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POTENTIAL OF PLANT GROWTH PROMOTING RHIZOBACTERIA AND CHEMICAL FERTILIZERS ON SOIL ENZYMES AND PLANT GROWTH

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Abstract

The present investigation deals with the role of Plant Growth Promoting Rhizobacteria and chemical fertilizers alone or in combination on urease, invertase and phosphatase activities of rhizospheric soil and also on general impact on growth of safflower cvv. Thori and Saif-32. The PGPR (*Azospirillum brasilense* and *Azotobacter vinelandii*) were applied at 10^6cells/mL as seed inoculation prior to sowing. Chemical fertilizers were applied at full (Urea 60 Kg ha^{-1} and Diammonium phosphate (DAP) 30 Kg ha^{-1}), half (Urea 30 Kg ha^{-1} and DAP 15 Kg ha^{-1}) and quarter doses (Urea 15 Kg ha^{-1} and DAP 7.5 Kg ha^{-1}) during sowing. The chemical fertilizers and PGPR enhanced urease and invertase activities of soil. Presence of PGPR in combination with quarter and half doses of chemical fertilizers further augmented their effect on soil enzymes activities. The soil phosphatase activity was greater in *Azospirillum* and *Azotobacter* in combination with half dose of chemical fertilizers. Maximum increase in leaf melondialdehyde content was recorded in full dose of chemical fertilizers whereas coinoculation treatment exhibited significant reduction in cv. Thori. Half and quarter dose of chemical fertilizers increased the shoot length of safflower whereas maximum increase in leaf protein was recorded in *Azotobacter* in combination with full dose of chemical fertilizers. Root length was improved by *Azospirillum* and *Azotobacter* in combination with quarter dose of chemical fertilizers. Leaf area and chlorophyll contents were significantly improved by *Azotobacter* in combination with half dose of chemical fertilizers. It is inferred that PGPR can supplement 50 % chemical fertilizers for better plant growth and soil health.

Introduction

Soil is an important ecological niche for microbial community. The excessive uses of chemical fertilizers not only affect the soil health and soil physicochemical properties but also pollute the ecosystem in addition to the depleting resources and high cost; therefore, efforts are being made to replace chemical fertilizers with more sustainable, environmental friendly and cost effective measures such as PGPR. Plant Growth Promoting Rhizobacteria (PGPR) constitutes a group of bacteria that colonize root surfaces and improve plant growth and development (Wu *et al.,* 2005). Previous studies demonstrate the positive role of PGPR inoculation on crop production (Egamberdiyeva, 2007).

PGPRs have been reported to boost the plant growth by bringing improvements in the soil quality. Soil enzymes are essential for turnover of organic matter and the metabolic activity of soil microorganisms (Nannipieri *et al.,* 2002). The most active enzymes in soil include protease, urease, pectinase, cellulase, dehydrogenase, catalase, amylase and phosphatase. Ureases catalyze the hydrolysis of urea to CO_2 and NH_3, which is a vital process in the regulation of N supply to plants after urea fertilization. It also makes the availability of the nitrogen that has been leached down. It is believed that urea is a slow releasing fertilizer and undergoes transformation through two steps. First step is the urea transformation into carbonate and ammonia in the presence of urease enzyme and in the second step ammonia is converted to nitrite and then to nitrates ions which are readily available for the direct use of plant (Falih, 2000).

In soil, ureases are tightly bound to soil organic matter and minerals, and were demonstrated to correlate with soil nutrients (Li *et al.,* 2006). Invertase is a hydrolase, cleaving sucrose into two monosaccharides and hence provides energy for germination. Phosphatases have been detected on root surfaces and in rhizosphere soil. Hydrolytic cleavage of P, by extracellular phosphatases of microbial or root origin is one mechanism of such mineralization. Previously it was reported that the application of arbuscular mycorrhizal fungi and PGPR enhanced the soil enzyme activities such as urease, dehydrogenase and phosphatase and ultimately improved soil quality (Mäder *et al.,* 2011). Wu *et al.* (2012) reported that nitrogen fixing bacteria improved the urease and phosphatase activity of the soil.

Malondialdehyde (MDA) is a major cytotoxic product of lipid peroxidation and has been used widely as an indicator of free radical production (Mohammadkhani & Heidari, 2007), the concentration of MDA in the cell or tissue shows the degree of cell macromolecules destruction resulting in the loss of the cell function and ultimately cell death (Stoparic & Maksimovic, 2008). Safflower is an important oil seed crop and is tolerant to drought and salt stress. It can also be grown on soil with poor fertility.

Keeping in view the importance of PGPR in improving plant growth and those of soil enzymes in maintaining soil fertility and in order to economize the use of commercial fertilizers, the present investigation was aimed to assess the role of PGPR alone and in combination with commercial fertilizers on safflower growth and their impact on some biologically important soil enzymes which take part in improving the soil health.

Material and Method

Plant material and growing conditions: Certified seeds of Safflower cv. Thori (spineless) and cv. Saif-32 (spiny) were obtained from National Agricultural Research Centre (NARC), Islamabad, were surface sterilized with 10% chlorox solution for 3 min and subsequently washed three times with sterilized distilled water and then sterilized with

95% ethanol and finally washed with distilled sterilized water. The seeds were sown in plastic pots (11x8 cm^2) filled with soil and sand (1:1) in the green house under controlled environmental conditions. The pots were arranged in complete randomized design (CRD).

The PGPR were applied as seed inoculation @10^6cells/mL and the number of bacterial cells/seed were measured as $4x10^5$. The chemical fertilizers were applied as aqueous solution at the time of sowing.
Following treatments were made.

