Ann Microbiol (2010) 60:735–745
DOI 10.1007/s13213-010-0124-2

ORIGINAL ARTICLE

Growth promotion and protection of lentil (*Lens esculenta*) against herbicide stress by *Rhizobium* species

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Received: 29 March 2010 / Accepted: 6 August 2010 / Published online: 29 August 2010

Abstract This study was designed to recover lentil-specific rhizobial strains tolerant to herbicides (quizalafop-p-ethyl and clodinafop) and synthesizing plant growth regulators even in the presence of herbicide stress. Furthermore, the impact of rhizobial strain was assessed on lentil plants grown in herbicide-treated soils. Quizalafop-p-ethyl- and clodinafop-tolerant *Rhizobium* sp. isolate MRL3 recovered from the nodules of lentil produced plant growth-promoting substances in substantial amount both in the absence and presence of herbicides. In addition, each herbicide at recommended, two and three times the recommended dose adversely affected lentil growth in pot trials. Both herbicides at recommended and higher rate generally decreased biomass, symbiotic properties, nutrients uptake and seed yield of lentil. Interestingly, the herbicide-tolerant *Rhizobium* isolate MRL3, when used with any concentration of the two herbicides, significantly increased the measured parameters compared to the plants grown in soils treated solely (without inoculant) with the same individual treatment of each herbicide. The present findings suggest that the rhizobial isolate MRL3 endowed with multiple properties could be used to facilitate the productivity of lentil under herbicide-stressed soils.

Keywords Quizalafop-p-ethyl · Clodinafop · Herbicide · Lentil · *Rhizobium* · PGPR

Introduction

Currently, the prime objective of the agronomists is how to expedite plant growth at unprecedented rates and to maximize the productivity of important crops including legumes (Fox et al. 2007). A major interference to attain this goal is, however, the pervasiveness of undesirable weeds growing along with the emergent crops. These unwanted and resistant plants create constraints upon the development of the desired crop plants due to their unusually greater potential to compete with the crops in absorption of soil nutrients, and resistance to fluctuating ecological factors like drought, salinity, temperature, humidity, toxic metals and agrochemicals (Ahemad et al. 2009; Powles 2008). Therefore, a wide range of herbicides are presently employed to overcome such nuisances. Injudicious and indiscriminate application of these chemicals, however, leads to their accumulation into soils up to a level that is not only detrimental for the beneficial plant growth-promoting rhizobacteria (PGPR) including rhizobia (Khan et al. 2006a; Gigliotti and Allievi 2001) but is also toxic for growing agronomically important crop plants (Khan et al. 2006b). Consequently, the crop productivity is adversely affected (Song et al. 2007).

Lentil, one of the important legume crops, fixes atmospheric N_2 in association with its microsymbiont *Rhizobium* through nodule formation (Athar 1998). Rhizobia in addition to their intrinsic N_2-fixing ability also facilitate plant growth by solubilizing soil phosphate (Alikhani et al. 2006), producing phytohormones (Spaepen et al. 2009), siderophores (Wani et al. 2007), exo-polysaccharides (Ahemad and Khan 2009) and ACC-deaminase (Duan et al. 2009). A great deal of information concerning the effects of herbicides on rhizobia and legumes is available. However, the reports are scanty where the effect of herbicides has been studied on both rhizobia and their host legume in parallel. Moreover, to the best of our knowledge, there is no report about the effect of the herbicides quizalafop-p-ethyl [Ethyl (RS)-2-(4-6-chloroquinoxolin-2-yloxy) phenoxy] propionate (CAS-No. 100646-51-3)], and clodinafop {(R)-2-[4-(5-chloro-3-fluoro-2-pyridyloxy) phenoxy] propionic acid (CAS-No. 105512-06-9)} (Fig. 1, on lentil. In view of this scenario, the present

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study was, therefore, designed to (1) isolate quizalafop-p-ethyl and clodinafop tolerant *Rhizobium* from lentil nodules, (2) determine the plant growth-promoting activities of *Rhizobium* isolates in the presence and absence of selected herbicides, and (3) assess the PGP potential of quizalafop-p-ethyl- and clodinafop-tolerant *Rhizobium* sp. isolate MRL3 using lentil as a test crop under herbicide stress.

