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Pesticide tolerant plant growth promoting rhizobacteria isolated from rhizosphere of okra

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Abstract

Pesticides are widely used in different parts of the world to control insect pests and to enhance crop production. However, these pesticides are found to have deleterious effects due to their non-degradable nature and residual effects on non-target organisms. Some microorganisms can develop resistance against these pesticides and use them as the carbon source. These microorganisms can successfully be used for bioremediation of pesticides contaminated sites in addition to their plant growth promoting attributes. Bifenthrin is the major pesticide used in vegetables especially okra, to control fruit borer. A study was conducted to isolate and characterize the pesticide-tolerant rhizobacteria from rhizosphere of okra (Abelmoschus esculentus L.). Total 20 bacterial strains were isolated from rhizosphere of okra plants where high dose of bifenthrin was applied. Pesticide tolerance of rhizobacterial strains was determined at three different levels (100, 250 and 500 mg L^{-1}) of bifenthrin. The results revealed that bacterial strains showed a variable response to increasing levels of bifenthrin and the growth of most of these strains decreased with increase in pesticide dose. The strain S10 showed the highest growth at 500 mg L^{-1} of bifenthrin followed by S20 and S6. These isolates were further characterized for plant growth promoting traits, such as Zn solubilization, urease and catalase activities, ammonia production, salinity stress tolerance, and growth at different pH levels. Out of 20 isolates, S1, S6, S8, S10, S13, S14 and S20 showed Zn solubilization ability while S3, S9, S12, S13, S14, S15, S19 and S20 were positive for urease activity. The isolates S6, S8, S14 and S20 showed ammonia production ability. All tested isolates showed the presence of catalase enzyme. The maximum root colonization ability was observed by S20. At highest salinity level, maximum cell growth was observed in the case of S20. All the rhizobacterial isolates showed maximum optical density at neutral (7), followed by alkaline pH (9). In general, S10 and S20 were better isolates and had the maximum pesticide tolerance along with other multifarious traits. These isolates may be evaluated for their ability to degrade bifenthrin pesticide and may have practical application for bioremediation of pesticide contaminated sites.

Keywords: Rhizobacteria, pesticide tolerance, stress, zinc solubilization, okra

Introduction

Okra, (*Abelmoschus esculentus* L.) is an important vegetable grown in the tropical and subtropical areas of the world. It is widely grown by the farmers, throughout Pakistan (Javed *et al.*, 2009). In Pakistan, its total cultivated area is 2.21×10^5 hectares with a production of 2.86×10^6 tons (Kashif *et al*., 2008; Anwar *et al.*, 2011). It is cultivated for its young edible pods which are consumed as a vegetable (Kashif *et al.*, 2008). It provides carbohydrates, proteins, vitamin C (Dilruba *et al*., 2009) vitamin A and B, phosphorus, iodine, minerals and salts to human diet (Khushk *et al*., 2003).

In modern agriculture, large number of pesticides are being used to improve crop production (Cycon *et al*., 2006) but their excessive and unreasonable use in okra causes stress and yield loss in addition to deterioration in soil health (Wagan *et al*., 2014). Bifenthrin is effective for control of insect pets of cotton (Ali and Karim, 1994), and vegetables (Gupta *et al.*, 2009). The LD_{50} for moderate toxicity of bifenthrin in human is > 50 – 500 mg kg^{-1}. Heavy use of pesticides reduces the fertility of soil as these accumulate in the environment (Mohammed, 2009). These chemicals have negative impact on microbial activities (Yousaf *et al*., 2013) and their high application negatively affects microbial population by reducing the activities of enzymes and cause stress in microbial communities (Muñoz-Leoe *et al*., 2013).

Some microorganisms develop resistance after a long-term exposure to agrochemicals and can successfully be used for bioremediation of pesticide contaminated soils (Khan *et al.*, 2009). Some microbes perform efficiently in the presence of specific pesticides by using them as a source of nutrients and energy (Qiu *et al*., 2009).

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Microorganisms degrade these pesticides and use them as a carbon source. Their ability to degrade pesticide is an important phenomenon through which these chemicals are eliminated from the environment and control the environmental pollution (Surekha *et al*., 2008).

