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RESEARCH ARTICLE

Screening for Crop Response to Diazotrophic Bacteria Isolated from Potato Rhizosphere

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Abstract: Microbe-plant interactions in the rhizosphere can be beneficial, neutral, or deleterious for plant growth. The present study aimed to characterize eight diazotrophic bacteria isolated from potato rhizosphere and to evaluate their effects on potato growth responses. Initially, soil bacterial isolates were screened for *in vitro* pH, nitrogenase activity, *in vitro* IAA production and seedling vigor. Then, they were further evaluated for their effects on growth responses of potato seed tubers using a pot experiment under greenhouse conditions. Out of the bacterial isolates, *Bacillus* sp., *Acidomonas* sp., *Serratia* sp. and *Bradyrhizobium* sp. responded positively to all screening and greenhouse experiments. The highest nitrogenase activity, IAA production, seedling vigor and the lowest medium pH were shown by *Bradyrhizobium* sp. ($P < 0.05$). Further, inoculation of potato with *Bradyrhizobium* sp. produced the highest tuber weight, tuber number and stolon number. Inoculum pH was inversely proportional to the production of IAA. Further, tuber weight and tuber number were increased with the reduction of soil pH. Positive relationships were observed between IAA production with seedling vigor, tuber weight, and tuber number, separately. Out of the bacterial isolates, *Bradyrhizobium* sp. was found to be the best since it showed the highest performance in all screening and pot experiments.

Keywords: biofertilizers, plant growth promoting rhizobacteria, potato, rhizosphere microbes.

INTRODUCTION

The diverse groups of living microorganisms in soil can be named collectively as the 'soil microbiome'. Soil contains many microhabitats that are suitable for microbial growth. Microbes are aggregated in the immediate surroundings of plant roots, which is an active region for root activity and metabolism, and known as the 'rhizosphere' (Saharan and Nehra, 2011). Plant-associated bacteria that are able to colonize roots are called rhizobacteria and can be classified into beneficial, deleterious, and neutral groups on the basis of their effects on plant growth. Rhizobacteria, expressing direct or indirect beneficial responses in soil nutrient enhancement and plant growth, are classified as plant growth promoting rhizobacteria (PGPRs, Hayat *et al.*, 2010). The direct plant growth promotion by PGPR entails either providing the plant with plant growth promoting substances that are synthesized by the bacterium or facilitating the uptake of certain plant nutrients from the environment. The indirect promotion of plant growth occurs when PGPR minimize or prevent deleterious effects of one or more phytopathogenic micro-organisms and help plants to withstand against environmental stresses (Glick *et al*., 2007). The use of PGPRs has been found to be beneficial in sustainable crop production (Dashti *et al*., 1997: Hayat *et al.*, 2010). Therefore, PGPRs are now being used as microbial inoculants or biofertilizers to enhance crop productivity and to reduce the use of chemical fertilizers (CF) in agriculture (Adesemoye *et al*., 2009; Buddhika *et al*., 2014). In this regard, indigenous PGPRs in soil are preferred since they are completely adapted to the environment and can be more competitive than introduced microbial inoculants (Hayat *et al*., 2010). Beneficial microbial inoculants of PGPRs have been used in different leguminous and non-leguminous crops for several decades (Bashan, 1998; Adesemoye *et al*., 2009). Enhanced PGPRs activity in the rhizosphere exerts beneficial effects on plants either symbiotically or non-symbiotically. It has been reported that 1-4% of microorganisms isolated

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from naturally existing potato rhizosphere showed significant plant growth promotion on potato i.e. enhancement of the stolon length, early tuber setting and increase of tuber yield (Suslow *et al*., 1979). Application of PGPR strains have been reported to improve N availability through the fixation of atmospheric nitrogen (N_2) (Seneviratne *et al*., 2008: Franche *et al*., 2009) in soil and solublization of minerals (Kloepper, 1997; Glick, 2007) while enhancing crop productivity of other non-leguminous tuber crops such as sweet potato (*Ipomoea batatas*) (Yasmin *et al.*, 2009).

