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ORIGINAL ARTICLE

# Ice nucleation active bacteria from pistachio in Kerman Province, Iran

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**Abstract**
Frost damage is one of the major factors threatening pistachio production in southern part of Iran. In the present study, an attempt was made to screen and characterize ice nucleation bacteria associated with frost damage among some predominant epiphytic microflora. A large number of epiphytic bacteria was isolated from different regions in Kerman province. 1200 bacterial isolates were obtained from bud, twig, leaf and green fruit surfaces. Sixty-four strains exhibited ice nucleation activity. Among these, a vast microbial diversity was observed. For preliminary characterization of the isolates, certain key biochemical and pathogenicity tests were performed. The preserved cultures were often checked for ice formation and loss of ice nucleation activity was found in most cultures. Furthermore, factors affecting the increment of ice nucleation activity were studied and the isolates that recovered their ice nucleation activity were selected for sequence analyses of 16SrDNA, *rpoD* and *recA* genes, then compared with other isolates in NCBI GenBank using blasts methods. *Pseudomonas fragi, P. putida, P. moraviensis*, *P. virdiflava* and *Pantoea agglomerans* were the major predominant ice nucleation active bacteria established on plant surfaces. This work reports for the first time the presence of two ice nucleation active bacteria, *P. fragi* and *P. moraviensis*, on pistachio plants.

**Keywords** Ice nucleation bacteria · Frost damage · Pistachio · Kerman province

## Introduction

Pistachio is one of the most valuable tree species which has economic importance in agricultural production of Iran and many other countries since it represents a large portion of the non-petroleum exportation. According to estimates made by World Food and Agriculture Organization (FAO) for the year 2002, Iran ranked first in the world in terms of pistachio production (300,000 metric tons) with Kerman province in the first position at the country level for the bulk and quality of production (Koshteh and Urutyan 2005).

Frost damage in pistachio trees is a serious problem in hot spot locations of the region, leading to a significant yield loss and economic problem for the growers. In the past decade, on average, 50% of pistachio was damaged by early spring frost. In Kerman province, the damage was estimated to be 420 million dollars for two consecutive years of 2004 and 2005 (Salary Sorkhan et al. 2011).

A somewhat neglected problem of this economic agricultural crop is the frost damage caused by ice nucleation active bacteria (INA) which probably predispose the plants to pathogen attacks with a much more serious outcome (Lindow 1982a; Ramstedt et al. 1994; Cambours 2004).

Ice nucleation active (INA) bacteria are common epiphyte on multiple plant species from different geographical areas, and several studies confirmed a correlation between the presence of INA bacteria and freezing of crops (Hirano et al. 1982; Lindow 1983; Ketabchi 2004; Mazarei and Ghasemi 1993; Sahragard et al. 1997).

Ice nucleation-active proteins (INAP) are on the outer bacterial cell wall that facilitates ice formation in inter- or intracellular plant tissues (Love and Lesser 1989). In the absence of INA bacteria, water in tissues of more sensitive freezing plants can be incredibly cool, i.e., tissues below 0 °C to −12 °C without freezing inter- or intracellular fluid (Maki and Willoughby 1978), but INAP as heterogeneous ice nucleus leads to ice formation at temperatures as high as 0 to −2 °C

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(Deininger et al. 1987). Therefore, ice nucleation active bacteria (INA) play significant role in freezing injuries of cold sensitive plants (Hirano and Upper 2000; Morris et al. 2004; Kennelly et al. 2007). To overcome this problem, various methods have been proposed. These include employment of different models of phonological timing in different plant varieties, control of INA bacteria by means of antagonists and bactericides, and investigating the microbiome of leaf surface (Prabha and Hoogenboom 2008; Burke et al. 1976; de Rodriguez Penaranda 1980; Lindow 1982a; Morris et al. 2004; Lindow 1995).

