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ACINETOBACTER CALCOACETICUS AMELIORATED PLANT GROWTH AND INFLUENCED GIBBERELLINS AND FUNCTIONAL BIOCHEMICALS

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Abstract

Acinetobacter calcoaceticus, a plant growth promoting rhizobacteria (PGPR) and gibberellins producing strain was investigated for the presence of organic acids in its culture and interactive effect on cucumber plant's growth, phytohormonal levels and functional biochemicals. *A. calcoaceticus* culture's analysis showed the presence of three organic acids viz. malic, succinic and citric acids. The quantity of malic acid was significantly high compared to succinic and citric acids. Besides that, the free phosphate level was highest at the fifth day of bacterial culture growth. In *A. calcoaceticus* and plant's association experiment, the strain has significantly ameliorated cucumber plants to higher growth. The PGPR culture application had higher shoot length; plant biomass and chlorophyll contents compared to controls (distill water-DW and nutrient broth-NB). The bacterial culture treated plants has higher amino acid and crude protein contents compared DW and NB. In amino acid analysis, threonine, alanine and proline quantities were significantly higher in PGPR treated plants than control plants. *A. calcoaceticus* treated plants had low endogenous abscisic acid contents but contrarily higher GAs (GA_1, GA_4, GA_9 and GA_{20}) compared to controls (DW and NB). The PGPR has activated the GAs biosynthesis pathway while promoting the cucumber plant's growth. Application of such eco-friendly PGPR can be a viable alternative to synthetic fertilizers.

Introduction

Rhizosphere is rich sources of various microorganisms that can benefit plant growth and survival. Among those, bacteria are of key importance to plants especially to crops. Various classes of bacteria have been reported to improve plant growth and metabolism, known as plant growth promoting rhizobacteria (PGPR). Various strains of *Bacillus, Enterobacter, Burkholderia, Acinetobacter, Alcaligenes, Arthrobacter, Azospirillium, Azotobacter, Beijerinckia, Erwinia, Flavobacterium, Rhizobium* and *Serratia* (Rodriguez & Fraga, 1999; Sturz & Nowak, 2000; Sahin *et al*., 2004) have been identified as PGPR. The beneficial effects of these rhizobacteria on plant growth can be direct or indirect. PGPR are residing in rhizoshpere and establishing a mutualistic association with the host plants. In the presence of PGPR, plant growth is facilitating in nutrient's uptake, water use efficiency, physical modification of rhizosphere and curtailing environmental stresses. In response, plant provides PGPR with a living sanctuary, nutrients, and reproduction to the next generation (Gulati *et al*., 2009; Lugtenberg & Kamilova 2009). Thus plant receives an extra strength to develop and grow more steadily compared to plants lacking such associations. The PGPR promote plant growth through numerous ways i.e. (i) secretion of phytohormones like gibberellins, auxins, etc (ii) changing chemo-physiology of rhizosphere by ensuring availability of functional mineral/nutrients, (iii) rescuing plant in abiotic stresses and (iv) decreasing pathogenic attacks by releasing antibiotics or toxins (Glick *et al*., 1995; Lugtenberg & Kamilova, 2009; Rodriguez & Fraga, 1999). They also compete with pathogens for nutrients or specific niches on the root and induce systemic resistance (Bloemberg & Lugtenber, 2001; O'Connell, 1992). In short, PGPR can act as biofertilizers as some other studies has also elaborated this (Adesemoye *et al.*, 2009; Lugtenberg & Kamilova, 2009).

With increasing human population, demands for higher crop production and food supply have persuaded farmers to use synthetic fertilizers in agricultural fields. Complying with such food demands while using synthetic fertilizers has not only affected the long-term fertility of agricultural lands but also has paved a way for development of numerous environmental problems. Although alternatives to synthetic fertilizers are also available however, bio-fertilizers, including microorganisms can be a viable opportunity to explore. PGPR may add nitrogen to the soil by symbiotic or asymbiotic N_2 fixation, while some bacteria such as *Bacillus* are widely used in plant production system and are important phosphorus-solubilizing microorganisms, resulting in improved growth and yield of crops (Dobereiner, 1997). The production of plant growth regulators such as auxin, cytokinin and gibberellin by these bacteria may also give an additional support to growth and development of host plant species (Joo *et al.,* 2009; Kang *et al.,* 2009). Previously research studies on *Acinetobacter* focused on growth potential, indole-3-acetic acid (IAA) production, inorganic phosphate-solubilization, and nitrogen fixation (Huddedar *et al.*, 2002; Indiragandhi *et al.*, 2008; Khan *et al.,* 2011b).