In addition to N_2 fixation, PGPRs enhance seed germination, seedling vigor and ultimately plant growth due to the production of plant growth hormones (Kumar *et al*., 2012). Generally, IAA has been reported to stimulate plant growth. It is one of the most important phytohormones and function as an important signal molecule in the regulation of growth promotion (Hayat *et al*., 2010). Production of IAA by microbial isolates varies greatly among different species and strains (Ngoma *et al*., 2012). For example, *Azospirillum* sp., a diazotroph enhanced plant growth through its ability to produce a high amount of IAA (Shahab *et al*., 2009). Further, the inductive role of IAA on stolon initiation and tuber induction in potato has been documented in previous studies (Dragicevic *et al.*, 2008). Therefore, prior characterization of beneficial effects of naturally existing rhizosphere microbial isolates is of great importance. This study was aimed at screening and characterizing the functions of beneficial microbial strains isolated from potato rhizosphere to evaluate their effects on crop responses.

METHODS AND MATERIALS

Isolation of rhizosphere microorganisms

Soil samples and plant specimens (*Solanum tuberosum* L.) were collected from a cultivated crop land at the Regional Agriculture Research and Development Center, Bandarawela, (6° 48' 0" N,80° 58' 0" E) [up country intermediate zone,IU_3, elevation 1,506 asl, temperature 22 ^oC, and annual rainfall 1,100 mm - 1,400 mm], Sri Lanka. These were transported to the laboratory of the National Institute of Fundamental Studies, Kandy, Sri Lanka. Root associated microorganisms were isolated using streak and spread plate techniques and soil microorganisms were isolated using soil dilution plate technique. The soil samples were serially diluted (10 fold) and inoculated on sterile Nutrient Agar [NA, (Himedia™, India) 20 g per liter of medium] plates followed by incubation for 24 hrs at 25 ºC. Bacterial colonies were differentiated on the basis of colony morphology. Colonies were sub cultured on the NA medium and re-incubated at 25 ºC for 48-72 hrs. This isolation process was repeated until monocultures were obtained. Isolated bacteria were identified based on preliminary biochemical tests (Gram's test, Catalase test, Oxidase test, Citrate test, Urease test, MR-VP test, Indole test and Motility test).

Screening of microbial isolates

Diazotrophic microorganisms were isolated using Combine Carbon Medium (CCM, Koomnok *et al*., 2007), a modified N free medium for N_2 fixing microorganisms (pH 6.8). Several screening experiments were carried out for the diazotrophic bacterial isolates as follows. Bacterial isolates were inoculated in 10 ml of CCM broth and incubated for five days at 25 ºC. Thereafter, pHs of broth media with different microbial inoculations were measured. Lettuce seed germination assay was used to examine pathogenecity and toxicity, and also the response of early plant growth to the isolates since lettuce is generally a highly responsive crop to environmental cues (Argyris *et al*., 2008). Lettuce seeds were treated separately by each microbial broth cultures under aseptic conditions (Mia *et al.*, 2012). Then, they were allowed to germinate in the dark for five days and seedling vigor was calculated using germination percentage, and root and shoot lengths. Nitrogenase activity of the microbial isolates was measured using the acetylene reduction assay (Husen, 2003; Piromyou *et al*., 2011). Production of IAA by the microbial isolates was measured colorimetrically using Salkowski regent (Husen, 2003; Shahab *et al.*, 2009; Buddhika *et al*., 2014). Selected bacterial isolates were evaluated for their growth responses on potato using a pot experiment under greenhouse conditions.

