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Research Article

Long-term adaptation study of bacterial isolates of plant growth-promoting bacteria in heat-stressed conditions

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Abstract

In many bacterial species, there is still a lack of comprehensive research and characterization of the basic mechanisms behind bacterial adaptation. Furthermore, it's still unclear if prokaryotes can learn by association and adaptation. Since Plant Growth Promoting Bacteria (PGPB) are essential to the preservation of plant physiology and growth across a range of stress scenarios, PGPB can be utilized to analyze this adaptation of bacteria under stress. This study examines the initial findings on adaptive flexibility in PGPB under conditions of heat stress. The performance of the isolated PGPB receiving both periodic and non-periodic heat stress was compared to that of the control group. Characteristics such as ammonia and siderophore production, phosphate utilization, and amount of indole-3-acetic acid produced, as well as antioxidant activities like DPPH activity, hydroxyl radical scavenging activity, and hydrogen peroxide scavenging activity were analysed. Following heat stress treatment, it was clear from the isolated PGPB that those under periodic stress were able to outperform the PGPB exposed to non-periodic stress in comparison to the control. When compared to the other isolates in our investigation, the two novel strains of *Paenibacillus alvei* SJ6 and *Paenibacillus alvei* SJ8, among the four isolated PGPB have demonstrated the greatest capacity to respond to sporadic heat stress. Therefore, preliminary evidence for the existence of history-dependent adaptation has been examined in this work.

Keywords

adaptability, memory, plant growth-promoting bacteria (PGPB), heat stress, stress physiology

Abbreviations

PGPB – Plant Growth-Promoting Bacteria; DPPH – 2,2-Diphenyl-1-Picrylhydrazyl; H₂O₂ – Hydrogen Peroxide; DMRT – Duncan's Multivariate Test; SPSS – Statistical Package for Social Science

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Introduction

The mechanisms behind bacterial memory in various species have not yet been thoroughly studied or characterized. Additionally, the ability of associative learning in prokaryotes is still unknown. Memory is essential to the organism, regardless of whether these learned events are genetically fixed and heritable or remain epigenetically changeable (Zhang et al. 2023). Despite having distinct evolutionary histories, bacteria and archaea are both "small but not stupid." Because eukaryotes can communicate, they can plan and coordinate their behaviour in a manner similar to that of a multicellular creature (Shapiro 2007).

One of the main factors that causes plants to become dehydrated and limit their growth is heat stress. Reduced photosynthetic output may result from heat stress's reduction of leaves' relative water content and water potential. Additionally, it has the ability to produce reactive oxygen species (ROS), which can harm the cell functioning (Hasanuzzaman et al. 2013). These PGPB seem to be good alternatives to agrochemicals because of their ability to strengthen plants' tolerance to a variety of unfavourable stress conditions, including drought, salt stress, heat stress, poor and degraded soils, and plant diseases (Yadav et al. 2015). Through the synthesis of proteins that are produced during heat shock and the activation of structural alterations in plants, plant growth-promoting microorganisms (PGPM) can develop thermotolerance in plants. Furthermore, the PGPM is responsible for nitrogen fixation, nutrient mobilization, and phytohormone synthesis (Hakim et al. 2021). Under heat stress, plants generate reactive oxygen species (ROS), which in turn trigger the production of antioxidants (Zandi and Schnug 2022). Under conditions of heat stress, inoculated plants exhibited subpar growth performance. Conversely, under heat stress, plants that had received PGPB inoculation grew more rapidly because the symbionts made it easier for the plants to absorb nutrients (Chan, Ariyawansa and Rho 2024).

In our current study, Plant Growth-Promoting Bacteria (PGPB) are able to be used to analyze this adaptation of bacteria under stress because PGPB is crucial to the maintenance of plant physiology and growth under a variety of stress scenarios, and

therefore the adaptiveness can be learned using them (Glick 2012). Gaining a deeper comprehension of plant-beneficial bacteria is anticipated to facilitate the development of environmentally conscious, climate-smart, and sustainable farming technologies that may be implemented in agriculture to overcome limited environmental conditions. PGPB enhance stress tolerance through mechanisms such as siderophore production, nitrogen fixation, phosphate solubilization, phytohormone synthesis, and enzyme activities including ACC deaminase (Kumar et al. 2020). Furthermore, PGPB stimulate antioxidant activity while promoting nutrient uptake and maintaining plant homeostasis under diverse stress conditions. They also inhabit healthy plant tissues, supporting growth without causing harm (Pajuelo Domínguez et al. 2021).

Evaluations are conducted for PGPB using growth characteristics like turbidity, colony shape, and bacterial size, as well as PGPB biochemical measures like ammonia and siderophore production, phosphate utilization, and amount of indole-3-acetic acid produced (Amaya-Gómez et al. 2020). The contrasting thermal response patterns observed among periodically and non-periodically stressed bacteria holds agronomic significance. Strains acclimated to predictable cyclic stress may be better suited for regions experiencing stable diurnal heat fluctuations, whereas isolates adapted to non-cyclic, erratic stress may exhibit improved resilience under unpredictable climatic extremes. This study examines the adaptive behaviour of historically dependent PGPB adaptation in a heat stressed setting.

Materials and Methods

Reagents. Luria-Bertani broth (HiMedia, Mumbai, India), Nessler's reagent (Merck, Darmstadt, Germany), Salkowski reagent (SRL Chemicals, India), and other analytical-grade chemicals were used for all experiments.

