Ecology and Epidemiology

Antagonistic Activity of An Isolate of *Candida* Species to Ice Nucleation-Active *Pseudomonas syringae*

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ABSTRACT

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An epiphytic yeast, an isolate of *Candida* sp. that was not active in ice nucleation, reduced the frost damage of corn (*Zea mays* L.) seedlings incited by an ice nucleation-active (INA) isolate of *Pseudomonas syringae*. The effectiveness of the antagonist in reducing frost damage increased with increasing antagonist cell density. When corn seedlings were sprayed with a mixed suspension of antagonist and INA bacteria in nutrient broth, both of the organisms multiplied together for 2 days and then maintained their populations nearly constant until 4 days after application. The presence of the antagonist did not affect the growth of coexisting INA bacteria on corn leaves. Nevertheless, frost damage incited by INA bacteria was greatly reduced by the antagonist even in the case of plants to which the antagonist had been applied 2 days after application of INA bacteria.

Epiphytic populations of ice nucleation-active (INA) bacteria exist on plant leaves in nature and are responsible for inciting frost damage to plants where they reside (9). A non-INA strain of *Erwinia herbicola* (Löhnis) Dye, antagonistic to INA bacteria, showed a significant protecting effect against frost damage of corn seedlings incited by INA bacteria (12). Lindow and co-workers reported that chemical mutants deficient in ice nucleation activity gave protection from frost damage under field conditions (8,11,13). Ice nucleation-deficient [INA(−)] mutant strains of *Pseudomonas syringae* van Hall and *P. fluorescens* competed with an INA wild type on blossoms of greenhouse-grown strawberry plants. The INA(−) strain of *P. syringae* protected blossoms against freezing by other INA(+) strains of *P. syringae* but did not inhibit or protect against INA(+) *P. fluorescens*. In addition, the INA(−) strain of *P. fluorescens* was much more effective as an inhibitor of strains of *P. syringae* than of other strains of *P. fluorescens* (7). Lindow also found that genetically engineered INA deletion mutants were effective for plant frost control in greenhouse tests (10). Recently, greenhouse evaluation on young corn plants showed that some strains of *P. fluorescens* and *P. putida* significantly suppressed the INA bacterial population (3). These bacterial antagonists might compete with native INA bacteria for nutrients in water films on leaf surfaces, and/or antibiosis might be important in interactions of microorganisms on leaves. This report deals with the protecting effect of corn seedlings against frost damage incited by the INA bacteria by an antagonistic yeast that is possibly present as epiphytes on frost-sensitive plants in Korea.

MATERIALS AND METHODS

The antagonistic non-INA yeast strain was obtained from washings of a field-grown Chinese quince leaf in late June 1985.

The yeast grew well in nutrient broth, with an optimum growth temperature of 25 C, and had a broad pH spectrum (5.0–9.0) for growth. The mean generation time was 8.4 hr. Important characteristics of the yeast, by which it was identified as *Candida* sp., included a globose to ovoid shape with a size of 2.5–3.5 × 3.0–4.5 μm; reproduction by multilateral budding; formation of a pseudomycelium; absence of true mycelium; possible presence of chalmydospore; absence of ascus. The yeast also gave a positive reaction for nitrate assimilation and for fermentation of glucose, galactose, maltose, and sucrose. It also assimilated galactose, sucrose, maltose, trehalose, D-xylose, D-mannitol, succinic acid, and citric acid. The following tests were negative: growth at 37 C, hydrolysis of urea; acid production; fermentation of lactose and raffinose; and assimilation of lactose, L-arabinose, rhamnose, salicin, inositol, raffinose, D-ribose, and D,L-lactic acid. On the basis of these tests, the antagonistic yeast most closely matched the description of *C. versatilis* except for a difference in assimilation of D-xylose, succinic acid, and citric acid.

In 1984, an isolate of ice nucleation-active *P. syringae* was obtained from the sprout of a sweet persimmon (*Diospyros kaki* T.) growing in Jeonnam province of Korea (5). Unless otherwise specified, INA bacterial and antagonistic yeast cultures were grown at 25 C and stored at 5 C on nutrient agar. Cells for application to plants were grown on nutrient agar plates for 2 days at 25 C, harvested with a loop, suspended in 0.1 M phosphate buffer (pH 7.0), and diluted to the desired cell densities, which were

verified by dilution plating.

