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IN VITRO INVESTIGATIONS TO EXPLORE THE TOXICITY OF FUNGICIDES FOR PLANT GROWTH PROMOTING RHIZOBACTERIA

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Abstract

Various pesticides are used to protect different crops from pest attack but very little is known about the damaging effects of pesticide application on non-target, beneficial soil microflora. Present study was designed to explore the effect of fungicides on Plant Growth Promoting Rhizobacteria (PGPR) isolated from rhizosphere of wheat and other cereals. Different PGPR strains were tested *In vitro* to check their resistance against commercial formulation of diverse fungicides, at dose recommended for field application. Results were taken on the basis of the number of viable bacterial cells through plate count, fungicide resistance pattern through disc diffusion method. Comet assay was used for the first time, to assess the genotoxicity of fungicides for PGPR. Results indicate that fungicides viz., Alert Plus, Darosal and Mancozeb do have the inhibitory effect while Benlate have stimulatory effect on growth though the effect varies from strain to strain. No tested fungicides cause the genotoxicity.

Introduction

The worldwide impel for sustainable agriculture systems involve optimizing agriculture resources to gratify human needs and simultaneously maintaining the quality of the environment and uphold natural resources. Pakistan being a developing country is facing constraints in agriculture such as low land area and immense use of expensive chemicals that not only increase the input cost but disturb the ecosystem as well. In an agriculture dependent country such constraints diminish crop yield that is unbearable for the low-income farmers. Due to all these factors the production of crop is inadequate to stay pace with increasing population. Biofertilizers or bacterial inoculants have been proved to be an effective alternate and economical substitute of chemical fertilizers to reduce the cost of production as well as to avoid the environmental hazards (Hafeez *et al.*, 2002).

Pesticide application is still the most effective and accepted means for the protection of plants from pest (Bolognesi, 2003). But the extensive use of pesticides over the past four decades has resulted in tribulations caused by the interaction with natural biological system (Ayansina & Oso, 2006). Fungicide seed treatment is frequently used to improve early plant emergence and to control the early attack by the pests. This strategy is familiar as useful in reducing fatalities from seed borne pathogens and seedling damping off agents (Phipps, 1984; Sinclair & Backman, 1989). These fungicides may harmfully effect the non-target soil microflora (Ayansina & Oso, 2006), specially when these fungicides are used in conjunction with microbial inoculants they cause damage to the inoculants by

affecting the bacterial infection to the root hair, nodule formation and bacterial growth hormone production. This important issue attracted a negligible amount of research in the past and not much more in recent years. The majority of work has been done under *in vitro* conditions (Ingham & Coleman, 1984; Bashan & Holguin, 1997) for leguminous crops on the basis of biochemical (Revellin *et al.,* 1993), as well as molecular technologies (Sigler, 1999; Sigler & Turco, 2002; Crecchio *et al.,* 2001).

The present study was designed to evaluate the adverse effect of fungicides, on growth survival and resistance of root associated PGPR under laboratory conditions using various toxicity experiments. The main aim of this study was to screen out fungicide tolerant strains for the improvement of biofertilizer for sustainable agriculture.

Materials and methods

Bacterial strains: Different PGPR strains, isolated from root and rhizosphere of cereal crops (Table 1) were obtained from the Biofertilizer Resource Center (BIRCEN) culture collection of National Institute for Biotechnology and Genetic Engineering (NIBGE) and maintained on yeast extract mannitol (YEM) and/or Lauria Bertani (LB) agar plates at 28 ± 2ºC for 2-3 days. Among these *Azospirillum* strains C-6, ER-20, *Pseudomonas* strains 96-51, Z2-7, K-1 and *Agrobacterium* strain Ca-18 are used in inoculum production for commercial wheat biofertilizer (*BioPower*) by the institute.

Table 1. Percent change in log. No. of PGPR colonies in LB* medium supplemented with different fungicides over the control as indicated by viable plate count.