Acinetobacter calcoaceticus was recently reported as gibberellins producing bacterium (Kang *et al.*, 2009). *A. calcoaceticus* is known as human pathogen revealing pathological features like DNase and hemolytic activity as biocontrol agents (Hebbar *et al.,* 1999). Recent literature indicates its positive role in plant growth enhancement and biologically active metabolites production (Indiragandhi *et al*., 2008; Kang *et al.,* 2009). Although, *A. calcoaceticus* strains from soybean and canola have been reported to

improve plant growth (Prashant *et al*., 2009), however, their effect on plant physiology and functional biochemicals still to be understood. Thus, using such beneficial strain as bio-fertilizers instead of synthetic chemicals will not only improve plant growth and development but will also help to enhance soil and crop productivity (O'Connell, 1992). The objective of the current study was to investigate the influence of *A. calcoaceticus* on growth attributes, endogenous gibberellins, abscisic acid, crude protein and amino acid contents of cucumber plants. The bacterial culture was also explored for the presence of organics acids and free phosphate.

Materials and Methods

Bacterial strain isolation and identification: The bacterial strain, *A. calcoaceticus* tested in the study was present with authors and has already been reported as gibberellins producing bacterial strain (Kang *et al*., 2009). Bacterial strain was isolated from soil sample as described by Kang *et al*., (2009). Isolate SE370 was identified as a new strain of *A. calcoaceticus* on the basis of 16S ribosomal DNA (rDNA) sequence and phylogenetic analysis and was give accession number KCTC11095BP (Kang *et al*., 2009).

***A. calcoaceticus* and cucumber plant's growth:** The bacteria culture suspension was incubated for 3 days at 30□ on a shaking incubator at 200 rpm to an estimated cell density of 10^8 CFU/ml. Cucumber seeds (Seminis Korea Co. Korea) were surface sterilized with NaOCl (5%) for 10 min and thoroughly rinsed with autoclaved distilled water. Seeds were sown in autoclaved plastic pots under controlled green house conditions at 30±2ºC. Cucumber seedlings were treated with 5 ml of bacterial suspension after 2 weeks of sowing and the growth attributes i.e., shoot length, shoot & root fresh weight, shoot & root dry weight, and chlorophyll contents were recorded after 21 days of treatment. The experiment comprised three treatments and four replicates. Each replicate consists of 24 plants. Distilled water and nutrient broth (NB) were used as negative and positive controls respectively during the experiment. The chlorophyll contents of all fully expanded leaves were analyzed with Chlorophyll Meter (Minolta Co., Ltd, Japan).

Endogenous phytohormonal analysis: After three weeks of *A. calcoaceticus* suspension treatment, the plants were harvested, immediately frozen in liquid nitrogen and lyophilized. The lyophilized plant samples were used for the extraction and quantification of endogenous gibberellins and absicic acid (ABA) based on the already established procedure of Lee *et al*., (1998) and Kamboj *et al.,* (1999) respectively. In GA analysis, 0.5g lyophilized sample was used. The GC (Hewlett-Packard 6890, 5973N Mass Selective Detector) with HA-1 capillary column (30m × 0.25mm i.d. 0.25µm film thickness) oven temperature was programmed for 1 min at 60°C, then a rise of 15°C min^{-1} to 200°C, followed by 5°C min^{-1} to 285°C. Helium carrier gas was maintained at a head pressure of 30 kPa. The GC was directly interfaced to a Mass Selective Detector with an interface and source temperature of 280°C, an ionizing voltage of 70 eV and a dwell time of 100 ms. Full scan mode (the first trial) and three major ions of the supplemented [$^2H_2$] GAs internal standards (the second trial) and the endogenous gibberellins were monitored simultaneously (standard GAs were purchased from Prof. Lewis N. Mander, Australian National University, Canberra, Australia). The endogenous GAs contents were calculated from the peak area ratios respectively. Retention time was determined by the hydrocarbon standards to calculate the KRI (Kovats Retention Indices) value (Kovàts, 1958). The data was calculated in nano-grams per gram dry weight and the analysis was repeated three times, using different sample each time.

