# Study of cognitive adaptiveness of isolated Plant Growth Promoting Bacteria in nutritionally stress condition

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***Abstract***
*The biological processes behind bacterial memory in different species are still under terra incognita. Additionally, the ability of learning through association in prokaryotes is still unknown. Cross-fertilization between the study of multicellular creatures' cognitive capacities and that of bacteria is possible. Therefore, Plant Growth Promoting Bacteria (PGPB) can be used to analyze this cognitive adaptation of bacteria under stress because PGPB is crucial to the maintenance of plant physiology and growth under a variety of stress scenarios. This study focuses on analyzing preliminary evidence of cognitive adaptability in PGPB under nutritional stress conditions. The isolated PGPB were treated with nutritional deprivation in both periodical and non-periodical manners and their performance was compared with the control group. The characteristics of PGPB, such as ammonia production, siderophore production, phosphate solubilization and indole-3-acetic acid, as well as anti-oxidant activities such as DPPH activity, hydroxyl radical scavenging activity and hydrogen peroxide scavenging activities, were analysed and compared to periodically and non-periodically stressed PGPB with control.*

*In the isolated PGPB post-nutrition deprivation treatment, it was evident that the periodically stressed performed better than the non-periodically stress-exposed PGPB compared to the control wherein the isolates produced as high as 2.551±0 µmol $mL^{-1}$ ammonia, 23.04±06 $mgL^{-1}$ indole-3-acetic acid, 69.16 ± 0.71 psu siderophore and 123.578±0.429$mgL^{-1}$ phosphate solubilised. Out of the four isolated PGPB, the two novel strains, Paenibacillus alvei SJ6 and Paenibacillus alvei SJ8, have shown to possess the supreme ability to adapt to periodic nutritional stress compared to the other isolates in our study.*

**Keywords:** Cognitive adaptability, Memory, Plant Growth Promoting Bacteria (PGPB), Nutritional stress.

## Introduction

The mechanisms underlying microbial adaptability have garnered a lot of interest lately. Different processes improve the performance of the strain when microorganisms adapt over the long and short terms. Genetic changes during adaptation may increase the strain's growth rate or product yield, improving fitness in the stressful environment[1]. Just as there is not a single, widely agreed-upon definition for behaviour, neither is there one for intellectual/cognitive ability. In contrast to animals, prokaryotes like bacteria and plants may also be capable of such cognitive capacity. The results of this study suggest that there may be some preliminary evidence supporting the existence of cognitive intelligence in bacteria as several studies have demonstrated the ability of these organisms to exhibit features that suggest memory and learning[2].

It is expected that the acquisition of an expanded understanding of plant-beneficial PGPR can help in designing methods for sustainable, environment-friendly and climate-smart agricultural technologies for adoption in agriculture to overcome constricted environmental conditions. Nitrogen fixation, phosphate solubilization, phytohormone synthesis, siderophore generation and enzyme activity such as ACC deaminase enhance tolerance for multiple stressors in PGPB[3]. The proliferation of microbiota is regulated by plants as part of the plant-bacterium interaction at the rhizosphere plane which facilitates adaptation to shifting environmental conditions[4]. Additionally, under various stress situations, PGPBs increase antioxidant activity and aid in the uptake of nutrients and plant homeostasis. Bacterial PGPBs that promote plant growth reside within healthy plant tissues without damaging them[5].

Given evidence of their capacity to increase plant resistance to a wide range of unfavorable stress conditions (drought, salinity, high temperature, poor and degraded soils) as well as against plant infections, these PGPBs appear to be viable substitutes for agrochemicals[6]. Furthermore, some research has looked into the possibility of PGPBs that have evolved to harsh settings like those with nutritional stress, pH stress, salinity, or cold temperatures. Because they can withstand harsh environments and encourage plant growth and survival, they have a great deal of potential for plant production and adaptation[7]. The growth parameters such as turbidity, colony morphology, bacterial size and PGPB biochemical parameters such as phosphate solubilization, ammonia production, indole-3-acetic acid and siderophore production are evaluated for PGPB[8]. In this study, the cognitive behaviour of historic dependent adaptiveness of PGPB in nutritionally stressed environment has been analysed.

## Material and Methods

**Isolation of plant growth promoting bacteria:** From our previously conducted study, a group of PGPB was isolated

from *Spinacia orlacea* (unpublished). The soil samples (in triplicate) were collected from the plant's rhizosphere and preserved at 4 °C in sterile zip-lock plastic bags while adhering to aseptic protocols. It was also labeled with the source and location. The samples that were gathered, were transported to the laboratory so that soil bacteria could be isolated using the serial dilution method[9]. Conventional methods such as morphological analyses, culture characteristics, staining methods and biochemical analyses such as the methyl red test, simmon citrate test, indole test, Voges Proskauer test and catalase test, were used to characterize the isolated bacteria [10]. The isolated bacterial isolates were identified by 16S rRNA gene sequencing[11].

With the use of universal primers for 16S rRNA-F and 16S rRNA-R primers having the sequence (forward 5′-AGAGTTTGATCMTGGCTCAG-3′) and (reverse 5′-CTGCTGCSYCCCGTAG-3′), the 16S rRNA gene fragment was amplified and the resulting PCR amplicon was put through forward and reverse sequencing processes. The sequencing was performed at Barcode Biosciences, Bangalore, Karnataka, India. These sequences were analysed using Basic Local Alignment Search Tool (BLAST) analysis and after that, the sequence data was uploaded to the NCBI database in order to receive an accession number.

**Bacterial cognitive adaptivity study:** This study possessed three sets of groups consisting of one study set and two control sets (positive control and negative control). The study group of rhizosphere bacterial culture was exposed to stress induced in a periodic manner for a period of 14 days. The purpose of the first control group was to observe and to compare the growth characteristics compared to the study set and the purpose of the second control group (negative control) was to rule out the possibility of an identical growth pattern to that of the study group in non-periodic growth conditions. All the experiments have been done in triplicate and the values are represented as mean ± standard deviation.

