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Stimulation of Plant Growth Promoting Rhizobacteria with ZnO Nanoparticles to Improve Growth and Development of Groundnut Plants (*Arachis Hypogaea* L.)

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Abstract

Rhizobacteria that stimulate plant growth have been extensively utilized as biofertilizers to improve the nutrient content of the soil for various crop plants. In the present study, we have isolated plant growth promoting rhizobacteria (PGPR) for rhizospheric soil of groundnut crops from Gujarat agricultural fields in the Saurashtra region. Fifty-two isolates with particular colony characteristics were obtained from rhizospheric soil. All strain was tested for their plant growth promoting (PGP) traits including chitinase assay, phosphate and potassium solubilisation, nitrification, zinc solubilization, indole acetic acid (IAA), ammonia and hydrogen cyanide (HCN) production, gibberellins production and siderophore production in order to explore its potential for improving plant growth. Only three isolates displayed all PGP traits positive and one of the potent isolate was identified using molecular identification. The strain RG12 has close evolutionary similarity with Bacillus haynesii. Furthermore, zinc oxide nanoparticles (ZnO NPs) were synthesized using sol gel method. The nanoparticles were characterized using UV visible spectrophotometer, scanning electron microscope (SEM) and transmission electron microscope (TEM). The ZnO NPs concentrations (100 to 800 ppm) were used evaluate the optimized concentration with Bacillus haynesii. The pot assay indicated that the combined application of RG12 and ZnO NPs (400 ppm) resulted in highest growth and development of groundnut plants in comparison to all other individual treatments.

Keywords: PGP traits, *Bacillus haynesii*, ZnO nanoparticles, optimization of ZnO NPs with PGPR (pot assay)

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INTRODUCTION

Among the various industries in the country, agriculture not only plays an important role in the country's survival but also paves the way to meet the demands of the growing population and economic exports. Agriculture is considered an important source of economic support for growth in developing countries. The global population continues to rise and is predicted to exceed 10 billion by 2050 [1]. Furthermore, ensuring that nutritious food is secure for future generations is an ambitious goal that necessitates innovative approaches for current agricultural production regulates in order to accomplish food and nutritional security [2]. Moreover, agricultural land is limited; hence, it is crucial to develop innovations to expand and increase crop production by controlling plant diseases and using sustainable agricultural practices. Groundnut is an essential oilseed crop grown in Gujarat that offers a high nutritional value, with 25–30% protein and 45–50% oil. India ranks second in global groundnut production and is one of the top three producers of groundnuts worldwide. The main groundnut producing states in India include Madhya Pradesh, Orissa, Gujarat, Tamil Nadu, Andhra Pradesh, Karnataka, and Maharashtra. One crop cycle harvested from groundnut typically removes approximately 112 kg of nitrogen, 27 kg of phosphorus, and 34 kg of potassium from land [3]. The excessive use of chemical fertilizers is possibly the fastest, shortest, and possibly the most extreme method for enhancing crop productivity.

Chemical fertilizers are currently a feasible option for boosting agricultural productivity. However, the exploitation of chemical fertilizers has led to the deterioration of soil, nitrogen leaching, compaction of the soil, and overall soil organic matter loss [4]. However, because of the hazardous effects of chemical fertilizers and development of disease resistance, there is a critical need to limit the use of agrochemicals in cultivation techniques to promote crop yield and maintain secondary metabolite quality.

In the last decade, several researchers have proposed the development of greener alternatives to chemical fertilizers. Furthermore, following the Green Revolution of the 1960s and Biotechnology Revolution of the 1990s, nanotechnology emerged as the sixth breakthrough technology [5]. Modern agriculture aims to transform traditional agricultural practices into real agriculture that provides food security to growing populations in a cost-effective and environmentally sustainable manner by integrating nanotechnology, biotechnology, and other scientific disciplines into agricultural science. Moreover, nano-and biofertilizers are gaining momentum as good alternatives and have gained immense popularity in the agricultural sector. The agricultural sectors in dire need an immediate reduction in chemical-based fertilizers and pesticides due to the ecological challenges caused by current agricultural practices. As a result, alternatives, such as the combined application of PGPR and nanoparticles, have been regarded as the technological and scientific future for sustainable development. The use of PGPRs as biofertilizers with nanoparticles is a biological approach toward the sustainable intensification of agriculture. PGPRs regulate plant hormones and nutritional balance, induce resistance against plant pathogens, and solubilize nutrients for easy uptake by plants [6]. Compared to chemical fertilizers, PGPRs are cost-effective and environmentally friendly. The application of PGPR with metal oxide nanoparticles can aid plants in a controlled manner by enhancing the efficiency of biofertilizers, minimizing volatilization and leaching, and reducing environmental hazards.

Zn is a vital micronutrient in plant cells for the synthesis of tryptophan, which is the precursor of indoleacetic acid, a phytohormone responsible for physiological and biochemical functions. Furthermore, the effects of ZnO NPs on plants depend on their size, concentration, and plant species [7]. Recently, foliar application of ZnO NPs (10 mg/L) has led to higher biomass and photosynthetic rates in plants. ZnO NPs slightly increased the dry and fresh weights of the biomass at a lower concentration [8]. Moreover, high concentrations of ZnO NPs inhibited root growth. Furthermore, elevated concentrations of ZnO NPs inhibited chlorophyll biosynthesis, leading to a reduction in the photosynthetic efficiency of plants.

In the present study, we investigated the potential of potent PGPR and ZnO NPs (100–800 ppm) and their combinations for groundnut plant growth in pot experiments.

MATERIALS AND METHODS

Isolation of Rhizobacteria

The locations of the study were four different agricultural fields in Saurashtra, Gujarat: Kotdapitha 21.966728,71.204532, Virnagar 22.043104,71.113215, Kalawad, District Jamnagar, and Garani 21.924582, 71.136719, District Amreli. Rhizospheric soil samples were collected and stored on nutrient agar at 4°C for isolation.

PGP Traits

Fifty-two isolates with different colony characteristics were isolated from the rhizospheric soil. The potential of all strains to showcase growth-promoting characteristics, such as the production of gibberellins, phosphate and potassium solubilization, zinc solubilization, nitrification, siderophore, hydrogen cyanide (HCN), ammonia production, IAA production (indole acetic acid) and chitinase hydrolysis, was examined.