In ABA analysis, plant samples were extracted with 30ml of extraction solution containing 95% isopropanol, 5% glacial acetic acid, and 10mg of [(±)-3,5,5,7,7,7-d6]-ABA. The filtrate was concentrated by a rotary evaporator. The residue was dissolved in four ml of 1 N NaOH solution, and then washed three times with three ml of methylene chloride to remove lipophilic materials. The aqueous phase was brought to approximately pH 3.5 with 6 N HCl and partitioned three times into ethyl acetate (EtOAc). EtOAc extracts were then combined and evaporated. The dried residue was dissolved in phosphate buffer (pH 8.0) and then run through a polyvinylpolypyrrolidone (PVPP) column. The phosphate buffer was adjusted to pH 3.5 with 6 N HCl and partitioned three times into EtOAc. The EtOAc extracts were combined again and evaporated. The residue was dissolved in dichloromethane, and passed through a silica cartridge (Sep-Pak; Waters Associates, Milford, MA, USA), which was pre washed with 10 ml of diethyl ether: methanol (3: 2, v/v) and 10 ml of dichloromethane. ABA was recovered from the cartridge by elution with 10 ml of diethyl ether: methanol (3: 2, v/v). The extract was dried and methylated by adding diazomethane for analysis through GC/MS SIM. For ABA quantification, the Lab-Base (ThermoQuset, Manchester, UK) data system software was used to monitor responses to ions of m/e 162 and 190 for Me-ABA and 166 and 194 for Me-[$^2H_6$]-ABA.

Organic acid and free phosphate contents: For the analysis of organic acids, bacterial cultures were filtrates through 0.22 µm Millipore filter and 10µℓ of filtrates were injected to HPLC (Model: Waters 600E) equipped with a Refractive Index Detector (Model: Waters 410). Column: RSpak KC-811(8.0 x 300mm), Eluent: 0.1% H3PO4/H2O, Flow rate: 1.0 *ml*/min, Temperature: 40ºC. Phosphate solubilizing A. calcoaceticus was inoculated into 100 ml containing tricalcium phosphate at 30ºC for 7 days; pH of the medium was recorded with a pH meter equipped with glass electrode. Quantitative spectrophotometric analysis of the soluble phosphate was performed according to the method of King (1932).

Amino acid and crude protein quantification: The amino acid composition of PGPR treated plants and controls was determined with a Biochrom 20 autoanalyzer (Pharmacia) after hydrolysis of 100 mg of protein with 6 M HCl at 110°C for 24 h. Crude protein contents of cucumber plants treated with DW, NB and PGPR was calculated according to % N×6.25.

Statistical analysis: The data was analyzed for standard error using sigma plot software (2004). The mean values were compared using Duncan's multiple range test at $p \leq 0.05$ (ANOVA SAS release 9.1; SAS Cary, NC, USA).

Results and discussion

Effect on cucumber plant's growth: The effect of bacterial culture suspension (*A. calcoaceticus*) on the growth attributes (shoot length, shoot fresh/dry biomass, root fresh/dry biomass and chlorophyll contents) of cucumber plants were significant ($p>0.05$) compared to both the positive (nutrient broth-NB) and negative (distill water-DW) controls (Table 1). Results indicate that *A. calcoaceticus* application resulted in 66.58% and 40.87% higher shoot lengths of cucumber plants in comparison with DW and NB. Fresh weight of PGPR treated plants were 22.17% and 39.98% higher than NB and DW respectively. A similar significant trend was also observed for the root dry weights and chlorophyll contents (Table 1). The beneficial effects of PGPR have been demonstrated for many agricultural crop species like wheat (Khalid *et al.,* 2004), tobacco (Kloepper *et al.,* 1991; Zhang *et al.,* 2004), *Brassica juncea* (Asghar *et al.*, 2002) tomato (Kidoglu et al., 2008), bell pepper and cucumber (Kidoglu et al., 2008) and barley (Cakmakci *et al.,* 2007). Present study also reflect the same findings however, the effects of *A. calcoaceticus* (accession no. KCTC11095BP) has been reported for the first time, while previously it was identified as a gibberellins producing strain. This growth promotion capacity of *A. calcoaceticus* might be attributed to its potential to secrete gibberellins. There are various studies, which suggested the ameliorative influence of PGPR application and plant growth while also synthesizing phytohormones (Gulati *et al.,* 2009; Lucy *et al*., 2004; Prashant *et al*., 2009). Cucumber plants being sensitive to varying environmental conditions and application of such strain will be helpful to rescue plant growth as previous reports also showed this (Yang *et al.*, 2008). The excessive use of chemical fertilizers, herbicides, and pesticides in modern agriculture has severely modified and polluted our existing ecosystems. Plant growth promoting rhizobacteria (PGPR) offer a valid alternative to chemical fertilizers, herbicides and pesticides but their effectiveness often lacks consistency (Bevivino *et al.,* 1998). However, gibberellins producing *A. calcoaceticus* can extend greater flexibility and performance in the field conditions compared to other strains.

