trifoliate orange appear promising. Trees on these stocks have performed well in other experiments (7, 8) and merit continued evaluation. Their lower yields in comparison to the larger trees could be compensated for by closer planting. Even though the trees were growing in a relatively fertile soil with a high organic matter content, a 10 or 12 x 20 ft spacing might have been more appropriate for the smaller trees.

Two commercially important rootstocks, Carrizo and Swingle, were included in this experiment. Trees on these 2 stocks appeared to be well-adapted to the local soil environment and performed similarly except for cold tolerance. As previously observed (10), trees on Swingle were more cold tolerant than those on Carrizo.

Lemons are not likely to be a major crop in Florida. Nevertheless, it is evident that lemon tree vigor under Florida conditions can be reduced through the use of selected rootstocks. Furthermore, the relative rootstock effects observed in this study may be similar with other scion cultivars.

Acknowledgments

Appreciation is extended to the Coca Cola Company, Foods Division who provided a site and care for the experiment. Michael Patterson of Coca Cola, assisted in planting the experiment and Dr. R. L. Phillips selected and propagated the plant material.

Literature Cited

1. Bitters, W. P., D. A. Cole, and C. D. McCarty. 1977. Citrus relatives are not irrelevant as dwarfing stocks or interstocks for citrus. Proc. Intern. Soc. Citriculture 2:561-567.
2. Boswell, S. B., R. M. Burns, and H. Z. Hield. 1976. Inhibition effects of localized growth regulator sprays on mature lemon trees. HortScience 11:115-117.
3. Bryan-Jones, J. and D. J. Finney. 1983. On an error in "Instructions to authors". HortScience 18:279-282.
4. Burns, R. M., S. B. Boswell, and H. Z. Hield. 1976. Effects of growth inhibitors on Ventura lemon trees. Citrograph 61(10):335, 356, 382.
5. Burns, R. M., S. B. Boswell, and S. F. Wear. 1981. Growing lemons and grapefruit at an angle. Citrograph 66(9):217, 218.
6. Carpenter, J. B., R. M. Burns, and R. F. Sedlacek. 1981. Phytophthora resistant rootstocks for lisbon lemons in California. Citrograph 66(12):287, 291, 292.
7. Castle, W. S. 1980. Citrus rootstocks for tree size control and higher density plantings in Florida. Proc. Fla. State Hort. Soc. 93:24-27.
8. Castle, W. S. and R. L. Phillips. 1980. Performance of 'Marsh' grapefruit and 'Valencia' orange trees on eighteen rootstocks in a closely spaced planting. J. Amer. Soc. Hort. Sci. 105:496-499.
9. Wear, S. F. and J. D. Kirkpatrick. 1974. The sleeping beauties: horizontal lemon plantings. Citrograph 60(1):7, 8, 32.
10. Yelenosky, G. 1976. Cold-hardening young 'Valencia' orange trees on 'Swingle' citrumelo (CPB-4475) and other rootstocks. Proc. Fla. State Hort. Soc. 89:9-10.

Proc. Fla. State Hort. Soc. 96:25-28. 1983.

BACTERIA-INDUCED FREEZING OF YOUNG CITRUS TREES[1]

GEORGE YELENOSKY
U.S. Department of Agriculture, Agricultural Research Service, 2120 Camden Road, Orlando, FL 32803

Additional index words. supercooling, freeze avoidance.

Abstract. **Leaves of 'Valencia' trees** (Citrus sinensis **(L.) Osb.) on sour orange** (C. aurantium **L.) rootstock that were sprayed with ice nucleation-active (INA) bacteria started to freeze sooner than unsprayed leaves during freeze trials in a controlled-temperature room. Leaves sprayed with INA bacteria usually started to freeze before 23°F (−5°C), whereas unsprayed leaves remained in a supercooled freeze-avoidance state. Moisture on the leaves during freezing temperatures increased the risk of early freezing. Neither antibacterial streptomycin sprays nor non-INA bacteria prevented the freezing associated with INA bacteria. Nonbacterial INA agents also were found to cause earlier freezing in citrus leaves. Data suggest that INA agents play an active role in minimizing supercooling (freeze avoidance) in agricultural crops during freezes in Florida, but do not yet support concerns that INA bacteria are the major cause of freezing in citrus tissues during natural freezes. Additional and more detailed studies are needed to determine whether eliminating or controlling INA bacteria will significantly reduce freeze damage to citrus in Florida.**