Materials and methods

Rhizobial isolates and herbicide tolerance

A total of 50 rhizobial isolates were recovered from nodules borne on the root system of lentil plants grown in experimental fields of Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh (27°29'N, 72°29'E), India, using yeast extract mannitol (YEM) medium (g l^{-1}: mannitol 10; K_2HPO_4 0.5; $MgSO_4 \cdot 7H_2O$ 0.2; NaCl 0.1; yeast extract 1; $CaCO_3$ 1; pH 7) (Vincent 1970). The rhizobial isolates were identified at genus level by biochemical tests following Holt et al. (1994) and host specificity (Somasegaran and Hoben 1994). The isolates 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, $MgSO_4 \cdot 7H_2O$ 0.2, $CaCl_2 \cdot 2H_2O$ 0.02, $FeSO_4 \cdot 7H_2O$ 0.01, pH 6.5). The freshly prepared agar plates were amended separately with increasing concentrations (0–3,200 μg ml^{-1}; at two fold dilution intervals) of both quizalafop-p-ethyl and clodinafop. Later, plates were spot inoculated with 10 μl of 10^8 cells ml^{-1} rhizobial isolates. Each experiment was replicated three times. Plates were incubated at 28±2°C for 72 h and the highest concentration of quizalafop-p-ethyl and clodinafop supporting rhizobial growth was defined as the maximum resistance level (MRL).

Effect of quizalafop-p-ethyl and clodinafop on plant growth promoting activities

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, rhizobial isolates exhibiting maximum 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 *Rhizobium* culture (10^8 cells ml^{-1}), grown in YEM broth. The inoculated LB broth was incubated at 28±2°C for 5 days with shaking at 125*g*. 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% perchloric acid) was added to the LB broth (100 ml) and incubated at 28±2°C in darkness for 1 h. 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 rhizobial isolates 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 prepared separately and divided into equal sectors. Plates were spot inoculated with 10 μl of 10^8 cells ml^{-1} and incubated at 28±2°C for 5 days. Development of yellow to orange halo around the bacterial growth was considered as positive for siderophore production. The siderophores produced by the test isolates 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 rhizobial isolates and incubated at 28±2°C for 5 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 rhizobial isolates was evaluated further under in vitro conditions. For this, the isolates were grown in 100-ml capacity flasks containing basal medium supplemented with 5% sucrose and incubated for 5 days at 28±2°C on a shaker (100*g*). Culture broth was centrifuged at 5,433*g* for 30 min and EPS was extracted by adding three volumes of chilled acetone to one volume of supernatant. The precipitated EPS

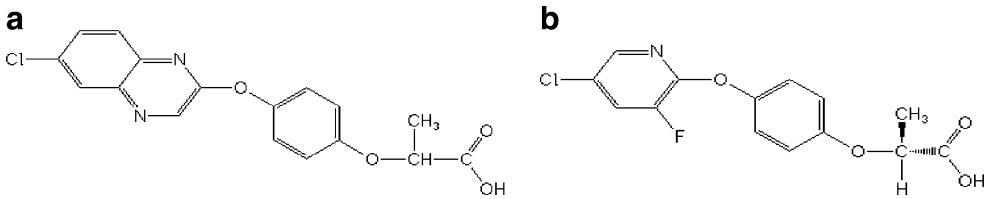

Fig. 1 Chemical structures of **a** quizalafop-p-ethyl and **b** clodinafop used in the present study

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. *Rhizobium* isolates were also screened for hydrogen cyanide (HCN) synthesis (Bakker and Schipper 1987). Briefly, rhizobial isolates 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 was incubated at 28±2°C for 4 days. Rhizobial isolates 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 4 days. Development of orange to red color indicated HCN production. Rhizobial isolates 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 rhizobial isolates (200 μl of 10^8 cells ml^{-1}) were inoculated in 20 ml peptone water in tubes and incubated at 28±2°C for 4 days. One milliliter of Nessler reagent 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

