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Improvement in the Growth and Symbiotic Attributes of Fungicide-Stressed Chickpea Plants Following Plant Growth Promoting Fungicide-Tolerant *Mesorhizobium* Inoculation

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Abstract: The aim of this study was to assess the pre-emergent impact of three concentrations [100 (recommended dose), 200 and 300 μg kg^{-1} soil] of technical-grade fungicide tebuconazole on chickpea plants vis-à-vis to assess the performance of tebuconazole-stressed chickpea plants inoculated with plant growth promoting tebuconazole-tolerant *Mesorhizobium* strain MRC4. Generally, the concentration-dependent phytotoxicity of tebuconazole was observed for plant dry weight, nodule numbers, dry nodule biomass, leghaemoglobin, nitrogen and phosphorus uptake, seed yield and grain protein. Interestingly, when the strain MRC4 was also used with any concentration of tebuconazole, it significantly increased the measured parameters when compared to the plants grown in soils treated solely (without inoculant) with the same individual treatment of tebuconazole. The study suggested that plant growth promoting *Mesorhizobium* strain MRC4 can be used as bio-inoculant to increase the productivity of chickpea in fungicide-stressed soils.

Key words: Chickpea · *Mesorhizobium* · Tebuconazole · Phytotoxicity

INTRODUCTION

Rhizobium-legume symbiosis is one of the most important characteristics for increasing the nitrogen (N) pool of both legumes and soils. This relationship between rhizobial species and their cognate host legume plant is invariably advantageous since it not only provides N in fixed forms to the host-plants but also nurture and maintain the N status and turnover of the soil ecosystem [1]. This biological interaction also occurs between the species of Gram negative *Mesorhizobium* bacterium and chickpea [2].

Various biological factors like, Nod factors, the extracellular polysaccharides, the lipopolysaccharides, the K-antigens and the cyclic glucans together with soil profile and agrichemicals including herbicides, insecticides, fungicides, biocides and fertilizers affects root nodulation and N_2 fixation [3,4]. In most of the agricultural operations, the fungicides of diverse chemical groups and spectrum are applied to leguminous plants either as seed-dressing or soil-drench injudiciously and indiscriminately to prevent losses due to seed borne pathogens.

Thus, the recurrent use of agrochemicals lead to their accumulation in soils to a level (higher than the recommended dose) that is detrimental to beneficial plant growth promoting rhizotrophic microflora including rhizobial species and also *Rhizobium*-legume symbiosis, which in turn cause loss to soil-N status [5]. For instance, in a study, the recommended and higher rates of fungicide captan reduced nodulation and N_2-fixation by *Trifolium repens* [6]. Similarly, other fungicides like, thiram and captan have also been reported to abolish nodulation and N_2-fixation of several grain and forage legumes [7,8].

Although, huge information on the toxic effect of fungicides on cultivated crops including legumes are available, there is inconsistency in the reported literature. Consequently, it becomes difficult to assess the actual effects of fungicides on legumes and their respective symbiotic partners. Additionally, information concerning the parallel study of fungicide tebuconazole on rhizobial sp. and chickpea (*Cicer arietinum*) is not reported. The present study was therefore, designed to evaluate the impact of technical grade fungicide, tebuconazole [(*RS*)-1-*p*-chlorophenyl-4,4-dimethyl-3-(1*H*-1,2,4-triazol-1-ylmethyl)pentan-3-ol] on the performance of plant growth

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promoting (PGP) and tebuconazole-tolerant *Mesorhizobium* strain MRC4-inoculated/un-inoculated chickpea plants sown in tebuconazole treated alluvial soils.

METHODOLOGY

Determination of Fungicide Tolerance and Plant Growth Promoting Activities: Rhizobial isolates were obtained from nodules borne on the root system of chickpea plants grown in experimental fields of Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, India, using yeast extract mannitol (YEM) medium [9]. These rhizobial isolates were not identified on molecular basis and are referred as *Mesorhizobium* only, due to their host specificity [10] and morphological and biochemical tests [11]. The rhizobial strains were tested for their sensitivity/resistance to technical grade tebuconazole (active ingredient 100%; Parijat Agrochemicals, New Delhi, India) by agar plate dilution method using minimal salt agar medium. The freshly prepared agar plates were amended separately with increasing concentrations of tebuconazole (0 to 3200 μg ml^{-1}; at two-fold dilution intervals). Later, these plates were spot inoculated with 10 μl of 10^8 cells ml^{-1} of rhizobial Strains. 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 resistance level (MRL).

