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Plant-Growth-Promoting Fungicide-Tolerant *Rhizobium* Improves Growth and Symbiotic Characteristics of Lentil (*Lens esculentus*) in Fungicide-Applied Soil

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Abstract The goal of this study was to identify lentil-specific rhizobial strains with the ability to tolerate fungicide and synthesize plant growth regulators even in soils contaminated with fungicides. A fungicide-tolerant and plant-growth-promoting rhizobial strain was used to assess its impact on lentil grown in fungicide-treated soils. The tebuconazole-tolerant *Rhizobium* sp. strain MRL3 produced plant-growth-promoting substances when grown in the presence and the absence of tebuconazole. Tebuconazole at the recommended and two and three times the recommended doses decreased consistently the dry biomass, symbiotic properties, nutrient uptake, and seed yields of lentil plants. In contrast, the fungicide-tolerant strain MRL3 significantly increased the measured parameters when lentil was grown in soils treated with varying concentrations of tebuconazole compared to uninoculated plants. As an example, strain MRL3 with 100 μg tebuconazole/kg soil significantly increased the root nitrogen, shoot nitrogen, root phosphorus, shoot phosphorus, and seed yield by 31, 10, 41, 21, and 117%, respectively, compared to the uninoculated plants grown in soil treated solely with 100 μg tebuconazole/kg soil. In conclusion, the *Rhizobium* strain MRL3 may be applied as biofertilizer to enhance the performance of lentil plants in fungicide-applied soils.

Keywords *Rhizobium* · Lentil · Plant-growth-promoting rhizobacteria · Fungicide

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Introduction

In contemporary agronomic practices, rhizobial cultures exhibiting various plant-growth-promoting (PGP) activities are commonly applied to legume seeds (Khan and others 2010). These bio-inoculants are often used in combination with fungicides that are applied to protect crops from the deleterious effect of soil-borne pathogens. Many of these fungicides, however, inhibit the nodulating competence of rhizobia and subsequently their N_2-fixing ability (Revellin and others 1993; Dunfield and others 2000). Reports on the impact of fungicides on rhizobia establishment, nodulation, and crop yields are, however, variable (Graham and others 1980; Kyei-Boahen and others 2001). Hence, understanding the effect of fungicides on rhizobia, their PGP activities, and ability to form symbioses with legumes in varied soil and climatic conditions is important for achieving optimum production. On the other hand, constancy and productive efficiency of a soil ecosystem are decidedly governed by soil microorganisms and their associated activities. Moreover, microbial activities of other soil microflora are equally vital for decomposition processes in soils. In many soils, however, the structure, composition, and activities of microbial populations are undesirably affected by fungicides, used frequently in high-input cultivation practices (Cernohlavkova and others 2009). In a manner similar to the effect of fungicides on microbial populations, legumes have also been found to be adversely affected by fungicide application. For example, Aamil and others (2004) have shown that carbendazim, captan, thiram, and mancozeb, applied at 2 g.a.i./kg soil, significantly reduced plant vitality, chlorophyll content, N uptake, protein content, nodulation, and seed yield of chickpea (*Cicer arietinun*). Taking these facts into consideration, it is pertinent to identify microbes that could alleviate the toxic

effects of agrochemicals so that crops can be grown in contaminated soils.

An alternative to overcoming the deleterious effects of fungicides could be the treatment of seeds with rhizobia as a bio-inoculant which displays a wide range of tolerance to fungicides, that is, exhibits PGP activities other than their inherent N_2-fixing attribute. Tebuconazole [(*RS*)-1-*p*-chlorophenyl-4,4-dimethyl-3-(1*H*-1,2,4-triazol-1-ylmethyl) pentan-3-ol] (Fig. 1) is a systemic triazole fungicide that is used widely in agricultural practices to manage phytopathogenic fungi such as *Curvularia* spp. and *Fusarium* spp. Tebuconazole disrupts the membrane functions of the target organism leading to the inhibition of sterol biosynthesis. Although tebuconazole is extensively applied, comprehensive data on its biological impact on rhizobia-legume symbiosis, specifically lentil plants, are very limited.

