Synergistic Roles of Zinc Oxide Nanoparticle and Plant Growth Promoting Rhizobacteria on the Growth of Groundnut (*Arachis hypogaea L.*)

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Abstract

A comprehensive study was conducted to explore the plant growth-promoting potential of soil microbes and the synergistic effects of nanoparticles on plant growth. A total of 84 PGPR isolates were obtained from soil samples in Saurashtra, Gujarat, India, with three strains—*Pseudomonas songnenensis, Bacillus haynesii*, and *Priestia megaterium*—selected for further research due to their positive results in promoting plant growth under in vitro conditions. The efficiency of PGPR was studied with the co-application of zinc oxide (ZnO) nanoparticles. ZnO NPs were produced via the sol-gel process and characterized through UV-visible spectroscopy, X-ray diffraction (XRD), and transmission electron microscopy (TEM). The experimental setup was aimed to study the effect of three effective microbial strains with an optimal concentration of ZnO nanoparticles (400 ppm) on the growth of groundnut plants. The co-application of PGPR and ZnO NP displayed a significant increase in the physic-chemical characteristics of the plants. The cumulative effect of isolate RGKP3+ZnO NPs treatment showed maximum carotenoid content (~ 77.4 µg/g), while chlorophyll content was 24.80 mg/g. The present study emphasized the potential of co-application of PGPR and ZnO nanoparticles for sustainable agriculture and improving crop yields in eco-friendly manner.

Keywords: PGP traits, PGPR, ZnO nanoparticles, PGPR nanoparticles

INTRODUCTION

Recent global challenges, including limited productive land, decreased soil fertility, and environmental issues, need to be approached with innovative, sustainable, and engaging strategies to enhance agricultural productivity. By 2050, the population is projected to reach over 10 billion people, demanding the development of novel approaches to ensure food and nutritional security for the next generation [1]. Among the crops that contribute significantly to these future demands, groundnut has a strategic position due to its nutritional and economic importance.

Groundnut is a crucial oilseed crop that has a significant role in the agricultural landscape of India, and assists the economy of the country, specifically in Gujarat. In

the year 2018-19, Gujarat produced nearly 8.5 million metric tons of groundnuts [2]. However, groundnut production declined to about 6.2 million metric tons by the year 2021-22. Additionally, the groundnut cultivation land has also decreased from 2.5 million hectares in 2018 to nearly 1.8 million hectares in 2022, resulting in a decreased average yield per acre from 3.4 tons in 2019 to 3.0 tons in 2022 [3].

The declining productivity is often compensated with the excessive employment of chemical fertilizers to improve and maximize the yield. However, it has numerous detrimental effects, including loss of organic matter, nitrogen leaching, soil compaction, and soil degradation [4]. Furthermore, these fertilizers also jeopardize soil health and endanger both human and environmental health. Hence, investigating alternative agricultural methods is crucial to sustaining the high yield of groundnut crops and advancing sustainability [5,6]. Furthermore, groundnut growth, development and yield are highly influenced by soil nutrient availability and rhizosphere interactions.

Plant growth-promoting rhizobacteria (PGPR) and nanomaterials are one of the potential approaches to sustainable agriculture as they are advantageous for plant development, nutrient availability, and environmental preservation [7]. PGPRs have been extensively studied for crop improvement in agricultural systems and have a remarkable future in sustainable crop production. Several PGPR genera have been reported to enhance the root growth and crop yields, such as *Bacillus*, *Serratia*, *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, and *Enterobacter*. Microbial inoculant along with nanomaterial is the emerging strategy for sustainable agriculture [8].

The promising applications of nanomaterials in agriculture includes improvement in photosynthesis and nutrient uptake, increased adaptability to stressors, and decreased reliance on chemical fertilizers and pesticides [9-10]. Furthermore, the employment of nanoparticles enables faster uptake by the plants than conventional methods [11]. ZnO nanoparticles are promising nanomaterials in agriculture as they play a crucial role in enzyme activation, stress tolerance, and zinc supplementation. In the present study, the combination of rhizobacteria and zinc oxide nanoparticles was investigated to determine their capabilities in enhancing the growth and productivity of groundnut plants. The study was designed to determine the impact of individual and combined treatments of microbial inoculants or ZnO NPs on enhancing the physiological, biochemical, and morphological attributes of groundnut plants under pot conditions.

