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Evaluation of plant growth promoting potential of four rhizobacterial species for indigenous system

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Abstract: The aim of this work was to see whether *Pseudomonas putida* NWU12, *Pseudomonas fluorescence* NWU65, *Vibrio fluvialis* NWU37 and *Ewingella americana* NWU59 are beneficial to plants and are able to promote plant growth and development when inoculated as plant growth-promoting rhizobacteria (PGPR). The four rhizobacteria were tested in vitro for PGPR activities and on spinach and pepper in pot experiments. The inoculants are all positive for ammonia (NH_3), catalase, hydrogen cyanide (HCN), phosphate solubilization and siderophore production. Among the inoculants, *E. americana* NWU59 is oxidase negative. *P. putida* NWU12 and *P. fluorescence* NWU65 are producing indole-3-acetic acid (IAA). The inoculants exhibit some PGPR activities and thus tested in the screen-house. Treatments are control (water) and the four inoculants. Rhizobacterial inoculants increase spinach (17.14% – 21.43%) and pepper (15.0% – 37.5%) plant heights over the control. Such inoculants have the potential of improving plant yield components and may be used as biofertilizer.

Key words: *Ewingella americana;* microbial inoculants; PGPR; *Pseudomonas* spp; screening; *Vibrio fluvialis*

1 Introduction

Our present climate conditions and modern agriculture are severely modifying and polluting the natural ecosystems. Rhizobacteria that exert beneficial effects on plants, called 'Plant Growth-Promoting Rhizobacteria (PGPR)', are considered to be an alternative to the use of chemicals. PGPR are a group of organisms that have a close association with plants and can help plants to establish in degraded ecosystems, protect plants from diseases and promote plant growth [1–3]. They are a heterogeneous group of bacteria that are found in the rhizosphere, that is, at root surface and in association with roots, and can improve the quality of plant growth directly or indirectly. PGPR activity has been reported in species belonging to several genera, such as *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Acetobacter*, *Burkholderia*, *Bacillus* and *Serratia* [2–5].

PGPR use one or more direct or indirect mechanisms to improve the growth and health of plants. These mechanisms can be active simultaneously or independently at different stages of plant growth. Among these, phosphate-solubilization, biological nitrogen fixation, improvement of other plant nutrients uptake, and phytohormone production like, indole-3-acetic acid (IAA) are some of the regulators that profoundly influence plant growth [6].

In the root zone, biocontrol is the outcome of multiple allelopathic events, a large proportion of which can be rhizobacterial in origin. Rhizobacterial antagonism can take many forms and is not directed at pathogens specifically, but at any group of organisms 'invading' an established root zone community. Thus, positive or neutral interactions among autochthonous (established) consortia of microflora and microfauna are more probable, while any invader (colonist or pathogen) is more likely to provoke a negative response.

Recently, more attention has been paid to the biological relationships that occur between plants and bacteria in the root zone. These relations can influence the growth of plants, both positively and negatively. Therefore, search, identification and selection of PGPR indigenous to North West Province, South Africa, could play a beneficial role to increase the growth productivity of economic crops during successive application in the farming system. Being successful in this will help us (researchers) to advise the manufacturers on which bacteria can be used as biofertiliser. That will be cheaper for the farmers because the bacteria are able to multiply and increase in number on their own unlike other chemical fertilizers.

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Over the past years, the PGPR have gained worldwide importance and acceptance for agricultural benefits. They seem to be the potential tools for sustainable agriculture and the movement for the future. Scientific researchers involve multiple disciplinary approaches to understand the adaptation of these rhizobacteria to the rhizosphere, mechanism of root colonization, effects of plant physiology and growth, biofertilization etc. Microorganisms are important in agriculture in order to promote the circulation of plant nutrients and reduce the need for chemical fertilizers as much as possible. They are able to exert a beneficial effect upon plant growth [7].