Indole-3-acetic acid (IAA) was quantitatively assayed by the method of Gordon and Weber [12] later modified by Brick et al. [13]. For this activity, rhizobial strains 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), 100 (recommended dose), 200 and 300 μg L^{-1} tebuconazole. A-100 ml of LB broth containing 100 μg ml^{-1} tryptophan was inoculated with one milliliter *Mesorhizobium* culture (10^8 cells ml^{-1}), grown in YEM broth. The inoculated LB broth was incubated at 28±2 °C for seven 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) was added to it and incubated at 28±2 °C in darkness for one 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. The experiments were repeated three times.

Siderophores synthesized by the rhizobial strains was assayed by the method of Alexander and Zuberer [14]. CAS agar plates treated with 0, 100, 200 and 300 μg L^{-1} tebuconazole were prepared separately and divided into equal sectors and spot inoculated with 10 μl of 10^8 cells ml^{-1}. Plates were incubated at 28±2 °C for 96 h. Development of yellow to orange halo around the growth was considered as positive for siderophore production. Each individual experiment was repeated three times. The siderophores produced by the test strains was 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 ammended with 0, 100, 200 and 300 μg L^{-1} tebuconazole were inoculated with 100 μl of 10^8 cells ml^{-1} of rhizobial strains and incubated at 28±2 °C for five days. Cultures were spun and the catechol type phenolates [salicylic acid (SA) and 2,3 dihydroxybenzoic acid (DHBA)] in the supernatant was measured [15].

Moreover, the exo-polysaccharide (EPS) produced by the rhizobial strains was detected under *in vitro* conditions. For this, the rhizobial strains were grown in 100 ml capacity flasks containing basal medium supplemented with 5 % sucrose and incubated for 120 h at 28±2 °C on shaker (100 rpm). Culture broth was spun (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 repeatedly washed three times alternately with distilled water and acetone, transferred to a filter paper and weighed after overnight drying [16]. Rhizobial strains were also screened for the synthesis of hydrogen cyanide (HCN) by the method of Bakker and Schipper [17].

Briefly, rhizobial strains 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 were incubated at 28±2 °C for four days. Rhizobial 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 and incubated at 28±2 °C for four days. Development of orange to red color indicated HCN production. Rhizobial strains 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 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 was added to each tube. Development of yellow color indicated a positive test for ammonia [18].

Rhizobial Inoculation and Fungicide Treatment: Seeds of chickpea (var. C 235) were surface sterilized with 70 % ethanol, 3 min. followed by 3 % sodium hypochlorite, 3 min, washed six times with sterile water and dried. The sterilized seeds were bioprimed with *Mesorhizobium* strain MRC4, grown in YEM (g l^{-1}: mannitol 10; K_2HPO_4 0.5; $MgSO_4.7H_2O$ 0.2; NaCl 0.1; yeast extract 1.0; $CaCO_3$ 1.0; pH 6.5) broth for seven days by soaking the seeds in liquid culture medium for 2 h using 10 % gum arabic as adhesive to deliver approximately 10^8 cells per seed. The non-coated sterilized seeds were soaked in sterile water only and served as control. A total of 10 non-inoculated and inoculated seeds were sown separately in clay pots (25 cm high, 22 cm internal diameter) using three kg unsterilized soils (alluvial sandy clay loam, sand 667 g kg^{-1}, silt 190 g kg^{-1}, clay143 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 and water holding capacity 0.44 ml g^{-1}, cation exchange capacity 11.7 cmol kg^{-1} and 5.1 cmol kg^{-1} anion exchange capacity) with control (without tebuconazole) and three treatments with 100 (recommended), 200 and 300 µg kg^{-1} soil tebuconazole. For each treatment, six pots were used and arranged in a complete randomized design. Three plants were maintained in each pot one week after emergence. The pots were watered with tap water when required and were maintained in an 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 leghaemoglobin (Lb) content in fresh nodules removed from the root system of plants was quantified at 90 DAS [19]. 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 N content in plant organs (roots and shoots) was measured at 135 DAS by micro-Kjeldahl [20]. Phosphorus (P) contents in such tissues were assayed at 135 DAS by the method of Jackson [21]. The remaining pots (three pots) for each treatment containing three plants per pot were maintained until harvest (135 DAS). Seed yield and grain protein [19] was determined at 135 DAS. Plants uprooted at 135 DAS were oven-dried (at 80°C) and the dry matter accumulated was measured.

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 analysis of variance (ANOVA). The difference among treatment means was compared by high range statistical domain (HSD) using two-way ANOVA at 5% probability level.

