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ANTAGONISTIC ACTIVITY OF BACTERIAL INOCULUM MULTIPLIED ON *RHIZOPHORA MUCRONATA* LAMK., IN THE CONTROL OF ROOT INFECTING FUNGI ON MASH BEAN AND OKRA

MARIUM TARIQ, SHAHNAZ DAWAR, FATIMA S. MEHDI AND M. JAVED ZAKI

Department of Botany,
University of Karachi, Karachi-75270, Pakistan.

Abstract

Microbial antagonists viz., *Bacillus subtilis, B. thuringiensis, B. cereus* and *Rhizobium meliloti* multiplied on dry powder of leaves and stem of *R. mucronata* were used in the control of root infecting fungi on mash bean and okra. Good growth of microbial antagonists was observed on all plant parts. Population increased with the increase in time. Highest population of *R. meliloti, B. subtilis, B. thuringiensis* was obtained on stem whereas *B. cereus* on leaves powder of *R. mucronata*. Of the four antagonists used *R. meliloti* showed highest population on *R. mucronata* plant parts.

Germination of seeds, shoot length, shoot weight, root length and root weight in okra and mung bean showed promising results when bacterial antagonists viz., *B. subtilis, B. thuringiensis, B. cereus* and *R. meliloti* after multiplication on mangrove plant parts was used @ 1% w/w. Infection of *R. solani* was significantly inhibited on okra when *R. meliloti* multiplied on leaves powder of *R. mucronata* was used @ 1 % w/w whereas all biocontrol bacteria viz., *B. subtilis, B. thuringiensis B. cereus* and *R. meliloti* completely suppressed the infection of *R. solani* and *M. phaseolina* on mung bean when used after multiplication on leaves and stem powder of *R. mucronata.*

Introduction

Rhizobium spp., the plant growth promoting rhizobacteria have a beneficial effect on plants including biological control of soil borne pathogens, induce systematic resistance to plant pathogen, improvement of nutrient, uptake of plant (Seuk Bae *et al*., 2000). The genus *Rhizobium* has an ability of nitrogen-fixation in leguminous plants (Hynes & Connel, 1990) and in the control of soil-borne root-infecting fungi both in leguminous and non-leguminous plants (Ehteshamul-Haque & Ghaffar, 1993; Siddiqui *et al*., 1998a; 1998b). Although most species of *Bacillus* are harmless saprophytes, two species viz., *B. thuringiensis* and *B. cereus* are considered medically significant. *B. thuringiensis* is a plant growth promoting bacterium which produces bacteriocin compounds (Gray *et al*., 2006). *B. thuringiensis* (commonly known as 'Bt') is an insecticidal bacterium, marketed worldwide for control of many important plant pests, mainly caterpillars of Lepidoptera, mosquito larvae and black flies etc. Application of bacteria either as seed dressing or as soil drenching has shown a significant suppression of root infecting pathogens on leguminous and non- leguminous plants (Zaki & Ghaffar, 1987; Ehtesham-ul-Haque *et al*., 1990; Shahzad & Ghaffar, 1992).

Mangroves are widespread in tropical and subtropical regions, growing in the saline intertidal zones of sheltered coast lines. Mangrove specie *R. mucronata* comprises less than 5% of the Indus Delta in Pakistan (Saifullah, 1982). The mangrove plant parts are of various uses like bark used for tanning and dye. Leaves are the source of a black or chestnut dye (Burkill, 1966). It is reported that mangrove is a folk remedy for angina, diabetes, diarrhea, dysentery, hematuria and haemorrhage (Duke & Wain, 1981).

Plant diseases causing organisms produce serious losses to crop plants and adversely affect the agricultural economy of a country (Hafeez, 1986). The soil borne root-infecting fungus, *Macrophomina phaseolina* is reported to produces charcoal rot over 500 species of plants (Sinclair, 1982). *Rhizoctonia solani* exists as active mycelium in the soil, attacks over 2000 species of plants (Parmeter, 1970) and *Fusarium* species (Booth, 1971) are known to attack a wide range of host plants in different parts of the world.