The present study was designed to (1) identify fungicide-tolerant *Rhizobium* species with multiple PGP traits and (2) determine the impact of PGP fungicide-tolerant *Rhizobium* on the performance of lentil plants grown in alluvial soils treated with the fungicide tebuconazole.

Materials and Methods

Fungicide Tolerance and PGP Activities

Rhizobial isolates were recovered from the nodules of lentil plants grown in the experimental fields of the Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, India, using yeast extract mannitol (YEM) medium (Vincent 1970). The rhizobial isolates were maintained on the same medium until use. The rhizobial isolates were identified on the basis of host specificity (Somasegaran and Hoben 1994) and morphological and biochemical tests (Holt and others 1994). The rhizobial isolates were tested for their sensitivity/resistance to technical-grade tebuconazole [active ingredient 100%; CAS No. 107534-96; Parijat Agrochemicals, New Delhi, India] by the agar plate dilution method using minimal salt agar medium (g/L: 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; agar, 15; pH 6.5). To prevent pesticide degradation, stock solutions of tebuconazole were prepared just prior to each experiment by dissolving the fungicide in double-distilled water. The freshly prepared agar plates were amended separately with increasing concentrations of tebuconazole (0, 100, 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800, 2000, 2200, 2400, 2600, 2800, 3000, and 3200 μg ml^{-1}). Later, plates were spot inoculated with 10 μl of 10^8 cells ml^{-1} of rhizobial isolates. Each experiment was replicated three times. Plates were incubated at 28 ± 2°C for 72 h. The highest concentration of tebuconazole supporting rhizobial growth was defined as the maximum tolerance level (MTL).

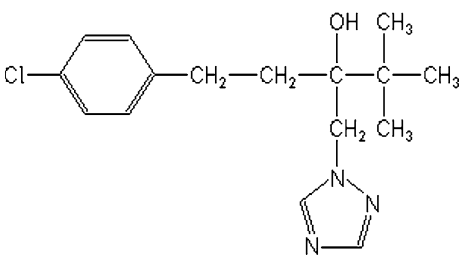

Fig. 1 Structure of the triazole fungicide tebuconazole

Indole-3-acetic acid (IAA) was quantitatively determined following the method of Gordon and Weber (1951), later modified by Brick and others (1991). For IAA quantification, rhizobial isolates exhibiting the highest MTL values were grown in Luria–Bertani (LB) broth (g l^{-1}: tryptone, 10; yeast extract, 5; NaCl, 10; pH 7.5) supplemented with 0 (control), 100 (recommended dose), 200, and 300 μg l^{-1} tebuconazole. A 100-ml sample of LB broth supplemented with 100 μg ml^{-1} tryptophan was inoculated with 1 ml *Rhizobium* culture (10^8 cells ml^{-1}) grown in yeast-extract-mannitol (YEM) broth. The inoculated LB broth was incubated at 28 ± 2°C for 7 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% 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. The IAA concentration in the supernatant was determined using a calibration curve of pure IAA as a standard. The experiments were repeated three times.

The siderophore-producing ability of rhizobial isolates was evaluated using chrome azurol S (CAS) agar medium (Alexander and Zuberer 1991). CAS agar plates supplemented with 0, 100, 200, and 300 μg l^{-1} tebuconazole were prepared separately and spot inoculated with 10 μl of 10^8 cells ml^{-1}. Plates were incubated at 28 ± 2°C for 96 h. Development of a yellow to orange halo around the growth indicated siderophore production. Each individual experiment was repeated three times. The siderophores produced by the test isolates were also quantitatively assayed using Modi medium (K_2HPO_4, 0.05%; $MgSO_4$, 0.04%; NaCl, 0.01%; mannitol, 1%; glutamine, 0.1%; NH_4NO_3, 0.1%). Modi medium containing 0, 100, 200, and 300 μg L^{-1} tebuconazole were 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 [salicylic acid (SA) and 2,3-dihydroxybenzoic acid (DHBA)] in the supernatant were measured (Reeves and

