Journal of Nuts
Journal homepage: ijnrs.damghaniau.ac.ir

Evaluation of Ice Nucleation Activity (INA) and INA Gene Detection in the Bacteria Isolated from Pistachio Trees in Kerman Province, Iran

Mahdieh Rostami[1], Nader Hasanzadeh[*1], Pezhman Khodaygan[2], Ali Riahi Madvar[3]

[1]*Department of Plant Protection, Faculty of Agricultural Sciences and Food Industries, Science and Research Branch, Islamic Azad University, Tehran, Iran*

[2]*Plant protection Department, Faculty of Agriculture, Vali- E- Asr University, Rafsanjan, Iran*

[3]*Department of Biotechnology, Institute of Science and High Technology and Environmental Sciences, Graduate University of Advanced Technology, Kerman, Iran*

ARTICLEINFO

Keywords:

Ice nucleation bacteria,
Ice nucleation frequency,
Kerman Province,
Pistachio

ABSTRACT

Ice nucleation active (INA) bacteria are common epiphytic inhabitants that cause frost damage in many plants in the near-zero temperatures. Yet, no studies were found in ice nucleation bacteria associated with pistachio trees. In our earlier study some INA strains were identified and reported. These were assigned as *Pseudomonas fragi*, *P. putida*, *P. moraviensis* and *Pantoea agglomerans*. In current work, two new strains namely *P. viridiflava* and *Entrobacter cloacea* were identified. Their ice nucleation frequency were evaluated and compared with above-mentioned ice positive strains isolated from pistachio trees. *Pseudomonas fragi* raf_3 was considered as the most ice nucleation active bacteria. This was followed by *P. putida* raf_6, *P. moraviensis* raf_1, *P. moraviensis* raf_5, *Pantoea agglomeranse* raf_7, *P. viridiflava* raf_2, *Entrobacter cloacea* raf_8 and *Pseudomonas* sp. raf_4, respectively. To detect INA genes, two sets of degenerate primers were used and partial INA gene sequences were amplified. INA gene sequence (425bp) for *Pseudomonas putida* raf_6, *Pantoea agglomerans* raf_7 and *P. fragi* raf_3 were amplified with primer pair of 3308/3463. Whereas, a fragment of 194bp was detected in *Pseudomonas* sp. raf_4, *P. moraviensis* raf_5 and *P. moraviensis* raf_1 using forward and reverse primer pair of 3076/3463. *Entrobacter cloacea* raf_8 has reported for the first time as epiphytic ice plus strain. The capability of the latter as a bacterial *biocontrol* agent against insect pests was reported.

Introduction

Ice nucleation active (INA) bacteria are common epiphytes on a vast variety of plant species and probably are widely distributed in the earth′s atmosphere (Wowk and Fahy, 2002). These bacteria act as nucleating centers for ice crystals by producing different ice nucleation-active proteins (INAPs) on their outer cell walls (Love and Lesser, 1989). This facilitates intercellular and/or intracellular ice formation in plant tissues and triggers frost damage. On the other hand, frost injury seems to predispose plants to infection by pathogenic microorganisms. Multiple researches have revealed a correlation between the presence of INA

[*]**Corresponding author**: Email address: hasanzadehr@yahoo.com
Received: 23 November 2017; Received in revised form: 7 February 2018; Accepted: 17 June 2018
DOI: 10.22034/jon.2018.542997

bacteria and crops freezing in widespread geographical locations (Maki *et al*., 1974; Hirano *et al*., 1982; Lindow, 1983).

Agricultural losses due to frost damage in Iran are remarkably significant. Pistachio trees, like many other trees, are sensitive and vulnerable to early spring frost (Hokmabadi, 2011). Saving this economical crop from frost damage is a top priority in Iran since it is one of the most valuable tree species and has important role in the agricultural production and non-petroleum exportation. According to the statistics released by the World Food and Agriculture Organization, Iran is one of the largest pistachio producers in the world and Kerman Province ranks the first in the terms of production, quality and export of pistachio nuts (Karimi, 2017). Spring frosts and freezes have been two annual threats to the pistachio crops over the last three decades. Early spring frost lost usually amounts to 50% every year. But, there is a record of more than 60% in two consecutive years (2004 and 2005) amounting to $420 million in Kerman Province (Hokmabadi, 2011).