In the nutritional deprivation study, the study set of culture was maintained in 100X diluted LB (Luria Bertani) media and was introduced to periodic exposure of rich nutritionally supplemented broth for 24 hours and then was nutritionally deprived for the next 24 hours for a total of 14 days. This routine was repeated and sub-cultured after each cycle. The cultural and biochemical characterization of this study group was compared to the positive control group, maintained in a well-nourished condition and the second control group was subjected to non-periodic intervals of nourishment such as 3 or 4 days for a total of 14 days.

**Bacterial colony morphology study:** The PGPB colonies after the periodic and non-periodic stress study were determined by identifying their morphological characteristics. The environment had a frequent impact on a colony's shape, edge, height, colour and texture. Changes in the morphological characteristics of colonies can be a macroscopic representation of the various biological methods that microbes adopt to deal with stressful situations such as starvation, oxygen deprivation, antibiotics and host defenses [12].The shape, size, colour and elevation differences amongst the study set and the positive and negative control were identified after the stress exposure study.

**Bacterial size imaging study:** The nutritional components of the growth medium determine the maximum size that a bacteria can reach, indicating that nutrients have an impact on one or more rate-limiting steps that regulate size and growth rate of bacteria[13].The bacterial image was captured using Leica microscope DMi8 inverted light microscope and the bacterial size was analysed.

Comparative study of biochemical characteristics of PGPB was exposed to periodic and non-periodic nutrition stress

**1. Ammonia production:** When under nutritional stress, the production of ammonia aids in the accumulation of nitrogen in plants which encourages the growth of biomass, roots and shoots[14].The comparative analysis of ammonia produced in both periodic, non-periodic and control exposure was identified by inoculating these PGPB in peptone media, which was incubated for 48–78 hours at 28 ± 2°C. Each tube was then added with 0.5 mL of Nessler's reagent. A positive test result for ammonia production was shown by the colour turning from brown to yellow and the absorbance was read at 420nm[15].

**2. Indole-3-acetic acid:** Plant functions influenced by IAA include senescence, flowering, tropism, apical dominance, elongation, differentiation and cell division during nutritional stress[16].The indole-3-acetic acid was quantified using Salkowski reagent. The PGPB post stress treatment was inoculated in Tryptophan concentrations of 2.5mg/mL and added to Luria Bertani broth. After that, the culture was kept at 28 ± 2 °C for 7 days. The culture was centrifuged for 30 minutes at 10,000 RCF. 2 mL of supernatant and 4 mL of Salkowski reagent were mixed. The qualitative evaluation of the formation of indole acetic acid was indicated by the presence of a pink colour. For the quantitative evaluation of the IAA production, visible spectrophotometry was used, with the optical density being measured at 530 nm in wavelength. Next, the amount of IAA produced was estimated using an IAA-standard curve[17].

**3. Siderophore production:** Many microbes, particularly bacteria that promote plant growth, create siderophores which are agents that chelate iron (PGPB). When iron is in short supply, these siderophores can assist plants in obtaining it, which can promote plant growth and can enhance nutrition[18]. The PGPBs were grown in nutrient broth for 24 hours at 37°C. Subsequently, the isolates were cultured in tryptophan (TY) broth and centrifuged at 10,000 rpm for 10 minutes. After adding the CAS test solution and ferric chloride solution, the absorbance was measured at 630 nm.

Siderophore production (psu) = $\left((Ar - As) \div Ar\right) * 100$

where Ar = absorbance of reference (CAS solution and uninoculated broth) and As = Absorbance of sample (CAS solution and cell free supernatant of sample)

**4. Phosphate solubilisation:** By solubilizing inorganic phosphate and mineralizing organic phosphate, PGPR can aid in the growth of plants by supplying phosphorus to them when their nutritional supply is limited[19]. To measure the phosphate solubilization, 1000 $mgL^{-1}$ of tricalcium phosphate was added to Pikovaskya's agar media. Due to the proximal phosphate usage, phosphate-solubilizing bacteria growing on this medium will solubilize the available phosphate which was then incubated at 28 °C for 10 to 15 days. After incubation, phospho-molybdate blue assay was performed to quantify the available free phosphate at 700nm.

Comparative study of antioxidant activities in PGPB exposed to periodic and non-periodic nutrition stress:

**(i) DPPH activity:** The activity of α,α-diphenyl-β-picrylhydrazyl (DPPH) in scavenging free radicals was measured. Following an overnight incubation period in LB medium, the PGPB isolates were centrifuged for 15 minutes at 10,000 rpm. The antioxidant activity of the sample was assessed using the DPPH test in accordance with the Shi et al[27] procedure. The reduction of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical to 2,2-diphenyl-1-picrylhydrazine (DPPH-H) is the basis of the DPPH radical technique. When an antioxidant is present, the color turns from purple to yellow. Freshly prepared methanol-based 0.3 mM DPPH solution was made. The combination of 1 mL DPPH and 1 mL sample was then prepared. After 30 minutes of dark storage, the mixture's absorbance at 517 nm was analyzed.

DPPH (%) = $\left((Ar - As) \div Ar\right) * 100$

where Ar = absorbance of reference (mixture solution and uninoculated broth) and As = Absorbance of sample (mixture solution and cell free supernatant of sample).

**(ii) Hydroxyl radical scavenging assay**

If the accumulation of hydroxyl radicals is not managed, this oxidative process has the potential to destroy cells. In order to prevent runaway death from mild, transitory stress, the formation of hydroxyl radicals will result in secondary macromolecular damage and subsequently will promote more ROS generation[21].

The resistance to hydroxyl radicals test was determined by Shi et al[27] method. Solution containing 1 mL of 1,10-phenanthroline (0.75 mM), 1.5 mL of sodium phosphate buffer, 1 mL of $FeSO_4$ (0.75 mM), 1 mL of $H_2O_2$ and 1.0 mL of sample was incubated at 37 °C after 30 minutes. The hydroxyl radical generated in the mixture was read at 536 nm and was determined using a spectrophotometer[22].