Despite these research efforts, frost injury still occurs in most regions. Annual reports confirm a significant increase in the risk of frost damage throughout the Kerman region. To minimize higher risk of frost damage to pistachio orchards, a preliminary work was done to isolate the bacteria probably involved in pistachio frost injury. So far, there is a little evidence if on pistachio plant frost damage proceeds from ice nucleation bacteria. Therefore, further research is needed to understand different aspects of this phenomenon in Iran climate condition.

## Material and methods

**Bacterial isolation** During autumn and spring seasons, seasonal surveys were carried out in different pistachio orchards of Kerman province and considerable plant samples were collected from aerial parts of trees, mainly leaves, buds and twigs. GPS coordinates were recorded. All the samples were placed in sterile plastic bags and transported to the laboratory in refrigerated boxes. For each sample, approximately 150 g of the tissues were soaked in 100 ml of sterile distilled water containing 1 ml of Tween-20 and shaken at 180 rpm for 1 h. A loopful sample suspension was streaked onto plates containing nutrient agar, KB and GYNA (glucose yeast extract nutrient agar) media (Sherafati et al. 2014). All the plates were incubated at room temperature (28–30 °C) for 2 days. The suspected colonies with different morphology were selected and re-streaked on tryptic soy agar (TSA) medium and checked for purity of the cultures (Nejad et al. 2006). Cultures for routine use were stored at 4 °C. For long-term storage, cultures were inoculated on LB + 30% glycerol and maintained at −80 °C.

**Detection of INA colonies** All colonies on each plate were picked up and tested for ice-nucleation assay (Fahy and Persley 1983). Distilled water and *Pseudomonas syringae* were used as negative and positive controls, respectively. The experiment was done in two replicates. A colony was suspended in 2 ml of distilled $H_2O$ with ca. $10^8$ CFU/ml and all test tubes were held in a bath in a solution containing ethanol and crushed ice in the ratio of 1:1 to maintain the temperature at about −9 °C (Fig. 1). Strains were scored positive for ice nucleation activity if immediate ice formation was formed in test tubes (Fahy and Persley 1983). In the control tubes with ice minus bacteria, no ice crystals developed even at −10 °C for 1 h period.

**The effect of growth medium composition on ice nucleation activity** The effect of different media composition on ice-nucleation activity of bacteria was assessed with (i) the standard nutrient agar (NA; 3.3 g Bacto peptone, 2.7 g Difco nutrient broth, 2.0 g yeast extract and 15.0 g Bacto agar/l distilled $H_2O$) amended with two carbon sources, 2.5% glycerol (*v*/v) plus 2.5% glucose or 2.5% glycerol (v/v) plus 4% glucose (*w*/*v*); (ii) King's medium B (20 g proteose peptone, 10 ml glycerol, 1.5 g $K_2HPO_4$, 1.5 g $MgSO_4.7H_2O$, 15 g agar in 1 l distilled $H_2O$) and (iii) minimal agar medium [1.0 g glucose, 7.0 g $K_2HPO_4$, 0.5 g sodium citrate, 0.1 g $MgSO_4.7H_20$ and 1.0 g $(NH_4)_2SO_4$, 15.0 g Bacto agar in 1 l distilled $H_2O$]. All the cultures were incubated at 22 °C and checked for their potential ice nucleation activity by the drop-freezing assay (Lindow et al. 1982c).

**Phenotypic characteristics** Fresh cultures were prepared from each strain and all were subjected to key phenotyping test (Krimm et al. 2005). The KOH test was used to determine the Gram reaction. Fluorescent pigmentation on King's B medium was visualized under UV light. O/F test was done to determine aerobic/facultative anaerobic growth. The main biochemical tests were levan production from sucrose, cytochrome oxidase reaction, arginine dihydrolyase, urease, and catalase (Schaad et al. 2001; Suslow et al. 1982). Hypersensitivity reaction was achieved with overnight cultures of the isolates which were injected into intercellular spaces of intact tobacco leaves. The leaves were examined for hypersensitive reaction after 24 and 48 h incubation time (Schaad et al. 2001).