There are bacteria in citrus plantings that decrease supercooling (cooling below 32°F (0°C) without ice forming) and cause early freezing in plant tissues (1, 9). These ice nucleation-active (INA) bacteria are strains of *Pseudomonas syringae* van Hall and *Erwinia herbicola* (Löhnis) Dye that induce ice to form at temperatures as warm as 30°F (−1°C). This small amount of supercooling precludes any beneficial effects of supercooling in avoiding damage in citrus plantings during natural freezes. Other researches (8, 12) have suggested that early freezing due to INA bacteria results in longer durations of ice in citrus tissues, which induces greater freeze damage. Citrus seemingly is not injured until ice forms in the tissues.

The importance of supercooling is not firmly established in citrus groves, but supercooling is probably the most energy-efficient freeze-avoidance mechanism that assures plant survival (4, 7). Supercooling is largely an unexplained and unpredictable event in the freezing of plants. Factors such as degree of vascular development (2), diameter of xylem vessels (3), and heterogenous nucleators (12) apparently play a role. Temperature-and/or water stress-induced cold-hardening regimes increase supercooling in citrus (14, 17, 20). But, supercooling does not segregate unhardened cold-hardy citrus types (18). The elimination or control of INA populations of bacteria supposedly would allow supercooling to develop and result in less freeze damage to citrus. Bactericides and antagonistic non-INA bacteria are 2 approaches to control INA bacteria below critical population levels (8).

The purpose of this study was to determine the activity of INA bacteria and other INA agents in freezing of citrus during controlled-temperature regimes. Interest was in determining frequency of freezing, critical temperatures, effectiveness of bactericides, and potential problem areas in citriculture.

[1]Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that also may be suitable.

Materials and Methods

Test trials were varied to include different situations and INA agents. Trees were 'Valencia' orange (*Citrus sinensis*) budded on 10-month-old sour orange (*C. aurantium*) rootstock grown from open-pollinated seed. Individual trees were grown in 2.5-liter plastic pots containing equal parts of sphagnum peat moss, vermiculite, and perlite, and were watered as needed under natural light in a greenhouse. Maximum light approached 1 x 10^3 microeinsteins/m^2/sec (PAR) with maximum temperature 95°F (35°C) and minimum 35% relative humidity during days, and minimum 68°F (20°C) and maximum 97% relative humidity during nights. Trees of uniform growth and appearance were arbitrarily selected for tests 8 to 10 months after budgrafting.

INA bacteria were slant cultures of *P. syringae* and *E. herbicola* and lypholized *P. syringae* (courtesy of Dr. S. Lindow, Univ. California, Berkeley) and frozen broth cultures of *E. herbicola* (courtesy of Microlife Technics, Sarasota, FL 33578). Slant cultures stored at 41°F (5°C) were periodically subcultured on nutrient agar containing 2.5% glycerol. Bacteria populations were grown in nutrient broth with continuous agitation at 86°F (30°C) for 8 hr and stored at 39°F (4°C) for 20 hr. Turbid broth cultures were centrifuged at 5,000 X *g* and bacteria pellets suspended in either 150 ml sterile water, 0.1 M phosphate buffer (pH 7), or in nutrient broth. Spray concentrations were based on pour plate dilutions. Estimates of bacterial populations prior to test sprays were determined by immersing single leaves, 3 to 5 per tree, each into 10 ml of sterile H_2O, shaking for 30 min on a vortex shaker, and making a series of pour plate dilutions. Similar samples were taken after test sprays immediately before freeze tests. Frozen broth cultures of *E. herbicola* (Microlife Technics) were stored at −76°F (−60°C) and contained 2 x 10^{10} colony-forming units (CFU) per ml. These were diluted as needed with sterile water. Non-INA bacteria (coded, and also from Microlife Technics) were treated similarly and used in conjunction with agricultural streptomycin sulfate (17% streptomycin) to inhibit INA bacteria. Lyopholized *P. syringae* at 3.6 x 10^{10} active ice nuclei per gram was used in 1% (w/v) freshly prepared aqueous suspensions. Similar suspensions were nonbacteria INA agents silver iodide, phenazine, and flurophlogopite (a synthetic mica, courtesy of Dr. C. Rajashekar, Univ. Minnesota, St. Paul).