S. No.	Bacterial Strains	Log No. Of Colonies (% Change)									
		Control	Vitavax		Darosal		Captan		Benlate		
1.	F2	9.20	8.12(12)	↓	7.10 (23)	↓	9.64 (05)	↑	8.64 (06)	↓	
2.	F3	8.20	8.50(04)	↑	8.10 (0.6)	↓	0.00 (100)	↓	9.10 (11)	↑	
3.	F4	9.10	7.80(14)	↓	9.60 (5.5)	↑	7.90 (14)	↓	9.11 (00)	=	
4.	F8	9.53	8.50(11)	↓	9.46 (01)	↓	9.53 (00)	=	9.53 (00)	=	
5.	F9	9.48	0.00 (100)	↓	8.20 (14)	↓	0.00 (100)	↓	8.66 (09)	↓	
6.	F13	9.60	1.00 (100)	↓	9.34 (03)	↓	7.40 (23)	↓	0.00 (100)	↓	
7.	F14	9.16	0.00 (100)	↓	6.83 (26)	↓	5.12 (44)	↓	0.00 (100)	↓	
8.	Wb3	9.65	5.95 (38)	↓	8.95 (07)	↓	0.00 (100)	↓	7.40 (24)	↓	
9.	96-51	9.69	0.00 (100)	↓	9.26 (05)	↓	0.00 (100)	↓	9.70 (0.1)	↑	
10.	K-1	8.00	0.00 (100)	↓	9.62 (21)	↑	0.00 (100)	↓	9.59 (20)	↑	
11.	KY1	8.00	8.34 (04)	↑	7.64 (05)	↓	6.64 (17)	↓	6.60 (16)	↓	
12.	N-4	9.35	9.79 (05)	↑	9.35 (00)	=	9.35 (00)	=	8.62 (08)	↓	
13.	ER-20	8.39	8.34 (0.6)	↓	8.34 (01)	↓	0.00 (100)	↓	8.38 (00)	=	
14.	BtJ # 8	7.83	7.77 (0.8)	↓	7.79 (01)	↓	8.75 (12)	↑	0.00 (100)	↓	
15.	BtJ # 16	9.46	9.89 (05)	↑	9.46 (00)	=	9.60 (02)	↑	9.49 (0.5)	↑	
16.	BtJ # 18	9.59	9.38 (02)	↓	0.0 (100)	↓	9.57(0.2)	↓	9.69 (1.1)	↑	
17.	5.1-A	9.69	9.83 (02)	↑	9.82 (02)	↑	9.34 (04)	↓	9.34 (04)	↓	
18.	Ca-18	9.64	9.64 (00)	=	9.34 (03)	↓	9.51 (1.4)	↓	9.92 (03)	↑	
19.	JCM-1270	9.34	9.12 (02)	↓	9.23 (02)	↓	6.65 (29)	↓	0.00 (100)	↓	

• *LB medium for PGPR strains except Ca-18 that was grown on YEM medium
• Values are average of four replicates
• Values outside the bracket indicate Log No of colonies
• Values in bracket showed: ( )% Decrease; ( ) % Increase and (=) No change, over control.

Fungicides: The fungicides Alert Plus, Benlate, Captan, Darosal, Mancozeb, and Vitavax, commonly used for wheat crop, were purchased from a local agriculture dealership store. Fungicides were used as recommended to the farmer by the manufacturers.

Bacterial enumeration through viable plate count: Purified PGPR strains were grown at 28 ± 2ºC for 2-3 days at 100 rpm in LB broth except strain Ca-18 (grown in YEM broth). Serial dilutions of individual broth culture were prepared (Somasegaran & Hoben, 1985) by taking 1 mL of each culture in 9mL of sterile saline (0.89% NaCl). Tenfold serial dilution was prepared from each of these suspensions up to eight levels (10^{-1}- 10^{-8}). Three maximum dilution levels i.e. 10^{-6}- 10^{-8} were taken for plate count by using Miles and Misra drop plate method (Somasegaran & Hoben, 1994). A 20µL drop from each dilution (in triplicate) was transferred aseptically to LB and YEM agar plates supplemented with 2 g/L of fungicides in triplicate. Plates without fungicides were used as control. After inoculation, plates were incubated at 28 ± 2º C for 48 hours. After incubation the number of bacterial colonies was recorded with the help of colony counter. The number of viable cells per mL was calculated by using the following formula:

No. of viable cells/mL = (number of colonies) x (dilution factor) x (vol. of inoculum) (Somasegaran & Hoben, 1994).

Fungicide resistance pattern-Disc diffusion method: PGPR strains were checked for their resistance towards different fungicides through disc diffusion method (Martensson, 1992). Stock solutions of test fungicide were prepared by adding 2 g/L of commercial formulation of fungicides in distilled water and filter sterilized using 0.45 µm filter papers. Filter paper discs (5 mm diameter) were prepared by adding four different concentrations of these stock solutions (equivalent to 1, 2, 4 and 6 g/Kg seeds) to evaluate the concentrations equivalent to, above and below the recommended dose of test fungicides. A 100 µL of individual bacterial culture (10^7 cells/mL harvested at early logarithmic growth) was spread on LB agar plates with sterile glass spreader aseptically. After drying these discs were placed at equidistance on the agar surface in duplicate. The plates were incubated for 2-3 days at 28 ± 2º C, and the diameter of the inhibition zones was measured in mm.