These bacterial strains can be used in modern agriculture as inoculants under stress conditions such as heavy metal stress (Bhatt and Vyas, 2014), herbicide stress (Ahemad and Khan, 2011a), insecticides stress (Ahemad and Khan, 2011b) fungicides stress (Ahemad and Khan, 2012), and salinity stress (Tank and Saraf, 2010). Therefore, these microbes are essential component in recycling process and important to maintain soil fertility (Glick, 2012). These microbes also have other plant growth promoting traits in addition to pesticide degradation and can be used to enhance the remediation process (Shahgoli, 2014) in addition to plant growth promotion.

The use of pesticide tolerant plant growth promoting rhizobacteria may help to degrade pesticides which are being used injudiciously by the vegetable growers in the country. So, the present study was conducted to isolate and characterize the pesticide tolerant rhizobacteria for multiple plant growth promoting traits.

Materials and Methods

Isolation of pesticide tolerant rhizobacteria

Rhizobacterial strains were isolated from rhizosphere of okra plants following the dilution plate technique (Dworkin and Foster, 1958) where heavy dose of bifenthrin was applied intentionally. The modified general purpose medium containing pesticide (10 mg L^{-1} bifenthrin) as sole source of carbon was used for isolation of bacterial strains. These strains were purified following the dilution plate technique on modified general purposes medium (GPM) also containing pesticide as sole source of carbon. Twenty strains were preserved in 80% glycerol at 4 °C for further use.

Pesticide tolerance

The sensitivity of rhizobacterial strains to bifenthrin was studied quantitatively in minimal salt medium without agar at three levels of bifenthrin (100, 250 and 500 mg L^{-1}) as carbon source. The glucose was used as carbon source in case of control. The medium was prepared, poured in glass tubes and inoculated with 1 mL of respective rhizobacterial strains having uniform cell density (10^6-10^7 cfu mL^{-1}). These tubes were incubated at 28 ± 2 °C for 72 h. The growth of bacterial strains at highest concentration of pesticide was considered as the maximum tolerance level (MTL).

Biochemical characterization

The bacterial strains were characterized for following plant growth promoting characteristics. For urease activity, the rhizobacterial strains were inoculated in sterilized urea broth and incubated for 48 h at 28 ± 2 °C. Catalase activity was determined by following the method as described by Schaad (1992). The ability of rhizobacterial strains to solubilize zinc was detected by following the protocol as described by Saravanan *et al*. (2007). For this purpose, the rhizobacterial isolates were inoculated into liquid medium containing dextrose, 10.0 g L^{-1}; $(NH_4)_2SO_4$, 1.0 g L^{-1}; KCl, 0.2 g L^{-1}; K_2HPO_4, 0.1 g L^{-1}; $MgSO_4$, 0.2 g L^{-1}; pH, 7.0, and insoluble Zn compound (ZnO 0.1%; Agar: 15.0 g L^{-1}). The media was autoclaved at 121°C for 20 min.

The actively growing cultures of each strain were spot-inoculated (3 µL) onto the agar plates and plates were incubated at 28°C for 48 h. The clearing zone around colony was recorded for the isolates with the ability to solubilize zinc on agar plates. Rhizobacterial isolates were characterized for NH_3 production ability by using Nessler's reagent (Dye, 1962). Urease activity of rhizobacterial strains was determined by growing them in sterilized urea broth for 24-48 h at 28 ± 2 °C and observations were recorded for pink color (Shruti *et al*., 2013). On the basis of plant growth promoting characteristics and zinc solubilization ability, five isolates were selected and root colonization ability of these rhizobacterial isolates was detected by following the protocol as described by Simons *et al*. (1996) using okra as test crop. Oxidase activity was determined by following the method described by (Steel, 1961). The chitinase activity was determined by adopting the method of Chernin *et al*. (1998).

Osmoadaptation assay

Salt tolerance of bacterial isolates was examined by osmoadaptation assay. The bacterial isolates were grown in nutrient broth with four different salt concentrations i.e. original (control) 4, 8 and 12 dS m^{-1}. These were incubated for three days at 28 ± 2 °C and optical density was measured at 540 nm by following the method of Zahir *et al*. (2010). Modified general purpose medium with 10 mg L^{-1} bifenthrin as carbon source was used for this assay.

