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RESEARCH ARTICLE

Role of phyllosphere microbes in Ice Nucleation Activity and Its impact on drought mitigation of tomato

Guna, M[1], Panneerselvam, S*[2], Subramanian, K.S[3], Krishnamoorthy, A.S.[4] and Lakshmanan, A[5]
[1]Agro Climate Research Centre,[2]Water Technology Centre, [3]Department of Nano Science and Technology,
[4]Departmnet of Plant Pathology,[5]Department of Nano Science and Technology, Tamil Nadu Agricultural University, Coimbatore-3

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ABSTRACT

Microbes play a key role in ice nucleation that facilitates bioprecipitation, which enables the plants to survive under limited water conditions. In order to test the hypothesis, a laboratory experiment was conducted to confirm ice nucleation using two phyllosphere microorganisms *viz., Pseudomonas fluorescens* and pink-pigmented facultative methylotrophs (PPFM). The bioprecipitation impacts on tomatoes were assessed using a set of physiological parameters such as relative water content, stomatal conductance, leaf temperature besides colony forming unit/cm^2 and tube nucleation test. A field experiment was carried out in tomato (PKM 1) involving spraying of these inoculants in 3 moisture regimes (0.6, 0.8, 1.0 IW/CPE ratio) replicated four times in a factorial RBD. The data revealed that both the microbial sprays did not significantly alter the physiological parameters of tomato with an exception of leaf temperature of *P. fluorescens* sprayed plants with the scheduling of irrigation at 1 IW / CPE which was significantly lower (19.7 °C) than PPFM sprayed ones. On the other hand, the higher relative water content of 77.4% was registered in PPFM sprayed plants irrigated at 1.0 IW/CPE ratio. In both microbial sprays, the RWC increased progressively with the advancement of the experiment from day 1 to 10 DAS and declined thereafter. However, the average stomatal conductance of the PPFM sprayed tomato plants (0.43 mmol/m^2/sec) at 15 DAS was significantly higher than *P. fluorescens* sprayed plant (0.34 mmol/m^2/sec) under all levels of moisture regimes. The PCR amplification confirms the presence of *inaW* genes (ice nucleation active W gene) in *P. fluorescens* while it was absent in PPFM. The tube nucleation tests were proved the ice crystallization induced by the *P. fluorescens.* Overall, the study suggests that *P. fluorescens* may assist in ice nucleation activity that enables tomato plants to maintain lower leaf temperature while RWC and stomatal conductance were comparable with PPFM.

Keywords: I*ce nucleation, Relative water content, Pseudomonas fluorescens, PPFM, Bioprecipitation*

INTRODUCTION

The rainwater is a major source of water to the Earth's planet. It provides water for all creatures in the world including humans, plants and animals. The rainwater is produced as a result of condensation of water vapour in the atmosphere. The evaporated liquid condenses in the cold air, forming moisture-filled rain clouds. In the climate change scenarios and extreme weather events such as Elnino and La Nino that are known to impede the rainfall pattern and cause irregular and erratic precipitation (Kakarla *et al., 2019*). Since the rainfall pattern in highly erratic and occurrence of frequent droughts is quite common, there is an urgent need to evolve a strategy to develop artificial rain to protect agriculture from devastation. Artificial rain was first reported in the globe in the year 1940 (Droessler, 1993) and it was demonstrated in Tamil Nadu, India during 1983 (CASWMT., 2011). Substances used for artificial rain include silver iodide, sodium chloride and dry ice. The condensation of water vapour led to the formation of ice crystals at extremely low sub-zero temperatures of below 40°C under natural conditions. Such condensation reaction will take place at - 20°C when artificial nucleating agents such as Ag+ ions are used to develop the rain water droplets (Prasadarao, 2015). Despite the fact that chemicals have been successfully demonstrated

*Corresponding author's e-mail: Panneer_selvam43@yahoo.com

for the production of artificial rains, the excessive quantities may cause associated ill-effects such as toxicities and salinity. Continuous use may lead to accumulation in the natural ecosystems affecting the flora and fauna. In this context, microbes being natural with no-side effects, scientists are looking for a biological strategy to produce artificial rains using aerobic microbes (Maki *et al*., 1974).

