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Response of Greengram [*Vigna radiata* (L.) Wilczek] Grown in Herbicide-Amended Soil to Inoculation with *Bradyrhizobium* sp. (vigna) MRM6

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ABSTRACT

The present study was conducted to determine the plant growth-promoting activities of *Bradyrhizobium* sp. (*vigna*) strain MRM6 grown in the presence and absence of the selected herbicides, quizalafop-p-ethyl and clodinafop. The herbicide tolerant *Bradyrhizobium* sp. (*vigna*) strain MRM6 was further tested for bioremediation and plant growth promoting potential using greengram as a test crop, grown in soils treated with quizalafop-p-ethyl and clodinafop, at both recommended and higher dose rates. The quizalafop-p-ethyl and clodinafop tolerant *Bradyrhizobium* sp. (*vigna*) strain MRM6 recovered from the nodules of greengram plants produced a substantial amount of indole acetic acid, siderophores, hydrogen cyanide and ammonia, both in the presence and absence of technical grade quizalafop-p-ethyl and clodinafop under *in vitro* conditions. Both quizalafop-p-ethyl [40 (recommended dose), 80, and 120 µg kg^{-1} soil] and clodinafop [400 (recommended dose), 800, and 1200 µg kg^{-1} soil] decreased the growth of *Bradyrhizobium* sp. MRM6-inoculated and un-inoculated plants. Quizalafop-p-ethyl at all concentrations showed more phytotoxicity and affected the growth in terms of nodulation, total dry biomass, nutrients (nitrogen and phosphorus) uptake and seed yield compared to clodinafop or un-inoculated control. When the inoculant strain MRM6 was used with any concentration of the two herbicides, the growth and nodulation parameters of the plants were relatively better compared to the plants grown in soils treated solely (without inoculant) with the same concentration of each herbicide. For example, when strain MRM6 was used with 1200 µg clodinafop kg^{-1} soil, it increased the symbiotic attributes (nodule number, nodule dry mass, leghaemoglobin), whole biomass, root N, shoot N, root P, shoot P, seed yield, and grain protein by 14%, 62%, 60%, 102%, 23%, 31%, 9%, 10%, 72% and 4%, respectively, compared to the un-inoculated treatment having the same concentration of clodinafop. The present findings suggest that the bradyrhizobial strain MRM6 endowed with multiple properties could be used to facilitate the productivity of greengram under herbicide-stressed soils.

Keywords: Bioremediation, Herbicide-stress, Legume, PGPR (Plant growth promoting rhizobacteria), Toxicity.

INTRODUCTION

Herbicides are commonly used for weed control in high-input crop production systems. Due to extensive and injudicious application, most of the unused fractions of herbicides, however, may persist within soils (Madhaiyan *et al.*, 2006). These plant protection measures with herbicides, thus, pose a potential threat to the survival and metabolic activities of rhizobacteria (Ahemad *et al.*, 2009) and, consequently, could indirectly affect soil fertility. While assessing the influence of selected herbicides on the symbiotic efficiency and nitrogenase activity of various rhizobial strains, Niewiadomska and Klama (2005)

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reported that herbicides in general reduced nitrogenase activity of metabolically active strains. Therefore, the nodulation and root growth of the tested plants were severely reduced, as also reported by others (Ahemad and Khan, 2009; Anderson *et al.*, 2004).

One of the most widely practiced strategies to maintain the fertility of soils is the cultivation of legumes either alone or in a rotation cropping system. Legumes increase N pool of soils by forming symbiosis with N_2-fixing rhizobia, which transform unavailable forms of N into other compounds utilizable by plants (Fox *et al.*, 2007). Because of this property, rhizobial inoculants are often applied to seeds of legumes/soils to ensure effective/competitive nodulation and subsequent nitrogen fixation (Ahemad and Khan, 2009; Zawoznik *et al.*, 2005). Such rhizobial inoculants are generally used in conjunction with stressors such as, herbicides, which may be detrimental to both legumes and its symbiotic partner (Eberbach and Douglas, 1989; Khan *et al.*, 2004).

Although a lot of information on the phytotoxic effects of herbicides on cognate rhizobia and agronomic crops (including legumes) is available, there is a great contradiction in the reported results. Henceforth, it becomes difficult to deduce a firm and logical conclusion about the parallel effects of herbicides on legumes and *vis-à-vis* their symbiotic associates. In addition, the toxicity of herbicides, quizalafop-p-ethyl [Ethyl (RS)-2-(4-6-chloroquinoxolin-2-yloxy) phenoxy] propionate] and clodinafop [(R)-2-[4-(5-chloro-3-fluoro-2-pyridyloxy) phenoxy] propionic acid] to plant growth promoting (PGP) traits of *Bradyrhizobium* sp. and greengram is not known. The aim of this study was, therefore, to assess the PGP potentials of quizalafop-p-ethyl and clodinafop tolerant *Bradyrhizobium* sp. in the presence of technical grade quizalafop-p-ethyl and clodinafop. The effect of herbicide tolerant strain on the biological and chemical properties of green gram plants, grown in sandy clay loam soils treated with different concentrations of quizalafop-p-ethyl and clodinafop was also evaluated.