RESULTS

Bio-inoculant: Fungicide Tolerance and Characterization: Among the total of 50 rhizobial strains isolated from the nodules of chickpea (90 days old), strain MRC4, was distinctively preferred because of its optimum growth and greater MRL (1400 µg ml^{-1}) to the technical-grade fungicide tebuconazole in minimal salts medium supplemented with increasing concentrations of this fungicide as a sole source of C and N and *in vitro* production of PGP substances (IAA, siderophores, EPS, HCN and ammonia) in considerable amount (Table 1-2) relative to other rhizobial isolates. The tebuconazole tolerant strain MRC4 was identified as *Mesorhizobium* sp. following biochemical and host-specificity tests (Table 1). *Mesorhizobium* sp. strain MRC4 exhibited a substantial production of PGP substances even in the presence of tebuconazole at recommended and higher doses (Table 2).

Table 1: Morphological and biochemical characteristics of *Mesorhizobium* sp. strain MRC4

Characteristics	Strain MRC4
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 resistance level (MRL)	
Tebuconazole	1400 µg ml^{-1}

+ indicates positive and – indicates negative reactions.

Table 2: Effect of three concentrations of tebuconazole on growth, symbiotic properties, nutrient-uptake and yield of chickpea plants grown in soil inoculated with *Mesorhizobium* sp. strain MRC4† and without bioinoculant

			Nodulation			N content		P content			
		Total dry		Nodule	Lb[a]	(mg/g)		(mg/g)		Seed	Grain
	Dose rate	biomass	Nodule	biomass	content					yield	protein
Treatment	(µg/ kg soil)	(g /plant)	no./ plant	(mg/ plant)	[mM (g f.m.)$^{-1}$]	Root	Shoot	Root	Shoot	(g/plant)	(mg/g)
Uninoculated	Control	4.99	21	180	0.13	18	27	0.17	0.21	2.7	241
	100	2.57	16	116	0.04	15	21	0.15	0.17	1.7	209
	200	1.97	14	97	0.03	13	20	0.13	0.15	1.5	196
	300	1.70	11	80	0.01	12	17	0.12	0.14	0.9	184
Inoculated	Control	5.63	38	325	0.19	24	32	0.23	0.27	3.7	260
	100	3.21	28	151	0.05	18	26	0.19	0.24	2.3	237
	200	2.63	25	121	0.03	16	24	0.17	0.22	1.9	232
	300	1.82	20	96	0.02	13	22	0.14	0.21	1.2	228
LSD		8.7	1.2	1.5	0.009	1.4	1.6	0.006	0.008	0.41	2.4
F value	Inoculation (df=1)	346.5*	2547*	4288*	152.3*	540.3*	142.3*	152*	2475*	272.4*	424*
	Fungicide (df=3)	62.4*	562*	443*	36.4*	85.4*	32.2*	38.2*	27.2*	45.6*	203.9*
	Inoculation×fungicide (df=3)	21.2*	459*	137*	6.2*	52.3*	7.4*	9.2*	90.2*	15.1*	38.8*

Values are mean of three replicates where each replicate constituted three plants/ pot. †Strain MRC4 at 0, 100, 200 and 300 µg l^{-1} of tebuconazole, produced 35, 26, 23 and 21 µg l^{-1} SA; 19, 13, 10 and 8 µg l^{-1} DHBA; 44, 17, 14 and 11 µg ml^{-1} IAA and 21, 22, 23 and 25 µg ml^{-1} EPS, respectively and was positive for HCN and ammonia production; [a]Leghaemoglobin; *Significantly different from the control at p≤ 0.05.

Effect of Fungicide and Bio-Inoculant on Chickpea Plants: The ability of tebuconazole-tolerant *Mesorhizobium* strain MRC4 to produce PGP substances significantly even at abnormally higher concentrations of tebuconazole impelled us to assess the effect of this strain on the performance of chickpea grown in fungicide-stressed sandy clay loam soils. The chickpea plants exposed to three concentrations of tebuconazole suffered a considerable decline in all measured growth parameters (plant dry biomass, nodulation, nutrient-uptake and grain-yield/ quality). A consistent and tebuconazole-concentration dependent decrease in plant growth parameters was observed both for the inoculated and un-inoculated plants. However, severity of phytotoxicity of tebuconazole was less prominent when inoculant *Mesorhizobium* strain MRC4 was also used along with tebuconazole.

In the absence of bio-inoculant, tebuconazole at 100 µg/ kg soil for example, decreased the total dry biomass, the nodule number, nodule dry biomass, Lb, root N, shoot N, root P, shoot P, seed yield and grain protein by 48, 24, 36, 69, 17, 22, 12, 19, 47 and 13%, respectively while at 300 µg/ kg soil it decreased the same parameters by 66, 48, 66, 92, 33, 47, 29, 33, 67 and 24%, respectively, relative to control. Also, in the presence of bio-inoculant, tebuconazole at 100 µg/ kg soil decreased the total dry biomass, nodule number, nodule dry mass, Lb, root N, shoot N, root P, shoot P, seed yield and grain protein by 43, 26, 54, 74, 25, 19, 17, 11, 38 and 9%, respectively and by 68, 47, 70, 89, 46, 31, 39, 22, 68 and 12% respectively, at 300 µg/ kg soil, over inoculated control (Table 2).