Isolation of plant growth-promoting bacteria. A group of PGPB was identified from *Spinacia oleracea* based on our earlier investigation (Swetha and Sayantan 2024). Following aseptic procedures, soil samples (in triplicates) were taken from the plant's rhizosphere and stored at 4°C in sterile zip-

lock plastic bags. The source and place were also labelled on it. The collected samples were brought to the lab so that the serial dilution procedure could be used to isolate soil bacteria (Amaya-Gómez et al. 2020). The isolated bacteria were characterized using conventional techniques like morphological studies, culture characteristics, staining techniques, and biochemical analyses like the Methyl Red test, Simmon citrate test, Indole test, Voges Proskauer test, and catalase test (Powers and Latt 1977). 16S rRNA gene sequencing was used to identify the isolated bacterial isolates utilizing the forward (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse (5'-CTGCTGCSYCCCGTAG-3') primers for universal 16S rRNA-F and 16S rRNA-R (Johnson et al. 2019). After the 16S rRNA gene fragment was amplified, forward and reverse sequencing were applied to the PCR amplicon. At Barcode Biosciences in Bangalore, Karnataka, India, the sequencing was done. After analysing these sequences using the Basic Local Alignment Search Tool (BLAST), the obtained data was submitted to the NCBI database to get an accession number.

Bacterial adaptivity study. Three groups were established: (1) Control - cultures maintained at 37°C without stress; (2) Periodically stressed - exposed to 55°C every 24 h for 14 days; and (3) non-periodically stressed- exposed to 55°C on irregular days. Non-periodic heat stress was applied irregularly on days 3, 7, and 10 (24 h each at 55°C) with intervening recovery at 37°C. After each cycle of this protocol was completed, it was sub-cultured. For the purpose of this study, long-term was defined as repeated stress exposure cycles maintained over a 14-day experimental period. During this time, the study group of rhizosphere bacterial cultures was subjected to periodic stress induction, while control groups were maintained without or with irregular stress exposure. The highest range of temperature at which these isolates thrive in was identified by plating them on LB media and incubated in different range of temperature (45°C, 50°C, 55°C and 60°C) for an overall time period of 24 h. The research set of cultures for the heat stress experiment was kept at its optimum temperature of 37°C, exposed to heat stress on a periodic basis for 24 h, and then grown

in its highest thriving temperature for a further 24 h, for a total of 14 days. After each cycle of this protocol was completed, it was sub-cultured. The study group's cultural and biochemical characteristics was compared to those of the control group, which received periodic stress, and the second group, which received stress at irregular intervals of three or four days for a total of fourteen days. Every experiment was conducted in triplicate, with the results shown as mean \pm standard deviation.

Bacterial colony morphology study. By analysing their features, the PGPB colonies following the periodic and non-periodic stress the bacterial colonies were examined. The height, colour, texture, edge, and shape of a colony are often influenced by its surroundings. A macroscopic illustration of the different biological strategies that microorganisms use to cope with stressful conditions, such as starvation, oxygen deprivation, antibiotics, and host defences, can be found in changes in the morphological features of colonies (Sousa et al. 2013). Following the stress exposure study, the changes in shape, size, colour, and elevation between the study set and the positive and negative control were determined.

Bacterial size imaging study. The bacterial size imaging was captured using a Leica DMI8 inverted light microscope. Bacterial cultures from each experimental group (Control, periodically stressed, non-periodically stressed) were prepared in triplicate and sampled on Day 0 (before stress), Day 7 and Day 14. At each time point, 1 mL aliquots were removed from each culture and fixed with 4% paraformaldehyde for 15 min at room temperature to preserve cell morphology, followed by two washes in sterile phosphate-buffered saline (PBS) and resuspension in 100 μ L PBS. Fixed cells were mounted on glass slides and imaged using a Leica DMI8 inverted light microscope (40 \times /100 \times objective as appropriate). For each biological replicate, images of at least 100 non-overlapping cells were captured across multiple fields to avoid bias (\approx 300 cells per treatment per time point). Microscope calibration was performed using a stage micrometer before image acquisition. Cell sizes

(length the isolate morphology) were measured from the micrographs using leica software. Since the isolates were maintained under vegetative growth conditions for 14 days, with nutrient availability that did not trigger sporulation, spore-specific staining was not performed. Measurements were conducted blinded to treatment to avoid observer bias. Size distributions were exported and expressed as mean \pm standard deviation.

PGPB characteristic assay to compare between periodic heat stress and non-periodic heat stress exposure study

Ammonia production. The PGPB were inoculated in peptone media and cultured for 48-78 h at 28°C to identify the ammonia produced in the periodic, non-periodic, and control exposures. Then, 0.5 mL of Nessler's reagent was incorporated to all tubes. The color change in the media was noted and the absorbance was read at 420 nm indicated a positive test result for ammonia production ([Ammonia Nessler Test Method 2022](#)).

Indole-3-acetic acid production. The Salkowski reagent was used to quantify the indole-3-acetic acid ([Rapparini et al. 2002](#)). Tryptophan was added to Luria Bertani broth at a dosage of 2.5 mg. mL⁻¹ to inoculate the PGPB post-stress treatment. Following that, the culture was maintained for seven days at 28 \pm 2°C. Subsequently, the culture was centrifuged at 10,000 RCF for 30 min. 4 mL of Salkowski reagent and 2 mL of supernatant were combined. The appearance of a pink color suggested the presence of indole acetic acid production. Visible spectrophotometry was utilized to quantify the IAA generation, with the optical density being measured at 530 nm wavelength. Next, an IAA-standard curve was used to estimate the amount of IAA produced ([Etesami and Glick 2024](#)).