Among the microbial communities, bacteria are well known to be involved in ice nucleation. Some bacteria's outer cell membrane contains a protein that can function at temperatures as warm as −1°C as an effective ice nucleator. A few bacterial species generate ice nucleation active proteins (INA). The INA bacteria were widely found in plants (as saprophytic epiphytes and/or as plant pathogens) that include *Pseudomonas syringe, P. viridiflava, P. fluorescens, Pantoea agglomerans* (formerly known as *Erwinia herbicola) and Xanthomonas campestris* (Wolber., 1993; Gaignard and Luisetti., 1993). One of the well-known bacteria, *Pseudomonas fluorescens* is a rod-shaped, gram-negative bacterium with multiple flagella. It belongs to the family pseudomonadaceae and class gamma proteobacteria. *P. fluorescens* also produces ice nucleation active (inaW) proteins which cause water (in plants) to freeze at high temperatures (-4 to -2°C). Since 1970s, *P. fluorescens* has been implicated as an atmospheric "biological ice nucleator", with airborne bacteria serving as cloud condensation nuclei (Turner *et al*., 1990). The INA proteins have been detected in artificial snow. Even the denatured INAs can also assist in ice nucleation (Hazra *et al*., 2004). The pink-pigmented facultative methylotrophs (PPFM) is a genus *Methylobacterium*, gram-negative bacteria playing an important role in drought tolerance at phyllosphere region. The PPFM was reported to increase the canopy water status of plants that were sprayed with microorganisms (Sivakumar *et al*., 2018). Such a strain is of novel ecological interest because the combinations of epiphytic growth habit and ice nucleation ability have previously been described in *P. fluorescens*. Our interest in this strain arises from both suitability of explaining the genetic component of *P. fluorescens* and PPFM ice nucleation in tomato leaves. We wanted to determine the genes needed to encode the phenotype of the ice nucleation and identify the gene product. This study hypothesizes that microbial spray carrying phyllosphere organisms such as *P. fluorescens* and PPFM assist in ice nucleation that enables the may tomato plant to survive under water deficit conditions.

MATERIAL AND METHODS

A field experiment was conducted at the Orchards, Tamil Nadu Agricultural University, Coimbatore, during rabi-summer (December 2018 to February 2019). Treatments consisted of two microbial sprays (M1 - *Pseudomonas fluorescens*; M2 - Pink Pigmented Facultative Methylotrophs) and three irrigation regimes (0.6, 0.8 and 1.0 IW/CPE ratio) replicated four times in an FRBD. Soil moisture regimes were maintained by drip irrigation using IW/CPE method to each treatment at regular intervals corresponding to the irrigation intervals of 12-14, 8-10 and 6-8 days, respectively. Microbial sprays (1 x 10^7 per ml) were given @ 2 per cent at the 45th day of transplanting. The crop was fertilized with NPK @ 100:60:60 kg per ha through drip fertigation. Observations on relative water content, stomatal conductance, leaf temperature, transpiration rate besides microbial counts were recorded on 1, 5, 10 and 15th day after the microbial sprays correspondingly to 46, 50, 55 and 60 days after transplanting, respectively.

The leaf RWC was estimated (Barrs and Weatherly, 1962) and calculated using the following formula and expressed as per cent. Turgid weight was measured after taking the fresh weight of the leaf samples, 10 ml of distilled water added in to test tubes and place the sample tubes in a refrigerator (at 4°C in darkness) for 24 h (to reach full turgor). The leaf samples were taken out of the tube, quickly and carefully blot dry with a paper towel and finally weigh the turgid weight of the leaf sample.

$$RWC = \frac{\text{Fresh weight - Dry weight}}{\text{Turgid weight - Dry weight}} \times 100$$

Stomatal conductance, transpiration rate and leaf temperature were measured using Portable Photosynthesis System (PPS) (Model LI-6400 of LICOR inc., Lincoln, Nebraska, USA). Totally, three measurements were taken in the same leaf. Leaves were inserted in a 3 cm^2 leaf chamber and Photosynthetic photon flux density (PPFD) at 1200 mmol photos/m^2/sec were set. The number of colony forming unit (CFU) per cm^2 of the tomato leaf sample was enumerated immediately after treatment using King's B medium (King *et al*., 1954) for *Pseudomonas fluorescens* and ammonium mineral salt (AMS) agar with 0.5 per cent methanol (Whittenburry *et al*., 1970) for PPFM.

