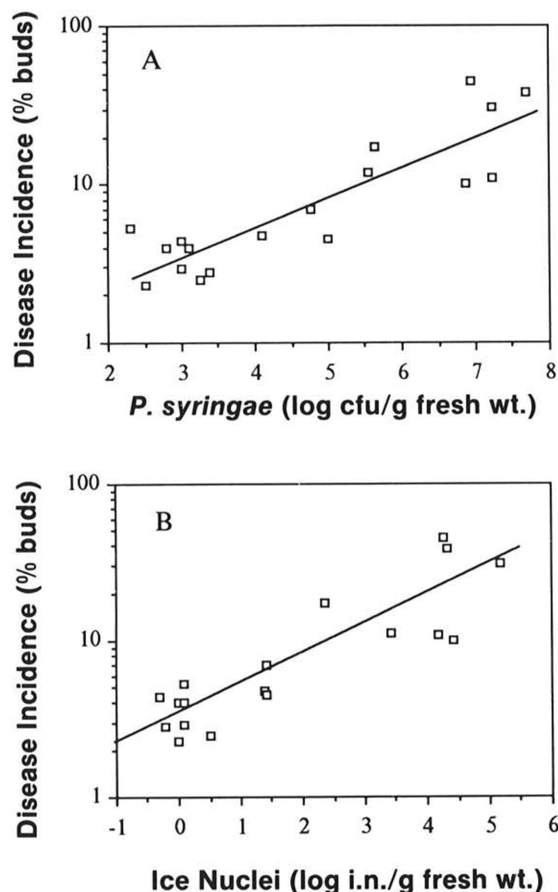


**Fig. 5.** Relationships among $\log_{10}$ flower bud population size of *Pseudomonas syringae* (**A**), $\log_{10}$ numbers of ice nuclei (i.n.) active at −5 C (**B**) assessed on December 1988, and mean disease incidence per tree in six commercial orchards (three cultivar types per orchard), determined the first week of March 1989. Regression equations were log disease incidence (DI) = 0.202 log colony-forming units (cfu)/g fresh weight − 0.103 ($r = 0.89$, $P < 0.01$) and log DI = 0.189 log i.n./g fresh weight + 0.507 ($r = 0.88$, $P < 0.01$).

A significant relationship between population levels of *P. syringae* and the amount of ice nuclei within dormant buds has been found for 1988 and 1989. This finding suggests that most of the ice nuclei detected may be of bacterial origin. The mean log nucleation frequency (log ice nuclei per colony-forming unit) observed for 95 samples was −3.5; namely, an average of $3.2 \times 10^3$ bacterial cells was needed to develop an ice nucleus at −5 C. Our results for dormant flower buds are consistent with the mean nucleation frequencies of −4.5 obtained by other researchers with isolated strains under laboratory growth conditions (22,34,35) and mean nucleation frequencies of −2 to −4 obtained from field samples of different plant material and origin (19,23). Although a single threshold value for nucleation frequency is difficult to establish because it is influenced by strain, growth temperature, and medium composition (36), it seems that, under field conditions and on an average basis, about $10^3$ bacterial cells are needed to develop an ice nucleus at −5 C in the orchards that we studied.

The nucleation temperatures of detached dormant flower buds of pear trees in this study depended on the population level of *P. syringae*. Several researchers have reported similar results on oat (23) and bean leaves (32) and on inoculated peach flower buds (22). It also has been described that nucleation temperatures are influenced by sample mass (1,4–6). However, in our case, orchard differences of 3.3 C in the $NT_{50}$ of dormant flower buds could not be attributed to differences in bud weight because the variability was low (0.10–0.12 g fresh wt.). Ice nucleation sites of nonbacterial origin occur within the woody stem tissues of *Prunus*, *Pyrus*, and *Malus* spp. and have been reported to be active within the range of nucleation temperatures promoted by INA bacteria (19,22). In spite of this evidence, we did not attempt to measure nucleation temperatures of attached dormant flower buds because experimental evidence indicates that wood-associated ice nuclei probably do not affect nucleation temperatures of dormant flower buds and are not found in detached buds, flowers, or leaves. A xylem discontinuity layer in the base of primordium tissues of dormant flower buds has been associated with avoidance of freezing injury in cold-acclimated *Prunus* species capable of deep supercooling (7). Therefore, in case of ice formation within the xylem, this barrier may prevent its propagation from the vascular system into the bud primordium tissues. The presence of this barrier makes dormant buds behave like detached buds. The xylem discontinuity disappears during spring deacclimation at budbreak, and the resulting flowers, shoots, and fruitlets supercool only to −1.2 to −3.8 C (3,5,42). Consequently, the presence of INA bacteria within buds during tree dormancy may govern its capacity of supercooling.