Resistance to hydroxyl radicals (%) = $\left((Ar - As) \div Ar\right) * 100$

where Ar = absorbance of reference (mixture solution and uninoculated broth) and As = Absorbance of sample (mixture solution and cell free supernatant of sample)

**(iii) Hydrogen peroxide activity:** Other bacterial species may also experience a growth stop due to released $H_2O_2$ [23]. Li et al[16] approach was used to test for resistance to this $H_2O_2$. For 16–18 hours, the PGPB isolates were cultured in LB broth at 37 °C[22]. This culture was then combined with 1.0 mM hydrogen peroxide and incubated for additional 8 hours at 37 °C. At 600 nm, the growth of the cells was evaluated using a spectrophotometer.

Resistance to hydrogen peroxide (%) = $\left((Ar - As) \div Ar\right) * 100$

where Ar = absorbance of reference (mixture solution and uninoculated broth) and As = Absorbance of sample (mixture solution and cell free supernatant of sample)

**Statistical Analysis:** All the values were represented as means with respective standard deviations (N=3). Analysis of Variance (ANOVA) was conducted for all the assays using statistical package for social sciences (SPSS) version 30 software, followed by the comparison of multiple treatment levels with the control, using Duncan's multivariate test (DMRT) at ($P \leq 0.05$).

## Results and Discussion

**Isolation of PGPB:** Four morphologically unique rhizosphere bacteria's namely P3, P5, P6 and P8 that were isolated from *Spinacia orlacea*, were characterised for potential PGPB traits. Through the use of Basic Local Alignment Search Tool (BLAST) analysis, the sequence was compared to others in the database, it was discovered that P3 and P5 were *Bacillus clarus* and *Bacillus licheniformis* whereas P6 and P8 belonged to *Penaebacillus* family (Table 1).

**Bacterial colony morphology study:** The difference in the colony morphology was observed to be significantly distorted in non-periodically stressed bacteria than the periodically stressed bacteria compared to the control in nutritionally starved stress condition at the end of treatment (Figure 1). *Bacillus clarus* had a smooth circular off white colony whereas *Bacillus licheniformis* had opaque circular smooth large colonies. Isolates belonging to *Paenibacillus* family had similar morphology of slight opaque smooth circular colonies.

After stress treatment in control and periodically stressed condition, the colonies maintained the same morphology whereas in non-periodically stressed condition, the colonies of *Bacillus clarus* were distorted rough with irregular

growth, similar pattern of distortion was observed in *Bacillus licheniformis* and isolates of *Paenibacillus* family.

Bacterial morphologies can also be influenced by different factors, motility needs, nutritional starvation and predation pressures. These aspects allow the bacteria to adapt their morphologies to obtain benefits in response to various environmental cues[24]. A previous report suggested that certain bacteria such as bacilli, *Yersinia enterocolitica* and Campylobacter spp., could produce donut cells with different colony morphologies when exposed to stressors like heat, freezing, radiation and exposure to copper. Staphylococcus spp. may also exhibit this phenomenon[25].

**Bacterial size imaging study:** There is a significant decrease in the size of the bacterial cell size in non-periodically stressed bacterial cells (2-fold decrease in *Bacillus clarus* and *Paenibacillus alvei SJ8*, 4-fold decrease in Bacillus licheniformis and a marginal decrease in *Paenibacillus alvei SJ6*) whereas periodically stressed bacteria more or less sustained the same size with marginal differences (Figures 2 and 3).

The similarity in bacterial size in periodically stressed bacteria indicates the ability to adapt to the regular exposure of stress treatment compared to the irregularly exposed bacterial isolates (Table 2). Bacterial adaptation is evaluated during multiple passage over the time ranging from days to months whereas bacterial memory lasts for a few seconds to a few generations. Bacteria must exhibit history-dependent behaviour in order to quickly adjust and adapt to changing conditions[26]. The nutritional components of the growth medium determine the maximum size that a bacteria can reach, indicating that nutrients have an impact on one or more rate-limiting steps that regulate size and growth rate of bacteria[13].

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

| Bacterial Isolate Code | Bacteria Identified | Percentage Identity | Accuracy Length | Accession Number |
|---|---|---|---|---|
| P3 | *Bacillus clarus* | 98.41% | 1552 | PP355449 |
| P5 | *Bacillus licheniformis* | 99.57% | 1545 | PP355450 |
| P6 | *Paenibacillus alvei SJ6* | 100 % | 907 | PP355538 |
| P8 | *Paenibacillus alvei SJ8* | 100% | 719 | PP355543 |

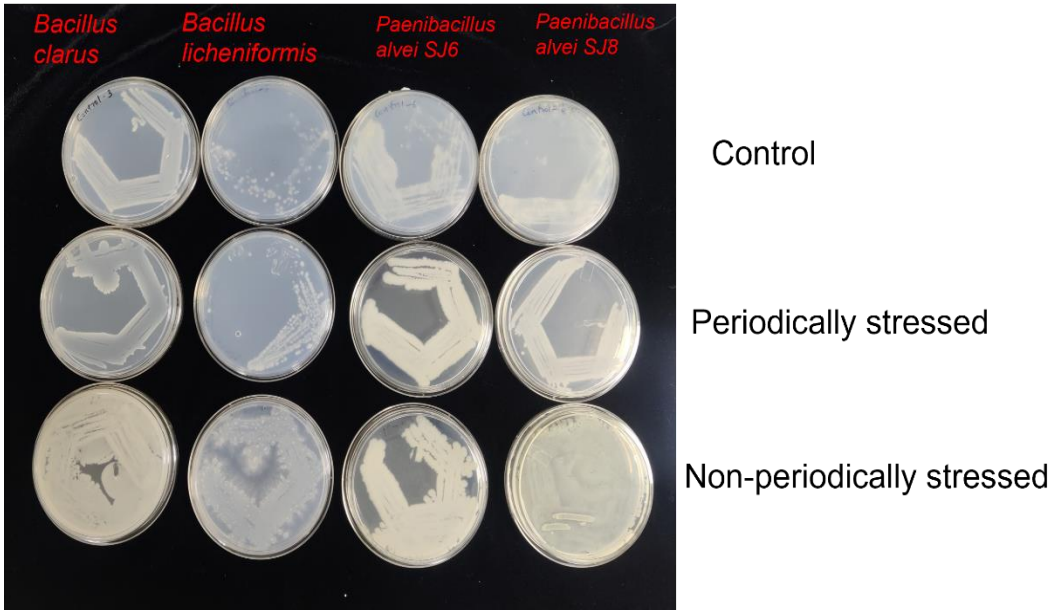


**Figure 1: Bacterial Trial with Nutritional starvation as stress factor. Control was treated with Full strength LB media and the periodically and non-periodically were treated in both 100X diluted LB media / full strength LB media**

Therefore, in our study, the periodically stressed bacteria have adapted to the stress whereas the non-periodically stressed PGPB have significantly reduced in size.