The main objective of this study was to examine the efficacy of biocontrol agents viz., *R. meliloti*, *B. subtilis*, *B. thuringiensis*, and *B. cereus* multiplied on *R. mucronata* plant parts for the control of root infecting fungi and growth promotion of mash bean and okra.

Materials and Methods

Mangrove plant parts viz., leaves and stem of *Rhizophora mucronata* Lamk., were collected from Sonmiyani. Cultures of different species of *Bacillus* viz., *B. thuringiensis* (Bt-10), *B. cereus* (Bc-20) and *B. subtilis* (Bs-12) were obtained from the Department of Microbiology, University of Karachi whereas *R. meliloti* (R-5) was obtained from the root nodules of leguminous plant like *Melilotus alba.* The cultures were grown on nutrient agar, PDA or Yeast Extract Mannitol Agar medium supplemented with antibiotics and incubated for 1-2 days. Leaves and stem of *R. mucronata* as an organic substrate were used for the growth of bacterial antagonists viz., *B. subtilis, B. thuringiensis, B. cereus* and *R. meliloti*. Different plant parts was air dried and powdered in an electric grinder. For the multiplication of microbial antagonists 100 gm of each part powder was transferred in polyethylene bag. The bag was sealed and then sterilized in an autoclaved at 15 psi. The substrates in polyethylene bags were inoculated by injecting with a syringe a cell suspension of biocontrol agents @ 2 ml/100 g plant parts. The inoculated substrates were stored at room temperature for growth.

Population of bacterial antagonists was determined by using soil dilution plate method (Waksman & Fred, 1922) with slight modification. After making dilutions, one ml suspension was poured on Yeast Extract Mannitol Agar medium and incubated at 28˚C for 2-4 days. Bacterial colonies growing on plates were counted and multiplied by the dilution factor which gave cfu/ml of bacteria. Colony forming unit/seed was calculated by taking ten seeds after treatment with suspension of microbial antagonists transferred in test tube containing 10 ml sterilized distilled water. The test tube was shaken and dilution series was made. One ml suspension was poured on PDA and YMA respectively for fungi and bacteria.

Seeds of okra and mung bean were surface sterilized with 1 % $Ca(OCl)_2$ for 3 min., rinsed thoroughly in running tap water and dried aseptically. Five surface disinfested seeds were treated with bacterial cultures using 1% gum Arabic. For soil drenching a 25 ml aqueous cell suspension of 5 day old bacterial strains were drenched in 8 cm diam., plastic pots, each containing 300 g soil. Treatments were replicated three times. Okra and mung bean seeds were used as test plants. Five treated seeds were sown in 8 cm diam., plastic pots, each containing 300 g soil. Seeds treated with sterile distilled water served as control. The soil used was sandy loam (Sand, Silt, Clay; 70, 19,11% respectively), pH ranged from 7.5-8.1 with moisture holding capacity (MHC) of 40% (Keen & Raczkowski, 1922), total nitrogen 0.077-0.099% (Mackenzie & Wallace, 1954), total organic matter 4.17-4.59%. Soil had natural infestation of 4-6 sclerotia/g soil of *Macrophomina phaseolina* as estimated by wet sieving dilution technique (Sheikh & Ghaffar, 1975); 3-5% colonization of *Rhizoctonia solani* on sorghum seeds used as baits (Wilhelm, 1955) and $8x10^6$ cfu/g soil of *Fusarium* sp, as assessed by soil dilution technique (Nash & Snyder, 1962). Treatments were replicated three times.

Table 1. Population of *Bacillus* spp., and *Rhizobium meliloti* multiplied on different plant parts of *Rhizophora mucronata* at different time interval.

