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RESEARCH ARTICLE

Impact of Chlorpyrifos on Plant Growth Promoting Rhizobacteria Isolated from *Abelmoschus esculentus*

Preeti Swarupa and Anil Kumar*

Department of Life Sciences, School of Natural Sciences, Central University of Jharkhand, Brambe, Ranchi - 835 205, Jharkhand, India.

Abstract

Chlorpyrifos (CP), an organophosphate insecticide, is massively used in agriculture for protecting cereal and vegetable crops from various types of pests. Its persistence and toxic nature towards non-target organisms has ensued in widespread contamination causing noxious effect on terrestrial and aquatic living entities including humans. Change in soil microflora biodiversity due to excessive CP use is a major concern nowadays as it will affect soil fertility ultimately leading to detrimental effect on plant health and yield. In the present study impact of CP on PGPRs from Okra (*Abelmoschus esculentus*) plant has been tested. Three bacterial isolates O-1, O-2 and O-3 were isolated, all showing either or combinations of selected plant growth promoting characters such as nitrogen fixation, phosphate solubilisation, IAA production and siderophore production. O-2 isolate was showing all the four selected plant growth promoting traits. Effects of CP (RADAR 20® EC) on growth of three isolates were checked on Luria Bertani (LB) agar and M9 minimal medium (M9MM) salts supplemented with CP (50, 500 and 5000 mg/L). O-1 and O-2 grew on LB agar and M9MM agar media supplemented with 50, 500 and 5000 mg/L CP revealing their tolerance to CP. O-2 isolate after repeated field trial and biodegradation study, can prove as potential candidate for PGPR possessing potential of bioremediation of pesticide (chlorpyrifos) contamination as well.

Keywords: Chlorpyrifos, organophosphate, microflora, PGPR, tolerance.

***Correspondence:** anil.kumar@cuj.ac.in; kumaranilvp@rediffmail.com; +91-9955273226

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INTRODUCTION

Soil is teeming with diverse group of microorganisms for example bacteria, fungi, actinomycetes, algae, nematodes etc. Among the various categories of microorganism, bacteria plays promising role by influencing different biotic factors of soil ecosystem like taking part in nutrient recycling and enhancement of plant growth and yield, formation of soil structure, bioremediation and biodegradation of toxic xenobiotics and heavy metals[1,2,3,4,5,6]**.** Plant growth promoting rhizobacteria (PGPR) hold a major position because these belongs to the group of root colonizing beneficial bacteria which enhances plant growth through various mechanisms such as acquisition and solubilisation of unavailable nutrients like nitrogen, phosphates, iron and production of plant hormones like IAA, gibberallins and cytokinins and acting as biocontrol agents and replaces explicit use of fertilizers and pesticides[7,8,9]. In addition farmers are obligated to use chemical means in the form of fertilizers, pesticides, herbicide etc. to increase the agricultural productivity to fulfil economic need and food security[10,11]**.** Pesticides (insecticide, nematicide, herbicide, fungicide) are a group of chemical compounds which are applied in agriculture fields to combat damage of crops, vegetables and fruit plants from pests. 80% of pesticides are used in the form of insecticide in India[12]. Injudicious use of pesticide has created turbulences in ecological balance of hydrosphere and lithosphere. Chlorpyrifos (CP) is a type of insecticide, acaricide and nematicide belonging to the class of organophosphate which is chemically O,O-diethyl O-(3,5,6-trichloro-2-pyridyl phosphorothioate). Dow chemicals, USA first introduced CP in 1965 for controlling insect pests in agricultural and domestic practises[13]. Commercially, it is available as Dursban, Lorsban, Agromil, Dorson, Dhanwan, Omexan across the world. CP has a wide application against an array of insect pests of economically valued crops. Its extravagant use and neurotoxic as well as immunotoxic nature has created a number of unavoidable and inevitable contamination of aquatic and terrestrial ecosystem causing serious threats to non-targeted organisms[14,15,16,17]. CP usage disturbs microflora of soil[18,19,20]**.** Loss of microbial diversity in soil ultimately leads to loss in soil fertility which is detrimental to plant growth and productivity.

