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The Effect of Leaf Water Variables on Ice Nucleating *Pseudomonas syringae* in Beans

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Abstract. **Pinto bean seedlings 'UI 114' (*Phaseolus vulgaris* L.) were subjected to temperatures between −2° and −5°C for periods ranging from 0.5 to 12 hr. The plants that were not sprayed with a suspension of the nucleating *Pseudomonas syringae* bacteria and those that were water-stressed to near wilting were most resistant to ice nucleation. Plants with dry leaf surfaces were much more apt to supercool than those with distilled water droplets on their leaves, whether inoculated with the bacteria or not. Spraying the freeze-dried bacteria suspended in distilled water on the leaves increased wettability and dew formation on the leaf surfaces. Tests with an oxytetracycline preparation, which also increased wetting, suggested that a hydrophobic leaf surface helps delay ice formation. Use of wetting agents in leaf sprays may be counterproductive so far as supercooling stability is concerned. It is obvious that leaf water relations interact with bacterial ice nucleation.**

Plants with tender growing tissue often survive mild freezes through supercooling of their internal water (4, 8). If the plant surfaces are relatively free of ice-nucleating bacteria, the chances of supercooling are greatly enhanced (7). Under field conditions, surfaces are seldom entirely free of ice-nucleating bacteria, but populations may be low enough to favor supercooling rather than ice nucleation (5). It has been shown that dew on leaves increases the likelihood of ice nucleation (1, 2, 4). The purpose of this experiment was to ascertain the effect of selected leaf water variables on ice-nucleating activity of *P. syringae* on bean leaves of pinto cv. UI-114.

Beans were grown in a growth chamber under 14 hr of light at 24°C and 10 hr of darkness at 15°. A peat moss, sand, and vermiculite mixture was used in the pots, which contained three seedlings each. After the first trifoliolate expanded, the seedlings were moved to the greenhouse for treatment and then subjected to controlled freezing temperatures in a chamber. All studies were completed before the 2nd trifoliolate was fully expanded. With the exception of the controls in the first four trials listed in Table 1, all the seedlings were sprayed with an aqueous suspension of freeze-dried *P. syringae* 16 to 24 hr before the freezing test. The suspension contained 4 mg of freeze-dried powder per 100 ml of water, or about 3.8×10^5 active ice nuclei per ml.

Some trials included antibiotics sprayed on both sides of the leaves several hours before the freeze. The concentrations were 2.2 g Terramycin (oxytetracycline hydrochloride injectable solution) and 0.5 g of a mixture of penicillin and streptomycin suspended in 500 ml of water. The antibiotics were also used individually as 2 g of Terramycin in 250 ml of water or 0.5 g of the penicillin with streptomycin in 250 ml. Plants were put in the dark so that the stomates closed before placing them in the freezing chamber, where the temperature was lowered from 0°C over the first hour to the desired level. When the desired minimum temperature was reached, some leaves were misted with distilled water to simulate dew formation. Otherwise, the leaf surfaces remained dry. When a significant number of the leaves showed dark green, water-soaked ice spots, the freeze treatment was ended. Several days after freezing, the number of plants that showed tissue damage was recorded. In two instances, the freeze was so severe that almost all plants showed some tissue damage, so the fresh weight of the tops in each pot was recorded several days after the freeze. The fresh weights were inversely proportional to the freeze damage because the damaged cells dried and reduced the water content of the tops.

All treatments, including controls, contained 12 pots with three plants each. Treatment differences were determined with a nonpaired sample *t* test.

The results of the freezing trials are summarized in Table 1. Both inoculation with the freeze-dried bacteria and free water standing on the leaf increased the risk of ice formation in the plants at −2.6°C (trials 1 and 2). Trials 3 and 4 were designed to evaluate possible causes for the difference between wet and dry leaf surfaces. With the stomata open, the underside of the first trifoliolates was sprayed with a fine mist of the freeze-dried suspension using enough pressure to cause the water to enter the internal leaf space. This was evidenced by a darkening of the tissue. The leaves' appearance returned to normal after a few hours as the water was absorbed from the internal air spaces. The control was treated in the same manner, except distilled water was forced into the underside of the trifoliolates. There was no difference in ice nucleation. In trial 4, we tested the inoculation of a cut leaf edge against controls in which the leaf edge was cut but not inoculated. Inoculation was performed by dipping the freshly cut leaf edge in the freeze-dried suspension about 14 hr before the freezing trial. The cut edge inoculation only slightly increased the nucleation of ice compared to the control. If the stability of the dry leaves in trial 2 was due to lack of liquid pathways between the nucleation sites and the bulk of the internal plant water, many more of the treated plants

Fig. 1. Droplets of water condensed on a bean leaf in the same spot where a drop of the freeze-dried bacteria suspension had been left to dry one day earlier.