1. *IAA production:* The bacterial isolates were screened for IAA production using the Salkowski colorimetric assay according to the protocol proposed by Gordon and Weber et al. (1951) [9].
2. *Ammonia production:* The qualitative and quantitative ammonia (-NH_3) producing potential of the isolates was investigated using Cappuccino and Sherman (1996) methodology [10].
3. *HCN production:* Qualitative HCN production was measured by Alstrom et al., 1989 [11] and the quantity of HCN produced by isolates was determined by the method described by Sherawat et al. (2022) [12].
4. *Gibberellin production:* Gibberellin production was determined using Folin-Ciocalteu reagent [13].
5. *Phosphate solubilization:* Using a modified technique that included the use of PVK agar, selected phosphate-solubilizing isolates were tested for their qualitative ability to solubilize calcium phosphate. The chlorostannous-modified molybdophosphoric acid blue technique was used to quantify the amount of phosphate released (Jackson, 1973) [14].
6. *Potassium Solubilization Test:* The potassium solubilization ability of all isolates was assessed using Aleksandrow agar medium [15]. The Aleksandrow agar was modified with bromothymol blue.
7. *Chitin hydrolysis Assay:* Colloidal chitin was prepared according to a modified method [16]. The chitinase assay was performed using 1% colloidal chitin in nutrient agar medium and incubated for five days at room temperature. Later, the bacterial cultures were inoculated in the center of the plate, and chitin hydrolysis was indicated by the presence of a clear zone.
8. *Nitrogen fixation:* rhizobacterial strain was studied for free-nitrogen fixation. This method was previously described by Jensen [17].
9. *Zinc solubilization:* All isolates were studied to determine their zinc solubilization efficacy. Pikovaskay's medium contains 0.1% insoluble zinc oxide compounds (ZnO, ZnS, and $ZnCO_3$) zinc oxide compounds [18]. Bacterial cultures were inoculated onto the spot to visualize the surrounding halo zone. After 48 h of incubation at 28 °C, halo zones around the colonies on the plates were measured.
10. *Siderophore production:* The CAS test was used to screen the bacterial strain for qualitative siderophore production. (Schwyn and Neilands, 1987) [19, 20].

Identification of Potent PGPR strain (RG12)

Following the primary and secondary screening, all three isolates showed positive PGP traits. A potent PGPR strain (RG12) was selected for further molecular identification.

1. *Molecular identification of PGPR isolates by 16s rRNA sequencing:* DNA was extracted from the RG12 overnight culture, DNA was quantified, and the gene fragment was amplified using PCR. Upon resolution on an Agarose Gel, a single distinct PCR amplicon band was observed. To remove contaminants, the PCR amplicons were subjected to column purification. Sequencing was carried out using the BDT v3.1 Cycle sequencing kit with an ABI 3730xl Genetic Analyzer, in accordance with the manufacturer's protocol provided by the company (SLS Research Private Limited Lab, Suart). The resulting gene sequences were examined against sequences in the GenBank database using NCBI and BLAST at https://blast.ncbi.nlm.nih.gov. Sequences were submitted to the NCBI GenBank database, and accession numbers were allocated.

Chemical synthesis and characterization of ZnO Nanoparticles

ZnO nanoparticles were synthesized using a sol-gel method [21]. Briefly, 0.02M zinc acetate is dissolved in 100mLmilliQ water was boiled with continuous stirring at 100ºC for 30-40 minutes. Later, aqueous 2M sodium hydroxide (NaOH) and 0.5 M sodium citrate were added at a constant flow rate until the color of the solution changed from colorless to white. Subsequently, the synthesized nanoparticles were placed in an ice bath for 2 h. Finally, the sol was centrifuged at 8000rpm for 10 min and characterized using a UV-visible spectrophotometer (Shimadzu UV-1800), scanning Electron Microscope (SEM, ZEISS- Model EVO 18), and TEM (JEOL JEM 2100 TEM HR LaB6 version). The nanoparticles were air-dried in a hot air oven overnight at 60 °C and stored at 4 °C until further use.

Optimization of effective concentration of ZnO NPs for plant growth

Various concentration of ZnO NPs (100-800 ppm) were inoculated with the PGPR *Bacillus haynesii* to determine the optimum concentration for plant growth and development. Briefly, 10peanut seeds were soaked in 100 mL of sterilized nutrient broth medium [22]. Three sets of seed-treatment trials were conducted in the laboratory. Laboratory tests were performed on a single batch of treated seeds to determine the synergistic effect of ZnO NPs and PGPR on plant growth. Four replicates of different sets of treated peanut seeds were planted in pots with the same amount of soil and watered to the capacity of the container. To reduce the impact of soil heterogeneity, efforts were made to use comparable soil in each pot.

Pot Assay

Seed treatment: The Nutrient broth medium was inoculated with a single isolated colony of *Bacillus haynesii,* and the optical density (OD)of the supernatant was measured at 660 nm after 10 min of centrifugation at 3000 rpm. ZnO nanoparticles and bacteria were used to coat the groundnut seedlings. Three seedlings per pot were placed in pots containing an autoclaved soil and sand combination (3:1), and each received 1mL of bacterium inoculum. The individual and combined treatments of PGPR and ZnO NPs were carried out accordingly (control=untreated plant, 100-800ppm NPs= only NPs with 100-800ppm concentration of ZnO NPs, PGPR+NP + NP = *Bacillus haynesii* combined with 100-800ppm ZnO NPs) and the treatments without PGPR and ZnO NPs application were termed as controls [23].

Physical and Biochemical parameters (Data Recording and Related Procedures)

Physical parameters: After 30 days, the plants were harvested, and their dry and fresh weights, as well as physical growth metrics such as the number of leaves, branches, roots, and shoot and root lengths, were measured.

Biochemical parameters: Following the completion of physical parameters, a biochemical assessment was carried out that involved the measurement of proline, total sugar and protein content, flavonoid content, and carotenoid and chlorophyll content.

a. *Chlorophyll and Carotenoids Content:* Arnon (1949) method was used to estimate the chlorophyll and carotenoid content [24]. Fresh leaves (0.1 g) were combined with 5mL of acetone (80% w/v). The homogenized mixture was centrifuged for five minutes at 2000 rpm in order to extract the suspension. Chlorophyll concentration was determined using the supernatant. The optical density (OD) of the solution was measured at 645 nm (chlorophyll a), 663 nm (chlorophyll b), and 480 nm (carotenoids). Acetone (80%) was used as a blank [25].

$$Chlorophyll\ a = (12.7 \times A_{663}) - (2.69 \times A_{645})$$

$$Chlorophyll\ b = (22.9 \times A_{645}) - (4.68 \times A_{663})$$

$$Total\ Chlorophyll = (12.7 \times A_{663}) + (22.9 \times A_{645})$$

$$Carotenoids\ (per\ 100\ g\ plant\ tissue) = \frac{4 \times O.D. \times Total\ Sample\ Volume}{Weight\ of\ fresh\ plant\ tissue}$$

b. *Flavonoids Content:* The flavonoid content was determined following the method of Zhishen et al. (1999) [26]. The 80% methanol homogenate containing the leaves was centrifuged for 10 min at 3000 rpm. The aluminum chloride ($AlCl_3$) reagent was prepared by dissolving 400 mg of crystalline sodium acetate and 133 mg of crystalline $AlCl_3$ in 100 mL of 80% methanol. Finally, 400 µL of water and 1 ml of the $AlCl_3$ reagent were added to 2 mL of the supernatant. The absorbance was measured at 430 nm against a blank [27].

c. *Proline Content:* The proline content of the leaves was assessed using the Bates method [28]. Leaves were homogenized in 5 mL of 3% sulfosalicylic acid. The extracts were centrifuged for 15 min at 3000 rpm. Then, 2 mL of the supernatant was added, followed by 2 mL of glacial acetic acid and 2 mL of ninhydrin reagent for 1 h. A mixture of chemicals was incubated at 100°C for 1 h. After cooling the reaction mixture, 4mL of toluene was added. The colour emerged. The toluene layer was then separated by vigorous mixing. The top layer of the reaction mixture was removed, and the absorbance of the supernatant at 520 nm was measured against a toluene blank [29].