PGPB characteristic assay is to compare between periodic nutritional stress and non-periodic nutritional stress exposure study:

**(a) Ammonia production:** The amount of ammonia produced by periodically stressed PGPB is more or less equal or higher than that of the control condition whereas the non-periodically stressed PGPBs are significantly less than that of control (Table 3). *Paenibacillus alvei SJ8* and *Bacillus clarus* are the highest ammonia producing isolates in nutritionally deprived stress condition. Nutritionally stressed isolates *Paenibacillus alvei SJ8* and *Bacillus clarus* have the highest ammonia producing isolates with 2.551±0 µmol $mL^{-1}$ and 2.439±0 µmol. $mL^{-1}$ respectively (Figure 4).

The microorganisms' generation of ammonia benefits the plants in two ways. One of the key characteristics of PGPRs that helps the crop even under plant stress, is the ammonia released by diazotrophic bacteria[27]. Therefore, continuous exposure to stress decreases the ammonia production but the periodically stressed PGPB adapted to the stress without marginal reduction in their ammonia production compared to control due to this stress.

In another study, it was found that four strains that were studied generated ammonia, albeit at different concentrations: 0.23 to 0.33 µmol·$mL^{-1}$. The results showed that *B. halotolerans J143* had the lowest value and *E. hormaechei J146* had the highest[28]. Compared to this, our isolates *Bacillus clarus, Bacillus licheniformis, Paenibacillus alvei SJ6 and Paenibacillus alvei SJ8* had significant production of ammonia.

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

| Bacterial Isolates | Day 1 size (µm) | Day 7 size (µm) | | Day 14 size (µm) | |
|---|---|---|---|---|---|
| Nutritional stress | Control | Regular | Irregular | Regular | Irregular |
| *Bacillus clarus* | 4.22 | 4.28 | 3.49 | 3.64 | 2.64 |
| *Bacillus licheniformis* | 4.01 | 3.63 | 3.21 | 3.39 | 1.49 |
| *Paenibacillus alvei SJ6* | 1.89 | 1.83 | 1.88 | 1.68 | 1.17 |
| *Paenibacillus alvei SJ8* | 3.99 | 3.99 | 3.38 | 4.2 | 2.41 |

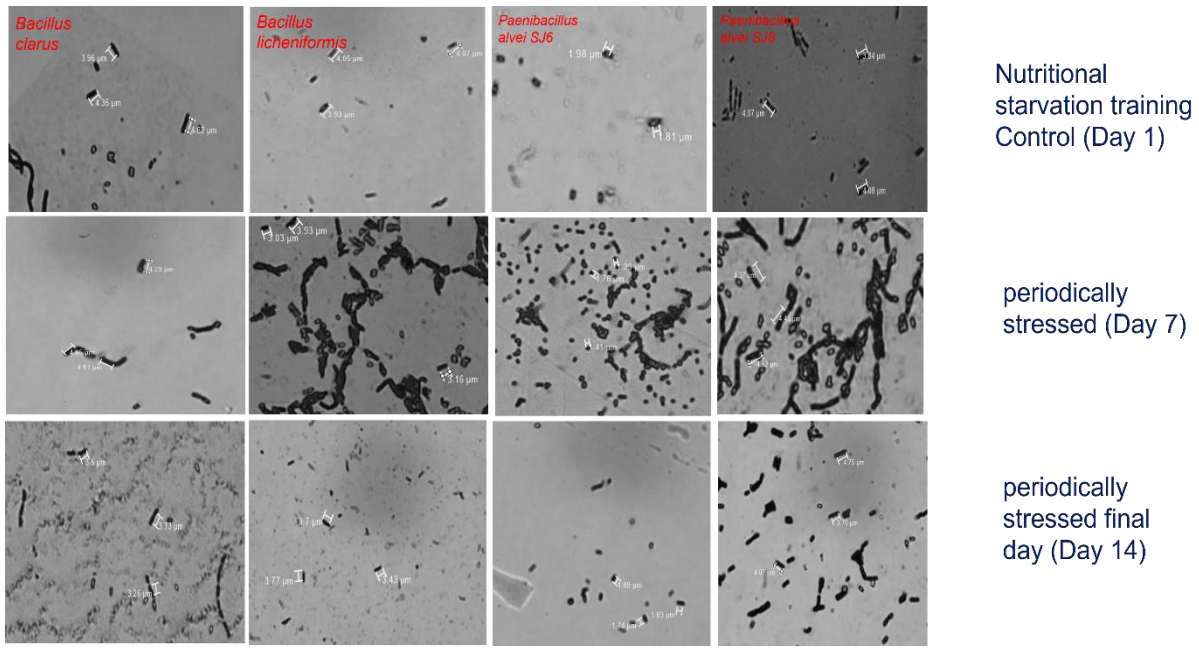


**Figure 2: Bacterial size of isolates under nutritional stress treatment with periodically stressed on days 1, 7 and 14**