Siderophore production. The PGPB were cultured in nutrient broth at 37°C for a full day. The isolates were then cultivated in TY broth and centrifuged for 10 minutes at 10,000 rpm. At 630 nm, the absorbance was measured following the addition of the ferric chloride solution and the CAS test solution ([Arora and Verma 2017](#)).

$$\text{Siderophore production, } \mu\text{su} = \frac{(Ar - As)}{Ar} \times 100$$

Where Ar = absorbance of reference (CAS solution and control broth), As = Absorbance of sample (CAS solution and sample).

Phosphate solubilisation. Pikovaskya's agar medium was supplemented with 1000 mg.L⁻¹ of tricalcium phosphate in order to quantify the phosphate solubilization. Phosphate-solubilizing bacteria will thrive on this medium and solubilize the available phosphate due to the proximal phosphate use. The medium was then incubated at 28°C for 10 to 15 days. The amount of free phosphate that was accessible at 700 nm was measured using the phospho-molybdate blue test after incubation.

Anti-oxidant activity of PGPB between periodic heat stress and non-periodic heat stress

DPPH activity. After being incubated in LB medium for the entire night, the PGPB isolates were centrifuged for 15 min at 10,000 rpm. The DPPH test was utilized to evaluate the sample's antioxidant activity in compliance with the [Shi et al.](#), protocol. The DPPH radical approach is based on the reduction of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical to 2,2-diphenyl-1-picrylhydrazine (DPPH-H). The colour changes from purple to yellow in the presence of an antioxidant ([Shi et al. 2019](#)).

$$\text{DPPH, \%} = \frac{(Ar - As)}{Ar} \times 100$$

Where Ar = absorbance of reference (mixture solution and control broth), As = Absorbance of sample (mixture solution and sample).

Hydroxyl radical scavenging assay. For plants, ROS buildup is fatal and exceedingly harmful. Different defensive mechanisms, depending on both non-enzyme and enzyme antioxidants, protect plants from reactive oxygen species. Cells may be destroyed by this oxidative process if the buildup of hydroxyl radicals is not controlled ([Hasanuzzaman, Nahar and Fujit 2013](#)). In a mixture comprising 1.0 mL of the sample, 1.5 mL of sodium phosphate buffer, 1 mL of FeSO₄ (0.75 mM), 1 mL of H₂O₂, and 1 mL of 1,10-phenanthroline (0.75 mM). Using a spectrophotometer, the amount of hydroxyl radical produced in the mixture was measured at

536 nm following 30 min of incubation at 37°C (Li et al. 2012).

$$\text{Resistance to hydroxyl radicals, \%} = \frac{(Ar - As)}{Ar} \times 100$$

Where, Ar = absorbance of reference (mixture solution and control broth), As = Absorbance of sample (mixture solution and sample).

Hydrogen peroxide activity. Resistance to this H₂O₂ was tested using the Li et al. method (Li et al. 2012). The PGPB isolates were cultivated in LB broth at 37°C for 16–18 h (Dholakiya et al. 2017). After that, this culture was mixed with 1.0 mM hydrogen peroxide and kept at 37°C for a further 8 h of incubation. With a spectrophotometer, the cells' growth was measured at 600 nm.

$$\text{Resistance to hydrogen peroxide, \%} = \frac{(Ar - As)}{Ar} \times 100$$

Where Ar = absorbance of reference (mixture solution and control broth), As = Absorbance of sample (mixture solution and sample).

Results and Discussion

Isolation of PGPB. Potential PGPB features were identified for four morphologically distinct rhizosphere bacteria P3, P5, P6, and P8 that were isolated from *Spinacia oleracea*. When the sequence was BLAST-analyzed against other sequences in the database, P3 and P5 were found to be *Bacillus clarus* and *Bacillus licheniformis*, whereas P6 and P8 were two novel strains identified as members of the *Paenibacillus* family identified as *Paenibacillus alvei* SJ6 and *Paenibacillus alvei* SJ8 (Table 1).

Table 1. Bacterial identification and accession number obtained for the bacterial isolates

Bacterial Isolate Code	Bacteria identified	Percentage Identity	Accuracy length	Accession Number
P3	<i>Bacillus clarus</i>	98.41%	1552	PP355449
P5	<i>Bacillus licheniformis</i>	99.57%	1545	PP355450
P6	<i>Paenibacillus alvei</i> SJ6	100%	907	PP355538
P8	<i>Paenibacillus alvei</i> SJ8	100%	719	PP355543

The isolates SJ6 and SJ8 displayed distinct colony morphologies, differential IAA and ammonia production, and enhanced thermal tolerance when compared to reference *Paenibacillus alvei* strains. These phenotypic distinctions substantiate their classification as novel *P. alvei* variants.

Bacterial colony morphology study. After growing the isolates in different ranges of high temperature (45°C, 50°C, 55°C and 60°C) it was found that these isolates could only survive till 55 °C therefore, this temperature was chosen as the highest range of temperature for rest of the experiment. At the conclusion of treatment, it was shown that, in comparison to periodically stressed bacteria, non-periodically stressed bacteria had

considerably more deformed colony morphology under heat stress.