$$Proline\ content\ (mg/g) = \frac{K\ value \times dilution\ factor \times O.D.}{Weight\ of\ the\ sample}$$

Where, K value =19.6

d. *Sugar Content:* The sugar content of the plant leaves was evaluated using the Dubois technique with glucose as a reference. Fresh plant material (0.5 g) was homogenized in 10 mL of distilled water in a clean mortar and pestle and centrifuged at 3000 rpm for 15 min. In 0.1 mL of supernatant and 1 mL of 0.5% (v/v) phenol was added. The samples were then incubated at room temperature for 1 h. After the incubation period, 5 mL saturated H_2SO_4 was added. The absorbance of the sample was measured at 420 nm in comparison with that of a blank [30].

e. *Protein Content:* The protein content of the leaves was assessed using Bradford's method. The leaves were homogenized in 10 mL of 0.1M phosphate buffer. The extract was centrifuged at 10,000 rpm for 15 min. The supernatant of the extract was evaluated for total soluble protein content. Briefly, 1mL of diluted extract with 0.1M phosphate buffer and 5mL of Bradford's reagent was added. The absorbance of the resultant blue-colored complex was recorded at 595 nm and the amount of total soluble protein was determined using a standard curve. A standard curve was constructed with BSA (bovine serum albumin) in the range of 20-100 mg/g [31].

RESULTS AND DISCUSSION

Rhizospheric soil samples were collected from Rajkot (RG), Jamnagar (RGK), and Amreli (RGKP) districts of Saurashtra, Gujarat. Fifty-two rhizobacteria were isolated from the rhizospheric soil. PGP traits were carried out to observe the various plant growth promoting potencies of all fifty-two isolates. Rhizospheric isolates assist plants in plant growth promotion by promoting the production of IAA, ammonia, HCN, gibberellins, siderophores, chitinase hydrolysis, phosphate, zinc, and potassium solubilization, and nitrification.

Chitinase acts as an endophyte enzyme and helps in degrading plant cell walls for entry through pores in roots, stems, flowers, cotyledons, and injuries [32]. Total 7 isolates displayed clear zone due to chitinase hydrolysis. Bhatt et al. (2020) reported that chitinase dissociates chitin, which is a component of the pathogenic fungal cell wall, and inhibits their growth in the rhizosphere. Out of fifty-two, only 5 supported chitinase hydrolysis [33].

Potassium-solubilizing bacteria help in soil enrichment of plants by providing K^+ ions for easier uptake. All isolates tested positive for potassium solubilization, except RGKP7. The halo zone for potassium solubilization was in the range of 1 to 4.5 cm. RGKP4 has a maximum halo zone diameter of 42 mm. RG2 and RG18 showed the lowest amount of potassium solubilization with a 0.5 cm halo zone. Effective K-solubilizing microorganisms (KSM) must be exploited in soils to facilitate the conversion of a fixed form of K into an accessible form [34]. Parmar and Sindhu (2013) also found that KSB isolated from wheat fields solubilizes potassium at pH 7 [35].

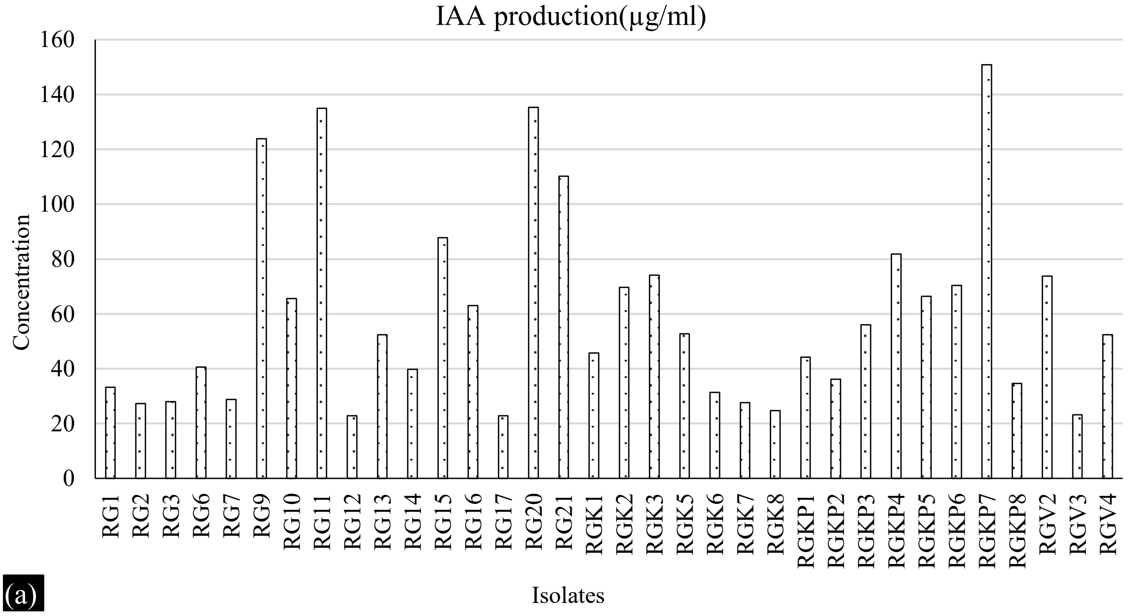
Nitrogen is particularly important because it is a key component of chlorophyll, which plants use to convert carbon dioxide and water into sugars during photosynthesis. It is also a crucial component of amino acids, which serve as building blocks of proteins. Plants deteriorate and perish without proteins. Twenty-two isolates were grown on Jensen's agar plates during the incubation period [36].

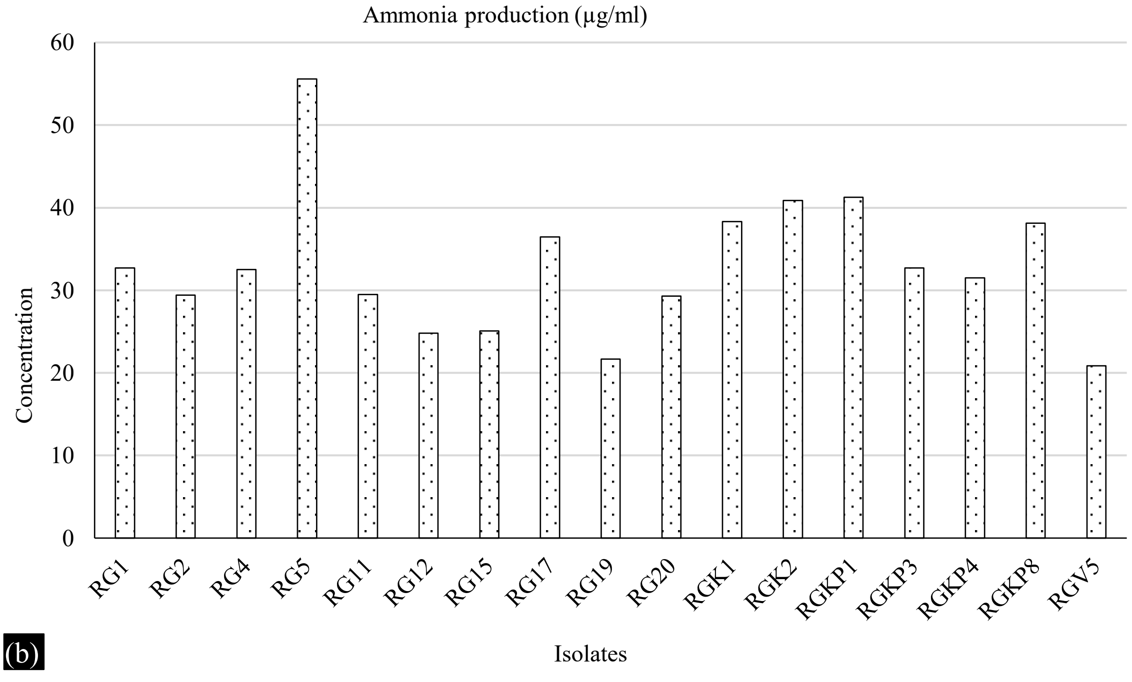
Zinc is an essential component of several enzymes that are involved in many metabolic events in all crops. Plant growth and development are halted if certain enzymes are not present in the plant tissue.