Growth at different pH levels

For pH optimization, the rhizobacterial strains were grown in nutrient broth with three pH levels (5, 7 and 9). The medium with different pH levels was prepared, poured in glass tubes and inoculated with respective rhizobacterial strains. These tubes were incubated at 28 ± 2 °C for 72 h and optical density was measured at 600 nm after three days by following the method of Pandey *et al.*

(2014). The data were analyzed statistically. The standard error of means was calculated using MS excel in Microsoft Office 10.

Results

Pesticide tolerance

The results showed that pesticide had a negative impact on growth of most isolates at higher levels (Table 1). Rhizobacterial isolates showed variation in growth with increasing levels of bifenthrin. Most of these strains showed high optical density at low pesticide level (up to 250 mg L^{-1}) and their growth decreased with increasing concentration of pesticide. Under control conditions, maximum growth was observed in the case of strain S20, followed by S2, S10, S12, S3 and S8, while minimum optical density was observed in the case of S7. Similarly, at highest pesticide level, maximum cell growth was observed in the case of S10, followed by S20, whereas minimum growth was shown by S5.

Table 1: Growth of rhizobacterial strains in broth culture after 3 days of incubation at different levels of bifenthrin

Strain	Pesticide tolerance (Optical density at 600 nm)			
	Control	100 mg L^{-1}	250 mg L^{-1}	500 mg L^{-1}
S1	0.56 ± 0.001	0.62 ± 0.006	0.63 ± 0.003	0.50 ± 0.005
S2	0.94 ± 0.008	0.67 ± 0.002	0.71 ± 0.007	0.63 ± 0.006
S3	0.79 ± 0.002	0.42 ± 0.002	0.57 ± 0.003	0.50 ± 0.003
S4	0.52 ± 0.002	0.60 ± 0.006	0.64 ± 0.002	0.51 ± 0.001
S5	0.62 ± 0.001	0.51 ± 0.003	0.44 ± 0.001	0.32 ± 0.002
S6	0.56 ± 0.001	0.55 ± 0.002	0.70 ± 0.002	0.71 ± 0.001
S7	0.50 ± 0.003	0.59 ± 0.003	0.61 ± 0.005	0.50 ± 0.003
S8	0.77 ± 0.001	0.61 ± 0.002	0.70 ± 0.003	0.64 ± 0.002
S9	0.63 ± 0.002	0.63 ± 0.003	0.64 ± 0.002	0.70 ± 0.008
S10	0.81 ± 0.003	0.66 ± 0.002	0.71 ± 0.001	0.75 ± 0.021
S11	0.61 ± 0.001	0.52 ± 0.002	0.71 ± 0.002	0.42 ± 0.001
S12	0.81 ± 0.002	0.69 ± 0.002	0.68 ± 0.002	0.69 ± 0.001
S13	0.63 ± 0.007	0.52 ± 0.005	0.58 ± 0.002	0.42 ± 0.002
S14	0.61 ± 0.002	0.62 ± 0.002	0.67 ± 0.002	0.62 ± 0.002
S15	0.63 ± 0.009	0.51 ± 0.003	0.54 ± 0.006	0.43 ± 0.001
S16	0.62 ± 0.001	0.51 ± 0.001	0.48 ± 0.002	0.44 ± 0.003
S17	0.61 ± 0.002	0.33 ± 0.003	0.45 ± 0.002	0.46 ± 0.007
S18	0.61 ± 0.004	0.50 ± 0.002	0.41 ± 0.002	0.40 ± 0.002
S19	0.62 ± 0.001	0.47 ± 0.002	0.53± 0.001	0.47 ± 0.002
S20	0.96 ± 0.001	0.59 ± 0.001	0.68± 0.002	0.72 ± 0.001

Note: The results are average of three replicates ± standard error

Biochemical characterization

The biochemical characters are presented in Table 2. Results showed that all 20 strains under study were found to be positive in catalase activity. Seven strains namely S1, S6, S8, S10, S13, S14 and S20 had Zn solubilizing ability as these developed clear zone in zinc oxide media during plate assay. The largest halo zone was observed by the strain S10 (Table 3) which exhibited 28 mm halo zone followed by S20 (26 mm). Eight isolates S3, S9, S12, S13, S14, S15, S19 and S20 showed positive response in urease test. Four isolates (S6, S8, S14 and S20) showed ammonia production ability. The isolates S14 and S20 showed positive results for all plant growth promoting traits under study.

