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Research Article

Ice nucleation active bacteria and its mitigation on tomato (*Solanum lycopersicum*)

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Abstract
Microbes play a vital role in ice nucleation, supporting bio-precipitation, and allowing plants to live in low-water environments. A field experiment was conducted during December 2018 with two phyllosphere microorganisms' spraying *viz.*, *Pseudomonas aeruginosa* and pink-pigmented facultative methylotrophs (PPFM) and under three moisture regimes (0.6, 0.8, 1.0 IW/CPE (irrigation water/ cumulative pan evaporation) ratio) on tomato (PKM 1) in Tamil Nadu Agricultural University, Coimbatore (Tamil Nadu). A laboratory experiment was conducted to confirm ice nucleation using two phyllosphere microorganisms' *P. aeruginosa* and PPFM. The bioprecipitation impacts on tomatoes were assessed using a set of physiological parameters such as photosynthetic rate, average chlorophyll Index and the ice nucleation activity (INA) assessed using tube nucleation test and scanning electron microscope (SEM). The results showed that the mean photosynthetic rate of PPFM sprayed tomato (*Solanum lycopersicum*) (PKM 1) plants (40.7 µmol $CO_2/m^2/s$) at 10 DAS was significantly higher than *P. aeruginosa* sprayed plant (38.7 µmol $CO_2/m^2/s$) under different irrigation regimes. The average chlorophyll Index value of the *P. aeruginosa* sprayed tomato plants (58.1) was higher than PPFM sprayed plants (56.4). The tube nucleation tests were proved that ice crystallization induced by *P. aeruginosa* in super-cooled buffer at - 2 to -10°C while PPFM not catalyze the buffer even after 3hours. The scanning electron microscope (SEM) indicated the *P. aeruginosa* growth at the upper surface of the leaf and PPFM growth more at the lower surface of the leaf compared to without inoculation of microbes on leaves. Overall, the result revealed that *P. aeruginosa* may assist in ice nucleation activity that will help to make artificial rain in the near future.

Keywords: Ice nucleation activity, *Pseudomonas aeruginosa*, Pink-pigmented facultative methylotrophs, Scanning electron microscope, Tube nucleation test

INTRODUCTION

Microbes play a key role in ice nucleation that facilitates bioprecipitation, which enables plants to survive under limited water conditions. In India, rains are received as precipitation as a result of water currents passing the sub-continent in a unique weather pattern as South-West and North-East Monsoons. Apart from monsoon rains, atmospheric disturbances and convectional processes lead to rain formation (Kakarla *et al*., 2019). In all the cases, there is a need for a nucleus in the form of dirt or microorganism to facilitate cloud condensing nuclei. Since the rainfall pattern is highly erratic and frequent droughts are quite common, there is an urgent need to develop a strategy to develop artificial rain to protect agriculture from devastation. Artificial rain was first reported globally in 1940 (Schaefer, 1946) and demonstrated in Tamil Nadu, India, in 1983 (CASWMT,

1983). Silver iodide, sodium chloride and dry ice were well-known substances used for artificial rain. The condensation of water vapour led to the formation of ice crystals at extremely low sub-zero temperatures of below 40^{o}C under natural conditions. Such condensation reaction will occur at - 20^{o}C when artificial nucleating agents such as AgI ions are used to develop the rainwater droplets (Prasada, 2015). Despite the fact that chemicals have been successfully demonstrated 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 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). In urban regions, the concentration of aerosols was around 3 x 10^9 to 5 x 10^{10} particles per cubic meter. Atmospheric aerosols contain more minerals dust. Soot particles are more in the polluted area. But anyone knows that pollens are the only biological components in atmospheric aerosols. A bacterium covers around 25 per cent of atmospheric aerosols (Perrino and Marcovecchio, 2016).

