

Original Research Article

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Assessment of Glyphosate and Quizalofop Mediated Toxicity to Greengram [*Vigna radiata* (L.) Wilczek], Stress Abatement and Growth Promotion by Herbicide Tolerant *Bradyrhizobium* and *Pseudomonas* species

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ABSTRACT

Continued and widespread use of herbicides for controlling weeds and indirectly enhancing crop production often results in reduction in soil fertility and via food chain, human health. Realizing this threat, herbicide tolerant plant growth promoting rhizobacterial strains were isolated and identified. Of the total 40 bacterial strains, *Bradyrhizobium* sp. strain R5 and *Pseudomonas* sp. strain PS6 tolerated 3200 and 800 $\mu\text{g ml}^{-1}$ of glyphosate and Quizalofop P ethyl, respectively. *Bradyrhizobium* sp. strain R5 and *Pseudomonas* sp. strain PS6 produced indoleacetic acid, exopolysaccharides, siderophores, ammonia and solubilized inorganic P even in the presence of herbicides. Scanning electron microscopic and CLSM images revealed a clear toxicity of herbicides to bacterial cells. The toxicity of herbicides to greengram plants increased with increasing rates of glyphosate and quizalofop. The herbicide tolerant *Bradyrhizobium* strain R5 and *Pseudomonas* sp. strain PS6 when used with herbicide, considerably reduced toxicity to greengram plants. For example, *Bradyrhizobium* strain R5 significantly increased the nodule number, nodule dry mass, root and shoot length, root and shoot weight and total chlorophyll content by 10, 33, 3, 16, 68, 23 and 6% respectively, whereas, inoculation of *Pseudomonas* sp. strain PS6 enhanced the measured parameters by 2, 7, 3, 12, 25, 22 and 20%, respectively, as compared to the plants grown solely with 1444 $\mu\text{g kg}^{-1}$ of glyphosate. Among the two bacterial strains, *Bradyrhizobium* R5 showed better results under identical herbicide stress as compared to the *Pseudomonas* PS6. In general, the present findings suggest that both *Bradyrhizobium* sp. strain R5 and *Pseudomonas* sp. strain PS6 could be exploited as an efficient microbial inoculant to increase the productivity of greengram while reducing the toxicity of glyphosate and quizalofop.

Keywords

Greengram,
Herbicide toxicity,
PGPR,
Bioremediation,
Growth promotion.

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Introduction

Environmental pollution due to wide spread use of agrochemicals is a major global threat to the sustainability of agro-ecosystems. Among various agrochemicals applied in agricultural practices, the intensive and uncontrolled use of herbicides over the years has caused serious problems (Ozkara *et al.*,

2016). The productivity of legumes on the other hand often suffers from weed competition. And hence, to control weeds and consequently to enhance crop production, herbicides are applied consistently. Among legumes, greengram is known for its detoxification activities and is used to refresh

mentality, alleviate heat stroke, and reduce swelling in the summer (Tang *et al.*, 2014). Greengram is a highly nutritious grain legume popularly cultivated in the tropics and forms a highly specific symbiosis with *Bradyrhizobium* sp. (*Vigna*). Like many legumes, greengram productivity often suffers from weed competition and therefore, requires chemical management (Jiddimani, 2017). Apart from affecting crops adversely at higher rates, herbicides also affect microbes (Zaller *et al.*, 2016) indirectly, causing physiological changes and increasing enzymatic production (Usman *et al.*, 2017) and /or leading eventually to the death of microorganisms (Prashar and Shah, 2016). Also, herbicides when applied had variable effects on greengram production (Gupta *et al.*, 2017). These toxic problems however, can be circumvented by applying naturally occurring soil microflora. Among diverse microbial communities, plant growth promoting rhizobacteria (PGPR) have been reported to reduce the agrochemical toxicity by different mechanism (Azubuikwe *et al.*, 2016) and has therefore, received considerable attention by the agronomists. Plant growth promoting rhizobacteria offers a viable and inexpensive option for safe detoxification/removal of agrochemicals from contaminated sites (Akbar and Sultan, 2016). Among PGPR, rhizobial species are reported to degrade many toxic pesticides including herbicides to non-toxic forms (Jaiswal *et al.*, 2017), synthesize antifungal compounds (Gao *et al.*, 2017), phytohormones (Porte, 2017) and siderophores (Kandel *et al.*, 2017), and solubilize insoluble phosphate (Alori *et al.*, 2017). Endowed with these properties, when rhizobia are applied, they have been found to enhance the growth of legumes in different ecological niches (Egamberdieva *et al.*, 2017). The toxicity of herbicide to greengram plants on one hand and the ability of PGPR to circumvent herbicide toxicity on the other hand prompted us to undertake this study with

the following specific objectives-(i) isolation and identification of rhizobacterial strains from root nodules of greengram plants and mustard rhizosphere (ii) assessment of herbicide tolerant potential of the isolated bacterial strains (iii) evaluation of plant growth promoting activity of herbicide tolerant strains exposed to herbicide stress and (iv) assay of toxic impact of herbicides and bioremediation potential of herbicide tolerant PGPR strains using greengram as a test legume.