The most tolerant isolates (S6, S9, S10, S12 and S20) were further characterized to find out their root colonization ability, oxidase and chitinase activity (Table 4). A rapid increase in bacterial population was observed in root colonization assay. All isolates showed strong root adherence ability but the maximum root colonization (5.10×10^{8} cfu mL^{-1}) was given by S20 followed by S10. In oxidase test, the strain S6 gave negative result while all others were found to be positive. In chitinase test, except S12, all the selected isolates showed the presence of chitinase enzyme.

Osmoadaptation assay

Results showed that rhizobacterial growth was negatively affected with increasing levels of salinity (Table 5). Minimum optical density was obtained at highest salinity level. Under control conditions, the maximum optical density was observed in the case of S2, followed

by S20, S12, and S10. At highest salinity level (12 dS m^{-1}), maximum cell growth was observed in the case of S20, followed by S3 and S10 while S15 gave poor cell density.

Table 3: Colony diameter and halo zone formation by zinc solubilizing rhizobacteria

Bacterial strain	Colony diameter (mm)	Halo zone diameter (mm)
S1	10	16
S6	13	20
S8	8	12
S10	15.5	28
S13	9	19
S14	7	15
S20	12	26

Growth at different pH levels

In the present study, all rhizobacterial strains showed maximum optical density at neutral pH (7), followed by alkaline pH (9). The optical density was decreased with decreasing pH level from neutral to acidic. All strains showed poor growth on acidic pH. The maximum growth at pH 9 was observed in the case of S10 strain followed by S20 (Table 6).

Discussion

The result of present study showed that bifenthrin adversely affected the growth of most rhizobacterial isolates at higher levels whereas stimulated the growth of same strains at lower level. The variable response of these isolates to different levels of bifenthrin was observed that may be due to their ability to use bifenthrin as carbon or energy source (Qiu *et al*., 2009). In case of pesticide tolerant rhizobacterial isolates, an increase in microbial growth was obtained by adding the pesticide up to 250 mg L^{-1}.

Table 2: Biochemical characterization of pesticide tolerant rhizobacteria

Strain	Plant growth promoting traits			
	Zn solubilization	Ammonia production	Urease activity	Catalase Activity
S1	+	-	-	+
S2	-	-	-	+
S3	-	-	+	+
S4	-	-	-	+
S5	-	-	-	+
S6	+	+	-	+
S7	-	-	-	+
S8	+	+	-	+
S9	-	-	+	+
S10	+	-	-	+
S11	-	-	-	+
S12	-	-	+	+
S13	+	-	+	+
S14	+	+	+	+
S15	-	-	+	+
S16	-	-	-	+
S17	-	-	-	+
S18	-	-	-	+
S19	-	-	+	+
S20	+	+	+	+

Table 4: Biochemical characterization of pesticide tolerant rhizobacteria

Strain	Plant growth promoting trait		
	Root colonization (cfu mg^{-1})	Oxidase activity	Chitinase activity
S6	1.43×10^{8}	-	+
S9	3.00×10^{7}	+	+
S10	2.48×10^{8}	+	+
S12	6.30×10^{7}	+	-
S20	5.10×10^{8}	+	+

(+) = growth; (-) = no growth

The increase in growth was determined by an increase in optical density. Our findings are supported by the finding of Ahmed and Ahmad (2006). They studied the effect of

Table 5: Growth of rhizobacterial strains in broth culture after 3 days of incubation under salt-stressed conditions