In zinc-deficient plants, carbohydrate, protein, and chlorophyll production is drastically decreased. Of all the isolates, only six PGPR showed a clear zone after 3 days.

IAA is a phytohormone that is required for plant cell and tissue division and differentiation. It also promotes root elongation in plants [37].

Figure 1(a) shows the results of IAA generation in comparison with the control. The results revealed that the isolates produced IAA ranging from 20.7 to 133 µg/mL. Isolate RGV1 produced the highest amount of IAA, which was 87% higher than that isolate RG9. The amount of IAA produced by RG12 was 21.6 µg/mL.





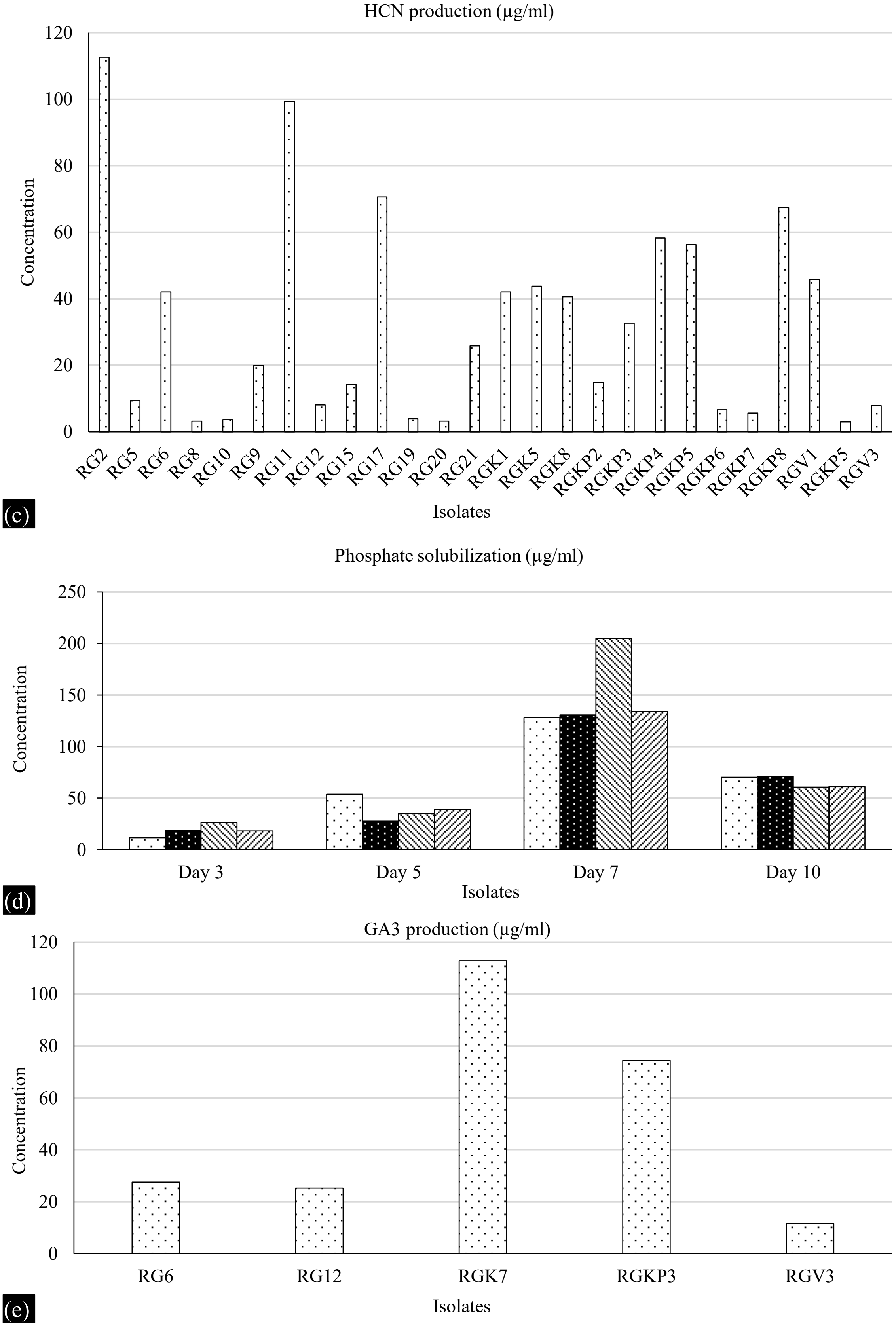


Figure 1. (a) IAA (Indole acetic acid) production by the isolates. Quantification of (b) ammonia production by the isolates; (c) HCN (hydrogen cyanide) production by the isolates; (d) phosphate solubilization of 4 isolates; and (e) Gibberellins (GA) production by positive isolates.

Ammonia production by PGPR has a cumulative effect on plant growth and development. The nitrogenous components of peptones break down into ammonia, which is released into the soil and used by plants as a source of sustenance. Only 17 isolates were brown in color and were analyzed spectrophotometrically. Figure 1(b) shows that isolate RG5 produced the highest amount of ammonia (55.5 µg/mL). The other isolates produced ammonia in the range of 21.4-55.5 µg/mL. Goswami et al. (2013) reported maximum ammonia production of 36 µg/mL, which was 36% lower than our findings [38]. Ammonia was generated using RG12 at a amount of 24.5 µg/mL.

HCN production is associated with bioremediation, and functions as a biocontrol for growth augmentation and antagonistic effects. Twenty-six isolates produced HCN (Figure 1(c)), and the solution was orange to reddish-brown in color; nevertheless, the remaining isolates did not. The remaining isolates produced HCN in the 2.34-112.08 µg/mL range. RG12 produced HCN at a rate of 5.91 µg/mL. Furthermore, Anwar et al. (2016) reported only two isolates that supported our HCN trait results with two *Streptomyces* strains (*Streptomyces* WA-1 and *S. djakartensis* TB-4) [39].

