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Effect of Gypsum on Plant Growth Promoting Rhizobacteria

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ABSTRACT

The present study was carried out to check the effect of gypsum on the plant growth promontory rhizobacteria. Ten bacterial isolates were isolated from agricultural field of the Shobhit University Gangoh, under onion cultivation in the year 2018. These bacterial isolates were checked for the plant growth promoting activities like phosphate, zinc solubilization and production of siderophore, hydrogen cyanide and Indole acetic acid. Two bacterial isolates (AP_1 and AP_2), were showed best plant growth properties and further checked for their growth in presence of gypsum which showed enhanced growth pattern in the presence of 20 mg L^{-1} gypsum. Results suggest that the application of gypsum enhances the bacterial growth nutrient broth as well as protein content in presence of gypsum and can be used in bioformulations to increase the shelf life of bioinoculants.

Keywords Gypsum, PGPR, Rhizobacteria, Protein.

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) play a vital role in agriculture production by increasing the nutrients uptake (Gonzalez *et al.* 2015, Chaudhary *et al.* 2021b). Plant growth by PGPR can be enhanced by direct or indirect mechanisms. In direct mechanism, plant growth may be boosted by mechanisms like nitrogen fixation, phosphate and potassium solubilization (Khan *et al.* 2014) and production of substances like Indole acetic acid, 1-amino-cyclopropane-1-carboxylate (ACC). While in indirect mechanism, enhanced plant growth by PGPR may be achieved by decreasing the harmful effects of phytopathogenic microorganism by production of antibiotics or development of systemic resistance in the plant (Kumar *et al.* 2018). There are two main types of PGPR, extracellular PGPR (ePGPR) and intracellular PGPR (iPGPR). Bacteria like *Azotobacter*, *Serratia*, *Bacillus*, *Agrobacterium* belong to the ePGPR class and microbes like All *orhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* belong to iPGPR category. Phosphorus in soil is present in soluble form, so it is not easily absorbed by plants. PGPR helps plant in absorption of

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phosphorus by converting it into soluble forms by mechanisms like mineralization and solubilization (Kumari *et al.* 2021). Examples of microorganisms involved in phosphorus solubilization include *Bacillus*, *Arthrobacter*, *Erwinia*, *Flavobacterium* and *Serratia* (Sharma *et al.* 2013, Otieno *et al.* 2015). Iron is an important micronutrient required by the microbes and being highly insoluble is often a limiting condition in the rhizosphere. Iron binding ligands (siderophores) for iron acquisition to have a competitive advantage over other microorganisms. These siderophores bind to ferric iron in the soil or the root zone and are then taken up using outer membrane receptors (Beneduzi *et al.* 2012). HCN is produced by bacteria such as fluorescent *Pseudomonas*, which has been long known for its beneficial effect on the plants in disease suppression (Sahu *et al.* 2018). Indole acetic acid (IAA) is a natural auxin which is also synthesized in many species of non-seeded plants, many bacteria, fungi and algae. The amino acid tryptophan is commonly regarded as the precursor for the biosynthesis of auxin in plants (Varalakshmi and Malliga 2012). There are various bacterial genera which are involved in IAA production. Bacteria *Pantoea agglomerans* is reported for the production of maximum IAA at pH 7(Apine and Jadhav 2011). *Azolla* could be a consistent provider of tryptophan for IAA producing microbe in its rhizosphere (Raut *et al.* 2017). *Nostoc* and *Anabaena* were also efficient in enhancing the germination and growth of wheat seeds and exhibited significantly high protein accumulation by IAA production (Prasanna *et al.* 2009). Ammonia accumulation is reported to increase pH of soil which helps in maintaining alkaline condition of the soil and inhibit growth of many fungi and Nitrobacter. Ammonia production is important traits which is beneficial for the crops.Gypsum is an essential source for plant nutrients like calcium and sulfur and can improve overall plant growth. Gypsum amendments can also improve the physical properties of some soils (especially heavy clay soils). Such amendments promote soil aggregation and thus can help prevent dispersion of soil particles, increase water infiltration rates and movement through the soil profile (Dontsova *et al.* 2005). Gypsum improves the chemical and physical properties of soil and makes agriculture more sustainable. Nano form of gypsum enhances the growth of *Pseudomonas taiwanensis* and *Pantoea agglomerans* @ 50 ppm concentration (Chaudhary and Sharma 2019). Present study was planned to isolate the best PGPRs from agriculture field and their growth pattern and protein content in the presence of gypsum.