Strain	Salinity tolerance (Optical density at 540 nm)			
	Control	4 dS m^{-1}	8 dS m^{-1}	12 dS m^{-1}
S1	0.57 ± 0.006	0.62 ± 0.004	0.46 ± 0.008	0.33 ± 0.008
S2	0.92 ± 0.009	0.61 ± 0.006	0.53 ± 0.008	0.33 ± 0.001
S3	0.78 ± 0.003	0.73 ± 0.005	0.70 ± 0.001	0.55 ± 0.004
S4	0.72 ± 0.003	0.60 ± 0.003	0.42 ± 0.001	0.32 ± 0.004
S5	0.72 ± 0.002	0.53 ± 0.003	0.51 ± 0.005	0.43 ± 0.002
S6	0.57 ± 0.003	0.50 ± 0.003	0.52 ± 0.003	0.49 ± 0.002
S7	0.52 ± 0.002	0.32 ± 0.002	0.20 ± 0.004	0.22 ± 0.001
S8	0.78 ± 0.002	0.55 ± 0.003	0.51 ± 0.003	0.39 ± 0.003
S9	0.62 ± 0.005	0.63 ± 0.001	0.50 ± 0.006	0.43 ± 0.007
S10	0.79 ± 0.001	0.62 ± 0.002	0.65 ± 0.001	0.50 ± 0.002
S11	0.61 ± 0.003	0.51 ± 0.003	0.41 ± 0.003	0.41 ± 0.002
S12	0.81 ± 0.002	0.50 ± 0.002	0.41 ± 0.005	0.21 ± 0.002
S13	0.63 ± 0.005	0.64 ± 0.001	0.52 ± 0.005	0.43 ± 0.005
S14	0.64 ± 0.001	0.67 ± 0.005	0.43 ± 0.001	0.43 ± 0.008
S15	0.61 ± 0.002	0.30 ± 0.002	0.21 ± 0.005	0.20 ± 0.002
S16	0.62 ± 0.007	0.72 ± 0.005	0.45 ± 0.002	0.43 ± 0.006
S17	0.61 ± 0.002	0.50 ± 0.002	0.24 ± 0.003	0.22 ± 0.002
S18	0.61 ± 0.003	0.41 ± 0.008	0.40 ± 0.002	0.30 ± 0.002
S19	0.62 ± 0.008	0.63 ± 0.005	0.53 ± 0.002	0.43 ± 0.006
S20	0.86 ± 0.001	0.63 ± 0.002	0.63 ± 0.002	0.58 ± 0.003

Note: The results are average of three replicates ± standard error

Table 6: Growth of rhizobacterial strains in broth culture after 3 days of incubation at different pH levels

Strain	pH (Optical density at 600 nm)		
	5	7	9
S1	0.22 ± 0.006	0.56 ± 0.005	0.41 ± 0.003
S2	0.25 ± 0.006	0.95 ± 0.003	0.64 ± 0.003
S3	0.22 ± 0.002	0.79 ± 0.005	0.72 ± 0.004
S4	0.32 ± 0.002	0.51 ± 0.003	0.49 ± 0.003
S5	0.31 ± 0.002	0.52 ± 0.003	0.46 ± 0.001
S6	0.29 ± 0.003	0.56 ± 0.003	0.55 ± 0.004
S7	0.21 ± 0.002	0.53 ± 0.002	0.50 ± 0.001
S8	0.32 ± 0.003	0.77 ± 0.003	0.53 ± 0.004
S9	0.24 ± 0.003	0.62 ± 0.003	0.51 ± 0.001
S10	0.33 ± 0.003	0.80 ± 0.004	0.73 ± 0.003
S11	0.25 ± 0.006	0.63 ± 0.002	0.53 ± 0.003
S12	0.20 ± 0.003	0.81 ± 0.003	0.51 ± 0.001
S13	0.21 ± 0.007	0.63 ± 0.005	0.64 ± 0.002
S14	0.22 ± 0.004	0.62 ± 0.002	0.51 ± 0.006
S15	0.21 ± 0.003	0.61 ± 0.004	0.48 ± 0.006
S16	0.23 ± 0.002	0.61 ± 0.003	0.53 ± 0.004
S17	0.21 ± 0.003	0.62 ± 0.003	0.51 ± 0.008
S18	0.21 ± 0.003	0.60 ± 0.002	0.39 ± 0.003
S19	0.21 ± 0.002	0.61 ± 0.002	0.60 ± 0.002
S20	0.27 ± 0.003	0.96 ± 0.007	0.72 ± 0.003

Note: The results are average of three replicates ± standard error

pesticides on bacteria and reported that bifenthrin increased the number of cells in terms of colony forming units at lower dose (up to 250 mg L^{-1}) of pesticide whereas decreased the bacterial population at higher dose (500 mg L^{-1}). The ability of bacterial strains to use pesticide for a carbon source has also been reported by Chen *et al*. (2012) and Sarat and Barathi (2013). Similarly, Pandey *et al*. (2014) reported that bacterial isolates growing on bifenthrin waste developed resistance up to 800 mg L^{-1} and were capable to use it as carbon and energy source.