Table 1. Effect of bacterial culture suspension application on the growth attributes of cucumber plants.

Treatments	Shoot length (cm/plant)	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)	Chlorophyll (SPAD)
Distilled H_2O	16.49 ± 1.07^{c}	4.49 ± 0.29^{c}	0.49 ± 0.07^{b}	4.46 ± 0.3^{c}	0.27 ± 0.05^{b}	30.29 ± 0.8^{b}
NB	19.51 ± 1.22^{b}	5.14 ± 0.31^{b}	0.53 ± 0.06^{b}	3.83 ± 0.5^{b}	0.34 ± 0.06^{b}	30.41 ± 1.7^{b}
A. calcoaceticus	27.47 ± 1.69^{a}	6.28 ± 0.43^{a}	0.73 ± 0.03^{a}	5.53 ± 1.02^{a}	0.47 ± 0.15^{a}	34.67 ± 1.42^{a}

In a column, treatment means having a different letter(s) are significantly different at the 5% level by DMRT. A 10 ml bacterial culture suspension was given to plant seedlings after 41 days of sowing and the resultant growth promotion was measured after 21 days of treatment. NB stands for nutrient broth.

Effect on plant's endogenous ABA and Gibberellins: Plants treated with *A. calcoaceticus* culture had lower endogenous ABA contents compared to NB and DW (Fig. 1). However, NB had significantly higher ($p>0.05$) ABA contents. In case of endogenous GAs analysis, the results showed that bioactive GA_1 and GA_4 quantities were significantly higher in *A. calcoaceticus* treated plants than both positive and negative controls (Fig. 2). GA_1 and GA_4 quantities were 0.73±0.04 and 6.95±0.13 respectively. The amount of physiologically inactive GAs i.e. GA_9 and GA_{20}, which are the immediate precursors of GA_1 and GA_4, indicated a similar trend (Fig. 2). Plant growth hormones play a vital role in plant growth and their responses are of significant importance in understanding acclimation mechanism of plants. Gibberellins (GAs) are plant growth hormones specialized for plant growth and development, while ABA is a well know plant stress hormone. Our study showed that application of *A. calcoaceticus* significantly promoted endogenous bioactive GA_1 and GA_4 and their immediate precursors. Presence of GA_1 and GA_4 showed that both early C-13 hydroxylation and non C-13 hydroxylation pathways are operational in cucumber, while a significantly higher amount of GA_4 suggests that the major GA biosynthesis pathway in Cucumber is the non C-13 hydroxylation pathway. On the other hand, ABA contents of Cucumber plants treated with *A. calcoaceticus* significantly lowered as compared to NB and DW treatments. It is well understood that ABA contents increase in response to abiotic stress and can inhibit plant growth by lowering leaf area and shoot length (Khan *et al.*, 2011a). A decrease in ABA contents in *A. calcoaceticus* inoculated plants suggests favorable environment provision by PGPR.

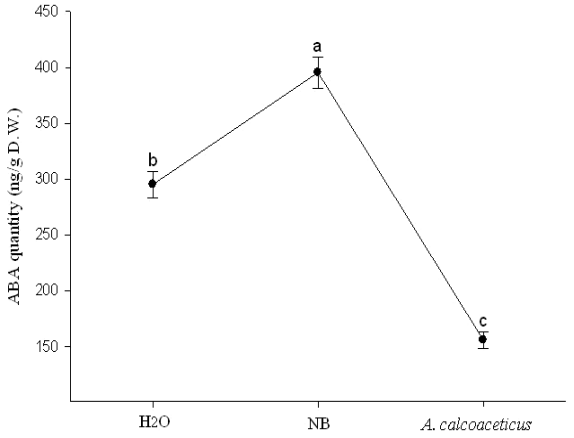

Fig. 1. Effect on the quantity of ABA biosynthesis in cucumber plants during *A. calcoaceticus* interaction in comparison with control. Cucumber plants were treated with PGPR (*A. calcoaceticus*), distilled water (H_2O) and nutrient broth (NB). a,b and c represents Duncan's Multiple Range Test (DMRT) analysis which refers significant difference between the control and PGPR treatments at $p<0.05$.