There was also a significant relationship between disease incidence after several winter frosts and the preceding population levels of *P. syringae* and amount of ice nuclei in buds. This finding is supported by the work of Lindow (29,31) and Lindow et al (36) which found a log-linear relationship between the size of INA bacterial population and the degree of spring frost injury. Also these studies reported that frost protection can be achieved by reducing INA bacterial populations on fruit trees. However, these studies contrast with direct measurements of frost injury on flower clusters, fruitlets, and shoots of fruit trees during spring vegetative growth under a wide range of population levels of INA bacteria (2,4,21,22,42). In these reports, there is evidence

that the unidentified indigenous wood-associated source of ice nuclei appears to govern frost susceptibility in fruit tree systems after budbreak, thus masking the effect of INA bacteria on nucleation termperature of these organs (22).

After all, cold-acclimated dormant flower buds and stems of peach and pear can avoid freezing by supercooling to near −20 C (44,47). Acclimated dormant flower buds avoid lethal intracellular ice formation in primordium tissues by increasing concentration of solutes, resulting in a decrease in their nucleation temperature (46). Gross et al (22) showed in *Prunus* flower buds that during the frost-tolerance phase (prebloom stages) temperatures of −8 to −10 C were required to give about 50% injury. When freezing occurs, it appears to be initiated in the scales and bud axes, and once it affects bud primordia, the whole bud is killed as a unit (45).

The lowest temperature recorded for several winter frosts that occurred during our study did not exceeded −4 C of minimum air temperature. Although it was sufficient to permit expression of ice nucleation activity in buds from some orchards having high populations of *P. syringae*, it may not be enough to kill properly acclimated dormant flower buds. Therefore, the relationships we observed among blast of dormant flower buds, population levels of *P. syringae*, and amount of ice nuclei could be explained only if the pathogen has a high potential to invade and develop necrosis of primordium tissues by conventional attack (exoenzymes, toxins, etc.) regardless of the expression of ice nucleation activity, and if there are some plant-associated factors (for example, improper cold acclimation or bud differentiation) that predispose dormant buds to freezing injury. We have shown that inoculation of dormant buds with doses comparable to those observed in the field, but under conditions that do not permit expression of bacterial ice nuclei, is sufficient to produce significant disease incidence. However, once primordium tissues become freeze damaged by expression of bacterial ice nuclei, subsequent invasion by resident *P. syringae* may be favored. This was shown during infectivity titration experiments with strain EUPG94 by the existence of a significant increase in injury on inoculated dormant buds after exposure to cold-shock treatment.

A threshold level for significant incidence of blast of dormant buds under field conditions could be derived from our data. Assuming that the mean fresh weight of a dormant bud for the different cultivar types was 0.1 g, the effective doses producing 5% disease ($ED_5$) calculated from the regression equations were 1 i.n./bud and $9.8 \times 10^2$ cfu/bud. The effective doses producing 50% disease ($ED_{50}$) were $2.0 \times 10^4$ i.n./bud and $8.3 \times 10^7$ cfu/bud. However, these data contrast with those from reports of other diseases caused by *P. syringae*. Rouse et al (50) on bacterial brown spot of beans obtained a slope of the log disease-log dose curve of 0.175, comparable to the value of 0.195 that we obtained, but the $ED_5$ and $ED_{50}$ were different: $10^2$ cfu/leaflet and $4 \times 10^5$ cfu/leaflet, respectively. This difference could be due to differences in strain virulence or in plant or organ material or to the fact that their data refer to colony-forming units of INA *P. syringae*. In our case the data are for colony-forming units of *P. syringae* regardless of whether or not they are INA.