MATERIALS AND METHODS

Rhizobial Strains and Herbicide Tolerance

A total of 50 rhizobial strains were isolated from nodules of greengram plants grown in the experimental fields of the Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh (27° 29' latitude and 72° 29' longitude), Uttar Pradesh, India, using yeast extract mannitol (YEM) medium (g l^{-1}: mannitol 10; K_2HPO_4 0.5; $MgSO_4.7H_2O$ 0.2; NaCl 0.1; yeast extract 1; $CaCO_3$ 1; pH 7) (Vincent, 1970). The rhizobial isolates were identified to genus level by biochemical tests following Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) and host specificity (Somasegaran and Hoben, 1994) and were referred to as bradyrhizobial strains. The strains were tested for their sensitivity/resistance to technical grade quizalafop-p-ethyl and clodinafop (a.i. 98% for both herbicides; Parijat Agrochemicals, New Delhi, India) by agar plate dilution method using minimal salt agar medium (g l^{-1}: KH_2PO_4 1, K_2HPO_4 1, NH_4NO_3 1, $MgSO_4.7H_2O$ 0.2, $CaCl_2.2H_2O$ 0.02, $FeSO_4.7H_2O$ 0.01, pH 6.5). The freshly prepared agar plates were separately amended with increasing concentrations (0 to 3200 µg ml^{-1}, at two-fold dilution intervals) of both quizalafop-p-ethyl and clodinafop. After that, plates were spot inoculated with 10 µl of 10^8 cells ml^{-1} bradyrhizobial strains. Each experiment was replicated three times. Plates were incubated at 28±2°C for 72 hours and the highest concentration of quizalafop-p-ethyl and clodinafop supporting bradyrhizobial growth was defined as the maximum resistance level (MRL).

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Effect of Quizalafop-P-Ethyl and Clodinafop on Plant Growth Promoting Compounds

Indole-3-acetic acid (IAA) was quantitatively assayed by the method of Gordon and Weber (1951) later modified by Brick *et al.* (1991). For this activity, bradyrhizobial strains exhibiting the highest MRL were grown in Luria Bertani (LB) broth (g l^{-1}: tryptone 10; yeast extract 5; NaCl 10 and pH 7.5) supplemented with 0 (control), 40 (recommended dose), 80 and 120 µg l^{-1} quizalafop-p-ethyl and 0, 400 (recommended dose), 800, and 1,200 µg l^{-1} clodinafop. A 100-ml of LB broth supplemented with 100 µg ml^{-1} tryptophan was inoculated with 1 ml of *Bradyrhizobium* culture (10^8 cells ml^{-1}), grown in YEM broth. The inoculated LB broth was incubated at 28±2°C for five days with shaking at 125 rpm. An aliquot of 2 ml supernatant was mixed with 100 µl orthophosphoric acid and 4 ml Salkowsky reagent (2% 0.5 M $FeCl_3$ in 35% per-chloric acid) and incubated at 28±2°C in darkness for 1 hour. The absorbance of pink color developed was read at 530 nm. The IAA concentration in the supernatant was determined using a calibration curve of pure IAA as a standard (Brick *et al.*, 1991). The experiments were repeated three times.

The bradyrhizobial strains were further assayed for qualitative production of siderophores using Chrome azurol S (CAS) agar medium. The method of Alexander and Zuberer (1991) and $FeCl_3$ test (Neiland, 1981) was followed. CAS agar plates supplemented with 0, 40, 80 and 120 µg l^{-1} quizalafop-p-ethyl and 0, 400, 800 and 1,200 µg l^{-1} clodinafop were separately prepared and equally divided into two sectors. Plates were spot inoculated with 10 µl of 10^8 cells ml^{-1} and incubated at 28±2°C for five days. Development of yellow to orange halo around the bacterial growth was considered as positive for siderophore production. The siderophores produced by the test strains were also quantitatively assayed using Modi medium (K_2HPO_4 0.05%; MgSO4 0.04%; NaCl 0.01%; mannitol 1%; glutamine 0.1%; NH_4NO_3 0.1%). Modi medium amended with quizalafop-p-ethyl (0, 40, 80 and 120 µg l^{-1}) and clodinafop (0, 400, 800 and 1,200 µg l^{-1}) was inoculated with 100 µl of 10^8 cells ml^{-1} of bradyrhizobial strains and incubated at 28±2°C for five days. Cultures were centrifuged and the catechol type phenolates [salicylate (SA) and 2,3-dihydroxy benzoic acid (DHBA)] in the supernatant were measured (Reeves *et al.*, 1983).