Treatments	Symbols
Control (without inoculation and without chemical fertilizers)	C
Chemical fertilizers full dose (urea 60 Kg ha^{-1} and DAP 30 Kg ha^{-1})	CFF
Chemical fertilizers half dose (urea 30 Kg ha^{-1} and DAP 15 Kg ha^{-1})	CFH
Chemical fertilizers quarter dose (urea 15 Kg ha^{-1} and DAP 7.5 Kg ha^{-1})	CFQ
Single inoculation of *Azospirillum brasilense* (accession no. GQ255949)	SP
Azospirillum brasilense + full dose of chemical fertilizers	SPF
Azospirillum brasilense + half dose of chemical fertilizers	SPH
Azospirillum brasilense + quarter dose of chemical fertilizers	SPQ
Single inoculation of *Azotobacter vinelandii* (accession no. GQ849485)	BT
Azotobacter vinelandii + full dose of chemical fertilizers	BTF
Azotobacter vinelandii + half dose of chemical fertilizers	BTH
Azotobacter vinelandii + quarter dose of chemical fertilizers	BTQ
Inoculation with consortium of *A. brasilense* + *A. vinelandii*	SPBT

The soil nutrient analysis was carried out according to the method of Soltanpour and Schwab (1977) by Ammonium Bicarbonate-DTPA method. Ten gram of oven dried soil was taken in a conical flask and then 20 mL of extraction solution DTPA was added. The mixture was shaken for 30 min. utes(remove) and then filtered by(remove) using filter paper (Whatman No. 40). The extracted aliquots were kept in capped bottles for analysis on Atomic Absorption Spectrophotometer (Spectra AA-100, Varian).

Soil organic matter was determined by the method of Nelson and Sommers (1982). Plant nitrogen analysis was carried out according to micro-kjeldahl digestion method. The plant material was digested with concentrated H_2SO_4 (20mL) and then it was allowed to cool. Digested mixture was transferred in 50 mL flask and digestion was carried out with 40% NaOH (20 mL) and it was collected in boric acid (5 mL). Then titration of distillate was carried out against H_2SO_4 (0.1 N) and $KMNO_4$ was used as indicator.

Soil phosphorus contents were measured by(remove) using the method of Olsen and Sommers (1982). The sample preparation was carried out by mixing of 2 g of soil with 0.5 M of NaHCO3 (40 mL) and then this mixture was mixed homogeneously on mechanical shaker for 30 minutes. After filtration, 10 mL of filtrate was taken in a flask and colouring reagent was added and finally volume was made upto 50 mL with distilled water. After development of colour, optical density was measured at wavelength of 880 nm.

Electrical conductivity was determined from a saturated paste of soil which was prepared by suspending soil into the water with constant stirring for 30 minutes, then after settling, the electrical conductivity was measured by conductivity meter (KL-138). Soil pH was measured by preparing the saturated paste of soil aas above method and then(remove) after settling, pH was measured by pH meter (Model 215).

Colony forming unit (CFU) count(remove) of *Azospirillum* and *Azotobacter* in 1g of soil was determined by plating 0.1 mL of tenfold serial dilution on Lauria Beratni (LB) media. Number of bacteria/g soil was calculated from the colony forming units obtained on plates by the given formula as proposed by James (1987).

$$\text{Number of Colony forming unit} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume of inoculum}}$$

The effect of PGPRs on soil enzymes in the rhizosphere was determined after harvest of the plants (30d after sowing). Soil invertase activity was measured by the method of Zhou & Zhang (1980). The sucrose (8%) was used as a substrate. Five mL of phosphoric acid buffer (pH 5.5) and 15 mL of substrate were mixed with 5 g of soil and were incubated at 37°C for 2 h. Next, 3 mL of 3, 5- dinitrosalicylic acid were added to 1 mL of the soil filtrate and heated for 5 min at 95°C in a water bath. The amount of 3-amino-5-nitrosalicylic acid formed was determined based on the absorbance at 508 nm using a spectrophotometer (Hitachi U-1500). Invertase activity was expressed as µg glu./g/h.

The soil urease was measured according to the method of Douglas & Bremner (1970). Soil sample (5g) was placed in a universal bottle and 4ml of 0.5 M sodium acetate buffer (pH 6.5), 1 mL of toluene and 10 mL of the substrate (5mM urea-N) solution was added followed by incubation of mixture for 8 h at 25°C. After the completion of incubation period, the reaction was stopped by the addition of 2 M potassium chloride-phenyl mercuric acetate reagent (20 mL). Further shaking of the flasks was carried out for 30 min, filtered through Whatman No. 1 filter paper and the urea concentration of the soil extract was analyzed at 525 nm using a spectrophotometer (Hitachi U-1500). Urea concentration was calculated from a urea-N standard curve (0-10 ug ml^{-1}) prepared on the day of analysis. Soil phosphatase activity was determined by the method of Tabatabai & Bremner (1969) using disodium paranitrophenyl phosphate as substrate.

The pots were separated into two sets each having three replica for all the treatments. From one set, melondialdehyde (MDA) contents were measured in emerging cotyledonary leaves at post germinating stage (emerged 72h after sowing). The MDA was estimated according to Hernandez & Almansa (2002). Emerging cotyledon leaves (0.2 g) were homogenized in 2 mL of

0.1% (w/v) trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 15,000 *g* for 10 min, and 0.5 mL of the obtained supernatant was added to 1.5 mL of thiobarbituric acid in 20% (w/v) TCA. The mixture was incubated at 90°C in a shaking water bath for 20 min, and the reaction was stopped by placing the reaction tubes in an ice water bath. The samples were centrifuged at 10,000 *g* for 5 min, and the light absorbance of the supernatant was read at 532 nm.

The plants in another set were harvested 30d after sowing when the plants were fully established. The soluble protein content of leaves was determined following the method of Lowry *et al*., (1951) using BSA as standards. The chlorophyll and carotenoids estimation of leaves were made following the method of Arnon (1949) as modified by Kirk (1968). Leaf area was measured according to Ahmed & Morsy (1999).

Statistics

The data was analyzed statistically by factorial ANOVA using Statistix software version 8.1 techniques and comparison among mean values of treatments was made by least significant difference (Steel and Torrie, 1980).

Results

Soil used for cultivation of safflower was sandy loam, having EC 0.52 dSm^{-1}, pH 7.3, soil organic matter content 0.42%, the available phosphorus 3.5 mg kg^{-1}, total nitrogen 0.021%, available potassium 100 mg kg^{-1} and sodium 1138 ppm.