It was observed in this study that pesticide tolerant rhizobacterial strains exhibited multiple plant growth promoting traits. In the present study, 7 isolates out of 20 could develop clear zone in zinc oxide medium during plate assay. This showed their ability to solubilize inorganic zinc source. Zinc solubilization by rhizobacterial strains has been reported Bapiri *et al*. (2012) who isolated 8 strains those were able to solubilize zinc oxide or zinc carbonate in agar plate or in broth assays. Similarly, Kumar *et al.* (2012) screened seven isolate of which four isolates showed zinc phosphate dissolution. Role of Zn solubilizing bacteria as a plant growth promoter has been reported by Abaid-Ullah *et al*. (2015). The possible mechanisms used by the microorganisms to solubilize Zn involves the acidification of the soil environment (Hafeez *et al.,* 2005) by the release of organic acids, gluconic acids or its other derivatives (Fasim *et al*., 2002) or by the production of siderophores (Abaid-Ullah *et al*., 2015).

In present findings, four isolates were found to be positive in ammonia production. Ammonia production by PGPR has been reported by Kumar *et al.* (2012). They isolated thirty bacterial strains and found that three isolates were positive in ammonia production. Similarly, Bhatt and Vyas *et al*. (2014) found 33 percent isolates to be positive in ammonia production. In this study, 7 isolates gave positive result in urease test. These bacteria might have used urea as a sole nitrogen source and hydrolyzed it to convert into ammonia and carbon dioxide. In present work, all strains showed catalase activity. This can be correlated to the ability of microbes for scavenging system of reactive oxygen species (Noctor and Foyer, 1998). The same results have been reported in the previous studies of Kumar *et al*. (2012). They studied thirty isolates and found that all were positive in catalase test. The catalase activity of the bacterial isolates can be used to induce stress tolerance in crop plants.

It was observed in our study that these strains have variation in their ability to colonize roots of okra. The root colonization ability also shows that these rhizobacterial strains give immediate response to their host by colonizing roots. Root colonization genes in *Enterobacter cloacae* UW5 bacterium have been identified by English *et al.* (2010) who reported that these genes were involved in colonization process and helped the plants for better growth.

The study showed that salinity had an adverse effect on growth of bacteria. The growth of rhizobacterial isolates decreased with each level increase in salinity. Some isolates showed more growth even at higher salt concentrations. This difference in their growth at higher salinity levels might be due to the ability of bacterial strains to tolerate salt stress. Ability of bacterial isolates to grow under salt stress has been reported by Shivakumar *et al*. (2013). They reported that growth of bacterial isolates increased rapidly up to 4.5 M NaCl and inversely proportional to increase in salinity levels. Similarly, Roohi *et al.* (2012) reported variable ability of bacterial isolates to tolerate salinity stress.

Growth of all bacterial isolates was negatively affected at acidic pH and showed variable response with increasing level of pH up to alkaline range. This variation in growth of rhizobacterial strains at different pH levels has been reported by Padan *et al*. (2005). They reported that some bacteria have shown higher growth at alkaline pH that may be attributed to metabolic modification with high acid production, raising the transporters and enzymes, and changes in the outer layers of cell for proton retention (Padan *et al.,* 2005).

Conclusion

In present work, most of the strains exhibited several plant growth promoting traits and tolerance to environmental stresses confirming their capability to improve plant growth as growth promoters. Out of twenty isolates, S10 and S20 were efficient isolate in terms of Zn-solubilization, catalase activity, root colonization ability and in reaction to environmental stresses. It is concluded that S10 and S20 are better strains and have the maximum pesticide tolerance along with other multifarious traits. These isolates may be evaluated for their ability of bioremediation under bifenthrin stress.

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