Bacillus licheniformis exhibited large, opaque, smooth colonies, while *Bacillus clarus* had smooth, round, off-white colonies. Isolate belonging to *Paenibacillus* family has similar shape- mild opaque smooth round colonies conditions (Fig. 1). Following stress treatment in the control and periodically stressed conditions, the colonies retained their original morphology. In contrast, in non-periodic stress treatment the colonies of *Bacillus clarus* showed rough distortion and irregular growth. *Bacillus licheniformis* and *Paenibacillus* family isolates also displayed a similar pattern of distortion.

Numerous elements, such as the necessity for motility, nutritional deprivation, temperature fluctuations and pressures from predators, can also affect the morphologies of bacteria. These characteristics enable the bacteria to modify their morphologies in response to different environmental stimuli in order to gain advantages (Young 2007).

Bacterial size imaging study. There is a significant decrease in the size of the bacterial cell size in non-periodically stressed bacterial cells (four-fold

decrease in *Bacillus clarus* and *Bacillus licheniformis* whereas two-fold decrease in *Paenibacillus alvei* SJ8 and *Paenibacillus alvei* SJ6) whereas periodically stressed bacteria more or less sustained the same size (Fig. 2 and 3). This pattern reflects adaptability rather than mere accession-specific resistance. If the isolates were intrinsically resistant, we would expect stable tolerance irrespective of the stress pattern. Instead, the periodically stressed PGPB demonstrated sustained performance across cycles, maintaining

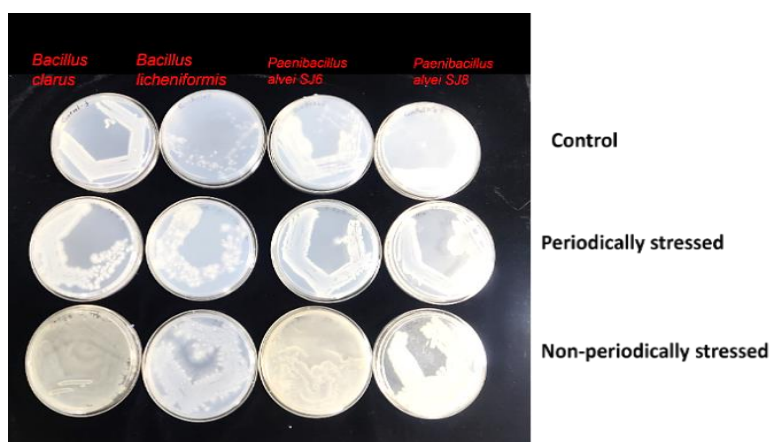


Figure 1. Bacterial Trial with high temperature as stress factor. Control was treated at 37°C and periodic and non-periodic stressed samples were exposed to 55°C/ 37°C

cell size and metabolic functions over 14 days. By contrast, the non-periodically stressed isolates exhibited progressive size reduction and metabolic

decline, indicating that adaptation was driven by repeated, predictable stress exposure rather than innate resistance (Table 2).

Table 2. Size of bacteria after periodic and non-periodic treatment under heat stress compared to control.

Bacterial Isolates	Day 1		Day 7		Day 14	
	Size, μm		Size, μm		Size, μm	
Heat stress	Control	Periodic stress	Non-periodic stress	Periodic stress	Non-periodic stress	
<i>Bacillus clarus</i>	4.22	4.28	3.49	3.64	2.64	
<i>Bacillus licheniformis</i>	4.01	3.63	3.21	3.39	1.49	
<i>Paenibacillus alvei</i> SJ6	1.89	1.83	1.88	1.68	1.17	
<i>Paenibacillus alvei</i> SJ8	3.99	3.99	3.38	4.20	2.41	

A variety of temperature fluctuations provide valuable data for investigating heat-adaptation, including phenotypic plasticity that bacteria may display in response to heat stress (Bullivant et al. 2024). Certain processes such as stress improve the strain performance in microorganisms.

Genetic changes during adaptation may increase the strain's growth rate or product yield, hence improving fitness in the stressful environment (Sheppard, Guttman and Fitzgerald 2018). A brief shift in the distribution of cell volume was caused by an abrupt temperature change within the middle

range (Shehata and Marr 1975). When comparing to our study the periodically stressed PGPB have sustained their bacterial size by adapting to the regular exposure compared to the control whereas the non-periodically stressed PGPB have significantly reduced in size.

Although periodically stressed isolates retained cell morphology and metabolic activity during extended exposure, minor post-stress reductions in metabolic rate were noted, suggesting a transient energetic trade-off associated with stress maintenance.

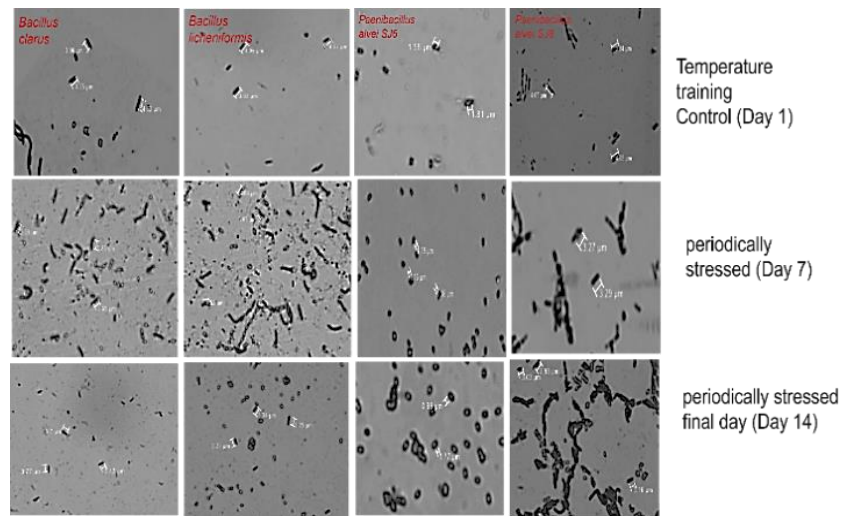


Figure 2. Bacterial size of isolates under heat stress treatment with periodically stressed on days 1, 7 and 14