Frost injury to three-leaf stage corn seedlings at various temperatures was measured by the method reported by Arny et al (1). Plants were sprayed with phosphate buffer alone or with suspensions of antagonist or INA bacteria at 72 hr before freezing. The plants were incubated in a mist chamber at 25 C in the dark until immediately before freezing. After incubation, the plants were cooled to −1 C at a rate of about 1.0 C/min, then to −5 C at a rate of 0.3 C/min, and held at −5 C for 5 min before being rewarmed to 25 C. Damage was assessed 24 hr later and expressed as the fraction of leaves per plant that showed any injury.

The effectiveness of the antagonist in reducing frost injury incited by INA bacteria was estimated in three different ways. First, plants were sprayed with antagonist in nutrient broth and held in a mist chamber for various periods of time before exposure to −5 C. Twenty-four hours after application with antagonist, the plants were sprayed with INA bacteria in nutrient broth and returned to the mist chamber until freezing. Second, plants were sprayed with a mixed suspension of antagonist and INA bacteria and held in a mist chamber for various periods of time before freezing. Third, plants were sprayed with INA bacteria, held in a mist chamber for 2 days, sprayed with antagonist, and returned to the mist chamber for various periods of time before freezing.

Mixed populations of antagonist and INA bacteria on leaves were measured by the method of Lindow et al (12). Leaves of corn seedlings were cut into 3-cm lengths and washed in 100 ml of 0.1 M phosphate buffer (pH 7.0) containing 1.0% Bacto peptone in 500-ml Erlenmeyer flasks on a reciprocal shaker. Appropriate dilutions of these washings were plated on King's B agar. Fluorescent pigment produced by *P. syringae* on the medium allowed quantitation of this isolate. *Candida* sp. on the same medium could be identified and quantitated by its colony morphology.

RESULTS AND DISCUSSION

Corn seedlings sprayed with cell suspensions (10^6 cfu/ml in phosphate buffer) of an INA strain of *P. syringae* began to be damaged at −2 C and were almost completely damaged at −5 C, whereas seedlings sprayed with phosphate buffer only or with antagonistic yeast alone were not completely injured until the temperature decreased to −9 C (Fig. 1). It was thus shown that the isolate of *Candida* sp. does not have ice nucleation activity. When plants were treated with different cell densities of antagonist 24 hr before application of INA bacteria, frost damage decreased with increasing antagonist cell density (Fig. 2). This result indicates that antagonism between leaf-surface INA bacteria and antagonistic yeast might be augmented by increasing the population of non-INA yeast, which reduce frost sensitivity of plants.

The antagonistic yeast isolate and the INA bacteria coexisted well on corn leaves when plants were sprayed with mixed suspensions of *P. syringae* and *Candida* sp. (Fig. 3). Both of the organisms grew logarithmically until 48 hr and thereafter maintained nearly constant populations until 4 days after application. The presence of the antagonist did not affect the growth of INA bacteria on leaves. However, frost damage of corn seedlings incited by INA bacteria was reduced by application of the antagonist (Fig. 4). The effectiveness of the antagonist in reducing frost damage increased as the time between application of antagonist and freezing (incubation time of antagonist) increased. The effectiveness was also greater when plants were pretreated with antagonist before application of INA bacteria than when both organisms were applied at the same time or when the antagonist