Spray volumes per tree ranged from 15 ml to runoff. Spraying prior to freeze tests ranged from 0.5 to 36 hr under greenhouse conditions, between 8 and 10 AM. Freezes were in dark and 50 ± 5% relative humidity in controlled-temperature facilities (17). Induced freezing was determined visually as watersoaking in the leaves (19). The moments of freezing or threshold temperatures were based on instant release of latent heat of fusion during exothermic scans using 36-gauge, copper-constantan thermocouples. Thermocouple leads were connected to digital multimeters (1 uv per digit resolution). Accuracy was rated ± 1 digit, less than 2 sec settling time. Reference junction was an insulated ice bath stable at 0 ± 0.2°F. Variable speed strip-chart recorders, 0 to 100 mv, were connected to digital multimeters.

Results and Discussion

In general, trees sprayed with relatively high concentrations of INA bacteria started to freeze before unsprayed trees. Earliest freezing induced was at 28°F (−2.2°C). The most noticeable differences in tests with INA isolates from California were in trials with *Pseudomonas syringae* (Fig. 1). In these instances, the effectiveness of the bacteria was evident only when citrus leaf surfaces were allowed to dry after sprays and before freeze tests. Freezing of citrus leaves with wet surfaces was similar regardless of INA bacteria. This reaffirms that frozen moisture on leaf surfaces is a very efficient ice nucleator. Leaves with dry surfaces supercooled to 24°F (−4.4°C), whereas wet leaves started to freeze at 28°F (−2.2°C). The number of trees starting to freeze after 15 min at 24°F averaged 1 tree every 6 min for INA bacteria-sprayed trees, and 1 tree every 18 min for control trees. This 3-fold frequency increase attributed to INA bacteria is considered significant in the freezing of citrus trees at a constant temperature. All of the 16 trees with INA bacteria were freezing after 2 hr at 24°F in contrast to 31% of the trees that were not sprayed with INA bacteria. Experience suggests that most of the remaining 69% of unfrozen trees would start to freeze with longer duration at 24°F and some trees would supercool to 22°F (−5.5°C), and even to 20°F (−6.7°C) (16). Freeze avoidance at temperatures lower than 20°F is infrequent and unpredictable in unhardened citrus trees. It is yet unclear whether such observations reflect INA agents such as bacteria on leaf surfaces and/or heterogenous nucleators within the cells of citrus. Similar results to those in Fig. 1 were also evident with *Erwinia herbicola* isolates from California (data not shown).

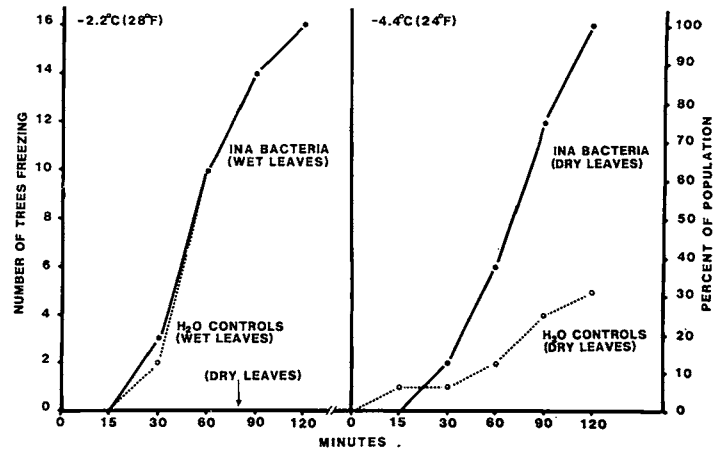

Fig. 1. Rate of freezing of 8-month-old potted 'Valencia' orange trees on sour orange rootstock with and without *Pseudomonas syringae* (California isolate) ice-nucleation-bacteria (INA) (4 x 10^7 CFU/ml) during 28°F and 24°F freezes for 2 hr each in controlled-temperature tests with leaves wet and dry after 100 ml spray/tree 0 and 15 hr, respectively, before free freeze trials.