MATERIALS AND METHODS

Collection of soil sample

Soil sample were collected from the agriculture field of Shobhit University, Gangoh, Saharanpur, Uttar Pradesh under onion cultivation in the year 2018.

Bacterial isolation

Soil samples were serially diluted up to 10^4 times and plated on nutrient agar medium. 1 ml of the diluted sample was poured on 20 ml of nutrient agar. Mixed properly in clockwise and anticlockwise directions. Then inoculated plates were incubated at 30^0C for 24 h. Ten colonies with different morphology were selected and purified on nutrient agar.

Plant growth properties of bacterial isolates

Bacterial isolates were screened qualitatively for solubilization of phosphorus and zinc, Indole acetic acid, siderophore, Hydrogen Cyanide and ammonia production.

Phosphate solubilization

Bacterial cultures were spot inoculated on Pikovskaya medium (HI media) and incubated for 4 to 6 days at 28^0C. Formation of halo zone around bacterial colony indicates phosphate solubilization by the bacteria (Pikovskaya 1948).

Zinc solubilization

Screening of Zn solubilizing bacteria was done on the basal medium supplemented with 0.1% ZnO by the method of (Saravanan *et al.* 2004). Recovered bacteria were tested for their Zn solubilizing potential based on halo zone formation around the bacterial colonies.

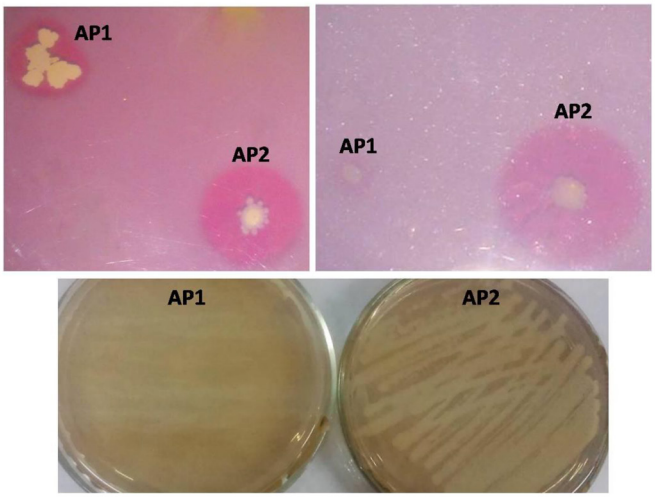

Fig. 1. PGPR properties of AP_1 and AP_2 (A) Phosphate solubilization (B) Zinc solubilization (C) HCN production.

HCN production

HCN production by bacterial isolates were streaked King's B agar medium supplemented with glycine. Sterile filter paper was soaked in picric acid solution and placed on the upper lid of the Petri plate. Plates were sealed with parafilm and incubated at 28°C for 48 h. Change in color of the filter paper from yellow to brown indicates HCN production (Bakker and Schippers 1987).

Ammonia production

Actively growing bacterial cultures were inoculated in 10 ml Peptone water and incubated for 72 h at 27°C in a rotatory shaker at 100 rpm. Production of ammonia was tested by adding Nessler's reagent (1ml) to the bacterial culture after 4 days of incubation (Cappuccino and Sherman 1992). Presence of yellow to brown color indicates production of ammonia.