Organic acid and free phosphate analysis: HPLC analysis of the culture filtrates was done to identify and quantify the organic acids produced during the growth of *A. calcoaceticus* in a culture medium. Succinic, malic and citric acids were found during the analysis. The chromatograms have been given in Figure 3. The results showed that the quantity of malic acid was significantly higher than the succinic and citric acids (Fig. 4). The NB medium was verified for the presence of organic acid but no peak was observed during HPLC analysis. Similar, study was also conducted on the *Pseudomonas fluorescens*, which produced oxalacetic, gluconic, fumaric and oxalic acids in different bacterial growth mediums (Henri et al., 2008) while it promoted the growth of maize plants. Same findings were also reported for different strains of *Pseudomonas* (Henri *et al.*, 2008, Vyas & Gulati, 2009) however, we couldn't found any reports of organic acid production by *A. calcoaceticus*. Previously we reported that *A. calcoaceticus* shows higher phosphate solubilization in National Botanical Research Institute's Phosphate (NBRIP) growth media plates (Kang *et al.,* 2009). In the present study, it was observed that during the growth of *A. calcoaceticus* in the culture medium, the amount of free phosphate increased. It was maximum on the fifth day of bacterial growth (Fig. 5).

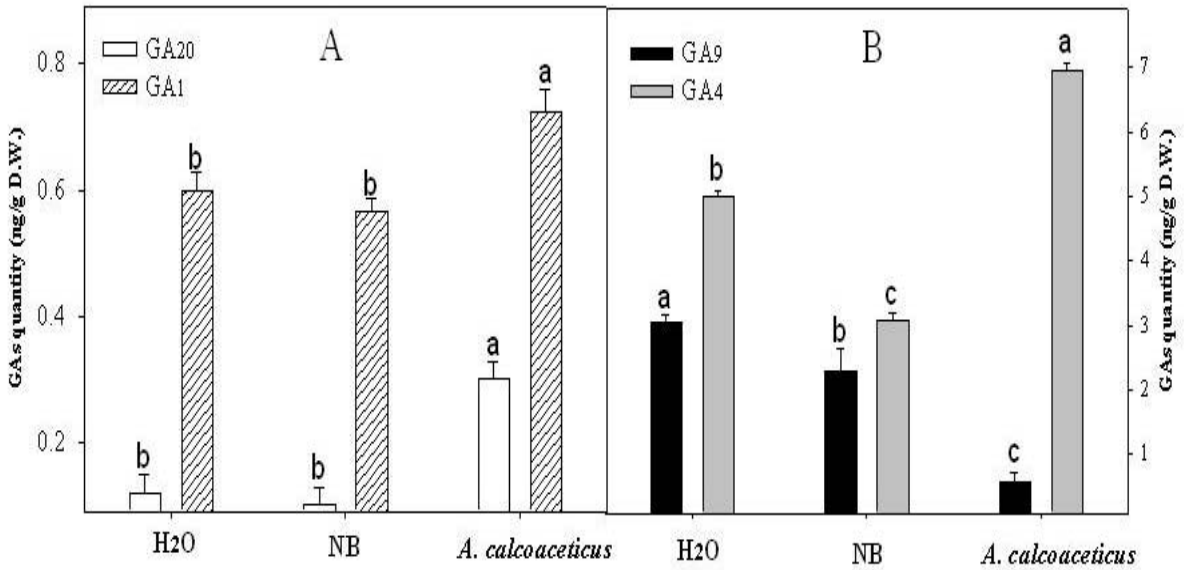

Fig. 2. Influence of *A. calcoaceticus* inoculation on endogenous gibberellins biosynthesis. Cucumber plants were applied with PGPR (*A. calcoaceticus*), distilled water (H_2O) and nutrient broth (NB). For each set of treatment, the different letter (s) indicates significant differences between plants treated with PGPR and controls at $p<0.05$ level by (DMRT).