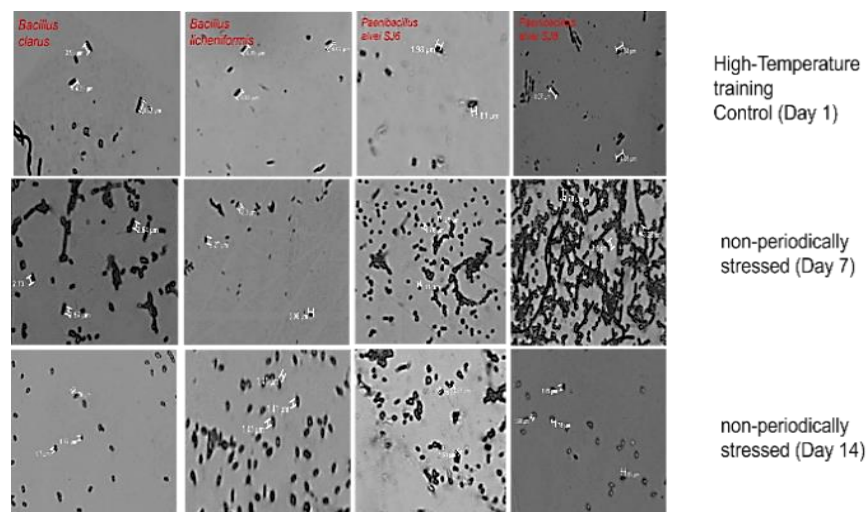


Figure 3. Bacterial size of isolates under heat stress treatment with periodically stressed on days 1, 7 and 14

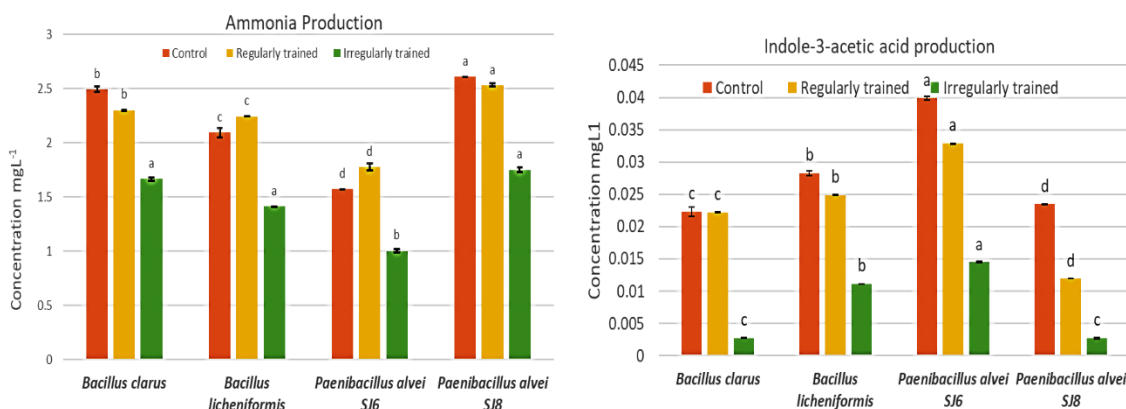


Figure 4. (a) Production of Ammonia ($\mu\text{mol. mL}^{-1}$) and (b) Indole-3-acetic acid (mg. L^{-1}) 2.5% L-tryptophan. The bar graphs represent the different concentrations of ammonia and IAA formed in both periodically and non-periodically stressed PGPB. Values are average of three replications and the results are shown as mean \pm SD. Error bars represent standard deviation. Means, followed by the same letter in a column are not significantly different ($p = 0.05$) by Duncan's multivariate test (DMRT) ($p \leq 0.05$).

PGPB characteristic assay to compare between periodic heat stress and non-periodic heat stress exposure study.

Ammonia production. Numerous microorganisms that are resistant to stress have been found, and they help plants cope with heat stress either directly or indirectly (Kapadia et al. 2022).

Through the production of metabolites that aid in plant growth and nutrient uptake, PGPB assist plants in overcome this heat stress (Mitra et al. 2021). Periodically stressed PGPB produced ammonia levels comparable to or greater than the control, whereas non-periodically stressed isolates showed a marked reduction. *Paenibacillus alvei* SJ8 and *Bacillus clarus* generated the greatest amounts of ammonia under heat stress, $2.533 \pm 0.016 \mu\text{mol. mL}^{-1}$ and $2.301 \pm 0.001 \mu\text{mol. mL}^{-1}$, respectively (Fig. 4). By generating ACC deaminase, which converts ACC into ammonia and α -ketobutyrate, plant growth-promoting bacteria (PGPB) can raise the levels of ammonia in stressed plants (Glick 2012).