PGPR have important beneficial effects on plant health and growth, suppress disease-causing microbes and accelerate nutrient availability and assimilation [2]. Therefore, in the research to improve soil fertility and crop yield and also to reduce the negative impacts of chemical fertilizers on the environment, there is a need to use PGPR for continued beneficial agricultural purposes [2]. Using the beneficial soil microorganisms such as the bacteria as agricultural inputs to improve crop production requires the selection of rhizosphere-competent microorganisms with plant growth-promoting traits [2]. These PGPR are disease-suppressive microorganisms that improve plant health and growth; unlike other chemical fertilizers which have some negative impacts on plants when they are used on plants, the PGPR seem to have no negative effects on plants. Therefore, there is a need to commercially exploit them (PGPR) as biofertilizers for their agricultural benefits.

Rhizobacteria are root-colonizing bacteria that form a symbiotic relationship with many plants, especially legumes. Though parasitic varieties of rhizobacteria are there, the term usually refers to the bacteria that form a relationship beneficial for both parties (mutualism) [8]. The bacteria grow at the expenses of carbohydrates from the host and in turn provide fixed nitrogen for amino acid biosynthesis. This symbiosis is a prime example of an intimate mutual relationship between the soil bacterium and its host plant and illustrates the concept behind the term ‘Plant Growth Promoting Rhizobacteria (PGPR)’ [9].

Besides all these biocontrol agents and/or other chemical fertilizers, the use of PGPR may be highly advantageous to plants as they are not like other biocontrol agents. Significant increases in growth and yield of agronomically important crops in response to inoculation with PGPR have been reported in various parts of the world [5]. These bacteria significantly affect plant growth by increasing nutrient cycling, suppressing pathogens by producing antibiotics and siderophores or bacterial and fungal antagonistic substances and/or by producing biologically active substances such as Auxins and other plant chemicals [5], change the concentration of the plant hormones IAA, cytokinins [10], and dissolve phosphates and other nutrients [11–12]. It is not all the plant growth promoting rhizobacteria that can be used as biofertilizers since many of them have different modes of action and some stimulate the growth of plants by helping to control pathogenic organisms, and maintain root health, nutrient uptake and the tolerance of environmental stress [9].

According to what has been stated above, the objective of this work was: 1) to see whether these selected bacterial inoculants are beneficial to plants and are able to promote plant growth and development when inoculated as PGPR under the screen house conditions. By identifying such, it will be easier to know and recommend the use of the ones which really work better than the others. As it is not all rhizobacteria that may be used as biofertilizers, screening them will be helpful in knowing the most effective ones to be used as biofertilizers and thus reduce the costs of plant-disease control by the farmers, reduce the costs of buying the expensive chemical fertilizers and also to substitute the use of chemicals which pollute the soil and ground water; 2) to see whether they all have the PGPR traits when tested in vitro; 3) to see whether one or more bacterial inoculants can promote the growth of one or two plant species. For example, it was to find out whether it can promote the growth of green pepper alone, spinach alone or the two of them.

2 Experimental

2.1 Preparation of inoculum

The bacterial isolates used in the experiments were isolated in a previous study [3]. There were four bacterial inoculants which were randomly selected for this work. Firstly, the Nutrient Agar (Biolab, RSA) media were prepared in which the bacterial cells were grown. Four Petri dishes were inoculated for each species and incubated at 37 °C for 24 h. After 24 h, sterile wire loop was used to inoculate some bacterial cells into flasks containing 100 mL of autoclaved Nutrient Broth, mixed well by shaking and placed into the Shaker machine set at 120 r/min at 30 °C for 24 h [13]. After 24 h, they were taken out of the machine and placed into the plastic tubes of 50 mL and centrifuge at 6 000 r/min for 10 min. The bacterial cells were harvested and washed with sterile water and then diluted with the sterile water to make pure bacterial solutions which were then used as inoculants.