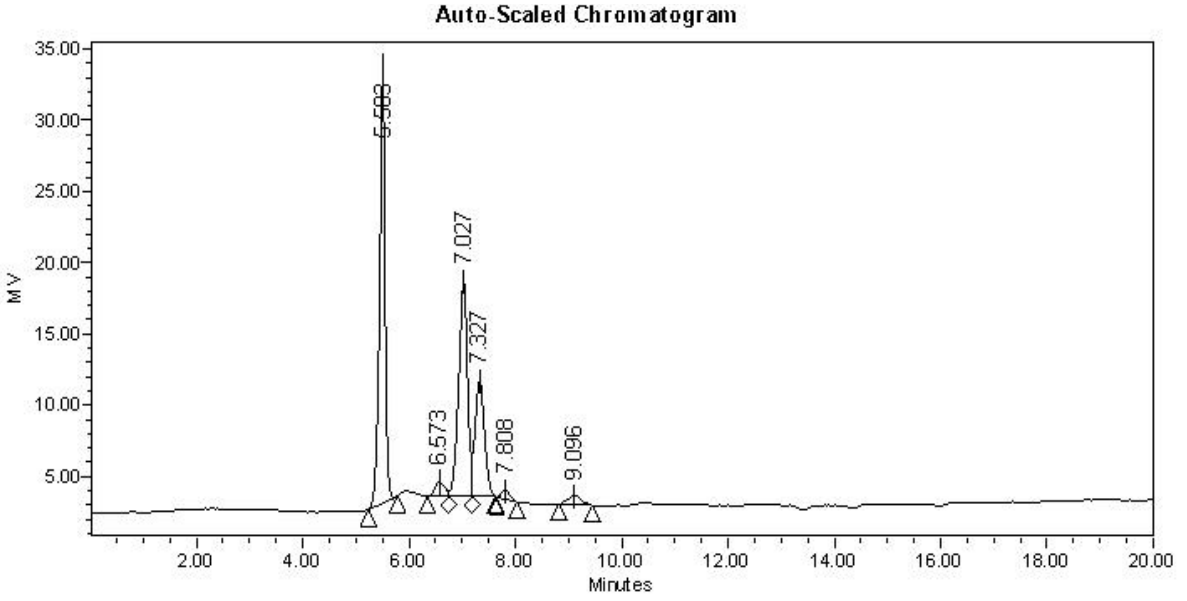

Fig. 3. Chromatographs of the three organic acids (citric acid – A, malic acid – B and succinic acid – C) found in the bacterial culture of *A. calcoaceticus.*

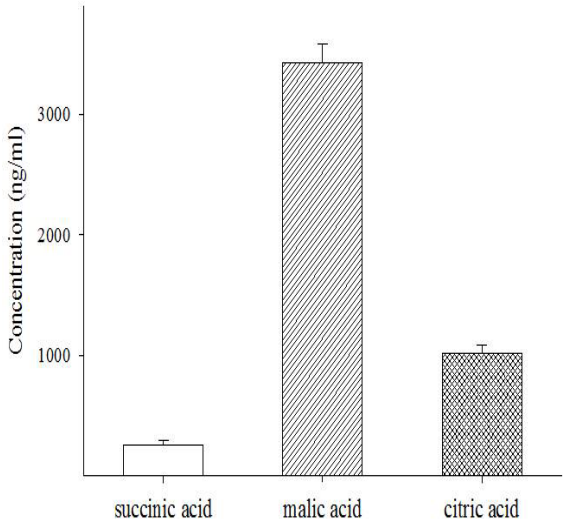

Fig. 4. Occurrence of organic acid in the bacterial culture. n=3.

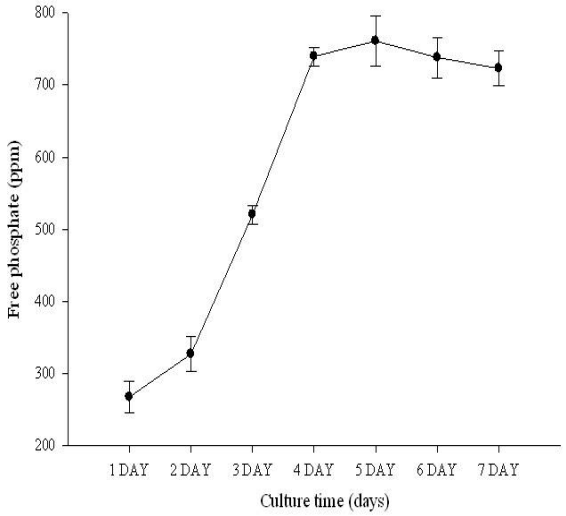

Fig. 5. The quantity of free phosphate in the bacterial culture. n=3.

Effects on plant's functional biochemicals: Amino acids are the main building blocks for synthesis of various essential biochemicals including proteins in plants (Lam *et al*., 1996). In amino acids analysis, the results indicate that the *A. calcoaceticus* inoculated cucumber plants had significantly higher amount of amino acids than DW and NB applied plants (Table 2). Among these amino acids, the quantity of threonine was much pronounced (212.86%) than NB treated plants. Alanine and proline were the second and third in this surge. The percentage of other amino acids in comparison to NB control plants have been mentioned in Figure 6. Similarly, in crude protein analysis, the amount of protein was higher in inoculated plants than controls (Fig. 7).