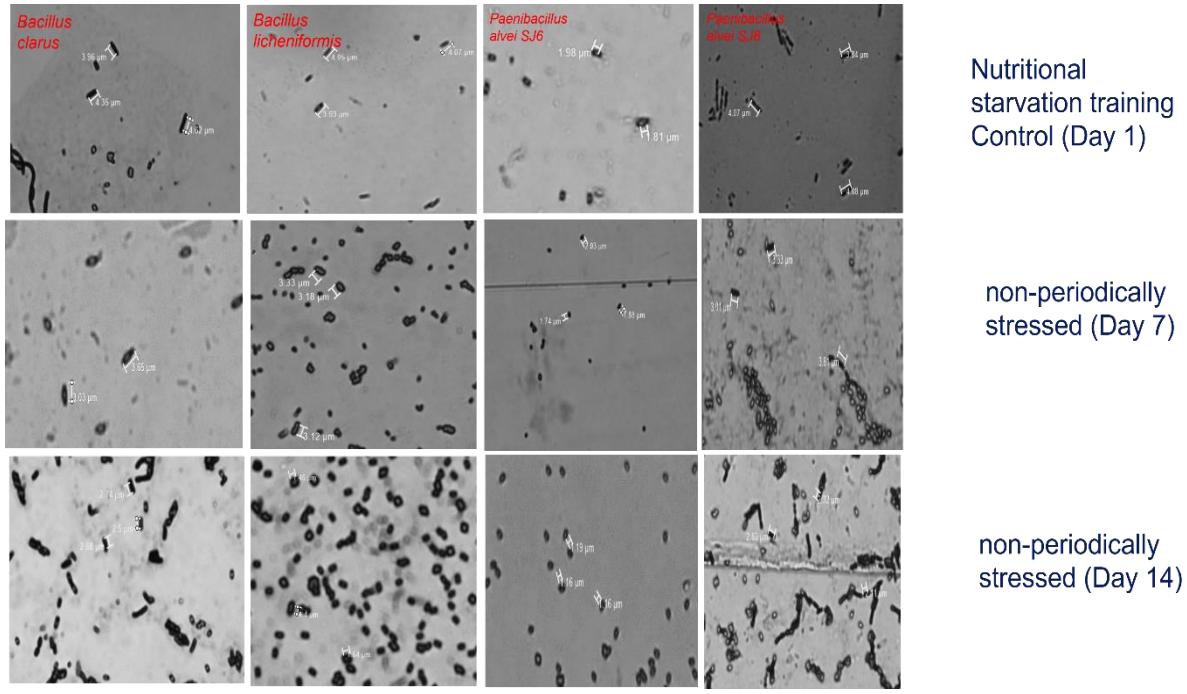


**Figure 3: Bacterial size of isolates under nutritional stress treatment with periodically stressed on days 1, 7 and 14**

**Table 3**
**PGPB characteristic assays performed with periodic and non-periodic nutritional stress treatments compared to control**

| Stress Treatment | Control | periodically stressed | non-periodically stressed |
|---|---|---|---|
| Nutritional Starvation | Concentration of Ammonia (Concentration µmol $mL^1$) | | |
| *1. Bacillus clarus* | 2.495±0.027 $^b$ | 2.439±0.002 $^b$ | 0.312±0.003 $^d$ |
| *2. Bacillus licheniformis* | 2.094±0.042 $^c$ | 1.842±0.016 $^c$ | 0.424±0.004 $^c$ |
| *3. Paenibacillus alvei SJ6* | 1.571±0.002 $^d$ | 1.553±0.042 $^d$ | 1.301±0.032 $^b$ |
| *4. Paenibacillus alvei SJ8* | 2.607±0.002 $^a$ | 2.551±0.002 $^a$ | 1.599±0.006 $^a$ |
| | Concentration of indole-3-acetic acid (Concentration $mgL^{-1}$) | | |
| *1. Bacillus clarus* | 22.312±0.735 $^d$ | 17.972±0.121 $^b$ | 11.146±0.002 $^a$ |
| *2. Bacillus licheniformis* | 28.263±0.368 $^b$ | 14.997±0.06 $^c$ | 10.061±0.06 $^b$ |
| *3. Paenibacillus alvei SJ6* | 39.884±0.264 $^a$ | 23.047±0.002 $^a$ | 7.541±0.06 $^c$ |
| *4. Paenibacillus alvei SJ8* | 23.467±0.105 $^c$ | 14.997±0.06 $^c$ | 10.201±0.303 $^d$ |
| | Concentration of phosphate solubilization (Concentration $mgL^{-1}$) | | |
| *1. Bacillus clarus* | 74.691±0.429 $^d$ | 75.187±2.393 $^d$ | 19.848±0.744 $^b$ |
| *2. Bacillus licheniformis* | 114.893±0.429 $^c$ | 111.418±3.286 $^c$ | 35.234±2.818 $^c$ |
| *3. Paenibacillus alvei SJ6* | 123.082±0.429 $^b$ | 123.578±0.429 $^b$ | 18.608±0.429 $^d$ |
| *4. Paenibacillus alvei SJ8* | 204.478±0.001 $^a$ | 112.659±0.429 $^a$ | 5.856±0.002 $^a$ |
| | Siderophore Producing unit (PSU) | | |
| *1. Bacillus clarus* | 9.25±0.002 $^d$ | 7.68±0.024 $^d$ | 7.68±0.024 $^c$ |
| *2. Bacillus licheniformis* | 11.972±0.096 $^b$ | 0.791±0.001 $^b$ | 0.791±0.002 $^d$ |
| *3. Paenibacillus alvei SJ6* | 11.444±0.048 $^c$ | 8.388±0.072 $^c$ | 8.388±0.072 $^b$ |
| *4. Paenibacillus alvei SJ8* | 14.222±0.024 $^a$ | 12.541±0.001 $^a$ | 12.541±0.002 $^a$ |

Values are average of three replications and the results are shown as mean ± 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 ≤ 0.05)]