Bacterial genotoxicity test through comet assay: Comet assay technique or micro gel electrophoresis was used to study the DNA double strand breaks in single PGPR DNA molecule under the influence of fungicides. Gel preparation, electrophoresis conditions and procedures were performed as described by Singh *et al.* (1999). PGPR strains were grown in 50 mL LB and or YEM broth supplemented with recommended doses of test fungicides; Alert Plus, Benlate, Darosal and Mancozeb @ 2 g/L at 37 ± 2 ºC overnight with constant shaking. PGPR strains grown without fungicides were used as respective negative control. Cells were harvested by centrifugation, from exponentially grown cultures (10^8 cell/mL).

Frosted or plain glass slides were pre-coated with 100µL of 0.5% agarose (1:3 high resolution agarose, Amresco, Solon, OH) in normal saline. First layer coating was made on slides using a 24 x 50 mm cover glass. Coated gel slides were air-dried at room temperature before use. 500µL of each exponentially growing bacterial culture

(containing approximately $2x10^8$ cell/mL) was mixed with 500 µL of 0.5% agarose (in normal saline) maintained at 45ºC. A micro gel was then prepared using 24 x 50 mm cover glass. Slides were placed on ice cold steel tray for rapid solidification of agarose. The cover glass were removed and a third layer of 200 µL 0.5% agarose having 5 µg/mL, RNAse A, 0.25% sodium N-lauroyel sarcosine and 0.5 mg/mL lysozyme was made using a cover glass. A high concentration of lysozyme was used for the complete lyses of bacterial cell wall, rich in peptidoglycane-lipopolysaccharide. Each slide contained approximately 200,000 cells. The slides were then incubated at 3ºC for 10 minutes (Leitao & Carvalho, 1988) and placed in a humid chamber at 37 ± 2 ºC for 30 min. Cover glass were removed and slides were immersed in lysing solution for 1 hour and then transferred to a solution of enzyme digestion and incubated at 37 ±2 ºC for 2h.

Electrophoresis: The slides were placed on horizontal slab of an electrophoresis unit and microgels were equilibrated with 300 mM sodium acetate and 100 mM tris (pH 9) for 20 minutes and allowed to run at 12 V (0.4 V/cm, approximately 100mA) for 1h. After electrophoresis, slides were fixed by immersing in 1 M ammonium acetate in ethanol (5 mL of 10 M ammonium acetate and 45 mL of absolute ethanol) for half an hour and then in absolute ethanol for an hour. The slides were air dried at room temperature and then immersed in 70% ethanol for 15 minutes and again air-dried. Slides were pretreated with 50 µL of a freshly prepared solution of 5% Dimethyl sulfoxide (DMSO) and 10 mL NaH_2PO_4, and stained with 50 µL of ethidium bromide for half an hour. Observations were made using fluorescent microscope Lieca equipped with (40X and 100X oil immersion objectives) having FITC filter combination. For the visual counting of breaks in individual bacterial DNA following criteria was adopted:

i. Any cell with observable migration was considered to have at least one DNA double strand break.
ii. Number of breaks was counted along the length of migrated or stretched DNA molecule, starting from original location of the cells in agarose.
iii. The presence of gap in the continuity of migrated molecule was considered a DNA double strand break..

Results and Discussions

Bacterial cell enumeration-viable plate count: Results obtained from enumeration of viable bacterial cells (Table 1) indicate the percent change in log number of bacterial colonies in the presence of fungicides. It was observed that most of the test fungicides reduced the bacterial counts as compared to control but growth stimulation was also observed in some cases. Fungicides negatively affected on *in vitro* bacterial growth in the following order: Benlate < Captan ≅ Darosal ≅ Vitavax, indicating that Benlate is less toxic at full-recommended dose. Data reveals that *Pseudomonas* strains F2, F9, F13, KY1, *Bacillus* strain F14, *Azospirillum* strains ER-20, Wb3, BtJ-8, BtJ-18, JCM-1270 and *Agrobacterium* strain Ca-18 are most sensitive to all test fungicides. *Bacillus* strains F3, is sensitive to Darosal, Captan, and resistant to Benlate and Vitavax while F4 is sensitive to Captan, Vitavax and resistant to Darosal and Benlate. *Azospirillum* strains BtJ-16 is most resistant and better survived in all fungicide supplemented medium, even its growth is stimulated in the presence of fungicides. Pell *et al.* (1998) in a similar study