Evaluation of screened microbial inoculants for growth responses in potato

Plant growth effects and pathogenicity of the isolated microbial cultures were evaluated using a pot experiment with potato seed tubers at the Regional Agriculture Research and Development Center, Bandarawela, Sri Lanka. Effect on

growth was evaluated using tuber fresh weight, tuber number, stolon number, shoot dry weight and root dry weight of potato plants. The emergence of bacterial wilt disease and late blight disease were evaluated using number of wilted plants and number of leaves with brown/black lesions, respectively. Heat sterilized (dry oven at 160 ^{o}C for 2 hrs) loamy soil was used (particle size $\leq$ 2mm) as the growth medium. Disease free potato seed tubers ('Granola' variety) were sown in black plastic pots (pot size- 6 inch diameter) with the sterilized growth medium. Five replicates were maintained for each treatment and the pots were arranged in complete randomized design (CRD) in the greenhouse. A mixture of urea (3.0 g), Triple Super Phosphate (TSP) (5.0 g) and Muriate of Potash (MOP) (2.0 g) was blended with the soil as the basal fertilizer mixture. Rate of the Chemical fertilizer (CF) application was calculated per plant basis according to the standard recommendations. After the establishment of the roots, CF mixture (3.0 g of urea and 2.0 g of MOP) and 20 ml of diluted diazoptrophic bacterial cultures in CCM broths (10^7 cfu/ml) were mixed separately with the soil medium. Potato plants applied with un-inoculated CCM broth was taken as the control for the experiment. Moisture level of the potting medium was maintained constantly by applying 250 ml of water for each pot every day. Plants were grown with a daily minimum-maximum temperature range of 20 ^{o}C – 30 ^{o}C.

Visual observations were made of the plants once a week to evaluate the emergence of any harmful pest attacks or diseases. Number of wilted plants and leaves with brown/ black lesions were counted once in two weeks to evaluate the emergence of bacterial wilt or late blight diseases due to inoculation. Section cuttings of infected leaves were observed under the high power of light microscope to confirm the disease infections. Results were compared with non-treated control to evaluate pathogenicity. After 90 days from seed sowing, plant responses i.e. number of tubers, dry and fresh weights of the tubers, roots and shoots and number of stolons and soil pH, were recorded. Statistical data analyses were performed using one-way Analysis of Variance (ANOVA) in MINITAB 16 Statistical Software. Treatment means were compared using the Tukey's HSD test at 5% probability level. A statistical ranking method for data on screening and plant growth response was used to select effective microbial strains. Relationships between different parameters were established using non-linear regression analysis.

RESULTS

Isolation, identification and preliminary screening of microbial monocultures

Fourteen bacterial strains were isolated initially from the rhizosphere of potato. Out of them, eight isolates were diazotrophic bacteria and identified as *Acinetobacter* sp., *Bacillus* sp. (two species; sp. 1 and 2), *Acidomonas* sp., *Pseudomonas* sp. (two species; sp. 1 and 2), *Serratia* sp. and *Bradyrhizobium* sp. All bacterial isolates, except *Acinetobacter* sp. and *Pseudomonas* sp., showed acidic pH in CCM (Fig. 1a). Out of eight different strains, the lowest pH ($P < 0.05$) and the highest nitrogenase activity ($P < 0.05$) were recorded by *Bradyrhizobium* sp. (Figures 1a and 1b). *Acinetobacter* sp. and *Pseudomonas* sp. showed very low nitrogenase activity compared to other isolates. The highest significant IAA production was shown by *Bradyrhizobium* sp. ($P < 0.05$) compared to all other bacterial isolates (Fig. 1c). Moreover, out of eight different bacterial isolates, the highest significant seedling vigor value ($P < 0.05$) was shown by *Bradyrhizobium* sp. (Figure 1d) and the seedling vigor was enhanced by approximately 35% compared to the un-inoculated control.

A significant negative correlation was observed ($R^2 = -0.78$, $P = 0.02$) between pH and IAA production by different bacterial isolates (Figure 2a). Further, a significant positive correlation ($R^2 = 0.94$, $P = 0.001$) was observed between seedling vigor and IAA production (Figure 2b). Therefore, the correlations revealed the effect of IAA production on medium pH of the medium and the seedling growth.