The survival efficiency measured in term of colony forming unit (cfu) of the PGPR isolates was enhanced in combination with quarter and half doses of chemical fertilizers in both the cultivars of safflower (Fig. 1). The maximum significant increase in cfu (44%) was recorded in *Azotobacter* in combination with quarter dose of chemical fertilizers (BTQ) treatment as compared to *Azotobacter* (BT) alone treatment in cv. Thori. *Azospirillum* in combination with full dose of chemical fertilizers (SPF) showed 25% reduction as compared to single inoculation of *Azospirillum* (SP) whereas in combination with half dose of chemical fertilizers (SPH) showed 18% increase over SP alone. Similarly *Azotobacter* in combination with full dose of chemical fertilizers (BTF) showed 2% reduction as compared to *Azotobacter* alone treatment (BT) however *Azotobacter* in combination with half dose of chemical fertilizer (BTH) showed 9% significant improvement in cfu. In cv. Saif-32 *Azospirillum* in combination with quarter dose of chemical fertilizers (SPQ) exhibited maximum increase in cfu that was 47% higher than SP. Treatment SPF showed reduction of 11% as compared to that of SP alone however SP in combination with half and quarter doses of CF (SPH and SPQ) the cfu counts were increased to 18% and 47% over SP treatment respectively. Treatment BTF exhibited 4% reduction in cfu counts as compared to BT alone. Magnitude of increase in BTH and BTQ was 20% and 22% higher over BT treatment. The cfu counts of treatment BT were 22% lower than treatment SP.

The urease activity of soil was significantly enhanced by almost all the treatments in both the varieties (Table 1). In cv. Thori maximum increase (82%) was due to BTQ as compared to untreated control. Treatment BTQ exhibited 61% increase over CFQ (quarter dose of chemical fertilizers) and 73% increase over BT treatment alone. Single inoculation of *Azospirillum* and *Azotobacter* exhibited 53% and 37% increased in urease activity over control and 30% and 5% significant increase over full dose of chemical fertilizers (CFF). In cv. Saif-32 treatment SPH showed maximum significant increase (74%) over untreated control. Treatment SPH showed 51% increase over CFH (half dose of chemical fertilizers) and 67% over SP treatment alone. Treatment BTQ showed significant increase (72%) over control and furthermore it showed 61% significant increase over CFQ and BT treatments. Single inoculation of SP and BT showed 22% and 28% significant increase over control however both the treatments showed reduction in urease activity as compared to CFF treatment. The cv. Thori is more responsive for soil urease activity as compared to that of cv. Saif-32.

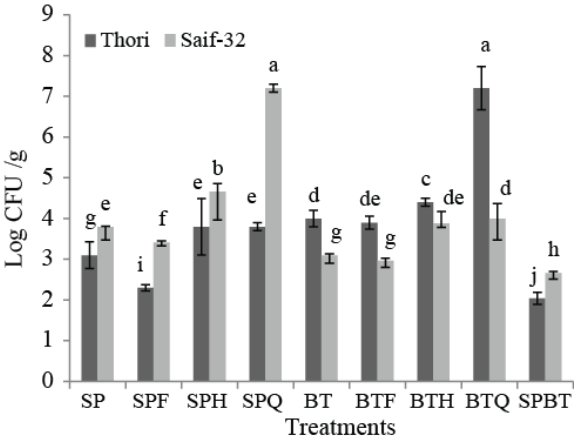

Fig. 1. Effect of PGPR and chemical fertilizers on CFU counts (Log cfu/g).
All such means which share a common English letter are similar; otherwise differ significantly at $p<0.05$
SP: *A. brasilense*, SPF: *A. brasilense* + full dose of chemical fertilizers, SPH: *A. brasilense* + half dose of chemical fertilizers, SPQ: *A. brasilense* + quarter dose of chemical fertilizers, BT: *A. vinelandii*, BTF: *A. vinelandii* + full dose of chemical fertilizers, BTH *A. vinelandii* + half dose of chemical fertilizers, BTQ: *A. vinelandii* + quarter dose of chemical fertilizers, SPBT: *A. brasilense* + *A. vinelandii*

All the treatments significantly enhanced the invertase activity except CFF and CFQ in the rhizosphere of cv. Thori and SP in cv. Saif-32 (Table 1). In cv. Thori maximum significant increase in invertase activity (67% and 66%) being recorded in SPF and BTF treatments as compared to untreated control. The % increase by SPF and BTF over respective SP and BT treatments was 24% and 43% respectively. Single inoculation of SP and BT showed 57% increase over control and 42% significant increase over CFF treatment. In cv. Saif-32 maximum increase (84%) was recorded in rhizospheric soil of plants inoculated with consortium of both PGPRs (SPBT) as compared to control which was 86% and 60% higher over SP and BT treatments. Single inoculation of SP showed reduction whereas single inoculation of BT showed 59% increase over control and 44% over CFF treatment. The soil invertase activity was greater in rhizospheric soil of cv. Saif-32 as compared to cv. Thori

Table 1. Effects of PGPR and chemical fertilizers on soil urease (ug urea/g/h) and invertase (µg glu./g/h) activity of safflower. The experiment was carried out in pots with three replicates.

Treatments	Soil urease activity (ug urea/g/h)		Soil invertase activity (µg glu./g/h)	
	cv. Thori	cv. Saif-32	cv. Thori	cv. Saif-32
C	53.78 ^{y}	55.56 ^{w}	36.33 ^{m}	24.00 ^{p}
CFF	79.44 ^{p}	103.33 ^{j}	36.33 ^{m}	33.67 ^{n}
CFH	70.56 ^{t}	104.44 ^{i}	70.00 ^{g}	48.33 ^{k}
CFQ	72.78 ^{s}	76.33 ^{r}	28.00 ^{o}	48.33 ^{k}
SP	114.44 ^{h}	70.00 ^{u}	85.00 ^{d}	20.00 ^{q}
SPF	126.67 ^{g}	92.22 ^{m}	113.00 ^{b}	75.00 ^{e}
SPH	76.67 ^{r}	215.56 ^{b}	54.00 ^{j}	114.00 ^{b}
SPQ	161.67 ^{e}	97.78 ^{l}	47.33 ^{k}	32.67 ^{n}
BT	84.67 ^{o}	77.22 ^{q}	62.00 ^{h}	59.33 ^{i}
BTF	56.11 ^{v}	87.22 ^{n}	109.00 ^{c}	84.00 ^{d}
BTH	150.56 ^{f}	182.78 ^{d}	39.33 ^{l}	62.33 ^{h}
BTQ	312.78 $_{a}$	198.89 ^{c}	48.00 ^{k}	61.33 ^{h}
SPBT	101.11 ^{k}	55.00 ^{x}	71.33 ^{f}	147.33 ^{a}
LSD	**0.4641**		**1.0970**	