Furthermore, after nitrogen, phosphorus is the second most important nutrient for plant development (Gyaneshwar et al., 1998) [40]. Only four of the fifty-two isolates displayed the ability to solubilize phosphorous from an insoluble form. The medium contained the phosphate source. Using the colorimetric method, four positive isolates were tested for phosphorous quantification. Compared to other isolates, RG12 solubilized the most phosphate on days 5th and 7th days. At the 7th day, RG12 solubilized phosphorus at a rate of 183.17 µg/mL. In isolate K7, phosphate solubilization was 259.5µg/mL (Figure 1(d)). After the 7th day of phosphate solubilization by the isolates, the amount of free phosphate steadily declined (Gupta et al., 2022) [41].

Gibberellins are physiological regulators crucial for germination and stem elongation. Gibberellin was produced by only five isolates, and gibberellin was produced in the range of 10.2–112.4µg/mL (Figure 1(e)). Gibberellin production was estimated by Lenin et al. (2012) to be between 6.14 and 6.64µg/mL, which is less than what we observed [42].

Molecular Identification

The most promising rhizobacterial isolate possesses several advantageous PGP properties. Gram staining revealed that PGPR was a gram-positive bacterium. The isolates were identified using partial 16S rRNA sequencing. GenBank accession number OP445813 has been specified to the 16S rRNA sequences of RG12 PGPR. Phylogenetic analysis of the PGPR RG12 isolate is shown in Figure 2.

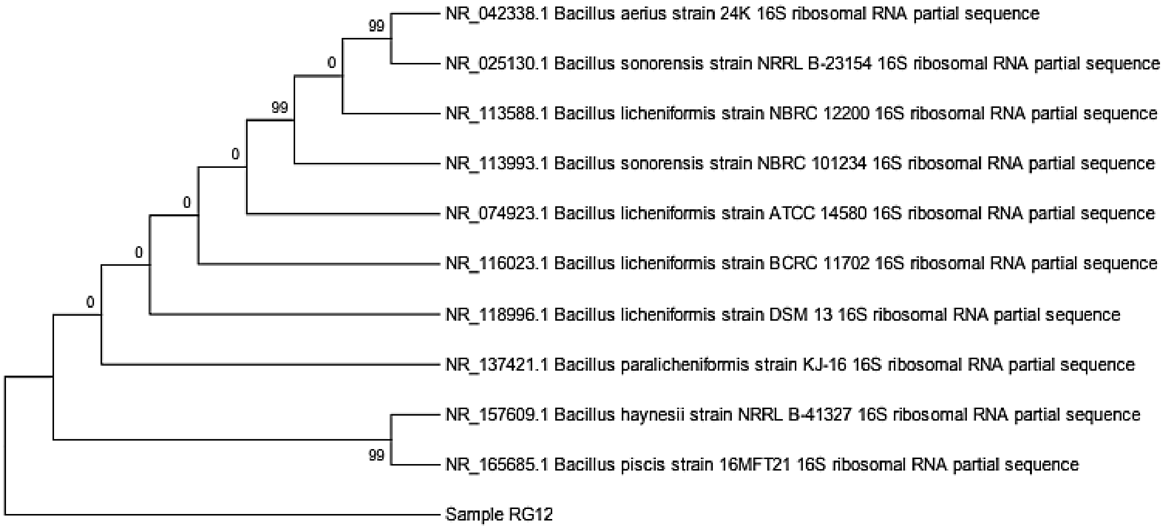


Figure 2. Phylogenetic tree showing the evolutionary relationship between RG12, a PGPR isolate and reference strains from GenBank database.

Characterization of ZnO NPs

ZnO NPs were successfully synthesized using the sol–gel method. The samples were characterized using a UV visible spectrophotometer (Figure 3(a)), SEM (Figure 3(b)), and HR-TEM (Figure 3(c)). The centrifuged samples were measured in the range–700 nm. The wavelength range is 300-700 nm for analysis. UV-vis absorption spectrophotometry showed a peak around–340-350 nm wavelengthand which is characteristic of well-dispersed ZnO NPs. Furthermore, PGPR was optimized using synthesized ZnO nanoparticles at various concentrations (100–800ppm), and it was discovered that the biological and physical characteristics of plants during their growth and development were influenced by the quantity of ZnO nanoparticles.

Optimization of Effective Concentration of ZnO NPs for Plant Growth Physical Parameters

Evaluation of physical parameters PGPR with 400ppm ZnO NPs showed the maximum number of roots, number of leaves, and root length. As a result (Figure 4(a)), we can say that PGPR with ZnO NPs increased the number of physical parameters compared to only NP-treated plants (Figure 4(a-c)). Compared to the control, only salt- and NP-containing plants also showed an increased number of physical parameters.

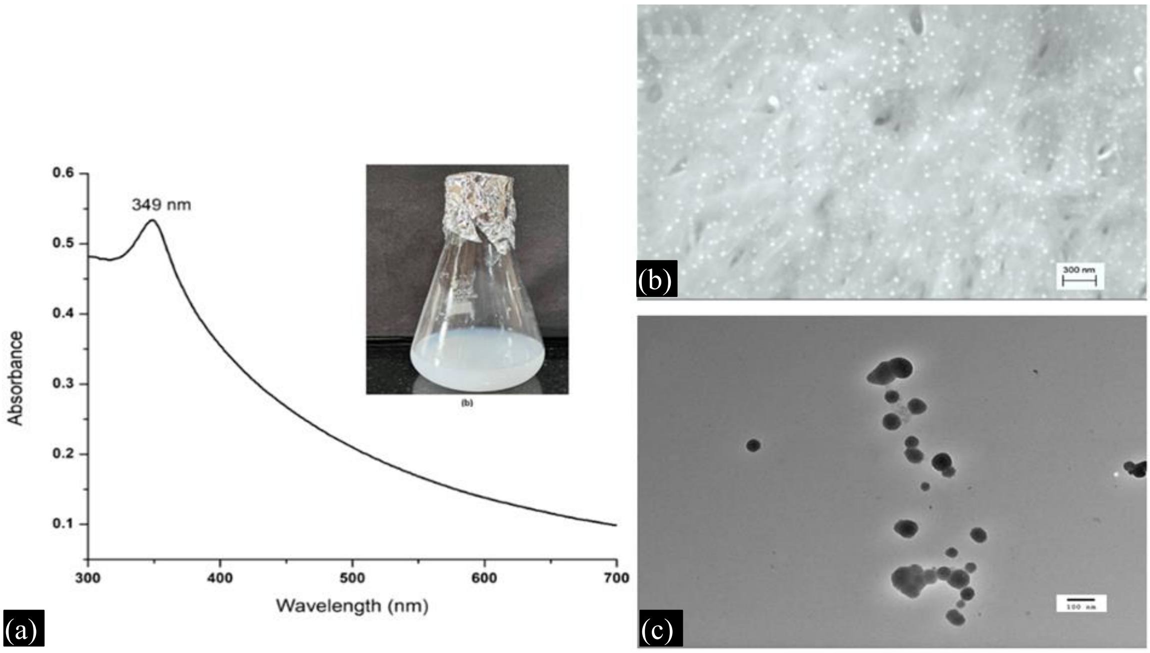
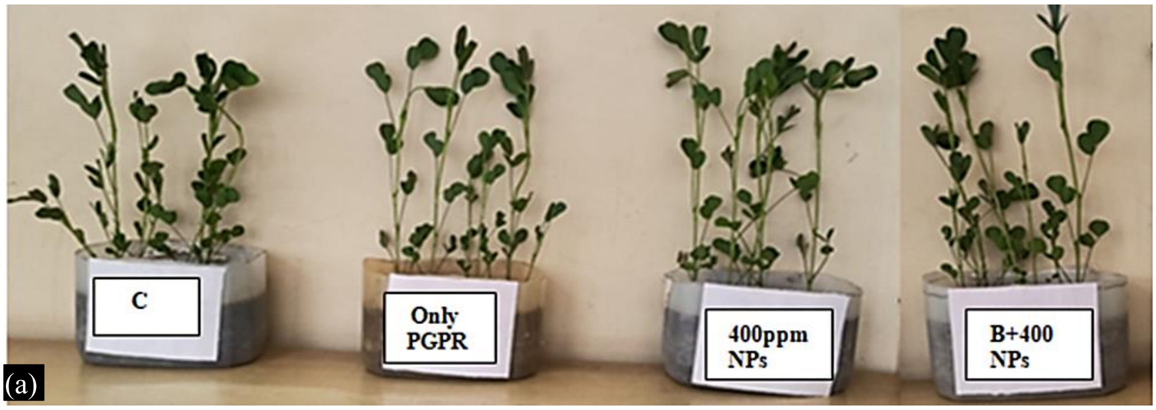