The exo-polysaccharides (EPS) produced by the bradyrhizobial strains was evaluated further under *in vitro* conditions. For this, the strains were grown in 100 ml capacity flasks containing basal medium supplemented with 5% sucrose and incubated for five days at 28±2°C on shaker (100 rpm). Culture broth was spun (5,433 g) for 30 minutes and EPS was extracted by adding three volumes of chilled acetone to one volume of supernatant. The precipitated EPS was repeatedly washed three times alternately with distilled water and acetone, transferred to a filter paper and weighed after overnight drying (Mody *et al.*, 1989). To detect catalase, bacterial cultures were grown in nutrient agar medium for 24 h at 28±2°C. The cultures were mixed with appropriate amount of H_2O_2 on a glass slide to observe the evolution of oxygen.

Bradyrhizobium strains were also screened for hydrogen cyanide (HCN) synthesis (Bakker and Schipper, 1987). Briefly, bradyrhizobial strains were grown in HCN induction medium (g l^{-1}: tryptic soy broth 30; glycine 4.4; agar 15) supplemented with 0, 40, 80 and 120 µg l^{-1} quizalafop-p-ethyl or 0, 400, 800 and 1,200 µg l^{-1} clodinafop and were incubated at 28±2°C for four days. Bradyrhizobial strains were streaked on HCN induction plates. A Whatman filter paper No.1 soaked in 2% sodium carbonate prepared in 0.5% picric acid solution was placed on the top of the plate and was sealed with parafilm. Plates were incubated at 28±2°C for four days. Development of orange to red color indicated HCN

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production. Bradyrhizobial strains were also tested for the excretion of ammonia in peptone water supplemented separately with 0, 40, 80 and 120 µg l^{-1} quizalafop-p-ethyl and 0, 400, 800 and 1,200 µg l^{-1} clodinafop. Freshly grown bradyrhizobial strains (200 µl of 10^8 cells ml^{-1}) were inoculated in 20 ml peptone water in tubes and incubated at 28±2°C for four days. One milliliter of Nessler reagent [potassium iodide 50 g; distilled water (ammonia free) 35 ml: add saturated aqueous solution of mercuric chloride until a slight precipitate persists; potassium hydroxide 400 ml: dilute the solution to 1,000 ml with ammonia free distilled water; allow to stand for one week, decant supernatant liquid and store in a tightly capped amber bottle] was added to each tube. Development of yellow color indicated a positive test for ammonia (Dye, 1962). Each individual experiment was repeated three times.

Plant Growth under Herbicide Stress in Pot Trials

The experimental soil was sandy clay loam (organic C 0.4%, Kjeldahl N 0.75 g kg^{-1}, Olsen P 16 mg kg^{-1}, pH 7.2, 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). Seeds of greengram *var.* K851 were surface sterilized (70% ethanol, 3 minutes; 3% sodium hypochlorite, 3 minutes), rinsed six times with sterile water and dried. The sterilized seeds were inoculated with *Bradyrhizobium* strain MRM6, grown in YEM broth. Seeds were soaked in liquid culture medium for 2 hours using 10% gum arabic as adhesive to deliver approximately 10^8 cells $seed^{-1}$. The non-coated sterilized seeds were soaked in sterile water only and served as control. The non-inoculated and inoculated seeds (10 seeds per pot) were sown in clay pots (25 cm high, 22 cm internal diameter) using three kg unsterilized soils with 0, 40 (1X- the recommended dose), 80 (2X), and 120 (3X) µg quizalafop-p-ethyl kg^{-1} soil or 0, 400 (1X- the recommended dose), 800 (2X), and 1,200 (3X) µg clodinafop kg^{-1} soil. Six pots used for each treatment were arranged in a complete randomized design. One week after emergence, plants in each pot were thinned to three plants. The pots were watered with tap water when required and were maintained under open field conditions. The experiment was conducted for two consecutive years.

All plants in the three pots for each treatment were removed 50 days after seeding (DAS) and were observed for the extent of nodulation. The roots were carefully washed and nodules were detached, counted, oven dried (at 80°C) and weighed. The leghaemoglobin (Lb) content of fresh nodules was quantified at 50 DAS (Sadasivam and Manikam, 1992). The Lb was extracted with sodium phosphate buffer (pH 7.4). The extract was divided equally into two glass tubes (5 ml/tube) and equal amount of alkaline pyridine reagent was added to each tube. The haemochrome formed was read at 556 and 539 nm after adding a few crystals of potassium hexacyanoferrate and sodium dithionite, respectively. Plants uprooted at 80 DAS were oven-dried (at 80°C) and dry matter accumulation in plants was measured. Total nitrogen (N) and total phosphorus (P) contents in roots and shoots were measured at 80 DAS by micro-Kjeldahl (Iswaran and Marwah, 1980) and Jackson (1967) methods, respectively. The remaining pots (three pots) for each treatment were maintained until harvest (80 DAS). Seed yield and grain protein (Sadasivam and Manikam, 1992) were determined at harvest.

Statistical Analysis

The experiment was conducted for two consecutive years under the identical environmental conditions using the same treatments. Since the data of the measured parameters obtained were homogenous, they were pooled together and subjected to ANOVA. The difference among treatment

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means was compared by high range statistical domain (HSD) using Tukey test by SPSS 10 for *in vitro* study and two-way ANOVA by Mini-Tab V11 for pot study at 5% probability level.