The characterization of ice nucleation active W gene (*inaW* gene) was isolated from the total genomic DNA of the bacteria that was performed using lysis buffer method (Chen and Kuo, 1993). The Genomic DNA will be isolated from *P. fluorescens* and PPFM using the standard cethyl trimethyl ammonium bromide (CTAB) protocol. In order to confirm the isolates as *inaW* gene, INAWF (ATGAATGCATAATGGGCACTG); INAWR (GTCTGCGTGCTGCCATAACC) primers (Operon, Inc., Alameda, CA) will be used to obtain an amplicon size of 1500 bp. Amplification was carried out with a

total reaction volume of 50 µl in Eppendorf- Master cycler, Germany. The PCR products will be resolved on a 2% agarose gel at 50 V, stained with ethidium bromide (0.5 µg/ml) and will be photographed and analyzed using gel documentation system (Alpha Infotech Corporation, San Leandro, California). The DNA sequencing was performed at Eurofins Genomics India Pvt. Ltd., Bangalore, India. Hence, the bacteria identified as inaW gene through the molecular tools were used for screening of ice nucleation active bacteria.

A tube nucleation test method was employed to determine the ice nucleation potential of the bacteria's (Hirano *et al., 1985)*. Test tubes (18 x 150 mm size) filled with 10 ml K-phosphate buffer (10 mmol, pH 7.0) were plugged with cotton and sterilized by autoclaving at 121°C. After cooling at room temperature, tubes super-cooled to -10°C and discarded the tubes in which liquid was frozen. Other tubes were used for further studies. The tubes that were not frozen, were placed in ambient temperature and inoculated with either 1 ml of *P. fluorescens* or PPFM. The *P. fluorescens* and PPFM isolates were cultivated on nutrient agar with 10^8 cells/ml density. The test tubes were mixed and placed at -10°C and observed freezing at 15 mins intervals. Wherever the treatment differences were significant, critical differences worked out at 5% probability level and the values were reported.

RESULTS AND DISCUSSION

Relative water content (RWC)

The mean values of leaf RWC at 1 DAS for different irrigation regimes ranged from 52.2 to 61.9 per cent and microbial sprays from 57.1 to 57.4 per cent (Table .1). The leaf RWCs increased with the progression of the experiment until 10th day and declined thereafter. RWCs registered were 60 to 68 per cent for irrigation regimes and microbial spraying 63.7 to 64.9 per cent. In general, there was a decline in RWC for all the treatments during 15 DAS. The PPFM sprayed plants had significantly lower RWC on day 5 and 15 than *P. fluorescens* while the values were comparable on day 1 and 10. The RWCs increased with the increasing frequencies of irrigations in the order of 1.0, 0.8 and 0.6 IW / CPE ratios. RWC is considered as one of the key parameters measuring the water status of leaves. The leaf RWC is closely associated with the productivity of crops (Haloi and Baldev, 1986). Microbial sprays assisted in an increase in concentrations of osmolytes like proline that enhanced the water uptake by plants. The higher RWC was also related to the osmotic adjustment (Katerji *et al*., 1997). The data are in conformity with the observations

Table 1.Impact of phyllosphere microbes on relative water content (RWC) o tomatoes

Treatments	Relative water content (%)											
	1DAS			5DAS			10DAS			15DAS		
	M1	M2	Mean	M1	M2	Mean	M1	M2	Mean	M1	M2	Mean
I1	52.9	51.5	**52.2**	72.9	65.8	69.4	68.3	63.7	**66**	60	60	60
I2	57.7	57.7	**57.7**	70.1	67.4	68.8	72.1	75.8	**73.9**	65.2	64.7	64.9
I3	61.6	62.2	**61.9**	75.5	76	75.8	76.4	77.4	**76.9**	69.5	66.6	68
Mean	**57.4**	**57.1**		72.8	69.7		72.2	72.3		64.9	63.7	
	M	I	M x I	M	I	M x I	M	I	M x I	M	I	M x I
SEd	0.45	0.55	0.78	0.55	0.67	0.95	0.55	0.68	0.96	0.49	0.61	0.86
CD (*P*=0.05)	NS	1.17	NS	1.17	1.44	2.04	NS	1.45	2.05	1.06	1.3	NS

Note: M1 – *P. fluorescens;* M2 – PPFM; I1 – 0.6 IW /CPE ratio; I2 – 0.8 IW /CPE ratio; I3 – 1.0 IW /CPE ratio