In view of above problem, the present study has investigated the isolation of PGPR which may enhance not only the plant growth but also tolerate or degrade superfluous pesticide CP.

MATERIALS AND METHODS

Sample collection and isolation of bacteria

Standing plant of Okra (35 days old, days were counted from planting of nursery plant into well puddled arable crop field) was aseptically collected on 25/08/2015 from agricultural field of Brambe, Ranchi, Jharkhand, India and brought to laboratory for sample processing and isolation of bacteria. Bacteria were isolated and purified in LB agar (HiMedia Laboratories Pvt. Ltd., India) media using standard pure culture techniques such as serial dilution, spread plate and streak plate[21]. Bacteria were assigned isolate names with an initial alphabet of source plant followed by Arabic numeral on the basis of colony, form, margin, colour, size, opacity, elevation and texture.

Morphological and biochemical characterization of bacterial isolates

The morphological and biochemical characterization of all the bacterial isolates were done by Gram staining, bacterial motility test, catalase test, citrate utilization test and Methyl Red-Voges Proskauer (MR-VP) test and nitrate reduction test[22].

In-vitro screening of plant growth promoting characters

Isolated bacteria were grown overnight in 10 ml LB broth (HiMedia Laboratories Pvt. Ltd., India) in culture tube. Cells were harvested by centrifugation (Mikro 200R, hettich, Zentrifugen, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) at 10,000 rpm for 5 minutes at room temperature. Cells were washed in 1 ml normal saline and centrifuged at 10,000 rpm for 5 minutes. Cells were re-suspended in normal saline up to optical density (OD)-1 at $\lambda_{600.}$ These cells were used for test of nitrogen fixation, phosphate solubilisation, indole acetic acid production and siderophore production.

Nitrogen fixation test

Nitrogen fixation by bacteria was tested by their ability to grow on nitrogen deficient

(except atmospheric N_2) nutrient media-JNFb (malic acid- 5g/L, K_2HPO_4- 0.6 g/L, KH_2PO_4- 1.8 g/L, $MgSO_4.7H_2O$- 0.2 g/L, NaCl- 0.1 g/L, $CaCl_2.H_2O$- 0.2 g/L, 1.4% Fe-EDTA- 4.0 ml/L, $Na_2MoO_4.2H_2O$- 0.002 g/L, bromothymol blue- 5 ml (0.5% in 0.2 N KOH)/L, and KOH- 4.5 g/L, pH 5.8) and their ability to change pH (as indication of ammonia production) of media[23]. 5 µl of each culture, suspended in normal saline, was drop inoculated on JNFb agar plates and incubated (Orbital Incubator Shaker, Optics Technology, Delhi, India) at 37°C for 48 hours. Growth in nitrogen deficient medium followed by change in colour of medium from light-yellow to blue was recorded as positive indication of nitrogen fixation.

Phosphate solubilisation

Phosphate solubilisation character of bacteria was tested in Pikovskaya's medium (HiMedia Laboratories Pvt. Ltd., India). 5 µl of each culture, suspended in normal saline, was inoculated on Pikovskayas agar medium followed by inoculum drying and incubation at 37°C for 48 hours. Formation of clear halo zone around the colony was observed and regarded as phosphate solubilization potential of respective bacteria.

Indole acetic acid production

Production of indole acetic acid (IAA) by bacteria was tested by method adopted from Gordon and Weber 1951[24]. Bacterial cultures were inoculated in JNFb broth supplemented with ammonium chloride (NH_4Cl) as nitrogen source in two sets with and without tryptophan (0.1 mg/ml). Cultures were incubated at 37°C for 108 hours. 2 ml samples, from each tube, were harvested aseptically at 36, 72 and 108 hours following inoculation. Samples were centrifuged at 8000 rpm. 2.0 ml of modified Salkowski reagent (2% of 0.5 M $FeCl_3$ in 35% $HClO_4$ solution) was added to 1.0 ml of culture supernatants and incubated at room temperature for 25 minutes. Development of pink colour was regarded as positive test for IAA.