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 lentil (var. K75) were surface sterilized (70% ethanol, 3 min; 3% sodium hypochlorite, 3 min), rinsed six times with sterile water and dried. The sterilized seeds were bacterized with *Rhizobium* sp. isolate MRL3, grown in YEM broth. Seeds were soaked in liquid culture medium for 2 h 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 3 kg un-sterilized soil with 0, 40 (recommended dose 1×), 80 (2×) and 120 (3×) μg quizalafop-p-ethyl kg^{-1} soil or 0, 400 (recommended dose 1×), 800 (2×), and 1,200 (3×) 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 three pots for each treatment were removed 90 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. Plants uprooted at 90 DAS were oven-dried (at 80°C) and dry matter accumulation in plants was measured. The leghaemoglobin (Lb) content in fresh nodules was quantified at 90 DAS (Sadasivam and Manikam 1992). The leghaemoglobin 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. Total nitrogen (N) content in roots and shoots was measured at 120 DAS by the micro-Kjeldahl method (Iswaran and Marwah 1980). The total phosphorus (P) content in roots and shoots at 120 DAS was estimated by the method of Jackson (1967). The remaining pots (three pots) for each treatment having three plants per pot were maintained until harvest (120 DAS). Seed yield and grain protein (Sadasivam and Manikam 1992) was assessed 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 and subjected to analysis of variance. The difference among treatment means was compared by high range statistical domain (HSD) using two-way ANOVA at 5% probability level.

Results

Herbicide tolerance and *in vitro* plant growth promoting activities

In this study, a total of 50 rhizobial isolates recovered from lentil nodules were presumptively identified following biochemical and host specificity tests. Of these, the isolate MRL3 was specifically selected because of tolerating the highest concentration of quizalafop-p-ethyl (1600 μg ml^{-1}) and clodinafop (1600 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 effect of quizalafop-p-ethyl (40, 80 and 120 μg l^{-1}) and clodinafop (400, 800 and 1,200 μg l^{-1}) on PGP traits like IAA, siderophores, EPS, HCN and

Table 1 Morphological and biochemical characteristics of *Rhizobium* sp. isolate MRL3

Characteristics	Isolate MRL3
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	1,600 μg ml^{-1}
Clodinafop	1,600 μg ml^{-1}

+ Positive, – negative reactions

ammonia was determined (Table 2). *Rhizobium* isolate MRL3 produced a maximum amount (37 μg ml^{-1}) of IAA. Generally, the synthesis of IAA by the rhizobial isolate decreased significantly ($P \leq .05$) as the concentration of quizalafop-p-ethyl and clodinafop was increased from recommended to three times the recommended rate. For example, maximum decline of 46 and 41% in IAA synthesis was observed at 120 μg ml^{-1} of quizalafop-p-ethyl and 1,200 μg ml^{-1} of clodinafop, respectively, over untreated control. Moreover, the isolate MRL3 also showed the siderophore activity through the formation of an orange colored zone (12 mm) around the bacterial growth on CAS agar plates. A gradual reduction in siderophore zone was observed with the increment of each herbicide. Likewise, in the absence of herbicides, rhizobial isolate MRL3 produced 29 μg ml^{-1} SA and 21 μg ml^{-1} DHBA. The synthesis of SA and DHBA decreased significantly ($P \leq .05$) 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 49 and 57%, respectively, while clodinafop (at 1,200 μg l^{-1}) decreased these substances by 41 and 52%, respectively over control. In contrast, the EPS secretion increased significantly ($P \leq .05$) with progressive increase of each herbicide. For example, when quizalafop-p-ethyl (120 μg l^{-1}) and clodinafop (1,200 μg l^{-1}) was added to the medium, the EPS was increased by 33 and 22%, respectively, relative to control. Further, MRL3 was positive for catalase, HCN and ammonia

Table 2 Plant growth-promoting activities of *Rhizobium* sp. isolate MRL3 both in the presence and absence of quizalafop-p-ethyl and clodinafop