In the absence of ice nucleation positive bacteria, liquids in tissues of plants can be supercooled to as low as -8 to -12°C without freezing within tissue (Maki and Willoughby, 1978; Lindow *et al.,* 1982b) but INAP as heterogeneous ice nucleus facilitates ice formation at high temperatures about 0 to -2°C (Deininger *et al*., 1987). Generally, the presence of INA bacteria has been detected in locations with the experience of yearly-frost injury (Wolber and Warren, 2012). Ice nucleation-active (INA) bacteria play a significant role in frost injury to both freezing-sensitive and even cold-adapted plants (Wisniewski *et al*., 2001).

There is little information about (INA) bacteria abundance in natural vegetation, factors that trigger their release, or persistence of their ice nucleation activity and whether treatments which reduce the number of ice nucleating bacteria or their activity can mitigate crop freezing losses (Burke *et al*., 1976).

To evaluate some of these aspects, a research work was carried out in Kerman province where the pistachio orchards are suffering from spring frost damages. In the previous study all dominant bacteria were isolated and identified (Rostami *et al*., 2018). In this study, ice nucleation active sequences were obtained and their nucleation activity was evaluated.

Materials and Methods

Bacterial isolation and identification

Isolation and identification of two new ice plus strains was done as has been described in (Rostami *et al*., 2018). In brief, epiphytic bacteria were isolated by washing foliar samples in Erlenmeyer flasks and culturing sample suspensions onto culture media. Then, the representative colonies were tested for their ice nucleation activity according to Lindow *et al.,* 1978. A fresh culture was prepared from each (INA^+) isolate. A series of biochemical tests including gram reaction, O/F test to determine aerobic/facultative anaerobic growth, cytochrome oxidase reaction, catalase, arginine dihydrolyase, levan production from sucrose, nitrate reductase, gelatin hydrolase, fluorescent pigmentation on King's B medium, pectolytic activity (potato soft rot) and hypersensitivity reaction on tobacco were conducted to characterize the (INA^+) isolated bacteria *according to Schaad et al*., (2001).

Measurement of ice-nucleating frequency

To determine ice-nucleation frequency, bacterial suspensions of 10^7cell/ml (OD_{660} = 0.1) were prepared by diluting in 50mM phosphate buffer (pH 7.0) and were further diluted by ten-fold serial dilutions (Obata *et al*., 1999).

Droplet freezing method was used to assess ice nucleation activity in INA^+ bacteria introduced by Vali (1971) and modified by Lagzian *et al*., (2014).

In brief, droplets were placed on the surface of an aluminum foil sheet coated with paraffin floating on a supercooled alcohol bath. A total of 20 droplets of equal volume (25µl) for each dilution and sterile D.H_2O as control was pipette onto its surface and the frozen drops were recorded. Ice-nucleation frequency was measured based on formula introduced by Lindow *et al*. (1982a).

$$N(T) = (-\ln f) / V$$

Where, N (*T*) is nucleation frequency at temperature T, *f* is the proportion of unfrozen droplets (i.e, the number of unfrozen droplets divided by the total number of examined droplets) at temperature T, and V is the volume of individual droplets in liters. Number of ice nucleators per gram was obtained from dividing N (T) by the density (g/L) of suspension as described by Block (2002).

Freezing test on wheat seedlings

For assessing frost injury in plant tissues by two new ice plus isolates (*P. viridiflava* raf_2 and *Entrobacter cloacea* raf_8), bacterial suspensions were prepared and sprayed on wheat plants as described below. Bread wheat (*Triticum aestivum* L.) seeds were planted in plastic pots (20 cm in height and 10 cm in diameter) filled with commercial potting soil mix. Seeds were germinated on sterile moist filter paper placed in Petri dishes. 20 g of germinated seeds were planted in each pot and grown under natural day and night temperatures (20 ± 4°C and 10 ± 4°C respectively) and sunlight. The relative humidity was set at 40%. The plants were watered every two days with 250 ml distilled water. In order to make the plants acclimate to cold conditions, 7-day-old seedlings were placed in an illuminated growth chamber at 375 microeinsteins (µE) at 25/5°C day/night temperature and 60-70% relative humidity for a week. All plants with three replicates per treatment were gently sprayed with 1×10^9 CFU/ml fresh suspension of ice positive strains until they were wet enough to runoff. Bacterial suspensions were prepared from overnight cultures grown on NA containing 2.5% glycerol at 22°C. The controls were treated with distilled water. Plants were maintained for two days at 22 ± 2°C in a plastic enclosure with elevated humidity to facilitate bacterial colonization. All colonized wheat plants were, then, subjected to the freezing test (-5°C for 40 min.) (Buttner and Amy, 1989). Plants under cold-acclimating conditions were removed from cold chamber and warmed to room temperature and assessed for frost damage. To estimate the extent of frost damage in each treatment, damaged (flaccidity/discoloration) and undamaged leaves were harvested from each pot after freezing and were counted. Leaves were cut from plants along a line from the edge of the pot to the center at approximately the same height above the soil. Then, the percentage damage (the ratio of the number of damaged leaves to the whole) was recorded.