Materials and Methods

Isolation, characterization and identification of rhizobacterial strains

A total of 40 rhizobacterial strains (N=20 *Bradyrhizobium*, N=20 *Pseudomonas*) were recovered from the experimental fields of Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh (27° 29' latitude and 72° 29' longitude; western district of Uttar Pradesh, India) which had previous history of pesticide application for the last 7 years. Rhizobial strains (N=20) were isolated from nodules of greengram plants using yeast extract mannitol (YEM) medium (g L⁻¹: mannitol 10, K₂HPO₄ 0.5, MgSO₄.7H₂O 0.2, NaCl 0.1, yeast extract 1.0, CaCO₃ 1, pH 7) (Somasegaran and Hoben 1985). For this, pink colored and healthy nodules were surface sterilized (sodium hypochlorite 2.5 % for 2 min.), rinsed in 95 % ethanol (v/v) and washed several times with distilled water and crushed gently in normal saline solutions (NSS). Nodule suspensions were serially diluted in NSS and 100 µL of each diluent was spread plated on YEM agar medium containing 2.5% Congo red dye. *Pseudomonas* strains (N=20) were isolated from mustard rhizosphere using serial dilution assay. A-100µl diluted soil suspension was spread plated on king's B agar medium (g L⁻¹: Proteose peptone 20, K₂HPO₄ 1.5,

MgSO₄.7H₂O 1.5, Agar 20 pH 7.2±0.2). The inoculated plates were incubated at 28±2 °C for 3 (*Pseudomonas*) to 5 days (rhizobia). A single bacterial colony from each plate was picked and streaked 4 times on the same medium to ascertain the purity of the cultures. The bacterial strains were maintained on their respective agar medium at 4°C until use. The rhizobial isolates were identified to genus level morphologically and biochemically (Table 1) following standard microbiological methods described in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) and host specific plant infection test (Somasegaran and Hoben, 1994).

Assessment of bacterial strains for herbicide-tolerance

The tolerance of rhizobacterial strains to herbicides was determined using commercial grade herbicides (Table 1); glyphosate and quizalofop-p-ethyl (Parijat Agrochemicals, New Delhi, India) by agar plate dilution method using minimal salt agar medium (g L⁻¹: KH₂PO₄ 1, K₂HPO₄ 1, NH₄NO₃ 1, MgSO₄.7H₂O 0.2, CaCl₂.2H₂O 0.02, FeSO₄.7H₂O 0.01, pH 6.5). The freshly prepared agar plates were amended individually with concentrations ranging from 0 to 6,400 µg mL⁻¹ at two-fold dilution intervals for both glyphosate and quizalofop-p-ethyl. The plates were spot inoculated with 10 µL of 10⁸ cells mL⁻¹ of bacterial strains and incubated at 28±2°C for 3 days and the highest concentration of herbicides showing no inhibitory effect was defined as the maximum tolerance level (MTL).

Assay of plant growth regulators under glyphosate and quizalofop stress

Plant growth promoting activity of selected PGPR strains was assessed under both normal and herbicide stressed conditions. Among plant promoting substances, indole acetic acid

(IAA) was quantitatively assayed by spectrophotometric method (Gordon and Weber 1951) later modified by Brick *et al.*, (1991). Bacterial strains believed to secrete IAA were grown in LB broth supplemented with 0, 600, 1200 and 1800 µg mL⁻¹ glyphosate and 0, 200, 400, and 600 µg mL⁻¹ quizalofop P ethyl. A-100 ml of LB broth treated with 100 µg mL⁻¹ tryptophan was inoculated with 1 mL (10⁸ cells mL⁻¹) each of *Bradyrhizobium* and *Pseudomonas* strains. The bioprimered LB broth was incubated at 28 ±2°C for 5 days with moderate shaking at 125 r/m. Rhizobacterial cultures were removed at exponential growth phase and centrifuged at 5,433g for 15 min. An aliquot of 2 ml supernatant was mixed with 100 µL orthophosphoric acid and 4 ml Salkowsky reagent (2% 0.5 M FeCl₃ in 35% perchloric acid) was added to it and incubated at 28±2°C in darkness for 1 h. The absorbance of pink color developed was read at 530 nm. IAA concentration in the supernatant was determined using a calibration curve of pure IAA as a standard. Siderophores released by the bacterial strains were detected by FeCl₃ test (Neiland, 1981).

Bacterial cultures were grown in NB supplemented with different concentrations of herbicides (0, 600, 1200 and 1800 µg mL⁻¹ glyphosate and 0, 200, 400 and 600 µg mL⁻¹ quizalofop P ethyl) and incubated at 28±2 °C for 4 days. After incubation, cultures were centrifuged (3000 rpm) for 20 min. One ml of supernatant was taken and 1 ml of 2% FeCl₃ was added to each tube. Change in color from reddish brown to orange was observed. For exopolysaccharide (EPS) estimation, rhizobial strains were grown in 100 mL capacity flasks containing basal medium containing 5% sucrose and different concentration of herbicides and incubated for 5 days at 28±2°C on rotary shaker. Culture broth was spun at 5,433×g for 30 min. and EPS was extracted using 3:1 ratio of chilled acetone. The

precipitated EPS was repeatedly washed 3 times alternately with distilled water and acetone, transferred to a filter paper and weighed after overnight drying (Mody *et al.*, 1989). Hydrogen cyanide (HCN) and ammonia production under herbicide stress was determined by the methods of Bakker and Schipper (1987) and Dye (1962), respectively.

The phosphate solubilization activity (PSA) was quantitatively estimated using liquid Pikovskaya medium amended with 0, 1X, 2X and 3X concentration of glyphosate and quizalofop. The amount of solubilized P was evaluated by chlorostannous reduced molybdophosphoric acid blue method (King, 1932; Jackson, 1976). Each individual experiment was repeated three times.

Cellular damage induced by herbicides under SEM and CLSM

Cellular damage to the bacterial strains R5 and PS6 were observed under SEM using the method described by Saleem *et al.*, (2017) after growing the cultures in nutrient broth (g l^{-1} : peptone 10; beef extract 10; NaCl 5; pH=7) treated with 1200 and 400 $\mu\text{g ml}^{-1}$ of glyphosate and quizalofop, respectively. Also, the toxicity of the herbicides to bacterial strains was validated under Confocal Laser Scanning Microscopy (CLSM).

