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Effect of Plant Growth Promoting Activities of PG-33 in Drought Stress on *Sorghum bicolor*

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ABSTRACT

The agricultural output is nevertheless impacted by elements including improper mechanisation, a shortage of arable land, and harmful abiotic stressors. Drought has a significant negative impact on agricultural output and may endanger sustainable farming practices. The global trend towards organic farming is expanding. The natural organic formulation known as fermented panchagavya, which is formed from five cow products (dung, urine, milk, curd, and ghee), is abundant in plant growth-promoting microbes (PGPM). PGPMs produce various plant growth-promoting substances even in the presence of drought stress. 64 bacteria were isolated from fermented panchagvaya. Their plant growth promoting activities were checked in drought stress. Selected PG-33 bacterial isolate showed various plant growth promoting activity even in the presence of PEG-6000 (drought stress). PG-33 showed 19.92 µg/ml IAA production in control and 18.72 µg/ml in the presence of PEG. The highest GA production by PG-33 was 6.11 mg/ml in control and 5.58 mg/ml in the presence of PEG. Ammonia production was 2.14 µmol/ml in the control and at 1.68 µmol/ml in the presence of PEG. The phosphate solubilization in PG-33 was 168.2 µg/ml in the control and 148.6 µg/ml in the presence of PEG. The seed germination index of Sorghum bicolor was observed 127.5 in the presence of PEG-6000 and PG-33. On the 15th day, PG-33 treated plants showed an increase in root (10%) and shoot (47%) length as well as an increase in leaf length (60%). The treated plants were longer than the untreated control plants in terms of their roots and shoots.

Keywords: Panchagavya, PEG-6000, drought, plant growth promoting microbes (PGPM), exopolysaccharides

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INTRODUCTION

Drought stress

Agriculture has been a vital part of human civilisation, supplying food, fibre, and other resources. People are dependent on agriculture to provide for their basic needs and to maintain their societies from the beginning of the history of humanity. Agriculture gained importance as human populations increased, allowing communities to maintain greater populations and create more complex economies (17). Agriculture is under a lot of stress worldwide because of rising population and corresponding rises in food demand (29). However, several issues, including improper mechanisation, a shortage of arable land, and the presence of numerous biotic and abiotic stresses, continue to have an impact on agricultural output (12). There will be over 9.7 billion people on earth (more than 65%) which rely completely on agriculture for their living by 2050. According to (10), this ratio could rise to 90% in developing countries. Agriculture will consequently be crucial to a nation's economy and food supply. Drought is a severe destructive abiotic stress that reduces agricultural output significantly and may be the biggest danger to sustainable agriculture. Food security is threatened by drought, one of the most destructive and detrimental abiotic forces (47). Drought stress has a negative impact on social, economic, and environmental systems as well as on grasslands, shrubs, and trees. According to recent findings, droughts appear to have a significant impact on the pools, processes, and fluxes of the terrestrial carbon and nitrogen cycles in ecosystem (14).

Drought is a major issue that farmers deal with every year. According to, (18), a drought is reportedly affecting 42% of the agricultural land in India (159.7 million hectares).

Drought stress on plant growth and development

Drought alters the physiological and morphological characteristics of plants by changing their water potential and turgor to the point that it interferes with normal activities (23). Numerous crops, including barley (48), maize (25), rice (31), and wheat, have been examined for growth reduction under drought stress (45). Drought reduces soil organic carbon decomposition, lowers microbial biomass, and causes less CO_2 production. It is one of the major agricultural problems reducing crop yield in arid and semiarid regions in the world. Drought stress causes several physiological and biochemical changes that may affect organ function and limit plant development (5). The scavenging of reactive oxygen species (ROS), signalling of kinase cascade, hormonal imbalance, regulation of gene expression, production of osmolytes, changes in cell structure, activation of ion channels, metabolism of carbohydrates and energy, metabolism of amino acids and fatty acids, assimilation of nitrogen, and other specific drought-regulating mechanisms are involved (24). High concentrations of reactive oxygen species may have a negative impact on the biochemical and physiological processes at various stages of molecular and cellular organisation during the plant's growth (50,22,37). Bark and twig cracking, branch dieback, thinning tree and shrub canopy, necrosis, and poor and stunted growth are also unusual symptoms. Lastly, plant death occurs under extreme conditions (15,49)