All such means which share a common English letter are similar; otherwise differ significantly at p<0.05

C: Control, CFF: Chemical fertilizers full dose, CFH: Chemical fertilizers half dose, CFQ: Chemical fertilizers quarter dose, SP: *A. brasilense*, SPF: *A. brasilense* + full dose of chemical fertilizers, SPH: *A. brasilense* + half dose of chemical fertilizers, SPQ: *A. brasilense* + quarter dose of chemical fertilizers, BT: *A. vinelandii*, BTF: *A. vinelandii* + full dose of chemical fertilizers, BTH *A. vinelandii* + half dose of chemical fertilizers, BTQ: *A. vinelandii* + quarter dose of chemical fertilizers, SPBT: *A. brasilense+A. vinelandii*

LSD: Least significant difference

Table 2. Effect of PGPR and chemical fertilizers on soil Phosphatase (ug p-Np/g/min) activity and leaf MDA (nmol/gFW) contents of Safflower. The experiment was carried out in pots with three replicates.

Treatments	Soil phosphatase activity (ug p-Np/g/min)		Leaf MDA (nmol/gFW)	
	cv. Thori	cv. Saif-32	cv. Thori	cv. Saif-32
C	5.44 ^{jk}	5.40 ^{k}	0.460 ^{efghi}	0.350 ^{ij}
CFF	6.29 ^{abcd}	5.85 ^{fghi}	0.807 ^{a}	0.362 ^{hij}
CFH	6.36 ^{abc}	5.45 ^{jk}	0.444 ^{efghi}	0.428 ^{fghi}
CFQ	6.05 ^{cdefg}	5.69 ^{hijk}	0.486 ^{defghi}	0.498 ^{cdefgh}
SP	6.26 ^{abcd}	5.93 ^{efgh}	0.416 ^{ghi}	0.560 ^{cdef}
SPF	6.22 ^{bcde}	5.58 ^{ijk}	0.545 ^{cdefg}	0.623 ^{cd}
SPH	6.58 ^{a}	5.75 ^{ghij}	0.550 ^{cdefg}	0.480 ^{efghi}
SPQ	6.10 ^{cdef}	5.59 ^{ijk}	0.476 ^{efghi}	0.414 ^{ghij}
BT	6.32 ^{abcd}	6.00 ^{defgh}	0.580 ^{cde}	0.514 ^{cdefg}
BTF	6.29 ^{abcd}	5.75 ^{ghij}	0.770 ^{ab}	0.434 ^{fghi}
BTH	6.44 ^{ab}	5.68 ^{hijk}	0.560 ^{cdef}	0.353 ^{ij}
BTQ	5.69 ^{hijk}	5.57 ^{ijk}	0.272 ^{jk}	0.483 ^{defghi}
SPBT	5.79 ^{fghi}	5.72 ^{hijk}	0.132 ^{k}	0.437 ^{fghi}
LSD	**0.3239**		**0.1426**	

All such means which share a common English letter are similar; otherwise differ significantly at p<0.05

C: Control, CFF: Chemical fertilizers full dose, CFH: Chemical fertilizers half dose, CFQ: Chemical fertilizers quarter dose, SP: *A. brasilense*, SPF: *A. brasilense* + full dose of chemical fertilizers, SPH: *A. brasilense* + half dose of chemical fertilizers, SPQ: *A. brasilense* + quarter dose of chemical fertilizers, BT: *A. vinelandii*, BTF: *A. vinelandii* + full dose of chemical fertilizers, BTH *A. vinelandii* + half dose of chemical fertilizers, BTQ: *A. vinelandii* + quarter dose of chemical fertilizers, SPBT: *A. brasilense* + *A. vinelandii*

LSD: Least Significant Difference

Results showed that all the treatments increased soil phosphatase activity in the rhizosphere of both the varieties as compared to control (Table 2). In cv. Thori, maximum significant increase in phosphatase activity (17%) was recorded in SPH treatment as compared to untreated control, further more SPH treatment exhibited 3% non-significant increase over CFH treatment and also differ non-significantly from SP treatment alone. The treatment BTH also showed significant increase (15%) over untreated control. The seed inoculated with SP and BT alone significantly increased (13% and 14%) the phosphatase activity in the rhizospheric soil as compared to control and magnitude of increase in phosphatase activity by these treatments was at par with that of CFF treatment. All the other treatments differ non-significant among each other. In cv. Saif-32, maximum significant increase in soil phosphatase activity was observed in BT (10%) followed by SP (9%) treatments over control and this increase was non-significantly higher than CFF treatment alone.

Treatments showed different response towards malondialdehyde (MDA) contents of safflower in both varieties (Table 2). Maximum significant increase (42%) in MDA content was due to CFF treatment as compared with untreated control in cv. Thori. Treatment BTF also showed significant increase (40%) over control however it showed non-significant reduction as compared to CFF but it showed 24% increase over BT treatment. All the other treatments differ non-significantly as compared to control however treatments BTQ showed significant reduction of 41% over control and 42% decrease as compared to CFQ treatment and SPBT exhibited maximum significant reduction (71%) that was 57% and 93% decrease as compared to that of SP and BT treatments respectively. In cv. Saif-32, maximum increase (43%) in MDA contents was recorded in SPF treatment that was 26% significant increase over CFF and 9% non-significant increase over SP treatment. Treatment SP and CFQ exhibited significant increase (37% and 28%) in MDA content as compared to that of untreated control. All the other treatments showed non-significant increase over control as well as among each other. Cultivar Saif-32 showed increase in MDA contents as compared to that of cv. Thori.

Root length was significantly increased in all the treatments in cv. Thori (Table 3). The maximum significant increase in root length was due to SPQ (66%) and BTQ (63%) treatments as compared to that of control in cv. Thori. The treatments SPQ exhibited 33% increase over CFQ and 44% increase over SP treatment whereas BTQ showed 29% increased over CFQ treatment and 47% increase over BT treatments respectively. The % increase by SP was 39% over control and 21% over CFF treatment and BT showed 32% significant increase over control and 12% significant increase over CFF treatment. All the other treatments exhibited significant increase as compared to that of control and also among each other. In cv. Saif-32 maximum significant increase (53%) was recorded in SPH Treatments which was 22% higher than CFH and 13% significant increase over SP treatment. Magnitude of increase by single inoculation of SP and BT was 46%, 11% as compared to that of untreated control respectively however SP showed 45% increase and BT showed 11% increase over CFF treatment All the other treatments significantly improved the root length except CFF treatment at $p<0.05$.