Seed inoculation, herbicide treatment and plant culture

Healthy seeds of greengram (var. K851) were surface sterilized with 70% ethanol for 3 min.; 3% sodium hypochlorite (NaOCl) for 3 min.; rinsed six times with sterile water and dried. Sterilized seeds were bacterized with *Bradyrhizobium* strain R5 and *Pseudomonas* sp. strain PS6, separately by soaking the seeds in liquid culture medium for 2 h using 10% gum arabic as sticker to maintain a bacterial load of 10^8 cells seed⁻¹. The un-inoculated

sterilized seeds soaked in sterile water only was used as control. A-total of 10 un-inoculated and inoculated seeds were sown in earthen pots (25 cm high, 22 cm internal diameter) using 3 kg unsterilized soils (sandy clay loam, organic C 6.2 g kg^{-1} , Kjeldahl N 0.75 g kg^{-1} , Olsen P 16 mg kg^{-1} , pH 7.2 and water holding capacity 0.44 mL g^{-1} , cation exchange capacity 11.7 cmol kg^{-1} and 5.1 cmol kg^{-1} anion exchange capacity). Pots were treated pre-sowing with 0 (control), 1444 (1X), 2888 (2X) and 4332 (3X) μg glyphosate kg^{-1} soil and 0 (control), 40 (1X), 80 (2X) and 120 (3X) μg quizalofop p ethyl kg^{-1} soil separately. Soils after adding the calculated concentration of each herbicide was mixed homogenously. Three pots for each treatment were arranged in a complete randomized design. Three plants were maintained in each pot 7 days after emergence. The pots were watered as and when required and maintained in an open field conditions.

Measurement of biological characteristics of plants

Length, dry matter and symbiotic attribute

Greengram plants grown in three pots for each treatment were uprooted and change in growth (length and height) of plants was recorded at 60 DAS. Plants detached at 60 DAS were dried at 80°C and dry biomass was determined. Plants removed at 60 days after seeding (DAS) were also used to score nodule numbers.

Roots were gently washed, and nodules were detached, counted, oven dried at 80°C and nodule dry mass were measured. Due to- (i) small size of experimental pots (ii) lodging problems of standing greengram plants and (iii) problem in maintaining adequate nutrients in pots, the plants were harvested before grain yield stage.

Total chlorophyll content

Total chlorophyll content of fresh foliage was estimated by the method of Arnon (1949). For this, fresh leaves (0.5g) were crushed in 20 ml of 80% acetone. The extract was centrifuged at 5000 r/min for 5 min. Supernatant was taken and the volume was made 50 ml with 80% acetone. Absorbance was read at 645 and 663nm on UV vis-spectrophotometer.

Total chlorophyll content was calculated as:

$$\text{Total chlorophyll} = \frac{[20.2 \times 0.D_{645} + 8.02 \times 0.D_{663}] \times V}{1000 \times W}$$

Where, V is the final volume of chlorophyll extraction in 80% acetone and W is the fresh weight of tissue extract.

Effect of herbicides on root morphology of greengram (SEM study)

The toxic and destructive impact of glyphosate and quizalofop on root morphology of greengram plants grown on soft agar plates treated with and without 1200 and 400 $\mu\text{g ml}^{-1}$ of glyphosate and quizalofop, respectively, was monitored under JSM 6510 LV scanning electron microscope (JEOL, Japan) as described by Ahmed *et al.*, (2017).

Statistical analysis

In vitro experiments were repeated three times and data were statistically analysed using Duncan's multiple range test (DMRT) at 5% probability level. Since the data of the measured parameters obtained were homogenous, they were pooled together and subjected to analysis of variance (ANOVA). The difference among treatment means was compared by high range statistical domain (HSD) using DMRT test. Plant based data were also analyzed by two-way ANOVA at 5% probability level.

Results and Discussion

Characterization and identification of herbicide tolerant rhizobacterial strains

In this study, the morphological and biochemical features of bacterial strains recovered from mustard rhizosphere and greengram nodules varied greatly (Table 2). Based on these properties, Gram negative strains R5 (from nodules of greengram) and PS6 (from mustard rhizosphere) were identified to genus level as *Bradyrhizobium* and *Pseudomonas*, respectively (Table 2). Also, the bacterial strains when grown on herbicide amended minimal salt agar plates showed significant tolerance to quizalofopethyl, and glyphosate, which ranged between 800 to 3200 $\mu\text{g ml}^{-1}$. Occurrence of tolerance or resistance to herbicides among PGPR herbicides is perhaps a unique feature which is regulated both genetically and physiologically. Hence, microorganisms that have developed resistance to pesticides are capable of frequently degrading them (Curutiu *et al.*, 2017). Resistance against pesticides in general is attributed due to physiological changes that induce microbial metabolism to follow a new metabolic pathway that help organisms to bypass a biochemical reaction which could otherwise be inhibited by some specific pesticides (Bellinaso *et al.*, 2003). Also, the resistance could be due to emergence of mutations inherited by successors of microbes (Herman *et al.*, 2005). Since the minimal salt media used in our study to evaluate the MTL values of herbicide tolerant of both rhizobacterial strains (R5 and PS6) did not contain any C and N sources except the tested herbicides, it is inferred that both the strains might have utilized these herbicides amended in minimal salt agar medium as a sole energy source through a biodegradation process and hence, showed high tolerance levels against herbicides. Similarly, in other report, P.

fluorescens tolerated higher level of herbicides like, Imazethapyr, 2,4-D and pendimethalin (Kurahde *et al.*, 2016).

Effect of herbicides on PGP activities of rhizobacterial strains

IAA, siderophores, HCN and ammonia

The effect of herbicides on plant growth promoting rhizobacteria including nitrogen fixers (Nithyakalyani *et al.*, 2016) and phosphate solubilizing microorganisms (Chandra *et al.*, 2016) have been variable. Here, both R5 and PS6 strains displayed significant PGP activities and produced IAA, siderophores, HCN and NH₃. It was observed that herbicides in general had deleterious effect which however, increased with increasing concentration of glyphosate and quizalofop-p ethyl. Of the different growth regulators, IAA produced by PGPR has been found to enhance the growth and other physiological activities of plants such as-(i) root morphogenesis and root elongation (ii) cell growth, division and symbiosis (iii) apical dominance and (iv) phototropism and geotropism seven in herbicide contaminated soils (Deinum *et al.*, 2016).