**Pathogenicity test** The abaxial leaf surfaces of three-month-old pistachio seedlings (*Pistacia vera* L. cv. Badami) were injected with 14 $HR^+$ bacterial cell suspensions at concentration of $10^8$CFU/ml. The treated seedlings were maintained in growth chamber under 12 h photoperiod with a mean

**Fig. 1** Ice nucleation test in microcentrifuge tubes. All 64 strains showed highly ice nucleation activity

temperature of 27 °C day/20 °C night and a relative humidity of 80%. All the seedlings were assessed daily for two weeks to record disease symptoms (Hoque and Mansfield 2005).

**Freezing test** Bread wheat (*Triticum aestivum* L.) seeds were planted in 10-cm-diameter pots containing commercial potting soil mix. Twenty grams of previously germinated seeds were planted in each pot and grown in a greenhouse under a 12-h photoperiod and average maximum and minimum temperatures were 26 and 16 °C, respectively, and the relative humidity was 40%. The pots were watered with distilled water every other day. 7-day-old seedlings were acclimated to cold in a growth chamber under illumination of 375 microeinsteins (μE) with 25 °C days, 5 °C nights, and average relative humidity of 70% for a week. All the pots with three replicates per treatment were sprayed with $1 \times 10^9$ CFU/ml fresh suspension of ice plus strains until plants were wet to runoff (Buttner and Amy 1989). Bacterial suspensions were prepared from cultures grown on NA containing 2.5% glycerol for 24 h at 22 °C. The controls were treated with distilled $H_2O$. Plants were maintained for two days for bacterial colonization as described above. All the colonized wheat plants were then subjected to the freezing test (−5 °C for 40 min.). Plants under cold-acclimating conditions were removed from cold chamber and warmed to room temperature and assessed for frost damage.

**PCR amplification and gene sequencing** Total DNA, from bacterial isolates was prepared using Qiagen DNeasyTM Kit. The extracted DNA was used as a template for PCR amplification of 16SrDNA, *rpoD* and *recA* genes. PCR primers used for amplification and sequencing of these genes were 63f (5′-CAGGCCTAACACATGCAAGTC-3′) and 1387r (5′-GGGCGGWGTG TACAAGGC-3′) for 16SrDNA (Marchesi et al. 1998). $Prpo_1$ (5′-TGAA GGCGARATCGAAATCGCCAA-3′) and $Prpo_2$ (5′-YGCMGWCAGCTTYTGCTGGCA-3′) for rpoD (Parkinson et al. 2011), recAr (5′-GGTAAAGGGTCTAT CATGCG-3′) and recAf (5′-CCTTCACCATACATAATTTG GA-3′) for recA (Waleron et al. 2002).

Reaction mixture of 16SrDNA template were placed in a thermocycler and the following program was run: initial denaturation at 94 °C for 4 min, 40 cycles of 94 °C for 60 s, 65 °C for 45 s, 72 °C for 60 s and a final extension step at 72 °C for 10 min (Marchesi et al. 1998).

A fragment of approximately 700 bp of *rpoD* gene was amplified in the reaction mixture with initial denaturation at 95 °C for 4 min, 30 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 2 min and a final extension step at 72 °C for 7 min (Parkinson et al. 2011).

Thermal cycling program for amplification of *recA* gene was: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min with a final extension at 72 °C for 10 min (Waleron et al. 2002). Positive and negative controls with and without DNA were included in every step of amplification, to test for the presence of contamination of reagents and reaction buffer.

The PCR products were monitored on 1% agarose (wt/vol). Gel electrophoresis was carried out at 80 V for 30 min. The ethidium bromide-stained gel was visualized using UV transillumination. PCR products obtained from bacterial strains were sent to Bionner Company (South Korea) for sequencing. The complete sequences (1300 bp) of 16SrDNA genes were retrieved by nucleotide Basic Local Alignment Search Tool (BLAST) program at the National Center of Biotechnology Information (NCBI) and compared (Altschul et al. 1997).