Substrates	Time interval (days)				
	0	**7**	**14**	**21**	**28**
	Rhizobium meliloti				
R. mucronata leaves	17.3 x10^6	30.2 x10^8	35.6 x10^8	42.7 x10^8	31.2 x10^8
R. mucronata stem	20.4 x10^6	31.3 x10^8	36.4 x10^8	44.6 x10^8	30.1 x10^8
	Bacillus thuringiensis				
R. mucronata leaves	15.2 x10^6	23.2 x10^8	26.5 x10^8	31.2 x10^8	17.2 x10^8
R. mucronata stem	17.6 x10^6	24.7 x10^8	32.1 x10^8	40.1 x10^8	28.3 x10^8
	Bacillus subtilis				
R. mucronata leaves	16.4 x10^6	24.4 x10^8	28.9 x10^8	30.2 x10^8	21.1 x10^8
R. mucronata stem	21.3 x10^6	28.2 x10^8	31.6 x10^8	38.2 x10^8	28.9 x10^8
	Bacillus cereus				
R. mucronata leaves	14.2 x10^6	22.1 x10^8	27.3 x10^8	31.7 x10^8	25.3 x10^8
R. mucronata stem	14.3 x10^6	19.7 x10^8	21.6 x10^8	28.2 x10^8	16.2 x10^8

Plants were uprooted after 30 days of emergence and growth parameters in terms of shoot length, shoot weight, root length and root weight were recorded. Roots were washed in running tap water and then cut five, 1 cm root pieces surface sterilized in 1% $Ca(OCl)_2$ for 3 min, were transferred on PDA plates containing penicillin @ 100,000/litre and streptomycin @ 20mg/l. Petri dishes were incubated for 5 days at 28±2˚C and after one week, infection of roots by fungi was recorded.

Data were analyzed and were subjected to analysis of variance (ANOVA) or factorial analysis of variance (FANOVA) depending upon the experimental design. According to Gomez & Gomez (1984), the follow up of FANOVA included least significant difference (LSD).

Results and Discussion

Good growth of *Bacillus* spp., viz., *B. subtilis, B. thuringiensis B. cereus* and *R. meliloti* on different plant parts viz., leaves and stem *R. mucronata* was observed. Population increased with the increase in time. Highest population of *R. meliloti*, *B. subtilis, B. thuringiensis* were obtained on stem of *R. mucronata* whereas *B. cereus* on leaves powder of *R. mucronata*. Of the 4 antagonists used *R. meliloti* showed highest population on *R. mucronata* plant parts (Table 1).

The seeds of mung bean were coated with *R. meliloti* @ 73.88 x 10^8 cells /seed and *Bacillus* spp viz; *B. subtilis* @ 84.54 x 10^8 cells /seed, *B. thuringiensis* @ 72.43 x 10^8 cells /seed and *B. cereus* @ 63.02 x 10^8 cells /seed whereas okra seeds were coated with *R. meliloti* @ 86.33 x 10^8 cells /seed, *B. subtilis* @ 105 x10^8 cells /seed, *B. thuringiensis* @ 93.68 x10^8 cells /seed and *B. cereus* @ 88.43 x 10^8 cells /seed. Maximum germination of mung bean seeds was observed where *B. thuringiensis* inoculum multiplied on leaves and stem powder of *R. mucronata* was used. Shoot length was significantly increased in okra where *R. meliloti* and *B. thuringiensis* after multiplication on *R. mucronata* stem powder was used @ 1 % w/w. Shoot weight, root length and weight were significantly increased in mung bean when *R. meliloti, B. cereus* and *B. subtilis* inoculum used on *R. mucronata* leaves and stem powder ($p<0.001$) was used. Seed dressing significantly increased growth parameters of okra and mung bean in all biocontrol bacteria viz., *B. subtilis, B. thuringiensis B. cereus* and *R. meliloti* ($p<0.001$) (Table 2).

Table 2. Effect of *R. meliloti*, *B. thuringiensis*, *B. subtilis* and *B. cereus variotii* multiplied on different parts of *Rhizophora mucronata* plant on plant growth of mash bean and okra.