DNA extraction, PCR amplification and sequencing

The total DNAs of all old and new two ice nucleate active strains were extracted using Qiagen DNeasy TM Tissue Kit. The quantity and quality of the extracted DNA was checked by spectrophotometry and agarose gel electrophoresis.

Two sets of degenerate primers (3308f, 3463r and 3076f, 3463r) were used to amplify partial sequences of INA genes from the most active ice nucleation bacteria isolated on pistachio trees (Table 1).

Table 1. Primers used in PCR analysis.

Gene	Primer name	5′ to 3′ sequences	Reference	Fragment size (pb)
16srDNA	63f	CAGGCCTAACACATGCAAGTC	Marchesi *et al*., 1998	1300
	1387r	GGGCGGWGTG TACAAGGC		
rpoD	Prpo1	TGAAGGCGARATCGAAATCGCCAA	Parkinson *et al.*, 2011	700
	Prpo2	YGCMGWCAGCTTYTGCTGGCA		
recA	RecAf	CCTTCACCATACATAATTTGGA	Waleron *et al*., 2002	800
	RecAr	GGTAAAGGGTCTATCATGCG		
ina	3076f	AGYTCGCTGATTGCGGGNC	Hill *et al*., 2014	425
	3463r	STGTAVCKTTTNCCGTCCCA		
ina	3308f	GGCGATMGVAGCAAACTSAC	Hill *et al*., 2014	194
	3463r	STGTAVCKTTTNCCGTCCCA		

INA gene sequences (approximately 425bp) for *Pseudomonas putida* Raf_6 and *P. fragi* Raf_3 were amplified with primers 3308 forward and 3463 reverse (Hill *et al*., 2014) using the following PCR conditions: an initial denaturation step at 95°C for 4 min, followed by 35 cycles of 94°C (30 s), 58°C (30s) and 72°C (1 min), with a final extension at 72°C for 5 min.

PCR primers that were used for partial sequences amplification of INA gene (approximately 196bp) in *Pseudomonas* sp. Raf_4, *P. moraviensis* raf_1 and *P. moraviensis* raf_5 were 3076 forward and 3463 reverse (Hill *et al*., 2014). The thermal program consisted of initial denaturation (3 min at 95°C) followed by 30 cycles of 1 min at 95°C, 30 s at 58°C and 1 min at 72°C with a final extension at 72°C for 5 min.

Bacterial identification by 16srDNA, rpoD and recA gene sequencing

Amplified DNAs of 16S rDNA, rpoD and recA obtained from bacterial strains were sent to Bionner Company (South Korea) for sequencing. Gene sequences were retrieved by nucleotide Basic Local Alignment Search Tool (BLAST) program at the National Center of Biotechnology Information (NCBI). The sequence data obtained in this study were deposited in GenBank under the accession numbers provided in Table 2.

Table 2. Blast result of *16S rDNA*, *rpoD* and *recA* Ice plus bacterium genes including Genbank Accession numbers.

Ice-Plus Bacterial strain	Accession numbers			Geographic origin of isolation
	16srDNA	rpoD	recA	
P. viridiflava Raf_2	KX640928	MF572932	*	Rafsanjan
Entrobacter cloacae Raf_8	KX640929	*	MF572931	Rafsanjani

Results

Bacterial isolates and their phenotypic characteristics

The phenotypic characteristics of the two newly isolated INA+ bacteria strains were determined. *Entrobacter cloacae* was a Gram-negative rod with negative oxidase and indole reaction and positive for nitrate reduction and catalase. Acid was produced from glucose aerobically and anaerobically. The other INA+

bacterium identified as *Pseudomonas viridiflava* was a *Gram negative*, rod shaped aerobe, producing fluorescent pigment on KB medium. Levan and oxidase were negative and potato soft rot was positive.

Measurement of ice-nucleating frequency

Numbers of active ice nucleates (N_T) and ice nucleation frequency for eight representative strains was determined at -7ºC. All eight strains were differed in ice nucleation frequency levels (Table 3 and Fig. 1.). *Pseudomonas fragi* raf_3 was considered as the most ice nucleation active bacteria. This was followed by *P. putida* raf_6, *P. moraviensis* raf_1, *P. moraviensis* raf_5, *Pantoea agglomeranse* raf_7, *P. viridiflava* raf_2, *Entrobacter cloacea* raf_8 and *Pseudomonas* sp., respectively.