reported that 15% out of 54 tested pesticides had inhibitory effects on bacterial activity in soil, whereas 11% had stimulatory effects. The present findings are supported by Gallori *et al.,* (1991), Revellin *et al.,* (1993), Taiwo & Oso (1997) and Dunfield *et al.,* (2000) who reported bacterial growth inhibition due to agrochemicals like Captan, Vitavax and Thiram in leguminous plants. The later two fungicides contain similar active ingredient (Tetramethylmethylthiuram disulphide) and all the three fungicides significantly reduced the number of rhizobia recovered from seeds as well as the nodulation and plant growth at high concentration. Direct application of fungicides can significantly reduce the growth but it will be offset by the presence of host plants. Study of Kel-Boahen *et al.,* (2001) regarding toxic effect of Captan on survival of *Rhizobium ciceri* and nodulation on chickpea plants also supports the present data. Captan decreased rhizobial viability and also reduced nodule dry weight. Zaharan (1999) also reported the deleterious effect of Captan and Vitavax on growth of root nodule bacteria in laboratory conditions. Similar trend was reported by Martensson (1992) who observed that fungicide treatment decreased the number of viable rhizobia on the seeds estimated by standard serial dilution and plate count techniques, whereas in some cases cell growth increased in the presence of fungicides due to different nutrient supplements in media that minimize the adverse effect of these chemicals. It was suggested that these fungicides triggered a growth stimulus when added to the medium at low concentrations. The results were also supported by Ingham & Coleman (1984) who tested different fungicide to see their effect on non target microorganisms and found that Fungizone was the most effective of the 4 fungicides tested in reducing active hyphae. Increased bacterial population was observed following fungal reductions.

Fungicide resistance pattern-Disc diffusion method: The paper disc method gave objective results and widely used to investigate the toxicity of chemical compounds (Martensson, 1992). The results regarding resistance pattern of PGPR strains against different commercially used fungicides showed that most of the PGPR strains were resistant towards the test fungicides (Table 2). Inhibition zone 2 mm diameter was observed around the filter paper discs impregnated with fungicide Mancozeb placed on bacterial lawn of *Pseudomonas* strains 96-51, Z2-7 and *Azospirillum lipoferum* strain ER-20. *Agrobacterium* strain Ca-18 showed clear inhibition zones against Mancozeb and Darosal on fungicide supplemented media plates. Control plate with filter paper discs without any fungicide showed no zone of inhibition for any strain. Castro *et al.,* (1997) reported that Mancozeb inhibited the rhizobial growth in pure culture. The data (Table- 2) indicates that most of the PGPR strains are showing resistance and can multiply around the discs impregnated with varying concentration of fungicides even against the concentration, which were recommended for field application. The factor responsible for this behavior may be the presence of agar in the medium because the presence of agar may influence the mode of action of the investigated compounds as described by Martensson (1992), regarding the effect of agrochemicals and heavy metals on fast growing rhizobia and their symbiosis with small seeded legumes. It was reported that some bacterial strains, were able to multiply at concentrations of agrochemicals equal to or higher than recommended field application rates. In his study, comparing three methods two agar-based technique gave similar results.

Table 2. Fungicide resistance pattern of PGPR strains used in wheat biofertilizer as studied by disc diffusion method.

Test fungicide	Conc. g/L	Resistant pattern of PGPR strains					
		96-51	Ca-18	K-1	C-6	Z2-7	ER-20
Alert Plus	1	+	+	+	+	+	+
	2*	+	+	+	+	+	+
	4	+	+	+	+	+	+
	6	+	+	+	+	+	+
Benlate	1	+	+	+	+	+	+
	2*	+	+	+	+	+	+
	4	+	+	+	+	+	+
	6	+	+	+	+	+	+
Mancozeb	1	+	--	+	+	--	+
	2*	--	--	+	+	--	--
	4	--	--	+	+	--	--
	6	--	--	+	+	--	--
Darosal	1	+	--	+	+	+	+
	2*	+	--	+	+	+	+
	4	+	--	+	+	+	+
	6	+	--	+	+	+	+
Control		+	+	+	+	+	+

-- ➔ Presence of zone of inhibition or bacteria are sensitive to test fungicide
+ ➔ No zone of inhibition or bacteria are resistant to test fungicide
* Recommended
• Values are average for four replicates

The paper disc method was used by Tu (1977) to test the acute toxicity of different pesticide against 17 *Rhizobium japonicum* cultures isolated from soybean nodules and reported that two insecticides viz., Lindane (gamma-1,2,3,4,5,6-hexachlorocyclohexane), Chlorpyrifos (O,O-diethyl, O-3,5,6-trichloro-2pyridyl phosphorothioate), and a fungicide, Thiram (tetramethylthiuram disulphide) individually or in combination caused significant inhibition of the growth of *R. japonicum* No. 16.