In a study by Agbodjato et al., the most prevalent PGPB mechanism is the generation of ammonia. A significant amount of ammonia was produced by a fermented panchagavya bacterial isolate in peptone broth. After 120 h, *Bacillus* sp. PG-8 produced the highest amount of ammonia ($6.51 \mu\text{mol. mL}^{-1}$), which reduced after 144 h (Agbodjato et al. 2015).

In a different study, Rhizobacteria, *Bacillus cereus*, and *Bacillus megaterium* produced ammonia after 72 h with corresponding concentrations of 2.3 and $6.2 \mu\text{mol. mL}^{-1}$ (Dutta and Thakur 2017).

Indole-3-acetic acid production (IAA). According to the simple theory of plant growth control, the higher the IAA concentration, the faster the plant grows when auxin is controlling growth. As a result, it is seen that growth rates increase as temperature rises, indicating that levels of free IAA should likewise rise as temperature rises (Gray et al. 1998). While the ammonia produced by non-periodically stressed PGPB is much lower than that of the control condition, the ammonia produced by periodically stressed PGPB is more or less equal to or higher than that of the control condition (Fig. 4). When subjected to heat stress, *Paenibacillus alvei* SJ6 is the isolate that produces the most ammonia- $32.88 \pm 0.06 \text{ mg. L}^{-1}$.

The PGPB isolates of *Bacillus* genus, namely the *Bacillus halotolerance* strain SI 339, was unable to exhibit this characteristic until *Bacillus megaterium* SI 404 and *Bacillus cabrialesii* SI 428 demonstrated 6.94 mg. L^{-1} (Barbaccia et al. 2022). The isolate utilized in the Gohil et al. investigation demonstrated the ability to produce organic acid, IAA, GA, and phosphate solubilization during heat stress. Through the activation of antioxidant

enzymes, control over gene expression, manufacture of the osmoprotectant proline, and improvement of photosynthetic pigment accumulation, IAA can mediate plants' tolerance to heat stress (Gohil et al. 2022; Siddiqui et al. 2017).

Siderophore production. By creating phytohormones, fixing atmospheric nitrogen, and manufacturing siderophores, PGPB can aid in the growth of plants. Additionally, by enhancing root development and altering plant defence mechanisms, they can help plants withstand stress. Temperature can affect the generation of siderophores (Kumari et al. 2022). Periodically stressed PGPB produce around the same quantity of siderophores as the control condition, while non-periodically stressed PGPB produce much less than the control. Under heat stress, the isolates that produce the most siderophores include *Paenibacillus alvei* SJ8 and *Bacillus licheniformis* with the ability to produce the most siderophore, 17.361 ± 0.24 psu (Fig. 5).

PGPB has the ability to invade the rhizosphere and encourage plant uptake of iron. They can also fix

atmospheric nitrogen, solubilize phosphates, and enhance plant nutrition. Bacteria develop organic ligands called siderophores in order to sequester iron (Timofeeva, Galyamova and Sedykh 2022). In the study conducted by Rehan et al., the siderophore production rates of four PGP strains produced in the range between 26.37- 35.51 psu (Rehan et al. 2023).

Phosphate solubilization. PGPB can assist plants in obtaining phosphate from insoluble substances. They accomplish this by producing organic acids that chelate phosphate-bound cations, turning them into soluble form. Temperature variations have an impact on plant growth-promoting bacteria's (PGPB) ability to solubilize phosphate (Aguilera-Torres et al. 2022). While the phosphate solubilized by non-periodically strained PGPB is much lower than that of the control condition, the amount solubilized by periodically stressed PGPB is more or less equivalent to or higher than that of the control condition. With 138.716 ± 0.429 mg.L⁻¹, *Paenibacillus alvei* SJ8 is the isolate that produces the most ammonia among those under temperature stress (Fig. 5).

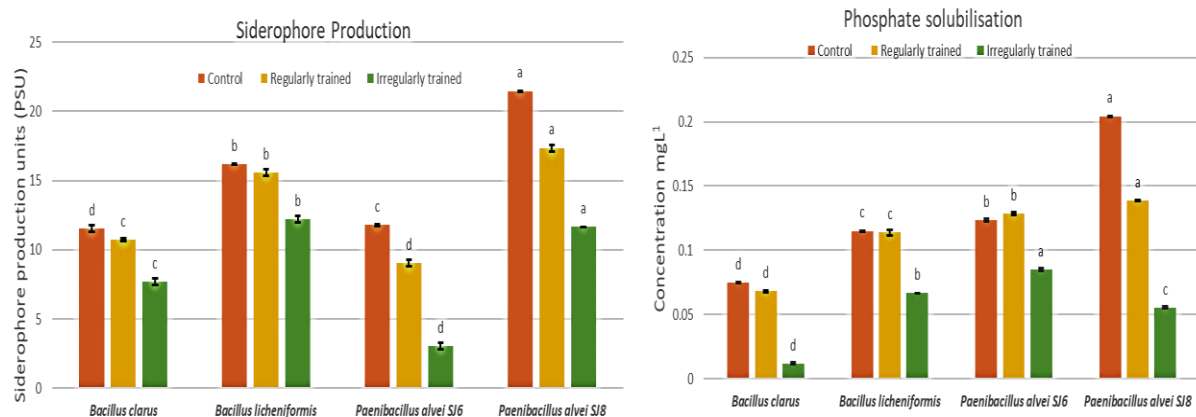


Figure 5. (a) Production of siderophore production (psu) and (b) phosphate solubilization (mg.L⁻¹). The bar graphs represent the different concentrations of siderophore formed and phosphate solubilized in both periodically and non-periodically stressed PGPB. Values are average of three replications and the results are shown as mean \pm SD. Error bars represent standard deviation. Means, followed by the same letter in a column are not significantly different ($p = 0.05$) by Duncan's multivariate test (DMRT) ($p \leq 0.05$).