Siderophore assay

Siderophore production by bacteria was done on CAS (Chrome Azurol S) agar plate as per the method of Schwyne and Neilands1987[25]. CAS agar plate was prepared by mixing 50 ml of Chrome Azurol S (60.5 mg/50 ml in water), 40 ml of Hexadecylmethyl ammonium bromide (72.9 mg/40 ml in water) and 10 ml $FeCl_3.6H_20$ (1mM in 10mM HCl). The volume of this final solution was made to 1000 ml by adding King's Medium B Base (HiMedia Laboratories Pvt. Ltd., India). 5 µl of each culture, suspended in phosphate buffer, was drop inoculated on CAS agar plate and incubated at 37°C for 72 hours. Formation of orange halozone around the bacterial colony was regarded as positive for siderophore production test of bacterial isolates.

Screening of impact of chlorpyrifos (RADAR 20® EC) on PGPR isolates

Commercial grade CP (RADAR 20® EC) was bought from Dharti Dhan, Lalpur, Ranchi, Jharkhand, India and used in this study. Concentration of chlorpyrifos in RADAR 20® EC was calculated as 192.3 mg/L and considered as stock solution which was sterilized by membrane filtration (0.45 mm) as per the method adopted from Chen *et al.,* 2012[26]. Effect of CP (RADAR 20® EC) on growth of bacterial isolates were checked on LB agar and M9 minimal medium (M9MM) salts (HiMedia Laboratories Pvt. Ltd., India) supplemented with three concentration (50, 500 and 5000 mg/L) of CP (RADAR 20® EC). Isolates were grown in 10 ml LB broth and incubated overnight. Cultures were centrifuged and washed twice with M9MM broth. 5µl of inoculum, OD-1 at λ_{600} was taken and spot inoculated on LB+CP (50 mg/L), LB+CP (500 mg/L), LB+CP (5000 mg/L) and on M9MM+CP (50 mg/L), M9MM+CP (500 mg/L), M9MM+CP (5000 mg/L) alongwith control plate of LB agar and M9MM prepared without chlorpyrifos. Observation was noted after 72 hours of incubation.

Growth of bacterial isolate on analytical standard chlorpyrifos PESTANAL®

As per the method adopted from Chen *et al.,* 2012[26], stock solution of CP PESTANAL®, analytical standard (Sigma Aldrich, USA) of 100 g/L was prepared in methanol and sterilized by membrane filteration (0.45 mm). Bacterial isolate was grown in 10 ml LB broth and incubated overnight. Cultures were centrifuged and washed twice with M9MM broth. 5 µl of inoculum, OD-1 at λ_{600} was spot inoculated on M9MM agar media plates supplemented with three different concentration of CP PESTANAL®, analytical standard i.e. 50 mg/L, 500 mg/L and 1000 mg/L along with control plate prepared without CP. 10 µl of washed culture was inoculated in M9MM broth with and without CP of 50 mg/L concentration and growth was measured qualitatively (turbidity) and

quantitatively by OD at λ_{600}.

Growth response of bacterial isolates on different concentrations of salt

LB agar was reconstituted in different concentration (0%, 1%, 2%, 3%, 4%, 5%, 6%, 7% and 8%) of sodium chloride (NaCl). Bacterial isolate O-2 was inoculated in 10 ml LB broth and incubated overnight at 37°C with shaking at 150 RPM. Cultures were washed twice with LB broth (without NaCl) and suspended in LB broth (without NaCl) at culture OD-1 at λ_{600}. 5 µl of bacterial culture was inoculated in reconstituted LB agar plates of different concentration of NaCl. Plates were incubated at 37°C and growth was recorded at 24 hours of incubation.