RESULTS

Herbicide Tolerance and *In Vitro* Plant Growth Promoting Activities

In the present study, a total of 50 rhizobial isolates recovered from greengram nodules were presumptively identified following biochemical and host specificity tests (Table 1). Of these, *Bradyrhizobium* strain MRM6 was specifically selected due to maximum tolerance to both quizalafop-p-ethyl (1,600 µg ml^{-1}) and clodinafop (1,600 µg ml^{-1}) in minimal salts medium supplemented with increasing concentrations of quizalafop-p-ethyl and clodinafop (as a sole source of C and N) (Table 1). Furthermore, the effects of quizalafop-p-ethyl (40, 80 and 120 µg l^{-1}) and clodinafop (400, 800 and 1,200 µg l^{-1}) on the production of plant growth promoting substances by bradyrhizobial strain MRM6 were determined (Table 2). *Bradyrhizobium* strain MRM6 produced 38 µg ml^{-1} IAA in the absence of herbicide. The synthesis of IAA by the bradyrhizobial strain decreased significantly ($P \leq 0.05$) with the addition of quizalafop-p-ethyl and clodinafop at all of the tested herbicide doses. For example, quizalafop-p-ethyl at 40, 80 and 120 µg l^{-1} decreased the IAA production by 82, 89, and 92%, respectively, while clodinafop at 400, 800 and 1200 µg l^{-1} reduced the bacterial IAA secretion by 55, 76, and 82%, respectively, compared with the control. Furthermore, the strain MRM6 exhibiting siderophore activity (preliminarily indicated by the formation of orange colored zone of 12 mm on CAS agar plates) also secreted the iron chelating molecules such as SA (32 µg ml^{-1}) and DHBA (18 µg ml^{-1}) in the absence of herbicide stress. The synthesis of SA and DHBA also decreased significantly ($P \leq 0.05$) as the concentration of both quizalafop-p-ethyl and clodinafop was increased. For instance, quizalafop-p-ethyl at 120 µg l^{-1} decreased SA and DHBA by 62 and 72%, respectively, while clodinafop at 1200 µg l^{-1} decreased the synthesis of the same molecules by 52 and 50%, respectively. In addition, the strain MRM6 produced 21 µg EPS ml^{-1} when no herbicide was added to the medium. The EPS production by the herbicide tolerant rhizobial strain was significantly ($P \leq 0.05$) increased by 19% and 14% in the presence of quizalafop-p-ethyl (120 µg l^{-1}) and clodinafop (1,200 µg l^{-1}), respectively, compared to the untreated control. In addition, MRM6 was positive for catalase, HCN, and ammonia in the absence and presence of both quizalafop-p-ethyl and clodinafop (Table 2).

Table 1. Morphological and biochemical characteristics of the *Bradyrhizobium* sp. (*vigna*) strain MRM6.

Characteristics	Strain MRM6
Morphology	
Gram reaction	-
Shape	Rods
Biochemical reactions	
Citrate utilization	-
Indole	+
Methyl red	+
Nitrate reduction	+
Oxidase	-
Voges Proskaur	+
Carbohydrate utilization	
Dextrose	-
Lactose	-
Mannitol	+
Sucrose	-
Hydrolysis	
Starch	+
Gelatin	-
Tolerance to	
Quizalafop-p-ethyl	1600 µg mL^{-1}
Clodinafop	1600 µg mL^{-1}

(+) Indicates positive and (–) indicates negative reactions.

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Table 2. Plant growth promoting activities of the *Bradyrhizobium* sp. (*vigna*) strain MRM6 both in the presence and absence of quizalafop-p-ethyl and clodinafop.

Herbicides	Dose ($\mu g\ L^{-1}$)	IAA[a] ($\mu g\ mL^{-1}$)	Siderophores				EPS[e] ($\mu g\ mL^{-1}$)	Catalase	HCN[f]	Ammonia
			CAS[b] Agar (mm)	$FeCl_3$ test	Phenolates ($\mu g\ mL^{-1}$)					
					SA[c]	DHBA[d]				
Control		38a	13a	+	32a	18a	21bc	+	+	+
Quizalafop-p-ethyl	40	7c	12a	+	21bc	14bc	22b	+	+	+
	80	4d	11b	+	19c	8e	24a	+	+	+
	120	3d	10b	+	12d	5e	25a	+	+	+
Clodinafop	400	17b	12a	+	21bc	15b	21bc	+	+	+
	800	9c	12a	+	19c	11cd	23b	+	+	+
	1200	7c	11b	+	14d	9d	24a	+	+	+
F value	-	170.8	37.3	-	69.4	243.7	204.5	-	-	-

Values indicate the mean of three replicates. Mean values followed by different letters are significantly different within a row or column, respectively at $P \leq 0.05$ according to Tukey test. a= Indole acetic acid; b= Chrome azurol S agar; c= Salicylic acid; d= 2, 3-dihydroxy benzoic acid; e= Exo-polysaccharides; f = Hydrogen cyanide.