Indole acetic acid production

Test bacterial cultures were inoculated in 5 ml of sodium succinate broth, supplemented with 100 µg /ml tryptophan (Gordon and Weber 1951). After incubation at 28±1°C for 48 h, broth was centrifuged for 10 min at 10,000 rpm. After centrifugation, one ml culture supernatant was mixed with Salkovaski reagent (2 ml) and incubated at 30°C for 25 min in dark to observe color change. Development of pink color indicates a positive test for IAA production by the test bacteria. Optical density of the colored mixture was recorded at 530 nm by using visible spectrophotometer.

Siderophore production

Production of siderophore in bacterial isolates were

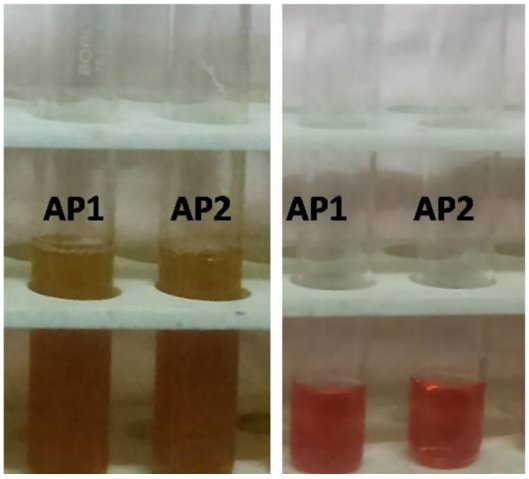

Fig. 2. Ammonia and IAA production by bacterial isolates AP_1 and AP_2.

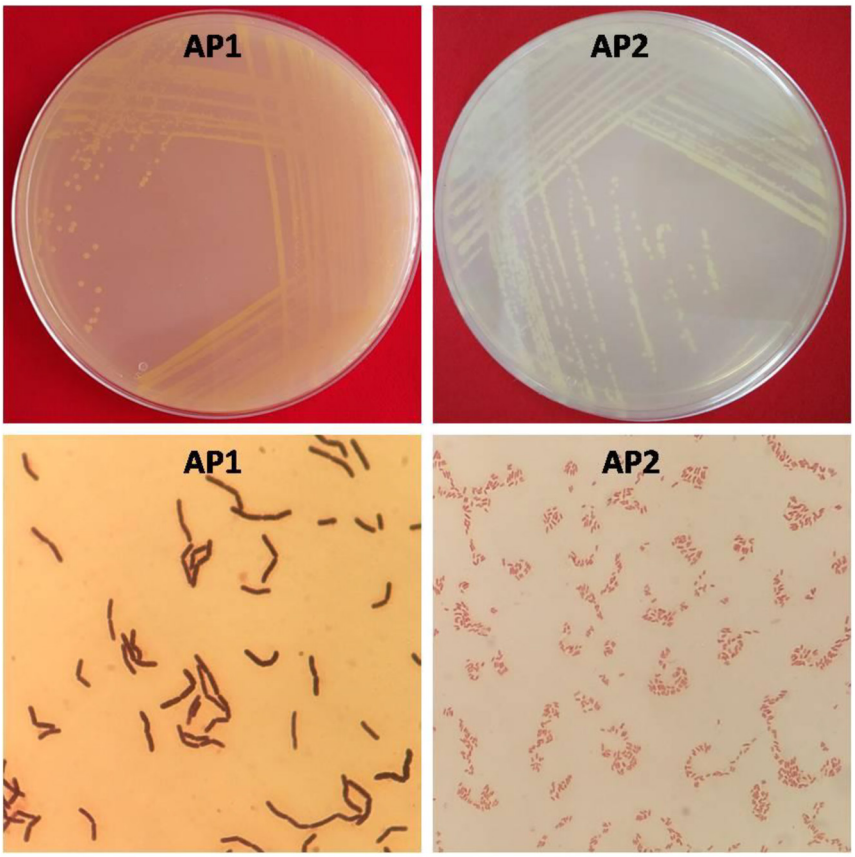

Fig. 3. Culture morphology and gram staining of AP_1 and AP_2.

examined by using the method of (Schwyn and Neilands 1987). Bacterial cultures were spot inoculated on CAS agar and then incubated at 30^0C for 60 to 72 h. Formation of orange or yellow halo zone around the bacterial colony indicates a positive test for siderophore production.

