

Production of ice nucleation deficient (Ice⁻) mutants of the epiphytic strains of *Erwinia herbicola*

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Abstract

To mutate the Ice Nucleation Active (INA) gene in *Erwinia herbicola* strains, Tn-5 transposon carried by P^{sup2021} plasmid was used. This plasmid was transferred to the bacterial cells by electroporation. Electrotransformation was carried out for 2.5 ms at 1800 v and 1 mm distance between the electrodes. Polymerase chain reaction was used for determination of presence or loss of INA gene, using a pair of primers designed for *inaZ* gene. The ice nucleation activity was tested by the freezing assay technique. The ice⁻ phenotype of the putative mutants were assayed through spraying sunflower seedlings, grown under aseptic condition, with 10⁷ to 10⁸ colony forming units per milliliter of the parent and transformant strains. The treated seedlings were kept at -8°C to -10°C for 8-10 hours and then transferred back to ambient (laboratory) condition for recovery or expression of freezing damage syndrome. Three out of ten electroporated strains yielded ice⁻ mutants that failed to freeze water droplets at -8°C and produce no freezing injury on sunflower seedlings.

Keywords: INA; *Erwinia herbicola*; *inaZ* gene; Transposon; Tn-5; Electroporation; Sunflower.

INTRODUCTION

Cold is one of the most important environmental stresses, affecting plant growth and crops productivity. Chilling (low temperatures above 0°C) and freezing

(temperatures below 0°C inducing extracellular ice formation) limit the geographical distribution and growing season of many crops and cause significant crops losses (Xin *et al.*, 2000). Freezing stress injuries have been observed intensively in many plants such as wheat, coffee, soybean, alfalfa, tobacco, potato, sugar beet, corn, rape, sunflower, citrus, strawberry, cherry, apricot, peach and pear (Beattie *et al.*, 1994).

Plants avoid freezing damage by using strategy, involved supercooling of some cells and tissues to temperatures as low as -40°C. Supercooling occurs because of lack of nucleating substances, necessary for ice initiation and by using barriers for ice growth in some tissues. Although all plants do not supercool to this extent, few degrees of protection afforded by supercooling is a significant avoidance mechanism (Burke *et al.*, 1976). Water in tissues of more sensitive freezing plants can supercool to -12°C without freezing intracellular or intercellular fluid (Maki *et al.*, 1978). Heterogeneous formation of ice nucleus induced by nucleating agents such as bacteria, fungi or particular chemical combinations can lead to low temperatures of ice nucleus about 0 to -2°C in organic and inorganic materials (Deininger *et al.*, 1987). Ice nucleation active bacteria can catalyze ice formation at temperatures as high as -2°C. Ice nucleation active bacteria (INA) have significant role in freezing injuries of cold sensitive plants. INA⁺ phenotype in Gram negative bacteria such as *Erwinia*, *Pseudomonas* and *Xanthomonas* has been identified by Maki, *et al* (1978). All INA bacteria are epiphytic and exist almost in all plants. Plant freezing injuries directly depend on logarithm of bacteria populations and logarithm of

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number of ice nucleus bacteria exist in plant at freezing time. In the presence of INA⁺ bacteria, freezing may occur at -1.2°C (Lindow, 1987). Burke and Lindow illustrated that, treatments which reduce the number of ice nucleating bacteria or their activity can decline crop freezing losses (Burke *et al.*, 1976). If genetic structure of *iceE* protein gene mutate, this protein is not synthesized in bacteria or synthesized incompletely and freezing and formation of ice nucleus is delayed therefore, growth season increase, the costs of freezing protection reduce leading to reduction in crop yields and quality reduction (Burke *et al.*, 1990).

In the present study, we identified INA⁺ *E. herbicola* isolates in Khorasan agronomical and horticultural crops and generated their INA⁻ mutants.