Differential ice nucleation frequency was calculated for *P. fragi* strain Raf_3 in three serial dilutions. The result showed that ice nucleation frequency decreases as the amount of ice nucleation is decreased in serial dilutions due to decline in the numbers of active ice nucleation in a given culture.

Linear inverse relationship was observed between serial dilutions of bacterial suspension and ice nucleation frequency (Fig. 2).

Table 3. Numbers of active ice nucleates (N_T) at -7°C after 24 hours growing on GYNA media. Initial cell density of all bacterial suspension were adjusted ca 1×10^7 cell/ml at 600nm.

Sample	$N_{(T)}$
Pseudomonas fragi strain raf_3	11×10^4
Pantoea agglomerans strain raf_7	3.6×10^4
Pseudomonas sp.strain raf_4	3.1×10^4
Pseudomonas putida strain raf_6	2.3×10^4
Pseudomonas viridiflava strain raf_2	1.7×10^4
Pseudomonas moraviensis strain raf_1	1.6×10^4
Pseudomonas moraviensis strain raf_5	1.4×10^4
Entrobacter cloacae raf_8	0.8×10^4

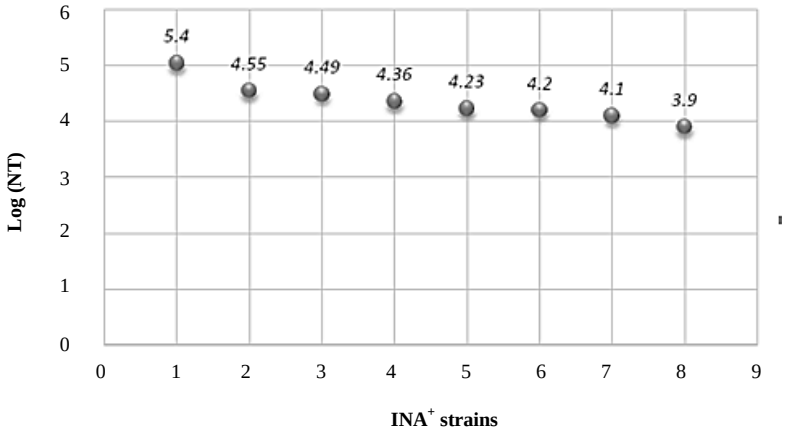

Fig.1. Comparison of ice nucleation frequency (NT) of INA^+ strains isolated from pistachio foliage. Numbers 1- 8 represent *P. fragi* strain raf_3, *Pantoea agglomerans* strain raf_7, *Pseudomonas* sp. strain raf_4, *P. putida* strain raf_6, *P. viridiflava* strain raf_2, *P. moraviensis* strain raf_1, *P. moraviensis* strain raf_5, and *Entrobacter cloacae* strain raf_8, respectively.

Fig.2. Linear inverse relationship between serial dilutions of bacterial suspension and ice nucleation frequencies of *P. fragi* strain raf3.

Freezing test on wheat seedlings

The seedlings showed different degrees of frost injury, when subjected to Ice plus *Pseudomonas viridiflava* raf_2 and *Entrobacter cloaceae* raf_8. Symptoms of frost damage was exhibited as water-soaking lesion and shoot tissue collapse. Damage was more sever in *Pseudomonas viridiflava* raf_2 than in *Entrobacter cloaceae* raf_8. No visible symptoms of frost damage was found in control plant (Fig. 3). The amount of frost damage was measured for eight INA strains as described above. Damage was the most sever in *P. fragi* (78%) and the least in *P. moraviensis* raf_5 (10 %) as shown in Table 4.

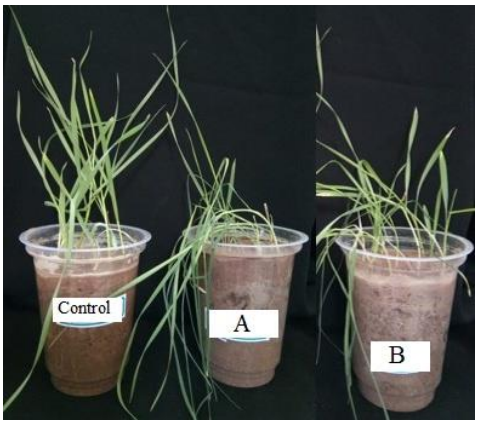

Fig. 3. Symptoms of frost damage (water-soaking and shoot tissue collapse) induced by INA positive strains at 5ºC for 40 min. Control treated with sterile distilled water, A: *Pseudomonas viridiflava* raf_2, B: *Entrobacter cloacae* raf_8.