Figure 3. (a) UV visible spectrophotometric analysis of ZnO NPs (inset: synthesized ZnO nanoparticles); (b) SEM micrograph of ZnO NPs (white dots represent nanoparticles); (c) TEM micrograph of ZnO NPs with average diameter of 50-70 nm.



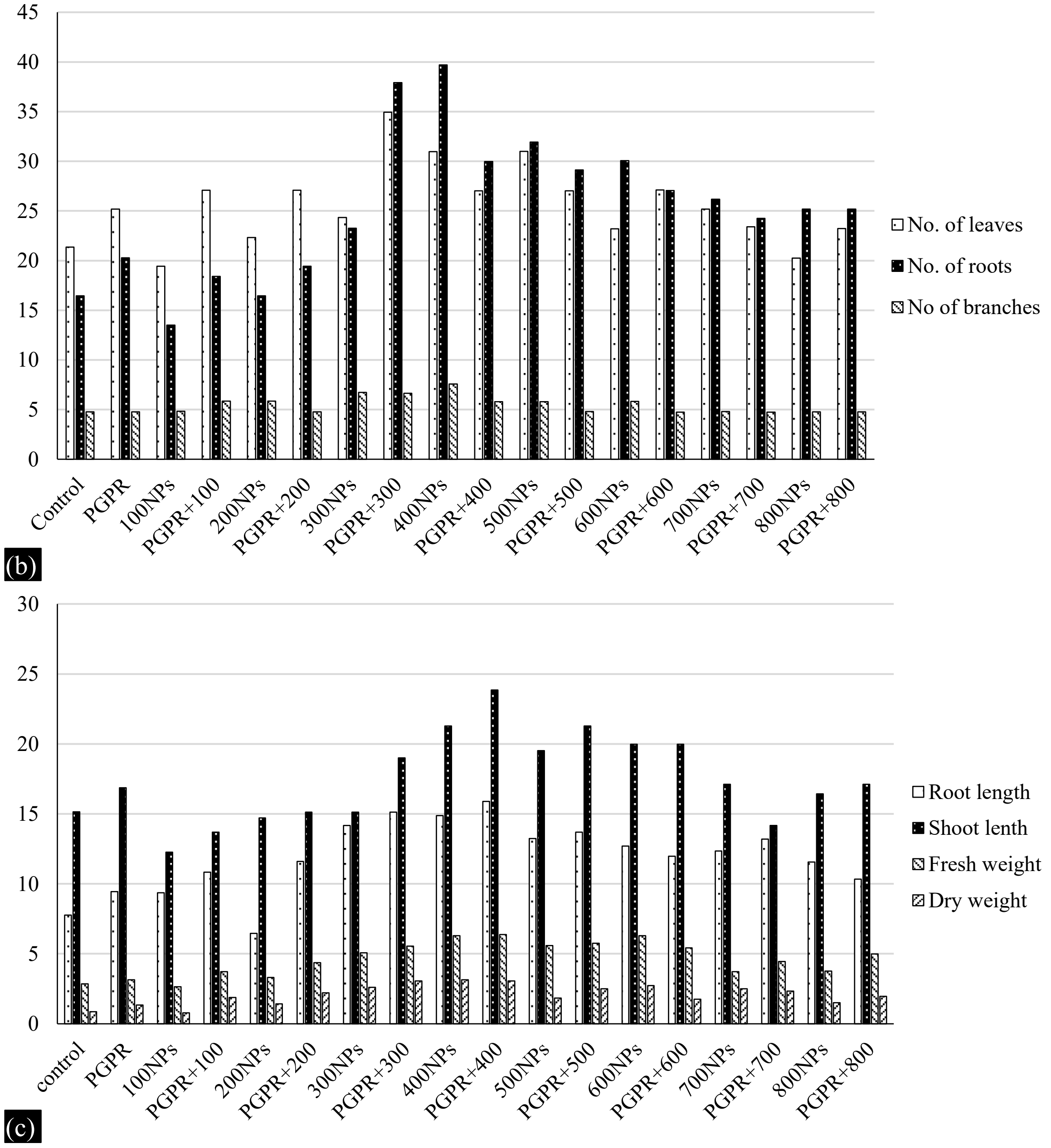


Figure 4. (a) Plants with different treatments in pot experiment after 1 month; Physical parameters of plants with combined PGPR and ZnO NPs at various concentration representing (b) number of leaves, roots, and branches; (c) root length, shoot length, fresh weight, and dry weight.