Effect of gypsum on the growth of bacterial isolates

Effect of gypsum (20 mg L^{-1}) was observed on the growth pattern of AP_1 and AP_2. Different treatments used are as follows.Blank (without bacterial culture and gypsum), AP_1 (only bacterial culture), AP_2 (bacterial culture), AP_1+G (bacterial culture with gypsum), AP_2+G (bacterial culture with gypsum).Stock solution of gypsum was sonicated at 20 KHz for 5–10 min. Aliquot from the stock solution of gypsum was added to nutrient broth. Broth was autoclaved at 15 lb psi for 20 min. 20 µl of the active bacterial culture was inoculated into 50 ml of sterile nutrient broth with or without gypsum. Aliquots of 3 ml were regularly with drawn at an interval of 0, 24, 36, 48 and 72 h, taking the absorbance at 600 nm under visible spectrophotometer.

Extraction of protein and their quantification

For this bacterial pellets were washed with Tris-Cl pH (6.8), centrifuged the pellets then suspended in extraction buffer (400 µl) and placed in water bath for 10 min. Protein samples were centrifuged and stored at -4^0C. Absorbance was taken at 595 nm to quantify the protein by using BSA as a standard (Bradford 1976).

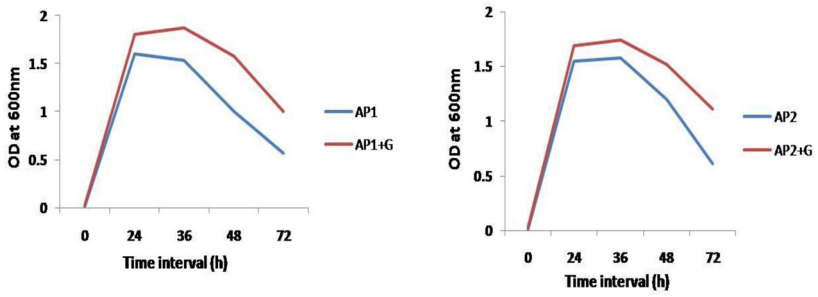

Fig. 4. Effect of gypsum on the growth of AP_1 and AP_2.

Statistical analysis

Analysis of variance (ANOVA) was done with statistical software SPSS Statistics (version 19.0). All the experiments were conducted in triplicate. Results are considered statistically significant at 95% confidence interval ($p < 0.05$).

RESULTS

Phosphate solubilization

Six bacterial isolates solubilized phosphate on Pikovaskaya medium. AP_1 and AP_2 showed maximum P solubilization. Four isolates did not solubilize phosphate compounds (Fig. 1).

Zinc solubilization

Out of ten bacterial isolates, three isolates did not show zone of clearance on basal medium supplemented with ZnO. AP1 and AP_2 showed maximum solubilization (Fig.1).

HCN production

HCN is produced by many rhizobacteria and helps in control of phytopathogens. Only two isolates were showed ($AP_1$and AP_2) HCN production (Fig. 1).

Ammonia production

All the bacterial isolates showed positive response for ammonia production. AP_1 and AP_2 gave best results for ammonia production (Fig. 2).

IAA production

All the ten bacterial cultures showed IAA production. Two isolates (AP1 and AP2) produced maximum Indole acetic acid. Eight isolates gave intermediate results (Fig. 2).

Siderophore production

Out of ten bacterial isolates, AP_1 and AP_2 showed maximum siderophore production. Two isolates showed moderate production range, six isolates showed minimum production of siderophore on CAS medium (Fig.1C).