All the treatments significantly improved the shoot length except BT treatment alone in cv. Thori (Table 3). Maximum significant increase (26%) was recorded in CFQ treatment over control. Treatment SPF showed (21%) significant increase in shoot length over control however it showed 12% significantly higher shoot length than CFF and SP treatment. Single inoculation of SP showed 9% significant increase over control but did not differ significantly from CFF, similarly BT did not differ significantly from control and showed reduction as compared to that of CFF. In cv. Saif-32 all the treatments showed significant increase over control however maximum significant increase (29%-30%) in shoot length was recorded in treatments CFH and CFQ as compared to untreated control. The single inoculation of SP and BT showed 12% and 4% significant increase over control. Variety Thori exhibited higher increase in shoot length than cv. Saif-32.

All the treatments of PGPR and chemical fertilizers significantly increased the leaf area of safflower in both the varieties (Table 4). Maximum increase (63%) in leaf area was recorded in BTH treatment over untreated control in cv. Thori, furthermore BTH showed 31% increase over CFH and 48% over BT alone. Single inoculation of SP and BT showed 21% and 32% significant increase over control however the respective treatments showed reduction in leaf area as compared to CFF treatment. In cv. Saif-32 maximum % increase (55%) in leaf area was recorded in BTF and SPH treatments and these treatments showed 11% significant increase over CFF and CFH treatments and 20% and 38% significant increase over BT and SP respectively. Single inoculation of SP and BT exhibited 26% and 43% increase over control but showed reduction as compared to CFF treatment. Cv. Thori showed higher response than cv. Saif-32 for leaf area.

Results indicated that different treatments showed different leaf chlorophyll contents of safflower in both varieties (Table 4). In cv. Thori maximum significant increase (64%) was recorded in BTF treatment over control and 37% and 23% increase over CFF and BT treatments respectively. Treatments SPF and SPH showed 50% significant increase as compared to control, however the respective treatments showed 12% and 22% non-significant increase over CFF and CFH treatments and 32% significant increase over SP treatment. CFF also exhibited 43% significant increase as compared to that of control. All the other treatments differ non-significantly from control. In cv. Saif-32 maximum significant increase (71%) in chlorophyll contents was observed in SPF treatment over control and the respective treatment showed 28% increase over CFF and 54% over SP treatment. All the other treatments showed significant increases over control but differ non-significantly among each other except CFH, CFQ and SP which showed non-significant increase as compared to control. Cv. Thori was more responsive than cv. Saif-32 for increase in chlorophyll contents.

Table 3. Effect of PGPR and chemical fertilizers on Root length (cm) and shoot length (cm) of safflower. The experiment was carried out in pots with three replicates.

Treatments	Root length (cm)		Shoot length (cm)	
	cv. Thori	cv. Saif-32	cv. Thori	cv. Saif-32
C	17.33 ^{t}	20.83 ^{rs}	12.66 ^{l}	13.03 ^{k}
CFF	22.34 ^{q}	21.00 ^{r}	14.66 ^{fg}	17.16 ^{b}
CFH	23.33 ^{p}	33.33 ^{ij}	14.60 ^{g}	18.36 ^{a}
CFQ	34.00 ^{i}	37.33 ^{h}	17.33 ^{b}	18.66 ^{a}
SP	28.33 ^{l}	38.66 ^{g}	14.06 ^{i}	14.83 ^{efg}
SPF	33.00 ^{j}	23.00 ^{pq}	16.20 ^{c}	14.60 ^{g}
SPH	37.33 ^{h}	44.33 ^{c}	14.16 ^{hi}	17.50 ^{b}
SPQ	51.33 ^{a}	42.33 ^{d}	13.30 ^{jk}	14.86 ^{efg}
BT	25.00 ^{n}	23.66 ^{op}	12.66 ^{l}	13.60 ^{j}
BTF	30.00 ^{k}	37.83 ^{gh}	15.0 ^{ef}	14.50 ^{gh}
BTH	40.00 ^{f}	41.33 ^{e}	13.66 ^{j}	16.50 ^{c}
BTQ	48.00 ^{b}	24.33 ^{no}	15.53 ^{d}	16.16 ^{c}
SPBT	20.00 ^{s}	26.33 ^{m}	15.16 ^{e}	10.83 ^{m}
LSD	**0.9631**		**0.3605**	

All such means which share a common English letter are similar; otherwise differ significantly at $p<0.05$

C: Control, CFF: Chemical fertilizers full dose, CFH: Chemical fertilizers half dose, CFQ: Chemical fertilizers quarter dose, SP: *A. brasilense*, SPF: *A. brasilense* + full dose of chemical fertilizers, SPH: *A. brasilense* + half dose of chemical fertilizers, SPQ: *A. brasilense* + quarter dose of chemical fertilizers, BT: *A. vinelandii*, BTF: *A. vinelandii* + full dose of chemical fertilizers, BTH *A. vinelandii* + half dose of chemical fertilizers, BTQ: *A. vinelandii* + quarter dose of chemical fertilizers, SPBT: *A. brasilense* + *A. vinelandii*

LSD: Least Significant Difference

Table 4. Effect of PGPR and chemical fertilizers on Leaf Area (cm^2) and Chlorophyll contents (mg/g) of safflower. The experiment was carried out in pots with three replicates.