Until now, only Lindow and co-workers have presented supportive data that INA bacteria are involved in freezing processes of fruit trees and that their control can significantly protect them from injurious spring frosts (29–36). However, simulated frost analysis done by Ashworth and co-workers (2,4) and field evaluations of frost injury done by Gross and co-workers (22,42,43) showed little or no involvement of INA bacteria in spring mild frost injury. Their work also demonstrated that the intrinsic wood-associated ice nuclei are the initial and primary source of ice nucleation in fruit trees during spring frosts.

Our results showed that INA bacteria that overwinter inside dormant buds may limit the buds' supercooling capacity and may induce ice formation which in turn favors primordium tissue invasion and subsequent blast development. The establishment of action thresholds based on the amount of ice nuclei and population levels of *P. syringae* will help provide predictive systems to be used in management programs for control of blast of dormant buds.

## LITERATURE CITED


1. Anderson, J. A., and Ashworth, E. N. 1985. Ice nucleation in tomato plants. J. Am. Soc. Hortic. Sci. 110:291-296.
2. Andrews, P. K., Proebsting, E. L., Jr., and Gross, D. C. 1983. Differential thermal analysis and freezing injury of deacclimating peach and sweet cherry reproductive organs. J. Am. Soc. Hortic. Sci. 108:755-759.
3. Ashworth, E. N. 1984. Xylem development in *Prunus* flower buds and the relationship to deep supercooling. Plant Physiol. 74:862-865.
4. Ashworth, E. N., Anderson, J. A., and Davis, G. A. 1985. Properties of ice nuclei associated with peach trees. J. Am. Soc. Hortic. Sci. 110:287-291.
5. Ashworth, E. N., and Davis, G. A. 1984. Ice nucleation within peach trees. J. Am. Soc. Hortic. Sci. 109:198-201.
6. Ashworth, E. N., Davis, G. A., and Anderson, J. A. 1985. Factors affecting ice nucleation in plant tissues. Plant Physiol. 79:1033-1037.
7. Ashworth, E. N., and Rowse, D. J. 1982. Vacular development in dormant *Prunus* flower buds and its relationship to supercooling. HortScience 17:790-791.
8. Barker, B. T. P., and Grove, O. 1914. A bacterial disease of fruit blossom. Ann. Appl. Biol. 1:85-97.
9. Burr, T. J., and Katz, B. H. 1984. Overwintering and distribution of *Pseudomonas syringae* pv. *papulans* in apple buds. Plant Dis. 68:383-385.
10. Cancino, L., Latorre, B., and Larach, W. 1974. Pear blast in Chile. Plant Dis. Rep. 58:568-570.
11. Canfield, M. L., Baca, S., and Moore, L. W. 1986. Isolation of *Pseudomonas syringae* from 40 cultivars of diseased woody plants with tip dieback in Pacific Northwest nurseries. Plant Dis. 70:647-650.