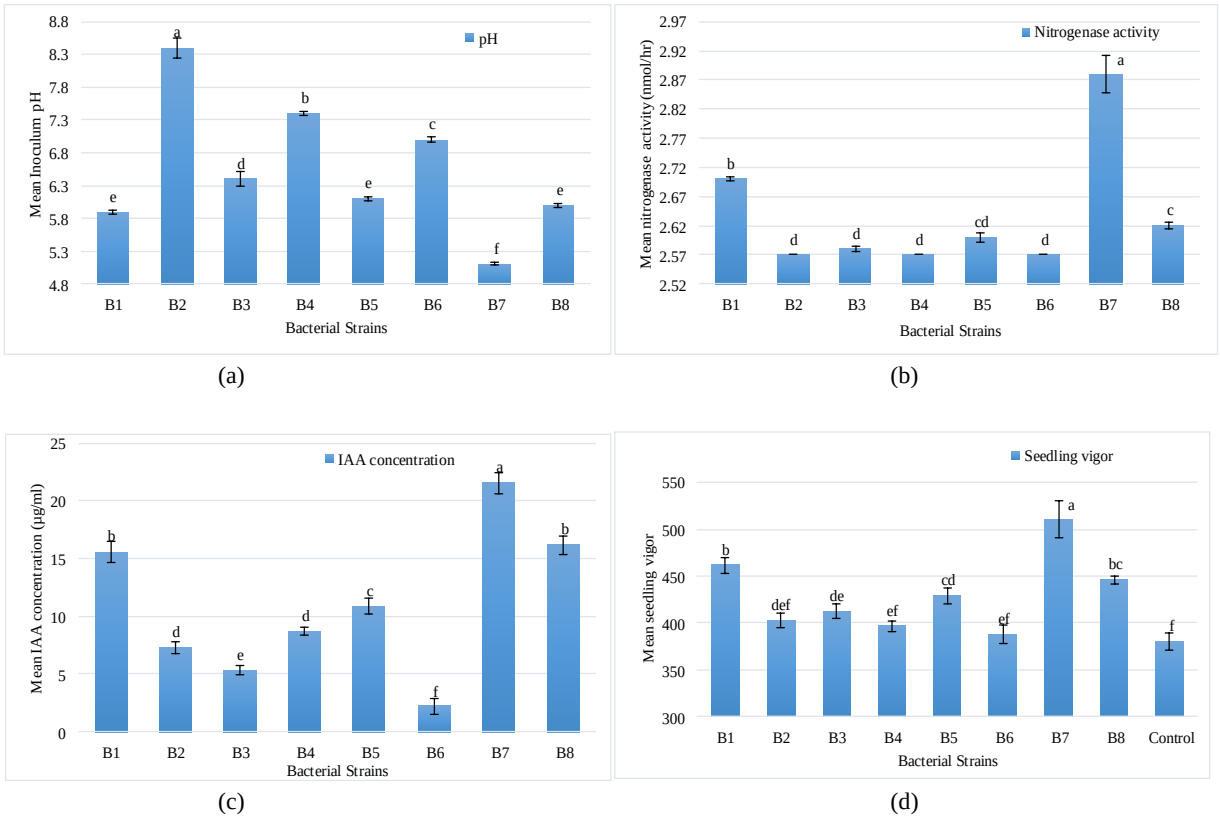


Figure 1: Responses of bacterial isolates to screening experiments. (a)- Broth medium pHs under different bacterial isolates. (b)- Nitrogenase activity based on ethylene production. (c)- IAA production by different bacterial isolates. (d)- Seedling vigor for lettuce seeds treated with different bacterial isolates (control – only ML medium). (B1 and B8- *Bacillus* sp., B2- *Acinetobacter* sp., B3- *Acidomonas sp*., B4- and B6- *Pseudomonas* sp., B5- *Serratia* sp., B7- *Bradyrhizobium* sp.) Columns with the same letter are not significantly different at 5% probability level. Vertical bars show standard errors.