In addition, some Ice plus bacterial strains were discriminated from each other using Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR. Primers used for ERIC-PCR were ERIC1 (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) (Versalovic et al. 1991). PCR amplification was performed using the following cycles: initial cycle at 95 °C for 3 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min with ERIC primers, and extension at 72 °C for 1 min with a single final extension cycle at 72 °C for 10 min. Amplified PCR product was separated by gel electrophoresis on 1.5% agarose gels for 5 h at 70 V, stained with ethidium bromide and photographed on a UV trans illuminator.

**Table 1** Effect of growth medium composition on ice nucleation activity of the bacteria

| Culture media | *Pseudomonas* sp. $Raf_4$ | *P. fragi* $Raf_3$ | *P. putida* $Raf_6$ | *P.moraviensis* $Raf_1$ | *P. moraviensis* $Raf_5$ | *Pantoea agglomerans* $Raf_7$ |
|---|---|---|---|---|---|---|
| NA + 2.5% glucose+4% glycerol) | – | + | – | – | – | + |
| (NA+ 2.5% glucose) | – | + | – | – | + | – |
| (NA+ 2. 5% glycerol) | – | + | + | + | – | + |
| Minimal medium | + | + | – | – | – | + |

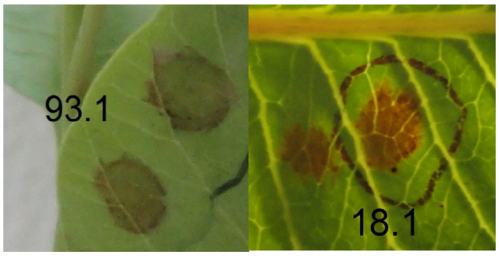


**Fig. 2** Necrotic local lesions developed on pistachio leaves followed by infiltration of two unidentified strains ($INA^+$ and $HR^+$) of 93.1 and 18.1

## Results

**Bacterial isolation and detection of ice nucleation active bacteria** In this study, a total of 1200 strains were isolated from the aerial parts of pistachio trees. All suspected colonies with different colonial characteristics were screened for ice nucleation activity. During the initial screening, 64 strains showed positive INA-reaction, but over time and at different intervals the number dropped to two isolates. Their potential ice nucleation activities were recovered by modifying growth temperature and media component.

**The effect of growth medium composition on ice nucleation activity** The role of glycerol and glucose as a main carbon source was investigated. Some bacteria with weak or lost ice nucleation activity recovered their ice nucleation activity after growth on nutrient agar plus glycerol and glucose at 22 °C. Similarly, when the bacteria were grown on minimal agar medium, a remarkable positive effect on ice nucleation activity of at least 10% of bacterial isolates was found (Table 1).

**Phenotypic characteristics** 64 isolates were considered as Ice-plus bacteria. Majority (70%) of these were Gram negative bacteria. There were distinct morphological and biochemical differences among these isolates.

**Pathogenicity test** None of the selected strains could induce typical disease symptoms on pistachio seedlings. Local injection-site necrosis was observed in some isolates such as 93.1 and 18.1 but none was progressive (Fig. 2).

**Freezing test** The young wheat seedlings when subjected to ice plus strains, showed variable degrees of cold damage at −5 °C. The control plants were intact and undamaged (Fig. 3). Damage was exhibited as flaccidity or discoloration (dark green or water-soaking) of the leaves upon rewarming of the plants (Figs. 4).

**PCR amplification and gene sequencing** The PCR products of six isolates including a strain with stable ice activity ($Raf_3$) and five strains with marked loss of ice nucleation activity ($Raf_1$, $Raf_3$, $Raf_4$, $Raf_5$, $Raf_6$, $Raf_7$) were purified by Bionner Company (South Korea) and sequenced. Results from BLAST by NCBI showed 99% identity between 16SrDNA sequences of the strains and deposited sequences, and 97% identity for *rpoD* and *recA* gene sequences. Accession numbers of *rpoD*, *recA* and 16SrDNA gene sequences are listed in Table 2. Based on these, five strains encoded $Raf_1$, $Raf_3$, $Raf_4$, $Raf_5$ and $Raf_6$ were assigned to the genus, *Pseudomonas*. Two strains, $Raf_1$ and $Raf_5$, showed 99% similarity with *Pseudomonas moraviensis*. Strains $Raf_3$, $Raf_4$ and $Raf_6$ belonged to *P. fragi*, *Pseudomonas* sp. and *P. putida*, respectively. The $Raf_7$ was closest NCBI match to *Pantoea agglomerans* (Table 2). Amplfication of 16SrDNA, rpoD, recA gene sequences are showen in Fig. 5 and 6.