Table 4. Percentage of freezing damage on wheat seedlings induced by INA positive strains.

INA strains	Percentage of freeze damage
Pseudomonas moraviensis raf_1	32%
P. viridiflava raf2	33%
P. fragi raf3	78%
Pseudomonas sp. raf4	49%
*P. moraviensis*raf5	10%
P. putida raf6	30%
Pantoea agglomerans raf7	75%
Entrobacter cloacea raf8	17%

PCR amplification and sequencing

In PCR amplifications of *16S rDNA* gene, a single band at ±1,300 bp was produced and 99% similarities were obtained from the alignment using BLAST (Fig. 4). Sequence analyses of *16SrDNA* and *rpoD* genes revealed that strain raf_2 was belonged to *Pseudomonas viridiflava*, whereas and the other strain was identified as *Entrobacter cloaceae* (raf_8) comparing both 16*SrDNA* and *recA* gene sequences. The results of BLAST showed 97% similarity between *rpoD* and *recA* gene sequences of the strains and deposited sequences. The GenBank accession numbers are shown in Table 2.

The PCR products amplified by rpodf and rpodr primer pair was identical to *P. viridiflava* with molecular length size about 730 bp (Fig 5). This was about 800 bp for *Entrobacter cloacae* raf_8 (Fig 6).

In order to characterize INA sequences in all INA positive isolates, two sets of degenerate primers were used as designed by Hill *et al*. (2014).

Primer pair 3076f/3463r amplified partial INA gene sequence (ca 425 bp) in *Pseudomonas putida* raf_6, *Pantoea agglomerans* raf_7 and *P. fragi* raf3 (Fig 7). The correctly sized DNA product (194 bp) with painted DNA band was observed for *Pseudomonas* sp. Raf4, *P. moraviensis* raf_5 and raf_1 using primer pair 3308f and 3463r (Fig 8). These primers successfully amplified partial INA sequences in mentioned species but failed for *Entrobacter cloacae* and *P. viridiflava*.

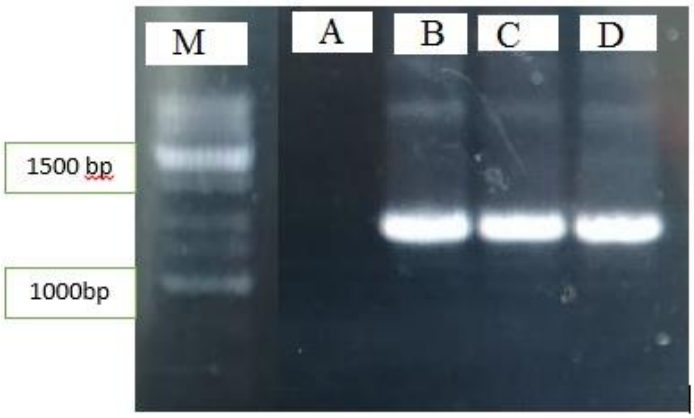

Fig 4. Amplification of *16SrDNA* partial gene (1300bp) using 63f and 1387r primers. A: Negative control, B: *P. viridiflava* raf_2, C: *Entrobacter cloacae* raf_8, D: Positive control (*P. syringae*), M: Lader 1000 bp.

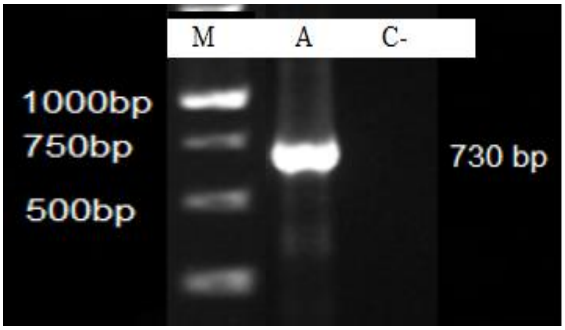

Fig. 5. Amplification of partial *rpoD* gene using $Prpo_1$ and $Prpo_2$ primers for *Pseudomonas viridiflava* raf_2 (LaneA). Approximately 730bp band was amplified. C-: Negative control. M: Lader 100bp.