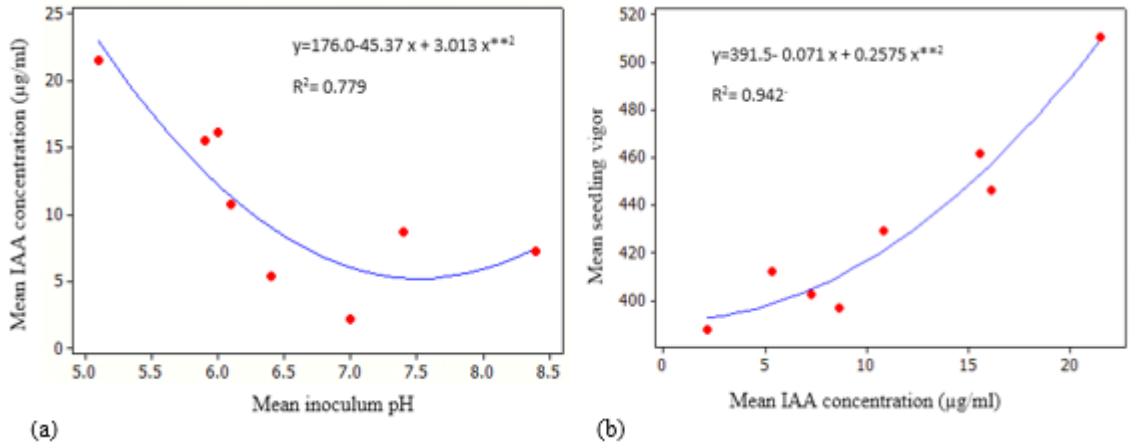
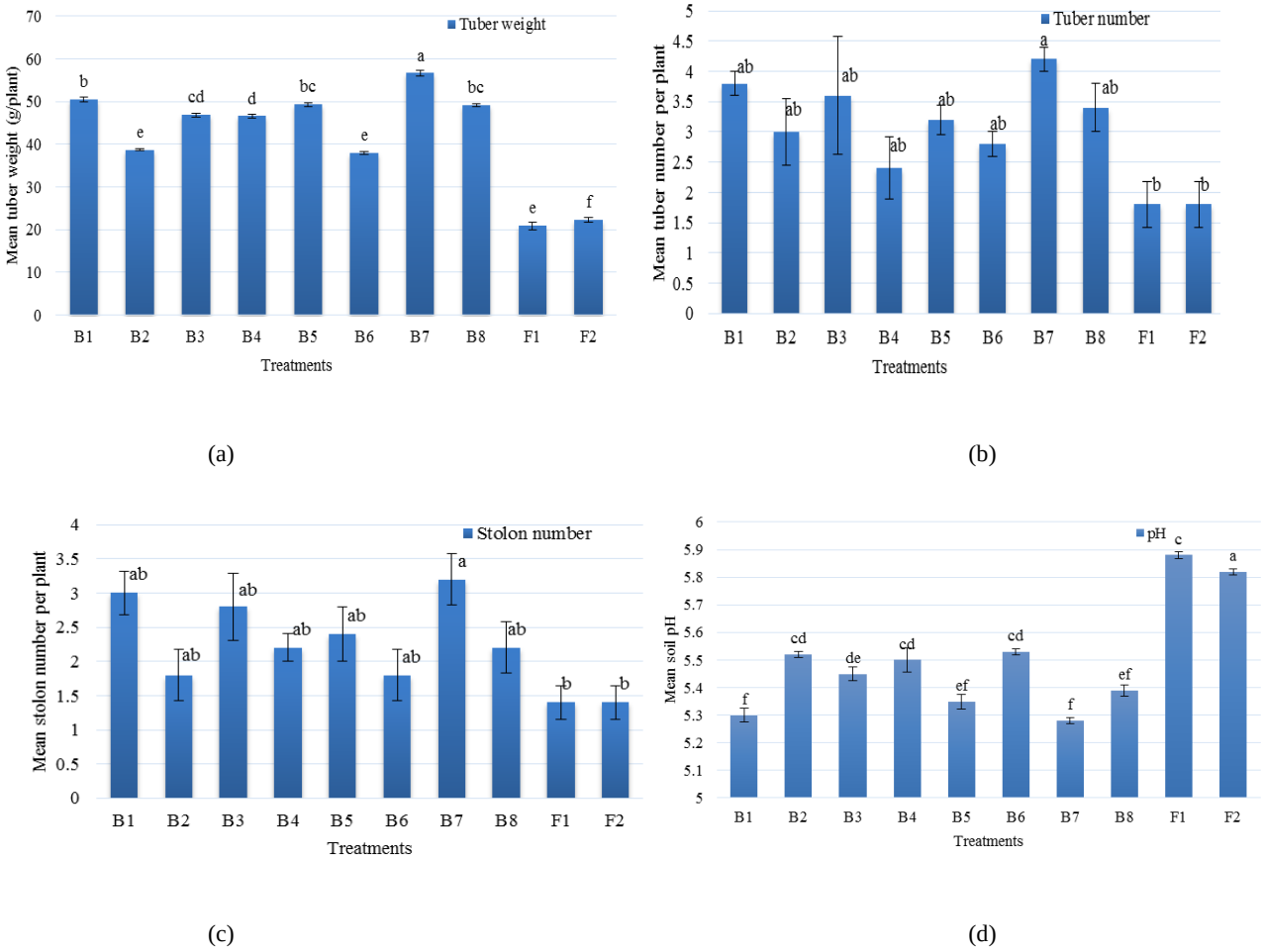