Effect of Herbicides and *Bradyrhizobium* Strain MRM6 on Greengram

The production of PGP substances (IAA, siderophores, EPS, HCN and ammonia) by the *Bradyrhizobium* strain MRM6 at concentrations higher than the recommended doses of both quizalafop-p-ethyl and clodinafop prompted us to assess the effect of this strain on the performance of greengram grown under herbicide stress. The inoculated and un-inoculated greengram plants exposed to three concentrations each of quizalafop-p-ethyl and clodinafop decreased the measured growth parameters of greengram plants. A consistent and herbicide dose dependent reduction in plant growth parameters was observed where the effect of herbicides was less severe in the presence of inoculant. In the absence of bio-inoculant, quizalafop-p-ethyl at 1X dose decreased the nodule number, nodule dry mass, Lb, total dry biomass, root N, shoot N, root P, shoot P, seed yield and grain protein by 29%, 38%, 25%, 46%, 25%, 24%, 33%, 22%, 51%, and 10%, respectively, compared with the control (Table 3). Similarly, in the presence of bio-inoculant, quizalafop-p-ethyl at 1X dose decreased the nodule number, nodule dry mass, Lb, total dry biomass, root N, shoot N, root P, shoot P, seed yield, and grain protein by 57%, 49%, 28%, 49%, 36%, 25%, 11%, 27%, 32% and 7%, respectively, compared with the control. It is important to mention that the bradyrhizobial inoculant with the 3X dose of quizalafop-p-ethyl increased the nodule number, nodule dry mass, Lb, total dry biomass, root N, shoot N, root P, shoot P, seed yield, and grain protein by 9%, 122%, 33%, 179%, 20%, 36%, 46%, 4%, 96% and 5%, respectively, when compared with the plants treated with the same herbicide dose and grown without inoculant (Table 3).

Similarly, without bio-inoculant, clodinafop at 1X dose reduced the nodule number, nodule dry mass, Lb, total dry biomass, root N, shoot N, root P, shoot P, seed yield, and grain protein by 10%, 8%, 12%, 14%, 3%, 4%, 4%, 6%, 14%, and 1%, respectively, compared with the control (Table 4). With bio-inoculant,

Table 3. Effect of three concentrations of quizalafop-p-ethyl on the growth and symbiotic properties of greengram plants grown in soil inoculated with the *Bradyrhizobium* sp. (*vigna*) strain MRM6 and without bioinoculant.

Treatment	Dose (µg kg^{-1} soil)	Nodulation			Total dry biomass (g plant^{-1})	N content (mg g^{-1})		P content (mg g^{-1})		Seed yield (g plant^{-1})	Grain protein (mg/g)
		No./ plant	Dry biomass (mg plant^{-1})	Leghaemoglobin [mM (g f.m)$^{-1}$]		Root	Shoot	Root	Shoot		
Uninoculated	Control	21	66	0.08	2.60	36	50	0.27	0.36	7.4	261
	40	15	41	0.06	1.40	27	38	0.18	0.28	3.6	235
	80	13	38	0.04	1.28	25	32	0.16	0.25	3.2	233
	120	11	35	0.03	1.15	20	28	0.13	0.23	2.8	230
Inoculated	Control	44	151	0.11	7.75	45	61	0.32	0.41	10.9	269
	40	19	93	0.08	3.94	29	46	0.24	0.30	7.4	249
	80	16	84	0.06	3.52	27	42	0.22	0.27	6.1	245
	120	12	78	0.04	3.21	24	38	0.19	0.24	5.5	241
LSD		2.1	0.64	0.005	2.8	1.1	1.5	0.04	0.03	0.16	1.7
F value	Inoculation (df= 1)	171*	111*	453.6*	361*	157.4*	217.3*	417*	379*	188.3*	219.3*
	Herbicide (df= 3)	63*	36.3*	2.4	65.3*	103.2*	19.2*	137.5*	72.3*	117.4*	96.2*
	Inoculation×herbicide (df= 3)	34*	21.3*	54.3*	54*	19.2*	21.3*	65.1*	42.4*	43.5*	34.4*

Values are mean of three replicates where each replicate had three plants/ pot. * Significantly different from the control at $P \leq 0.05$.

Table 4. Effect of three concentrations of clodinafop on the growth and symbiotic properties of greengram plants grown in soil inoculated with the *Bradyrhizobium* sp. (*vigna*) strain MRM6 and without bioinoculant.

