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ORIGINAL ARTICLE

Assessment of Antioxidant Activity and Anticancer Activity of Plant Leaves of *Amaranthus Spinosus*

P. Chidambara Rajan[1], R.Megala[*1], P.Sudhakar
[1] Department of Biotechnology, Dr. N.G.P. Arts and Science College, Coimbatore, Tamilnadu.
[2] Department of chemical engineering Annamalai university, Annamalainagar, Tamilnadu.
Corresponding author email:megu.rbmp@gmail.com

ABSTRACT
The aim of this research paper was to evaluate antioxidant activity and anticancer activity of Amaranthus spinous using the respective standard drug. The antioxidant activity and phenolic composition of the aerial part of Amaranthus spinous were investigated. Based on the experiments, the extract of Amaranthus spinous showed higher activity was identified with their IC_{50} values. Amaranthus spinous extract exhibited a better antioxidant activity against the ascorbic acid used as control in the present study. The results of antioxidant assays also showed significant activity. Similarly, MTT assay also exhibited higher viability percentage.
Keywords: *Amaranthus spinosus, Antioxidant activity, Nanomaterials, Anticancer activity, Medicinal plant, Silver nanoparticles.*

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INTRODUCTION

The numerous both abiotic and biotic variables have a significant impact on agricultural practices all over the world. Farming practices are significantly hampered by a number of these abiotic variables. The growth of plants is influenced by abiotic elements such as temperature, soil salinity, drought stress, and heavy metal stress. The salinity of the soil has emerged as one of the major problems limiting crop productivity globally. Due to anthropogenic activity, the rate of soil salinization has accelerated in recent years (23). Crop productivity is lost, and salt levels are high in areas of soil where there is an excessive input of fertilizer (32). The application of fertilizers with a high salt index has an osmotic influence that makes it challenging to access the water required for plant growth (14). It is also claimed that agricultural techniques have an impact on crop productivity on saline soils. In these circumstances, more salt is accumulated because deeper tilling or plugging of the soil causes more water to evaporate from its surface. Additionally, according to (8), salts in irrigation water may increase the salinity of the soil, making it less able to support plant growth. Most plants in nature are sensitive to salt stress because it may hinder microbial growth in their surrounding habitat and disrupt the normal plant-microbe interaction in soil (1). Salinization has an impact on all phases of plant development, including germination, physiological development, reproductive development, and senescence. Salinity in the soil has a negative effect on the general health of plants. Salinity has an impact on the physicochemical properties of the soil, the ecological balance of the area, and the agricultural production of most crops. Excessive salt consumption reduces leaf size, chlorophyll content, and the efficiency of photosynthetic activities. An essential part of the electron transport chain (ETC), photosystem II (PSII), is suppressed when subjected to salt stress (25,17).The growth and development of ST-PGPB (salt-tolerant plant growth promoting bacteria) may be crucial in rescuing crops from such stressful situations (20). The

natural defence against salinity stress conditions is the presence of microorganisms that promote plant growth (9). Under stressful circumstances, plant-microbe interaction is essential for maintaining soil characteristics, crop productivity, and microbial diversity. The interaction between plants and microorganisms is also crucial for plant growth and development in a dynamic environment. Plant growth-promoting microorganisms (PGPM) are a variety of bacteria that cluster around plant roots and promote the growth and development of the plant when it is exposed to salinity stress (19). According to (30), some halophilic bacteria have been shown to be effective at lowering plant salt stress, which can help to improve the growth and productivity of crops grown in salinity. Numerous studies have shown that bacteria like *Achromobacter, Arthrobacter, Bacillus, Chryseobacterium, Enterobacter, Ochrobactrum,* and *Pseudomonas*, which have been isolated from saline soil, can facilitate plant growth in saline environments (33, 16, 35, 4). Salinity control and the promotion of plant growth and development can both benefit from PGPB-mediated amelioration of salinity stress.The global trend towards organic farming is expanding. Currently, there are 0.7 million organic farms spread across 30.4 million hectares in more than 130 countries. Panchagavya is an organic formulation, which in Sanskrit means the blend of five cow products i.e., milk, ghee, curd, dung, and urine (all these products are individually called as "Gavya" and collectively named as panchagavya). "Panchagavya" has got reference in the scripts of Vedas (devine scripts of Indian wisdom) and Vrikshayurveda (31). They are a plentiful supply of microbial communities, macronutrients, micronutrients, and chemicals that support plant growth, such as immune boosters (21).PG-63 bacterial isolate was obtained from fermented panchagavya. The present study is focused on the characterization of PG-63 bacterial isolate and to check its effect on *Trigonella foenum-graecum* for salt tolerance. PG-63 shows good salt tolerance even at high concentrations of NaCl. In this study, we are aiming to find out the potential roles of PG-63 in salinity stress.