Role of plant growth promoting bacteria (PGPB) in growth of plants under drought Stress

Organic farming depends mainly on cow-based manure. Several crops have benefited from the use of panchagavya, a mixture of five cow-derived products, including dung, urine, milk, curd, and ghee (29). Agricultural activities (biocontrol, biofertilizer, plant growth enhancer, etc.), pharmaceutical value, probiotic and antibacterial potential, and pharmacological value have all been associated with panchagavya (38). South Indian farmers utilise Panchagavya for organic farming (39). The fermented panchagavya contains so many beneficial microbes (plant growth-promoting bacteria) that it is plant growth-promoting. The enzymes secreted by bacteria break down the intricate, soluble chemical molecules into simpler ones. This might make it easier for plants to acquire nutrients through microbial activities. Plants are assisted in growing by the compounds that microbes secrete that encourage plant growth. The fermented Panchagavya mixture contains macronutrients, micronutrients, amino acids, and substances that encourage plant growth, such as indole acetic acid, gibberellic acid, exopolysaccharides, and ammonia. Panchagavya is a biofertilizer that may be used in the field and is certain to have a population of bacteria that is helpful to the plant. This panchagavya isolate bacteria is helpful for plants to survive adverse conditions like drought stress.

In drought stress, PGPB shows induced systemic tolerance (IST), which generates chemical and physical changes in plants at the physical, biochemical, and molecular level (57). PGPBs can induce IST by releasing a variety of chemicals, including as lipopolysaccharides from their outer membranes, volatile organic compounds (VOC), biosurfactants, siderophores, antibiotics, along with other metabolites (16,56). This process also modifies the profile of phytohormones, activates an antioxidant defence system, generates osmoprotectants, and switches on stress-response proteins. The synthesis of the enzyme ACC deaminase by PGPBs to offset the negative effects of ethylene is one of the most common IST mechanisms (11).

In the present study, PG-33 bacteria were isolated from fermented panchagvaya, which shows tolerance towards drought stress. We are aiming to find out the effect of PG-33 on a *Sorghum bicolor* plant under drought stress.

MATERIAL AND METHODS

Isolation of bacterial isolates for fermented panchagavya

64 bacterial cultures were isolated from fermented panchagavya (19). Various plant growth promoting traits like IAA production, GA production, ammonia production, phosphate solubilization, EPS production were checked in drought stress condition. PG-33 bacterial isolate showed better survival in the presence of PEG-6000.

Inoculum preparation

The bacterial cultures from slants were inoculated in sterile nutrient broth and incubated at 30°C for 24 h. Cells were separated by centrifugation at 5000 rpm for 10 min and the supernatant was removed. The cell pellet was resuspended in sterile normal saline to get optical density of 1.0. The prepared culture suspension was used as a 1.0% (v/v) inoculum to study the plant growth-promoting parameters like IAA, GA, NH_3, EPS production and phosphate solubilization.

The absorbance was measured in spectrophotometer at 600nm after 24 hours. The standard calibration curve of $BaCl_2$ (1 O.D ~ 8×10^8 cells/ml) was prepared to calculate the number of cells.

Drought stress response of PG-33

The drought tolerance ability of PG-33 bacterial isolate was checked by observing their growth on the nutrient broth medium supplemented with different polyethylene glycol (PEG-6000) concentrations (1, 3, 5, 7, 10 and 15%; w/v). PG-33 was inoculated on nutrient broth and incubated at 28°C for 24 h.

Plant growth promoting traits

The selected bacterial cultures were tested qualitatively and quantitatively for multiple plant growth promoting activities like indole-3-acetic acid production (IAA), gibberellic acid production (GA), ammonia production (NH_3), phosphate solubilization (P).

- **Indole-3-acetic acid production**

For IAA production, 50.0 ml of Luria-Bertani broth (pH 7.5) containing 0.1% (w/v) L-tryptophan in 250 ml flask was inoculated with 1.0% (v/v) inoculum and incubated at 30°C in the dark (as IAA is light sensitive), 120 rpm on an orbital shaker. IAA production was estimated from samples withdrawn at 24-h intervals up to 24–144 h until production was decreased.

The spectrophotometric estimation of IAA was done according to (8). Culture supernatant (1 ml) was mixed with 2.0 ml of Salkowski reagent and incubated at room temperature (RT) in the dark for 30 min. Development of pink colour shows the production of IAA and absorbance was recorded at 530 nm. The standard calibration curve of IAA (100 µg/ml) was prepared to calculate the IAA production.