Treatments	Leaf area (cm^2)		Leaf chlorophyll (mg/g)	
	cv. Thori	cv. Saif-32	cv. Thori	cv. Saif32
C	10.67 ^{r}	8.54 ^{t}	0.35 ^{fgh}	0.22 ^{h}
CFF	17.40 ^{j}	16.14 ^{n}	0.62 ^{bcde}	0.55 ^{cdef}
CFH	20.10 ^{e}	16.70 ^{lm}	0.54 ^{cdef}	0.42 ^{efgh}
CFQ	18.48 ^{fg}	14.08 ^{p}	0.38 ^{fgh}	0.36 ^{fgh}
SP	13.64 ^{p}	11.60 ^{q}	0.48 ^{defg}	0.35 ^{fgh}
SPF	18.00 ^{ghi}	17.60 ^{ij}	0.71 ^{bc}	0.77 ^{ab}
SPH	21.32 ^{d}	18.13 ^{gh}	0.70 ^{bcd}	0.51 ^{cdefg}
SPQ	18.70 ^{f}	17.07 ^{kl}	0.49 ^{cdefg}	0.34 ^{fgh}
BT	15.88 ^{n}	15.00 ^{o}	0.43 ^{efgh}	0.36 ^{fgh}
BTF	26.47 ^{b}	18.73 ^{f}	0.99 ^{a}	0.47 ^{efg}
BTH	29.17 ^{a}	16.18 ^{n}	0.40 ^{efgh}	0.39 ^{fgh}
BTQ	16.25 ^{mn}	17.78 ^{hij}	0.30 ^{gh}	0.36 ^{fgh}
SPBT	22.06 ^{c}	10.13 ^{s}	0.35 ^{fgh}	0.42 ^{efgh}
LSD	**0.4995**		**0. 224**	

All such means which share a common English letter are similar; otherwise differ significantly at $p<0.05$

C: Control, CFF: Chemical fertilizers full dose, CFH: Chemical fertilizers half dose, CFQ: Chemical fertilizers quarter dose, SP: *A. brasilense*, SPF: *A. brasilense* + full dose of chemical fertilizers, SPH: *A. brasilense* + half dose of chemical fertilizers, SPQ: *A. brasilense* + quarter dose of chemical fertilizers, BT: *A. vinelandii*, BTF: *A. vinelandii* + full dose of chemical fertilizers, BTH *A. vinelandii* + half dose of chemical fertilizers, BTQ: *A. vinelandii* + quarter dose of chemical fertilizers, SPBT: *A. brasilense* + *A. vinelandii.*

LSD: Least Significant Difference

Table 5. Effect of PGPR and chemical fertilizers on Leaf Carotenoid (mg/g) and Protein contents (mg/g) of safflower. The experiment was carried out in pots with three replicates.

Treatments	Leaf carotenoid (mg/g)		Leaf protein (mg/g)	
	cv. Thori	cv. Saif-32	cv. Thori	cv. Saif-32
C	1.47 ^{hi}	1.34 ^{i}	239.39 ^{efg}	242.48 ^{efg}
CFF	2.80 ^{bcde}	3.55 ^{ab}	263.85 ^{cdef}	296.56 ^{cde}
CFH	2.21 ^{defghi}	2.18 ^{defghi}	242.36 ^{efg}	273.31 ^{cdef}
CFQ	1.70 ^{ghi}	1.96 ^{efghi}	299.59 ^{cde}	289.31 ^{cde}
SP	2.23 ^{defgh}	1.86 ^{fghi}	295.50 ^{cde}	267.94 ^{cdef}
SPF	3.33 ^{bc}	2.49 ^{cdefg}	403.08 ^{a}	325.17 ^{bc}
SPH	2.88 ^{bcd}	1.80 ^{fghi}	315.83 ^{bcd}	252.05 ^{def}
SPQ	2.59 ^{cdef}	1.85 ^{fghi}	258.36 ^{cdef}	256.84 ^{def}
BT	1.86 ^{fghi}	1.95 ^{efghi}	231.96 ^{efg}	270.39 ^{cdef}
BTF	4.37 ^{a}	2.23 ^{defgh}	261.87 ^{cdef}	317.11 ^{bcd}
BTH	2.13 ^{defghi}	2.32 ^{efgh}	277.17 ^{cdef}	384.04 ^{ab}
BTQ	1.85 ^{fghi}	2.02 ^{defghi}	271.44 ^{cdef}	249.25 ^{defg}
SPBT	1.64 ^{ghi}	1.91 ^{fghi}	205.10 ^{fg}	178.94 ^{g}
LSD	**0.8823**		**72.482**	

All such means which share a common English letter are similar; otherwise differ significantly at $p<0.05$

C: Control, CFF: Chemical fertilizers full dose, CFH: Chemical fertilizers half dose, CFQ: Chemical fertilizers quarter dose, SP: *A. brasilense*, SPF: *A. brasilense* + full dose of chemical fertilizers, SPH: *A. brasilense* + half dose of chemical fertilizers, SPQ: *A. brasilense* + quarter dose of chemical fertilizers, BT: *A. vinelandii*, BTF: *A. vinelandii* + full dose of chemical fertilizers, BTH *A. vinelandii* + half dose of chemical fertilizers, BTQ: *A. vinelandii* + quarter dose of chemical fertilizers, SPBT: *A. brasilense* + *A. vinelandii*

LSD: Least Significant Difference

All the treatments of PGPR and chemical fertilizers increased the carotenoids contents of safflower leaf in both the varieties (Table 5). In cv. Thori maximum increase of 66% was recorded in BTF as compared to that of control. Magnitude of increase by BTF was 35% higher than CFF treatment and 57% increase over BT treatment. The SPF treatment also showed 55% significantly higher increase in carotenoid contents as compared to control however SPF exhibited 15% increase over CFF and 33% over SP treatment alone. Treatments CFF, SPH and SPQ showed significant increase over control while all the other treatments exhibited non-significant increase over control. In cv. Saif-32 maximum significant increase (62%) was observed in CFF treatment however SPF, BTF and BTH also showed significant increase but all the other treatments exhibited non-significant increase as compared to untreated control and also among each other.

Different treatments had different effect on leaf protein contents of both the cultivars (Table 5). In cv. Thori higher increase (41%) in leaf proteins was recorded in SPF over untreated control. Treatments SPF showed 33% significant increase over CFF and 26% over SP. Treatment SPH also showed significant increase (24%) as compared to untreated control; furthermore it exhibited 23% increase over CFH and non-significant increase over SP treatment alone. All the other treatments showed non-significant increase over control. In cv. Saif-32 maximum % increase (36%) was recorded in BTH treatment which was 13% higher than CFH treatment and 29% increase over BT treatment. Treatments SPF and BTF showed significant increase (25% and 23%) over control however SPF exhibited 8% higher protein contents than CFF and 17% higher than SP whereas BTF showed 6% increase over CFF and 14% increase over BT treatment. Rest of the treatments showed non-significant results as compared to that of control.