MATERIALS AND METHODS

Isolation and identification of bacteria: Plant samples were collected from Mashhad and Tehran suburb from different nipped cherry, sour cherry, plum, apricot, wheat and barley plants in spring 2002. Infected plant tissues such as leaves and branch skins were cut to 0.5-1 cm segments and placed into Erlenmeyer flask in distilled water and shaken for 15-20 minutes. After that, plant samples removed from the flasks and 1-2 drop of the suspension spread on nutrient agar sucrose medium (NAS). After a day, *E. herbicola* suspect yellow colonies were selected from NAS medium and eliminate subcultured in NAS medium for purification. Identification of *E. herbicola* strains was done through their biochemical and physiological characteristics (Wendt-Potthof *et al.*, 1992).

Droplet-freezing method: Ice nucleation activity levels measured by droplet freezing method as previously described (Rajashakar *et al.*, 1983). Sterile-distilled water was used as negative control and ice⁺ *P. putida* (WCS358pvd-inaZ strain) (De Boer *et al.*, 2003) isolate was used as positive control.

Polymerase chain reaction: Polymerase chain reactions for amplification of INA gene in bacterial isolates carried out using 20 nucleotides specific primers, 5'-ATCCAGTCATCGTCCTCGTC-3' and 5'-CAAGTGTCACGTTACCGGTG-3' sequences (Castrillo *et al.*, 2000). *Pseudomonas putida* (strain WCS358pvd-inaZ) contains *inaZ* cloned gene was used as positive control. Electrophoresis was performed on 1% agarose gel at 30watt constant power.

PCR amplifications were performed in a total volume of 20 µl, containing 1X PCR buffer, 15 ng of genomic DNA (prepared by using alkaline method), 0.5 mM of each primer, 0.2 mM dNTP, 2.5 mM MgCl₂ and 1 units of *Taq* DNA polymerase. PCR amplifications were hot-started for pre-denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 57°C for 1 min and extension at 74°C for 2 min and then a final extension at 74°C for 10 min.

Preparation of bacteria for electroporation:

Bacterial isolates were cultured in test tubes in STOLP medium (10 g pepton, 3 g yeast extract, 5 g NaCl prepared in 1 liter distilled water) or L-broth (LB) medium (10 g tripton, 3 g yeast extract, 10 g NaCl and 1 liter distilled water) for overnight. Bacterial cells were precipitated by centrifugation after reaching to the logarithmic growth and then the sediments were washed in sucrose 10% for 3 times. The cells were dissolved in glycerol 10% after final centrifugation.

Plasmid extraction: *E. coli* cells contain p^{sup2021} plasmid were grown in LB liquid medium and precipitated by centrifugation. Cell sediments were dissolved in 200 µl of 0.05 M glucose, 0.01 M EDTA and 0.025 M tris (pH 8). Then 400 µl 1% SDS solution prepared in 0.2 N fresh NaOH (pH 10-12) were added to the solution and kept in ice for 2 min. The suspension was added by 300 µl 3 M cold sodium acetate and kept on ice for 5-15 min. The supernatant was transferred into new tubes after centrifugation at 10000-13000 rpm for 5 min. One volume of waterlogged phenol was added to solution and mixed carefully. Then tubes were centrifuged at 5000 rpm for 2 min. An equal volume of phenol-chloroform was added to supernatant solution and shaken vigorously for 1 minute after removing the pellet. Centrifugation was done at 6000 rpm for 15 min. The supernatant was transferred to a new tube and 2 volumes of ethanol 96% was added. Samples were centrifuged at 1000 rpm for 10 min after keeping at -20°C to precipitate nucleic acids. Then ethanol was discarded and tubes were allowed to place under the fume hood for drying completely. After drying, 100-200 µl TE buffer was added to DNA precipitate. Determination of quality and quantity of plasmid DNA was performed using spectrophotometer and electrophoresis.

Electroporation: Mutation in *E. herbicola* isolates, containing ice nucleating activity, was done using

electroporation method at 1800 v, 1mm distance between electrodes, 100 µl cell volume and 2.5 ms Pulse time. *Tn5* transposon used for *ice* gene mutation.