Figure 2: Correlations between different screening parameters. (a)- Non-Linear regression plot between mean ethylene production by different bacterial strains and mean inoculum pH. (b)- Non-linear regression plot between mean seedling vigor and mean IAA production by different bacterial isolates.

Evaluation of the effect of screened microbial isolates on potato tuber development

In comparison with all other treatments, the highest significant tuber weight ($P < 0.05$) was shown by plants inoculated with *Bradyrhizobium* sp. (Figure 3a). All bacterial isolates, except *Acinetobacter* sp. and *Pseudomonas* sp., enhanced the tuber weight compared to non-treated control. The highest mean number of tubers (4.2 ± 0.45) and stolon (3.2 ± 0.84), and the lowest mean soil pH (5.28 ± 0.12) were recorded with *Bradyrhizobium* sp. (Figures 3 b, c and d). Further, two sample t-test confirmed that *Bradyrhizobium* sp. significantly enhanced the mean number of tubers ($P < 0.05$) and stolon ($P < 0.05$) compared to the non- treated control. All bacterial isolates did not significantly influence the shoot ($P > 0.05$) and root dry weights ($P > 0.05$) of potato.

Significant negative relationships were observed between mean pH of soil and mean tuber weight (Figure 4a) ($R^2 = 0.94$, $P = 0.00$) and mean tuber number (Figure 4b) ($R^2 = 0.83$, $P = 0.00$). Figures 4c and 4d showed significant positive relationships of IAA concentration against tuber weight ($R^2 = 0.76$, $P = 0.03$), and tuber number ($R^2 = 0.58$, $P = 0.04$). An enhanced tuberization was recorded with the application of selected microbial inoculants.



(a) (b) (c) (d)

Figure 3: The effect of different microbial isolates on growth parameters of potato and soil pH. (a) Mean weight of potato tubers for different treatments. (b) Mean tuber number for different treatments. (c) Mean stolon number for different treatments. (d) Mean soil pH for different treatments. B1 to B8- different bacterial isolates (B1 and B8- *Bacillus* sp., B2- *Acinetobacter* sp., B3- *Acidomonas* sp., B4- and B6- *Pseudomonas* sp., B5- *Serratia* sp., B7- *Bradyrhizobium* sp.). Control – only CCM. Columns with the same letter are not significantly different at 5% probability level. Vertical bars show standard errors.