- **Gibberellic acid production**

Gibberellic acid production was carried out in 50.0 ml nutrient broth (pH 7.4) in 250 ml flask, 1.0% (v/v) inoculum, at 30°C, 120 rpm on an orbital shaker for 96 h. GA production was estimated from samples withdrawn at 24 h intervals up to 24–96 h until it was decreased.

An equal volume of cell-free supernatant and ethyl acetate (EA) was taken in a test tube and shook vigorously. The EA extract was collected separately in a glass beaker, and the extraction was repeated three times. The separated EA extract was evaporated by rotary evaporator at 45°C. The residues were dissolved in 2.0 ml methanol. To this, 1.0 ml DNPH (2, 4 – dinitrophenylhydrazine) was added and incubated in a boiling water bath for 5 min. After incubation, it was cooled in an ice-water bath, and 5.0 ml of 10% (w/v) alcoholic potassium hydroxide was added, allowed to stand for 10 min at RT. To this, 15.0 ml of sterile distilled water was added, and the intensity of the color (red wine) produced was measured at 430 nm (21). A standard calibration curve of GA (0.8 mg/ml) was prepared.

- **Ammonia production**

Ammonia production was determined in a 50-ml flask containing 20.0 ml sterile peptone water, pH 7.2. It was inoculated with 1.0% (v/v) inoculum and incubated at 30°C, 120 rpm on an orbital shaker for 144 h. The amount of NH_3 produced was estimated from samples withdrawn at 24 h intervals up to 24–144 h until it was decreased.

Spectrophotometric estimation of ammonia was done as described by (9). Culture supernatant (1 ml) was mixed with 0.1 ml Nessler's reagent, and the final volume was made to 5.0 ml by adding ammonia-free distilled water. NH_3 production is indicated by a change in color from yellow to brown, and absorbance was measured at 425 nm. The NH_3 produced was calculated using the ammonium hydroxide (0.2 µmol/ml) standard calibration curve.

- **Phosphate solubilization**

The qualitative phosphate solubilization test was carried out using spot inoculation technique on an agar plate containing an insoluble tricalcium phosphate (TCP) and incubated at 30°C for 120 h (44). A zone develops surrounding the colony because of phosphate solubilization. The phosphate solubilization index (PSI) was calculated using the formula below.

$$\text{PSI} = \frac{\text{Colony diameter} + \text{Zone of clearance}}{\text{Colony diameter}}$$

For quantitative phosphate solubilization, 100 ml nutrient broth in 250 ml flasks were inoculated and incubated at 30°C, at 120 rpm on an orbital shaker for 15 days. The amount of phosphate solubilization was measured at 5 days interval for up to 15 days. A 2.0 ml sample was centrifuged at 10,000 rpm for 20 min to separate the cells. To 0.1 ml supernatant, 0.9 ml double distilled water and 1.0 ml chloromolybdic acid was added. Contents were diluted by adding 4.0 ml double distilled water. To this, 25.0 µL chlorostannous acid reagent was added, and the test tubes were mixed well till blue color developed. Absorbance was measured at 600 nm. The amount of phosphate solubilized was calculated against K_2HPO_4 (100.0µg/ml) standard calibration curve.

Seed germination test

For the germination test, seeds were first surface sterilized by gently shaking in 70% methanol for 2 minutes, then the seeds were washed with sterile distilled water. The seeds were dipped in 0.1%(v/v) $HgCl_2$ for 1 minute and again seeds were washed twice with sterile distilled water. After surface sterilization, the seeds were placed on sterile cotton in a sterile Petri plate. The experiment was performed in 2 sets.