ERIC-PCR patterns could differentiate Ice plus *Pseudomonas* species from each other but failed to discriminate *P. moraviensis* strains $Raf_5$ and $Raf_1$ (Fig. 7).

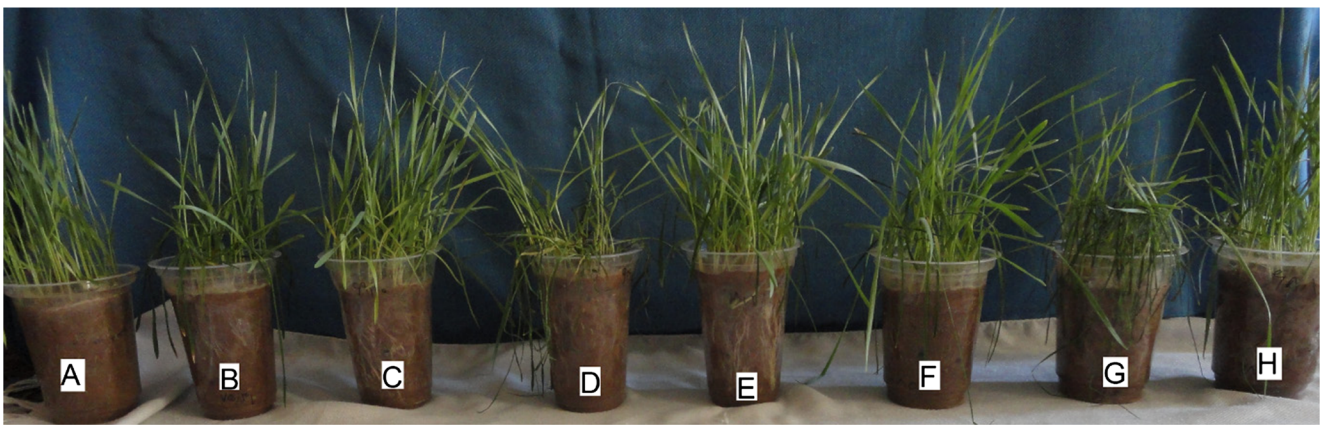


**Fig. 3** Different degrees of frost injury were observed on wheat seedlings treated with different bacteria in an ambient temperature of 5 °C. A, Negative control treated with sterile distilled water: B, *Pseudomonas* sp. $Raf_4$; C, *P. putida* $raf_6$; D, *Pantoea agglomerans* $raf_7$; E, *P. moraviensis* $raf_1$; F, *P. putida* $raf_6$; G, *P. fragi* $raf_3$; H, *P. moraviensis* $raf_5$