Tn5 transposon in (*E. coli* S17-1 p^{sup2021}) p^{sup2021} plasmid had kanamycin resistant gene that used for selection of mutants. Selective medium (NAS+ kanamycin) used for selection of the resistant mutants. Droplet-freezing test was used to evaluate the ice nucleation activity of mutants. the suspension of mutants sprayed on sunflower seedlings (as a sample of cold sensitive plant) to investigate effects of mutants on sunflower seedlings.

Determination of mutant effects on sunflower seedlings: A reasonable concentration of mutant bacteria suspension (10^8 - 10^9 cfu, OD₆₀₀ : 0.2-0.3) in sterile distilled water was sprayed over 2-5 leaves of sunflower seedlings. Seedlings were exposed to -8 to -10°C for 8-10 hours. The seedlings were placed at laboratory environment for drying, before evaluating the treatments. Sterile distilled water and bacteria with no mutation were used as negative and positive control respectively.

RESULTS

Isolation and identification of bacterial isolates: Based on biochemical and physiological characteristics, 10 isolates of *E. hebricola* were selected and used for further analyses (Table1).

Ice nucleation active isolates: *E. hebricola* isolates with ice nucleation activity selected as ice+ isolates in droplet-freezing test. 70% of isolates (7 isolates out of 10) had ice nucleation activity (ice+) (Figure 1,2A).

PCR amplified sequence: The size of amplified sequence in PCR was about 1400 bp.(Figure 3). This band was appeared for *P. putida* and all isolates that had positive result in droplet-freezing test.

Electroporated cells: Electroporated cells by *Tn5* transposon, cultured in selective medium contains kanamycin. After one week incubating, 28 resistant mutant cells were growth on selective medium and selected for further eliminate in vitro assays (Figure 4). Selected isolates were subjected to droplet-freezing test for mutants. Among 28 kanamycin resistant mutants, some of them never showed freezing and the rest of them showed just 10 to 50% freezing in various

Table 1. Comparison of biochemical and physiological characteristics of yellow colonies grown on NAS medium and *Erwinia hebricola* isolate.

Traits	positive isolates(%)	<i>Erwinia hebricola</i> (Pantoea agglomerans)
Produce of yellow pigment	100	+
Oxidase	0	-
Gram reaction	0	-
Anaerobic growth	100	+
Indol produce	0	-
Hydrolysis of scolin	100	+
Fluorescent colour	0	-
Use of:		
Maltose	100	+
Salisin	100	+
Melibiose	0	-
Lactose	0	-
Arbotin	100	+
Starch	66	- or +
Threhalose	100	+
Adonithole	0	-
Dolsitol	0	-
Rafinose	50	- or +

replications of Droplet-freezing test (Figure 2B).

Effects of mutants on sunflower seedlings: All 28 kanamycin resistant mutants suspension (10^8 - 10^9 cfu, OD₆₀₀: 0.2-0.3) were used to spray sunflower seedlings. After 8-10 hours of incubation, 1375-1584 bp the sprayed seedlings in -8 to -10°C, frozen seedlings became dusky brown but non-frozen seedlings remained green and fresh. Freezing was not

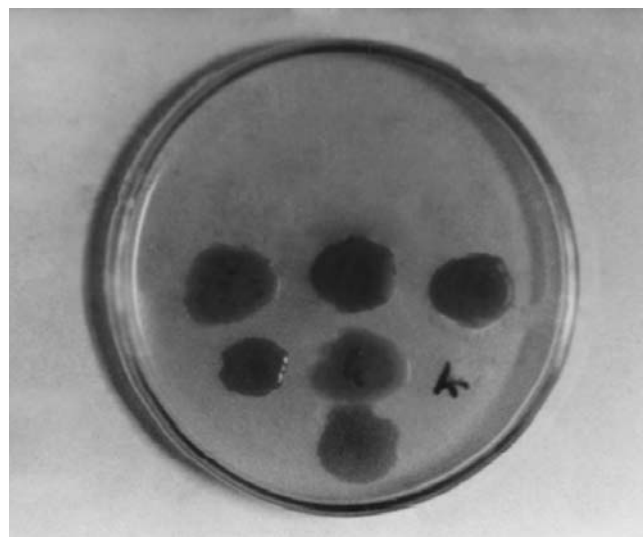


Figure 1. *E. hebricola* isolate colonies on NAS medium.