Table 2.Impact of phyllosphere microbes on stomatal conductance of tomatoes

Treatments	Stomatal conductance (mmol $H_2O/m^2/sec$)											
	1DAS			5DAS			10DAS			15DAS		
	M1	M2	Mean	M1	M2	Mean	M1	M2	Mean	M1	M2	Mean
I1	0.39	0.43	**0.41**	0.37	0.39	0.38	0.37	0.28	**0.32**	0.32	0.46	0.39
I2	0.38	0.33	**0.36**	0.35	0.33	0.34	0.33	0.39	**0.36**	0.35	0.43	0.39
I3	0.41	0.38	**0.40**	0.33	0.3	0.32	0.38	0.43	**0.40**	0.35	0.42	0.38
Mean	**0.39**	**0.38**		0.35	0.34		0.36	0.36		0.34	0.43	
	M	I	M x I	M	I	M x I	M	I	M x I	M	I	M x I
SEd	0.003	0.004	0.005	0.003	0.003	0.005	0.002	0.003	0.004	0.003	0.004	0.006
CD (*P*=0.05)	0.007	0.008	0.012	0.006	0.008	0.011	0.006	0.007	0.01	0.007	NS	0.013

Note: M1 – *P. fluorescens;* M2 – PPFM; I1 – 0.6 IW /CPE ratio; I2 – 0.8 IW /CPE ratio; I3 – 1.0 IW /CPE ratio

of Maggio *et al*. (2010) and Li *et al*. (2009) in tomato who have shown that the foliar spray of microbial inoculants like PPFM increased the RWC under the constraints of moisture. This study in combination with the reported literature clearly suggests that microbial spray facilitated condensing of leaf moisture through an inevitable physiological process.

Table 3.Impact of phyllosphere microbes on transpiration rate of tomatoes

Treatments	Transpiration rate (mol/m²/sec)											
	1DAS			5DAS			10DAS			15DAS		
	M1	M2	Mean	M1	M2	Mean	M1	M2	Mean	M1	M2	Mean
I1	6.38	10.66	8.52	9.25	8.64	8.95	9.62	5.91	7.77	8.85	9.6	9.22
I2	9.87	9.59	9.73	10.33	9.45	9.89	9.55	7.51	8.53	8.05	9.2	8.62
I3	11.56	7.83	9.70	9.45	8	8.73	7.41	7.38	7.40	9.8	8.48	9.14
Mean	9.27	9.36		9.68	8.70		8.86	6.93		8.9	9.09	
	M	I	M x I	M	I	M x I	M	I	M x I	M	I	M x I
SEd	0.074	0.090	0.128	0.068	0.083	0.118	0.056	0.069	0.98	0.07	0.086	0.122
CD (*P*=0.05)	NS	0.190	0.270	0.14	0.17	0.25	0.12	0.14	0.20	0.15	0.18	0.26

Note: M1 – *P. fluorescens;* M2 – PPFM; I1 – 0.6 IW /CPE ratio; I2 – 0.8 IW /CPE ratio; I3 – 1.0 IW /CPE ratio

Stomatal conductance

The microbial spray carrying *P. fluorescens* assisted the tomato plants to register significantly higher stomatal conductance at 1 and 5 DAS while PPFM sprayed plants had higher stomatal conductance at 10 and 15 DAS (Table.2). On the 15th day of microbial spray, tomato plants had the stomatal conductance of 0.43 while it was just 0.34 mmol $H_2O/m^2/sec$. The data clearly demonstrated that microbial spray enabled the plants to perform normal physiological functions such as stomatal conductance even after 15 DAS especially with PPFM. On the other hand, Sivakumar et al. (2018) have shown that PPFM induces stomatal closure on tomato leaves that enables reduced transpiration rate which protects the plants from extreme drought conditions. In our study, the tomato plants were irrigated optimally or sub-optimally and therefore, the plants had maintained higher stomatal conductance indicating the opening of stomata. Irrigation regimes had a phenomenal impact on stomatal conductance and the values decreased with increasing intensities of drought stress from 1.0 to 0.6 IW / CPE ratio. Such responses were observed throughout the experimental period of 1 to 15 DAS. Interaction was also significant. Despite the trend of responses were similar, PPFM sprayed plants had higher stomatal conductance even at the high intensity of drought stress at 0.6 IW / CPE throughout the experimental period from 1 to 15 DAS. The results are in line with Haloi and Baldev (1986) who have reported that the PPFM induces stomatal closure that enables the plant to survive under limited water environments (Sivakumar *et al.,* 2018). Further, Haloi and Baldev (1986) reported that crop productivity closely coincides with stomatal conductance.