Herbicides	Dose rate (μg l^{-1})	IAA[a] (μg ml^{-1})	Siderophores				EPS[e] (μg ml^{-1})	Catalase	HCN[f]	Ammonia
			CAS[b] Agar (mm)	$FeCl_3$ test	Phenolates (μg ml^{-1})					
					SA[c]	2,3-DHBA[d]				
Control		37 a	12 a	+	29 a	21 a	18 bc	+	+	+
Quizalafop-p-ethyl	40	27 bc	11 ab	+	21 bc	16 bc	20 b	+	+	+
	80	23 c	10 b	+	17 c	10 d	21 ab	+	+	+
	120	20 d	9 b	+	15 cd	9 de	24 a	+	+	+
Clodinafop	400	33 b	11 ab	+	25 ab	17 b	19 bc	+	+	+
	800	27 bc	10 b	+	22 b	14 c	20 b	+	+	+
	1200	22 c	9 b	+	17 c	10 d	22 ab	+	+	+
F value		244.6	15.5	–	311.2	68.4	131.3	–	–	–

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-polysaccharide

[f] Hydrogen cyanide

in the absence and presence of both quizalafop-p-ethyl and clodinafop (Table 2).

Lentil growth in the presence of herbicides and *Rhizobium* sp. isolate MRL3

The production of PGP substances by the rhizobial isolate MRL3 both in the presence and absence of quizalafop-p-ethyl and clodinafop prompted us to assess the effect of this isolate on the performance of lentil in quizalafop-p-ethyl- and clodinafop-stressed soils. The inoculated and non-inoculated lentil plants subjected to three levels each of quizalafop-p-ethyl and clodinafop decreased the measured growth parameters of lentil plants. Although a consistent and concentration-dependent reduction following herbicide application was recorded, the effect of herbicides was generally less severe in the presence of inoculant. In the absence of bio-inoculant, recommended dose of quizalafop-p-ethyl reduced the root length, shoot length, root dry biomass, shoot dry biomass and total plant dry biomass by 53, 35, 40, 44 and 44%, respectively, while 3× of quizalafop-p-ethyl decreased these parameters by 82, 80, 67, 65 and 66%, respectively, compared to control at 90 DAS. Moreover, all tested concentrations of quizalafop-p-ethyl at 90 DAS so adversely affected the nodulation that not a single nodule was recovered from the root system of lentil plants. Similarly, at 120 DAS, recommended dose of quizalafop-p-ethyl decreased the root length, shoot length, root dry biomass, shoot dry biomass, nodule number, nodule dry biomass and total plant dry biomass by 24, 42, 51, 41, 32, 51 and 43%, respectively, whereas 3× of quizalafop-p-ethyl by 48, 68, 70, 63, 63, 64 and 64%, respectively, over control. The plant growth parameters also consistently decreased in the presence of *Rhizobium* sp. isolate MRL3 as the concentration of each herbicide increased from the recommended to three times the recommended rate. Interestingly, when inoculated and un-inoculated treatments of the same concentrations of either herbicide were compared to each other, a substantial increase in plant growth parameters was observed. For instance, when inoculated treatments at three times the recommended rate (3×) of quizalafop-p-ethyl were compared to the uninoculated ones at the same rate of quizalafop-p-ethyl, inoculant *Rhizobium* isolate MRL3 increased the root length, shoot length, root dry biomass, shoot dry biomass, nodule number, nodule dry biomass and total plant dry biomass by 18, 10, 101, 69, 14, 11 and 73%, respectively, at 120 DAS (Table 3). On the other hand, the recommended dose of clodinafop, in the absence of bio-inoculant, reduced the root length, shoot length, root dry biomass, shoot dry biomass, nodule number, nodule dry biomass and total plant dry biomass marginally at both 90 and 120 DAS while 3× of clodinafop at 90 DAS decreased the same parameters by 47, 25, 41, 28, 37, 30 and 31%, respectively, and at 120 DAS by 24, 29, 33, 22, 26, 31 and 25%, respectively, compared to control. Moreover, when inoculated treatments at 3× of clodinafop were compared to the uninoculated ones at the same rate of clodinafop, inoculant *Rhizobium* isolate MRL3 increased the root length, shoot length, root dry biomass, shoot dry biomass, nodule number, nodule dry biomass and total plant dry biomass by 55, 27, 27, 67, 58, 104 and 65%, respectively, at 90 DAS, and at 120 DAS by 19, 9, 64, 81, 14, 35 and 77%, respectively (Table 4).