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Table 1. Results of the freezing trials expressed as the average number of seedlings per pot that nucleated ice, or the average fresh weight per pot in grams of the remaining plant tops. P-S is penicillin and dihydrostreptomycin while T indicates terramycin.

					Treated[z]		Control[x]	
Trial	Treatment	Leaf surface	Mean temp (°C)	Duration (hrs)	No. of seedlings/ pot	Weight of tops (g)	No. of seedlings/ pot	Weight of tops (g)
1.	Inoculated[z]	Wet	−2.6	0.75		7.1**		11.1
2.	Inoculated	Dry	−2.6	12	1.7**		0.1	
3.	Internal inoculation	Dry	−2.0	4.75	0.1		0.1	
4.	Cut inoculation	Dry	−1.8	4.5	0.2*		0	
5.	Water-stressed	Dry	−5.0	0.5	1.7*		2.7	
6.	Water-stressed	Wet	−2.0	2.5	0.1*		0.9	
7.	P-S and T	Wet	−2.0	1.5		10.6		10.7
8.	P-S and T	Dry	−4.2	1.0	1.5**		0.3	
9.	P-S	Dry	−5.0	0.5	2.0		1.8	
10.	T	Dry	−5.0	0.5	2.1		1.8	

[z]Treatment differences significant at the 95% (*); or 99% (**) confidence levels, respectively.
[x]Control plants in trials 1, 2, 3, and 4 were the only ones in the study that were not inoculated.

in trials 3 and 4 should have been damaged similar to those in the "wet surface" controls.

Trials 5 and 6 were to see whether internal leaf water content was also a factor in the supercooling. Soil water was withheld until the seedlings were visually water-stressed. In treatment 5, the control plant top water content was 86.92% compared to 85.95% for the stressed. Treatment 6 was 88.56% vs. 86.04%. The water-stressed plants proved less apt to freeze than the controls. Psychrometer measurements of the osmotic pressure of the cell sap of the driest leaves was never greater than 14 bars. This pressure corresponds to a freezing point depression of about 1.1°C. Therefore, the reduction of the freezing point of the sap was never great enough to prevent freezing at the minimum air temperatures used (3).

It has been shown that antibiotics may reduce the number of ice-nucleating bacteria and thus improve the chances of the plant water supercooling (6, 7). In this study there was no significant effect of the antibiotic treatment on wet leaves (trial 7). The antibiotic mixture actually increased freezing on dry leaves (trial 8). This effect prompted a more detailed investigation. Ten milliliters of the freeze-dried suspension was placed in test tubes with either 5 ml of penicillin and dihydrostreptomycin or 5 ml of the Terramycin solution. The test tubes were then submerged in a −3°C water bath and the resultant supercooling observed. The Terramycin was obviously effective in promoting supercooling and the distilled water controls nearly always remained unfrozen. On the other hand, the mixture of the freeze-dried cells, penicillin, and dihydrostreptomycin behaved no differently than the rapid nucleation that occurred in the untreated freeze-dried bacteria suspensions.

The penicillin and dihydrostreptomycin did not promote supercooling (trial 9), and, in contrast to the test tubes in a water bath, neither did the Terramycin (trial 10). The Terramycin solution acted as a wetting agent for the leaf, while the penicillin and dihydrostreptomycin antibiotic did not. Inoculation and colonization of the leaf with *P. syringae* also increased the wettability of the leaves and promoted rapid condensation of dew (Fig. 1). In this instance, a single drop of the freeze-dried suspension was placed on the leaf, allowed to dry, and then, 24 hr later, the leaf was cooled below the dewpoint and the rapid condensation of water occurred as shown. Water also condensed more or less over all of the leaf surface, but these droplets were too small to see without magnification. When the dry leaf was misted with distilled water, droplets were adsorbed at the point where the inoculation with the freeze-dried material occurred, indicating a preferential wetting of that area. *P. syringae* does produce compounds in culture that are hydroscopic, and rinsing the leaf with distilled water did not overcome the tendency of water to condense on the spots that were inoculated. It seems likely that the anomaly shown in trial 8 and the failure of Terramycin in trial 10 to promote supercooling was caused by a wetting agent in the Terramycin antibiotic that changed the water relations of the leaf surface. This effect would support Thomas and Barber's (9) suggestion that water repellency may help reduce leaf freezing during mild frost conditions of −2° or −3°C.

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