MATERIALS AND METHODS

Isolation of Rhizobacteria

Rhizospheric soil samples were obtained from four different agriculture fields of Kotdapitha, Virnagar, District Rajkot, Kalawad, District Jamnagar and Garani, District Amreli, from the Saurashtra, Gujarat. The plants were uprooted and stored in sterile sample bags and the soil samples were stored at 4ºC for further examination. Root inhabiting bacteria were isolated on nutrient agar plants by serial dilution method and after confirming pureness, isolates were preserved in the refrigerator for further studies.

PGP Traits

The bacterial isolates were screened qualitatively and quantitatively to evaluate their plant growth promoting (PGP) traits. Indole-3-acetic acid (IAA) production was estimated using Salkowski colorimetric assay as described by Gordon and Weber, et al., (1951) [12]. Ammonia (-NH_3) production was assessed following the method of Cappuccino and Sherman (1996) [13]. The qualitative estimation of HCN production was performed using protocol described by Sherawat et al., (2022) [14], while the quantitative HCN production was estimated using the method described by Alstrom et al., (1989) [15]. Gibberellin production was determined with the Folin-Ciocalteu reagent [16]. The chlorostannous modified molybdophosphoric acid blue technique was employed to quantify the amount of phosphate released (Jackson, 1973) [17]. The potassium solubilization ability of all isolates was evaluated using the Aleksandrow agar medium, which was modified with the addition of bromothymol blue [18]. Colloidal chitin was prepared following a modified method reported in literature to assess chitinase activity [19]. Free nitrogen fixation ability of all the rhizobacterial strains were analyzed by using the method described by Jensen [20]. All the isolates were studied for their zinc solubilization efficacy by inoculation onto zinc containing media. Finally, the CAS test was used to screen bacterial strains for qualitative siderophore production (Schwyn and Neilands, 1987) [21].

Identification of Potent PGPR strain (RG8, RG12 & RGKP3)

The primary and secondary screening yielded only three isolates exhibiting all PGP traits positive were identified. The most promising PGPR strains (RG8, RG12, and RGKP3) were selected for further molecular characterization.

Molecular identification of potent PGPR isolates by 16s rRNA sequencing

The DNA from RG8, RG12, and RGKP3 overnight cultures was extracted, amplified, purified, and sequenced as part of the process. The isolated DNA was quantified, and the gene fragment was amplified using PCR. The resultant sequences were analyzed against existing databases using the technique supplied by the supplier (SLS Research Private Limited Lab, Suart). The results, which have been published in the NCBI GenBank database.

Growth Curve of the bacterial isolates

Nutrient broth (NB) was prepared, sterilized by autoclaving, and subsequently inoculated with the bacterial strain under aseptic conditions. The inoculated culture was incubated in a shaking incubator at the appropriate temperature to ensure optimal bacterial growth. At predetermined time intervals, aliquots were withdrawn and the optical density (OD) of the culture was measured at 600 nm using a spectrophotometer. Measurements were continued until the culture reached the stationary phase of growth.

Chemical synthesis and characterization of ZnO Nanoparticles

The zinc oxide nanoparticles were synthesized using the sol-gel method [22]. Briefly, 100 mL of zinc acetate (0.002 M) was heated to 100^{o}C and trisodium citrate (0.1 M) and sodium hydroxide (2 N) was added to the solution at a constant flow rate. The change of coloration of the solution from colorless to white indicated formation of zinc oxide nanoparticles. The solution was centrifuged at 8000 rpm for 10 minutes and the pellet was collected in fresh Eppendorf tubes. The chemically synthesized ZnO NPs were characterized using a UV-visible spectrophotometer (Shimadzu UV-1800), X-ray

diffraction and a transmission electron microscope (TEM, JEOL JEM 2100 TEM HR LaB6 version) at Central University of Gujarat (Gandhinagar).