1) *Sorghum bicolor* seeds plus distilled water
2) *Sorghum bicolor* seeds plus PEG (5%w/v) + PG-33

After 48 hours, germinated seeds were counted and the germination percent, seed vigour index was calculated by the following formula:

$$\text{Seed vigour index} = \text{seedling length} \times \text{germination perecent}$$

$$\text{Germination percentage} = \frac{\text{Number of germinated seeds} \times 100}{\text{Number of seeds}}$$

Pot study

Pot study was carried out in plastic bags having 18×18 cm size in the month of February to March 2023, when the average temperature was below 30°C. For preparation of pots, collected farm soil was sun dried for 5 days. Approximately 4.0 kilograms of sun-dried soil was filled in the pots. Drought tolerance of the PG-33 was tested at 5%w/v PEG-6000. The seeds were surface sterilized by gently shaking them in 70% methanol for 2 minutes, then the seeds were washed with sterile distilled water, next the seeds were dipped in 0.1% $HgCl_2$; for 1 minute, then the seeds were again washed twice with sterile distilled water. The 10 numbers of seeds were sown in the pot at 1 cm depth. Irrigation was done with tap water. For preparation of bacterial suspension for pot treatment, the PG-33 was scraped from preserved slant and inoculated in 100ml sterile nutrient broth, incubated at 30°C, 120 rpm for 24 hours. After 20 days, plants were uprooted and vegetative parameters like root length, shoot length, total plant length, wet weight, dry weight was measured. Table-1 includes pot design on *Sorghum bicolor* plant.

RESULTS AND DISCUSSION

PG-33 was studied for their drought stress tolerance. PG-33 shows better growth in the presence of different polyethylene glycol (%w/v) concentrations. PG-33 shows better growth in the presence of different PEG-6000 (%w/v) concentrations. PG-33 shows plant growth promoting characteristics like indole-3-acetic acid production, gibberellic acid production, ammonia production, phosphate solubilization etc. in the presence of adverse conditions. Qualitative plant growth promoting characteristics of PG-33 were checked for further study.

Drought stress tolerance ability

PG-33 has grown well in nutrient medium with 1.0 to 15.0% (w/v) PEG concentrations (Table-1). It was observed that the number of bacteria decreased with increasing PEG concentration, but it could survive at higher PEG concentrations. PG-33 showed the best growth (about 50%) up to 5% PEG concentration. PGPB (plant growth-promoting bacteria) *B. amyloliquefaciens 5113* showed higher than 40 % drought tolerance (up to 5% w/v PEG) even after 7 days without water (26).

Plant growth promoting traits

PG-33 show various plant growth promoting activities like IAA production, GA production, NH_3 production, phosphate solubilization in the presence of PEG concentration. All the plant growth promoting activities were done with 2%w/v PEG concentration and 1%v/v inoculum.

Screening of PG-33 for plant growth promoting parameters

The PG-33 was screened for plant growth promoting activities like indole acetic acid (IAA), gibberellic acid (GA), ammonia production, exopolysaccharide production (EPS) and phosphate solubilization. (**Table 2**). Qualitative analysis of PG-33 was done for its various plant growth promoting activities. PG-33 shows positive results for IAA production, GA production, ammonia production and phosphate solubilization. PG-33 shows negative result for exopolysaccharide (EPS) production.

- **IAA production**

Figure-1 shows IAA production of PG-33 at different time intervals in PEG concentration. PG-33 shows the maximum IAA production (19.92 µg/ml) at 96 hours in control. While it shows higher production (18.72 µg/ml) after 96 hours with 5% PEG concentration. (36) observed potent IAA production in *P. simiae* strain AU in drought stress (up to 10% w/v PEG concentration). Indole acetic acid production is very common among the PGPB because it helps in root expansion and uptake of nutrients. (13) reported two isolates from rhizosphere which were able to produce 12.0 µg/mL and 7.0 µg/mL IAA after 72 hours. (41) reported the

bacteria isolated from panchagavya producing 75.86 µg/mL IAA after 72 hours. (53) reported a wide range of IAA, 35-217 µg/mL produced by bacteria. Rhizobacteria strain *23-B* produced 22.8 µg/mL IAA after 72 hours in drought stress (51). IAA formation with *Bacillus* strains *Cha43* (29.4 µg/mL), *ZM39* (28.7 µg/mL) and *Cha21* (26.9 µg/mL) (7).

- **GA production**

Figure-2 shows GA production of PG-33 at different time intervals in PEG concentration. Gibberellic acid is a phytohormone that helps in seed germination and plant growth (35).

PG-33 showed GA production (6.11 mg/ml) in control and 5.58 mg/ml in the presence of PEG after 48 hours. *DS9* strain which is plant growth promoting rhizobacteria (PGPR) shows potent GA production even in drought stress (6). Rhizospheric isolate *Bacillus siamensis* produced 0.24 mg/ml GA after 96 h in broth (4). *Bacillus cereus* isolated from the rhizosphere produced 0.39 mg/ml GA in a nutrient broth after 216 h (43). (33) reported drought-stressed strains produced significantly higher amounts of GA which is *Cha41* (0.0943 mg/mL), *Haw20* (0.0867 mg/mL) and *ZM39* (0.0854 mg/mL).