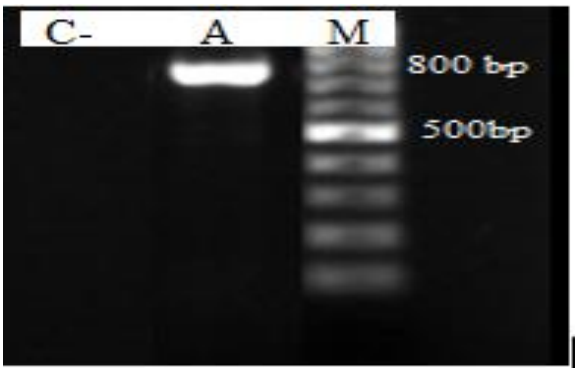

Fig.6. Amplification of partial *recA* gene using recAr and recAf primers for *Entrobacter cloacae* raf_8 (lane A). Approximately 800bp band was amplified. C-: Negative control. . M: Lader 100bp.

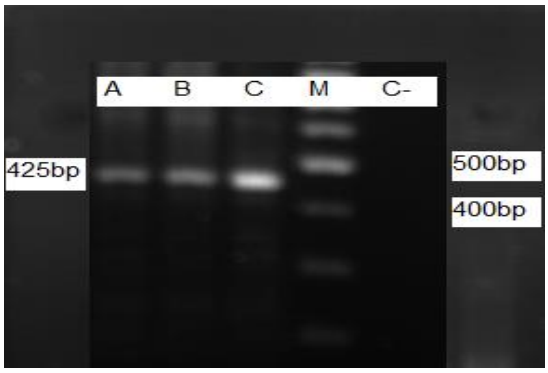

Fig. 7. PCR products of partial ina gene amplified with degenerated primers pair 3076f/3463r (Approximately 425bp band was amplified) in *Pseudomonas fragi* Raf_3, *P. putida* raf_6 and *Pantoea agglomerans* raf_7 (Lanes A, B, C). C-: Negative control. M: Lader 100bp.

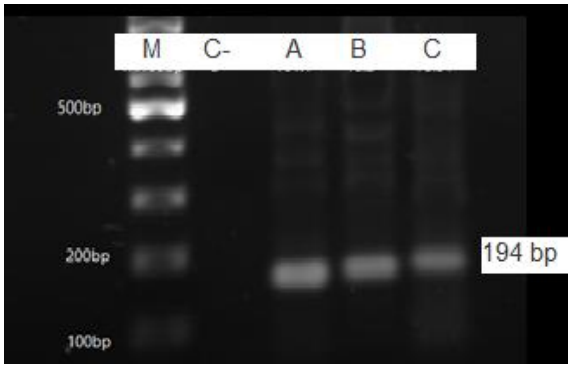

Fig. 8. PCR products of partial ina gene amplified with degenerated primers set, 3308f and 3463r (Approximately 194bp band was amplified) in *Pseudomonas* sp. Raf4, *P. moraviensis* raf_5 and *P. moraviensis* raf_1 (Lanes A, B, C). C-: Negative control. M: Lader 100bp.

Discussion

It has been reported that ice nucleation active bacteria play an important role in pistachio frost damage in Kerman Province (Rostami *et al*., 2018). In Follow up the previous study, the present work was carried out to determine ice nucleation frequency and detect INA genes in INA bacteria isolated from pistachio trees in these areas.

As mentioned earlier, in our previous work, several hundred bacterial strains from foliar parts of pistachio in different regions of Kerman Province were isolated. Eight out of 64 INA plus strains had fixed ice nucleation

activity so they were selected for further research in PCR analysis and ice nucleation activity of the two new cultured bacterial isolates.

These two new strains which tentatively were identified as *Pseudomonas viridiflava* and *Entrobacter cloacae* exhibited ice nucleation activity with different frequencies. Sequence analysis of partial *16SrDNA* gene confirmed our initial diagnosis. *rpoD* and *recA* genes analyses were made as alternative to the 16S rRNA gene sequences.

Ice nucleation activity of the INA strains was compared with each other. *P. fragi* and *E. cloacae* have the most and least ice nucleation frequency, respectively. All representative ice plus strains were subjected to freezing test and all of them induced different frost damage rates to wheat seedlings. As expected, with regards to ice nucleation frequency, *P. fragi* induced the highest frost damage rate in wheat seedlings and *E. cloacae* stimulated the least. However, degree of frost damage depends on host plant, tissue type, plant age, environmental conditions and the ability of these bacteria to colonize the host surface.

It is well documented that *Pseudomonas* spp. have a widespread distribution as epiphytes on plants and possess the ability to catalysts for ice formation at temperatures above -5°C (Sarris *et al*., 2012; *et al*., 2010; Parkinson *et al*., 2011; Morris *et al*., 2010; Varvaro *et al*., 1992). Among these, *P. syringae* and *P.viridiflava* are typical due to their most destructive frost damage to frost-labile plants.