Treatments	Mung bean					Okra				
	Germination %	Shoot length (cm)	Shoot weight (g)	Root length (cm)	Root weight (g)	Germination %	Shoot length (cm)	Shoot weight (g)	Root length (cm)	Root weight (g)
Control	66.66	19.46	0.30	3.88	0.026	60.00	15.45	0.25	3.78	0.023
R. meliloti (soil drenching)	66.66	20.72	0.32	4.64	0.033	66.66	17.19	0.27	4.52	0.025
R. meliloti (seed dressing)	80.00	21.23	0.36	5.15	0.036	73.33	19.62	0.29	4.56	0.025
R. mucronata leaves powder alone	100.00	23.91	0.5	5.18	0.058	86.66	21.05	0.31	5.64	0.026
R. meliloti inoculum on *R. mucronata* leaves	93.33	24.96	0.53	6.63	0.066	80.00	21.47	0.36	5.80	0.034
B. thuringiensis (soil drenching)	66.66	19.67	0.37	4.11	0.038	73.33	18.65	0.28	4.15	0.023
B. thuringiensis (soil dressing)	80.00	21.19	0.43	4.78	0.043	73.33	20.11	0.29	4.19	0.024
B. thuringiensis inoculum on *R. mucronata* leaves	100.00	24.6	0.53	5.49	0.677	93.33	22.08	0.38	5.83	0.036
B. subtilis (soil drenching)	80.00	22.18	0.34	4.42	0.055	80.00	20.58	0.28	4.08	0.023
B. subtilis (seed dressing)	80.00	21.66	0.39	4.95	0.056	73.33	20.77	0.30	4.88	0.026
B. subtilis inoculum on *R. mucronata* leaves	93.33	24.29	0.55	5.38	0.099	86.66	21.94	0.50	5.68	0.038
B. cereus (soil drenching)	80.00	20.55	0.44	4.64	0.044	66.66	19.05	0.28	4.32	0.023
B. cereus (seed dressing)	86.66	21.14	0.43	5.11	0.047	66.66	20.11	0.26	4.94	0.026
B. cereus inoculum on *R. mucronata* leaves	93.33	23.97	0.50	6.63	0.068	93.33	22.77	0.45	5.77	0.037
R. mucronata stem powder alone	80.00	23.68	0.54	5.08	0.077	86.66	22.36	0.51	5.44	0.035
R. meliloti inoculum on *R. mucronata* stem	80.00	23.71	0.57	5.32	0.076	86.66	25.55	0.56	5.77	0.038
B. thuringiensis inoculum on *R. mucronata* stem	100.00	25.53	0.58	5.50	0.086	93.33	26.88	0.54	5.68	0.035
B. subtilis inoculum on *R. mucronata* stem	80.00	23.59	0.68	5.83	0.084	86.66	22.42	0.58	5.60	0.035
B. cereus inoculum on *R. mucronata* stem	86.66	23.83	0.61	6.15	0.081	80.00	22.83	0.53	5.47	0.037
LSD0.05=	**30.67**	**2.987**	**0.16**	**2.03**	**0.03**	**36.68**	**5.86**	**0.33**	**2.72**	**0.05**

Table 3. Effect of *R. meliloti*, *B. thuringiensis*, *B. subtilis* and *B. cereus variotii* multiplied on different parts of *Rhizophora mucronata* plant in the infection % of root infecting fungi of mash bean and okra.