Moreover, quizalafop-p-ethyl (1×) when applied alone (without bio-inoculant), decreased total chlorophyll, root N, shoot N, root P, shoot P, seed yield and seed protein by 22, 29, 16, 24, 25, 57 and 5% respectively, while 3× of this herbicide decreased by 41, 47, 24, 43, 49, 80 and 9%, respectively, over control. The bio-inoculant used along with 3× of quizalafop-p-ethyl when compared with un-inoculated ones, increased total chlorophyll, root N, shoot N, root P, shoot P, seed yield and seed protein by 5, 22, 6, 58, 35, 166 and 4%, respectively (Table 5).

Although, clodinafop at recommended rate and without inoculant, decreased Lb, chlorophyll, root N, shoot N, root P, shoot P, seed yield and seed protein marginally yet 3X of clodinafop decreased the same parameters by 33, 16, 24, 11, 19, 18, 33 and 4%, respectively compared to control. However, when inoculated treatment at three times the recommended rate of clodinafop was compared to the un-inoculated ones at the same rate of clodinafop, *Rhizobium* isolate MRL3 increased Lb, total chlorophyll, root N, shoot N, root P, shoot P, seed yield and seed protein by 25, 15, 31, 8, 41, 22, 55 and 6%, respectively (Table 6).

Discussion

Plant growth promoting activities in the presence of herbicides

In the present study, *Rhizobium* isolate MRL3 showed the great resistance to quizalafop-p-ethyl and clodinafop which could probably be due to the fact that PGPR adopt diverse strategies to overcome the toxic effects of pesticides like biodegradation (Yang and Lee 2008) and enzymatic hydrolysis (Herman et al. 2005). Our study, however, showed that the MRL of the selected isolate (MRL3) was considerably higher for both quizalafop-p-ethyl and clodinafop.

The ability of herbicide tolerant N_2-fixing bacteria to provide N to the legumes in herbicide-contaminated soils could serve as a most suitable alternative strategy for detoxification of herbicides. In addition to N_2 fixation, the nodule bacteria could also exert their effect on legumes by other mechanisms, such as the production of plant growth-promoting (PGP) substances and siderophores (Wani et al.

Table 3 Effect of three concentrations of quizalafop-p-ethyl on growth and nodulation of lentil plants grown in soil inoculated with *Rhizobium* sp. isolate MRL3 and without bioinoculant

Treatment	Dose rate (μg kg^{-1} soil)	Length/ plant (cm)				Dry biomass (mg/ plant)				Nodulation				Total dry biomass (g/plant)	
		Root		Shoot		Root		Shoot		No./plant		Dry biomass (mg/plant)			
		90 DAS	120 DAS	90 DAS	120 DAS	90 DAS	120 DAS	90 DAS	120 DAS	90 DAS	120 DAS	90 DAS	120 DAS	90 DAS	120 DAS
Uninoculated	Control	17	21	20	31	366	536	1,076	1,966	19	38	30	74	1.47	2.57
	40	8	16	13	18	220	260	600	1,166	–	26	–	36	0.82	1.46
	80	6	13	9	12	173	220	486	833	–	21	–	31	0.66	1.08
	120	3	11	4	10	120	160	380	730	–	14	–	27	0.50	0.92
Inoculated	Control	20	24	28	34	570	706	2,130	3,433	28	42	72	84	2.77	4.23
	40	12	19	16	19	213	477	1,130	1,800	27	33	25	48	1.37	2.33
	80	9	17	12	14	173	445	730	1,533	20	31	18	39	0.92	2.02
	120	5	13	5	11	126	323	500	1,233	18	16	12	30	0.64	1.59
LSD		1.8	1.2	1.4	2.2	3.2	3.0	7.5	9.2	1.3	1.9	0.8	1.3	7.4	10.5
F value	Inoculation (*df*=1)	639*	346*	428*	517*	3,487*	2,182*	518*	1,649*	613*	872.4*	712*	544*	6,912*	2,019*
	Herbicide (*df*=3)	171.4*	35.3*	125*	64.8*	829*	239*	105*	412*	85.6*	65.4*	105.3*	104.1*	1,306*	276*
	Inoculation × herbicide (*df*=3)	25.4*	27.6*	51.4*	21.2*	539*	472*	214*	127*	27.4*	32.2*	91.5*	34.6*	1,203*	519.2*