- **NH_3 production**

Figure-3 shows ammonia production of PG-33 at different time intervals in the presence of PEG concentration. Ammonia production is the most common mechanism of PGPB. Abiotic stress may restrict the ability of plants to reduce and assimilate nitrogen through the inhibition of enzymes implicated in nitrogen metabolism, such as Nitrite Reductase. Use of PGPB that produces ammonia may provide a biological alternative for fixing atmospheric N_2.

PG-33 shows higher production (2.14 µmol/ml) and 1.68 µmol/ml in the presence of PEG after 72 hours. *A. brasilense NO40* and S. *maltophilia* show potent ammonia production in drought stress (40). Ammonia production was reported 15.21 µg/mL after 72 hours by a bacterial culture isolated from chickpea rhizosphere (3,34).

- **Phosphate solubilization**

Figure-4 shows phosphate solubilization of PG-33 at different time intervals in the presence of PEG concentration, respectively. The phosphate-solubilizing microorganisms (PSMs) such as phosphate-solubilizing bacteria (PSB) present in most soils which can solubilize these insoluble forms of phosphates. PG-33 shows higher production (168.2 µg/ml) in control and 148.6 µg/ml in the presence of PEG concentration after 10 days. *Pseudomonas koreensis* strain AK-1 shows phosphate solubilization in the presence of different PEG (%w/v) concentrations (27). (42) reported 2 bacterial isolates *C1* and *H6*, which were able to solubilize 305.49 µg/mL and 282.38 µg/mL tri calcium phosphate after 8 days. (30) isolated 5 isolates from the rhizosphere of French bean plants growing at different sites at Solan and Shimla of Himachal Pradesh in India, which were able to solubilize tri calcium phosphate in the range of 15 µg/mL to 60 µg/mL. According to (1) the maximum amount 98.3 ± 3.5 µg/mL of solubilized phosphate was recorded at a high PEG concentration 15.0%. At 5.0% PEG concentration it produced 81.6± 3.2 µg/mL phosphate after 7 days.

Seed germination test

Table-6 shows seed germination percentage, seedling length (cm) and seed vigour index of *Sorghum bicolor.* One of the most crucial stages of the plant growth cycle is seed germination.

The percentage germination ability of PG-33 is shown in Table-4 and figure-5. *Klebsiella sp. PG-64* treated *Vigna radiata* seedling showed 92% seed germination (20).

Sorghum bicolor seeds were treated with distilled water showed 100% seed germination with 150 seed vigor index. Seeds treated with PEG-6000 showed 50% seed germination with 25 seed vigor index. When seeds treated with PEG-6000 along with PG-33 bacterial culture it showed 75% seed germination with 127.5 seed vigor index.

Bacillus licheniformis culture-treated *Arachis hypogea* seedlings showed 85% germination (52). Biofuel crop *Pongamia pinnata* seeds were soaked in 2% and 5% panchagavya solution for 8h showed germination percentage 88% and 70% respectively while in control 68% (54). Seed germination is a parameter of prime significance and fundamental to total biomass and yield production. (55) reported effect of the potential drought-tolerant bacterial strains isolated from the rhizosphere of *S.acmella Murr.* on *Triticum aestivum L.* The germination of wheat seedlings (*Triticum aestivum L.)* was reduced under 60% PEG compared to without PEG (control). A maximum of 1.28-fold rise in shoot length was found in the seedlings treated with H3S3A bacteria (*Pantoea sp.).* Similarly, the root length was increased by ~ 1.23 to 2.8-fold compared to control. Around 2.8-fold rise in the root, the length was found in seedlings treated with C3S3E strain (*Burkholderia sp.)* and a minimum 1.23-fold increase in the case of Pa2S2E bacteria (*Klebsiella sp*.). According to, (2) the efficiency of *V. radiata* seed germination in pot soils amended with different doses of PEG and inoculated with PAB19 was recorded at 6 DAS. Almost all seeds planted in untreated soil

germinated, whereas PEG at 15% maximally retarded germination efficiency and SVI by 50 and 56%, respectively, over non-treated controls.