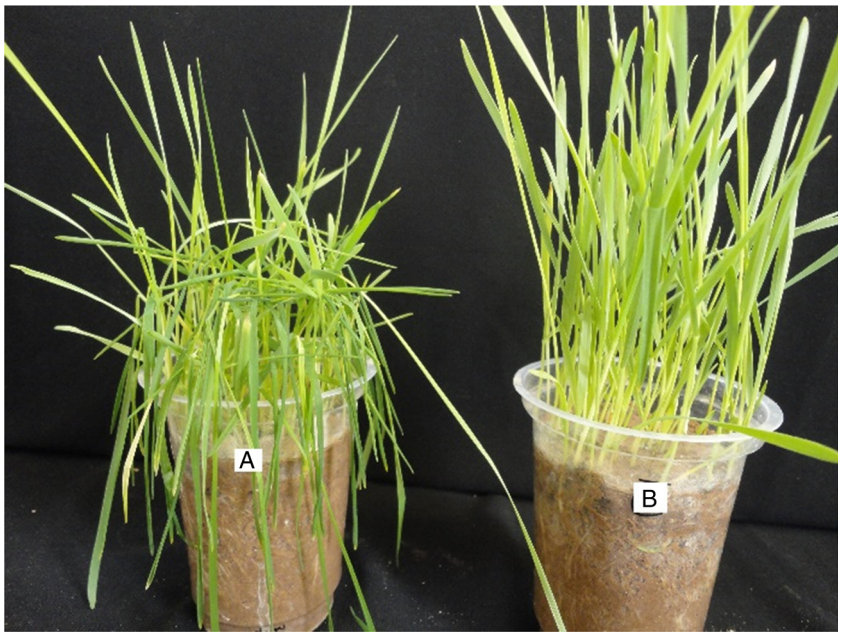


**Fig. 4** Freeze damage causes flaccidity/discoloration of leaves after incubation at −5 °C for 40 min. A: Wheat treated with *P. fragi* raf3. B: Negative control treated with sterile distilled water

## Discussion

Reports in the last three decades indicated a close relationship between ice nucleation active bacteria (INA) and frost damage in plants (Hirano et al. 1985; Lindow 1983; Lindow et al. 1978, 1982c; Yankofsky et al. 1981). Several methods have been employed to reduce frost damage caused by ice nucleating bacteria, which include the usage of bactericides, selective breeding of INA bacteria-resistant crops, and the employment of antagonistic non-ice nucleating bacteria to compete with ice nucleating bacteria. Still, much information is required to reduce the damages caused by Ice-plus bacteria.

In this study, a consistent number of bacteria associated with frost damage on pistachio was isolated. Initially, 64 bacterial isolates (ca. 5%) showed ice nucleation activity, but the majority of them lost their capability for crystal formation due to prolonged incubation period at 4 or −20 °C. This indicated that some factors affect INA genes expression. It was well documented that production of ice nucleation proteins is mostly influenced by several environmental conditions such as light intensity, ambient humidity and cultural factors (Attard et al. 2012; Ashworth et al. 1985). Some of the strains also exhibited variable freezing characters in their cell suspensions with different environmental and cultural conditions such as temperature and medium composition (Lindow et al. 1982c). Different kinds of glycerol feed stocks and experimental conditions have an important impact on distribution of metabolic products (Metsoviti et al. 2012; Lindow et al. 1982c). Here it was shown that culture media containing glycerol and glucose could activate the potential ice nuclei in certain strains (Table 1).

In addition, sensitivity to environmental changes such as light intensity and ambient humidity was more distinguished (O'Brien and Lindow 1988; Cambours et al. 2005; Attard et al. 2012). Cambours et al. (2005) reported that ice active epiphytes are the most prevalent in the spring. The same phenomenon was observed under Kerman orchard conditions where the population of INA bacteria increased from March to the end of April and steadily decline onwards.

**Table 2** GenBank sequence accession numbers and geographic origin of ice-plus bacterial strains

| Ice-plus bacterial strain | Accession numbers | | | Geographic origin of isolation |
|---|---|---|---|---|
| | 16srDNA | rpoD | recA | |
| *Pseudomonas moraviensis* $Raf_1$ | KX644088 | KY782323 | * | Raien |
| *P. fragi* $Raf_3$ | KX632157 | KY782324 | * | Marvast |
| *Pseudomonas* sp. $Raf_4$ | KX643369 | KY782325 | * | Rafsanjan |
| *P. moraviensis* $Raf_5$ | KX636092 | KY914483 | * | Marvast |
| *P. putida* $Raf_6$ | KX643375 | KY914484 | * | Rafsanjan |
| *Pantoea agglomerans* $Raf_7$ | KX63082 | * | KY782326 | Rafsanjan |