Among the bacterial strains, *Pseudomonas* PS6 produced significantly more amount of IAA (51.48 ug/ml) as compared to *Bradyrhizobium* strain R5 (43.8 ug/ml). However, under herbicide stress the level of IAA was considerably reduced in both the strains. Comparing the effects of the herbicides, quizalofop-p-ethyl at two time more concentration, reduced the IAA secretion maximally by 70% in case of PS6. Glyphosate at the same rate showed least toxicity and decreased IAA by 55% in case of *Bradyrhizobium* R5 while and it was 36% in case of *Pseudomonas* PS6 over untreated control (Table 3). In an identical experiment, Nityakalyani *et al.*, (2016) observed similar

results for *Bradyrhizobium* when grown in stress free medium. Reduction in IAA production by PGPR strains under herbicides stress has also been reported recently by Tripathi *et al.*, (2015). Siderophores is yet another important microbial metabolite secreted by majority of bacteria (Sicairos, 2015) and fungi (Aziz *et al.*, 2016). Siderophores synthesized by microbial communities' supply iron to plants when grown under iron-deficient conditions (Kurth *et al.*, 2016). Also, the microbial siderophores for example pyoverdine produced by many *Pseudomonas* species has been reported to play an important role in the management of phytopathogens (Premachandra, 2016). Considering these, the siderophores production potential of *Bradyrhizobium*R5 and *Pseudomonas*PS6 was evaluated in this study.

As the concentration of herbicides increased, it affected negatively the production of siderophores by both bacterial strains. Another important but indirect plant growth promoting activity of HCN production has been found to be a common trait of *Pseudomonas* and *Bacillus* (Thakur *et al.*, 2017). Cyanide is a secondary metabolite of several microorganisms, which can be produced directly from glycine and cyanogenic glycosides (Rijavec and Lapanje, 2016).

Though the strains demonstrated significant production of HCN and ammonia, but at the highest concentration (3X) of herbicides, these activities were completely inhibited. Reduction in HCN and ammonia production by rhizobacterial strains under stressed environment is reported probably be due to the impairment of various metabolic activities (Azarmi *et al.*, 2016). Similarly, inhibition in HCN and ammonia produced by rhizosphere microorganisms under stressed environment is reported (Rani and Kumar, 2017).

Exopolysaccharide (EPS) production and phosphate solubilization

Exopolysaccharide is a major biochemical component which protects bacteria from harsher environment (Kaushal and Wani, 2016). In our study, *Bradyrhizobium* strain R5 and *Pseudomonas* strain PS6 produced considerable amounts of EPS both under herbicide free and stressed conditions. However, as the concentration of herbicides was increased, the quantity of EPS was decreased (Fig. 2). At the highest concentration of herbicides, the percent decrease in EPS by *Bradyrhizobium* strain R5 followed the order: quizalofop-p-ethyl (89) > glyphosate (56) while in case of strain *Pseudomonas* strain PS6, it was: quizalofop-p-ethyl (81) > glyphosate (33).

Inorganic phosphate solubilization is yet another major PGP activity associated with PGPR that facilitate plant growth by supplying soluble P to plants. This P-solubilizing activity of rhizobacteria is coupled to drop in pH, which could be due to secretion of low molecular weight organic acids such as gluconic, α -2 ketogluconic, oxalic, citric, acetic, malic and succinic acids etc. (Patel *et al.*, 2015). In the present study, the amount of P-solubilized by the herbicide tolerant strains decreased with increasing concentration of herbicides. The maximum decrease in the P-solubilization was observed in *Bradyrhizobium* sp. R5 at quizalofop-p-ethyl, as compared to *Pseudomonas* sp. PS6 at all the concentrations tested. However, at the maximum concentration of herbicide added to media, the order of herbicide-toxicity in case of both strains (percent decline relative to control) was quizalofop-p-ethyl > glyphosate.

Similar production and reduction in phosphate solubilizing activities of bacterial strains under herbicides stress has been reported (Tripathi *et al.*, 2015).

Evaluation of herbicide induced toxicity to bacterial strains under SEM and CLSM

After assessing the lethal effect of glyphosate and quizalofop on viable growth of both bacterial strains under *in vitro* conditions, the toxicity of herbicides to strains R5 and PS6 was evaluated further by SEM. The SEM images showed a dissimilar but un-ruptured bacterial cell when grown in the absence of herbicide (Fig. 1A and D). However, bacterial strains when grown in the presence of 1200 and 400 $\mu\text{g ml}^{-1}$ of glyphosate and quizalofop, respectively, had damaged and disintegrated cell surface (Fig. 1B, C, E and F). The SEM analysis therefore, confirmed the herbicidal toxicity and validated the inhibitory effect of glyphosate and quizalofop observed under *in vitro* condition. To the best of our information, no similar reports on herbicidal toxicity to cellular damage of bacterial cells observed under SEM are available. CLSM images further validated the toxicity of herbicides resulting in increasing number of dead cells which increased with increasing concentrations of herbicides (data not shown). And hence, this is also perhaps the first report on herbicidal toxicity to agronomically important PGPR (*Bradyrhizobium* and *Pseudomonas*) observed under CLSM.