Growth response of bacterial isolates at different pH

LB agar was reconstituted in sodium phosphate buffer of different pH range 5.8, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0[27]. O-2 isolate was inoculated in 10 ml LB broth and incubated overnight at 37°C with shaking at 150 rpm. Culture was washed twice and resuspended in normal saline maintaining OD-1 at λ_{600}. 5 µl of culture was drop inoculated in reconstituted LB agar plates of different pH. Plates were incubated at 37°C and growth was recorded at 24 hours of incubation.

RESULTS

Three bacterial isolates were purified from root surface of Okra plant and were named O-1, O-2 and O-3 based on colony morphology. Isolates were further confirmed and characterized on the basis of Gram's staining, motility in semisolid LB agar medium, catalase, citrate utilization, nitrate reduction and MRVP tests (Table 1).

Three isolates, subjected to test, showed

Table 1. Colony morphology and biochemical characters of isolated bacteria

Isolates	Colony characteristics							Biochemical tests						
	Form	Margin	Colour	Elevation	Surface	Opacity	Texture	Catalase	NR	Motility	CU	GR	Cell shape	MR-VP
O-1	C	E	D	CX	S&G	OP	B	+	-	NM	+	+	SR	$MR^{+}VP^{-}$
O-2	C	E	O	RD	S&G	TL	B	+	-	NM	+	-	SR	$MR^{+}VP^{-}$
O-3	I	F	O	FT	S&G	TL	B	+	-	M	+	+	MR	$MR^{-}VP^{+}$

(+) = positive results, (-) = negative results, C-Circular, I-Irregular, E-Entire, F-Filiform, D- Dirty yellow, O- Off white, CX-Convex, RD- Raised, FT- Flat, S&G- Smooth and glistening, OP-Opaque, TL-Translucent, B- Butyrous, NM-Non-motile, M-Motile, SR-Short rods, MR- Medium Rods NR-Nitrate reduction test, GR-Gram reaction CU-Citrate utilization test, MR -VP- Methyl red -Voges Proskauer test

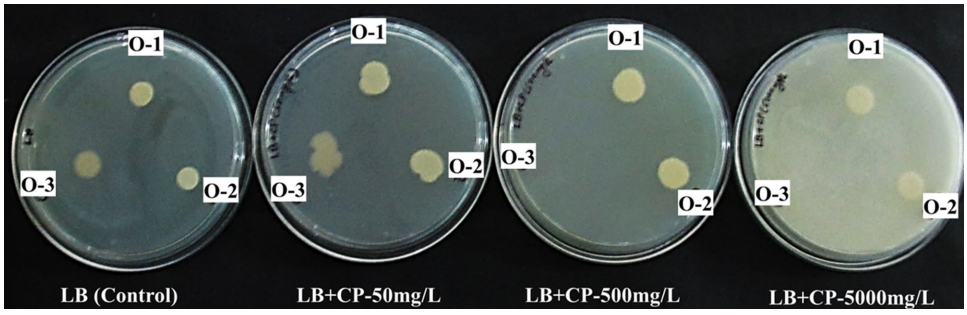

Fig. 1. Growth of O-1, O-2 and O-3 on LB agar media supplemented with different concentration of CP along with controll without CP

different plant growth promoting activity such as nitrogen fixation, phosphate solubilization, IAA production and siderophore production (Table 2). One isolate i.e. O-2 was found to show all the four properties of plant growth promotion i.e. nitrogen fixation, phosphate solubilization, IAA production and siderophore production.

Growth of all the three isolates i.e. O-1, O-2 and O-3 was observed on LB agar supplemented with different concentration of CP i.e. 50, 500, 5000 mg/L (Figure 1) except O-3 isolate which showed growth only on 50 mg/L (Fig. 1, Table 3).

O-1 and O-2 grew on the given concentration (50, 500, 5000 mg/L) of CP in M9MM agar plates but O-3 did not grow on any of the M9MM agar plates containing CP (Figure 2, Table 3).