Pot experiment

Seed treatment

The Nutrient broth medium was inoculated with the bacterium *Pseudomonas songnenensis, Bacillus haynesii, and Priestia megaterium,* and growth was assessed by the optical density (OD) at 660 nm of about 0.6. ZnO nanoparticles and bacteria were used to coat the groundnut seedlings. Three seedlings per pot were placed in pots with an autoclaved soil and sand combination (3:1), and each received 1 mL of bacterium inoculum. The individual and combined treatments of PGPR and ZnO NPs were carried out accordingly: control = untreated plant, only 400ppm NPs, Zinc salt, only RG8*, (Pseudomonas songnenensis)*, RG8 with 400 ppm, RG12 *(Bacillus haynesii)*, RG12 with 400 ppm, Only RGKP3 (*Priestia megaterium)*, RGKP3 with 400 ppm concentration of ZnO NPs [23]. For each treatment, three replicates (each pot containing three seedlings) were used.

Physical and Biochemical Parameters

Physical parameters

After 30 days, the plants were harvested, and their dry and fresh weights were measured. The physical growth metrics were also recorded, including the number of leaves, branches, roots, and the lengths of both shoots and roots [24].

Biochemical parameters

The assessment focuses on measuring key biochemical components, including proline, total sugar, protein content, flavonoid content, and the levels of carotenoids and chlorophyll. The Arnon method employs the measurement of optical density at three distinct wavelengths: 645 nm for chlorophyll a, 663 nm for chlorophyll b, and 480 nm for carotenoids [25]. The content of flavonoids was determined following the method of Zhishen et al. (1999) [26]. A blank was used to measure the absorbance at 430 nm. Using the Bates method, the proline content of leaves was calculated [27]. The reducing sugars were measured by Dubois method [28]. Bradford's method was used to determine the protein content of the leaves. Bovine serum albumin (BSA) was used in a standard curve with concentrations ranging from 20 to 100 mg/g [29].

RESULTS AND DISCUSSION

Isolation, characterization and Identification of PGPR

A total of 84 morphologically diverse isolates were obtained from the rhizospheric soil samples from groundnut. All the isolates were screened for plant growth-promoting (PGP) traits such as IAA, ammonia, HCN, gibberellins production, and phosphate solubilization (Table 1). Among them, three promising isolates RG8, RG12 and RGKP3 demonstrated significant *in vitro* plant growth-promoting traits.

Table 1: Quantitative Analysis of Plant Growth-Promoting Traits Produced by Bacterial Isolates- RG8, RG12, and RGKP3.

PGP Trait	RG8	RG12	RGKP3
IAA (µg/mL)	31.2	21.5	42.8

Ammonia production (µg/mL)	30.2	24.5	32.4
HCN production (µg/mL)	22.9	42.45	101.08
Phosphate solubilization (Day 7) (µg/mL)	125	204.9	129.5
Gibberellic acid (GA3) (µg/mL)	30.2	24.5	80.09

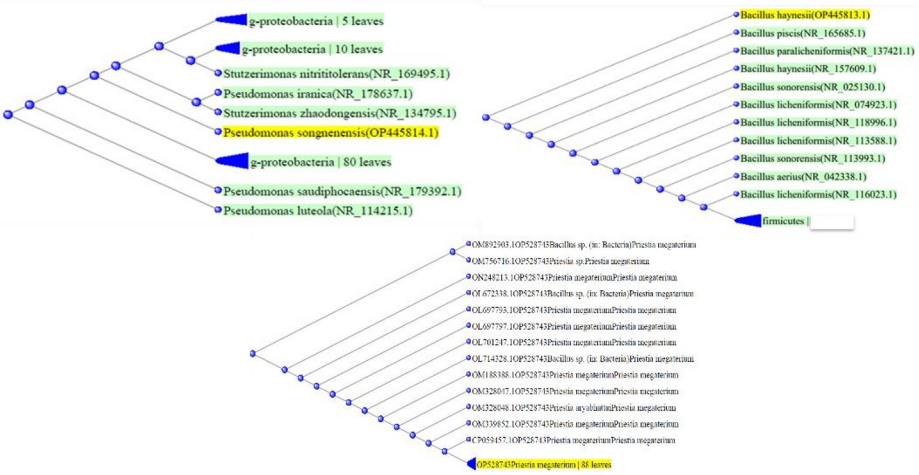

Fig. 1. Phylogenetic tree showing the evolutionary relationship of isolate RG8, RG12 and RGKP3 with reference strain from GenBank database.