Assessment of bioremediation potential of bacterial strains

Inoculation effects on length and dry biomass of plant organs

The change in growth of plant organs (roots and shoots) of greengram plant grown in sandy clay loam soil treated with the recommended (1X), two (2X) and three (3X) times more of recommended rates of commercial grade herbicides (quizalofop-p-ethyl and glyphosate) varied at 60 DAS (Table 4). Generally, a progressive decline of variable magnitude was observed for both

root and shoot length as the concentration of quizalofop-p-ethyl and glyphosate was increased from 1X to 3X in soil. Among herbicide, 4332 µg/ kg soil of glyphosate displayed the most toxic and obvious effect and decreased root and shoot length by 34% (5 cm) and 37% (15 cm), respectively while quizalofop at 120 µg/kg soil (3X) decreased root and shoot length by 44% (cm) and 32% (cm), respectively, at 60 DAS. However, when used as inoculant, *Bradyrhizobium* and *Pseudomonas* reduced significantly the toxicity of herbicides. For example, *Bradyrhizobium* sp. R5 inoculated greengram plants grown in soils treated with 1X of quizalofop had 8% more shoot length relative to uninoculated and untreated control plants. While, *Pseudomonas* sp. PS6 increased the root and shoot length by 3% and 12% when plants were grown with normal rates of glyphosate in comparison to uninoculated, but glyphosate treated greengram plants.

The dry biomass accumulation in plant organs (roots and shoots) and whole plants grown in sandy clay loam soil consistently decreased with increasing concentrations of both herbicides. In general, all concentrations of quizalofop and glyphosate decreased the dry matter accumulation in root and shoots removed at 60 DAS relative to the control. Quizalofop and glyphosate at 3X maximally declined the dry biomass of roots by 37 and 24% and shoots by 40 and 70% at 60 DAS, respectively. In contrast, the bioinoculants in general reduced the phytotoxic effect of herbicides to greengram plants. For example, maximum growth promotory effect was recorded when *Bradyrhizobium* and *Pseudomonas* inoculated plants were grown in soils treated with normal rates of glyphosate and the total shoot dry biomass of greengram plant was increased by 23% and 10%, respectively as compare to control at 60 DAS.

Table.1 Herbicides used in the present study

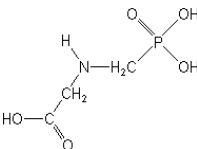
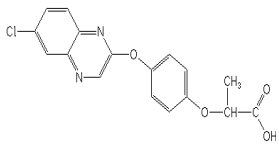
Characteristics	Herbicide used	
	Glyphosate	Quizalofop-ethyl
Common name	Glyphosate	Quizalofop-ethyl
Chemical name	N-(phosphonomethyl) glycine	(RS)-2-[4-6-chloroquinoxalin-2-yloxy) phenoxy] propionate
Chemical family	Organophosphorous	Quinoxaline
Trade name	Round up	Targa
Manufacturer	Monsanto, Excel	Nissan
Molecular weight	169.08	372.8
Empirical formula	C ₃ H ₈ NO ₅ P	C ₁₉ H ₁₇ ClN ₂ O ₄
Solubility	Water	Water, benzene, xylene etc.
Melting point	200 °C	91.7 °C
Recommended Dose (µg/kg)	1444	40
Structure		

Table.2 Morphological and biochemical properties of bacterial strains PS6 and R5

Characteristics	<i>Pseudomonas</i> sp. strain PS6	<i>Bradyrhizobium</i> sp. strain R5
Morphology	Margin entire, mucoid and dull green colony	Irregular margin, transparent, slimy and mucilaginous colony
Gram reaction	-ve	-ve
Shape	Short rods	Short rods
Biochemical reactions		
Citrate utilization	+	-
Indole	-	-
Methyl red	-	+
Nitrate reduction	+	+
Oxidase	-	+
Voges Proskaur	-	-
Carbohydrate utilization		
Glucose	+	+
Lactose	+	+
Fructose	+	+
Sucrose	+	+
Hydrolysis		
Starch	+	-
Gelatin	+	+
Tolerance to herbicides (MTL)		
Glyphosate	3200 µg mL	3200 µg mL
Quizalofop p ethyl	800 µg mL	800 µg mL

Table.3 Plant growth promoting activities of *Bradyrhizobium* strain R5 and *Pseudomonas* PS6 under glyphosate and quizalofop stress

Bacterial strains	Treatments	Dose rate (µg/ml)	Plant growth promoting substances					
			IAA (µg/ml)	Siderophores (FeCl ₃ test)	Ammonia HCN	P solubilized (µg/ml)	EPS (µg/ml)	
<i>Bradyrhizobium</i> sp. strain R5	Control	0	43.8±1.4b	++	+	+++	4.6±0.4c	1.9±0.4a
		600	39.8±0.2c	++	+	++	3.5±0.4d	1.3±0.2b
	Glyphosate	1200	32.7±0.6e	+	+	++	2.8±0.2fg	1.0±0.04bcd
		1800	21.6±0.9h	+	ND	+	2.0±0.06h	0.8±0.04def
	Quizalofop-p-ethyl	200	34.8±0.9e	++	+	++	1.9±0.07h	0.4±0.02ghi
		400	26.2±0.5g	+	+	+	1.4±0.04i	0.3±0.02hi
<i>Pseudomonas</i> sp. strain PS6	Control	0	51.4±1.2a	++	+	+++	5.5±0.5a	1.3±0.1b
		600	45.7±3.1b	+	+	++	4.9±0.2b	1.1±0.06bc
	Glyphosate	1200	40.8±1c	+	ND	++	3.8±0.2d	1.1±0.005bcd
		1800	32.9±1.4e	+	+	+	3.0±0.1e	0.9±0.07cdef
	Quizalofop-p-ethyl	200	33.9±0.9e	++	+	+	3.7±0.1d	1±0.1cde
		400	23.3±1.5h	+	+	+	2.6±0.1fg	0.7±0.04efg
LSD		600	12.8±1k	+	ND	+	1.6±0.2i	0.2±0.04hi
			1.9569	-	-	-	0.05182	0.02890

Values are mean of three replicates where each replicate constituted three plants pot⁻¹. Mean values followed by different letters are significantly different within a row or column at $p \leq 0.05$ according to DMR test following two-way ANOVA