Table 2. Result for *in-vitro* plant growth promoting activity

Isolates	Tests			
	Phosphate solubilization	Nitrogen Fixation	Siderophore production	IAA production
O-1	PS^+	NF^+	SP^-	IP^+
O-2	PS^+	NF^+	SP^+	IP^+
O-3	PS^-	NF^+	SP^-	IP^-

PS^+- Phosphate solubilization positive, PS^--Phosphate solubilization negative, NF^+-Nitrogenfixation positive, SP^+-Siderophore production positive, SP^--Siderophore production negative, IP^+-IAA production positive, IP^--IAA production negative

Table 3. Bacterial growth on LB agar and M9MM agar media having different concentration of CP following 72 hours of incubation

Isolates	LB	M9MM	LB+CP (50 mg/L)	M9MM+CP (50 mg/L)	LB+CP (500 mg/L)	M9MM+CP (500 mg/L)	LB+CP (5000 mg/L)	M9MM+CP (5000 mg/L)
O-1	+++	+	+++	+	+++	+	+++	+
O-2	+++	+	+++	+	+++	+	+++	+
O-3	+++	-	+++	-	-	-	-	-

(+) = presence of growth, (-) = no growth

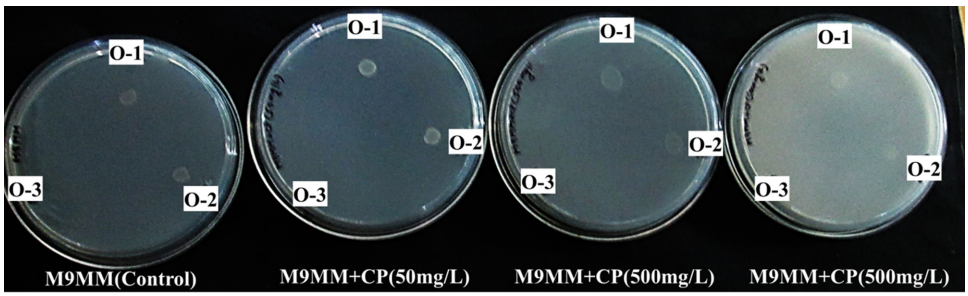

Fig. 2. Growth of O-1, O-2 and O-3 on M9MM agar media supplemented with different concentration of CP along with controll without CP

O-2 was selected for study its growth in CP PESTANAL®, analytical standard as it was exhibiting all the selected plant growth promoting characters. Growth of O-2 isolate was detected on three different concentration of CP PESTANAL®, analytical standard i.e. 50 mg/L, 500 mg/L and 1000 mg/L. Growth of O-2 isolate in M9MM broth with and without CP reveals there was more turbidity i.e. growth observed in M9MM broth containing CP as compared to M9MM broth without CP which is also apparent from OD (Table 4).

O-2 isolate were found to grow in a pH range of 6.6-8.0 and salinity 0.0-6% NaCl though the growth was slightly inhibited at pH below 6.6 and higher salt concentration beyond 6% NaCl (Figure 3).

Table 4. Growth of O-2 isolate on M9MM broth supplemented with and without CP PESTANAL® analytical standard

DAY	SET	Control	Visual turbidity of isolate	OD of isolates[a,b]
Day 7	SET A	-	++	0.101
	SET B	-	+++	0.144

SET A - M9MM, SET B -M9MM+CP (50 mg/L), (+) = presence of growth, (-) = no growth, control - without inoculation. [a] OD was recorded following 7 days of incubation. [b]water was taken as blank, OD in the table corresponds to corrected OD value (subtracting OD of control from all sample and control OD values). Value of control over blank (water) is 0.001 of control SET A and 0.007 of control SET B.