The impact of biological particles in the atmosphere is the only feature of aerobiology. According to the bacterium, ice nucleation active proteins originate from the soil or plants. For example, above the frost and wheat fields have been detected thousands of bacteria per cubic meter in the air (Harding *et al*., 2011). Agricultural practices such as bailing and combining are shoot values up to 10^5 to 10^9 bacteria per cubic meter (Morris *et al*., 2004). Cloud water droplets persist in a metastable liquid state at even below 0°C temperature in the absenteeism of freezing nuclei. Water in the fluid state at less 0°C temperature is supercooled water. Ice formation of supercooled water normally happens below -39°C. Henceforth, freezing nuclei are crucial for natural ice crystal formation outside of Polar Regions clouds, which is happening much on the troposphere. Supercooled water change into ice crystals that involves the clustering of a few water molecules are changed into a condensed phase. Clusters reach a critical size that grows as an ice nucleus. This ice nucleus initiates the rapid growth of the water drops into solid ice crystals. The development of clusters into nuclei is required a quantity of energy for the detachment of the water molecules from the clusters. For example, pure water forms ice crystals at -40°C, and roughly 75 water molecules are required to form the clusters (Harrison, 2019). Virtual molecular simulation of ice formation explains the schematic representation of the ice nucleation process (Matsumoto *et al*., 2002).

Different substances are able to form heterogeneous ice nucleation. Supercooled water molecules can be freezed by inorganic substances such as AgI (Silver iodide), single layers of long chain alcohols, amino acids, and organic substances such as metaldehyde phloroglucinol can freeze the (Wilson., 1994). Soot, minerals dust and metallic compounds are also catalyzing ice nucleation process (DeMott *et al*., 2003; Pandey *et al*., 2016). Bacteria are connected with the most extensively studied ice nuclei of biological origin. Some bacteria's outer cell membrane contains a protein that can function at temperatures as warm as -1°C as an effective ice nucleator. It's also known that only a few bacterial species generate INA proteins. INA bacteria were widely found in plants (as saprophytic epiphytes and/or as plant pathogens) that include *Pseudomonas syringe, P. viridiflava, P. aeruginosa, Pantoea agglomerans* (formerly known as *Erwinia herbicola) and Xanthomonas campestris* (Wolber and Paul, 1993; Gaignard and Luisetti., 1993). The very active ice nucleation through the plants' bacteria is also called leaf derived nucleai (LDN). The *Pseudomonas* genus was a generally diverse microorganism that most abundant particles in the upper atmospheric conditions and well-known ice nucleation active protein (Morris *et al*., 2013; Vasebi *et al*., 2019). The interest in the present strain arises from both suitability of explaining the genetic component of *P. aeruginosa* and pink-pigmented facultative methylotrophs (PPFM) ice nucleation in tomato leaves. The genes are 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.aeruginosa* and PPFM assist in ice nucleation, enabling the tomato (*Solanum lycopersicum*) plant to survive under water deficit conditions.

MATERIALS AND METHODS

The research trial was laid out at the Tamil Nadu Agricultural University (TNAU) Orchard, Tamil Nadu Agricultural University, Coimbatore from December 2018 to February 2019. The experiment had two factors, namely irrigation regimes (I) and microbial spray (M), with four replicates in a factorial randomized block design (FRBD). The tomato (PKM 1) crop was drip-fertigated throughout the crop period and the irrigations were scheduled as per IW / CPE ratio. To treat Factor M and I, microbial spray (M_1-*Pseudomonas aeruginosa*; M_2-Pink Pigmented Facultative Methylotrops (PPFM)) and irrigation regimes (I_1-0.6 IW/CPE ratio; I_2-0.8 IW/CPE ratio; I_3-1.0 IW/CPE ratio) were adopted. Soil moisture levels were maintained by drip irrigation using the IW/CPE method for each treatment at regular intervals corresponding to 12-14, 8-10 and 6-8 days, respectively. Microbial sprays (1 x 10^7 per ml) were given @ 2 per cent on the 45th day of transplanting. The crop was fertilized with NPK @ 100:60:60 kg per ha through drip fertigation. Observations on photosynthetic rate and

average chlorophyll Index 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 photosynthetic rate was measured by means of Portable Photosynthesis System (PPS) (Model LI-6400 of LICOR inc., Lincoln, Nebraska, USA). Three measurements were engaged 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.