Table.4 Effect of *Bradyrhizobium* sp. (Vigna) R5 and *Pseudomonas* sp. PS6 bio- inoculants on chemical and biological properties of greengram raised in sandy clay loam soil treated with three concentrations of herbicides

Treatment	Dose rate (µg/kg)	% seed germination	Symbiotic attributes			Plant height (cm)		Fresh weight (gm)		Dry biomass (gm)	
			Nodule number/plant	Nodule fresh weight (mg)	Dry biomass (mg)	Root	Shoot	Root	Shoot	Root	Shoot
Uninoculated Control	0	100	10	125b	44b	7.8a	25.7a	0.47c	1.33f	0.16b	0.7b
G (1X)	1444	90	9	111e	35fg	7.0bcd	19b	0.21e	1.1g	0.1efg	0.6d
G (2X)	2888	90	8	91.3g	29.3ij	6.2efg	18.7cd	0.10h	0.6j	0.06hi	0.3i
G (3X)	4332	70	6	70i	251m	5.07hi	15.9def	0.07i	0.4k	0.04ij	0.2j
Q (1X)	40	90	9	111e	40cd	7.6ab	18.7c	0.42a	1.3f	0.12de	0.8ab
Q (2X)	80	80	8	90g	40cd	6.1efg	16.1de	0.34b	0.8h	0.10efg	0.5f
Q (3X)	120	80	5	81h	24m	4.1j	15.4a	0.20ef	0.7i	0.06hi	0.4h
Bio inoculant Bradyrhizobium sp. R5	0	100	12	148a	54.6a	7.5ab	23b	0.33b	1.7c	0.17b	0.8a
G (1X)+R5	1444	90	10	146a	53a	6.2efg	22.7cd	0.23de	1.5e	0.13	0.8b
G (2X)+R5	2888	80	10	121b	38.6cde	5.05hi	13.1f	0.12h	1.08g	0.11def	0.7c
G (3X)+R5	4332	70	8	110e	33gh	4.4ij	22.4b	0.09hi	0.8h	0.09fg	0.5fg
Q (1X)+R5	40	90	8	114cd	36.3ef	6.04efg	20.3bc	0.28c	1.6d	0.03j	0.6de
Q (2X)+R5	80	80	7	92g	28.3jk	7.6b	18.6cd	0.26cd	1.3f	0.13d	0.5ef
Q (3X)+R5	120	80	6	82.3h	23.6m	6.0efg	21.9bc	0.18fg	0.9h	0.10ef	0.4h
Bio inoculant Pseudomonas sp. PS6	0	90	10	116c	41c	7.7a	21.6b	0.32b	1.9b	0.22a	0.8ab
G (1X)+PS6	1444	90	9	113cde	37.5def	7.2ab	15.6def	0.28c	1.4e	0.09gh	0.7c
G (2X)+PS6	2888	90	7	97f	35.3fg	6.6cde	12.9f	0.22e	1.3f	0.07h	0.4gh
G (3X)+PS6	4332	70	6	78.3h	27.3jkl	5.7gh	10fg	0.16g	0.8f	0.14cd	0.3i
Q (1X)+PS6	40	80	7	90.3g	321hi	6.5def	21.7bc	0.28c	2.06a	0.09gh	0.7c
Q (2X)+PS6	80	80	6	78h	28jk	5.8fg	17.8d	0.20ef	1.7c	0.07h	0.7c
Q (3x)+PS6	120	70	4	70i	25.6klm	4.1j	12.9f	0.11h	0.7i	0.04j	0.2i
LSD				4.07	2.45	1.08	2.57	0.03	0.08	0.02	0.05
F value				348.4	86.3	1.0	0.9	3.9	547.2	8.0	61.2
Inoculation (df=3)											
Herbicides (df=1)				1075.6	233.5	67.1	53.4	178.3	610.9	33.4	283.1

Each value is a mean of three replicate where each replicate constituted three plants/pot. Mean values are significant at $p \leq 0.05$. Means followed by similar alphabets not significantly different from each other according to DMRT test.

Fig.1 Scanning electron micrographs of *Pseudomonas* sp. strain PS6 (A) control cells, and treated with (B) 1200 µg/ml glyphosate and 400 µg/ml quizalofop. *Bradyrhizobium* sp. strain R5 (D) control cells (E) treated with 1200 µg/ml glyphosate and (F) treated with 400 µg/ml quizalofop. The area encircled indicates damage/rupture caused by the herbicides