Interestingly, the rhizobial inoculant (*Mesorhizobium* strain MRC4) with 100 µg/ kg soil tebuconazole, increased significantly ($p \leq 0.05$) the total dry biomass, nodule number, nodule dry mass, Lb, root N, shoot N, root P, shoot P, seed yield and grain protein by 25, 75, 30, 25, 20, 24, 27, 41, 35 and 13%, respectively, when compared to the plants treated with 100 µg/ kg soil of tebuconazole but without inoculant. Instead, the significant ($p \leq 0.05$) increment in the same plant growth parameters with 300 µg/ kg soil of tebuconazole was 7, 82, 20, 100, 8, 29, 17, 50, 33 and 24%, respectively, compared to the un-inoculated treatment having the same concentration of tebuconazole (Table 2).

Statistically, the two-way ANOVA revealed that the individual effects of bio-inoculant, *Mesorhizobium* strain MRC4, tebuconazole and their interactive effect (inoculation × fungicide) were found significant ($p \leq 0.05$) for plant dry biomass, nodule number, nodule dry biomass, Lb content, N and P uptake, seed yield and grain protein (Table 2).

DISCUSSION

In the absence of bio-inoculant, tebuconazole at all tested doses, in this study, led to a considerable decline in the plant growth parameters including symbiotic properties (nodulation and Lb), nutrient-uptake and grain yield of chickpea. Proportionately, the higher doses of the fungicide from recommended one showed more injurious effects on chickpea plants. Such enormous reduction in chickpea dry biomass following fungicide application may possibly be due to the fact that pesticides including fungicides inhibit the activity of enzymes involved in regulation of plant growth and their metabolic activities [22]. Therefore, the decline in plant growth, in this study, was observed in a proportion to the concentration of tebuconazole. Moreover, the reason for concomitant adverse effect of tebuconazole on chickpea-*Mesorhizobium* symbiosis and its attributes is that organic fungicides used in order to protect seeds against fungal diseases, are toxic to rhizobial growth and viability; in most cases, even if rhizobia remain viable, their host-plant nodulating efficiency or N_2-fixing ability is reduced [23,24]. Inhibition in symbiosis following tebuconazole application may also possibly be due to disruption of signaling between legume-derived phytochemicals and *Rhizobium* Nod D receptors that is essential for initiation of nodulation and N_2 fixation [3]. In other studies, Kyei-Boahen *et al.* [25] reported decreased nodulation, percent N derived from the atmosphere (% Ndfa) and plant growth in chickpea in response to pre-sowing seed treatment with commercial fungicides.

Interestingly, seed-inoculation of tebuconazole-tolerant plant growth promoting *Mesorhizobium* strain MRC4 in this study stimulated the growth of chickpea plants. In general, the plant growth parameters in the presence of tebuconazole were augmented following inoculant application, compared to plants grown in soils treated solely with tebuconazole. Plant growth promoting rhizobacteria including symbiotic N_2-fixers expedite plant development by protecting the growing plants from the toxic effects of pesticides through biodegradation of these agrochemicals [26] or by synthesizing the plant growth regulating substances like IAA, siderophores [2]. Moreover, the bio-inoculant significantly increased the nodulation compared to un-inoculated control consolidating the fact that the strain MRC4 might have reduced the toxicity of tebuconazole in sandy loam soil, as was evident through the growth of this strain on minimal media using tebuconazole as C source. In the present study, strain MRC4 produced a considerable amount of EPS even in the presence of tested fungicide. It might be possible that the production of EPS by the mesorhizobial strain resulted in more nodulation in inoculated treatments, as the role of EPS in legume-*Rhizobium* interaction is well reported [27,28].

CONCLUSION

We demonstrated the phytotoxicity of tebuconazole at different doses including recommended one to chickpea plants. Fungicide-tolerant *Mesorhizobium* sp. strain MRC4 used as seed inoculant not only protected the chickpea plants from the phytotoxic effects of tebuconazole but also increased plant dry biomass, nodulation, nutrients and yield possibly due to the synthesis and release of phytohormones, siderophores and EPS. The mesorhizobial strain with such multiple traits can be used as bio-inoculant to increase the productivity of chickpea in soil contaminated with fungicide.

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