Pot study on *Sorghum bicolor*

Table-5 shows physico-vegetative parameters of *Sorghum bicolor*. The biological contribution of PG-33 on the growth of *Sorghum bicolor* was investigated in the presence of PEG concentration.

The results were recorded with positive and negative control. An increase in root length and shoot length was observed on 15th day along with increase in leaf length. It was observed that treated plants had longer roots (10%) and shoots (47%) than the untreated control plants (Figure-6). In addition, treated plants had a higher fresh weight and dry weight.

Bacillus species, such as *Bacillus subtilis*, *Bacillus amyloliquoefaciens*, *Bacillus cereus*, *Bacillus pumilus*, and *Bacillus polymyxa*, are well known for their plant growth and development abilities because they produce antibiotics and produce phytohormones and phosphate solubilization in drought stress (46). Inoculation of soil or crops with PGPB is a potential technique for boosting plant growth, thus reducing the usage of environmentally harmful chemical fertilizers (32).

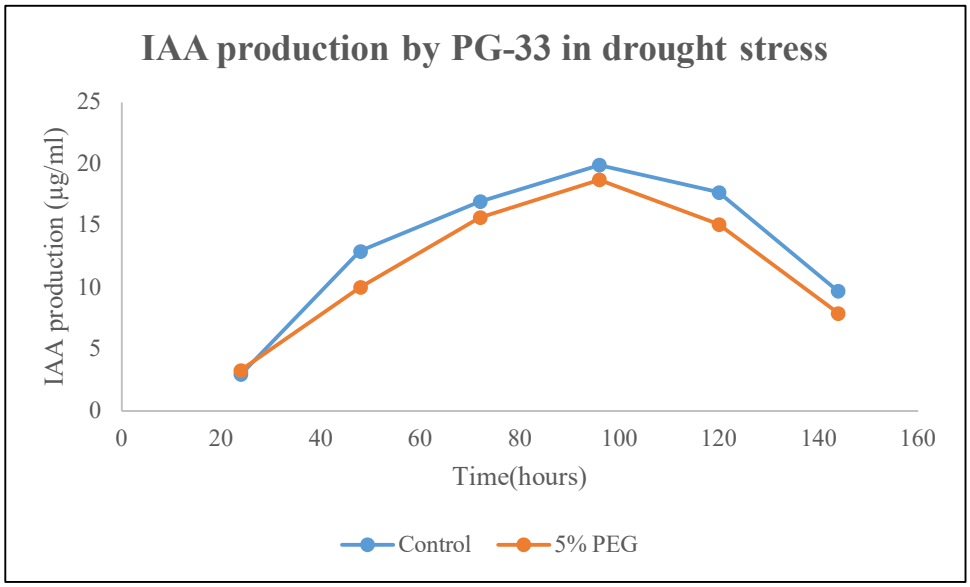

Figure-1: IAA production by PG-33 in drought stress along with control

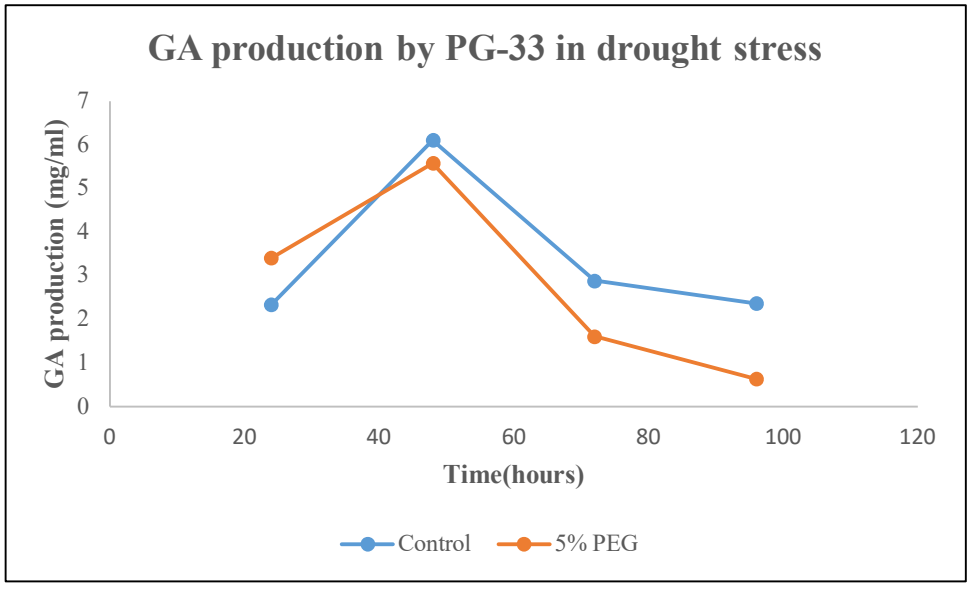

Figure-2: GA production by PG-33 in drought stress along with control

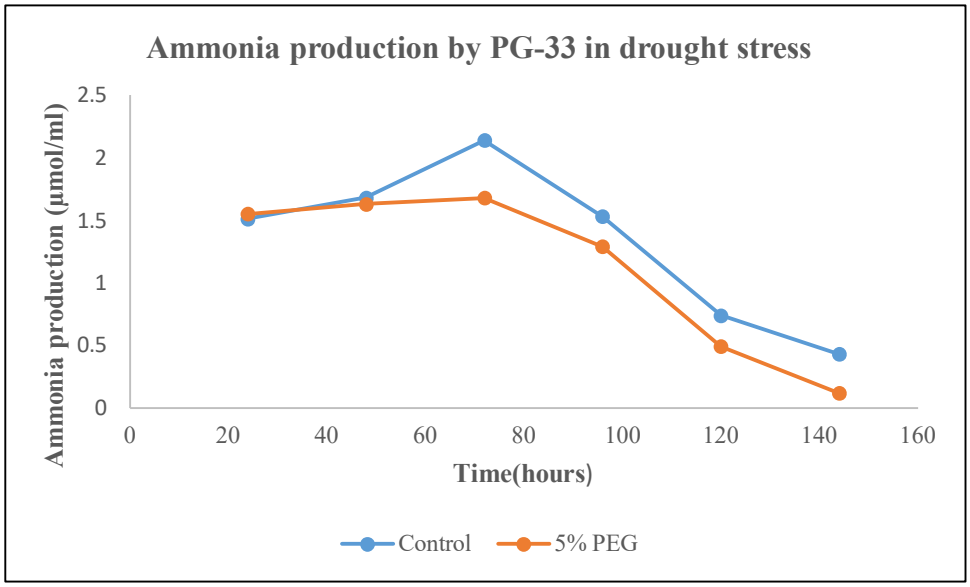

Figure-3: Ammonia production by PG-33 in drought stress along with control

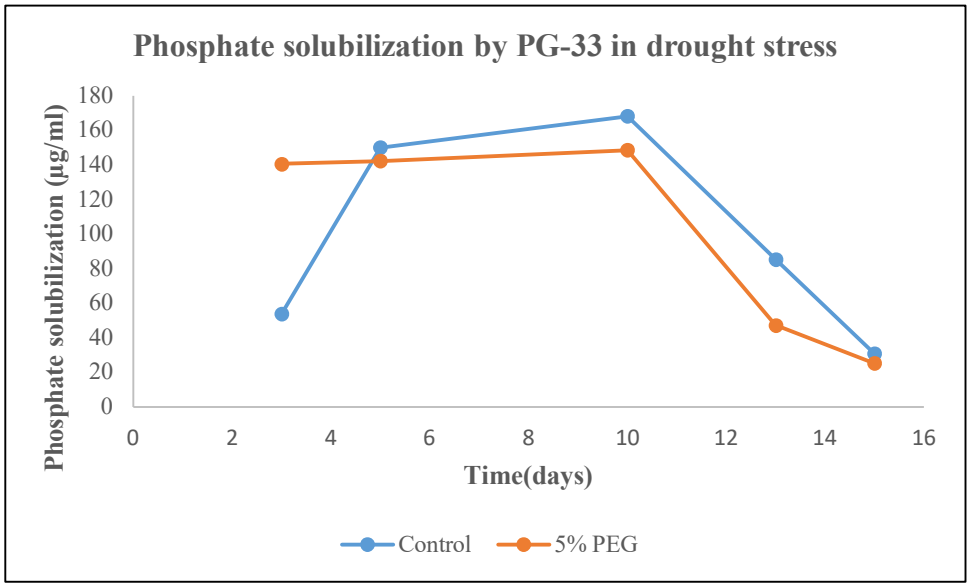

Figure-4: Phosphate solubilization by PG-33 in drought stress along with control

a) Control **b) Treated**

Figure-5: *Sorghum bicolor* seed germination a) Only seeds (distilled water) b) seeds+PEG+PG-33