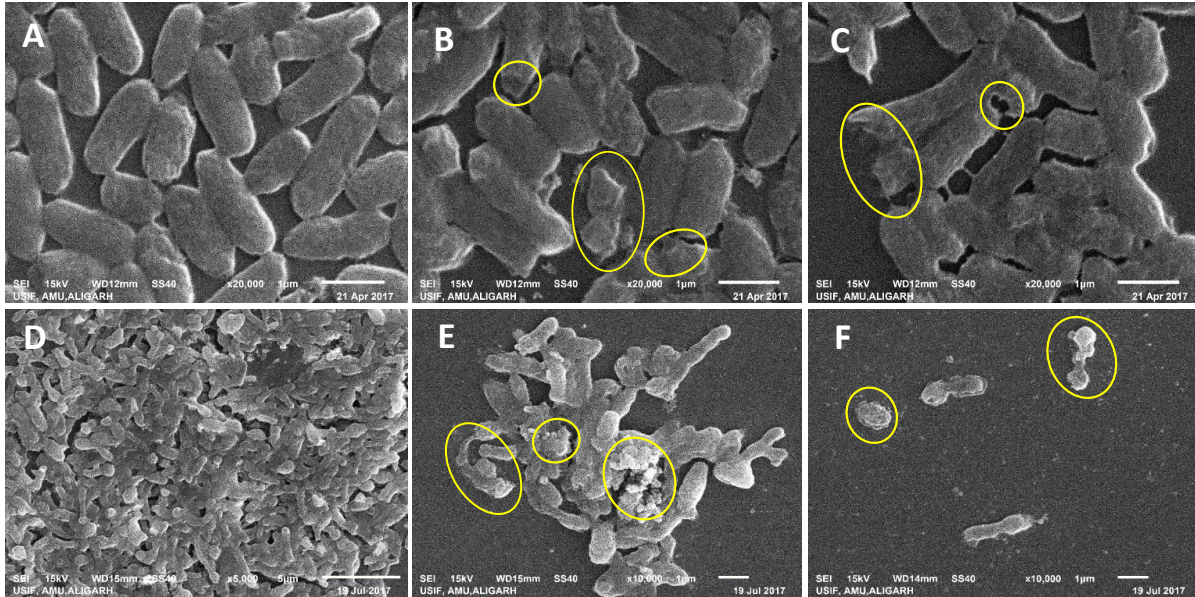


Fig.2 Inhibitory effect of herbicides glyphosate and quizalofop on EPS production by bacterial strain R5 and PS6

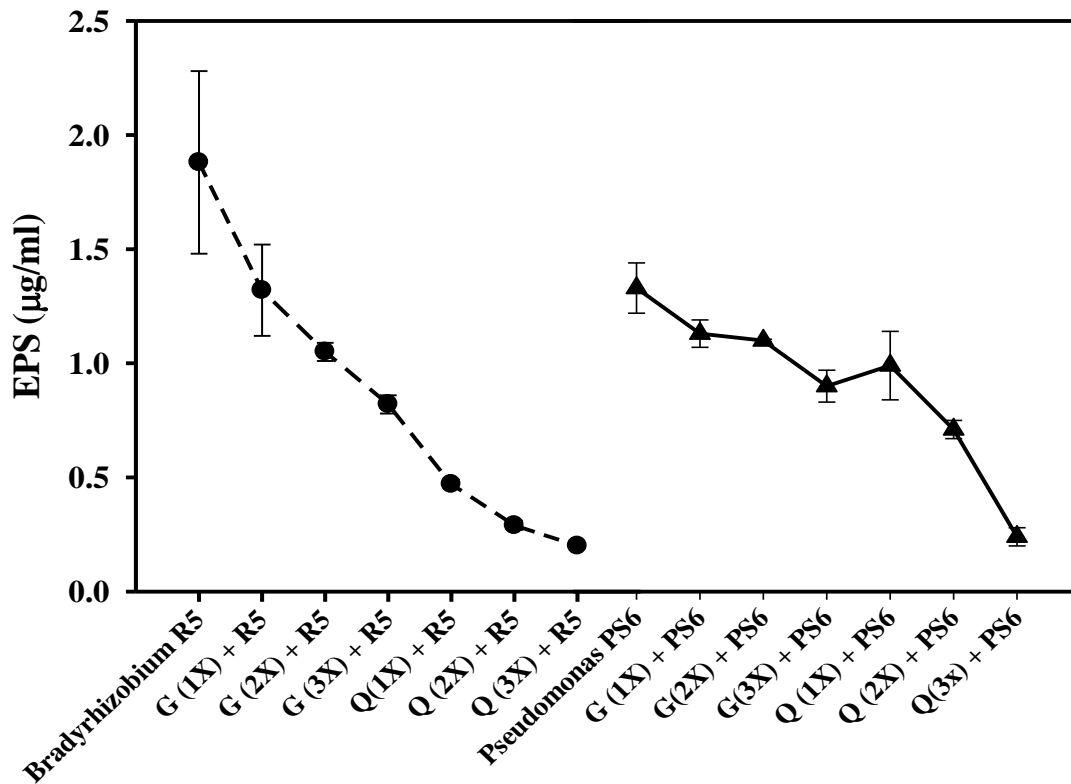


Fig.3 Chlorophyll content in fresh foliage of greengram plants grown in presence of glyphosate and quizalofop and herbicide tolerant bacterial strains (R5 and PS6)

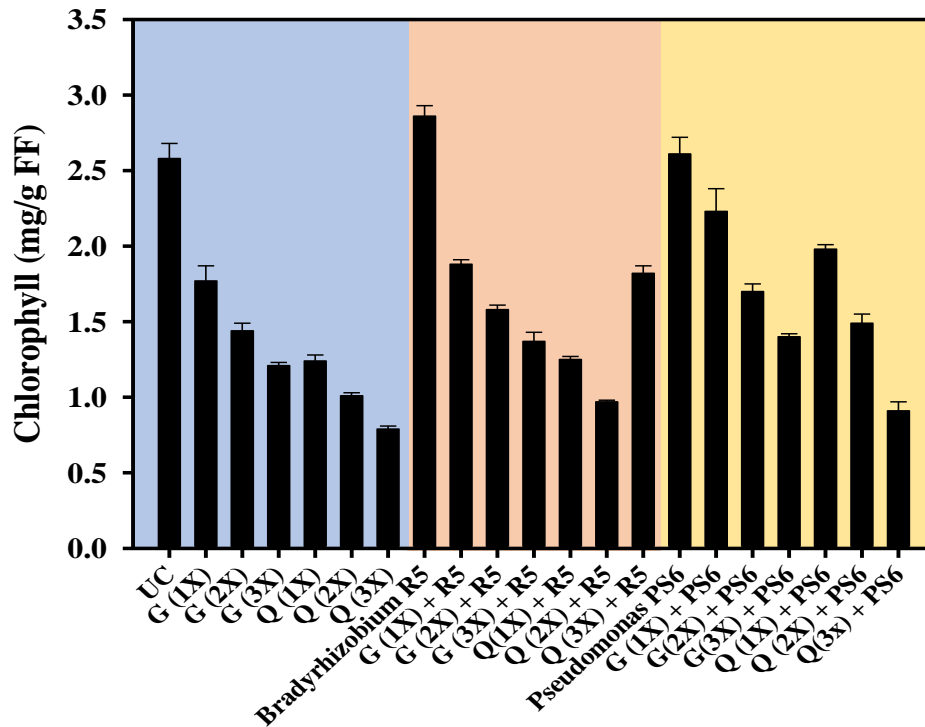
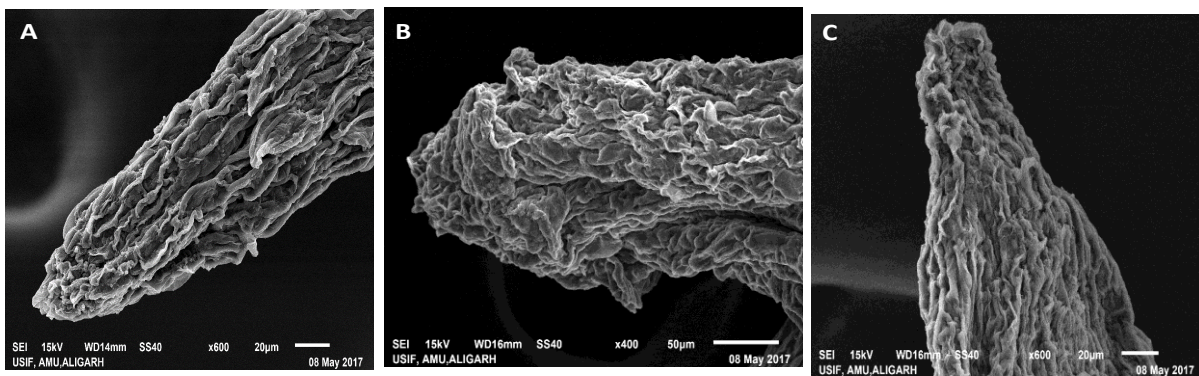