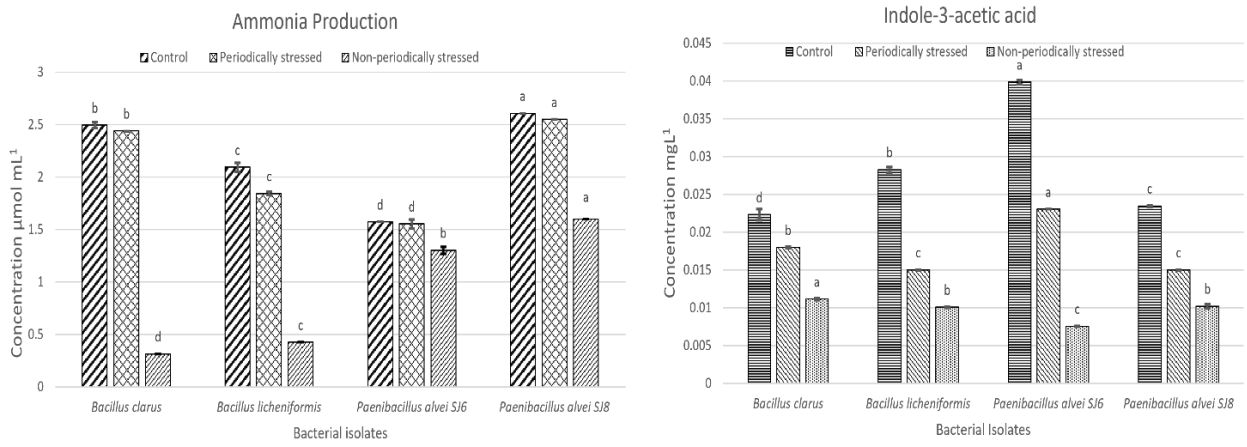


**Figure 4: (a) Production of Ammonia (µmol. mL$^{-1}$) and (b) Indole-3-acetic acid (mgL$^{-1}$) 2.5% L-tryptophan. The bar graphs represent the different concentrations of ammonia and IAA formed in both periodically and non-periodically stressed PGPB. Values are average of three replications and the results are shown as mean ± 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 ≤ 0.05)]**

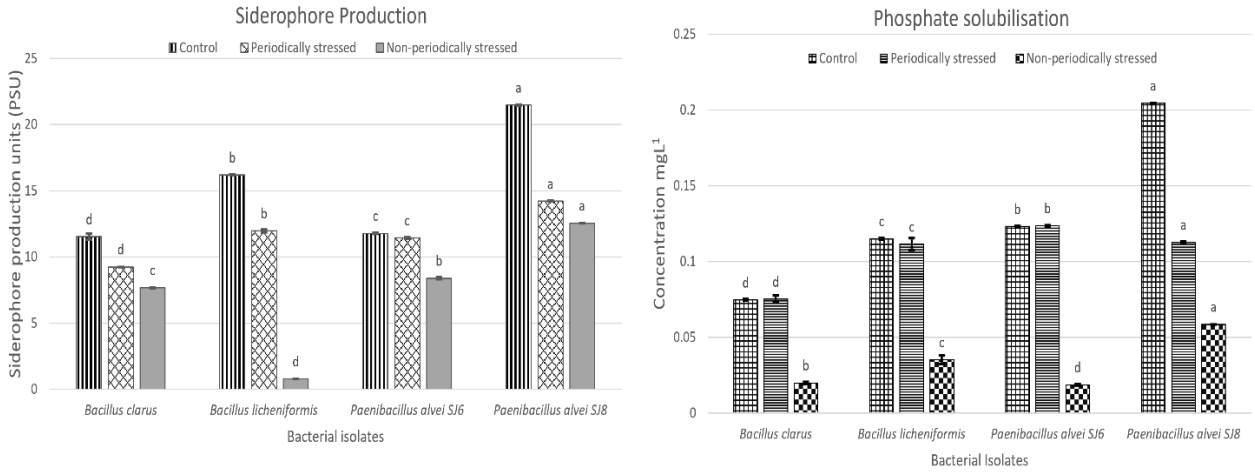


**Figure 5: (a) Production of siderophore production (psu) and (b) phosphate solubilization (mgL$^{-1}$). The bar graphs represent the different concentrations of siderophore formed and phosphate solubilized in both periodically and non-periodically stressed PGPB. Values are average of three replications and the results are shown as mean ± 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 ≤ 0.05)].**

**1. Indole-3-acetic acid production (IAA):** Periodically strained PGPBs create about the same amount of IAA as the control condition, but non-periodically stressed PGPB produce much less than that of the control. *Paenibacillus alvei SJ6* is the highest ammonia producing isolates in nutritionally stressed isolates with highest ammonia production at 23.04±0 mgL$^{-1}$ (Figure 4). IAA is used by beneficial bacteria to enhance nutrient utilization efficiency by promoting plant growth and to mitigate abiotic stressors. These bacteria increase plant resistance by producing more IAA when under abiotic stress[29].

Therefore, it is evidently seen that in our study, regularly stressed the PGPBs solubilized more phosphate than the irregularly stressed PGPBs. In another study, it was found that IAA was produced by six identified PGPB isolates at quantities as high as 36.93 mgL$^{-1}$ *Bacillus sp. NCTB5I* had the highest value followed by *Pseudomonas sp. CHTB 5B* (6.65 mgL$^{-1}$) and *Brevundimonas sp. CHTB 2C* (13.13 mgL$^{-1}$)[30]. Compared to this study, our isolate *Paenibacillus alvei SJ6* was produced as high as 23.04±06 mgL$^{-1}$.

**2. Siderophore production:** *Paenibacillus alvei SJ8* is the highest siderophore producing isolates in nutritionally stress condition. Isolate *Paenibacillus alvei SJ8* produced 12.541±006 psu of siderophore respectively (Table 3). While non-periodically stressed PGPBs produced significantly less siderophore than the control, periodically strained PGPBs produced around the same amount as the control condition (Figure 5). Conversely, various iron-chelating chemicals are produced by siderophore-producing microorganisms (SPM) which contribute to reducing plant

stress[31]. Likewise, in our study when the isolates were stressed regularly, they produced comparatively more siderophore than the non-periodically stressed PGPB. In another study, it was observed that the concentration of siderophore generated by the bacterial strains in salinity stress ranged from 7.97 ± 0.58 to 69.16 ± 0.71 psu [32]. Thus, compared to this study, our isolates *Bacillus clarus, Bacillus licheniformis, Paenibacillus alvei SJ6* and *Paenibacillus alvei SJ8* had considerable amount of siderophore production.