others 1983). The exopolysaccharide (EPS) produced by the rhizobial isolates was evaluated under in vitro conditions by growing rhizobial cultures in 100-ml capacity flasks containing basal medium supplemented with 5% sucrose. Cultures were incubated for 120 h at 28 ± 2°C on a rotary shaker (100 rpm). Culture broth was centrifuged at 5433*g* for 30 min. and EPS was extracted by adding three volumes of chilled acetone to one volume of supernatant. The precipitated EPS was washed three times alternately with distilled water and acetone, transferred to a filter paper, and weighed after overnight drying (Mody and others 1989).

Rhizobial isolates were also probed for the synthesis of hydrogen cyanide (HCN) by the method of 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, 100, 200, and 300 μg l^{-1} tebuconazole and 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 top of the plate. The plate and paper were sealed with parafilm and 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, 100, 200, and 300 μg L^{-1} tebuconazole. 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).

Rhizobial Inoculation and Fungicide Treatment

Seeds of lentil (var. K75) were surface sterilized with 70% ethanol for 3 min, followed by 3% sodium hypochlorite for 3 min, washed six times with sterile water, and dried. The sterilized seeds were soaked for 2 h in the *Rhizobium* strain MRL3-inoculated YEM broth (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 6.5) (incubated for 7 days), using 10% gum arabic as adhesive, to deliver approximately 10^8 cells per seed. The noncoated sterilized seeds were soaked in sterile water only and served as control. The uninoculated and inoculated seeds were sown in clay pots (25 cm high and 22 cm internal diameter; 10 seeds per pot) using 3 kg unsterilized soil (alluvial sandy clay loam: sand, 667 g kg^{-1}; silt, 190 g kg^{-1}; clay, 143 g kg^{-1}; organic matter, 6.2 g kg^{-1}; 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 anion exchange capacity, 5.1 cmol kg^{-1}) with control (without tebuconazole) and three treatments with 100 (recommended), 200, and 300 μg tebuconazole/kg soil. For each treatment, six pots were used and arranged in a completely randomized design. Three plants were maintained in each pot 1 week after emergence. The pots were watered with tap water when required and were maintained in open field conditions. The experiments were conducted for two successive years to ensure the reproducibility of the results.

All plants in three pots for each treatment were removed 90 days after seeding (DAS) and were observed for growth and symbiotic properties. The roots were carefully washed and nodules were removed, counted, oven-dried at 80°C, and weighed. The leghemoglobin (Lb) content in fresh nodules removed at 90 DAS was quantified (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 an equal amount of alkaline pyridine reagent was added to each tube. The hemochrome formed was read at 556 and 539 nm after adding a few crystals of potassium hexacyanoferrate and sodium dithionite, respectively. Total N content in plant organs (roots and shoots) was measured at 120 DAS by micro-Kjeldahl (Iswaran and Marwah 1980). Phosphorus (P) content in roots and shoots was assayed at 120 DAS (Jackson 1967). The remaining three pots for each treatment, containing three plants per pot, were maintained until harvest (120 DAS). Seed yield and grain protein (Sadasivam and Manikam 1992) were determined at 120 DAS. Plants uprooted at 120 DAS were oven-dried (at 80°C) and the dry matter accumulated was measured.

Statistical Analysis

In vitro experiments were carried out in three replicates and statistical analysis was performed using the Tukey test at the 5% probability level. The pot experiments were conducted for two consecutive years under identical environmental conditions using the same treatments. Because the data of the measured parameters obtained were homogeneous, 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 the Tukey test for in vitro experiments and both Tukey test and two-way ANOVA for pot trials at the 5% probability level.