Treatment	Dose (µg kg^{-1} soil)	Nodulation			Total dry biomass (g plant^{-1})	N content (mg g^{-1})		P content (mg g^{-1})		Seed yield (g plant^{-1})	Grain protein (mg/g)
		No./ plant	Dry biomass (mg plant^{-1})	Leghaemoglobin [mM (g f.m)$^{-1}$]		Root	Shoot	Root	Shoot		
Uninoculated	Control	21	66	0.08	2.60	36	50	0.27	0.36	7.4	261
	400	19	61	0.07	2.23	35	48	0.26	0.34	6.4	258
	800	18	58	0.06	2.06	32	45	0.25	0.31	6.1	255
	1200	16	55	0.05	1.82	30	42	0.23	0.29	5.3	251
Inoculated	Control	44	151	0.11	7.75	45	61	0.32	0.41	10.9	269
	400	21	116	0.10	4.54	42	58	0.29	0.38	10.2	267
	800	18	100	0.09	4.05	39	55	0.27	0.35	9.5	263
	1200	14	89	0.08	3.67	37	55	0.25	0.32	9.1	260
LSD		1.1	0.9	0.007	2.7	1.4	1.8	0.03	0.04	0.09	1.2
F value	Inoculation (df= 1)	211*	211*	443.6*	415*	145.6*	168.7*	344.2*	378*	115.8*	194.1*
	Herbicide (df= 3)	44.5*	65.4*	1.9	119*	76.6*	16.2*	127.7*	45.7*	91.4*	112.3*
	Inoculation×herbicide (df= 3)	5.3	28.1*	39.2*	63.5*	15.2*	15.2*	41.3*	35.4*	27.3*	21.6*

Values are mean of three replicates where each replicate had three plants/pot. * Significantly different from the control at $P \leq 0.05$.

clodinafop at 1X dose decreased the same parameters by 52%, 23%, 9%, 41%, 7%, 5%, 9%, 7%, 6%, and 1%, respectively, compared with the inoculated control. Interestingly, when the strain MRM6 was used with the 3X dose of clodinafop, it increased the nodule dry mass, Lb, total dry biomass, root N, shoot N, root P, shoot P, seed yield, and grain protein by 62%, 60%, 102%, 23%, 31%, 9%, 10%, 72%, and 4%, respectively, compared with the un-inoculated treatment having the same concentration of clodinafop (Table 4).

DISCUSSION

Herbicide Tolerance and *In Vitro* Plant Growth Promoting Activities

In the present study, *Bradyrhizobium* strain MRM6 tolerated the highest concentrations of quizalafop-p-ethyl and clodinafop (Table 1). To nullify the inhibitory effect exerted by the pesticides, rhizobacteria may biodegrade (Yang and Lee, 2008) or hydrolyze those chemicals enzymatically (Herman *et al.*, 2005). The variation in sensitivity/tolerance level of a wide array of rhizobacteria to a specific pesticide may be attributed to their different and diverse metabolic pathways that detoxify these xenobiotic compounds or break up/modify them into other forms that may be further more or less deleterious (Johnsen *et al.*, 2001, Kumar *et al.*, 1996).

The ability of herbicide resistant N_2-fixing bacteria to provide N to the legumes is perhaps one of the greatest strengths of rhizobia that could be exploited for raising the productivity of legumes in herbicide contaminated soils. Additionally, the nodule bacteria could also exert beneficial effects on legumes by synthesizing PGP substances and siderophores, as reported by Wani *et al*, (2008). Accordingly, the PGP activity of *Bradyrhizobium* strain MRM6 was also assessed. Quizalafop-p-ethyl and clodinafop-tolerant *Bradyrhizobium* strain MRM6 used in this study produced a substantial amount of PGP substances both in the absence and presence of quizalafop-p-ethyl and clodinafop (Table 2). A similar evidence of phytohormone production by other rhizobia such as *Mesorhizobium ciceri* (Wani *et al.*, 2008) and *Bradyrhizobium* (Pattan and Glick, 1996; Wani *et al.*, 2007) under conventional growth environment is reported. Plant growth hormones, like, IAA synthesized by plant growth promoting rhizobacteria (Sridevi *et al.*, 2008) is reported to affect many physiological activities of plants including cell enlargement, cell division, root initiation, growth rate, phototropism, geotropisms, and apical dominance (Frankenberger and Arshad, 1995; Karadeniz *et al.*, 2006; Remans *et al.*, 2008). Moreover, these phytohormones also act as signaling molecules during the development of symbiosis (Barker and Tagu, 2000) and are also reported to be involved in nodulation (Mathesius *et al.*, 1998; van Noorden *et al.*, 2006). Siderophores is another metabolite synthesized by microbial communities of soil. Siderophores supply iron to plants under iron-deficient conditions (Indiragandhi *et al.*, 2008). Furthermore, siderophores chelate iron and other metals. Indirectly, siderophores suppress the disease causing pathogens by limiting the supply of essential trace minerals to them. Siderophores may also directly stimulate the biosynthesis of other antimicrobial compounds by bacteria and may function in local and systematic host resistance in plants (Joseph *et al.*, 2007; Sinha and Mukherjee, 2008). The ability of bradyrhizobial strain to produce siderophores suggests that such strain could also be used as biological control agents.