Values are mean of three replicates where each replicate constituted three plants/pot

*Significantly different from the control at $P \leq 0.05$

Table 4 Effect of three concentrations of clodinafop on growth and nodulation of lentil plants grown in soil inoculated with *Rhizobium* sp. isolate MRL3 and without bioinoculant

Treatment	Dose rate (μg kg^{-1} soil)	Length/plant (cm)				Dry biomass (mg/plant)				Nodulation				Total dry biomass (g/plant)	
		Root		Shoot		Root		Shoot		No./plant		Dry biomass (mg/plant)			
		90 DAS	120 DAS	90 DAS	120 DAS	90 DAS	120 DAS	90 DAS	120 DAS	90 DAS	120 DAS	90 DAS	120 DAS	90 DAS	120 DAS
Uninoculated	Control	17	21	20	31	0.37	0.54	1.08	1.97	19	38	30	74	1.47	2.57
	400	16	21	19	30	0.33	0.50	0.99	1.87	18	37	28	69	1.35	2.44
	800	13	18	17	27	0.27	0.39	0.89	1.67	17	31	24	62	1.18	2.12
	1200	9	16	15	22	0.22	0.36	0.78	1.53	12	28	21	51	1.02	1.94
Inoculated	Control	20	24	28	34	0.57	0.71	2.13	3.43	28	42	72	84	2.77	4.23
	400	19	23	24	32	0.50	0.68	1.66	3.30	27	40	63	80	2.22	4.06
	800	17	21	22	28	0.38	0.65	1.60	2.93	24	36	54	74	2.04	3.66
	1200	14	19	19	24	0.28	0.59	1.30	2.77	19	24	43	69	62	3.43
LSD		1.7	1.5	1.8	2.2	2.7	2.4	7.1	8.3	0.5	0.8	1.2	0.9	6.5	6.9
F value	Inoculation (*df*=1)	609*	667*	242*	714.6*	4717*	549*	637*	190.2*	2,680*	455*	1,145*	1,616*	1,456*	1,584*
	Herbicide (*df*=3)	43.3*	131.4*	28.4*	117.5*	968*	53.6*	85*	87.4*	235*	209*	475*	353*	295.3*	568*
	Inoculation × herbicide (*df*=3)	21.2*	65.5*	17*	27.7*	487*	31.3*	118.6*	14.2*	69*	29.5*	205*	104.7*	804*	102*

Values are mean of three replicates where each replicate constituted three plants/pot

*Significantly different from the control at $P \leq 0.05$

Table 5 Effect of three concentrations of quizalafop-p-ethyl on chlorophyll, leghaemoglobin, N and P content, seed yield and grain protein of lentil plants grown in soil inoculated with *Rhizobium* sp. isolate MRL3 and without bioinoculant