MATERIALS AND METHODS

Isolation of bacterial isolates for fermented panchagavya

64 bacterial cultures were isolated from fermented panchagavya (12). Various plant growth promoting traits like IAA production, GA production, ammonia production, phosphate solubilization, EPS production were checked in salinity stress condition. PG-63 bacterial isolate showed better survival in the presence of NaCl. Results were shown in table-2.

Inoculum preparation

The bacterial cultures from slants were inoculated in sterile nutrient broth and incubated at 30°Cfor 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 production and phosphate solubilization.

The absorbance was measured in spectrophotometer at 600nm after 24 hours. The standard calibration curve of $BaCl_2$ (1.0 O.D ~8×10^8 cells/ml) was prepared to calculate the number of cells.

Salinity stress response of PG-63

The salt tolerance of PG-63 was checked by observing as their growth in the nutrient broth medium supplemented with different NaCl concentrations (1, 3, 5, 7, 10 and 15% w/v). PG-63 was inoculated in sterile nutrient broth and incubated at 28°Cfor 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 (5). 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 (11). 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 (6). 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 (29). 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) *Trigonella foenum-graecum* seeds plus distilled water
2) *Trigonella foenum-graecum* seeds plus NaCl(2%w/v) + PG-63

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. Salt tolerance of the PG-63 was tested at 1% NaCl. 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-63 bacterial isolate was scraped from preserved slant and inoculated in 100 ml sterile nutrient broth, incubated at 30°C, at 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. The pot experiment design is shown in table-1.

RESULTS AND DISCUSSION

PG-63 was studied for its salt tolerance. PG-63 shows better growth in the presence of different NaCl (%w/v) concentrations. PG-63 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. Plant growth promoting activities are helpful to the plants for their growth and survival in salinity stress condition.

Salinity stress tolerance ability

PG-63 bacterial isolate has grown well in nutrient medium with 1.0 to 15.0% (w/v) NaCl (Table-2). It was observed that the number of bacteria decreased with increased NaCl concentrations, but it could survive at higher NaCl concentrations. PG-63 show the best growth (about 50%) up to 5% NaCl concentration. (37) observed that panchagavya isolates, *Bacillus tequilensis,* and *Bacillus aryabhattai* can be employed in higher saline soils due to their capacity to survive at higher salt concentrations (12% w/v).

Plant growth promoting traits

PG-63 was screened for various plant growth promoting activities like IAA production, GA production, NH_3 production, phosphate solubilization in the presence of NaCl concentration. All the plant growth promoting activities were done with 2%w/v NaCl concentration and 1%v/v inoculum in PG-63.

Qualitative estimation of plant growth promoting traits

Qualitative estimation was done for the primary screening of plant growth-promoting traits (Figure-1). A pink colour was observed, which indicates positive IAA production. The wine-red colour indicated positive GA production. Positive ammonia production indicated a dark yellow colour. The blue colour indicated a positive result for phosphate solubilization.

IAA production

Figure-2 shows IAA production of PG-63 at different time intervals with 2%w/v NaCl concentration. In abiotic stress conditions, PGPB produces an appropriate amount of IAA hormone, which affects root development and growth.

PG-63 shows higher production (22.67 µg/ml) after 48 hours in control, while it shows higher production (25.15 µg/ml) after 120 hours in the presence of 2% NaCl concentration. *JPVS11* strain shows IAA activity even in the presence of high salt concentration (22). (7) reported two PGPB isolates producing 12.0 µg/ml and 7.0 µg/ml IAA after 72 hours. (3) also reported a rhizospheric bacteria that was able to produce 55.90 µg/ml IAA. Phytohormonal analysis of *AF7* revealed 36.41 µg/ml IAA production at 2.5%(w/v) NaCl (10).

