Survivability and plant growth-promoting traits of *Rhizobium aegyptiacum* under the stress of fungicides and insecticides

Sukriti Chandra and *Dipjyoti Chakraborty

Department of Bioscience and Biotechnology, Banasthali Vidyapith, Rajasthan-304022, India

**Corresponding author's email: cdipjyoti@banasthali.in*

Abstract

Chickpea (*Cicer arietinum* L.) production relies heavily on chemical fertilizers and pesticides which have several drawbacks. Biofertilizers provide an eco-friendly alternative, however, growth of the bio-inoculant is often hindered by the chemical residues of insecticides and fungicides present in the soil. In this study, we evaluated the growth of a potential plant growth-promoting bacterium *Rhizobium aegyptiacum*, in the presence of the fungicide bavistin (50% carbendazim) and the insecticide chlorpyriphos 20 EC. A decrease in the growth of *R. aegyptiacum* was observed with increase in concentration of the fungicide and the insecticide. A 33.7% of growth reduction was observed under 3 times of the recommended dose of carbendazim. Likewise, in the presence of chlorpyriphos 20 EC, 10.6, 21.7 and 50.01% growth was inhibited at 1X, 2X and 3X of recommended dose, respectively. In the absence of fungicide and insecticide, the phosphate solubilization index was determined to be 3.21, which reduced to 2.53 under 3X chlorpyriphos treatment and 2.90 under 3X carbendazim treatments. The lowest IAA production (17.8 µg mL^{-1}) was observed in 3X chlorpyriphos treatment. HCN production was also detected in the presence of both insecticide and fungicide. Thus, *R. aegyptiacum* was found to retain phosphate solubilization, HCN production and IAA production capacity in the presence of up to three times the recommended dosage of the bavistin and chlorpyriphos 20 EC. *R. aegyptiacum* can be recommended as bio-inoculum in chickpea cultivation in agricultural fields contaminated with high concentrations of insecticides and pesticides.

Keywords: Bavistin, Bio-pesticide, Chickpea, Chlorpyriphos 20 EC, Fungicide, Insecticide, *Rhizobium. aegyptiacum*.

Introduction

Chickpea (*Cicer arietinum* L.) is one of the major legumes cultivated in arid and semi-arid regions, under rainfed conditions. India has large areas under chickpea cultivation but comes in sixth position in productivity (1063 kg ha^{-1}) (Sharma and Sharma, 2020). Chickpea production is adversely affected due to the attack of several phytopathogens including three bacteria, 22 viruses, 67 fungi and 80 nematodes (Nene *et al*., 1996; Gurjar et *al*., 2010). Some of the major diseases in chickpea are dry root rot (*Macrophomina phaseolina*), wilt (*Fusarium oxysporum*), collar rot (*Sclerotium rolfsii*), root rot (*Fusarium solani*), blight (*Ascochyta* blight), and gram pod borer (*Helicover paarmigera*) (Garg *et al*., 2014; Javaid and Khan, 2016; Javaid *et al.*, 2020; Lokesh *et al*., 2020; Fatima *et al*., 2022). To control these diseases in chickpea fields, farmers are using insecticides such as chlorpyriphos 20 EC and fungicides namely bavistin, captan and mancozeb (Lokesh *et al*., 2020; Mansotra *et al*., 2022), which lead to accumulation of the chemical residues in the soil that contribute to agrochemical stress (Al Abboud *et al*., 2014; Kumar *et al*., 2019). These also have adverse effects on beneficial soil and symbiotic microorganisms (Cevheri *et al*., 2011), disturb plant growth-promoting activity, and result in poor symbiotic relations with host plant and a low nodulation rate (Ramirez and Damo, 2023; Yadav *et al*., 2023). Microorganisms with plant growth-promoting activities that can better adapt to biotic and agrochemical stresses can be used as biofertilizers (Ahemad and Khan, 2011; Sharf *et al*., 2021).

While insecticides and pesticides have detrimental effects on soil microorganisms, few symbiotic microorganisms, especially root nodulating bacteria in legumes can provide better tolerance to fungicide and insecticide contaminated soil (Khan *et al*., 2020; Meena *et al*., 2020; Gustavo *et al*., 2021). Selecting these effective stress-tolerant microbial strains could help to restore soil fertility and aid in plant development (Anjum *et al*., 2017; Sarvani *et al*., 2021). *Rhizobium* biofertiliser has been reported to have potential to bio-degrade pesticides while promoting plant growth (Kulkarni *et al*., 2022). Shoman *et al*., 2022, reported that the bacterial strains *Pseudomonas fluorescens*, *Rhizobium leguminosarum*, and *Bacillus megaterium* were able to degraded the pesticide chlorpyrifos to its non-toxic components 3,5,6-trichloro-2-pyridinol and chlorpyrifos oxon. The present study was

designed to evaluate the tolerance of a chickpea root nodulating bacterial strain against the commonly used fungicide bavistin (50% carbendazim) and the insecticide chlorpyriphos 20EC.