Results

Characterization of Fungicide-Tolerant *Rhizobium*

In this study, a total of 50 rhizobial strains were isolated from the nodules of lentil plants grown in the experimental

fields of the Faculty of Agricultural Sciences, A.M.U., Aligarh. Each rhizobial strain was tested for sensitivity or tolerance to tebuconazole on minimal salt agar medium. Of the 50 isolates, 8 rhizobial strains, namely, MRL1, MRL2, MRL3, MRL4, MRL5, MRL6, MRL7, and MRL8, that demonstrated higher tolerance to triazole fungicide were selected and their PGP traits were determined. In general, the rhizobial strain with the maximum fungicide tolerance exhibited the highest number of PGP traits and produced the maximum amount of PGP substances (Fig. 2). Among these 8 strains, *Rhizobium* strain MRL3 was preferably selected due to its ability to (1) tolerate tebuconazole up to 1600 μg ml^{-1} on minimal salt agar medium (Table 1); (2) grow well in minimal salts medium supplemented with tebuconazole at the recommended and two and three times the recommended rate (Fig. 3); (3) synthesize maximum amounts of PGP substances like IAA and siderophores; and (4) release EPS, HCN, and ammonia. The tebuconazole-tolerant strain MRL3 was further identified following biochemical and nodulation tests (Table 1). Strain MRL3 produced a significant amount of PGP substances in the presence of the recommended and two and three times the recommended field rate of tebuconazole; however, a tebuconazole-concentration-dependent progressive decline in the production of PGP substances (except HCN and ammonia) by the strain MRL3 was manifested (Table 2). Interestingly, HCN and ammonia production remained unaffected when the strain MRL3 was exposed to tebuconazole. Thus, the most promising *Rhizobium* strain MRL3 was used as a bio-inoculant in pot trials.

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

Characteristics	Strain 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	−
Maximum tolerance level (MTL) to tebuconazole	1600 μg ml^{-1}

+ indicates positive and − indicates negative reactions

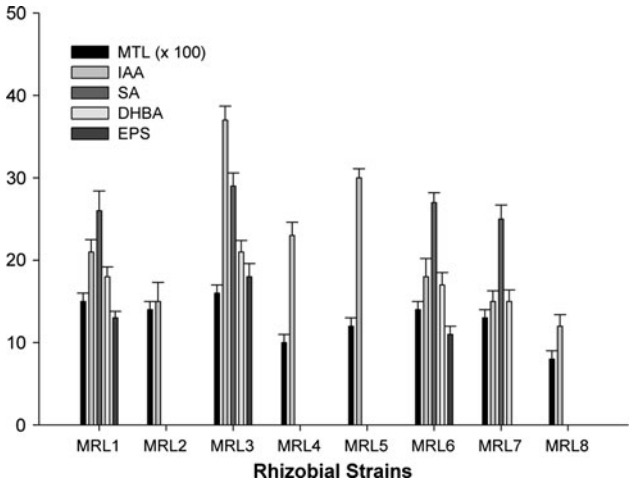

Fig. 2 Tolerance levels and plant-growth-promoting activities of rhizobial strains recovered from nodules of lentil plants. *Vertical bars* represent means of three replicates ± standard error. *MTL* maximum tolerance level, *IAA* indole acetic acid, *SA* salicylic acid, *DHBA* 2,3-dihydroxybenzoic acid, *EPS* exopolysaccharides