GA production

Figure-3 shows GA production of PG-63 different time intervals with NaCl concentration, respectively. GA has a crucial role in cell growth and division, root, stem, and leaf expansion, seed germination or dormancy, fruit senescence and flowering.

PG-63 shows higher production (3.38 mg/ml) in control and 2.19 mg/ml in the presence of NaCl after 48 hours. (10) observed GA production in the *AF7* strain (*Alcaligenes* sp.) in the presence of NaCl concentration. (36) reported two bacterial isolates from the rhizosphere which produced 0.48 mg/mL and 0.419 mg/mL gibberellic acid after 72 hours. *Pseudomonas stutzeri* isolated from rhizospheric soil produced 0.03 mg/ml GA after 72 h (28).

NH_3 production

Figure-4 shows ammonia production of PG-63 at different time intervals in the presence of NaCl concentration. 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-63 shows higher production (36.0 µmol/ml) in control and 33.8 µmol/ml in the presence of NaCl after 48 hours. Bacterial strains *P. reactans* and *P. alli* show ammonia production in the presence of different NaCl concentrations (27). *Azotobacter* isolates obtained from commercial biofertilizers reported 4.75 µg/mL ammonia production (38). *Bacillus megaterium* isolated from soil produced 2.04 µmol/ml ammonia after 96 h (18). *Klebsiella pneumoniae* from chickpea rhizosphere 1.00 µmol/ml ammonia after 96 h (24).

Phosphate solubilization

Figure-5 shows phosphate solubilization of PG-63 at different time intervals in the presence of NaCl concentration. The phosphate-solubilizing microorganisms (PSMs) such as phosphate-solubilizing bacteria (PSB) present in most soils which can solubilize insoluble forms of phosphates.

PG-63 shows higher production (64.40 µg/ml) in control after 10 days, while it shows higher production

(70.67 µg/ml) in the presence of NaCl after 3 days. BAM-4, BAM-6, BAM-12, and BAM-77 (soil bacteria) show phosphate solubilization at different NaCl concentrations (15). The *Bacillus cereus* was able to solubilize 159 µg/ml tri-calcium phosphate after 10 days in Pikovskaya's broth (34).

Seed germination test

Table-3 shows seed germination percentage, seedling length (cm) and seed vigour index of *Trigonella foenum-graecum.* One of the most crucial stages of the plant growth cycle is seed germination.

The percentage germination ability of PG-63 was shown in figure-6. *Trigonella foenum-graecum bicolor* seeds were treated with distilled water andshowed 82% seed germination with 106.6 seed vigor index. Seeds treated with NaCl showed 45% seed germination with 4.5 seed vigor index. When seeds treated with NaCl along with PG-63 bacterial culture it showed 90% seed germination with 81 seed vigor index. Alfalfa (*Medicago sativa*) showed better seed germination after application of PGPB (*Rhizobia strain*) in salinity stress (2).

Pot study on Trigonella foenum-graecum

Table-4 shows physico-vegetative parameters of *Trigonella foenum-graecum*.The biological contribution of PG-63 on the growth of *Trigonella foenum-graecum* was investigated in the presence of NaCl 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 (figure-7). It was observed that treated plants had longer roots (12%) and shoots (12.5%) than the untreated control plants (Figure-10).In addition, treated plants had a higher fresh weight and dry weight.

Arachis hypogea that has been exposed to Bacillus sp. PG-8 showed significant plant growth. A potential method for increasing plant growth and decreasing the use of hazardous chemical fertilisers is to inoculate soil or crops with PGPB. Arachis hypogea may have better growth characteristics because of Bacillus sp. PG-8 in the production of auxins, gibberellic acid, and phosphate solubilization, which increases the availability of nutrients (13).