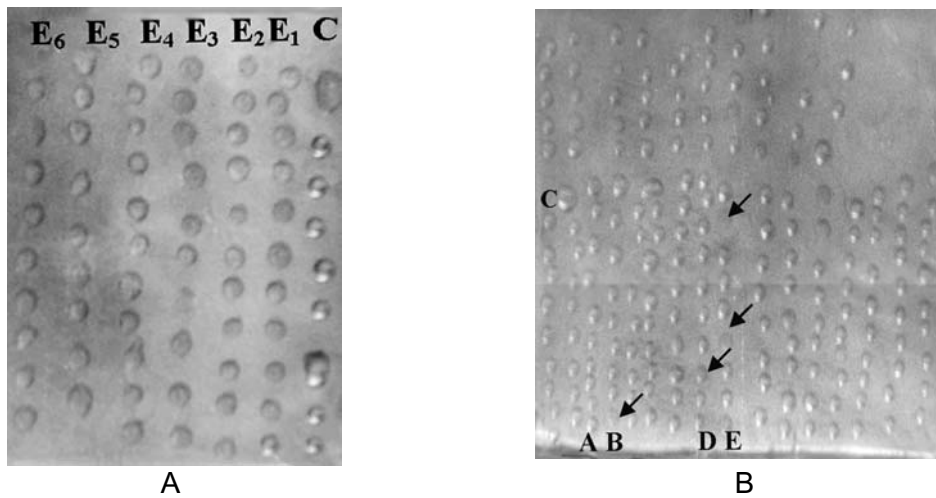


Figure 2. (A) Droplet-freezing test of *E. herbicola* mutants in cold bath. C, is negative control sterile distilled water. E1,E2,E3,E4,E5,E6 are the frozen suspensions of *E. herbicola* isolates. **(B)** Droplet-freezing test of *E. herbicola* mutants in cold bath. C, is negative control sterile distilled water. The arrows show the frozen drops in B,D,E samples. There was no frozen drop in A sample.

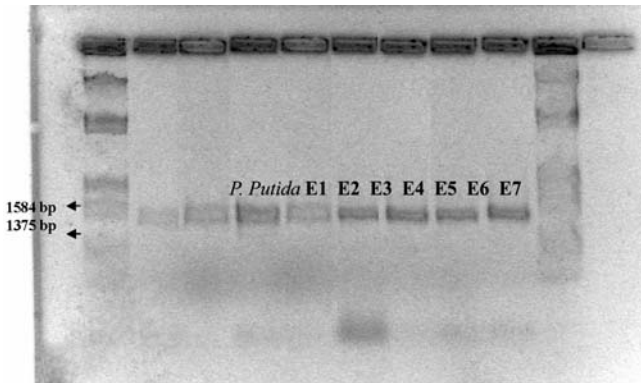


Figure 3. PCR products on 1% agarose gel. E1-E7 show different isolates of *E. herbicola*. *P. putida* is positive control carrying InaZ cloned gene. The size of bands are between 1375-1584 bp (average 1400 bp).

observed in three mutant isolates whereas, it occurred in other isolates as well as in positive control treatment (Figure 5).

DISCUSSION

Bacterial ice nucleus has low activity at high temperatures. Even under ideal conditions, the all of bacterial cells did not show ice nucleation activity. Hirano *et al.* (2000) reported that even various strains of a bacterial species, are different in their ability to produce ice nucleus. In this study, the *Erwinia herbicola* bacteria isolates from plant samples showed different ice nucleation activity in droplet-freezing test. Probably, it was because of differences between strains, inactivity of ice nucleus in all cells of a strain for the same conditions and various amounts of bacteria with ice nucleation activity.