The strains were selected for molecular characterization and identification. Further the amplification and sequencing of the 16S rDNA gene sequences, was performed. The obtained sequences were submitted to NCBI and, together with other relevant information, their accession numbers were provided (Fig. 1). According to molecular assessments, RG8, RG12 and RGKP 3 strains were identified as *Pseudomonas songnenensis, Bacillus haynesii, and Priestia megaterium* with accession numbers OP445814, OP445813, and OP528743 respectively (Fig. 1).

Synthesis and Characterization of ZnO NPs

ZnO nanoparticles were effectively synthesized using the sol-gel technique, and their formation and dispersion have been thoroughly characterized. The synthesized nanoparticles were characterized using SEM (Fig. 2a), HR-TEM (Fig. 2b), and XRD (Fig. 2c) for comprehensive understanding of the characteristics of the synthesized ZnO NPs.

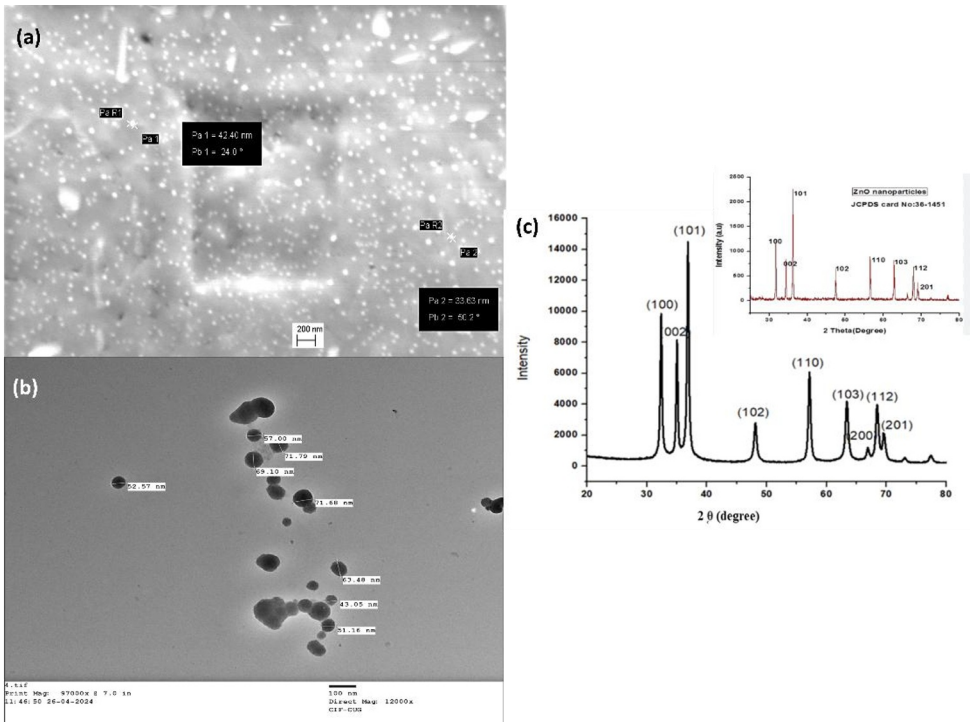

Fig. 2. (a) SEM micrograph of ZnO NPs (white dots represent nanoparticles); (b) TEM micrograph of ZnO NPs with an average diameter of 50-70 nm; (c) XRD analysis of chemically synthesized zinc oxide nanoparticles at 400 ppm concentration Reference with JCPDS NO. 36-1451

Furthermore, zinc oxide nanoparticles at 400 ppm increased plant growth and development. SEM analysis of ZnO nanoparticles (NPs) showed well-dispersed particles with sizes of ~33 nm and 42 nm and angular orientations of 71.6° and 39.8°, indicating possible structural alignment. No significant aggregation was observed, and the 300 nm scale confirmed their nanoscale size. TEM analysis revealed spherical NPs (43–72 nm) with slight agglomeration. The agglomeration visible in the TEM micrograph could be due to Van der Waals forces and solvent evaporation during the preparation of the samples.