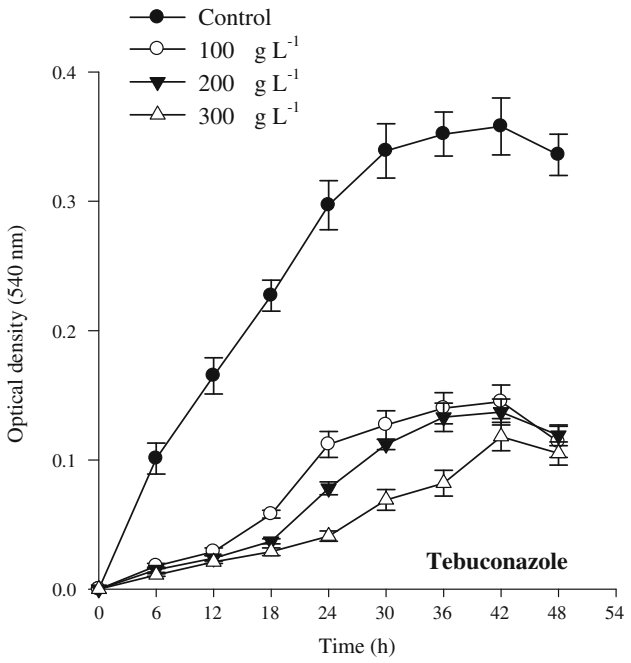

Fig. 3 Impact of the recommended (*open circle*), double (*filled inverted triangle*), and three times (*open triangle*) the recommended rate of tebuconazole on *Rhizobium* strain MRL3 (in terms of optical density) grown in minimal salt agar medium (devoid of carbon and nitrogen sources)

Table 2 Plant-growth-promoting activities of *Rhizobium* sp. strain MRL3 in the presence and the absence of tebuconazole

Dose rate (μg l^{-1}) of tebuconazole	IAA (μg ml^{-1})	Siderophores			EPS (μg ml^{-1})
		CAS agar (mm)	Phenolates (μg ml^{-1})		
			SA	2,3-DHBA	
0 (control)	37a (1.15)	12a (0.25)	29a (1.67)	21a (1.52)	18d (0.73)
100	21b (1.43)	10b (0.22)	20b (1.03)	14b (0.73)	20c (0.95)
200	17c (1.03)	10b (0.16)	16c (1.21)	12c (0.21)	23b (0.62)
300	15d (0.87)	9c (0.12)	14d (1.05)	10d (0.34)	27a (1.05)
F value	67.4	12.5	54.7	17.6	36.5

IAA indole acetic acid, *CAS* chrome azurol s agar, *SA* salicylic acid, *DHBA* dihydroxybenzoic acid, *EPS* exopolysaccharide

Values indicate the mean of three replicates. Mean values followed by different letters are significantly different within a row or column at $p \leq 0.05$ according to the Tukey test. Values in parenthesis indicate standard errors

Rhizobium sp. strain MRL3 also produced hydrogen cyanide (HCN) and ammonia at all three concentrations of tebuconzole

Growth of Uninoculated Lentil Plants in Fungicide-Applied Soils

The effect of three concentrations of tebuconazole on the performance of lentil plants differed considerably. In the presence of the three concentrations of tebuconazole, the dry biomass, nodulation, nutrient-uptake, and yield of uninoculated lentil plants declined significantly ($p \leq 0.05$). In general, the measured parameters decreased progressively as the concentration of tebuconazole was increased from 100 μg/kg soil (recommended dose) to 300 μg/kg soil (three times the recommended rate) in soils. Moreover, 200 and 300 μg tebuconazole/kg soil completely abolished nodulation of lentil plants. For example, tebuconazole at 100 μg/kg soil decreased the total dry biomass, nodule numbers, nodule dry biomass, and Lb significantly ($p \leq 0.05$) by 26, 21, 53, and 33%, respectively, which at 300 μg/kg soil were 54, 100, 100, and 100%, respectively, relative to uninoculated controls (Table 3). The recommended dose of tebuconazole also reduced the root N, shoot N, root P, shoot P, seed yield, and grain protein by 24, 13, 19, 14, 60, and 4%, respectively, whereas three times the recommended dose of tebuconazole reduced the same parameters by 47, 22, 38, 32, 70, and 7%, respectively, compared to uninoculated controls (Table 4).

Inoculation Effects of *Rhizobium* sp. Strain MRL3 on Lentil in Fungicide Stress

The effect of tebuconazole-tolerant and PGP *Rhizobium* sp. strain MRL3 on the growth and symbiotic attributes of lentil grown in tebuconazole-applied sandy clay loam soils varied considerably. Like uninoculated plants, a significant ($p \leq 0.05$) and tebuconazole-concentration-dependent and consistent decline in all biological and chemical characteristics of *Rhizobium* sp. strain MRL3-inoculated lentil plants was also observed when lentil was grown in soils treated with varying concentrations of tebuconazole. However, the reduction in plant growth parameters was less severe when *Rhizobium* sp. strain MRL3 was used with tebuconazole.