The EPS production is another important trait of bacteria because it provides protection to cells against desiccation, phagocytosis and phage attack and also helps in N_2 fixation by preventing high oxygen tension (Tank and Saraf, 2003). Interestingly, the amount of EPS secreted by the bradyrhizobial strain in this study increased progressively with gradual increase in quizalafop-p-ethyl and

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clodinafop concentrations (Table 2). Generally, the increase in EPS following elevated concentration of herbicides suggested that the herbicides might have acted as inducer for EPS synthesis. The EPS synthesized by rhizobia (Courtois *et al.*, 1994; Ghosh *et al.*, 2005) is likely to provide protection to the rhizobia while inhabiting the stressed environments. The release of HCN by rhizospheric bacteria into the soil can be toxic to subterranean animals and phytopathogenic organisms (Guo *et al.*, 2007). In agreement with our finding, Devi *et al.* (2007) also reported the excretion of HCN by the rhizobacterial strains into the rhizosphere. Similarly, ammonia production by rhizobial strains is reported elsewhere (Wani *et al.*, 2007). However, we are not aware of such reports where the effects of quizalafop-p-ethyl and clodinafop on the PGP activities of bradyrhizobia are assessed.

Effect of Quizalafop-P-Ethyl and Clodinafop on Greengram and the Role of Strain MRM6

The reduction in growth of greengram plants following herbicide applications could be due to the adverse effects of quizalafop-p-ethyl and clodinafop on plant organs, especially the function of nodules, which, consequently, diminishes N_2 fixation (Ahemad and Khan, 2009). Such inhibitory effect following herbicide applications might possibly be due to the inhibition of enzymes involved in growth and metabolisms (Zablotowicz and Reddy, 2004) or due to disruption of signaling between legume (host) plant-derived phytochemicals (luteolin, apigenin) and *Rhizobium* Nod D receptors that is necessary for initiation of nodulation and N_2 fixation (Fox *et al.*, 2007). Reports on the effect of herbicides on effective symbiosis of rhizobia with the legume host plants are, however, contradictory. For example, sethoxydim, alachlor, fluazifop butyl and metolachlor at recommended doses did not result in detrimental effects on seed yields or N_2 fixation in soybean (*Glycine max*), while paraquat significantly reduced the amount of N_2 fixed (Kucey *et al.*, 1988). Similarly, the adverse effects of terbutryn/terbuthylazine and bentazone on the growth and nodulation of pea (*Pisum sativum*) (Singh and Wright, 2002) and the phytotoxic effects of chlorimuron-ethyl on soybean inoculated with *Bradyrhizobium japonicum* (Zawoznik *et al.*, 2005) are reported. Numerous studies have shown that herbicides may inhibit nodulation (Datta *et al.*, 2009; Eberbach and Douglas, 1989, Mårtensson and Nilsson, 1989; Isoi and Yoshida, 1990) and N_2 fixation (Datta *et al.*, 2009; Mårtensson, 1992; Koopman *et al.*, 1995). For example, Mårtensson (1992) examined the impact of bentazon (photosynthetic inhibitor), chlorosulfuron (ALS/AHAS inhibitor) and MCPA (IAA mimic) on red clover (*Trifolium pretense* L.cv. Britta), lucerne (*Medicago sativa* L.cv. Vertus) and birdsfoot-trefoil (*Lotus corniculatus* L.) and reported that these herbicides triggered growth disorders such as root hair deformations that inhibited symbiosis and resulted in fewer nodules. The reason for the inhibitory effect was that these chemicals inhibited photosynthesis and acetolactate synthesis, both of which are important for N_2-fixation. However, the low number of nodules associated with chlorosulfuron application was attributed to an impedance of nodule formation and not nodule. Anderson *et al.* (2004) claimed that herbicides may negatively affect the legume-*Rhizobium* relationship by: (i) directly affecting root and shoot biomass of the host plant thereby limiting the number of available sites for rhizobia to attach to, or by decreasing the carbohydrate supply to existing nodules, (ii) directly affecting rhizobial survival or growth that leads to a decreased potential for rhizobial infection on root hairs, (iii) inhibiting or inactivating the biochemical signaling that plants require to initiate nodule development; this inhibition could affect either rhizobia or plants, and (iv) inhibiting nodule development by reducing the capacity for cell division.

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Plant growth promoting rhizobacteria (PGPR) including symbiotic N_2 fixers can affect plant development either indirectly by circumventing the toxic effects of pesticides (Yang and Lee, 2008) or directly by synthesizing the plant growth regulating substances (Ma *et al.*, 2009; Kumar *et al.*, 2009; Wani *et al.*, 2008). Therefore, inoculation of quizalafop-p-ethyl and clodinafop tolerant and phytohormone producing *Bradyrhizobium* strain MRM6 increased the growth parameters of greengram grown in herbicide treated soils (Tables 3 and 4). The present investigation suggests that the ability of the strain MRM6 to tolerate higher concentrations of quizalafop-p-ethyl and clodinafop could probably be due to entrapment of herbicides within the exo-polysaccharides released by the inoculant strain. Exo-polysaccharides are known to play an important role in concentrating nutrients, protecting the bacteria from antibacterial agents (Costerton, 1985) and improve N_2-fixation by preventing nitrogen metabolism related enzymes (e.g., nitrogenase) from high oxygen tension (Tank and Saraf, 2003). Experimental observations have also demonstrated that amendment of soil with microbial EPS resulted in enhanced soil aggregation (Dobbelaere *et al.*, 2003). Hence, it is possible that herbicides were entrapped and failed to exert their toxic effects on the overall performance of the green gram plants. In addition, the synthesis of siderophore and IAA by the strain MRM6 might also have enhanced root growth and uptake of soil minerals by the host plants. Moreover, the bio-inoculant significantly increased the nodulation compared to the un-inoculated control, consolidating the fact that the strain MRM6 might compensate/equalize the toxicity of quizalafop-p-ethyl and clodinafop in sandy loam soil, as was evident through the growth of this strain on minimal media using quizalafop-p-ethyl and clodinafop as C source.