Erwinia herbicola from frozen broth cultures was an effective INA agent when concentrations reached 2 x 10^8 CFU/ml and 15 ml were sprayed on each tree. Three days after such sprays in a greenhouse, none of 15 trees sprayed with *E. herbicola* escaped injury after 3 hr at 24°F (−4.4°C). Leaf kill was essentially complete at 98 ± 2%. In contrast, 12 of 15 trees sprayed with water and 13 of 15 trees sprayed with media escaped injury, and the few trees that were injured averaged less than 20% leaf kill. We were not able to inhibit the activity of INA *E. herbicola* with a strain (M232A) of a non-INA antagonistic bacterium. Concentrations of 2 x 10^8 CFU/ml used alone on trees did not show any ice-nucleating activity, and activity of INA *E. herbicola* was similar with or without the non-INA strain regardless if sprayed immediately before or after INA *E. herbicola* sprays. All *E. herbicola*-sprayed trees were freezing after 3 hr at 26°F (−3.3°C), while only 1 out of every 10 control trees was freezing after 5 hr. It is postulated by other researchers (8) that non-INA antagonistic bacteria would help keep INA populations below critical levels through natural competition for leaf surface area that otherwise would be colonized by INA strains. Supposedly, only 0.1 to 10% of the bacteria found on plant leaf surfaces harbor ice nuclei adequate to

induce early freezing. In our work, sufficient time may not have lapsed to adequately colonize the non-INA strain on leaf surfaces, or greenhouse conditions favored growth of INA *E. herbicola* over the non-INA strain.

We were also unable to reduce the nucleation activity of *E. herbicola* with antibiotic sprays of agricultural streptomycin sulfate (200 ppm of active ingredient) applied 1 hr after INA bacteria sprays. Two days after such sprays, all 15 *E. herbicola*-sprayed trees as well as 15 trees with *E. herbicola* followed by streptomycin sprays were freezing after 4 hr at 24°F (−4.4°C). Most of the control trees, 53% to 60%, remained supercooled and avoided freeze damage. The failure to reduce the activity of INA *E. herbicola* with streptomycin suggests dead cells continue to harbor effective ice nuclei although dead cells no longer contribute additional nuclei to existing populations. Living cells apparently are not needed for INA bacteria to induce early freezing in plant tissues, but cell disruption is critical in some instances (10). Wind and rainfall would help dislodge dead bacteria from leaves of trees in the field, which would help to control INA populations below critical levels. Whether INA bacteria would penetrate stomates and thus be less vulnerable to antibiotic sprays and removal poses yet another possible obstacle in population control.

Our results with lyopholized INA *Pseudomonas syringae* reinforces the supposition that dead INA bacteria cells serve as effective ice nucleators. Lyopholized INA *P. syringae* competed favorably with some of the most effective nonbacteria INA agents used in our work (Table 1). Lyopholization may have some economic advantages in the preparation, storage, and use of INA bacteria. Phenazine, which competes favorably with silver iodide as an ice nucleator in cloud seeding (5) and is produced as a secondary metabolite in fluorescent pseudomonads (*P. syringae*), can be manipulated with stress and media nutrition (6). Phenazine concentrations may play a role in the effectiveness of INA bacteria. Flurophlogopite, a synthetic mica found by other researchers (11) to have superior ice-nucleating activity, was also very effective in our work. Lyopholized INA bacteria probably could substitute as an INA agent to nucleate water in plastic bags (Table 2), in order to protect lower stems of young citrus trees with tree wraps during severe freezes (15).

Both INA bacteria and nonbacterial agents froze equally well on the top and underside of citrus leaves in freezing trials using single drops of aqueous suspensions of INA agents. Droplets froze as early as 28°F (−2.2°C), but watersoaking was observed sooner and was more evident on the underside than on top of leaf surfaces. Frequency of initial watersoaking at 24°F (−4.4°C) for 1 hr averaged 83% when drops were suspended on the underside and only 23% when drops were on the top surfaces of leaves. No differences in moment of freezing were apparent regardless of leaf surface position. Apparently, the cuticular layer offers some resistance to ice nuclei on the top surfaces of citrus leaves and/or stomates provide a favorable niche for ice nucleation.