Materials and Methods

The bacterial strain used in the study

A root-nodulating strain *Rhizobium aegyptiacum* Strain D (Genebank Acc No. MT980913) previously isolated from *Cicer arietinum* GNG 1958, a widely grown highly productive chickpea variety in India was used for this study.

Determination of fungicide and insecticide tolerance

Bacterial survival was determined in the presence of two commonly used fungicides and pesticides to treat chickpea plants viz., the fungicide, Bavistin (ai-50% carbendazim) and the insecticide, Chlorpyrifos 20 EC. Agrochemical concentrations were prepared equivalent to the recommended dose of pesticides under field condition following method of Mansotra *et al*., 2022 with some modifications.10 ml YEM broth, pH 6.8±0.2 was prepared and autoclaved. Thereafter, different concentrations of pesticides were prepared for bavistin *viz.* 1X, 2X, 3X with equivalent concentration of 3, 6 and 9 µg mL^{-1}, respectively, and for chlorpyriphos 20 EC *viz.* 1X, 2X, 3X with equivalent concentration of 1.8, 3.6 and 5.4 µL mL^{-1} respectively (Table 1), and added to the YEM broth separately under the laminar air-flow. One-day-old bacterial culture (100 µL) at log phase was inoculated to it and incubated for 78 h at 28 ± 2 ºC. Bacterial growth was determined at 420 nm and percentage inhibition was calculated by using the following formula:

Inhibition (%) = [{Growth in control (OD) - Growth in treated (OD)}/ Growth in control (OD)}] ×100

Determination of plant growth-promoting activity under fungicide and insecticide stress

The highest concentration of each test fungicide (3X, 9 µg mL^{-1} equivalent concentration) and insecticide (3X, 5.4 µL mL^{-1} equivalent concentration) in which select strain survived was used to evaluate plant growth-promoting activities. Phosphate solubilization was evaluated in Pikovskaya agar media supplemented with 0.5 g yeast extract, 10 g glucose, 5 g calcium phosphate, 0.5 g ammonium phosphate, 0.1 g magnesium sulphate, 0.002 g manganese sulphate, 0.002 g ferrous sulphate, 0.2 g potassium chloride, 0.2 g sodium chloride and 15 g agar L^{-1} and pH was maintained 7 ± 0.2. 0.1 µL of one day old bacterial suspension was spot inoculated and incubated for 8-10 days at 30 ± 2 ºC. The Phosphate solubilisation index (PSI) was calculated (Karpagam and Nagalakshmi, 2014) as follows:

PSI = (colony diameter halo + zone diameter)/ colony diameter

For the determination of HCN production, nutrient agar was prepared, amended with 4.4 g L^{-1} glycine and bacterial isolate was inoculated on it (Lorck, 2004). The streaked portion was covered with Whatman filter paper dipped in picric acid solution in 2% Na_2CO_3 and incubated for 3 days at 30 ± 2 ºC. HCN production is indicated by the change in color of the filter paper from yellow to red brown. To determine IAA production, Salkowski's reagent was used (Gordan and Weber, 1951). Bacterial culture supernatant (1 mL), 2 mL of Salkowski's reagent (0.5 M $FeCl_3$ and 35% Perchloric acid) was added and kept for 30 min at room temperature and absorbance observed at 530 nm.

Statistical analysis

All the experiments were conducted in three replicates. Analysis of variance (ANOVA) was determined by using SPSS 20 software. To calculate the significant difference among the means, Duncan test was applied ($P \leq 0.05$).

Results and Discussion

In order to reduce crop loss to pathogens and vectors carrying them, several effective insecticides and pesticides are available. However, indiscriminate use of such chemicals adversely affects beneficial microorganisms and deteriorates soil health (Ramirez and Damo, 2023; Yadav *et al*., 2023). Natural insecticides and pesticides are proposed to be better and sustainable, but they face unique challenges in implementation (Ames *et al*., 1990; Bahlai *et al*., 2010; Costantini and La Torre, 2022). The present study was thus undertaken to isolation and characterisation of microorganisms for compatibility with commonly used insecticide and pesticide in chickpea that could help in restoring soil fertility and aid in plant development.

A decrease in the growth of *R. aegyptiacum* was observed in the present study with increase in the concentration of fungicide and insecticide. Bacterial growth was observed in all doses of carbendazim although a 33.7% of growth reduction was observed under 3X treatment (Table 2). In the presence of the insecticide chlorpyriphos 20 EC, 10.6, 21.7 and 50.01% growth was inhibited at 1X, 2X and 3X of recommended dose, respectively (Table 3).