**3. Phosphate solubilisation:** The isolate *Paenibacillus alvei SJ6* is the highest phosphate solubilizing isolate in nutritional stress conditions (Table 3). The isolate *Paenibacillus alvei SJ6* has the highest amount of phosphate solubilized as 123.578±0.429mgL$^{-1}$ supplemented with 1000 mgL$^{-1}$ of tricalcium phosphate added to Pikovaskya's agar media. This inferes that the amount of phosphate solubilized by periodically stressed PGPB is considerably equal to that of the control condition whereas the non-periodically stressed PGPBs are significantly less than that of control (Figure 5). The performance of PGPB in terms of phosphate solubilization is greatly impacted by environmental circumstances, particularly in times of stress.

Due to stress from high salt, high pH and high temperatures, bacteria thriving in alkaline soils in India during the summer months have higher solubilization ability[26]. 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. In another study, estimated amount of dissolved phosphate in the NBRIP supernatant ranged from 85 mgL$^{-1}$ to 1312 mg L$^{-1}$, with two isolates L228 and L132 PGPB exhibiting the maximum solubilization[33]. The amount of phosphate solubilised in our study by *Bacillus clarus, Bacillus licheniformis, Paenibacillus alvei SJ6* and *Paenibacillus alvei SJ8* had significant amount of phosphate solubilisation potential.

Anti-oxidant activity of PGPB between periodic nutritional stress and non-periodic nutritional stress:

**(a) DPPH scavenging activity:** The isolates *Paenibacillus alvei SJ6* and *Bacillus licheniformis* have the highest scavenging activity in nutritionally stressed condition. They produced around 70.35±0.082 % and 63.96±0.021% scavenging activity (Table 4). The control group has relatively lesser scavenging activity whereas the periodically stressed PGPBs 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 (Figure 6).

When plants are exposed to various biotic and abiotic stressors, PGPBs can greatly aid in their adaptation and growth, as well as can stimulate the synthesis of phytohormones, antioxidants and siderophores[34]. By enhancing stress tolerance and stimulating plant development, bacterial DPPH scavenging can benefit plant growth.

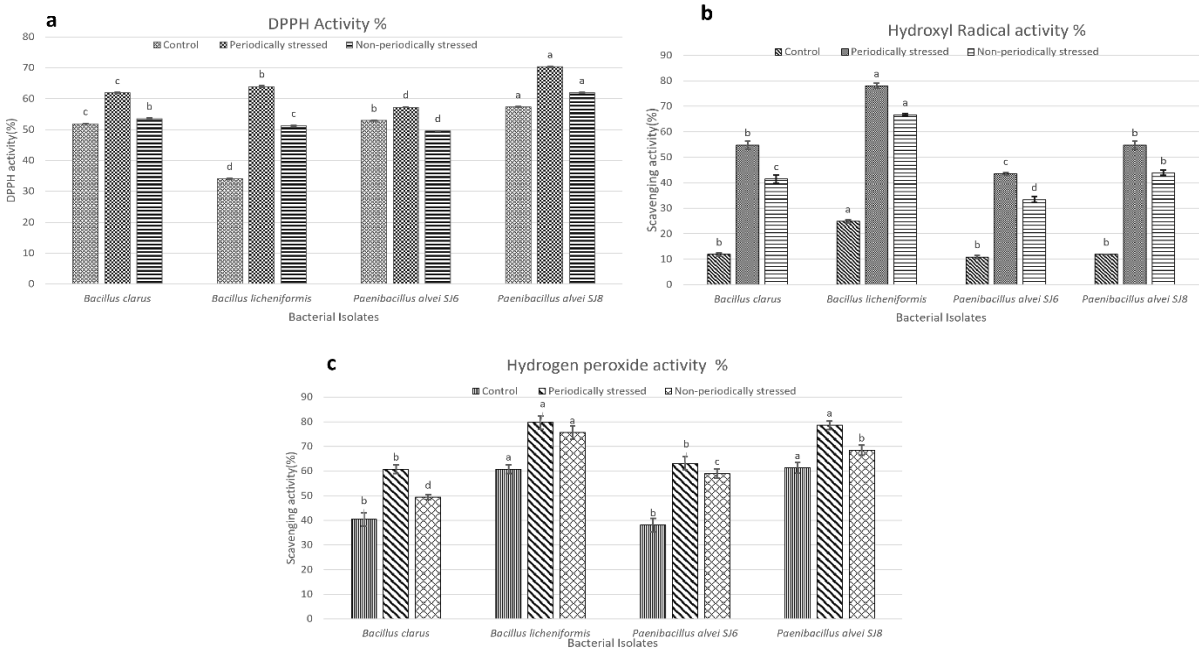


**Figure 6: (a) Production of DPPH activity (%), (b) Hydroxyl radical scavenging activity (%) and (c) Hydrogen peroxide scavenging activity (%). The bar graphs represent the different concentrations of radicals scavenged in both periodically and non-periodically stressed PGPB. Values are average of three replications and the results are shown as mean ± 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 ≤ 0.05)].**