*not sequenced

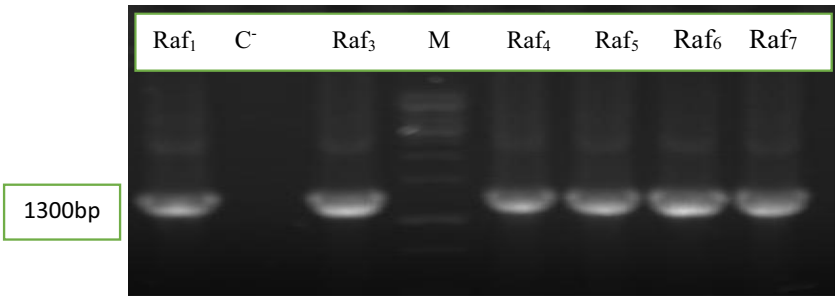


**Fig. 5** The PCR product of 1300 bp was obtained by amplification of 16SrDNA gene of six strains $Raf_1$, $Raf_3$ and $Raf_7$ with the primer pair 63F and 1387R. $C^-$, Negative control. M, Molecular 1 kb DNA Ladder

Most ice nucleator strains in the culture collection belong to the *Pseudomonas* genus. This agrees with the conclusion that *Pseudomonas* species as epiphytes, may affect plants negatively through induction frost damage (Lindow 1995).

The importance of 16SrDNA gene sequences in the identification of bacteria is well known. Sequences of this gene are extensively used as a framework for bacterial identification (Garcia-Martinez et al. 2001). At the same time, the identification based on 16SrDNA lacks resolution at intragenic level. Therefore, a highly selective *rpoD* gene encoding the principal sigma factor in many bacteria including the genus *Pseudomonas,* was employed. An additional discriminatory marker, recombinase A (*recA*) was also used for certain facultative anaerobes.

Based on these analyses, five ice-plus bacteria namely, *Pseudomonas moraviensis*, *P. putida, P. fragi, Pseudomonas* sp. and *Pantoea agglomerans* were isolated and identified from pistachio trees in Kerman province.

*Pseudomonas putida, P. fragi* and *Pantoea agglomerans* have been already reported as INA strains in nature (Costanzo and Lee 1996; Rutherford et al. 2000; Zdorovenko et al. 2004; Hasegawa et al. 1990; Ohgama et al. 1992; Rostami 2012; Hill et al. 2014; Morris et al. 2004; Lindow et al. 1982b), but the presence of *Pseudomonas moraviensis* as ice-plus bacterium from aerial parts of pistachio trees was reported here for the first time. The latter was introduced as biocontrol strain of *Rhizoctonia solani* in potato (Mrabet et al. 2013).

*P. putida* $Raf_6$ is another bacterium which was frequently isolated from the leaf surfaces of pistachio in Rafsanjan. *P. putida* isolated from the Canadian Arctic is a peculiar strain capable of proliferating at low temperatures (5 °C) and able to survive freezing temperatures (−20 and −50 °C), without the aid of cryoprotectants. *P. putida* GR12–2 could express AfpA, an antifreeze protein, and nucleation activity simultaneously (Sun et al. 1995; Muryoi et al. 2004). Besides, the bacterium could survive long-term in the environment (Molina et al. 2000). Therefore, it could be a good candidate for controlling pathogenic and INA bacteria in plants.

*P. fragi* $Raf_3$ is a typical isolate screened so far. A psychrophilic strain of the same species was reported to cause dairy spoilage (Pereira and Morgan 1957). Temperature for its growth and reproduction ranges from −20 to +10 °C. The strain, $Raf_3$ was found to be a strong ice-plus bacterium. The strain, $Raf_3$ was isolated in Marvast region, 200 km from the city of Rafsanjan in April 2015. This region is considered as a high-risk area of pistachio frost in Kerman (Omidvar and Banadaki 2012).

*Pantoea agglomerans* $MR_1$ showed 99% similarity with *16SrDNA* and 97% with *recA* sequences as compared to other GenBank sequences. It is often isolated from plant surfaces, seeds and fruits. According to Feng et al. (2006), the bacterium was associated with plants and acts as an important ice-nucleating bacterium. A novel cryoprotective protein was purified and identified from the *P. agglomerans* IFO12686 (Koda et al. 2001).