2.2 Microbial count

A serial dilution was done in order to be able to estimate the number of the bacterial cells given to each plant as test inoculants. A 100 μL of each bacterial solution was mixed with 900 μL (1:9 respectively) of sterile water into a small tube for each sample (isolate). A 10^{-1}–10^{-5} serial dilution was made and from each dilution a 50 μm of the solution was withdrawn and cultured (spread) into the agar plate (Nutrient Agar, Biolab, RSA) and incubated at 37 °C for 24 h. After 24 h, the bacterial colonies were visible and easy to be counted. They were counted using SC6 digital colony counter (Stuart Scientific, RSA) and the total number of bacterial cells was estimated by the ratio of the number of bacteria on dilution plate to the dilution factor of plate. Each plant was given the bacterial cells ranging from 1 890 000 to 36 000 000 cells according to the estimates.

2.3 Characterization of inoculum

The bacterial isolates were characterized based on their cultural conditions, morphological features (size, shape and colour of the colonies) and biochemical characteristics (catalase and oxidase reactions).

2.4 Assay for indole-3-acetic acid (IAA) production

Microbial inoculants were tested to see if they were able to produce an indoleacetic acid as it is needed by plants to grow well. The IAA production was detected by previously described method [14]. Quantitative analysis of IAA was performed using different concentrations of tryptophan (0, 50, 150, 300, 400 and 500 μg/mL). Bacterial cultures were grown in their respective media for a certain time and temperature; fully grown cultures were centrifuged at 3 000 r/min for 30 min. The supernatant (2 mL) was mixed with two drops of orthophosphoric acid and 4 mL of the Salkowski reagent (50 mL, 35% of perchloric acid, 1 mL 0.5 mol/L $FeCl_3$ solution). The development of pink colour was the indication that the bacteria were able to produce IAA. Optical density was taken at 530 nm with the aid of spectrophotometer (Spectronic $20D^+$). Concentration of IAA produced by cultures was measured with the help of standard graph of IAA (Hi-media) obtained in the range of 10–100 mg/mL.

2.5 NH_3 production test

The bacterial isolates used in this experiment were tested for the production of ammonia in peptone water. The freshly grown cultures were inoculated in 10 mL peptone water in each tube and incubated for 48–72 h at 28.2 °C. Nessler's reagent (0.5 mL) was added in each tube, and the development of brown to yellow colour was a positive test for ammonia production.

2.6 Hydrogen cyanide production (HCN) test

All the inoculants were screened for the production of hydrogen cyanide. Nutrient Broth was amended with 4.4 g (glycine)/L and each bacterium was streaked on modified agar plate. A Whatman filter paper No.1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed on the top of the plate. Plates sealed with parafilm were incubated at 28.2 °C for 4 d. The development of orange to red colour indicates HCN production.

2.7 Siderophore production test

The inoculants used were assessed for siderophore production on the Chrome azurol S (CAS) agar medium. CAS agar plates were prepared and divided into equal sectors, spot inoculated with test organisms (10 mL of 10^6 CFU/mL) and incubated at 28.2 °C for 48–72 h. The development of yellow to orange halo around the growth was considered to be a positive test for siderophore production [14].

2.8 Phosphate solubilization test

All isolates were tested for the solubilization of phosphate. The test isolates were inoculated in 25 mL Pikovskaya's (PVK) broth and incubated for 4 d at 28.2 °C. Thereafter, the bacterial cultures were centrifuged at 15 000 r/min for 30 min. The supernatant of 1 mL was mixed with 10 mL of chloromolibidic acid and the volume was made up to 45 mL with distilled water. Chlorostannous acid (0.25 mL) was added and the volume was made up to 50 mL with distilled water. The development of a blue colour was taken as positive test.

2.9 Potting medium

The soil used in the experiment was a mixture of 6 part Canadian Peat, 3 part Perlite and 4 part Vermiculite [15] mixed together and filled in the 5 L pots. All the soil components were sterile from the manufacturer and as a result there was no need to sterilize the soil. The pH of the potting medium was neutral for all the three components mixed.

2.10 Seeds

The seeds used in the work were the mature seeds of Stark Ayres-Swiss Chard of Fordhook Giant Spinach and the Sweet Pepper-California-Wonder which were bought from NWK-Agricultural Centre at Mafikeng industrial area in the North West Province, South Africa.