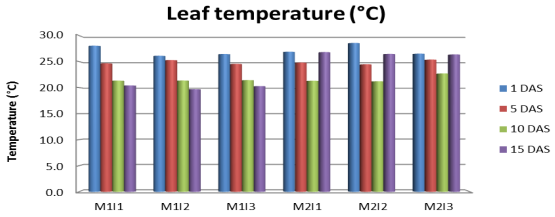

Figure 1.Impact of phyllosphere microbes on leaf temperature in tomatoes

(M1 – P. fluorescens; M2 – PPFM; I1 – 0.6 IW /CPE ratio; I2 – 0.8 IW /CPE ratio; I3 – 1.0 IW /CPE ratio)

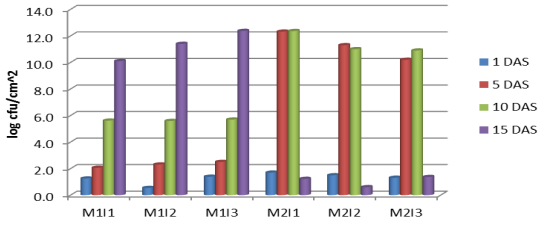

Figure 2.Colonies of *Pseudomonas fluorescens* and PPFM (10^7) cfu/ cm^2 in tomato leaves

(M_1 – P. fluorescens; M_2 – PPFM; I_1 – 0.6 IW /CPE ratio; I_2 – 0.8 IW /CPE ratio; I3 – 1.0 IW /CPE ratio)

Transpiration rate

Tomato plants that received the microbial spray of *P. fluorescens* had significantly higher transpiration rate during the day 1 till 10 DAS but the PPFM sprayed plants had higher transpiration rate at 15 DAS (Table.3). On the last day of experimentation, PPFM sprayed tomato plants had higher transpiration rate of 9.09 than *P. fluorescens* sprayed ones (8.9 $mol/m^2/s$). The data clearly demonstrated that PPFM spray had enabled the plants to perform normal physiological functions and the response had persisted even after 15 DAS. The intensities of frequencies of irrigations had decreased the transpiration rate in correspondence with the moisture regimes from 1.0 to 0.6 IW / CPE ratio regardless of microbial sprays and such trend was observed throughout the experimental period of 1 to 15 DAS. Interaction was also significant. Despite the trend of responses were similar, *P. fluorescens* sprayed plants had higher transpiration even at the

high intensity of drought stress at 0.6 IW / CPE throughout the experimental period

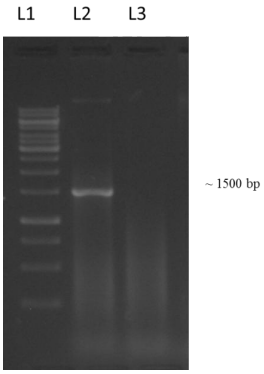

Figure 3.Detection of *inaW* gene in the *Pseudomonas fluorescens* using genus specific primers. L1= 5kb bp ladder, L2= *Pseudomonas fluorescens*, L3= PPFM

from 1 to 15 DAS. Microbe mediated physiological changes under varying moisture regimes have already been reported in maize-mycorrhizal symbiotic interactions (Subramanian et al., 1995; 1997). Mycorrhizas assist in improving host plant water relations as a secondary consequence of improved nutritional status particularly P. In this study, phyllosphere microorganisms facilitate condensation of evaporated water vapour leading to maintenance of leaf turgidity and normal physiological functions including transpiration rate.

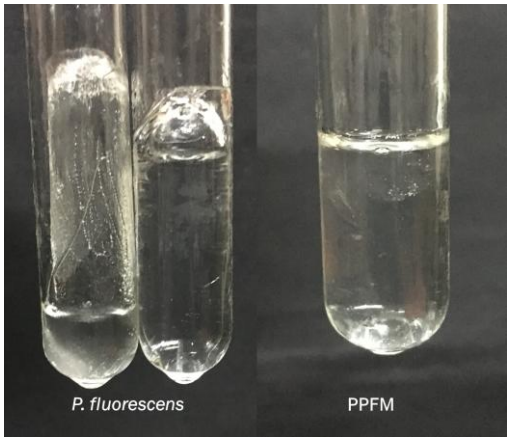

Figure 4.Tube nucleation test shows that Pseudomonas fluorescens frozen the liquid buffer at -10°C while PPFM not frozen the liquid buffer at -10°C

Jaiswal *et al*. (2017) reported that biochar controls the transpiration rate both the presence and absence of microbes in the tomato plants. In this present study, also state that the transpiration rate of tomato controlled by microbes. Our data closely coincided with the reported literature and bring out variations in response to the microbial sprays.