CONCLUSIONS

Inoculation of the herbicide tolerant *Bradyrhizobium* strain MRM6 demonstrated the following benefits to the green gram plants: (i) protected the greengram plants from the potential toxicity of herbicides, (ii) increased the overall growth, symbiotic properties, and nutrient uptake, and (iii) improved both quantity and quality of the green gram seeds. The increased growth of inoculated greengram plants, even in the presence of herbicides, might have possibly been due to (i) the synthesis and release of various plant growth promoting substances by the *Bradyrhizobium* strain MRM6 and (ii) the inherent N_2-fixing ability of the strain. This study indicates that the bradyrhizobial strain MRM6 could be developed as rhizo-bio-inoculant for enhancing the performance of greengram grown in herbicide-stressed soil.

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واکنش ماش سبز [*Vigna radiata* (L.) Wilczek] به تلقیح با باکتری برادی رایزوبیوم ریسه MRM6 کاشته شده در یک خاک دارای علف کش

م. احمد و م. صغیرخان

چکیده

این تحقیق برای تعیین فعالیتهای محرک رشدگیاه به وسیله ریسه MRM6 باکتری *Bradyrhizobium* sp. (*vigna*) در حضور و در نبود دو علف کش انتخابی Quizalafop-P-Ethyl وClodinafop انجام شد. ریسه مزبور که به این علف کشها مقاوم بود از نظر زیست-بهسازی و پتانسیل آن برای تحریک رشد گیاه با کاربرد ماش سبز به عنوان گیاه آزمایشی در یک خاک دارای دو علف کش-Quizalafop P-Ethyl وClodinafop (که به مقادیر توصیه شده و بیشتر از توصیه به خاک افزوده شده بودند) مطالعه شد. این ریسه از گره های ماش سبز به دست آمد و در شرایط آزمایشگاهی مقادیر زیادی ایندول استیک اسید، سیدو فور، هیدروژن سایانید، و آمونیاک را در حضور و درنبود علف کشهای مزبور(که

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با خلوص بالا مصرف شده بودند) تولید کرد. علف کشQuizalafop-P-Ethyl در مقادیر ۴۰ میکروگرم در کیلوگرم خاک (مقدار ثوصیه شده)، ۸۰ و ۱۲۰ میکروگرم درکیلوگرم خاک و علف کشClodinafop در مقادیر ۴۰۰ میکروگرم در کیلوگرم خاک (مقدار ثوصیه شده)، ۸۰۰ و ۱۲۰۰ میکروگرم درکیلوگرم خاک رشد گیاهان تلقیح شده و تلقیح نشده را کاهش دادند. در مقایسه با clodinafop یا تیمار شاهد، علف کشQuizalafop-P-Ethyl در تمام غلظت های افزوده شد اثرات سمیتی بیشتری روی رشد گیاه از نظر گره بندی، بیوماس خشک، جذب نیتروژن و فسفر، و تولید بذر نشان داد . هنگامی که ریسه مزبور در هر کدام از غلظت های دو علف کش به کار رفت، رشد و گره بندی گیاه در مقایسه با گیاهان تلقیح نشده بهبود نسبی یافت. به عنوان مثال، هنگامی که ریسه MRM6 همراه با ۱۲۰۰ میکروگرم درکیلوگرم خاک clodinafop به کار رفت ویژگیهای همزیستی گیاه (شامل تعداد گره ها، وزن خشک گره ها، و لگاهموگلوبین)، و بیوماس کل، نیتروژن ریشه، Nریشه، P ریشه، P ساقه، وزن تولید دانه، و پروتئین دانه را در مقایسه با گیاه تلقیح نشده که همان غلظت علف کش را داشت به ترتیب به مقدار۱۴٪، ۶۲٪، ۶۰٪،۱۰۲٪، ۲۳٪، ۳۱٪، ۹٪، ۱۰٪، ۷۲٪، و ۴٪ افزایش داد. از مطالعه حاضر چنین بر میآید که ریسه MRM6 برادی ریزوبیوم با خواص متعدد را می توان برای تسهیل تولید ماش سبز در زمینی که خاک آن درتنش علف کش است به کار برد.

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