2.11 Seed germination test

Before planting (sowing), the seeds were tested for germination. Some few seed particles were placed inside a Petri dish and covered with a wet cotton wool and incubated for 2–5 d at 35 °C and observed thereafter to check whether they germinated or not.

2.12 Planting

Two seeds of the green/bell pepper seeds were sowed in each pot at 3–6 cm deep hole with the distance ranging 5–8 cm apart. For spinach, planting was done in such a way that there were four seeds each in its own hole of 1.3–1.5 cm in depth with the distance ranging 5–10 cm apart. At planting, the bacterial cells were inoculated from the first day of sowing and maintained at seven days interval until the plants reach maturity. Thinning and weeding were done by hand at the time of growth. The plants were kept in a relatively moist condition at approximately water-holding capacity by watering daily to allow the bacteria to thrive.

2.13 Experimental design

In the screen-house, the pots used were washed clean and weighed before they were filled with the soil. Then, the pots were arranged in order, labeled accurately and placed at different locations where they were throughout the whole experiment. There were 40 pots whereby 20 (50%) of them were used to plant spinach and the other 20 (50%) to plant green pepper. The experimental design consisted of two locations whereby each location had four sets of replicates in a factorial arrangement having four bacterial inoculants as well as a control treatment, that is, without bacterial application. Each set of replicate had five pots including the control pot. The experiments were repeated twice.

2.14 Data collection

After planting was done, data on plant height and the number of developing leaves were collected at seven days interval and recorded as plants start to grow. Watering and weeding were part of care for the plants as to ensure their survival. As they started to grow, plant's survival was assessed daily and data were recorded accurately. At maturity, the plant height (cm) and fresh and dry mass (g) were determined. All the above mentioned parameters were determined and compared among these plants in order to see which bacterial works better than the others.

2.15 Statistical analysis

Plant growth measurement was expressed as the mean ± standard error (SE) for each treatment or group. Significance differences between treatments or groups for each crop were determined. All statistical analyses were conducted at α=0.05 using PASW statistics 18 [16].

3 Results and discussion

3.1 Characterization of inocula

The identification tests showed that isolates obtained and used in the work belong to the various isolates including *Pseudomonas fluorescence* NWU65, *V. fluvialis* NWU37, *E. americana* NWU59 and *P. putida* NWU12 (Table 1).

3.2 Laboratory assays

The biochemical tests were performed to check their reaction towards NH_3, catalase, oxidase, IAA, HCN, phosphate solubilization and siderophore production. All the isolates screened for their ability to produce siderophores formed an orange halo surrounding bacterial colonies on CAS agar. This occurred because iron was removed from the blue CAS-Fe(III) complex during siderophore production.

The inoculants have produced IAA-like substances. For this reason, it is important to study native inoculants isolated in the same region where they may be used as crop inoculants. This research was carried out to identify and evaluate the efficiency of PGPR as plant growth promotion agents of spinach and pepper. The effect of the various isolates on tested crops growth was evaluated.

All the four inoculants could solubilize inorganic phosphate, due to the excretion of organic acids. Plant growth parameters were generally enhanced by PGPR inoculation, since they have shown varied responses. It is clear that plant–microbe interaction within the

Table 1 Bacterial isolates and their features

Isolate	Gram staining	Source crop	NH_3	Catalase	Oxidase	IAA	HCN	Phosphate solubilization	Siderophore production
Pseudomonas fluorescence NWU65	Negative, Rod-shaped	Spinach	+	+	+	+	+	+	+
Vibrio fluvialis NWU37	Negative, Rod-shaped	Cauliflower	+	+	+	+	+	+	+
Ewingella americana NWU59	Negative, Rod-shaped	Bush bean	+	+	–	+	+	+	+
Pseudomonas putida NWU12	Negative		+	+	–	+	+	+	+

(+) indicates the positive reaction; (–) indicates negative reaction; IAA: Indole acetic acid; HCN: Hydrogen cyanide.

rhizosphere is different in all individual plants, and therefore, the results prove that they may have played an important role in allowing or restricting the expression of growth promotion.