Further, experimental data suggested that *Bacillus haynesii, Pseudomonas songnenensis*, and *Priestia megaterium* were not notably inhibited by 400 ppm ZnO nanoparticles (NPs). All strains exhibited typical S-shaped growth patterns with steady population increases, Figure 3 indicating that ZnO NPs at this concentration are non-toxic and do not suppress microbial proliferation.

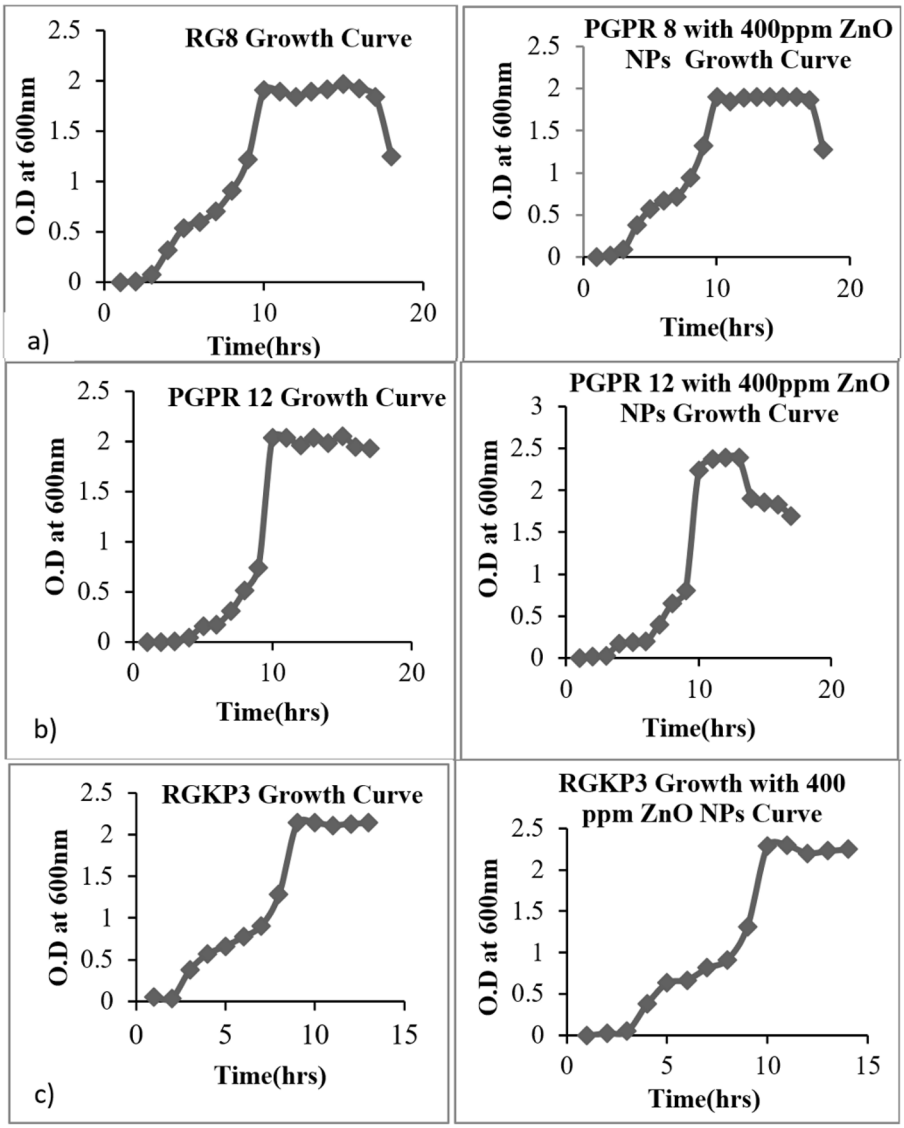

Fig. 3. Bacterial growth curve of (a) Only RG8 and RG8 with 400 ppm ZnO NPs; (b) Only RG12 and RG12 with 400 ppm ZnO NPs; (c) Only RGKP3 and RGKP3 with 400 ppm ZnO NPs

Pot experiment

Physical parameters

The physical characteristics are examined. PGPR with 400 ppm ZnO NPs resulted in more roots, leaves, and root length compared to the control set. Plants having only zinc salt, only PGPR, and only NPs likewise exhibited lower physical parameters.