Table-1: Pot study design for *Trigonella foenum-graecum*

Plant	Pot number	Pot design
Trigonella foenum-graecum	Pot-1	Only seeds
	Pot-2	Seeds+NaCl
	Pot-3	Seeds+PG-63
	Pot-4	Seeds+NaCl+PG-63

Table-2: The percentage growth of PG-63 in salinity stress condition

NaCl (%W/V)	Number of cells	Percent growth (%)
Control	94.67×10^8	100
1%	87.29×10^8	92.2
3%	56.51×10^8	59.7
5%	48.50×10^8	51.2
7%	31.31×10^8	33.0
10%	2.400×10^8	2.53
15%	0.150×10^8	0.12

Table-3: Seed germination and seed vigour index

Treatment	Number of seeds sown	Number of germinated seeds	Germination %	Seedling length (cm)	Vigour index
Only seeds(distilled water)	100	82	82	1.3	106.6
Seeds + 2% NaCl	100	45	45	0.1	4.500
Seeds+2% NaCl + PG-63	100	90	90	0.9	81.00

Table-4: Physico-vegetative parameters of *Trigonella foenum-graecum*

Plant parameters of *Trigonella foenum-graecum*after 15 days	Control	NaCl (Negative control)	PG-63 (Positive Control)	NaCl+PG-63
Shoot length (cm)	4.000	2.900	4.500	3.000
Root length (cm)	7.000	5.450	7.900	5.570
Leaf length (cm)	3.230	2.430	3.550	2.430
Fresh weight (gm)	0.200	0.120	0.165	0.134
Dry weight (gm)	0.030	0.010	0.025	0.018

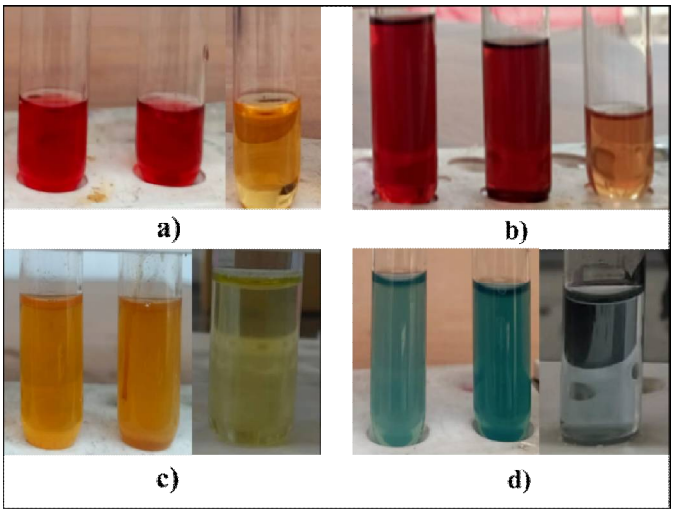


Figure-1: Qualitative estimation of plant growth promoting traits along with blank a) IAA production b) GA production c) Ammonia production d) Phosphate solubilization