Overall chlorophyll content was measured using Minolta chlorophyll meter SPAD-502 (Minolta Camera Co., Ltd). Minolta SPAD (Soil and Plant Analysis Division) utilized the light source and detectors to estimate the relative quantity of chlorophyll in leaves by measuring the light transmitted by a plant leaf at two diverse wavelengths, namely red (650nm, where absorption was extremely high) and near-infrared (940nm, where absorption was extremely low). The light transmitted by the leaf is transformed into electrical signals. The ratio of intensities of transmitted light at the two wavelengths corresponds to SPAD readings in SPAD units, which are values defined by Minolta. The SPAD values were occupied from the base of the leaf lamina of the second and fourth leaf from the top. The data were analyzed using SPSS software.

A tube nucleation test method was employed to determine the ice nucleation potential of the bacteria (Hirano *et al*., 1985). Test tubes (18 x 150 mm size) filled with 10 ml K-phosphate buffer (10 mmol, pH 7.0) were closed with cotton and decontaminated by autoclaving at 121°C. After cooling at room temperature, the tubes were 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. aeruginosa* or PPFM. The *P. aeruginosa* 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.

Scanning Electron Microscope (SEM) is an electron microscope that produces sample images by scanning the surface with a focused, high-energy electron beam. The sample atoms intermingle with the electrons in the system, generating numerous signals of a surface arrangement and sample placement. The size and morphology of the leaf sample were investigated by a scanning electron microscope (FEI Quanta 250, Netherlands). On the scanning sample stage, the sample stub was positioned (Kliewer, 2009).

RESULTS AND DISCUSSION

Photosynthetic rate

There was an increase in photosynthesis in different irrigation regimes on 1, 5, and 10 DAS and there was a sight decline on 15 DAS **(**Table 1). The 1.0 IW / CPE ratio had significantly (p=0.05) higher value (41.8 µmol $CO_2/m^2/s$) than 0.8 and 0.6 IW CPE ratio. Among the irrigation regimes 0.6 IW / CPE ratio recorded lower values on all different days. Among the microbial sprayed plants PPFM recorded a higher value (40.7 µmol $CO_2/m^2/s$), followed by *P. aeruginosa* on 10 DAS. The interaction was also significant. Among the microbial spraying, PPFM had higher photosynthetic rate than *P. aeruginosa.* The microbial spray may increase the light-saturated net CO_2 assimilation rate (Rodrigues *et al*., 2021). The amplified net CO_2 assimilation in tomato leaves may upsurge in the maximum carboxylation rate of PSII electron transport (Jiang *et al.,* 2012).

SPAD value

The microbial spray carrying *P. aeruginosa* assisted the tomato plants in registering significantly (p=0.05) higher SPAD values at 1 and 10 DAS while PPFM sprayed plants had higher SPAD values at 5 and 15

Table 1. Impact of irrigation regimes and phyllosphere microbes on photosynthetic rate of tomato

Treatments	Photosynthetic rate (µmol $CO_2/m^2/s$)											
	1 DAS			5 DAS			10 DAS			15 DAS		
	M1	M2	Mean	M1	M2	Mean	M1	M2	Mean	M1	M2	Mean
I1	30.1	30.6	30.3	32.9	31.1	32	40.1	36.5	38.3	34.8	31.0	32.9
I2	31.2	30.8	31.0	31.9	31.9	31.9	39.5	38.6	39.1	39.5	37.2	38.3
I3	30.6	32.7	31.6	32.9	35.8	34.35	36.6	47	41.8	32.7	31.2	31.9
Mean	30.6	31.6		32.5	32.9		38.7	40.7		35.6	33.1	
	M	I	M x I	M	I	M x I	M	I	M x I	M	I	M x I
Sed	0.23	0.28	0.40	0.24	0.3	0.42	0.29	0.36	0.51	0.25	0.3	0.43
CD (*P*=0.05)	0.50	0.61	0.87	0.52	0.64	0.91	0.63	0.77	1.1	0.53	0.65	0.92