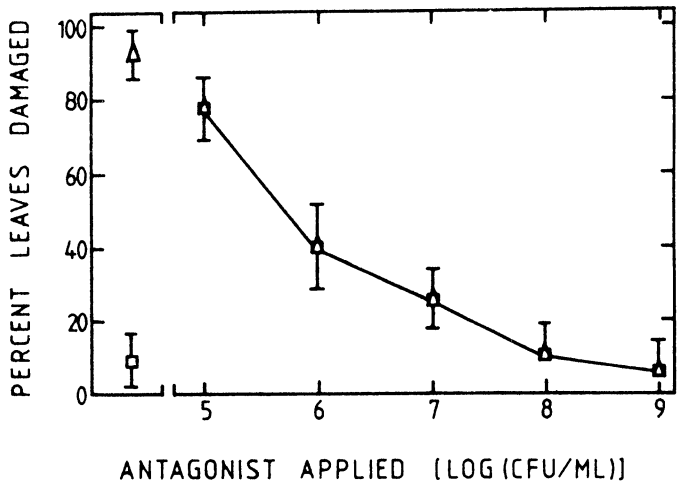

Fig. 2. Increase of protecting effect of antagonist *Candida* sp. on frost injury to corn seedlings incited by ice nucleation-active *Pseudomonas syringae* as the antagonist cell numbers that were applied increased. Corn seedlings were sprayed with *Candida* sp. at the cell densities (in nutrient broth) shown on the abscissa and held in a mist chamber at 25 C for 72 hr before exposure to −5 C. Forty-eight hours before exposure to −5 C, plants were sprayed with *P. syringae* (10^6 cfu/ml) in nutrient broth and returned to the mist chamber. □ = *Candida* sp. (10^8 cfu/ml) alone, △ = *P. syringae* (10^6 cfu/ml) alone. The vertical bars represent standard deviations.

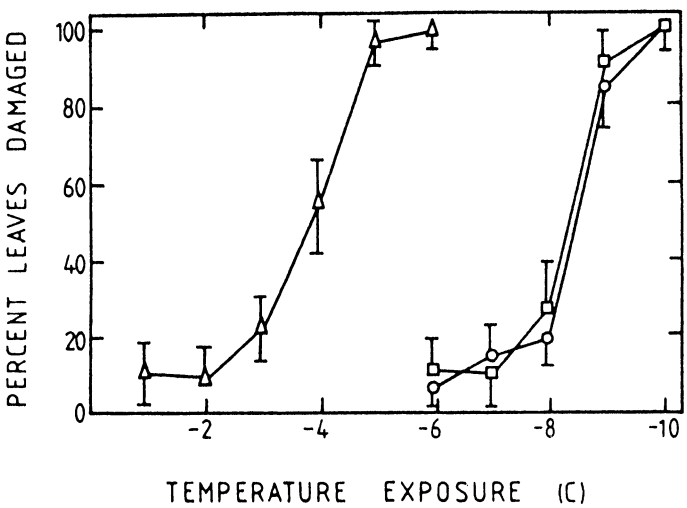

Fig. 1. Frost injury of corn seedlings pretreated with phosphate buffer alone (o), with suspension of antagonist *Candida* sp. (10^8 cfu/ml) in phosphate buffer (□), and with suspension of ice nucleation-active *Pseudomonas syringae* (10^6 cfu/ml) in phosphate buffer (△), respectively. After application, plants were held in a mist chamber at 25 C for 72 hr before exposure to the temperatures shown on the abscissa. The vertical bars represent standard deviations.

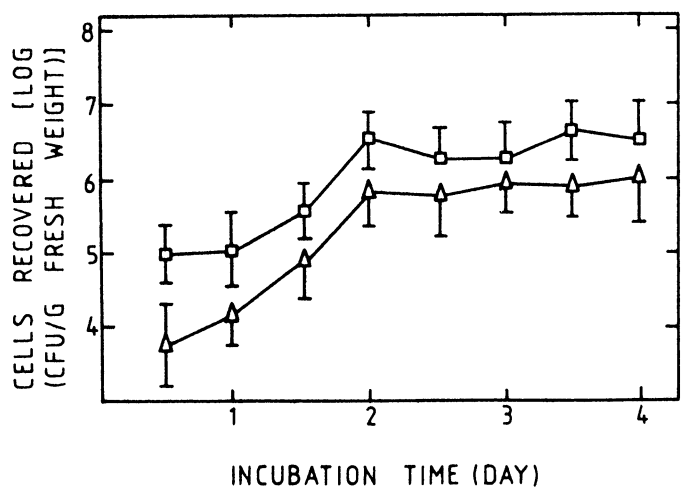

Fig. 3. Growth of mixed populations of antagonist *Candida* sp. (□) and ice nucleation-active *Pseudomonas syringae* (△) on corn seedlings. Seedlings were sprayed with a mixed suspension of *Candida* sp. (10^8 cfu/ml) and *P. syringae* (10^6 cfu/ml) in nutrient broth and held in a mist chamber at 25 C until assay. Each point represents the average value of three determinations with standard deviation.