In order to increase plant growth, the direct strategy of PGPB involves nitrogen fixation, phosphate solubilization, and the synthesis of phytohormones and siderophores these processes stimulate plant

metabolism (Abdelaal et al. 2021). It may be possible to lessen the negative impacts of abiotic stressors on plants, such as salt, drought, and extreme temperatures, by using the phosphorus-

solubilizing bacteria. Additionally, PGPB has the ability to dissolve insoluble potassium from silicate and rock (Rajawat et al. 2020). In a different investigation, the NBRIP supernatant was found to contain an estimated 85 mg.L⁻¹ to 1312 mg.L⁻¹ of dissolved phosphate, with isolates L228 and L132 PGPB showing the highest solubilization (Schoebitz, Ceballos, and Ciamp 2013). In our study when the isolates were regularly stressed they adapted to this historic exposure creating short term memory and thereby increasing the phosphate solubilization whereas non-periodically stressed PGPB had reduced solubility.

Anti-oxidant activity of PGPB between periodic heat stress and non-periodic heat stress

DPPH scavenging activity. Plants under heat stress responded favourably to PGPB inoculation, exhibiting improved antioxidant capacity, photosynthetic pigment accumulation, and enhanced biomass (Zhang et al. 2023). The isolates *Bacillus clarus* and *Paenibacillus alvei* SJ6 have the highest scavenging activity in heat stressed control group has relatively lesser scavenging activity whereas the periodically stressed PGPB have more capability to scavenge the free radicals compared to the non-periodically stressed PGPB indicating the ability of the periodically stressed PGPB ability to adapt to the historic dependent adaptation to stress (Fig. 6).

According to a study by Hassan et al. (Al-Zahrani, Alharby and Fahad 2022) heat stress especially high temperatures at night significantly decreased the synthesis of hormones and metabolites, changed antioxidant levels, and increased the accumulation of reactive oxygen species (ROS) in several plant sections in both rice cultivars (Al-Zahrani, Alharby, and Fahad 2022). When plants are stressed by cold or heat, photosynthesis system protection, ion homeostasis maintenance, and other benefits are all enhanced by PGPB (Sarkar, Chakraborty and Chakraborty 2021).

In another study by Kang et al. (Kang and Saltveit 2002) under all circumstances, the heat shock treatment dramatically raised the DPPH radical scavenging activity. When non chilled tissue was tested two hours after the heat shock, DPPH activity in HS tissue was 23% higher than in non-HS tissue (Karthik, Kumar and Bhaskara Rao 2013).

Hydroxyl radical scavenging assay. Heat stress causes oxidative stress in plants by accelerating the production of reactive oxygen species (ROS), such as singlet oxygen, superoxide radical, hydrogen peroxide and hydroxyl radical (Maury et al. 2020). The greatest activity of all the PGPB isolates is exhibited by *Paenibacillus alvei* SJ6, while all of them are capable of scavenging hydroxyl radicals. condition.

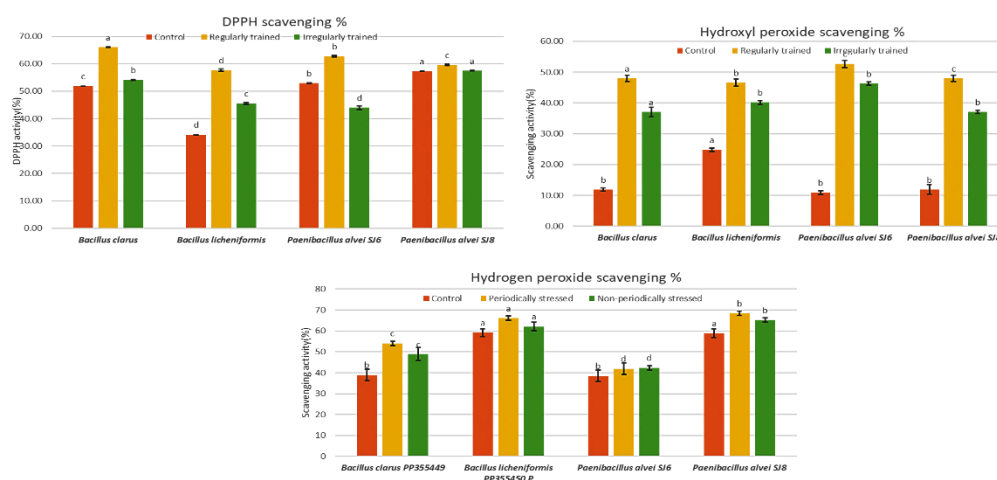


Figure 6. (a) Production of DPPH activity (%), (b) Hydroxyl radical scavenging activity (%) and (c) Hydrogen peroxide scavenging activity (%). The bar graphs represent the different concentrations of radicals scavenged in both periodically and non-periodically stressed PGPB. Values are average of

three replications and the results are shown as mean \pm SD. Error bars represent standard deviation. Means, followed by the same letter in a column are not significantly different ($p = 0.05$) by Duncan's multivariate test (DMRT) ($p \leq 0.05$).