In the presence of the bio-inoculant *Rhizobium* sp. strain MRL3, tebuconazole at 100 μg/kg soil decreased the total dry biomass, nodule numbers, nodule dry mass, and Lb by 22, 14, 54, and 27%, respectively. At 300 μg/kg soil, the decrease in the total dry biomass, nodule numbers, nodule dry mass, and Lb was 43, 31, 67, and 53% over inoculated control. Interestingly, strain MRL3 significantly ($p \leq 0.05$) increased the total dry biomass, nodule numbers, nodule dry mass, and Lb with all tested doses of tebuconazole when compared to the uninoculated plants grown with the same concentration of tebuconazole. For example, when *Rhizobium* sp. strain MRL3 was applied with 100 μg tebuconazole/kg soil, the total dry biomass, nodule numbers, nodule dry mass, and Lb increased significantly ($p \leq 0.05$) by 72, 60, 135, and 38%, respectively, relative to the uninoculated plants grown in soil treated solely with 100 μg tebuconazole/kg soil (Table 3).

Correspondingly, in the presence of the bio-inoculant *Rhizobium* sp. strain MRL3, tebuconazole at 100 μg/kg soil decreased the root N, shoot N, root P, shoot P, seed yield, and grain protein by 19, 12, 17, 15, 37, and 4%, respectively, whereas 300 μg tebuconazole/kg soil reduced the same parameters by 43, 22, 28, 29, 54, and 7%, respectively, above the inoculated controls. Interestingly, the bio-inoculant with the recommended dose of tebuconazole significantly ($p \leq 0.05$) increased the root N, shoot N, root P, shoot P, seed yield, and grain protein by 31, 10, 41, 21, 117, and 6%, respectively, compared to the plants treated with 100 μg tebuconazole/kg soil but lacking inoculant. Likewise, the increase in the same plant growth parameters by the rhizobial inoculant with three times the

Table 3 Effect of three concentrations of tebuconazole on growth and nodulation of lentil plants grown in soil inoculated with *Rhizobium* sp. strain MRL3 and without bio-inoculant

Treatment	Dose rate (μg/kg soil)	Total dry biomass (g/plant)	Nodulation		
			Nodule no./plant	Nodule biomass (mg/plant)	Leghemoglobin content [mM (g f.m.)$^{-1}$]
Uninoculated	Control	2.57d (0.31)	19d (1.05)	30c (2.30)	0.12b (0.005)
	100	1.91f (0.23)	15e (1.50)	14e (1.57)	0.08d (0.002)
	200	1.55 g (0.21)	–	–	–
	300	1.18 h (0.07)	–	–	–
Inoculated	Control	4.23a (0.12)	28a (1.14)	72a (2.12)	0.15a (0.002)
	100	3.29b (0.14)	24b (1.05)	33b (1.25)	0.11c (0.004)
	200	2.85c (0.22)	22c (1.35)	30c (0.95)	0.08d (0.003)
	300	2.41e (0.05)	19d (0.65)	24d (1.07)	0.07e (0.004)
LSD		1.7	1.6	0.95	0.006
F value	Inoculation (df = 1)	423*	234*	163*	43.6*
	Fungicide (df = 3)	14.5*	45.4*	41.4*	1.5
	Inoculation × fungicide (df = 3)	27.9*	73*	19.2*	1.2

Values are mean of three replicates where each replicate constituted three plants per pot. Mean values followed by different letters are significantly different within a row or column at $p \leq 0.05$ according to the Tukey test. Values in parenthesis indicate standard errors

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

Table 4 Effect of three concentrations of tebuconazole on N and P content, seed yield, and grain protein of lentil plants grown in soil inoculated with *Rhizobium* sp. strain MRL3 and without bio-inoculant