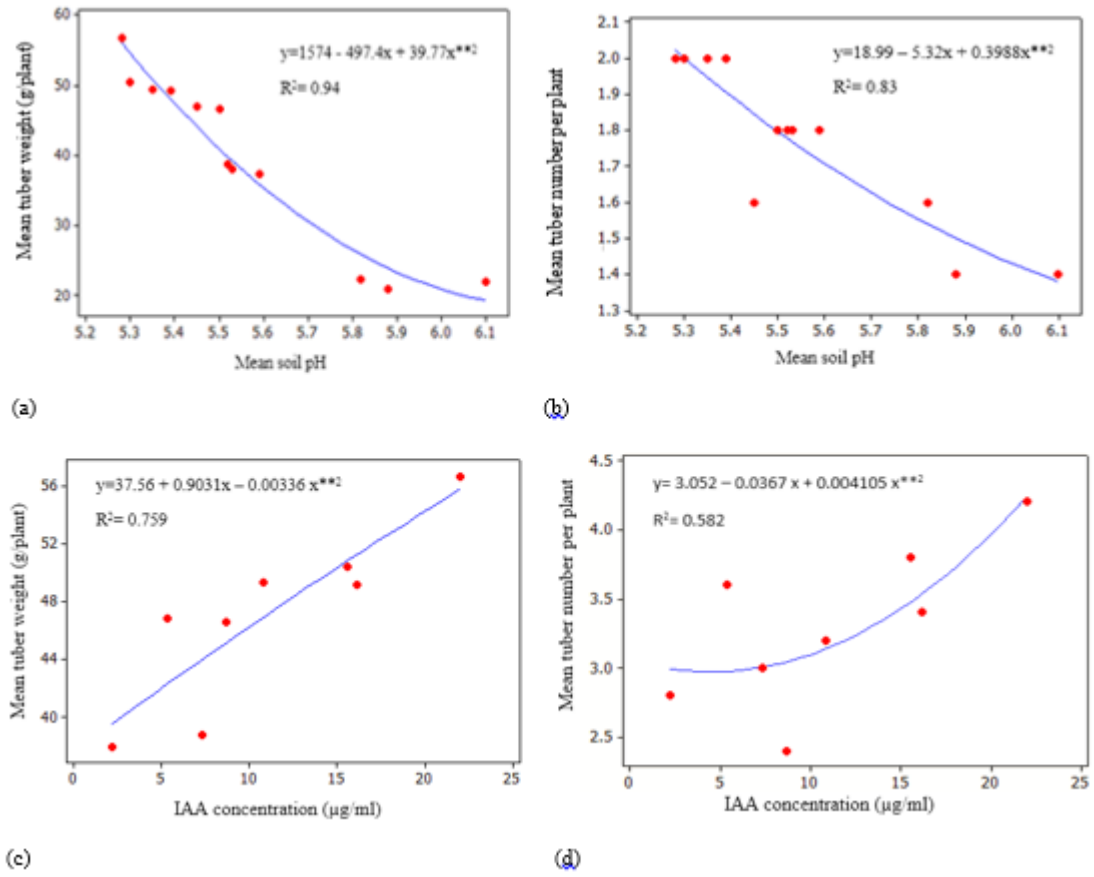


Figure 4: Non-linear regression relationships between (a) mean soil pH and mean potato tuber weight (b) the mean soil pH and mean tuber number (c) mean IAA concentration of bacterial inocula and mean potato tuber weight (d) the mean IAA concentration and mean tuber number when inoculated in a soil pot experiment.

DISCUSSION

Reduction of the pH of the medium was recorded by most of the microbial isolates. The microbial acidity is generally attributed to the production of plant growth promoting hormones like IAA (Shahab *et al*., 2009). However, reduction of pH in the growth medium can also be attributed to production of different organic and inorganic acids such as gluconic acid and 2-ketogluconic acid (Walpola and Yoon, 2012) by soil microorganisms. Production of chelating substances or inorganic acids such as sulphidric, nitric and carbonic acids may also contribute to the process (Vessey, 2003; Martinez-Viveros *et al*., 2010). Another *in vitro* study with some bacterial species showed a relationship among improved N_2 fixation, high IAA production and decreased pH (Bianco and Defez, 2010).