Castrillo (2000) used *inaZ* specific primers for amplification of INA gene in *Pseudomonas syringae* (*inaZ*), *Pseudomonas fluorescence* (*inaW*) and *Pseudomonas putida*. The size of amplified sequence by those primers was more than 4500 bp. in the present study, the size of amplified INA gene sequences by the same primers was 1400 bp both for *Erwinia herbicola* (*iceE*) and *Pseudomonas putida* (strainWCS358pvd-

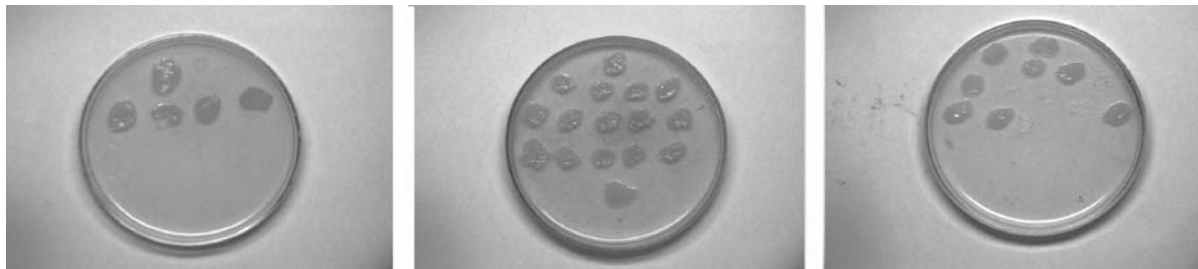


Figure 4. Kanamycin resistant mutants of *Erwinia herbicola* isolates, grown on selective NAS medium supplemented with kanamycin.

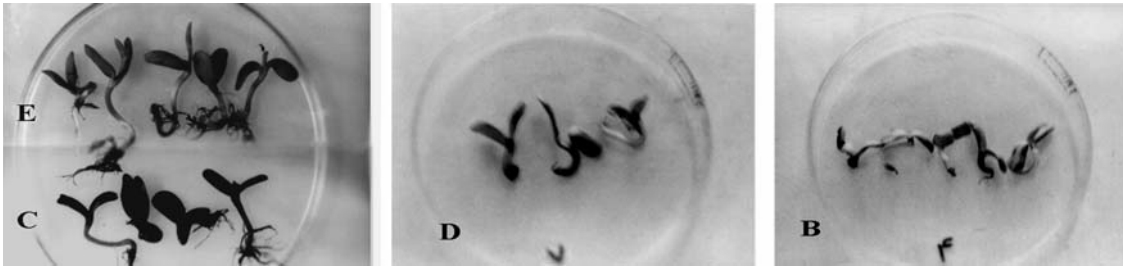


Figure 5. Sunflower seedlings after sprayed with mutant *E. herbicola* suspensions and sorted at -8°C – -10°C for 8 hours. The suspension sprayed on **B**, **D**, **E** samples didn't freeze the seedlings. **C**, is the control that sprayed with Ice+ strain.

inaZ) INA gene. According to Wolber studies (1992), INA genes in various species are different in some aspects but their general structure is similar to each other (Wendt-Potthof *et al.*, 1992). Thus, the differences between size of *iceE* gene from *Erwinia herbicola* and *Pseudomonas putida* (strain WCS358pvd-inaZ) in this study and size of *inaZ* gene from *Pseudomonas syringae*, *Pseudomonas fluorescence* and *Pseudomonas putida* in Castrillo (2000) studies may be explained by small differences among *inaZ*, *inaW* and *iceE* gene, although a precise analysis is required to be done on various isolates of these bacteria simultaneously. (The differences between *Pseudomonas syringae* INA gene (*inaZ*) and *Pseudomonas putida* which contains *inaZ* cloned gene, require further investigation.

Analysis of the effects of mutants on sunflower seedlings, after repeating the tests twice showed that, three isolates which had 10-15% freezing activity in droplet-freezing test, didn't cause any decay on sunflower seedlings as it was predictable. There was one isolate with 0% freezing activity in droplet-freezing test which caused freezing on sunflower seedlings. Thus, about 11% (3 of 28 isolates) of mutant produced by electroporation were ice-.

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