Leaf nitrogen contents were significantly increased by all the treatments in both the varieties however maximum significant increase (76%) was recorded in SPQ and BTQ treatments in cv. Thori as compared to control (Fig. 2). The SPQ exhibited 66% increase over CFQ and 40% increase over SP treatment whereas BTQ showed 66% increase over CFQ and 59% over BT treatment. Single application of SP and BT showed 35% and 29% increase over control however both treatments showed reduction in leaf nitrogen contents as compared to CFF treatment. In cv. Saif-32 maximum significant increase (72%) in leaf nitrogen contents was recorded in BTQ treatment over control. Treatment BTQ showed 51% higher nitrogen contents than CFQ and 55% as compared to BT treatment. Single inoculation of SP and BT showed 47% and 39% significant increase over control but showed significant reduction as compared to CFF treatment. Cv. Thori enhanced more leaf nitrogen contents as compared to cv. Saif-32.

Discussion

PGPR play important role in improving the plant growth. Maximum cfu counts were recorded in SPQ and BTQ treatments this may be due to the compatibility of PGPR with the respective dose of chemical fertilizer. Supplementing quarter dose of CF with *Azospirillum* (SP) for cv. Thori and with *A. vinelandii* (BT) for cv. Saif-32 may be implicated to promote growth economizing the use of CF. According to the current results the doses of chemical fertilizers favor the survival efficiency of microbes in the rhizospheric soil. Winget and Gold (2007)

reported low cfu counts in chemical fertilizers treatments as compared to PGPR treatments in *Brassica rapa* and this decrease is attributed due to the fact that increase input of chemical fertilizers decrease the soil pH which make the environment unsuitable for microbial population. The coinoculation of SPBT was less stimulatory than single inoculation of either of the PGPR, indicating that these two microbes SP or BT act antagonistically not synergistically. These results indicate that there are differences in the degree of PGPR association with the varieties. Jain and Patriquin (1984) reported similar results that association of PGPR depends upon the cultivar of wheat as well as on the type of PGPR strain used.

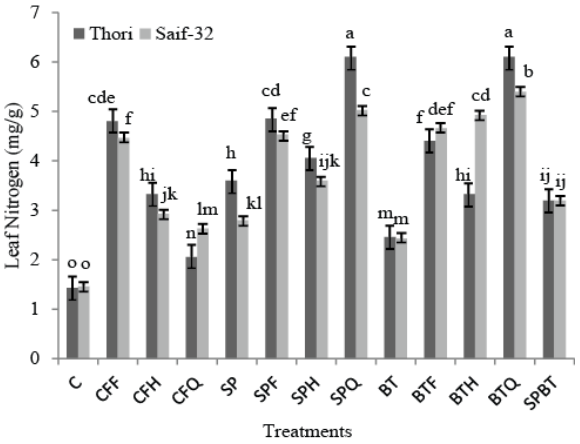

Fig. 2. Effect of PGPR and chemical fertilizers on Leaf Nitrogen (mg/g) contents of safflower.
All such means which share a common English letter are similar; otherwise differ significantly at $p<0.05$
C: Control, CFF: Chemical fertilizers full dose, CFH: Chemical fertilizers half dose, CFQ: Chemical fertilizers quarter dose, SP: *A. brasilense*, SPF: *A. brasilense* + full dose of chemical fertilizers, SPH: *A. brasilense* + half dose of chemical fertilizers, SPQ: *A. brasilense* + quarter dose of chemical fertilizers, BT: *A. vinelandii*, BTF: *A. vinelandii* + full dose of chemical fertilizers, BTH *A. vinelandii* + half dose of chemical fertilizers, BTQ: *A. vinelandii* + quarter dose of chemical fertilizers, SPBT: *A. brasilense* + *A. vinelandii*

Urease has been involved in urea hydrolysis and increases the utilization rate of nitrogen fertilizer (Klose & Tabatabai, 1999). Madhaiyan *et al.,* (2010) reported that co-inoculation of *A. brasilense* CW903 and *Methylobacterium oryzae* CBMB20 significantly increased the soil urease activity. Previous studies indicated that urease activity of *A. vinelandii* spp. was very closely connected with N inputs (Mikanová *et al.,* 2009). It is evident that fertilizers assist the PGPR in stimulating the urease activity. Root length of cv. Thori was positively and significantly correlated (r=0.529) with soil urease activity and it is evident that increase in urease activity in SPQ and BTQ treatments provides optimum amount of nitrogen to the plant which helps in root proliferation and increase in root length. Urease activity is correlated with cfu in the current study because cfu and urease activity both were enhanced in SPQ and BTQ treatments. The urease activity is also directly correlated with plant nitrogen contents (r=0.431) and it is clearly indicated that urease activity increased the nitrogen uptake of the plant which help in root growth and plant development. The plant nitrogen contents were also increased by various treatments of chemical fertilizers and PGPR. Treatments SPQ, SPH and BTQ exhibited maximum increase however full dose of chemical fertilizers and PGPR in combination with full dose of CF also improved nitrogen contents. It means that PGPR in combination with lower doses of chemical fertilizers showed better efficiency and ultimately improved plant growth. Zhang *et al.* (1996) reported that PGPR increased the plant nitrogen contents in soybean. Previously it was reported that application of nitrogen fertilizers significantly increased the nitrogen contents in the safflower plant (Soliman *et al.,* 2012). The lower doses of chemical fertilizers favor the survival efficiency of microbes which increased the nitrogen uptake by improving the urease activity. Soil invertase is an important indicator of soil quality and varies with land type. Invertase cleaves sucrose into hexoses to supply cells with fuel for respiration and with carbon and energy for the synthesis of several diverse compounds. PGPR markedly increased the invertase activity in rhizosphere of both cvv. Thori and Saif-32 and its activity was further augmented in the presence of half dose of CF. Hui *et al.* (2004) reported that inorganic fertilizers increased the soil invertase and urease activities being positively correlated with increased microbial activities in the rhizosphere. The co-inoculation of *A. brasilense* and *A. vinelandii* (SPBT) was more effective than single inoculation of either of the microbes and it may be due to synergistic effect of both microbes. Previous studies showed that *Azotobacter chroococcum* secreted invertase into the medium (Vega *et al.,* 1991). Soil phosphatase activity has often been anticipated as an indicator of the soil potential for organic phosphorus mineralization and biological activity. The soil phosphatase activity was increased in cv. Thori in all the treatments with PGPR and CF. Higher phosphatase activity in cv. Thori in the treatments SPH and BTH is worth mentioning. Noteworthy in cv. Thori, CF at all doses and even single application of SP and BT alone effectively enhanced phosphatase activity. It has been reported that application of *Enterobacter agglomerans* and *Bacillus subtilis* significantly improved the phosphatase and urease activities of the rhizospheric soil of tomato and lettuce (Kohler *at al.,* 2007; Kim *et al.,* 1998). It was reported previously that *Azotobacter vinelandii* and *Bacillus cereus* isolates *Pseudomonas* sp. and *Azospirillum* sp exhibit phosphate solubilising ability in vitro study (Husen, 2003; Ramachandran, 2007).