Leaf temperature

The microbial spray did not alter the leaf temperature during 1-10 DAS with an exception of 15 DAS. The *P. fluorescens* spray assisted the tomato plants in maintaining lower leaf temperature (19.7 °C) than PPFM sprayed plants (26.8 °C) at 15 DAS (Figure.1). The data clearly demonstrated that microbial spray enabled the plants to lower leaf temperature even after 15 DAS especially with *P. fluorescens* The moisture regimes had little impact on leaf temperature. The cooling of the leaf surface may be associated with a loss of water through transpiration leading to lower leaf temperature (Chartzoulakis *et al.,* 1993). Our data corroborate with the observations of Sivakumar *et al.* (2018) who have reported that phyllosphere microbes are capable of reducing the leaf temperatures in extreme weather conditions.

Phyllosphere population

The microbial sprays had increased the phyllosphere population by 3.3, 181 and 167 CFU/cm^2 in PPFM when compare to *P. fluorescens* (1.5; 21; 43 CFU/cm^2) on day 1, 5 and 10 DAS, respectively (Figure 2.). Conversely, on the 15 DAS, the PPFM population was extremely lower while *P. fluorescens* had the abundance of population. The data have clearly shown that the PPFM multiplied faster and reached the highest within 10 DAS while *P. fluorescens* reaches in 15 DAS. Moisture regimes had variable responses in microbial populations of both PPFM and *P. fluorescens* Our data are in conformity with the observations of Lindemann *et al*. (1982) who had reported a linear increase in population of *P. syringae*. Similar observations were reported for other microbes (Pietrarelli *et al.* 2006).

Characterization of inaW gene

Amplification of inaW gene isolates with INAWF (ATGAATGCATAATGGGCACTG) and INAWR (GTCTGCGTGCTGCCATAACC) region-specific primer yielded an expected amplicon of 1500 bp amplicon suggesting isolate belong to *P. fluorescens.* The amplified PCR products of inaW of *P. fluorescens* were sequenced partially. The rDNA homology was performed using nucleotide BLAST program though the internet server at the National Center for Biotechnology Information (NCBI), USA. The comparison of sequences from the GenBank database to determine the species level homology (Figure. 3). Wolber and Warren (1989) confirmed the inaW gene associate with the *Pseudomonas fluorescens*. The PPFM did not amplify with the fragment of primers. The PPFMs were detected in leaves surface, not in leaf buds. Phyllosphere strains of *Methylobacterium extorquens*, *Methylobacterium mesophilicum*, *Methylobacterium fujisawaense* did not catalyze the ice nucleation. Despite the fact that

the ice nucleation assists in moisture conservation in leaves and maintenance of physiological functions under drought conditions, it may cause frost injury. Methylobacterium are well known to protect plants from environmental factors (Romanovskaya *et al.,* 1999).

Tube nucleation test

The ice nucleation potential tested for two bacterias, of which *P. fluorescens* was more efficient than PPFM. *P. fluorescens* catalyzed the super-cooled buffer within the 30 min at - 9 °C, while PPFM was unable to catalyze the supercooled buffer even after 6 h (Figure 4.).Our study clearly demonstrated that *P. fluorescens* assists in ice nucleation that helped the plants to retain leaf moisture status and physiological functions as reported in the earlier section of this paper. Our data are in conformity with the observations of Wolber (1993) who reported that the outer membrane of the *P. fluorescens* causes ice crystallization at below 0 °C. Further, one of the other Pseudomonas species *p.aeruginosa* is well known to facilitate ice nucleation formation (Michaud *et al*. (2004). However, it is potentially a human pathogen and thus it was excluded in our study. Our study in combination with the reported literature, it is confirmed that the maintenance of moisture status of leaves closely associated with ice nucleation activity.

CONCLUSION

This is one of the pioneering works demonstrating that the spray of microbial inoculants such as *P. fluorescens* assisted in ice nucleation and facilitated maintenance of physiological activities normally under varying intensities of drought stress. Among the microbial sprays tested, *P. fluorescens* was found to exhibit higher ice nucleation and retain higher RWC and stomatal conductance while lower leaf temperature. The ice nucleation activity of *P. fluorescens* confirmed with the PCR and further by tube nucleation test. On the other hand, PPFM found to improve the drought tolerance of the plants by maintaining physiological activity under severe drought conditions. Overall, our data suggest that *P. fluroscens* possesses ice nucleation activities that facilitate retention of leaf moisture and lower temperature which may of practical significance to develop microbe – mediated artificial rain in the near future. Further, the use of PPFM spray assists in drought tolerance of the plants by the closure of stomata but lack of ice nucleation activity.

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