Additional support for flurophlogopite as an effective ice nucleator was evident in test trials using drops of aqueous suspensions of INA agents not on leaf surfaces (Table 3). The supercooling level for flurophlogopite averaged somewhere between 28°F (−2.2°C) and 29°F (−1.7°C). Other agents averaged lower than 25°F (−3.9°C) with water supercooling to 21°F (−6.1°C). Sucrose, proline, and expressed sap from leaves are all implicated in cold hardening of citrus trees (13) and seemingly promote freeze avoidance with supercooling approaching 18°F (−7.8°C) for 1% (w/v) proline.

Results of this study express the ice nucleation activity of both INA bacteria and nonbacteria agents and the vulnerability of citrus leaves to different INA agents. Data indirectly support concerns that heterogenous nucleators contribute to freeze damage of citrus trees during natural freezes. But as yet, there is insufficient data to support concerns that INA bacteria are largely responsible for freezing of citrus trees in Florida. Major problem areas remain in determining and identifying critical populations of INA

Table 1. Number of 10 8-month-old 'Valencia' orange trees starting to freeze at different temperatures with and without ice nucleation active (INA) agents sprayed on the leaves to runoff before the freeze.

Agent applied	Concn	Temperature (°F) 28	26	24 (2-hr duration)	22	20	Total no. of plants
Erwinia herbicola	2 X 10^8 CFU[z]/ml	0	2	8	—	—	10
Pseudomonas syringae (lyopholized)	1% (w/v)	0	1	5	4	—	10
Silver iodide	1% (w/v)	0	2	6	2	—	10
Phenazine	1% (w/v)	0	0	4	6	—	10
Flurophlogopite	1% (w/v)	0	1	4	5	0	10
Nutrient broth	1% (v/v)	0	0	0	3	7	10
Water	—	0	0	0	4	6	10
None	—	0	0	0	2	8	10

[z]CFU = colony forming units.

Table 2. Freezing of 100 ml of water in plastic bags with ice nucleation active (INA) agents.

Agent applied	Concn	Amount per bag	No. of bags	Lowest nucleation (°F)[z]	Percentage of bags nucleated
Erwinia herbicola	(2 x 10^8 CFU/ml)	1 ml	7	28.0	100
Silver iodide		10 g	7	28.2	100
Phenazine		10 g	7	28.2	100
Nutrient media		1 ml	7	21.0	0
Water		—	7	21.0	0

[z]Temperature decrease of 7°F per hr from 35°F to 21°F.

Table 3. Moment of freezing in 0.05-ml droplets with and without ice nucleation active (INA) agents.[z]

Comparison no.	Agent	Concn	Moment of freezing (°F)	ΔT
1	Flurophlogopite (INA)	1% (w/v)	28.2	−7.0**
	Water	000 MOSM	21.2	
2	*Erwinia herbicola* (INA)	2 x 10^8 CFU/ml	26.1	−4.7**
	Water	000 MOSM	21.4	
3	Lyopholized *Pseudomonas syringae* (INA)	1% (w/v)	26.1	−4.7**
	Water	000 MOSM	21.4	
4	Silver iodide (INA)	1% (w/v)	25.1	−4.1**
	Water	000 MOSM	21.0	
5	Phenazine (INA)	1% (w/v)	25.1	−4.7**
	Water	000 MOSM	21.4	
6	Nutrient broth	1% (v/v)	21.3	−0.0
	Water	000 MOSM	21.3	
7	Sucrose	1% (w/v)	20.0	1.3**
	Water	000 MOSM	21.3	
8	Proline	1% (w/v)	18.1	3.2**
	Water	000 MOSM	21.3	
	Expressed sap			
9	Unhardened 'Valencia' leaves	424 MOSM	21.4	−0.1
	Water	000 MOSM	21.3	
10	Cold-hardened 'Valencia' leaves	642 MOSM	19.0	2.0**
	Water	000 MOSM	21.0	

[z]Comparison of means, n = 5; ** significant at 1% level, temperature decrease 9°F per hr, continuous temperature monitoring of drops on a suspended 45.7 cm^2 x 0.6 mm thick copper sheet in controlled-temperature facility used to freeze whole plants.

agents in citrus groves, the advantages and reliability of bactericides in freeze protection, the role of antagonistic non-INA bacteria, and the amount of supercooling and/or freeze avoidance in citrus groves. Considerably more definitive studies on INA agents are needed in the field, along with basic work on the mode and mechanisms of freezing in citrus trees and fruit during natural freezes.