12. Dowler, W. M., and Weaver, D. J. 1975. Isolation and characterization of fluorescent pseudomonas from apparently healthy peach trees. Phytopathology 65:233-236.
13. Dye, D. W. 1956. Blast of pear. Orchardist N. Z. 29:5-7.
14. English, H., DeVay, J. E., and Ogawa, J. M. 1980. Bacterial canker and blast of deciduous fruits. Univ. Calif. Leafl. 2155.
15. Ercolani, G. L. 1967. Aspetti di *Pseudomonas syringae* nei frutteti emiliani. Inf. Fitopatol. 17:205-213.
16. Ercolani, G. L. 1969. Sopravivenza di *Pseudomonas syringae* van Hall sul pero in rapporto all'epoca della contaminazione in Emilia. Phytopathol. Mediterr. 8:207-216.
17. Fleckinger, J. 1965. Stades repérés des Pomacées. Les champignons parasites des arbres fruitiers à pepins. Coll. G. Viennot-Bourginn, I.
18. Gross, D. C., and Cody, Y. S. 1985. Mechanisms of plant pathogenesis by *Pseudomonas* species. Can. J. Microbiol. 31:403-410.
19. Gross, D. C., Cody, Y. S., Ice, E. L., Radamaker, G. K., and Spotts, R. A. 1983. Distribution, population dynamics, and characteristics of ice nucleation-active bacteria in deciduous fruit tree orchards. Appl. Environ. Microbiol. 46:1370-1379.
20. Gross, D. C., Cody, Y. S., Proebsting, E. L., Radamaker, G. K., and Spotts, R. A. 1984. Ecotypes and pathogenicity of ice-nucleation-active *Pseudomonas syringae* isolated from deciduous fruit tree orchards. Phytopathology 74:241-248.
21. Gross, D. C., and Proebsting, E. L., Jr. 1984. The role of ice nucleation-active *Pseudomonas syringae* in frost injury to floral buds of deciduous fruit trees. Pages 34-36 in: Proc. Working Group *Pseudomonas syringae* Pathovars, 2nd. Hellenic Phytopathological Society, Athens, Greece. 117 pp.
22. Gross, D. C., Proebsting, E. L., Jr., and Andrews, P. K. 1984. The effects of ice nucleation-active bacteria on temperatures of ice nucleation and freeze injury of *Prunus* flower buds at various stages of development. J. Am. Soc. Hortic. Sci. 109:375-380.
23. Hirano, S. S., Baker, L. S., and Upper, C. D. 1985. Ice nucleation temperature of individual leaves in relation to population sizes of ice nucleation active bacteria and frost injury. Plant Physiol. 77:259-265.
24. Hirano, S. S., Nordheim, E. V., Arny, D. C., and Upper, C. D. 1982. Lognormal distribution of epiphytic bacterial populations on leaf surfaces. Appl. Environ. Microbiol. 44:695-700.
25. King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44:301-307.
26. Leben, C. 1965. Epiphytic microorganisms in relation to plant disease. Annu. Rev. Phytopathol. 3:209-230.
27. Leben, C. 1988. Relative humidity and the survival of epiphytic