Table 2. Functional amino acid composition of cucumber plant samples.

mg/g D.W.	H2O	NB	*A. calcoaceticus*
Asp	15.81 ± 0.15	18.16 ± 1.85	18.15 ± 0.65
Thr	8.61 ± 0.52	7.93 ± 0.13	24.81 ± 0.42
Ser	8.71 ± 0.34	7.92 ± 0.19	9.39 ± 0.76
Glu	21.07 ± 1.5	24.32 ± 0.08	30.33 ± 0.71
Pro	8.15 ± 0.32	7.63 ± 0.15	13.13 ± 1.19
Gly	9.62 ± 0.41	8.27 ± 1.41	10.88 ± 1.57
Ala	11.52±0.912	10.31±0.85	21.10 ± 0.18
Cys	17.39 ± 0.45	17.43 ± 0.59	20.37 ± 0.19
Val	5.65 ± 0.62	5.75 ± 0.11	6.03 ± 0.18
Met	3.95 ± 0.356	3.99 ± 0.71	4.95 ± 0.09
Ile	6.16 ± 0.71	7.17 ± 0.85	7.26 ± 1.44
Leu	11.92 ± 2.05	12.27 ± 0.29	15.11 ± 2.21
Tyr	3.62 ±1.11	4.68 ± 1.23	5.69 ± 0.47
Phe	7.48 ± 0.106	8.76 ± 2.09	10.66 ± 0.15
His	3.13 ± 0.27	3.17 ± 0.64	4.16 ± 0.56
Lys	9.01 ± 0.98	8.94 ± 0.61	11.32 ± 0.41
Arg	7.38 ± 0.27	7.55 ± 0.78	9.09 ± 0.87

An increase in amino acid contents in the leaves of cucumber might be due to an increase in the rate of photosynthesis and metabolism activated by the application of *A. calcoaceticus*. Previously, Kim & Leustek (2000) reported that threonine levels in Arabidopsis plants increased from four- to sevenfold in comparison with the wild type in which methionine levels were reduced to 35%. Threonine is an essential amino acid and plays a pivotal role in human health. Plants synthesize threonine from aspartate using two different branches of the aspartate family pathway (Galili, 1995). Similarly, the increased proline contents after the application of *A. calcoaceticus* is an indicative adjustment of leaf osmotic potential, required for enhanced intracellular osmotic balance (Evelin *et al.,* 2009; Khan *et al.,* 2011ab). Phenylalanine is a precursor of various phenylpropanoids, which are important in plant metabolism. Salicylic acid formation in plants was believed to occur via a branch of phenylpropanoid metabolism, involving side-chain shortening of cinnamic acid by either an oxidative route analogous to the β-oxidation of fatty acids (Dixon *et al.,* 2002). Salicylic acid has been known to play a vital role in systemic acquired resistance (SAR) upon microbial infection with plants and can improve plant growth in stressful situation (Khan *et al.*, 2011ab).

Conclusion

Results of the study suggest that *A. calcoaceticus* is a novel PGPR due to its capacity to promote plant growth and metabolism, cause ameliorative effect on plant's endogenous ABA and GAs, amino acids and crude protein contents. Besides production of various physiologically active and inactive gibberellins (Kang *et al.,* 2009), the strain produces succinic, malic and citric acids in the growth culture. The strains shows favorable affect to cucumber plant growth and hence indicates broader field trails as a bio-fertilizer for higher crop production and eco-friendly farming systems.