In this study, the use of Enterobacterial Repetitive Intergenic Consensus as ERIC sequences in PCR on the DNAs of Ice plus *Pseudomonas* species was examined. ERIC analysis showed that DNA fragment lengths between the repetitive elements were variable, whereas the two ice

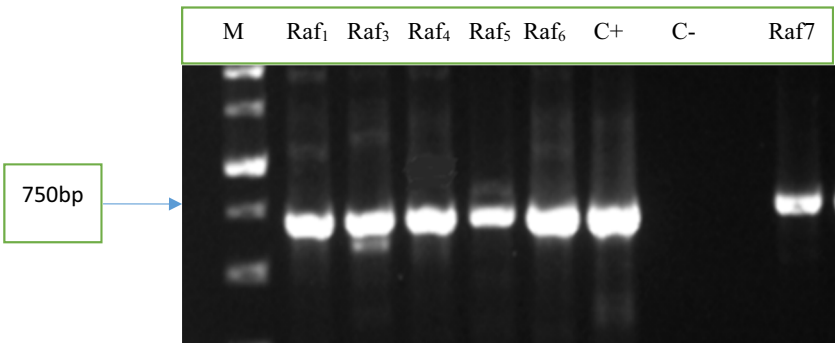


**Fig. 6** $Prpo_1$ and $Prpo_2$ were used to amplify rpoD gene for strains $Raf_1$, $Raf_3$, $Raf_4$, $Raf_5$ and $Raf_6$ (lanes 2–5). A fragment of approximately 730 bp was amplified from all strains except for recA strain $Raf_7$ (lane 9, 800 bp). $C^+$, *Pseudomonas syringae* and M, 1 kb DNA Ladder

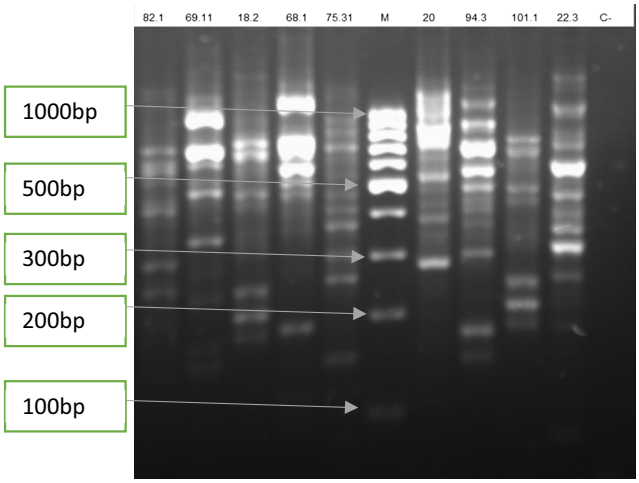


**Fig. 7** Patterns of ERIC-PCR on agarose gel electrophoresis. Lanes 1–11, isolates 82.1 (*P. putida* $Raf_6$), 69.11 (not identified), 18.2 (*P. moraviensis* $Raf_1$), 68.1 (not identified), 75.31 (*Pseudomonas* sp. $Raf_4$), M (Lader 100 bp), 20 (not identified), 94.3 (*Pantoea agglomerans* $Raf_7$), 101.1 (*P. moraviensis* $Raf_5$), 22.3 (*P. fragi* $Raf_3$). C- (negative control)

nucleation active strains of *P. moraviensis,* that is, $Raf_1$ and $Raf_5$, despite the different geographical locations, showed the same finger printing pattern (Fig. 7).

It may be concluded that abundance and variability of bacteria in different regions correlate with climatic conditions. Also, expression of ice-nucleating proteins in certain Ice-plus bacteria has close relationship with certain conditions such as culture media and temperature. These findings may be applicable in the production of natural pesticides (Tang et al. 2004.; Lee et al. 2016), frost prevention (Lorv et al. 2014), food uses (James et al. 2015) and artificial snow (Cid et al. 2016).

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