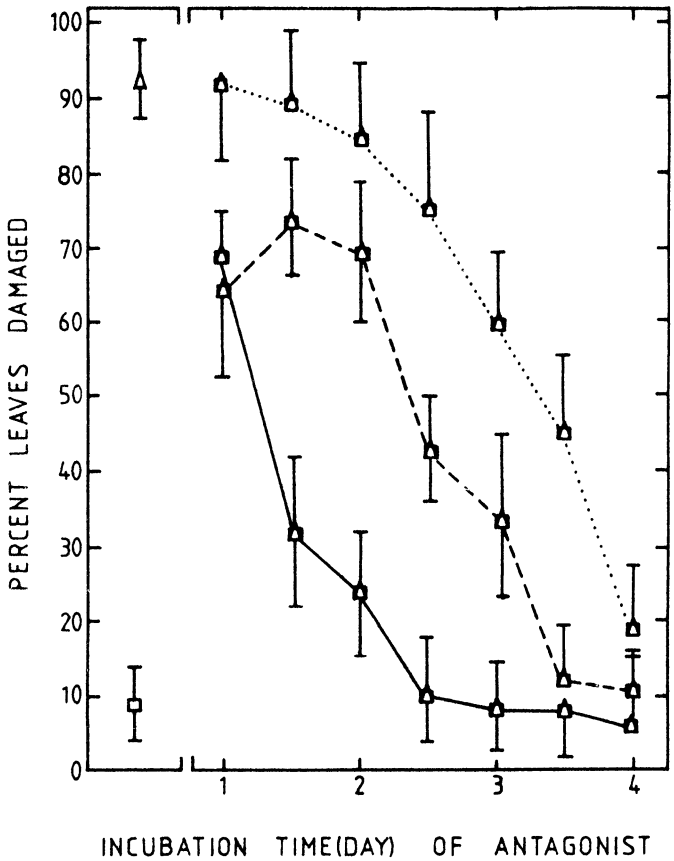

Fig. 4. Effect of antagonist *Candida* sp. on the decrease in frost injury of corn seedlings incited by ice nucleation-active *Pseudomonas syringae*. The first set of seedlings (straight line) were sprayed with *Candida* sp. (10^8 cfu/ml) in nutrient broth and held in a mist chamber at 25 C for the periods of time shown on the abscissa before exposure to −5 C. Twenty-four hours after application with antagonist, plants were sprayed with *P. syringae* (10^6 cfu/ml) in nutrient broth and returned to the mist chamber until −5 C treatment. The second set (dashed line) were sprayed with a mixed suspension of *Candida* sp. (10^8 cfu/ml) and *P. syringae* (10^6 cfu/ml) and held in a mist chamber at 25 C for the periods of time shown on the abscissa before exposure to −5 C. The third set (dotted line) were sprayed with *P. syringae* (10^6 cfu/ml) and held in a mist chamber at 25 C for 48 hr before application with antagonist. Then plants were sprayed with *Candida* sp. (10^8 cfu/ml) and returned to the mist chamber for the periods of time shown on the abscissa before exposure to −5 C. □ = *Candida* sp. (10^8 cfu/ml) alone, △ = *P. syringae* (10^6 cfu/ml) alone. The vertical bars represent standard deviations.

was applied after the INA bacteria. Establishment of the antagonist before the INA bacteria on leaves was needed for optimum frost damage protection. But frost damage was even reduced when the antagonist was applied 48 hr after application of INA bacteria (Fig. 4).

From the results, it appeared probable that the antagonistic effect of *Candida* sp. to *P. syringae* was neither due to nutritional competition nor to antibiosis since the presence of the yeast did not affect the population of *P. syringae*. The effect of the yeast on ice nucleation activity of *P. syringae* appeared to depend on growth of the yeast, since the longer the yeast grew in contact with *P. syringae*, the greater the reduction in ice nucleation activity (Fig. 4). Alteration of the chemical environment of leaf surfaces, such as by acid production after use of a certain subtrate, might play a role in antagonism on leaf surfaces (2,4). Such a chemical environment produced by the antagonist might not affect the survival of INA bacteria but might change the ice nucleation activity. Ice nucleation activity has been reported to be sensitive to heating, to heavy metal ions, and to a variety of positively charged organic molecules (14). Lectins, sulfhydryl reagents, and substituted borates are also found to inhibit the ice nucleation activity of bacteria (6).

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