Treatment	Dose rate (μg kg^{-1} soil)	Leghaemoglobin content [mM (g f.m.)$^{-1}$]	Chlorophyll content (mg g^{-1})	N content (mg g^{-1})		P content (mg g^{-1})		Seed yield (g/plant)	Seed protein (mg g^{-1})
				Root	Shoot	Root	Shoot		
Uninoculated	Control	0.12	0.32	17	45	0.21	0.28	3.0	232
	40	–	0.25	12	38	0.16	0.21	1.3	221
	80	–	0.21	10	36	0.14	0.19	0.8	216
	120	–	0.19	9	34	0.12	0.17	0.6	212
Inoculated	Control	0.15	0.38	21	49	0.29	0.34	4.1	245
	40	0.09	0.27	17	42	0.23	0.28	2.2	229
	80	0.08	0.24	14	38	0.21	0.26	1.9	225
	120	0.06	0.20	11	36	0.19	0.23	1.6	221
LSD		0.004	0.12	1.4	1.8	0.004	0.007	0.06	3.6
F value	Inoculation (*df*=1)	416.2*	256.1*	164.2*	1,217.2*	287.9*	1,141*	170.1*	510.2*
	Herbicide (*df*=3)	74.5*	108.4*	43.2*	512.3*	67.2*	217*	28.1*	127.5*
	Inoculation × herbicide (*df*=3)	152.1*	11.4*	12.5*	117.4*	38.1*	67.5*	11.8*	417.4*

Values are mean of three replicates where each replicate constituted three plants/pot

*Significantly different from the control at $P \leq 0.05$

2008). Therefore, the PGP activity of *Rhizobium* MRL3 was assessed further. Quizalafop-p-ethyl and clodinafop-tolerant *Rhizobium* isolate MRL3 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). Similar evidence of phytohormone production by *Mesorhizobium ciceri* (Wani et al. 2008) and *Bradyrhizobium* (Pattan and Glick 1996; Wani et al. 2007) under conventional medium has been reported. Plant growth hormones like IAA synthesized by plant growth-promoting rhizobacteria (Sridevi et al. 2008) are reported to affect many physiological activities of plants, such as cell enlargement, cell division, root initiation, growth rate, phototropism, geotropisms and apical dominance, etc.

Table 6 Effect of three concentrations of clodinafop on chlorophyll, leghaemoglobin, N and P content, seed yield and grain protein of lentil plants grown in soil inoculated with *Rhizobium* sp. isolate MRL3 and without bioinoculant

Treatment	Dose rate (μg kg^{-1} soil)	Leghaemoglobin content [mM (g f.m.)$^{-1}$]	Chlorophyll content (mg g^{-1})	N content (mg g^{-1})		P content (mg g^{-1})		Seed yield (g/plant)	Seed protein (mg g^{-1})
				Root	Shoot	Root	Shoot		
Uninoculated	Control	0.12	0.32	17	45	0.21	0.28	3.0	232
	400	0.11	0.31	16	43	0.20	0.26	2.6	229
	800	0.09	0.29	15	41	0.19	0.25	2.2	226
	1200	0.08	0.27	13	40	0.17	0.23	2.0	222
Inoculated	Control	0.15	0.38	21	49	0.29	0.34	4.1	245
	400	0.13	0.36	20	47	0.27	0.33	3.9	242
	800	0.12	0.33	19	45	0.26	0.31	3.4	238
	1200	0.10	0.31	17	43	0.24	0.28	3.1	236
LSD		0.003	0.05	1.3	2.2	0.004	0.005	0.06	2.5
F value	Inoculation (df=1)	144.2*	652*	1,029*	984.2*	407.3*	225.5*	2,550*	456.8*
	Herbicide (df=3)	16*	101*	186*	127.4*	87.2*	45.2*	317.5*	108.2*
	Inoculation × herbicide (df=3)	5.1*	36.1*	42.3*	26.5*	18.4*	12.5*	78.1*	51.4*

Values are mean of three replicates where each replicate constituted three plants/pot