Culture identification

On the basis of best PGPR properties AP_1 and AP_2 were selected for further studies. AP_1 was gram positive and long rods while AP_2 was gram negative and had short rods (Fig. 3).

Effect of gypsum on the growth of bacterial isolates

Both bacterial isolates AP_1 and AP_2 increased the growth in presence of gypsum 20 mg L^{-1} concentration. Optical density of bacterial isolates taken at 600 nm and supported positive response of gypsum on bacterial growth (Fig. 5).

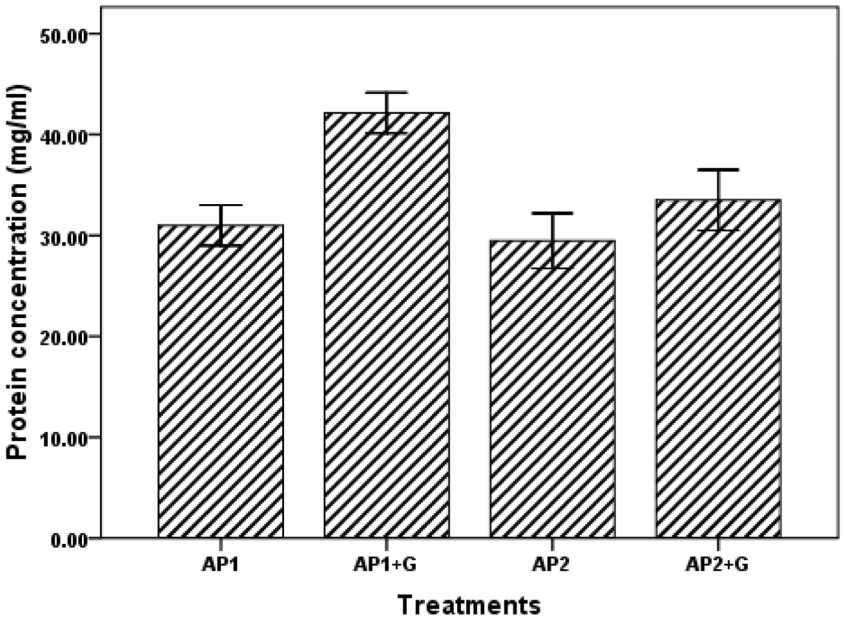

Fig. 5. Protein content in bacterial isolates in presence of gypsum.

Effect of gypsum on bacterial protein concentration

Expression of proteins were increased in presence of gypsum in bacterial cultures. Maximum protein content was observed in AP_1+G (42.13 mg/ml) followed by AP_2+G (33.50mg/mL) (Fig. 5).

DISCUSSION

PGPRs are the soil microbes play an important role in agricultural field to improve plant growth, productivity and soil health (Khati *et al.* 2017, Chaudhary *et al.* 2021a). Application of different bacterial groups have the ability to induce growth promotion on diverse crop production (Nieto-Jacobo *et al.* 2017, Chaudhary *et al.* 2021c). Various plant and fungal species such as *Bacillus, Pseudomonas*, *Rhizobium* and *Aspergillus, Alternaria* and *Trichoderma* can be used as PGPR (Khati *et al.* 2018; Kumari *et al.* 2020). In this study two bacterial isolates AP_1 and AP_2 isolated from agriculture field showed best PGPR properties.

Phosphorus is a major plant growth limiting nutrient. It is a structural component of phosphoproteins and phospholipids involved in photosynthesis (Anand *et al.* 2016). It helps in root elongation and proliferation. Phosphate solubilization by PGPRs occurs by lowering the pH by the production of low molecular weight organic acids, liberation of extracellular enzymes like acid phosphatase and the release of P during substrate degradation (McGill and Cole 1981, Khan *et al.* 2014). *Pseudomonas putida*, *Bacillus* sp., *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Paenibacillus polymxa* were reported to show solubilization of phosphate compounds by production of organic acids (Agri *et al*. 2021 ; Khati *et al.* 2019a). AP_1 and AP_2 showed maximum phosphate solubilization.