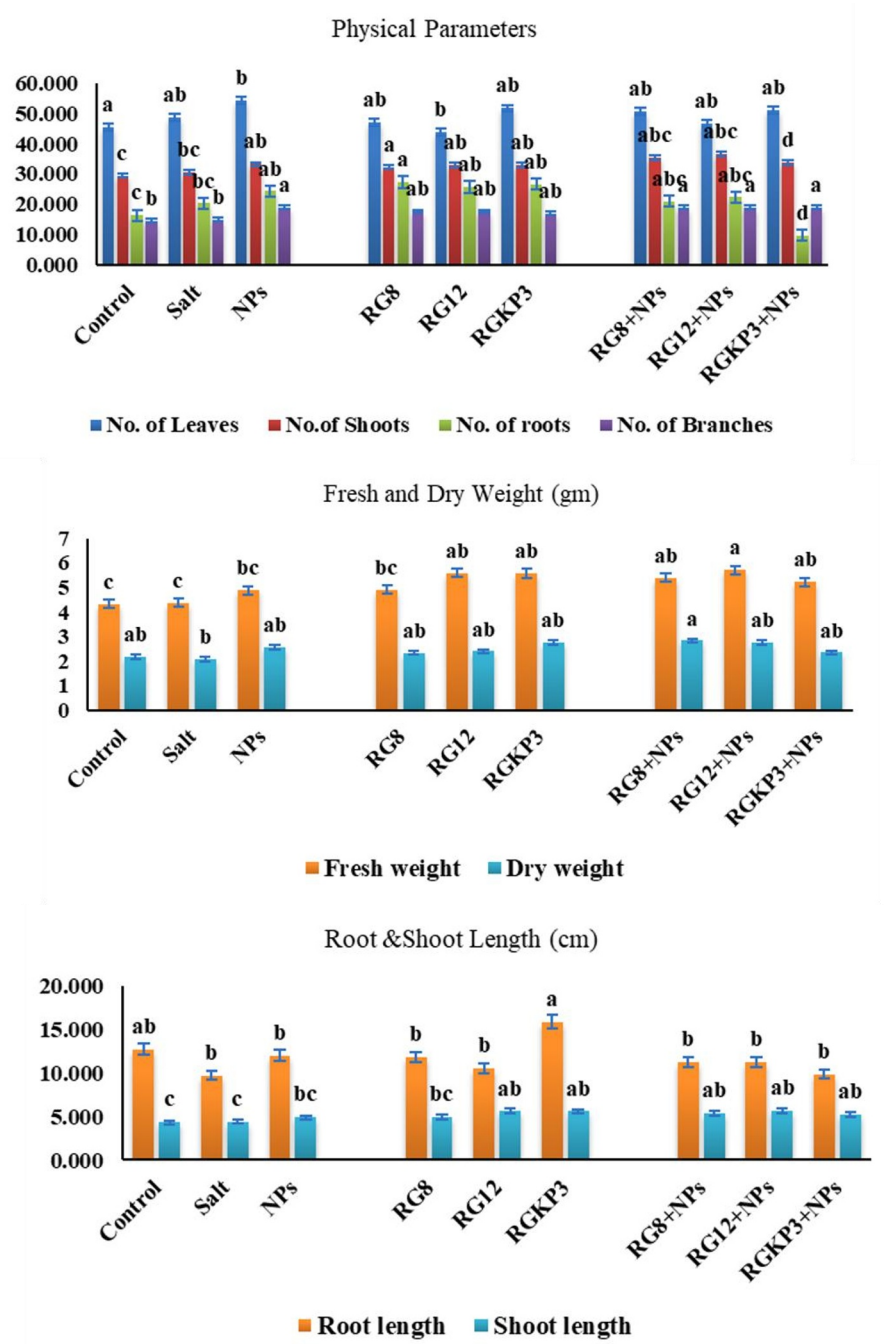

Fig. 4. Plants with different treatments in pot experiment after 1 month; Physical parameters number of leaves, roots, branches, and shoots of plants; fresh weight and dry weight; and root length and shoot length with combined PGPR and ZnO NPs, Only PGPRs, Only NPs, zinc salt and control (untreated)