*Significantly different from the control at $P \leq 0.05$

(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). For instance, auxin is reported to participate in nodulation process as evidenced by the presence of higher concentration of auxin within nodules (Mathesius et al. 1998; van Noorden et al. 2006). Siderophores, also synthesized by heterogenous microbial communities inhabiting soil, supplies iron to growing 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 rhizobial isolate to produce siderophores suggests that such isolate could also help to manage the pests affecting lentil plants. 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). Furthermore, the bacteria producing higher amounts of EPS exhibit a stronger ability of P-solubilization compared to EPS non-producing strains (Yi et al. 2007). Interestingly, the amount of EPS secreted by the rhizobial isolate in this study increased progressively with the gradual increase in quizalafop-p-ethyl and clodinafop concentration (Table 2) for reasons not yet explained. However, it is likely that the herbicides might have induced the synthesis of EPS leading to increase in EPS by the rhizobial strain grown in chemically defined medium supplemented with varying concentrations of these herbicides. The EPS so excessively synthesized by rhizobia (Courtois et al. 1994; Ghosh et al. 2005) is likely to provide protection to the rhizobia by masking the effect of other agrochemicals while growing in 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 effect of quizalafop-p-ethyl and clodinafop on the PGP activities of rhizobia is assessed.

Effect of quizalafop-p-ethyl and clodinafop on lentil and the role of isolate MRL3

The reduction in growth of lentil plants following herbicide application observed in this study could be due to the adverse effects of quizalafop-p-ethyl and clodinafop on plant organs, especially the function of nodules which consequently diminishes the N_2 fixation. Such inhibitory effects following herbicide applications may 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 rates did not result in detrimental effects on seed yields or N_2 fixation in soybean while paraquat significantly reduced the amount of N_2 fixed as measured by ^{15}N dilution methods (Kucey et al. 1988). Similarly, the adverse effects of terbutryn/terbuthylazine and bentazone on the performance of pea (Singh and Wright 2002) and the phytotoxic effects of chlorimuron-ethyl on *Bradyrhizobium japonicum*-inoculated soybean (Zawoznik and Tomaro 2005) has been reported.

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 (Wani et al. 2008). Therefore, inoculation of quizalafop-p-ethyl- and clodinafop-tolerant and phyto-hormone-producing *Rhizobium* isolate MRL3 in this study increased the growth including all the measured parameters of lentil grown in herbicide-treated soils. The present investigation suggests that the ability of isolate MRL3 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. Exo-polysaccharides are known to play an important role in concentrating nutrients, protecting the bacteria from antibacterial agents (Costerton 1985) and improving nitrogen fixation by preventing nitrogen metabolism-related enzymes (e.g., nitrogenase) from high oxygen tension (Tank and Saraf 2003). Experimental observations have also demonstrated that the amendment of soil with microbial EPS resulted in enhanced soil aggregation (Dobbelaere et al. 2003). And, hence, the entrapped herbicides might have failed to exert their toxic effects on the overall performance of lentil. In addition, the synthesis of siderophore and IAA by the isolate MRL3 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 uninoculated control consolidating the fact that the isolate MRL3 might have reduced the toxicity of quizalafop-p-ethyl and clodinafop in sandy loam soil, as was evident through the growth of this isolate on minimal media using quizalafop-p-ethyl and clodinafop as C source.

Conclusion

In this study, we demonstrated the harmful effects of quizalafop-p-ethyl and clodinafop on the performance of lentil plants grown in soils treated with the same herbicides. The inoculation of *Rhizobium* sp. isolate MRL3 used as seed inoculant, however, not only protected the lentil plants from the toxicity of the herbicides but also increased the growth, symbiotic properties, nutrient status and quantity and quality of lentil seeds. The increased growth of inoculated lentil plants even in the presence of herbicides as observed in this study might possibly have been due to the synthesis of plant growth-promoting substances by the isolate MRL3 and reduced availability of herbicides to plants as a result of EPS secretion by rhizobial isolate in addition to its inherent N_2-fixing ability. The multifaceted rhizobial isolate MRL3 endowed with properties of growth promotion and phytotoxicity reduction could be exploited as a bio-inoculant for the better performance of lentil even under herbicide stress.

Acknowledgments The authors acknowledge Dr. Nakhat Ara Naqvi, Parijat Agrochemicals, New Delhi, India, for providing technical grade insecticides and University Grants Commission, New Delhi, India, for providing fellowships.

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