Bacterial genotoxicity studies-comet assay technique: Present study is the first report on the use of Comet assay technique for the visual counting of DNA double strand breaks in single electrostreched PGPR DNA molecule. Double-strand breaks may lead to chromosomal breaks lethal to bacterial cell (Singh *et al.,* 1999). It was reported that fungicides; Captan (Carbonell *et al.,* 1995), Mancozeb (Jablonika *et al.,* 1989: Pasiquini *et al.,* 1996), Zineb (Solonesky *et al.,* 2002), Lindane (Rita *et al.,* 1987) Benomyl (Benlate) (Carbonell *et al.,* 1995: Pasiquini *et al.,* 1996) are responsible for cytogenetic effect in human population after a prolong exposure. In case of prokaryote genotoxicity of Captan was reported (Ruiz & Marzin, 1997) for *Salmonella* using *In vitro* Ames test and SOS chromotest.

In present study regarding genotoxicity due to fungicides through comet assay no significant difference was observed under fluorescence microscope in slides with fungicide treatment as compared to control. No distinct comet like structure was observed in any slide reveals that there is no DNA double strand break in any PGPR strain by any of the fungicide treatment (Fig. 1). Though these results are not depicting the toxic effect

of fungicide as compared to the effect on viable count and resistant pattern experiments but it is clear that growth inhibition of PGPR strains is not due to the damage of bacterial DNA. Results of comet assay deduced that the test fungicides do not cause bacterial DNA damage. This might be due to the fact that the test fungicides do not belong to the chlorinated hydrocarbons, which cause significant DNA damage. Cytogenetic toxicity of Chlorinated pesticides; Alachlor (Garaj-Vrhovac & Zeljezic, 2001: Zeljezic & Garaj-Vrhovac, 2001), organochlorines (Nehnez *et al.,* 1981: Paldy *et al.,* 1987: Kourakis *et al.,* 1992), DDT, Dichlorv (Rita *et al.,* 1987) and other chlorinated hydrocarbons (Amr, 1999) was reported in human beings after long exposure. Short-term genotoxicity assay indicated that pesticide Chlorpyriphos is the causal agent of mutation (Wildemauve *et al.,* 1983) and DNA damage (Klopman, 1985) in prokaryotes. Genotoxicity of chlorinated toxaphene mixture for *Salmonella* strain was reported (Schrader *et al.,* 1998) and confirmed by Bartos *et al.,* (2005) in a dose-dependent toxicity study using the *umuC* test. Results from the present study conclusively demonstrated that the test compounds do not cause DNA damage in PGPR strains.

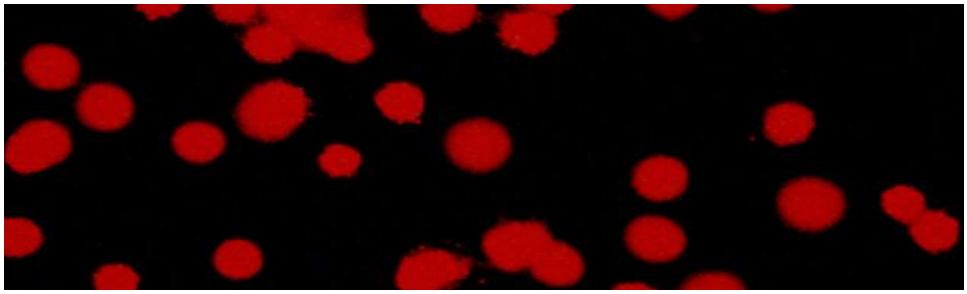

Fig.1. A photomicrograph showing DNA molecule from PGPR (cells treated with recommended dose of fungicides) lysed and electrophoresed for 1 h at 12 V in neutral condition and visualized using ethidium bromide dye. No DNA damage was observed by forming comet like structure.

Conclusion

It was inferred on the basis of viable counts and disc diffusion experiment that Darosal inhibits the growth of *Agrobacterium* strain Ca-18 and Mancozeb inhibits the growth of *Pseudomonas* and *Azospirillum* whereas all other strains were resistant to test fungicides. Comet assay clearly exhibited that none of the fungicide cause significant DNA damage. It can be concluded that different fungicides exert different effect on soil micro flora in standard laboratory experiments.

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