bacteria with buds and leaves of cucumber plants. Phytopathology 78:179-185.
28. Lindemann, J., Arny, D. C., and Upper, C. D. 1984. Use of an apparent infection threshold population of *Pseudomonas syringae* to predict incidence and severity of brown spot of bean. Phytopathology 74:1334-1339.
29. Lindow, S. E. 1982. Population dynamics of epiphytic ice nucleation active bacteria on frost sensitive plants and frost control by means of antagonistic bacteria. Pages 395-416 in: Plant Cold Hardiness and Freezing Stress: Mechanisms and Crop Implications. Vol. 2. P. H. Li and A. Sakai, eds. Academic Press, New York.
30. Lindow, S. E. 1982. Epiphytic ice-nucleation active bacteria. Pages 335-362 in: Phytopathogenic Prokaryotes. Vol. 1. M. S. Mount and G. H. Lacy, eds. Academic Press, New York.
31. Lindow, S. E. 1983. Methods of preventing frost injury caused by epiphytic ice-nucleation-active bacteria. Plant Dis. 67:327-333.
32. Lindow, S. E. 1986. Construction of isogenic ice strains of *Pseudomonas syringae* for evaluation of specificity of competition on leaf surfaces. Pages 509-515 in: Proc. I.S.M.E. Symp., 4th.
33. Lindow, S. E. 1987. Competitive exclusion of epiphytic bacteria by Ice-*Pseudomonas syringae* mutants. Appl. Environ. Microbiol. 53:2520-2527.
34. Lindow, S. E., Arny, D. C., and Upper, C. D. 1982. Bacterial ice nucleation: A factor in frost injury to plants. Plant Physiol. 70:1084-1089.
35. Lindow, S. E., and Connell, J. H. 1984. Reduction of frost injury to almond by control of ice nucleation active bacteria. J. Am. Soc. Hortic. Sci. 109:48-53.
36. Lindow, S. E., Hirano, S. S., Barchet, W. R., Arny, D. C., and Upper, C. D. 1982. Relationship between ice nucleation frequency of bacteria and frost injury. Plant Physiol. 70:1090-1093.
37. Luisetti, J., and Paulin, J. P. 1972. Etudes sur les bactérioses des arbres fruitiers. III. Recherche du *Pseudomonas syringae* (Van Hall) à la surface des organes aériens du poirier et étude de ses variations quantitatives. Ann. Phytopathol. 4:215-227.
38. Mansvelt, E. L., and Hattingh, M. J. 1986. Pear blossom blast in South Africa caused by *Pseudomonas syringae* pv. *syringae*. Plant Pathol. 35:337-343.
39. McKeen, W. E. 1955. Pear blast in Vancouver Island. Phytopathology 45:629-632.
40. Montesinos, E., and Vilardell, P. 1988. El desecamiento bacteriano del peral producido por *Pseudomonas syringae*. Fruticultura Profesional 18:56-59.
41. Paulin, J. P., and Luisetti, J. 1978. Ice nucleation activity among phytopathogenic bacteria. Pages 725-731 in: Proc. Int. Conf. Plant Pathog. Bact., 4th, Angers, France.
42. Proebsting, E. L., Jr., Andrews, P. K., and Gross, D. C. 1982. Supercooling young developing fruit and floral buds in deciduous orchards. HortScience 17:67-68.
43. Proebsting, E. L., Jr., and Gross, D. C. 1988. Field evaluations of frost injury to deciduous fruit trees as influenced by ice-nucleation-active *Pseudomonas syringae*. J. Am. Soc. Hortic. Sci. 113:498-506.
44. Quamme, H. A. 1974. An exothermic process involved in freezing injury to flower buds of several *Prunus* species. J. Am. Soc. Hortic. Sci. 99:315-317.
45. Quamme, H. A. 1978. Mechanism of supercooling in overwintering peach flower buds. J. Am. Soc. Hortic. Sci. 103:57-61.
46. Quamme, H. A., and Gusta, L. V. 1987. Relationship of ice nucleation and water status to freezing patterns in dormant peach flower buds. HortScience 22:465-467.
47. Rajashekar, C., Westwood, M. N., and Burke, M. J. 1982. Deep supercooling and cold hardiness in genus *Pyrus*. J. Am. Soc. Hortic. Sci. 107:968-972.
48. Ramos, A. H. 1979. Bacterial blight of coffee: The inoculum supply and avenues of infection. Plant Dis. Rep. 63:6-10.
49. Roos, I. M. M., and Hattingh, M. J. 1986. Pathogenic *Pseudomonas* spp. in stone fruits buds. Phytophylactica 18:7-9.
50. Rouse, D. I., Nordheim, E. V., Hirano, S. S., and Upper, C. D. 1985. A model relating the probability of foliar disease incidence to the population frequencies of bacterial plant pathogens. Phytopathology 75:505-509.
51. Sarejanni, J. A., Démétriadès, S. D., Zachos, D. G., and Papaïonnou, A. I. 1953. Rapport sommaire sur les principales maladies des plantes observés en Grèce au cours de l'année 1952. Ann. Inst. Phytopathol. Benaki 7:5-10.
52. Schaad, N. W., ed. 1988. Laboratory Guide for Identification of Plant Pathogenic Bacteria. 2nd ed. American Phytopathological Society, St. Paul, MN.
53. Szkolnik, M., and Hickey, K. D. 1986. Testing chemical sprays on blossoms of deciduous fruit trees. Pages 112-115 in: Methods for Evaluating Pesticides for Control of Plant Pathogens. K. D. Hickey, ed. American Phytopathological Society, St. Paul, MN.
54. Teng, P.S., ed. 1987. Crop Loss Assessment and Pest Management. American Phytopathological Society, St. Paul, MN.