Treatment	Dose rate (μg/kg soil)	N content (mg/g)		P content(mg/g)		Seed yield (g/plant)	Grain protein (mg/g)
		Root	Shoot	Root	Shoot		
Uninoculated	Control	17b (1.05)	45b (1.65)	0.21d (0.004)	0.28 cd (0.004)	3.0b (0.10)	232 cd (1.21)
	100	13d (0.74)	39e (1.25)	0.17e (0.006)	0.24e (0.003)	1.2f (0.05)	223f (1.05)
	200	11f (0.85)	37 fg (1.32)	0.15f (0.002)	0.22f (0.004)	1.0 g (0.01)	219 g (1.15)
	300	9 g (0.57)	35 g (1.05)	0.13 g (0.002)	0.19 g (0.005)	0.9 h (0.02)	216 h (1.25)
Inoculated	Control	21a (1.63)	49a (2.05)	0.29a (0.005)	0.34a (0.003)	4.1a (0.14)	245a (1.07)
	100	17b (0.78)	43c (1.46)	0.24b (0.003)	0.29bc (0.008)	2.6c (0.08)	236b (1.15)
	200	15c (0.83)	41d (1.02)	0.22c (0.002)	0.27d (0.005)	2.2d (0.02)	231d (1.36)
	300	12e (0.58)	38ef (0.56)	0.21d (0.004)	0.24e (0.003)	1.9e (0.04)	228e (1.12)
LSD		1.3	1.4	0.06	0.008	0.07	1.4
F value	Inoculation (df = 1)	416.3*	620*	318.7*	217.4*	327.4*	219.7*
	Fungicides (df = 3)	87.4*	163*	33.7*	127.2*	112.5*	144.2*
	Inoculation × fungicide (df = 3)	37.5*	82*	79.5*	32.4*	38.6*	63.5*

Values are mean of three replicates where each replicate constituted three plants per pot. Mean values followed by different letters are significantly different within a row or column at $p \leq 0.05$ according to the Tukey test. Values in parenthesis indicate standard errors

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

recommended dose of tebuconazole was 33, 9, 62, 26, 111, and 6%, respectively, compared to the uninoculated treatment with 300 μg tebuconazole/kg soil (Table 4).

Statistically, the two-factor ANOVA revealed that the individual effects of inoculation and fungicide and their interactive effect (inoculation × fungicide) were significant ($p \leq 0.05$) for plant dry biomass, nodulation, nutrient uptake, seed yield, and grain protein, except the effect of fungicide and its interaction with inoculant MRL3 for Lb content (Tables 2, 3).

Discussion

In modern agricultural practices, organic fungicides are used to protect seeds from fungal diseases and

consequently to enhance crop production. However, some organic fungicides like carbendazim are toxic to rhizobia whereas others like captan have been found to be compatible with nodule bacteria (Kaur and others 2007). It is reported that the viability of rhizobia exposed to fungicides is adversely affected and their nodulating and N_2-fixing abilities vanish (Zahran 1999; Guene and others 2003). Despite these conflicting reports, many studies have documented the benefit of rhizobia to legume production (McKenzie and others 2001; Zaidi and others 2003). In agreement with these findings, application of *Rhizobium*, which showed multiple plant-growth-promoting activities in the presence and the absence of fungicide, was generally beneficial. As reported by others (Tesfai and Mallik 1986), a significant reduction in the growth of lentil plants following fungicide application was observed in this study; it was probably due to the adverse effects of tebuconazole on plant organs, especially the function of nodules, which consequently diminishes the N_2 fixation (Fox and others 2007). Such inhibitory effects after fungicide application may also be due to the inhibition of nitrogenase activity or chlorophyll contents, as observed for soybean (*Glycine max*) when grown with varying concentrations of thiram (Bikrol and others 2005). Statistically significant interactions between fungicide and lentil were detected for nodulation and seed yield. The pattern of the interactions observed for nodulation and seed yield was a consistent poor nodulation and yield in the absence of rhizobial inoculants when tebuconazole was applied alone. In similar studies, recommended and higher rates of fungicide, for example, captan, reduced nodulation and N_2 fixation by *Trifolium repens* (Fisher and Hayes 1981). Fungicides like thiram and captan have also been reported to be harmful to nodulation and N_2 fixation of several grain and forage legumes (Heinonen-Taski and others 1982; Rennie and others 1985; Aamil and others 2004). In another study, Kyei-Boahen and others (2001) reported decreased nodulation, percent N derived from the atmosphere (%Ndfa), and plant growth in chickpea in response to a presowing seed treatment with commercial fungicides. In addition, our study showed that the toxicity of two and three times the recommended dose of the fungicide tebuconazole to lentil growth was more severe than that of the recommended dose. Fungicides at lower rates are not toxic possibly because the soil environment acts as a buffer and thus reduces the potentially toxic effects of the fungicides by dilution of the chemicals. However, at higher concentrations, fungicides cause a depressive effect on both microorganisms and plants (Ayansina 2009).