Further, it has been reported by several other studies that the nitrogenase activities shown by PGPRs vary between 0.25 nmoles/hr to 15.8 nmoles/hr (Sloger and Berkum, 1988; Khan and Doty, 2009). However, most of the bacterial isolates in the current study showed moderate nitrogenase activity (2.5 – 2.9 nmol/hr) compared to the previous studies. Nitrogenase activity is considered as an indirect measurement of N_2 fixation by the prokaryotes (Tissue *et al*., 1997). However, several studies have reported that PGPRs may simultaneously use mechanism besides biological N_2 fixation such as producing phytohormones like IAA, cytokinins and gibberellins to enhance plant growth stimulation (Zahir *et al*., 2004; Van Loon, 2007; Bhattacharyya and Jha, 2012).

A diverse group of microbes, including soil, epiphytic and tissue colonizing bacteria synthesizes IAA and the production is greatly varied among soil bacterial species (Patten and Glick, 1996; Xie *et al*., 1996; Acuna *et al*., 2011). It has been reported that plant growth regulators like IAA can be produced by more

than 80% of bacteria isolated from rhizosphere (Patten and Glick, 1996: Hayat *et al*., 2010). A study has shown that rhizobacteria isolated from sweet potato have the capability of producing 3.98-13.33 µg/ml IAA (Yasmin *et al.,* 2009). However, most of the bacterial isolates in the current study showed higher IAA productions ranging between 2.2-21.54 µg/ml higher than that of microbial strains reported in the literature. A similar relationship between high IAA production and decreased medium pH has been reported by another study (Ameur and Ghoul, 2012). In concurrence with that, relationship between low pH environment and IAA production by the bacterial strains like *Bacillus* sp. and *Paenibacillus* sp., has also been reported (Acuna *et al*., 2011).

Moreover, beneficial soil microorganisms which produce different phytohormones, promote plant growth while increasing germination rate of seeds (Bhattacharyya and Jha, 2012). Evidences suggest that the enhancement of seedling vigor of plants and faster germination of seeds with the inoculation of beneficial rhizobacterial strains like *Rhizobium* sp. due to the production of different phytohormones including IAA (Mia *et al.*, 2012). Further, it has been reported that the exogenous IAA produced by PGPRs enhance plant growth and development by regulating seedling vigor through stimulating root elongation and formation of lateral roots (Ahemad and Kibret, 2014).

It has been reported by previous studies that vegetative growth, tuber number and tuber yield can be enhanced (Nookaraju *et al*., 2011) by the inoculation of PGPRs with potato seed tubers before planting (Vrany and Fiker 1984; Sturz, 1995) in soil. It has been reported that beneficial microbial inocula like PGPRs in the soil solution near root hairs increase plant growth promotion through the secretion of plant growth promoters like IAA (Acuna *et al*., 2011). The application of exogenous IAA can acts as signaling molecule which can lead to early tuber initiation and tuber enlargement in potato (Harmey *et al.*, 1966; Patten and Glick, 1996). The inductive role of IAA on stolon initiation and tuber induction in potato has been documented in a previous study (Dragicevic *et al.*, 2008). Therefore, the high tuberization shown by *Bradyrhizobium* sp. inoculated potato in the present study may be due to cumulative effects of elevated IAA production and high nitrogenase activity (Figures 1c and 1b). Further, statistical ranking of the data obtained from screening and pot experiments confirmed the beneficial role of *Bacillus* sp., *Acidomonas* sp., *Serratia* sp. and *Bradyrhizobium* sp.

CONCLUSIONS

Out of the isolated bacterial species, *Bacillus* sp., *Acidomonas* sp., *Serratia* sp. and *Bradyrhizobium* sp. showed lower pHs and higher nitrogenase activities. , They also reported higher IAA production, seedling vigor and growth performance in greenhouse pot experiments with potato seed tubers. Therefore, they can be considered as beneficial PGPRs with the ability to stimulate tuberization in potato. *Bradyrhizobium* sp. showed the highest PGPRs activity in potato tuberization. Thus, *Bradyrhizobium* sp. can be considered as the best isolate to use as an inoculum to improve growth and tuberization in potato.

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