All the selected isolates were tested and were able to exhibit all the PGPR traits tested in vitro. PGPR isolates successfully produced plant growth promotion hormones including IAA, siderophore, HCN, phosphate-solubilizing and NH_3. All tested isolates showed positive for catalase. *E. americana* NWU59 tested negative for oxidase. The inocula and their traits indicate good candidates for pot trials.

3.3 Potting assay

The plant height and the number of the developing leaves were measured at different stages of seven days interval. As evaluated, there was a significant difference between the plants inoculated with the PGPR as compared to the controls which were not inoculated. Also the difference was seen amongst the PGPR inoculated plants which shows that their impact/effects are not equal though they have the same characteristics under the same conditions. All the values presented are averages and the standard error was also calculated as to measure a degree of errors that could have been incurred in the work.

Unfortunately, pepper was harvested at the sixty-sixth day after germination, before it produced its fruits because it was starting to shrink and the leaves were now falling off due to the coldness of winter but the parameters taken were still significant to evaluate the effects of PGPR on plants. The results presented in Table 2 clearly show the positive approach of PGPR application. The plant height and number of plant leaves were greatly increased by PGPR application. It should be noted in this work that the plant growth parameters did not show any significant changes after 45 d after planting (DAP). However, some plant growth parameters were slightly increased in the case of some bacterial inoculants.

Among all four bacterial inoculants, *P. fluorescence* NWU65 produced slightly more enhancement in growth parameters of spinach (Table 2, Figs. 1(a) and (b)). The maximum plant height was 21.25 cm, while number of leaves data was observed as 5.25 after 59 d from the date of inoculations. Other test bacterial inoculants also significantly increased the growth parameters as compared to controls. The obtained results proved the performance of isolated soil bacteria in this work. Similar results were also obtained when the inoculants were applied to pepper plant (Table 3, Figs. 2(a) and (b)). All the four inoculants have significantly produced positive effect on growth parameters as compared to control. The maximum plant height observed was 13.75 cm when *P. putida* NWU12 was used as PGPR. The number of leaves was the maximum in the case of *P. fluorescence* NWU65 treatment. However, the changes were not significantly different as compared to other inoculants in this work.

The fresh as well as dry plant weights were calculated after harvesting the plants from green-house.

Table 2 Effect of rhizobacterial inoculants on growth of spinach as measured on plant height and number of developing leaves at different intervals

Isolate	Time/d	Plant height/ cm	Number of leaves developing leaves
Pseudomonas fluorescence NWU65	10	2.25	2.00
	17	7.50	5.00
	24	12.25	6.25
	31	17.00	6.25
	38	18.25	6.25
	45	21.00	5.25
	52	21.25	5.25
	59	21.25	5.25
Standard error		(15.09)±2.52	±0.71
Vibrio fluvialis NWU37	10	1.80	2.25
	17	5.88	5.00
	24	12.88	6.25
	31	16.75	5.25
	38	18.25	5.75
	45	20.50	5.75
	52	21.00	5.75
	59	21.00	5.75
Standard error		(14.76)±2.60	±0.73
Ewingella americana NWU59	10	2.05	2.25
	17	5.00	4.00
	24	11.50	5.75
	31	16.00	6.00
	38	18.00	5.50
	45	19.50	5.50
	52	20.50	5.50
	59	20.50	5.50
Standard error		(14.13)±2.55	±0.52
Pseudomonas Putida Nwu12	10	2.33	2.25
	17	7.38	4.75
	24	11.63	5.50
	31	12.05	7.00
	38	17.50	5.75
	45	20.25	5.75
	52	21.00	5.75
	59	21.00	5.75
Standard error		(14.14)±2.46	±0.83
Control	10	1.95	2.00
	17	5.68	4.50
	24	11.50	5.25
	31	16.00	5.00
	38	16.25	3.50
	45	17.00	3.50
	52	17.00	3.00
	59	17.50	3.00
Standard error		(12.86)±2.11	± 0.67