Biochemical Parameters

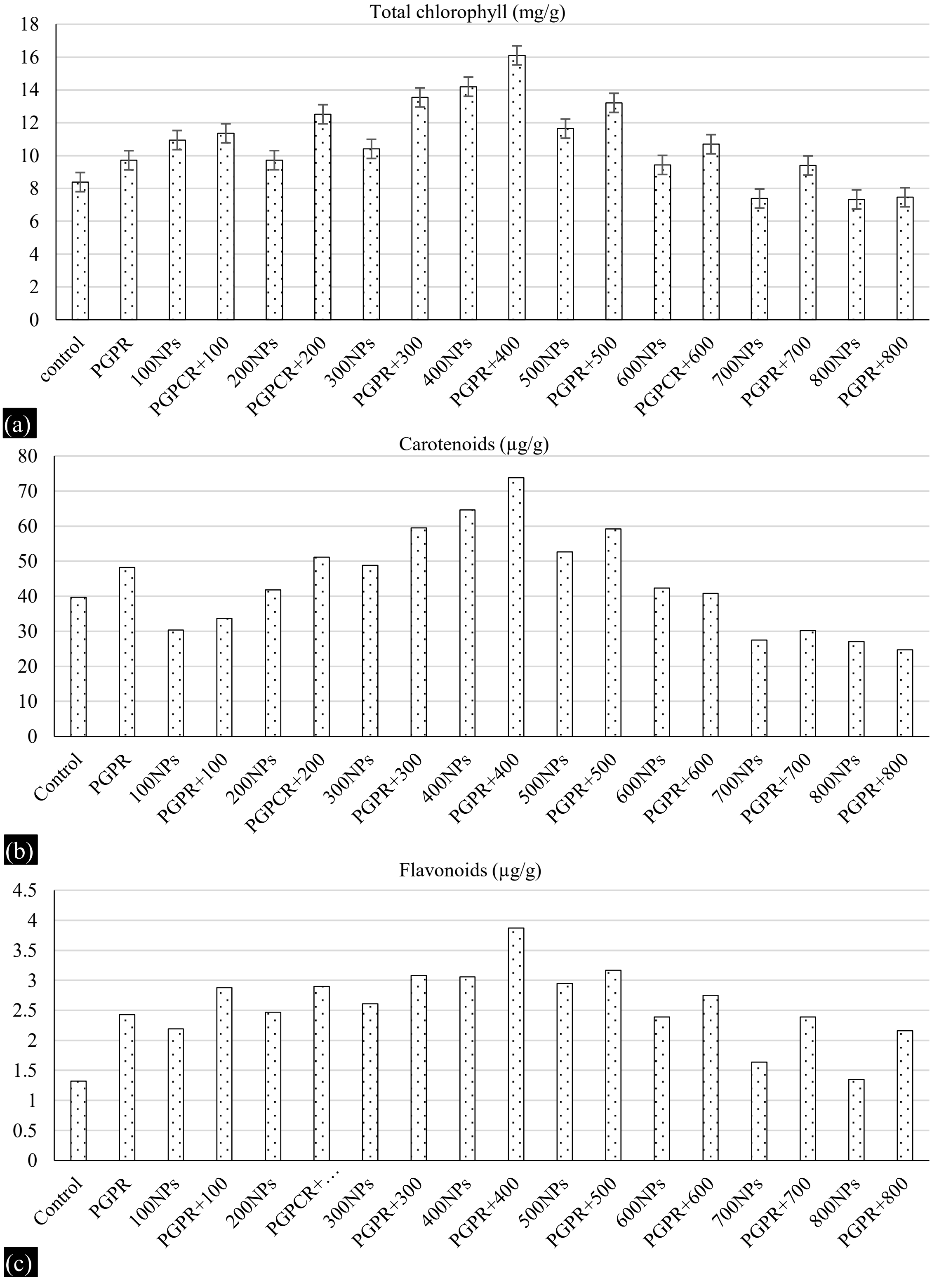
The assessment of the biochemical parameters of all 18 treated plants displayed increment in biochemical content in the PGPR + ZnO NPs treated plants compared to untreated plants.

Chlorophyll Content

Chlorophylls a and b play crucial roles in photosynthesis and are effective singlet oxygen photosensitizers. As compared to other treated plants, Figure 5(a) shows a significant increase in chlorophyll content in PGPR with 400ppm zinc oxide nanoparticles. The chlorophyll content of the plants decreases from 16.17 mg/g 7.6 mg/g. Similarly, the zinc oxide concentration decreased from 400 to 100 ppm and then from 400ppm to 800ppm. AlKahtani et al. (2020) determined that PGPR treatment promotes chlorophyll fluorescence and pigmentation in sweet pepper plants [43].

Carotenoids Content

Carotenoid synthesis can be enhanced in response to stress owing to its protective role in photosynthetic regulation. Carotenoids can quench the excited triplet chlorophyll as well as the unique oxygen, indirectly inhibiting the formation of reactive oxygen species. Plant leaves displayed an increased carotenoids content from 27.92 µg/g to 76.64 µg/g(Figure 5(b)). With the availability of iron, PGPRs have been found to enhance the chlorophyll synthesis pathway (Sharma 2017) [44].



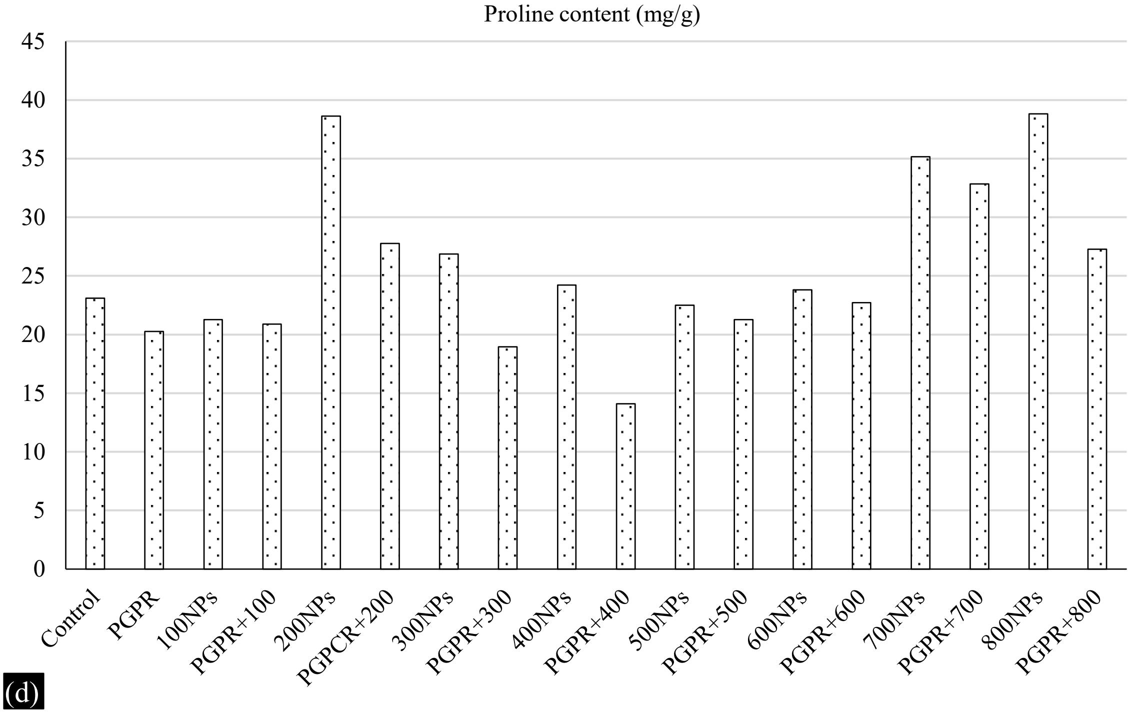


Figure 5. Estimation of (a) total chlorophyll content;(b) carotenoids content; (c) flavonoids content; and (d) produced proline content for different treated plants.

Flavonoids Content

Flavonoids play several roles in plants, including controlling cell development, attracting pollinators and insects, and defending against biotic and abiotic stressors. As shown in Figure 5(c), flavonoids were also the highest in plants treated with the combination (PGPR RG12 and ZnO NPs) compared to other treatments. Flavonoid content decreased from 500 ppm to 800 ppm with only NPs and combined treatment (PGPR + NPs). As a reference to the control, only PGPR produced higher flavonoid content. Rezazadeh et al. (2019) reported an increase in TFC during drought stress, especially under mild stress, which is consistent with the findings of Liu et al. (2013) and Yuan et al. (2012) [45,46,47].