Previous studies reflect a strain-specific response to carbendazim. Sarvani *et al*., 2021, reported that carbendazim is less toxic to rhizosphere isolates but in contrary Kaur *et al*., 2007 reported that it is more toxic to rhizobia isolates. Meena *et al*., 2020, reported the negative impact of pesticides on legume-rhizobia symbiosis due to interference with cell division resulting in poor nodule development.

Mansotra *et al*. (2022) observed that insecticides followed by fungicides strongly inhibit *Mesorhizobium* strains.

In the absence of fungicide and insecticide, the phosphate solubilization index was determined to be 3.21 which was reduced to 2.53 under 3X chlorpyriphos treatment and 2.90 under 3X carbendazim treatments (Table 3). The lowest IAA production (17.8 µg mL^{-1}) was observed on 3X chlorpyriphos treatment. HCN production was also detected in the presence of both insecticide and fungicide (Fig. 1). Shahid *et al*. (2019) reported a reduction of phosphate solubilising activity in *Azotobacter vinelandii* in the presence of different pesticides. Kulandaivel and Nagarajan (2014) reported that at 1.25% of endosulfan concentration, *Azospirillum* sp. and *Pseudomonas* sp. was able to produce IAA whereas *Klebsiella* sp. was not able to produce IAA. Hydrogen cyanide is volatile compound secret by microorganism which indirectly minimizes phytopathogen attack. Khan *et al*. (2020) reported HCN production in bacteria even at high concentration of pesticides. HCN is also effective in controlling weeds (Sivasakthi *et al*., 2014). Microorganisms that produce HCN can be used as biological agents to control pathogens (Al-Enazi *et al*., 2022). Thus, in treatment of *R. aegyptiacum* with bavistin (50% carbendazim) and the insecticide chlorpyriphos 20 EC at the recommended dose in chickpea, normal growth, phosphate solubilisation, IAA and HCN production was not affected, however, at higher concentrations, adverse effects were observed.

Conclusion

In arid regions, microorganisms residing in leguminous crops are considered as remarkable bio-inoculant for improving plant development under adverse environmental conditions. In the present study, *R. aegyptiacum* exhibited normal growth, phosphate solubilisation, IAA and HCN production when grown in the presence of the recommended dose of Bavistin and the insecticide Chlorpyriphos 20 EC. As such, the findings of the study will help in formulation of bio-fertiliser with *R. aegyptiacum* to be used in chickpea cultivation in agricultural fields contaminated with high concentrations of insecticides and pesticides.

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Table 1: Dosage and equivalent concentrations of bavistin and chlorpyriphos 20 EC used in the study.

Agrochemicals	Mode of application	Recommended dose concentration at field level (a.i. kg^{-1} seeds)	Dosage	Equivalent concentration of agrochemicals
Bavistin (50% carbendazim)	Seed treatment	3 g	1X	3 µg mL^{-1}
		6 g	2X	6 µg mL^{-1}
		9 g	3X	9 µg mL^{-1}
Chlorpyriphos 20 EC	Seed treatment	10 mL	1X	1.8 µL mL^{-1}
		20 mL	2X	3.6 µL mL^{-1}
		30 mL	3X	5.4 µL mL^{-1}

*a.i. = active ingredient

Table 2: Inhibition of growth after 48h of bavistin and chlorpyriphos treatment separately in *R. aegyptiacum.*

Agrochemicals	Dose	Growth inhibition (%)*
Control	-	-
Bavistin- 50%	1X	4.5
Carbendazim	2X	10.5
	3X	33.6
Chlorpyriphos 20 EC	1X	10.6
	2X	21.7
	3X	50.1

*Significant at $P \leq 0.05$, $LSD_{0.05} = 8.63$

Table 3: Phosphate solubilisation and IAA production of *R. aegyptiacum* under 3X recommended dose of bavistin and chlorpyriphos.

Agrochemicals	Phosphate solubilization index	IAA Production (µg mL^{-1})
Control	3.21± 0	28.7±0.1
Bavistin (50% carbendazim)	2.90± 0.15	20.65±0.25
Chlorpyriphos 20 EC	2.53± 0.1	17.8±0.3
LSD	1.68	3.29

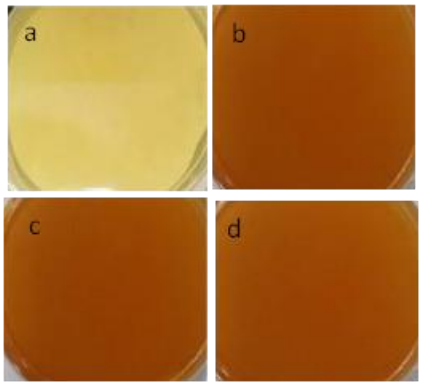

Fig. 1: HCN production by *R. aegyptiacum* control group: **a:** uninoculated, **b:** untreated-inoculated; treated group **c:** inoculated-treated with Bavistin; **d:** inoculated-treated with chlorpyriphos.

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