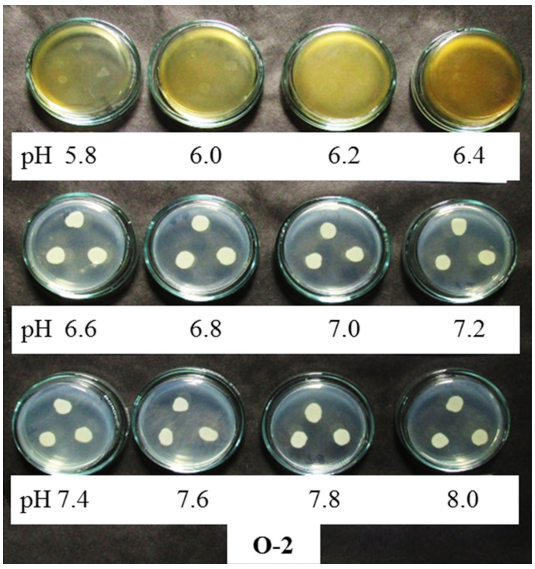

Fig. 3. Growth of O-2 at different pH

DISCUSSION

Reclamation of pesticide contamination through the use of biodegradation ability of microorganisms is crucial and indispensible approach for environmental clean-up in current scenario. Use of PGPR strains for biodegradation of pesticide will be one of the greatest strategies to be adopted not only for decontamination of pesticide but also restricting the use of fertilizers which is vastly used for plant growth. In the present study, three different bacterial isolates were isolated from root surface of *A. esculentus* and found to be having one or more of the selected plant growth promoting characters i.e. nitrogen fixation, phosphate solubilisation, IAA production and siderophore production. Occurrence of huge number of plant growth promoting bacteria around the root surface is due to the presence of exudates of plant roots which helps to flourish many different types bacteria which in exchange helps in plant growth promotion[28]. So, reports of many researchers elucidates the isolation and characterization of PGPR from rhizoplane and rhizosphere of plants[29,30,31,32,33,34].

Walia *et al.,* 2018[18] have reported inhibitory impact of CP (1000 mg/L) on plant growth promoting bacteria (PGPB) which certainly reduce the crop yield by preventing PGPB action. Growth experiments conducted on LB media and M9MM media supplemented with different concentration of CP (RADAR 20® EC) shows that O-1, O-2 can tolerate high concentration of CP i.e. upto 5000 mg/L which is validated from similar reports of other researchers[35,36,37,38,39] where they

have reported PGPR tolerance to various pesticides upto 600 mg/L. Verma *et al.,* 2016[40] have also reported CP tolerance of isolated PGPR, but low as compared to the current study.

O-2 was selected to study its growth on analytical grade of CP as it was exhibiting all the four selected plant growth promoting traits and was able to tolerate and grow at highest concentration of CP RADAR®20 EC (5000 mg/L) in LB agar media and minimal media.O-2 isolate showed presence of growth at all the given concentration (50 mg/L, 500 mg/L and 1000 mg/L) of analytical grade CP supplemented in M9MM agar media. Intensity of growth of O-2 isolate was also found to be more in M9MM broth containing (50 mg/L) CP, analytical grade as compared to M9MM broth without chlorpyrifos. The result shows that O-2 isolate may be utilizing CP as their carbon source and will help in biodegradation of CP but further biodegradation study through technique such as high performance liquid chromatography (HPLC), gas chromatography-mass spectrophotometry (GC-MS), gel permeation chromatography (GPC) is needed to be done to prove its biodegradation potential as done by many researchers in their study[41,42,26,19].

Growth of O-2 isolate on higher pH i.e. upto pH 8.0 is supplementary and favourable factor which will help in CP degradation as found in the study of Singh *et al.,*2003[43] who found enhanced rate of biodegradation of CP in higher pH. The capacity of O-2 isolate to survive and grow in the presence of high concentration of the commercial and analytical grade chlorpyrifos shows that the isolate after field trials and biodegradation study may be used as promising candidates for raising the productivity of plants even in the pesticide contaminated soils.

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