Proline Content

Proline is an amino acid that is essential for plants. It protects plants from various stressors and aids in plant recovery from stress. Proline content was reduced in all PGPR treatments, along with NPs+PGPR. The maximum increase in proline content was 52.8% compared to the treatment with PGPR+NPs. The proline content of the plant leaves increased from 14.112 to 38.612 mg/g. However, both the single and combined applications of PGPR and zinc oxide nanoparticles at concentrations of 100 ppm to 800 ppm demonstrated a decrease in proline content when compared to the 200 ppm and 800 ppm NPs concentrations. Treatment with 400 ppm PGPR and zinc oxide nanoparticles resulted in the lowest proline content. Figure 5(d) reveals that the maximum decrease was 14.112 mg/g in PGPR treated with 400 ppm ZnO NPs, which was 3.26% less than that of the control (untreated plant). Sardar et al. (2022) reported that treatment with SeNPs increased the proline content in both control and Cd-stressed plants [48].

Sugar Content

Sugars affect all stages of the plant life cycle, interact with other signaling molecules such as phytohormones, and regulate plant growth and development. High proline and sugar contents were also identified in paddy and maize plants under salt stress in the presence of PGPRs (Sandhya et al. 2010; Jha and Subramanian 2013). The minimum amount of sugar produced (34.6 mg/g) at 800ppm NPs concentration of ZnO. The greatest amount of total sugar (66.066 mg/g) was obtained by applying combined RG12+400 ppm NPs of zinc oxide NPs (Figure 6(a)). According to Marius et al. (2013), PGPR strains increase the total reducing carbohydrate (sugar) content by up to 49.28%, which increases the nutritional value of the harvested runner bean grains [49].

Protein Content

Plant proteins exhibit a variety of enzymatic, structural, and functional activities. They also serve as storage media for growing the development and nutritional needs of the seedlings. Figure 6(b) shows that the total protein content in the plant leaves increased as the concentration of ZnO NPs increased. Different concentrations of ZnO NPs produced different amounts of protein. Only PGPR-treated plants showed a higher total protein content than untreated plants. PGPR at 400 ppm concentration of ZnO NPs produced maximum protein, which was 89% more than that produced by the control. When inoculants were used individually and in combination, Aishwath et al. (2013) concluded that the protein content of coriander straw improved 60 DAS [50].

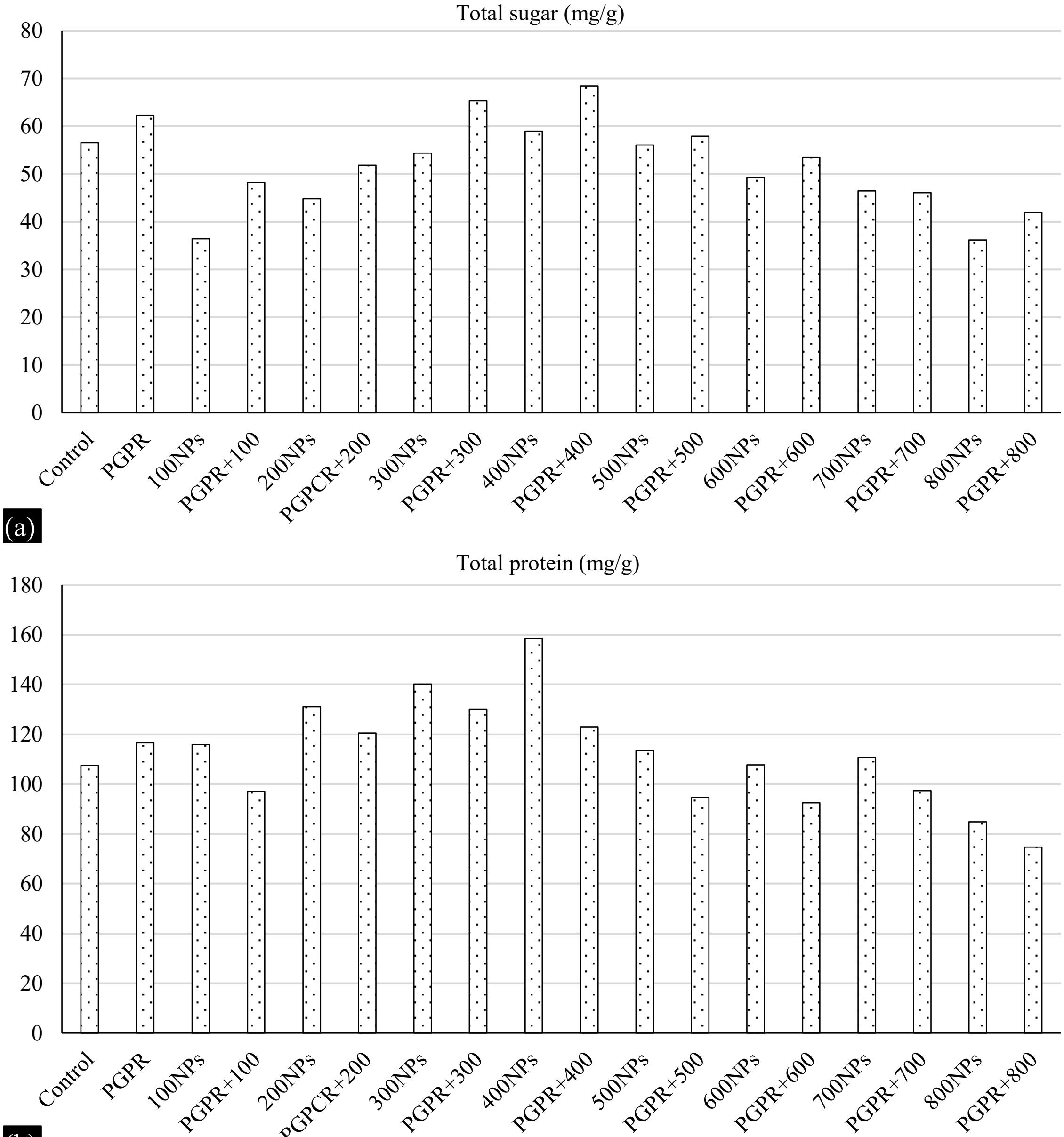


Figure 6. Quantification of (a) total sugar content and (b) total protein content in plants with various concentration of ZnO NPs in pot experiment.

Effect of PGPR(RG12) and ZnO NPs (400 ppm) on Plant Growth and Development

The PGPR strain *Bacillus haynesii* was combined with chemically synthesized ZnO oxide nanoparticles at 400 ppm to assess the growth and development of groundnut plants. PGPR (RG12) with a previously optimized NPs value helps to improve physicochemical metrics as well as produce higher amounts of biochemical content compared to untreated plants.

CONCLUSION

Previously, several PGPR have been isolated from rhizospheric soil in the Saurashtra region. They were then characterized for different PGP traits. Potent PGPR strains identified by molecular characterization (16S-rRNA sequencing). The accession number for RG12(*Bacillus haynesii*) was OP445813. ZnO nanoparticles were chemically synthesized and analyzed by SEM, HR-TEM, and UV spectrophotometric analysis. The combined application of *Bacillus haynesii* with an optimized concentration (400 ppm) of ZnO NPs enhanced the physical and biochemical parameters of groundnut plants. The intelligent associations between PGPR and nanoparticles on plants have great applications in sustainable agriculture.

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