The position and abilities of *Entrobacter cloacae*as INA^+ bacterium is quite different with *P.viridiflava*. In some papers, the *Entrobacter cloacae*as has been introduced as a dominant microbiota inhabiting and colonizing well in the gut of insects. According to Tang *et al*., 2003, this species is an efficient colonizer of insect guts and has weak plant epiphytic ability. This indicates its potential role as a suitable candidate for the control of agricultural insect pests. On the other hand, some investigators have reported that INA bacteria can reduce the cold hardiness of overwintering insects or parasite-vectors (Yadav *et al*., 2015). Therefore, a little fitness of *E. cloacaea* on leaves can be used as biocontrol agents. This was opposed by Watanabe *et al*., 2000, who believe *E. cloacaea* is rarely isolated from the leaves and attempts to isolate an INA strain of *E. cloacae* from natural environments have been unsuccessful. We here introducing *E. cloacaea* as ice plus bacterium which for the first time isolated from pistachio leaves. It is obvious that the population of bacteria increases after cold injury in plant tissues, maybe because host nutrients are made available (Lindow, 1983). It has been also shown that plants which have been already suffering frost damage are more susceptible to diseases (Weaver, 1978). Therefore, INA bacteria may play a role in virulence (Edwards *et al*., 1994). So, ice nucleation bacteria have been studied up because of their impact on agricultural products.

As the first survey of pistachio INA bacteria, this study offers new insights into the decrement frost damage induced by ice nucleation bacteria in this economic agriculture product. Nevertheless, further research will be required for various aspects of ice nucleation activity of phylloplane microflora of pistachio.

Acknowledgements

We would like to thank Mr. Naser Taheri, the Director of the Plant Protection Management of Kerman Province, and stuff members of the laboratory for their kind collaboration. We also would like to give gratitude to students of the laboratory of vali-e-Asr university of Rafsanjani, especially Mr. Basavand for his technical assistance in this project.

References

Block W (2002) Interactions of water, ice nucleators and desiccation in invertebrate cold survival.

European Journal of Entomology. 99(2), 259-266.

Burke MJ, Gusta LV, Quamme HA, Weiser CJ, Li PH (1976) Freezing and injury in plants. Annual Review of Plant Physiology. 27, 507-528.

Buttner MP, Amy PS (1989) Survival of ice nucleation-active and genetically engineered non-ice-nucleating *Pseudomonas syringae* strains after freezing. Applied and Environmental Microbiology. 55(7), 1690-1694.

Deininger CA, Mueller GM, Wolber PK (1987) Immunological characterization of ice nucleation proteins from *Pseudomonas syringae*, *Pseudomonas fluorescens*, and *Erwinia herbicola.* Journal of Bacteriology. 170(2), 669- 675.

Edwards AR, Van Den Bussche RA, Wichman HA, Orser CS (1994) Unusual pattern of bacterial ice nucleation gene evolution. *Molecular Biology and Evolution.* 11(6), 911-920.

Fahy PC, Persley GJ (20002) Plant Bacterial Diseases A Diagnostic Guide. Sydney, Australia. Academic Press. pp. 393. 337-375.

Hill TC, Moffett BF, DeMott PJ, Georgakopoulos DG, Stump WL, Franc GD (2014) Measurement of ice nucleation-active bacteria on plants and in precipitation by quantitative PCR. Applied and Environmental Microbiology. 80(4), 1256-1267.

Hirano SS, Nordheim EV, Arny DC, Upper CD (1982) Lognormal distribution of epiphytic bacterial populations on leaf surfaces. Applied and Environmental Microbiology. 44(3), 695-700.

Hokmabadi H (2011) Pistachio frost damage in Iran and new methods of frost protection. In XIV GREMPA Meeting on Pistachios and Almonds Zaragoza: CIHEAM-IAMZ/FAO/AUA/TEI Kalamatas/NAGREF. pp. 71-78.

Karimi E (2017) Solid state fermentation effects on pistachio hulls antioxidant activities. Asia-Pacific Journal of Science and Technology. 15(5), 360-366.

Lagzian M, Latifi AM, Bassami MR, Mirzaei M (2014) An ice nucleation protein from *Fusarium acuminatum*: cloning, expression, biochemical characterization and computational modeling. Biotechnology letters. 36(10), 2043-2051.

Lindow SE, Arny DC, Upper CD (1982a) Bacterial ice nucleation: a factor in frost injury to plants. Plant Physiology. 70(4), 1084-1089.