Zinc is an essential micronutrient which is required for plant growth. Zinc compounds in the soil present in unavailable form which are solubilized by the zinc solubilizing bacteria. These microbes convert applied inorganic zinc to available forms (Kamran *et al.* 2017). There are various zinc solubilizing bacterial species like *Bacillus* sp., *Pseudomonas* sp., *Gluconacetobacter* sp. and *Acinetobacter* sp. etc.

have been reported by (Vidyashree 2016). In this study seven bacterial isolates were positive for zinc solubilization, maximum was solubilized by AP_2.

HCN is a secondary metabolite and HCN producing bacteria have antagonistic property against the fungal pathogens and has biocontrol activity (Audrain *et al.* 2015). (Kumar *et al*. 2014) reported that HCN producing organism inhibited the sclerotia of *Macrophomina phaseolina.* In our study, HCN production was found in lesser extent only 2 isolates exhibited HCN production. Ammonia production also plays an important role for plant growth. Ammonia is ubiquitous in nature and acts as a substrate to produce nitrate (Amoo and Babalola 2017). Ammonia producing bacterial species makes it available to plants. Different bacteria are reported which are good ammonia producers like *Bacillus* sp., *Pseudomonas fluorescens*, *Rhizobium* and *Azotobacter* sp. (Mishra *et al.* 2010; Khati *et al.* 2019b). All the 10 bacterial isolates were found positive for NH_3 production.

Phytohormones play an important role as signals and regulators of growthand development in plants(Marques et al. 2010). Auxins, among them in particular, indole-3-acetic acid (IAA), are the most studied plant growth regulatorsand this includes physiological, biochemical and genetic aspects(Kukreti *et al.* 2020; Agri *et al.* 2021).Among all the isolates AP_1 and AP_2 showed higher IAA production 20.13 and 23.26 µg/ml respectively. *Azotobacter vinelandii*, *Azotobacter brasilense* and *Rhizobium* also reported for IAA production found that *Mesorhizobium loti* produced 24 µg/ml IAA.

Iron is an important micronutrient required by most of the living systems including microbes. Some bacteria promote plant growth by producing low density iron chelator biomolecules called siderophore which act as chelating agents for iron. A potent siderophore such as ferric siderophorecomplex, plays an important role in iron uptake by plants (Beneduzi *et al.* 2012). Several bacterial isolates were reported to facilitate siderophore production like *Bacillus megaterium*, *Bacillus cereus* and *Azotobacter* which promotes growth of plants and suppress disease (Shilev 2013). In the present study, out of ten bacterial cultures 8 isolates were found positive for siderophore production.

Bacterial cultures AP_1 and AP_2 showed enhanced growth in the presence gypsum when added @ 20 m L^{-1} in nutrient broth. The observation shows an interaction between gypsum and bacteria, which strongly supports the viability of PGPR isolates. Application of nanogypsum increases the beneficial population in the roots of different plants (Chaudhary *et al.* 2021d). It was concluded that the gypsum supports the growth of bacterial isolates and does not have any toxic effect on bacterial cultures. Effect of gypsum was checked on the protein content of the bacterial isolates. It was found that protein content was high in both bacterial isolates when treated with gypsum. This suggests that gypsum helps in growth and protein production which helps in sustenance.

CONCLUSION

In the present study, two bacteria isolated from the agriculture field showed best PGPRs activities i.e., AP_1 and AP_2 respectively and also enhance in the growth pattern in presence of gypsum. It can be concluded that application of gypsum at 20 mg L^{-1} concentrations does not cause any harmful effect on bacterial growth. Hence these bacterial isolates can be used as in different crops for enhanced plant, soil health and productivity for managing the nutrients deficiency in agriculture soils.

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