Biochemical parameters

The assessment of biochemical parameters across all treated plants revealed that the combination of PGPR and ZnO NPs leads to a notable increase in the production of these parameters. The combined effect of PGPR and ZnO NPs resulted in highest growth parameters, increment in the shoot length, root length, fresh weight, and dry weight, indicating synergetic effect of co-application of zinc oxide nanoparticles and PGPRs compared to the control and only salt treatments (Fig. 4 & 5).

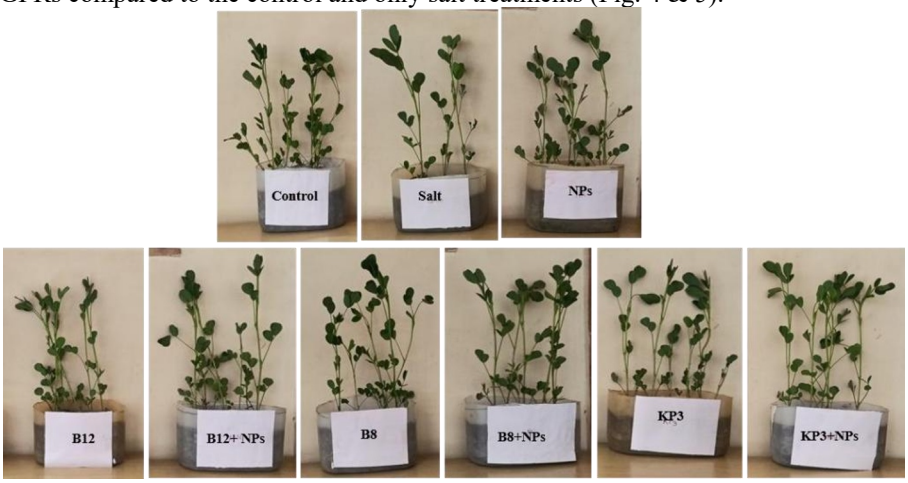

Fig. 5. Groundnut plants with different treatments in pot experiment after 1 month of in vitro experiment with combined application of three potent PGPR (RG8, RG12 and RGKP3) strain with ZnO NPs

Furthermore, the biochemical parameters were also evaluated with different treatment conditions and it was observed that chlorophyll a, b, and total chlorophyll of plants with PGPR + ZnO NPs improved chlorophyll content by almost two-fold value compared to only treated with PGPR (RG8, RG12 and RGKP3) (Fig. 6). Comparison data indicated that RGKP3 (*Priestia megaterium*) with an optimized amount of ZnO NPs produced the highest amount (24.80 mg/g) of total chlorophyll as compared to the other two potent strains. Komal et al. (2022) observed that Chlorophyll content was highest in the positive control were 6.119 mg/g in the chickpea plant [30]. The production of carotenoids ranged from 34.4 to 77.4 µg/g in leaves. Only RG 8 treatment showed 72.56 µg/g of carotenoids, which is more than other treated plants. Out of these, potent *Priestia megaterium* (RGKP3) showed the maximum amount of total carotenoid in the pot experiment. Nanoparticles contributed to the production of more carotenoids in plants at the ideal concentration. In addition, compared to other treated plants, the production of flavonoids was highest in plants treated with PGPR and NPs co-application (RG8+ ZnONPs, RG12+ZnONPs, and RGKP3+ZnONPs). PGPR + NPs combined to generate 2.44-2.51 µg/g of flavonoids, which was 68.5% higher than untreated plants. Only PGPR-treated plants were also showed elevated number of flavonoids compared to untreated and zinc salt-treated plants. Ham et al. (2022) reported that the total flavonoid content was 35.5 mg/g in the methanol extract of *G. aleppicum* [31]. The proline content of plant leaves shot up from 25.67 to 92.51 mg/g