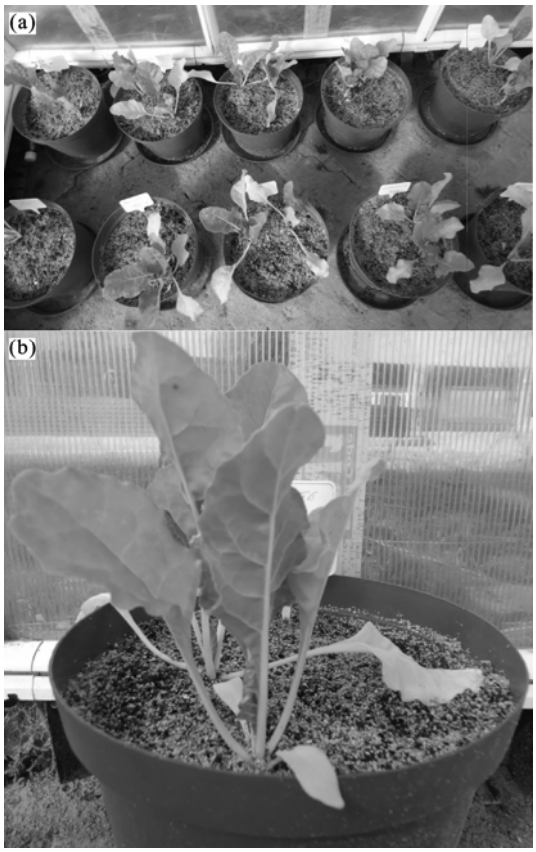

Fig. 1 Two replicate sets of spinach at 59 days after planting (DAP) (a) and close up view of size of spinach at 59 DAP (b)

The calculated results have clearly shown the increase in both plant parameters. All tested isolates have significantly improved the fresh and dry mass of tested plants viz., spinach and pepper. The results revealed that *P. putida* NWU12 greatly enhanced the mass of spinach as compared to other isolates. However, all other isolates have also significantly enhanced the plant mass as compared to control (Table 4). While, the plant mass of pepper was found more in pots treated with *V. fluvialis* NWU37 but the results revealed that other isolates have also significantly improved the above mentioned parameters as compared to control (Table 4). However, the isolated inoculants have produced slightly different results with respect to test crops. Some isolates were more effective in the case of spinach, while some of them showed greater effectiveness in the case of pepper. The obtained results revealed that the effectiveness of isolates varied with plant species. This was in agreement with previous findings that bacterial type significantly influenced the plant species with better performance over the non-inoculated control [17]. The results revealed that inoculated plants grew faster with higher height as compared to control.

These results indicate that the inoculants promote plant growth and the plant protection will remain high during the early stages of growth after sowing which is a period when young seedlings and plants are so vulnerable to the environmental stresses. As all the selected PGPR had shown promising positive effects on growth parameters on spinach and pepper under the

Table 3 Effect of bacterial inoculants on growth of pepper as measured on plant height and number of developing leaves at different intervals

Isolate	Time/d	Plant height/cm	Number of developing leaves leaves
Pseudomonas fluorescence NWU65	17	1.80	4.00
	24	5.25	3.50
	31	8.50	7.50
	38	10.0	8.50
	45	10.7	9.00
	52	13.0	10.25
	59	13.0	10.25
	66	13.0	10.25
Standard error		(9.41)±1.44	±0.37
Vibrio fluvialis NWU37	17	2.20	4.00
	24	5.00	4.00
	31	8.25	7.75
	38	10.7	9.50
	45	11.0	9.75
	52	11.5	9.75
	59	11.5	9.75
	66	12.0	9.75
Standard error		(9.02)±1.28	±0.34
Ewingella Americana NWU59	17	2.15	4.00
	24	5.88	4.00
	31	7.50	7.75
	38	11.50	9.00
	45	11.25	9.25
	52	13.5	11.5
	59	13.5	11.5
	66	12.5	11.5
Standard error		(9.72)±1.46	±0.42
Pseudomonas Putida NWU12	17	2.00	4.00
	24	5.83	4.00
	31	7.50	7.25
	38	9.75	8.25
	45	11.75	9.25
	52	13.75	10.00
	59	13.75	10.00
	66	13.75	10.00
Standard error		(9.76)±1.53	±0.34
Control	17	1.80	4.00
	24	4.75	4.00
	31	6.50	6.50
	38	8.75	6.50
	45	9.50	8.75
	52	10.0	9.50
	59	9.00	9.50
	66	8.50	10.0
Standard error		(7.35)±0.50	±0.38