Lindow SE, Hirano SS, Barchet WR, Arny DC, Upper CD (1982b) Relationship between ice nucleation frequency of bacteria and frost injury. Plant Physiology. 70(4), 1090-1093.

Lindow SE (1983) The role of bacterial ice nucleation in frost injury to plants. Annual Review of Phytopathology. 21, 363-384.

Lindow SE, Arny DC, Upper CD (1978) Distribution of ice nucleation-active bacteria on plants in nature. Applied and Environmental Microbiology. 36(6), 831-838.

Love J, Lesser W (1989)The Potential Impact of Ice-Minus Bacteria as a Frost Protestant in New York Tree Fruit Production. Northeastern Journal of Agricultural and Resource Economics. 18(1), 26-34.

Maki LR, Galyan EL, Chang-Chien MM, Caldwell DR (1974) Ice nucleation induced by *Pseudomonas syringae*. Journal of Applied Microbiology. 28(3), 456-459.

Maki LR, Willoughby Kj (1978) Bacteria as biogenic sources of freezing nuclei. Journal of *Applied Meteorology*. 17(7), 1049-1053.

Morris CE, Sands DC, Vanneste JL, Montarry J, Oakley B, Guilbaud C (2010).Inferring the evolutionary history of the plant pathogen

Pseudomonas syringae from its biogeography in headwaters of rivers in North America, Europe and New Zealand. mBio. 1, 00107–10.

Mulet M, Lalucat J, García-Valdés E (2010) DNA sequence-based analysis of the *Pseudomonas* species. Environ Microbiol. 12, 1513–30.

Obata H, Muryoi N, Kawahara H, Yamade K, Nishikawa J (1999) Identification of a novel ice-nucleating bacterium of Antarctic origin and its ice nucleation properties. Cryobiology. 38(2), 131-139.

Parkinson N, Bryant R, Bew J, Elphinstone J (2011) Rapid phylogenetic identification of members of the *Pseudomonas syringae* species complex using the rpoD locus. Plant Pathology. 60(2), 338–344.

Rostami M, Hasanzadeh N, Khodaygan P, Riahi-Madvar A (2018) Ice nucleation active bacteria from pistachio in Kerman Province, Iran. Journal of Plant Pathology. 51-58.

Sarris PF, Trantas EA, Mpalantinaki E, Ververidis F, Goumas DE (2012) Pseudomonas viridiflava, a multi host plant pathogen with significant genetic variation at the molecular level. PloS One 7: e36090.

Schaad NW, Jones JB, Chun W (2001) Laboratory Guide for Identification of Plant Pathogenic Bacteria. 3rd ed. Saint Paul Minnesota. American phytopathological society (APS) Press. pp. 373.

Tang C, Sun F, Zhao T (2003) Construction of ice nucleation active *Enterobacter cloacae* for control of insect pests. Chinese Science Bulletin. 48(2), 175-180.

Vali G (1971) Quantitative evaluation of experimental results the heterogeneous freezing nucleation of supercooled liquids. Journal of the Atmospheric Sciences. 28(3), 402-409.

Varvaro L, Fabi A (1992) The role of ice nucleation active *Pseudomonas viridiflava* in frost injury to kiwifruit plants. Rivista di Patologia Vegetale. 85-90.

Watanabe K, Abe K, Sato M (2000) Biological control of an insect pest by gut-colonizing *Enterobacter cloacae* transformed with ice nucleation gene. Journal of Applied Microbiology. 88(1), 90-97.

Weaver DJ (1978) Interaction of *Pseudomonas syringae* and freezing in bacterial canker on excised peach twigs. Phytopathology. 68(10), 1460-1463.

Wisniewski, M, Fuller M, Glenn DM, Palta J, Carter J, Gusta L, Duman J (2001) Factors involved in ice nucleation and propagation in plants: an overview based on new insights gained from the use of infrared thermography. Icelandic Agricultural Sciences. 14, 41-47.

Wolber PK, Warren GJ (2012) Evolutionary Perspective on the Ice Nucleation Gene-Encoded Membrane Protein. In: Andrews JH, Hirano SS (eds) Microbial Ecology of Leaves. Springer, New York.

Wowk B, Fahy GM (2002) Inhibition of bacterial ice nucleation by polyglycerol polymers. Cryobiology. 44(1), 14-23.

Yadav KK, Bora A, Datta S, Chandel K, Gogoi HK, Prasad GBKS, Veer V (2015) Molecular characterization of midgut microbiota of *Aedes albopictus* and *Aedes aegypti* from Arunachal Pradesh, India. Parasites Vectors. 8(1), 641-649.