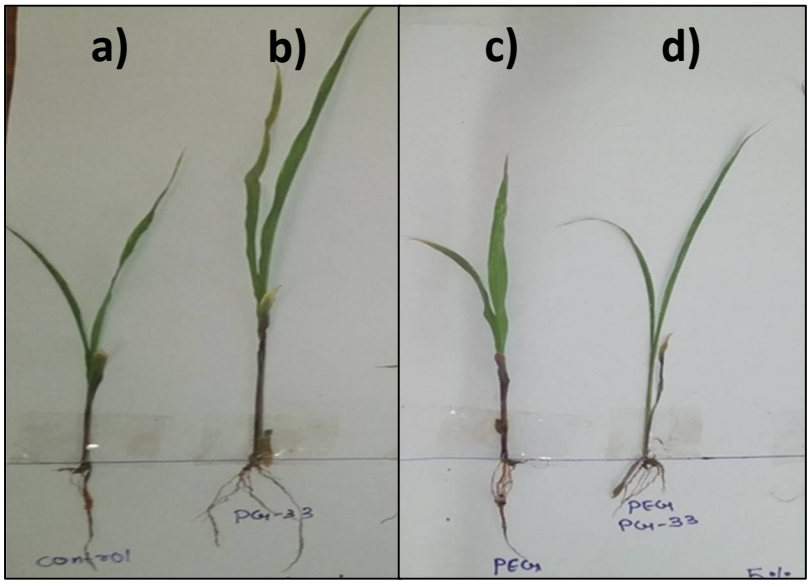

Figure-6: *Sorghum bicolor* plant growth after 15 days a) control (only seeds) b) positive control (seeds+PG-33) c) negative control (seeds+PEG) d) seeds+PEG+PG-33

Table-1: Pot study experiment design

Plant	Pot number	Pot design
Sorghum bicolor	Pot-1	Only seeds
	Pot-2	Seeds+PEG
	Pot-3	Seeds+PG-33
	Pot-4	Seeds+PEG+PG-33

Table-2: The growth of PG-33 in drought stress condition

PEG-6000 (%W/V)	Number of cells	Percent growth (%)
Control	4.97×10^8	100
1%	3.99×10^8	80.0
3%	2.99×10^8	60.1
5%	2.43×10^8	48.9
7%	2.42×10^8	48.7
10%	2.07×10^8	41.6
15%	1.45×10^8	29.2

Table 3: Qualitative analysis for plant growth promoting traits

PGP trait	Result
IAA Production	Positive
GA Production	Positive
NH_3 Production	Positive
EPS Production	Negative
Phosphate solubilization	Positive

Table-4: Seed germination for *Sorghum bicolor*

Treatment	Number of seeds sown	Number of germinated seeds	Germination %	Seedling length (cm)	Seed vigour index
Only seeds(distilled water)	100	100	100	1.5	150.0
Seeds + 5% PEG	100	50	50	0.5	25.00
Seeds+5% PEG + PG-33	100	75	75	1.7	127.5

Table-5: Physico-vegetative parameters of *Sorghum bicolor*

Plant parameters of *Sorghum bicolor* after 15 days	Control	PEG-6000 (Negative control)	PG-33 (Positive Control)	PEG+PG-33
Shoot length (cm)	3.400	3.000	5.000	1.900
Root length (cm)	5.000	4.500	5.500	2.300
Leaf length (cm)	11.50	10.20	18.50	10.00
Fresh weight (gm)	0.162	0.122	0.155	0.134
Dry weight (gm)	0.040	0.024	0.033	0.029

CONCLUSION

The current study indicates the importance of plant growth promoting bacteria isolated from fermented panchagavya. PG-33 helps plants to survive in drought condition because it shows many plant growth promoting activities (IAA production, GA production, ammonia production, phosphate solubilization etc.) even in the presence of drought conditions. The application of PG-33 on plants improved the plant growth with PEG. PG-33 may be used as biofertilizer. This study can be further explored far application of PG-33 in the field study.

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COMPETING INTERESTS

The authors have declared that no competing interest exists.

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