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

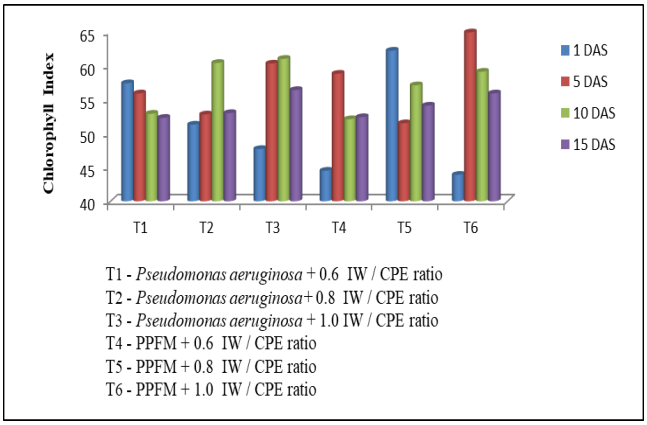

Fig. 1. *SPAD values of tomato as influence of irrigation regimes and microbial spraying at 1, 5, 10 and 15 DAS*

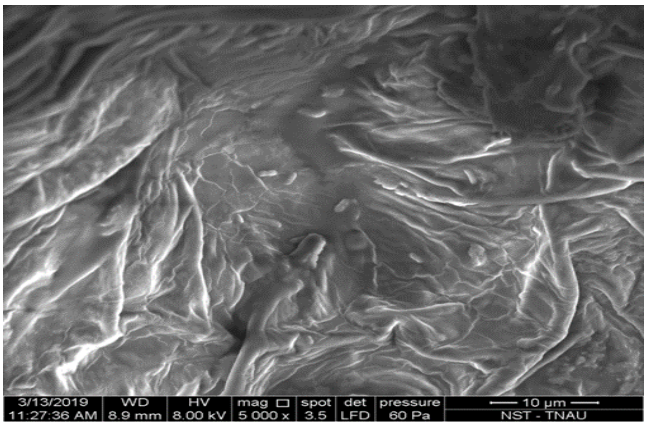

Fig. 2. *SEM image of upper leaf without inoculation of Pseudomonas aeruginosa*

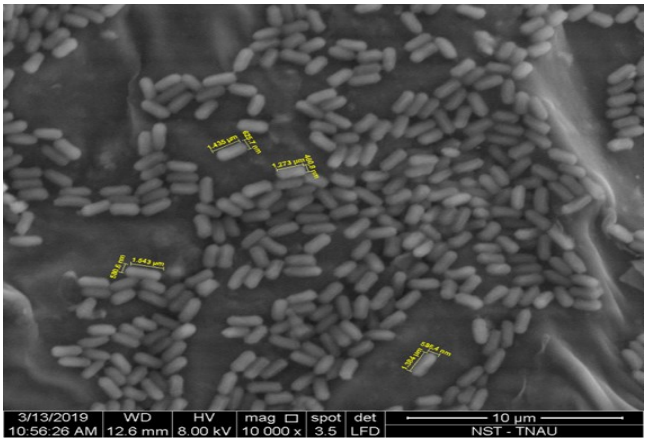

Fig. 3. *SEM image of upper leaf after inoculation of Pseudomonas aeruginosa*

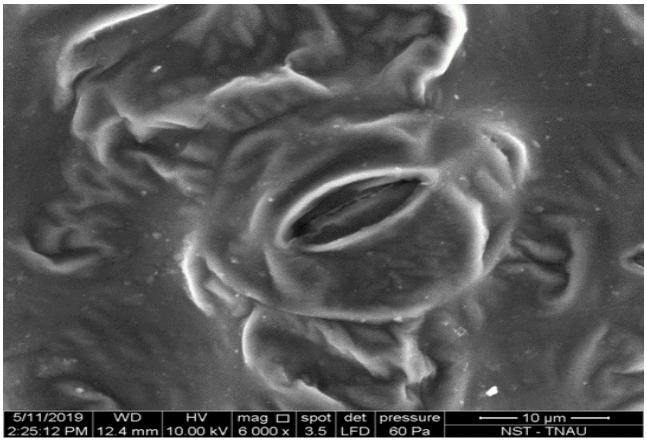

Fig. 4. *SEM image of lower leaf without inoculation of PPFM*

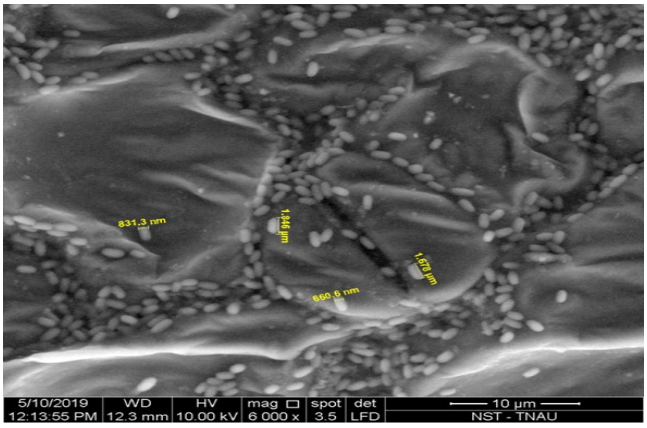

Fig. 5. *SEM image of lower leaf after inoculation of PPFM*

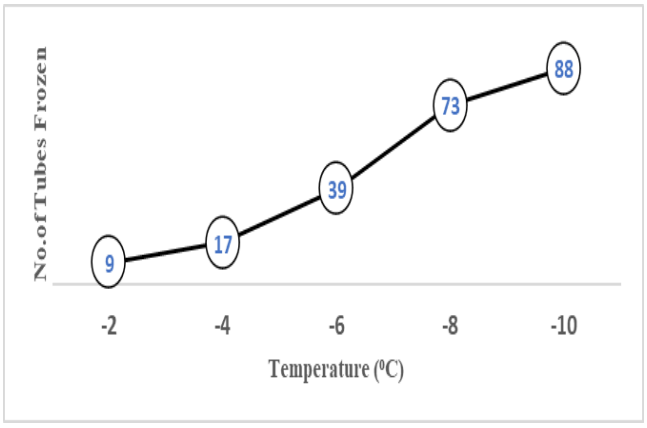

Fig. 6. *P. aeruginosa catalyzed the super-cooled buffer, while PPFM was unable to catalyze the super cooled buffer even after 3 h.*

DAS. On the 10th day of *P. aeruginosa* spray, tomato plants had the SPAD value of 58.1 while PPFM was 56.1. Among the irrigation regimes, the 1.0 IW / CPE ratio had a higher SPAD value and was significant (Fig. 1**)**. This outcome is similar to Misratia *et al*. (2013) in rice. Naik *et al.* (2020) found that microbial spray can increase chlorophyll content on different crops.

Tube nucleation test

The ice nucleation potential was tested for two bacteria's, of which *P. aeruginosa* was more efficient than PPFM. *P. aeruginosa* catalyzed the super-cooled buffer at – 2 to -10°C (Fig. 6), while PPFM was unable to catalyze the super-cooled buffer even after 3 h. *Pseudomonas* species *P.aeruginosa* is well known to facilitate

ice nucleation formation (Vanderveer *et al*., 2014).

Scanning electron microscope

The scanning electron microscope was performed in environmental scanning electron microscope (ESEM) mode to discover the bacterial size, shape and phyllosphere population. The SEM clearly showed that *P. aeruginosa* had grown on the upper leaf surface and PPFM was mostly present in the stomata region (Figs. 2, 3, 4 and 5). The *P. aeruginosa* SEM image was confirmed with a similar finding already demonstrated for *P. syringae* by Sarron *et al*. (2013). The bacteria like PPFM, SEM image furnished that colonies induced the stomatal closure over the lower surface of the rice leaves, and it is already reported in rice by Kim *et al*. (2010).

Conclusion

Among the microbial sprays tested, *P. aeruginosa* exhibited higher ice nucleation and retained a higher photosynthetic rate and chlorophyll content. The ice nucleation activity of *P. aeruginosa* was confirmed with the PCR and further by tube nucleation test and PPFM was found to improve the drought tolerance of the tomato plants by maintaining physiological activity under severe drought conditions. Overall, the present data suggest that *P. aeruginosa* possesses ice nucleation activities that may be of practical significance to develop microbe–mediated artificial rain in the near future.

Conflict of interest

The authors declare that they have no conflict of interest.

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