They produced around $66.14 \pm 0.082\%$ and $62.68 \pm 0.246\%$ scavenging activity. The *Bacillus clarus*, *Bacillus licheniformis*, *Paenibacillus alvei* SJ6, and *Paenibacillus alvei* SJ8 are isolates that exhibit scavenging activity percentages of $47.95 \pm 1.02\%$, $46.59 \pm 1.17\%$, $52.72 \pm 1.17\%$, and $47.95 \pm 1.002\%$ respectively. In comparison to the study's control group, plants under periodic stress are better able to scavenge more free radicals than non-periodically stressed plants (Fig. 6). This implies that plants under recurrent stress are able to learn and adjust to the stress by repetition. Plants under heat stress produce more reactive oxygen species (ROS), oxidative damage, misfolded proteins, and denaturation (Sarkar, Chakraborty and Chakraborty 2018). 16 *Lactobacillus* strains showed varying hydroxyl radical scavenging capabilities in research by Hu et al., ranging from $21.07 \pm 4.53\%$ to $62.80 \pm 5.72\%$. Peroxidases, superoxide dismutase, NADPH oxidases, and transition metal catalysts can all create these radicals in plant cells. Plant cell death, germination, growth, and other processes are negatively influenced by them (Richards et al. 2015).

Hydrogen peroxide scavenging activity. A study found that plants treated with PGPB exhibited lower levels of increased H_2O_2 than other plants when exposed to heat stress (Shi et al. 2019). With H_2O_2 scavenging activities of $79.16 \pm 1.03\%$ and $73.80 \pm 1.09\%$, respectively, the two isolates *Bacillus licheniformis* and *Paenibacillus alvei* SJ8 exhibited the highest levels (Fig. 6). In our investigation, every isolate exhibited noteworthy hydrogen peroxide scavenging capacity, suggesting that these PGPB are capable of adjusting to the severe periodic changes in their surroundings.

A signalling molecule termed H_2O_2 aids plants in surviving a range of environmental stressors, such as salt, drought, cold, high temperatures, and heavy metal stress (Hossain et al. 2015). The primary regulator of plant growth and environmental responses is H_2O_2 . They interact with other signalling molecules and phytohormones to control plant growth, development, and stress tolerance.

Plants can withstand environmental cues better when antioxidant defence mechanisms are upregulated, which is triggered by H_2O_2 (Anjum et al. 2022).

In contrast, the plants with PGPB showed correspondingly, under these treatments. In a study by Batool et al., under moderate and severe stress treatments, H_2O_2 increased by 68% in plants with PGPB and up to 75% in plants with no PGPB (Batool et al. 2020).

Conclusions

The current work shows that when exposed to a hostile environment, the PGPB may adjust to periodic stress and build historic dependent adaptivity. Consequently, selection of inoculant strains can be guided by regional climatic regimes: periodically conditioned bacteria for environments with stable daily temperature oscillations, and non-periodically conditioned isolates for regions facing sporadic heat waves.

The findings contribute to an improved understanding of bacterial adaptation and provide an empirical framework for the climate-specific application of PGPB inoculants in sustainable agriculture. With comparison to the control group, the PGPB under periodic stress in the overall research were more able to adjust to the change. Compared to the other isolates, *Paenibacillus alvei* SJ6 and *Paenibacillus alvei* SJ8 have demonstrated the greatest capacity for adaptation to recurrent heat stress. When exposed to periodic stress, all isolates exhibited increased activity of various PGPB features, including the synthesis of siderophores, phosphate solubilization, indole-3-acetic acid, ammonia, and antioxidants.

Importantly, the superior performance of periodically stressed isolates reflects an adaptive response rather than simple accession-specific resistance. While intrinsic resistance would manifest as uniform tolerance across conditions, the sustained functionality and metabolic stability observed under repeated stress cycles point to history-dependent adaptation, or 'stress memory.'

Such adaptability enables PGPB to fine-tune their physiology in response to predictable challenges, thereby maintaining plant growth-promoting traits under long-term heat stress. This distinction highlights the potential of PGPB not only as resilient microbes but also as dynamic partners in sustainable agriculture under climate stress (Bullivant et al. 2024; Sheppard, Guttman, and Fitzgerald 2018). The observed memory-like adaptive responses are likely governed by a combination of reversible gene-regulatory mechanisms, persistent stress-responsive proteins, and limited cell-wall remodelling rather than permanent genomic alterations. Such multilayered physiological plasticity enables bacterial populations to preserve functional stability under recurring heat stress. The findings of this investigation point to early evidence in favour of bacterial adaptive intelligence. It is necessary to carry out further study to fully investigate its potential, examine how it affects plant development parameters under various stress scenarios, and examine how plants adjust to stress.

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Author Contributions

Conceptualization: S.J. and S.D.; methodology: S.J.; formal analysis: S.J.; investigation: S.J.; data curation: S.J.; writing- original draft preparation: S.J.; writing- review and editing: S.J. and S.D.; visualization: S.J.; supervision: S.D.; project administration: S.D. All authors have read and agreed to the published version of the manuscript. All authors read and approved the manuscript.

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Institutional Review Board Statement

Ethical review and approval were waived for this study, as the research did not involve human participants or animals. The study was conducted using microbial isolates and plant-based experimental systems in accordance with the research guidelines of CHRIST (Deemed to be University), Bengaluru, India.

Informed Consent Statement

Not applicable.

Data Availability Statement

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

Conflicts of Interest

The authors declare no conflicts of interest.

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