Interestingly, the growth of lentil plants was stimulated when the PGP *Rhizobium* strain MRL3, which is tolerant to tebuconazole up to 1600 μg ml^{-1}, was applied as seed inoculant in fungicide-treated soil. In general, the measured parameters were increased following inoculant application with tebuconazole compared to plants grown in soils treated solely with tebuconazole. Plant-growth-promoting rhizobacteria, including symbiotic N_2 fixers, can facilitate 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 and others 2008). This fact is further shown in that the rhizobial strains that showed higher tolerance to tebuconazole were also found to exhibit better PGP activities (Fig. 2). In addition, the most tolerant rhizobial strain, MRL3, also grew well in carbon (C) and nitrogen (N) source-deficient minimal salt medium amended even with three times the recommended dose of tebuconazole (Fig. 3). Consequently, MRL3 as a bio-inoculant not only protected lentil plants from fungicide toxicity but also increased their growth and symbiotic attributes substantially in fungicide-amended soils. Such an increase in plant growth may be due to the fact that the introduced compound might have acted as a new source of C, N, P, and S, which if utilized by one microbial community allows them to proliferate and out-compete other microbial communities (Ayansina 2009).

To the best of our knowledge, no information is available in the literature regarding the relationship between pesticide/fungicide tolerance (resistance) and PGP activities of rhizobia/soil bacteria. Moreover, it has been reported that exopolysaccharides (EPS) are known to influence legume root infection and nodulation (Chen and others 1985; Leigh and others 1988). In the present study, strain MRL3 produced a substantial amount of EPS even in the presence of tested fungicide. It might be possible that the production of EPS by the rhizobial strain resulted in more nodulation in inoculated treatments, as the role of EPS in legume–*Rhizobium* interaction is also reported by others (Spaink 2000; Mithöfer 2002). Similarly, the N content in plant organs (root and shoot) was also higher in bioprimed plants, probably due to increased N_2 fixation that led to a considerable increase in N uptake (Joshi and others 1990). In addition, the synthesis of iron-chelator siderophores and IAA by the test strain MRL3 might also have enhanced root growth and uptake of soil minerals by the host plant. Moreover, the bio-inoculant significantly increased the nodulation compared to uninoculated controls, confirming the fact that MRL3 might have reduced the toxicity of tebuconazole in sandy loam soil, as was evident by the growth of this strain on minimal media using tebuconazole as a source of C and N (Fig. 3).

In conclusion, we demonstrated the phytotoxic effects of tebuconazole on the performance of lentil plants grown in fungicide-treated sandy clay loam soils. Fungicide-tolerant *Rhizobium* sp. strain MRL3, used as a seed inoculant, protected the lentil plants from the toxic effects of

tebuconazole and also increased the biological and chemical characteristics of the test plants. The increased growth of inoculated lentil plants in this study, even in the presence of fungicide, might have possibly been due to the availability of plant-growth-promoting substances like phytohormones, siderophores, and EPS to the lentil plants, synthesized by *Rhizobium* strain MRL3, in addition to providing N to plants through nitrogen fixation. The rhizobial strain expressing such multiple physiological traits can be used as a bio-inoculant to optimize the lentil production in fungicide-treated soils.

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