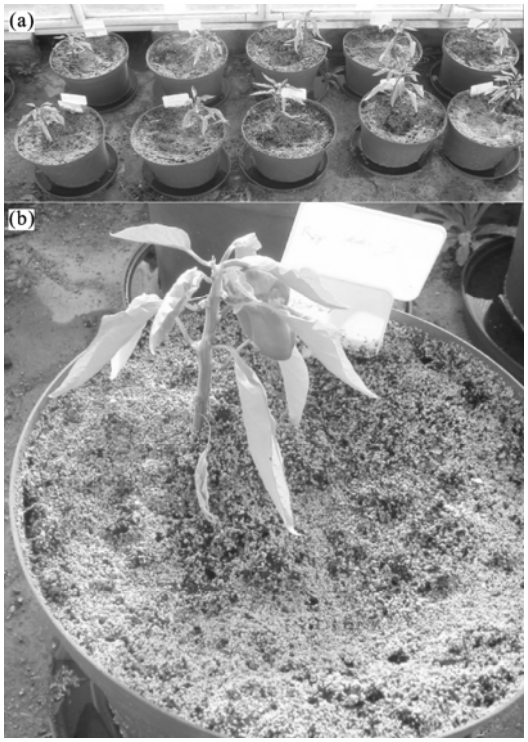

Fig. 2 Two replicate sets (a) and size (b) of pepper at 59 DAP

Table 4 Beneficial role of PGPR bacterial isolates on growth parameters of pepper (*Capsicum annum*) and spinach (*Spinacia oleracea*) under green house conditions

Bacterial isolate	Pepper		Spinach	
	Fresh mass/g	Dry mass /g	Fresh mass/g	Dry mass/g
Pseudomonas fluorescence NWU65	22.11	6.31	36.39	6.73
Vibrio fluvialis NWU37	31.82	7.41	35.73	6.79
Ewingella americana NWU59	31.63	7.22	39.35	7.11
Pseudomonas putida NWU12	23.8	5.73	43.01	7.44
Control	18.10	5.25	29.86	5.04
SE	(25.49)±2.71	(6.38)±0.42	(36.87)±2.17	(6.622)±0.42

green house conditions, according to the obtained results, it now provides a platform to test them in the field conditions and evaluate their performance. Some additional important capabilities of practical utility have been shown by some of these bacterial isolates as IAA production and P-solubilization, which suggested that these bacteria could be used as plant growth promoters [18]. PGPR inoculation has successfully influenced the mass of the roots and shoots of tested crops using the current approach. These properties might be responsible for the significant differences in growth provided by the different bacterial inoculants, suggesting that these bacteria could be used as inoculants for spinach and pepper to improve plant health and yield.

4 Conclusions

Increase in public concern about the environment has increased the need to develop and implement effective biocontrol agents for crop protection. An effective PGPR could be developed for growth productivity as well as disease control only after understanding its performance in the environment. In nature, agricultural crops are exposed to diverse environmental conditions such as solar radiation, aridity and the temperature changes in winter and summer. The fluctuations alter the microclimatic conditions existing around the plant. A thorough knowledge on the mechanisms and performance related to growth activity will help in the selection of promising candidates that suits research institute to produce reliable useful products. However, further studies are needed in order to study other ecological aspects of this topic, such as root colonization sites (epiphytic and/or endophytic) and whether some sort of competition for these microsites exists between inoculants. Besides, all the four tested isolates have shown to be effective in promoting the growth and development of plants under the screen house conditions and can be used as PGPR in agriculture.

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