Malonyldialdehyde (MDA) is a cytotoxic product and indicates the degree of lipid peroxidation. The full dose of chemical fertilizers and *Azotobacter* in combination with full dose of chemical fertilizers (BTF) was stimulatory to MDA contents. PGPR inoculation has been observed to decrease the MDA content under stressful conditions which indicates their positive role in preventing lipid peroxidation (Habibi *et al.,* 2010). This attribute of PGPR is important in oxidative stress and other stresses leading to ROS generation. But current findings are contradictory to the previous work who reported that increase in nitrogen fertilizers increased chlorophyll content, photosynthetic performance and decreased the MDA contents (Zhang *et al.,* 2010). There are reports that combined application of chemical fertilizers and biofertilizers decreased the electrolyte leakage and ultimately leads to decreased in MDA contents in ginger plant (Bo, 2007). Shukla et al. (2012) reported that application of PGPR isolates such as JG-02, JG-06, JG-07 and JG-11 on

Arachis hypogaea at different salinity levels significantly reduced the MDA content as compared to that of uninoculated plants. The PGPR induced the scavenging action of ROS and enhance the activity of antioxidant enzymes (Han & Li, 2005) and reduce the MDA contents.

Present data demonstrated that the stimulatory affect of PGPR on root length were higher in presence of half and quarter doses of chemical fertilizers. This might be because that PGPR enhance the effect of organic and chemical fertilizers on agricultural production by increasing the activity of microbial biomass (Shata *et al.,* 2007) in which case the PGPR use chemical fertilizers as C, N and P source. Mia *et al.* (2010**)** reported substantial increase in root length following PGPR inoculation. The beneficial effects of PGPR were also higher on shoot length in the presence of half and quarter doses of chemical fertilizers. This may be due to the fact that these bacteria directly affect the growth of the plants by improving the nitrogen absorption, the synthesis of phytohormones and the dissolving of minerals (Herman *et al.,* 2008). Shoot length was higher in full, half and quarter doses of chemical fertilizers and results for SPH were at almost at par to that of full dose of chemical fertilizers. Mishra & Jain (2013) reported that combined application of biofertilizers and 50% nitrogen, phosphorus and potassium fertilizers significantly increased the shoot length of *Andrographis paniculata* which is in accordance to our results. Zhang *et al.,* (1996) reported that PGPR increased the nitrogen transport from root to shoot in soybean which ultimately increases the shoot length at later growth stages of soybean. Similarly, Ilyas & Bano (2010) reported increased shoot length in wheat when inoculated with *Azospirillum brasilense*.

PGPR supplemented with full dose of chemical fertilizers was found significantly stimulatory for chlorophyll production; this was true for *Azotobacter* sp. in cv. Thori. The effect of *Azospirillum* supplemented with full dose of chemical fertilizers was highly effective in improving the chlorophyll contents of safflower. Previously it was reported that nitrogen supply increased the chlorophyll contents of the leaves and chlorophyll contents have direct relation with the nitrogen contents (Schlemmer *et al.,* 2005). The presence of PGPR further assists the increase by making the nutrient available to the plants (Liu *et al.,* 2013). The leaf area in current study was significantly increased in the BTF and BTH treatments; it means that the following combinations of PGPR are effective in improving leaf area. It is obvious from the results that leaf area and chlorophyll contents are correlated with each other and both the parameters are directly correlated with nitrogen supply to the plants. The growth parameters like leaf area, contents of pigment fractions in plant seedlings have been reported by bacterial inoculation (Karakurt & Aslantas, 2010). During present study the maximum improvement in leaf protein was recorded in SPF treatment in cv. Thori and BTH treatment in Cv. Saif-32. The increase in protein contents in the respective treatments might be due to the adequate supply of nitrogen by nitrogen fixation to the plant for the synthesis of amino acid and ultimately building up of protein structure. The phosphate fertilizers play an important role in providing enough energy in the form of ATP for the synthesis of protein in physiological processes (Soliman *et al.,* 2012). Malik *et al.* (1997) found that *Azospirillum* inoculation could contribute about 70% of the total N requirement of the host plant which play an important role in protein build up process of the plant. *Azotobacter* appeared more effective in cv. Saif-32 and responded better to chemical fertilizers whereas; *Azospirillum* was effective for cv. Thori. Noteworthy, the half and quarter dose of CF being most effective when applied alone, the effect of which was further enhanced with *Azospirillum* and *Azotobacter* inoculation. It has been reported that application of PGPR in combination with recommended doses of chemical fertilizers enhanced the growth and yield of the crop (Akhtar *et al*., 2009).

Conclusion

The chemical fertilizers can be supplemented with the PGPR to improve plant growth and soil health. The response of PGPR to applied dose of chemical fertilizers depends on the variety as well as the strain of PGPR and parameter studied. The *Azospirillum* and *Azotobacter* can be supplemented with half and quarter dose of chemical fertilizers for improving soil enzymes activities and better plant growth. It is inferred that 50%-75% of chemical fertilizers can be saved by the application of PGPR. Therefore, the application of these PGPR in crop fields may be much beneficial for the agriculturist and can be recommended as biofertilizing agents in the sustainable and environment friendly management of agricultural practices.

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