Literature Cited

1. Anderson, J. A., D. W. Buchanan, R. E. Stall, and C. B. Hall. 1982. Frost injury of tender plants increased by *Pseudomonas syringae* van Hall. J. Amer. Soc. Hort. Sci. 107:123-125.
2. Asworth, E. N. and D. J. Rowse. 1982. Vascular development in dormant *Prunus* flower buds and its relationship to supercooling. HortScience 17:790-791.
3. Bilanski, W. K. and R. C. Tzeng. 1980. Supercooling as related to diameter of conducting vessels in plants. Trans. Amer. Soc. Agr. Eng. 23:1334-1336.
4. George, M. F., M. R. Becwar, and M. J. Burke. 1982. Freezing avoidance by deep undercooling of tissue water in winter-hardy plants. Cryobiology 19:628-639.
5. Head, R. B. 1962. Ice nucleation by α-phenazine. Nature 196:736-738.
6. Leisinger, T. and R. Margraff. 1979. Secondary metabolites of the fluorescent pseudomonads. Microbiol. Rev. 43:422-442.
7. Levitt, J. 1980. Avoidance of freezing. p. 119-125. *In:* Responses of Plants to Environmental Stress, 2nd ed. Academic Press, New York.
8. Lindow, S. E. 1983. Methods of preventing frost injury caused by epiphytic ice-nucleation-active bacteria. Plant Dis. 67:327-333.
9. Lindow, S. E., D. C. Arny, and C. D. Upper. 1982. Bacterial ice nucleation: A factor in frost injury to plants. Plant Physiol. 70: 1084-1089.
10. Maki, L. R. and K. J. Willoughby. 1978. Bacteria as biogenic sources of freezing nuclei. J. Applied Meteorol. 17:1049-1053.
11. Rajashekar, C., P. H. Li, and J. V. Carter. 1983. Frost injury and heterogeneous ice nucleation in leaves of tuber-bearing *Solanum* species. Plant Physiol. 71:749-755.
12. Yankofsky, S. A., Z. Levin, and A. Moshe. 1981. Association with citrus of ice-nucleating bacteria and their possible role as causative agents of frost damage. Current Microbiol. 5:213-217.
13. Yelenosky, G. 1982. Seasonal variations in physiological factors implicated in cold hardiness of citrus trees. p. 561-573. *In:* P. H. Li and A. Sakai (eds.). Plant Cold Hardiness and Freezing Stress, Mechanisms and Crop Implications, Vol. 2. Academic Press, New York.
14. Yelenosky, G. 1979. Water-stress-induced cold hardening of young citrus trees. J. Amer. Soc. Hort. Sci. 104:270-273.
15. Yelenosky, G., 1979. Self-heating tree wraps in freeze protection. Proc. Fla. State Hort. Soc. 92:25-27.
16. Yelenosky, G. 1979. The potential of citrus to survive freezes. Proc. Intern. Soc. Citriculture 1:199-203.
17. Yelenosky, G. 1975. Cold hardening in citrus stems. Plant Physiol. 56:540-543.
18. Yelenosky, G. and G. Horanic. 1969. Subcooling in wood of citrus seedlings. Cryobiology 5:281-283.
19. Young, R. and A. Peynado. 1967. Freezing points and water-soaking in citrus leaves. Proc. Amer. Soc. Hort. Sci. 91:157-162.
20. Young, R. and A. Peynado. 1965. Changes in cold hardiness and certain physiological factors of Red Blush grapefruit seedlings as affected by exposure to artificial hardening temperatures. Proc. Amer. Soc. Hort. Sci. 86:244-252.