during the pot assessment. The treatment with RG12 and ZnO NPs produced the least proline content. The lower proline levels in treated plants suggest stress mitigation compared to control. Zinc oxide salts produced the least amount of proline when it is compared to NPs and bacteria only. So, nutritional products may help to relieve stress. Zainab et al. (2021) reported *B. xiamenensis* enhanced the proline content up to 117%, and *B. gibsonii* increased the proline content up to 112% [32]. Moreover, the individual treatment of RG8, RG12, and RGKP3 showed almost similar ranges (110.64, 112.41 and 110.64 mg/g respectively) of total sugar produced over control. In a comparison study, Fig.10 (f) Shows that the RG8 (*Pseudomonas songnenensis*) with 400 ppm ZnO NPs produced the highest sugar content. Marius et al., (2013) reported that the PGPR strains improve the nutritive value of the harvested runner bean grains by enhancing the total reducing carbohydrate content up to 49.28% [33].

RG8 + NPs, RG12 + NPs, and RGKSP3 + NPs treated plants produced high amounts of total protein 134.19, 133.70, and 116.45 mg/g respectively as compared to other treated plants. RG8+NPs (*Pseudomonas songnenensis*) treated plants produced the highest protein as compared to the other two potent strains. Kumar et al. (2017) concluded that the protein content of coriander straw improved at 60 DAS [34].

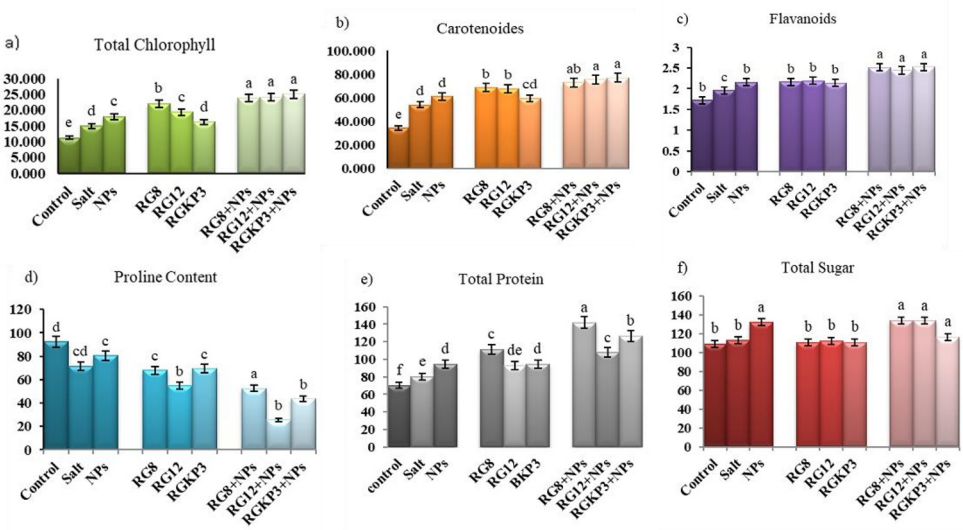

Figure 6: Estimation of (a) total chlorophyll content (b) carotenoids content; (c) flavonoids content; (d) proline content for differently treated plants (e) produced total protein content and (f) total sugar content in plants with a combination of PGPR RGKP3 & RG8 and ZnO NPs in a pot experiment.

PGPR strain (*Pseudomonas songnenensis, Bacillus haynesii, and Priestia megaterium*) combined with chemically synthesized zinc oxide nanoparticles at 400ppm for assessment of the groundnut plant's growth and development. The *Priestia megaterium* (RGKP3) and *Pseudomonas songnenensis* (RG8) are more supportive of promoting groundnut plant growth and development compared to *Bacillus haynesii* (RG12) strain.

CONCLUSION

The importance of integrating PGPR with ZnO NPs optimizes plant growth and biochemical production, paving the way for more effective agricultural practices that leverage beneficial microorganisms and nanotechnology. Seed inoculation with PGPR and ZnO NPs significantly boosted all yield traits. In the pot experiment, a comparison study suggested that the best-performing strain is *Priestia megaterium* (RGKP3) and and *Pseudomonas songnenensis* (RG8) as compared to *Bacillus haynesii* (RG12) to promote groundnut plant growth and development with optimized value of zinc oxide nanoparticles.

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