**Table 4**
**Anti-oxidant assays performed with periodic and non-periodic nutritional stress treatments compared to control.**

| Stress Treatment | Control | periodically stressed | non-periodically stressed |
|---|---|---|---|
| Nutritional Starvation | DPPH scavenging activity (%) | | |
| *1. Bacillus clarus* | 51.85±0.008 $^{c}$ | 61.88±0.001 $^{c}$ | 53.55±0.02 $^{b}$ |
| *2. Bacillus licheniformis* | 34.14±0.008 $^{d}$ | 63.97±0.01 $^{b}$ | 51.23±0.01 $^{c}$ |
| *3. Paenibacillus alvei SJ6* | 52.94±0.007 $^{b}$ | 57.24±0.009 $^{d}$ | 49.57±0.008 $^{d}$ |
| *4. Paenibacillus alvei SJ8* | 57.29±0.003 $^{a}$ | 70.36±0.008 $^{a}$ | 61.93±0.009 $^{a}$ |
| | Hydroxyl radical scavenging activity (%) | | |
| *1. Bacillus clarus* | 11.90±0.058 $^{b}$ | 54.76±1.55 $^{b}$ | 41.50±1.45 $^{c}$ |
| *2. Bacillus licheniformis* | 24.83±0.066 $^{a}$ | 78.06±1.02 $^{a}$ | 66.67±0.59 $^{a}$ |
| *3. Paenibacillus alvei SJ6* | 10.88±0.003 $^{b}$ | 43.54±0.58 $^{c}$ | 33.33±1.17 $^{d}$ |
| *4. Paenibacillus alvei SJ8* | 11.90±0.056 $^{b}$ | 54.76±1.55 $^{b}$ | 43.88±1.02 $^{b}$ |
| | Hydrogen peroxide scavenging activity(%) | | |
| *1. Bacillus clarus* | 40.48±2.72 $^{b}$ | 60.71±1.78 $^{b}$ | 49.40±1.03 $^{d}$ |
| *2. Bacillus licheniformis* | 60.71±1.78 $^{a}$ | 79.76±2.72 $^{a}$ | 75.60±2.72 $^{a}$ |
| *3. Paenibacillus alvei SJ6* | 38.10±2.72 $^{b}$ | 63.10±2.72 $^{b}$ | 58.93±1.77 $^{c}$ |
| *4. Paenibacillus alvei SJ8* | 61.31±2.06 $^{a}$ | 78.57±1.78 $^{a}$ | 68.45±2.06 $^{b}$ |

Values are average of three replications and the results are shown as mean ± 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 ≤ 0.05)]

The reason for this is because genes that produce secondary metabolites, which have antipathogenic and antipest characteristics, can be activated by DPPH scavenging. Reactive oxygen species (ROS) are also scavenged by these metabolites[35].Three marine Actinobacteria isolated from Nicobar Islands maritime sediments were found to have antioxidant activity in a study by Karthik et al[12] whereas phenolic compounds recovered from *Streptomyces sp. LK-3* had 76% DPPH scavenging activity. In a different investigation, *Actinobacteria* extracts from Vishakhapatnam region mangrove soil showed 46–70% DPPH scavenging ability, according to Rao and Rao[23].

**(b) Hydroxyl radical scavenging assay:** All the PGPB isolates have significant hydroxyl radical scavenging ability with *Bacillus licheniformis* having the highest activity (Table 4). Isolates *Bacillus clarus, Bacillus licheniformis, Paenibacillus alvei SJ6* and *Paenibacillus alvei SJ8* are having 54.76±1.55 %, 78.06±1.02%, 43.58±0.58% and 54.76±1.55% of scavenging activity. Periodically stressed plants are able to scavenge more free radicals than non-periodically stressed plants when compared to the study's control group (Figure 6). This suggests that periodically stressed plants are able to adapt and memorize to the periodically exposed stress.

Reduced uptake of carbon dioxide ($CO_2$) and partial reduction of molecular oxygen i.e. increased generation of ROS such as hydroxyl radicals and $H_2O_2$ led to oxidative stress in plant tissues such as mitochondria, chloroplasts and peroxisomes when exposed to various stresses like phytopathogens, salinity, drought and nutritional deprivation[38]. Therefore, the scavenging activity of these PGPB will help to reduce the radical stress to plants. The results of a study by Abdelaal et al[1] showed that *L. plantarum IH16L* (82.25 ± 1.60%) had the highest hydroxyl radical scavenging activity, followed by *L. sake IH15L* strain (82.20 ± 1.32%) and *L. cruvatus IH1L* strain (78.86 ± 0.73%). Compared to this, our study showed that isolates *Bacillus clarus, Bacillus licheniformis, Paenibacillus alvei SJ6* and *Paenibacillus alvei SJ8* had significant amount of hydroxyl radical scavenging capability with *Bacillus licheniformis* having the highest of 78.06±1.02% activity.

**(c) Hydrogen peroxide scavenging activity:** The two isolates *Bacillus licheniformis* and *Paenibacillus alvei SJ8* had the highest amount of $H_2O_2$ scavenging activity of 79.76±2.72% and 78.57±1.78% (Table 4). All isolates had significant hydrogen peroxide scavenging activity in our study, indicating the ability of these PGPB to adapt to the harsh periodic change in environment (Figure 6). In order to protect themselves from oxidative stress brought on by reactive oxygen species (ROS), plants upregulate their antioxidant activity. This mechanism makes plants more resilient to stress. Additionally, this species demonstrates strong stress-tolerance traits since stress treatment increases the activity of antioxidant enzymes like catalase, peroxidases and superoxide dismutase (SOD). Due to its strong hydrogen peroxide activity, *S. variabilis RD-5* is a possible source of antioxidants that can help to stop the progression of a number of conditions connected to oxidative stress. $H_2O_2$ scavenging activity of the *S. variabilis strain RD-5* extract was found to be 74.5%. So according to the present study, our isolates have higher activity of hydrogen peroxide scavenging ranging from 60.71±1.78% to 79.76±2.72%.

## Conclusion

In this present study, the ability of the PGPB to adapt to periodic stress and to form cognitive historic dependent memory when exposed to harsh environment is witnessed.

In the overall study, the PGPBs in periodic stress condition adapted to the change better than the non-periodic stress exposed PGPB compared to the control. The novel strains *Paenibacillus alvei SJ6* and *Paenibacillus alvei SJ8* have shown to have the supreme ability to adapt to periodic nutritional stress compared to the other isolates. All isolates under periodic stress exposure showed higher activities of different PGPB characteristics such as siderophore production, phosphate solubilisation, indole-3-acetic acid production, ammonia production and anti-oxidant activities.

The results of this study implicate preliminary evidence supporting the existence of cognitive intelligence in bacteria. Further study has to be conducted to explore its potential and effect on their application on plant growth parameters under different stress conditions and to further analyse their inter-cognitive adaptation in stress.

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