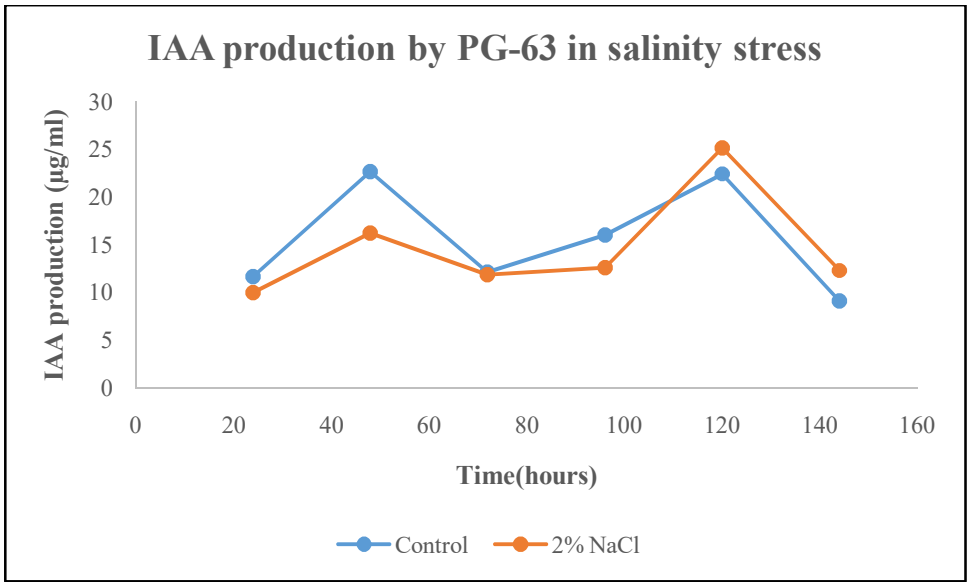


Figure-2: IAA production by PG-63 in salinity stress along with control

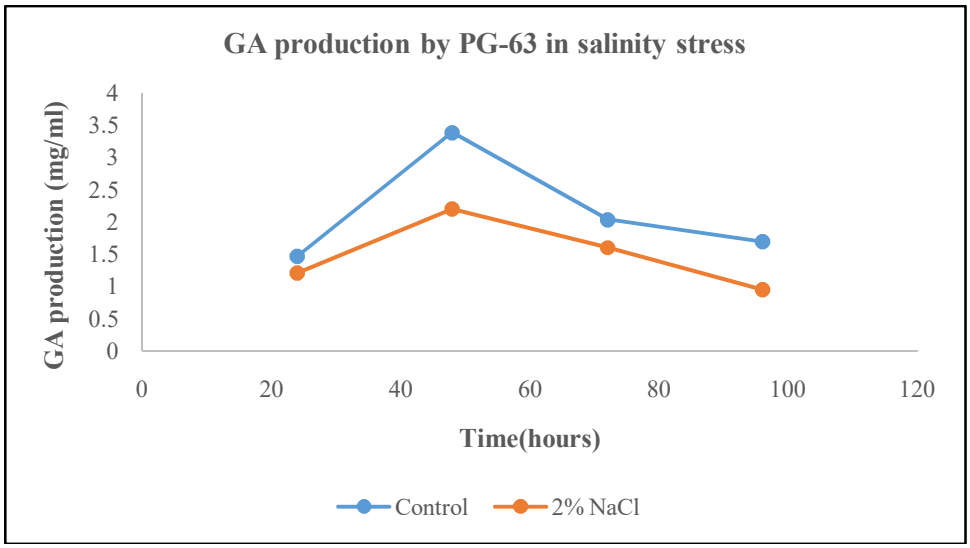


Figure-3: GA production by PG-63 in salinity stress along with control

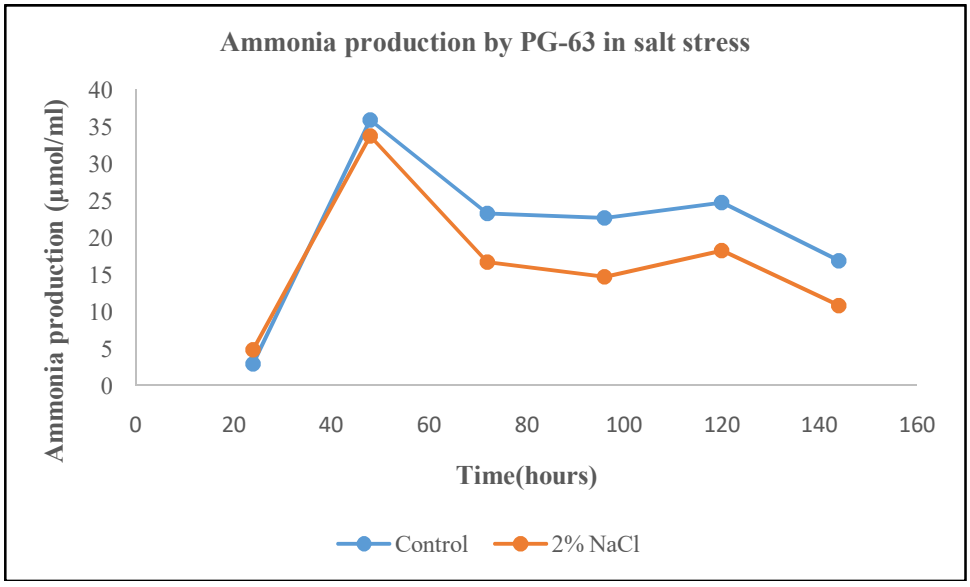


Figure-4: Ammonia production by PG-63 in salinity stress along with control

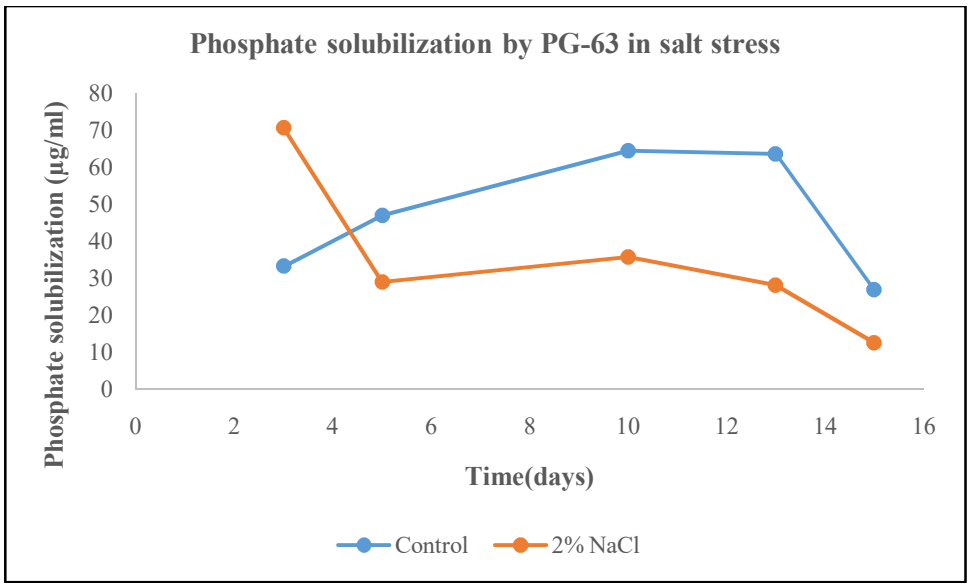
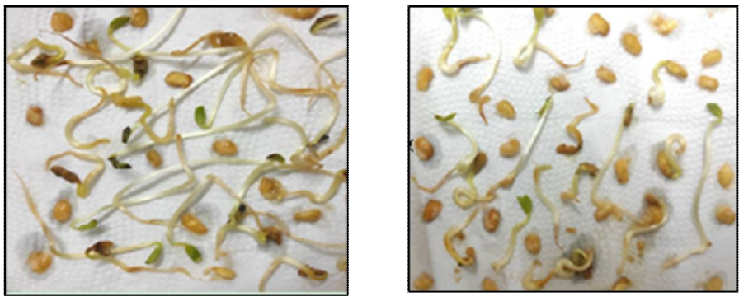


Figure-5: Phosphate solubilization by PG-63 in salinity stress along with control



a) Control **b) Treated**

Figure-6: *Trigonella foenum-graecum* seeds a) Only seeds (distilled water) b) seeds+NaCl+PG-63

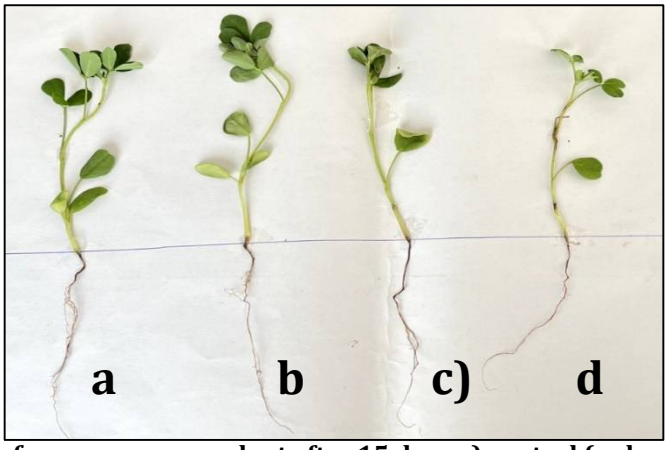


Figure-7: *Trigonella foenum-graecum* plant after 15 days a) control (only seeds) b) positive control (seeds+PG-63) c) negative control (seeds+NaCl) d) seeds+NaCl+PG-63

CONCLUSION

The current study indicates the importance of PG-63 isolated from fermented panchagavya. PG-63 helps plants to survive in adverse condition like salinity stress because it shows many plant growth promoting activities (IAA production, GA production, ammonia production, phosphate solubilization etc.) even in the salinity stress. The application of PG-63 on plants improved the growth with NaCl concentration. This study can be further explored by application of PG-63 in the field study. PG-63 may be used as biofertilizer. PG-63 isolate may have the potential to re-fertile the saline and sodic soils.

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Competing interests

The authors have declared that no competing interest exists.

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