Fig.4 Scanning electron micrograph of greengram roots. Panel A shows root tip of greengram grown without herbicide whereas, panel B and C indicate root morphology altered by glyphosate and quizalofop, respectively



Effect of herbicides on chlorophyll content and symbiotic attributes

The impact of three concentrations of glyphosate and quizalofop on photosynthetic pigments (chlorophyll content) of fresh leaves of greengram measured at pod fill stage (60

DAS) was variable (Fig. 3). The total chlorophyll content however, consistently declined with increasing rates of herbicides irrespective of inoculant application. For example, the 120 µg/kg soil of quizalofop decreased the chlorophyll content by 80% over control as compared to control. In

addition, when herbicide tolerant inocula (R5 and PS6 strains) were applied with three concentrations of herbicides, the chlorophyll content was increased as compared to greengram plants developed in soils treated with only herbicides. The chlorophyll content in fresh foliage of *Pseudomonas* sp. strain PS6 inoculated greengram plants increased by 46% at 2X concentration of quizalofop as compared to un-inoculated plants grown solely with 2X concentration of quizalofop. Similarly, glyphosate, followed a trend like those observed for quizalofop. When *Bradyrhizobium* sp. strain R5 was used as bio-inoculant, it increased the chlorophyll content maximally by 23%, at normal rates of glyphosate.

The inoculated and un-inoculated greengram plants exposed to three concentrations each of quizalofop P ethyl and glyphosate had variable symbiotic properties. As an example, glyphosate at normal rate decreased the nodule number and nodule dry mass by 10% and 20%, respectively, compared with the uninoculated and untreated control (Table 4). The bio-inoculant *Bradyrhizobium* strain R5 on the contrary, increased the nodule number and nodule dry mass by 10% and 33%, respectively, compared with the plants grown in soil treated only with glyphosate (1X dose). A similar trend was observed for *Pseudomonas* strain PS6. Moreover, it was interesting to note that the rhizobacterial inoculants substantially increased the nodule number and dry mass nodule recovered from root systems of plants grown even with 3X doses of glyphosate and quizalofop-p-ethyl (Table 4).

Morphological distortion in greengram roots

Distortion/damage to root tips was observed under SEM when greengram plants were raised in the presence of 1200 and 400 $\mu\text{g ml}^{-1}$ of glyphosate and quizalofop, respectively

(Fig. 4A, B and C). The inhibitory effect induced by both the herbicides was more distinct in the radical regions of growing roots. The destruction to root morphology of greengram plants grown under herbicide stress and observed under SEM is perhaps the first report by us in this study. However, a significant damage in the morphological structure of chickpea roots grown in the stressed condition is reported by Mondal *et al.*, (2013). Similar structural damage in roots of wheat plants grown in metal treated soils has been reported (Rizvi and Khan, 2017).

In conclusion, the inhibitory effects of glyphosate and quizalofop on survival, cellular morphology and growth regulators synthesizing ability of *Bradyrhizobium* sp. R5 and *Pseudomonas* sp. PS6 differed significantly. Interestingly, the ability of both rhizobacterial strains to excrete plant growth facilitators was not completely lost even when they were exposed to higher concentrations of herbicides; however, the quantum of secretion declined progressively.

Additionally, the inhibitory impact of glyphosate and quizalofop on greengram plants was reduced substantially when both *Bradyrhizobium* sp. R5 and *Pseudomonas* sp. PS6 were used as inoculants against greengram crops. The inclusive enhancement in greengram production due to inoculation of strains R5 and PS6 even in the presence of glyphosate and quizalofop could probably be due to multiple reasons such as-(i) capability of rhizobacterial strains to tolerate higher concentrations of herbicides (ii) secretion of phytohormone, siderophores and exopolysaccharides by bacterial strains (iii) bio-reduction of herbicides by herbicide-tolerant rhizobacteria. Due to these multiple properties, the herbicide-tolerant PGPR strains can be promoted as bio-fertilizer for accelerating the production of greengram even under herbicide-enriched soils.

Abbreviations

MIC- Minimum inhibitory concentration;
IAA- Indole-3-acetic acid; HCN- Hydrogen cyanide; DAS- Days after sowing

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