Treatments	Mung bean			Okra		
	Fusarium spp.,	*Rhizoctonia solani*	*Macrophomina phaseolina*	*Fusarium* spp.,	*Rhizoctonia solani*	*Macrophomina phaseolina*
Control	100.00	95.55	100	91.10	100	93.33
R. meliloti (soil drenching)	86.66	93.33	91.10	75.55	82.22	82.22
R. meliloti (seed dressing)	73.33	84.44	73.33	46.66	64.44	75.55
R. mucronata leaves powder alone	4.44	11.11	2.22	19.99	15.55	13.33
R. meliloti inoculum on *R. mucronata* leaves	4.44	0	0	6.66	0	8.88
B. thuringiensis (soil drenching)	88.88	91.10	91.11	66.66	60	79.99
B. thuringiensis (soil dressing)	62.22	57.77	62.22	31.11	48.88	66.66
B. thuringiensis inoculum on *R. mucronata* leaves	2.22	4.44	6.66	6.66	4.44	2.22
B. subtilis (soil drenching)	71.10	84.44	95.55	77.77	75.55	79.99
B. subtilis (seed dressing)	66.66	73.33	62.22	48.88	68.88	44.44
B. subtilis inoculum on *R. mucronata* leaves	2.22	4.44	0	11.11	6.66	11.11
B. cereus (soil drenching)	77.77	68.88	55.55	64.44	71.10	62.22
B. cereus (seed dressing)	31.10	33.33	42.22	53.33	42.22	66.66
B. cereus inoculum on *R. mucronata* leaves	4.44	8.88	0	8.88	4.44	11.10
R. mucronata stem powder alone	8.88	6.66	11.10	70.77	11.10	13.33
R. meliloti inoculum on *R. mucronata* stem	6.66	0	8.88	6.66	2.22	4.44
B. thuringiensis inoculum on *R. mucronata* stem	2.22	0	0	4.44	4.44	2.22
B. subtilis inoculum on *R. mucronata* stem	4.44	6.66	6.66	4.44	8.88	4.44
B. cereus inoculum on *R. mucronata* stem	8.88	6.66	2.22	8.88	6.66	2.22
LSD0.05=	**19.59**	**22.53**	**34.28**	**29.54**	**29.16**	**28.89**

Infection of *R. solani* on okra was significantly inhibited by *R. meliloti* inoculum multiplied on *R. mucronata* leaves powder used @ 1%w/w whereas all biocontrol bacteria viz., *B. subtilis, B. thuringiensis B. cereus* and *R. meliloti* after multiplication on leaves and stem powder of *R. mucronata* showed complete suppression of *R. solani* and *M. phaseolina* on mung bean ($p<0.001$) (Table 3). Infection of *Fusarium* spp., was significantly reduced ($p<0.001$) in mung bean and okra where *B. subtilis* inoculated on leaves and *B. thuringiensis* inoculum on stem was used @ 1 % w/w (Table 3). Due to the cost of chemicals and their hazardous effect, use of microorganism in the control of root rot fungi is another alternate method (Lumsden & Locke, 1989). Several bacteria have received considerable attention in the control of soil borne root infecting fungi. In the present study shoot weight, root length and weight were significantly increased in mung bean when *R. meliloti, B. cereus* and *B. subtilis* used after multiplication on *R. mucronata* leaves and stem powder @ 1 % w/w. Similar report was made by Dawar *et al*., (2005) on mung bean and Siddiqui *et al*., (2000) on okra where *Rhizobia* used as seed dressing and soil drenching significantly increased growth parameters and infection of root rot fungi. Ehteshamul-Haque & Ghaffar (1993) reported that some strains of *Rhizobia* which fix atmospheric nitrogen in association with leguminous plants showed significant control of *M. phaseolina*, *R. solani* and *Fusarium* spp., infection on both leguminous plants like soybean and mung bean and non-leguminous plants like sunflower and okra.

The ability of *Rhizobia* to inhibit certain soil borne plant pathogens (Chakraborty & Purkayastha, 1984; Zaki & Ghaffar, 1987) has increased the importance of *Rhizobia* in addition to their use in nitrogen fixation. Present study showed that seed dressing was more effective in reduction of root rot fungi in mash bean plants than the soil drenching treatment. It was also previously observed that *B. thuringiensis* applied as seed dressing and soil drenching showed a significant increase in seed germination, shoot length, shoot weight, root length and root weight on mung bean and okra (Sheikh *et al.*, 2006). There is therefore need to characterize nematicidal and fungicidal compound (s) produced by biological antagonists in the control of root rot instead of use of pesticides, which are costly and hazardous.

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