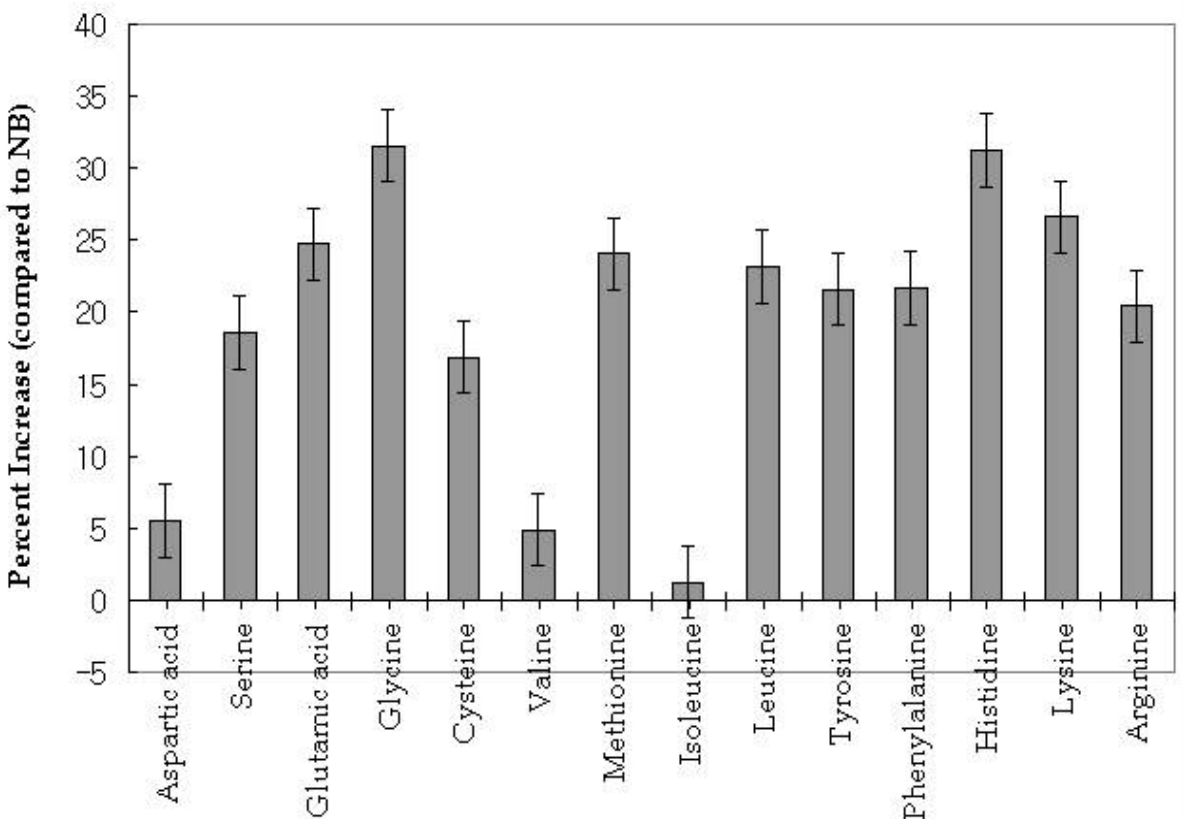

Fig. 6. Effect of *A. calcoaceticus* application on the quantity of various amino acids. The graph shows the percent amount in comparison to the amount of amino acids found in positive control (NB treated plants). n=3.

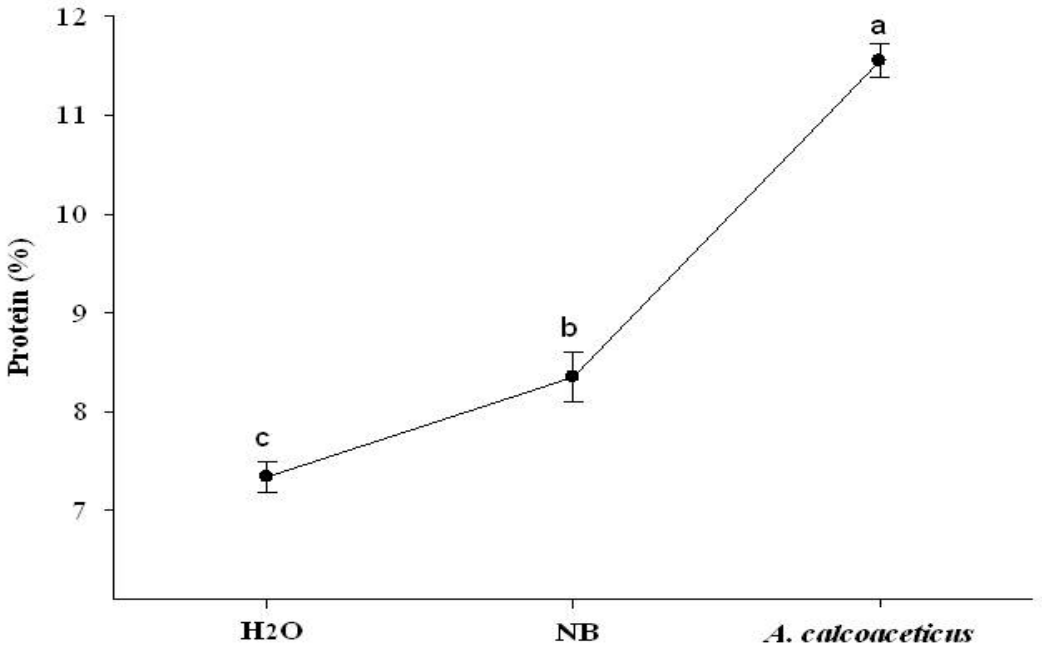

Fig. 7. Crude protein contents analysis in the cucumber plants treated with *A. calcoaceticus* and controls. Cucumber plants were applied with PGPR (*A. calcoaceticus*), distilled water (H_2O) and nutrient broth (NB). For each set of treatment, the different letter (s) indicates significant differences between plants treated with PGPR and controls at $P < 0.05$ level by (DMRT). n=3

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