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Antagonistic and growth promotion potential of endophytic bacteria of mulberry (*Morus* spp.)

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Abstract

Endophytes provide multifarious benefits such as promotion of plant growth and yield, suppression of phyto-pathogens, phosphate solubilising and fixation nitrogen. A study has been carried out to explore growth promotion and antifungal activities of endophytes of mulberry (*Morus* spp.). Endophytic bacteria were isolated from mulberry plants and studied their cultural, morphological characters, growth promotion as well as their antifungal activity against *Rhizoctonia bataticola* and *Fusarium oxysporum*, two mulberry root rot associated pathogens. Except two isolates, all bacteria were colourless and the colony size of eight isolates was small. The margin of five isolates was irregular and the consistency of three isolates was creamy, six isolates was slimy and one was mucoid. Texture of seven isolates was convex and others were flat. Eight isolates were gram positive and the rest Gram negative, five were cocci and others were bacilli (rod shaped). Four isolates were motile and all were catalase positive and only three isolates were oxidase positive. Spore staining was positive only for two isolates. The growth promotion study showed that there was significant difference in root length and seedling length. The antagonistic effect of the bacterial isolates was tested against *R. bataticola* showed significant (p<0.05) influence of the bacteria, days after inoculation and their interaction on the inhibition of fungal growth. The isolate En-7 completely inhibited the fungus followed by En-5 (66.67%). The bacterial isolates significantly (p<0.05) inhibited growth of *F. oxysporum* in PDA. The mean inhibition was higher (70.45%) in case of En-7 followed by En-8 (68.65%) and En-10 (66.44%). The study reveals that some endophytic bacteria associated with mulberry have growth promotion and antifungal activity and could be explored for promotion of mulberry growth and managing root rot disease.

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Introduction

Plant growth promotion and plant protection through plant microbe interaction has been given considerable interest by researchers and phylloplane microorganisms were explored for the last two decades. Recent studies showed that plants

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constitute diverse niches of endophytic microorganisms inhabiting in tissues without damaging their hosts. The relatively stable environment inside the plant tissues bioactive than the rhizosphere makes endophytes more associated microorganisms (He *et al*., 2009). In turn endophytes provide multifarious benefits such as promotion of plant growth and yield, phosphate solubilising, fixation nitrogen (Rosenblueth and Martinez Romero, 2006). Further, endophytic bacteria contribute close symbiotic association with the host which makes them more valuable biocontrol agents (Compant *et al.,* 2005; Bakker *et al.,* 2013). Compared to rhizospheric antagonist, endophytes have ability to enter the host system without stimulating pathogen induced vulnerability responses but triggering host defense pathways (Conn *et al.,* 2008; Podolich *et al.,* 2015). They could provide a barrier against the invading pathogens directly or through the production of bio-active compounds (Thomas and Upreti, 2014; Podolich *et al.,* 2015).

In sugarcane endophytic bacteria inoculation is reported to increase biological nitrogen fixation, promotion of root development, increased biomass and productivity (Oliveira *et al*. 2003). Barreti *et al.* (2008) reported increase in vegetative growth and fresh and dry weight in tomato. Endophytic fungi have been found to protect tomatoes (Hallman and Sikora, 1995) and bananas (Pocasangre *et al*., 2001; Sikora *et al*., 2008) from nematodes, and beans and barley (Boyle *et al*., 2001) from fungal pathogens. Similarly, the prevalence of higher endophytic bacterial diversity and more antagonistic organisms associated with the seedling roots of resistant cultivars over susceptible genotypes suggest a possible role by the root-associated endophytes in natural defense against the pathogen of tomato wilt caused by *Ralstonia solanacearum* (Upreti and Thomas, 2015). Exploration of endophytes therefore suggested to reduce the chemicals for plant protection especially when considering sustainable agriculture and environmental protection (Vale *et al*., 2010).

In mulberry, plethora of chemical are recommended to combat various diseases. However increased environmental awareness, cost of chemicals and highly sensitive nature of silkworm to plant protection chemicals that a minute chemical residue sufficient to result huge crop loss, make farmers reluctant to use chemicals. The alternative methods are therefore very essential to contain the situation. Considering the broad spectrum beneficial effect of endophytic bacteria, the present study attempt to isolate and evaluate few mulberry endophytic bacteria and evaluate their potential to control two fungal pathogens associated with root rot disease of mulberry.

Materials and Methods

Isolation of mulberry endophytic bacteria from mulberry

The nutrient agar medium with pH 7.0 was used for isolation of endophytic bacteria. The medium and plates were sterilized at 121ºC for 20 min and cooled to 50ºC. The sterile plates were exposed to UV light for 15 min. About 15 mL media were poured in 9 cm dia. Petri plates, the plates were then exposed to UV light for 15 min to ensure the sterility. The isolation of endophytes was done according to the procedure by Bacon *et al*. (2000). The mulberry plants were randomly selected from healthy plantation. The plants were uprooted and the collar region of the plant is taken for isolation of endophytes. The cut collar region of the plant is washed under running tap water. Sections of 3cm length were excised using flame sterilized secateurs from 3cm above and below the soil line. All the samples were washed thoroughly and allowed to dry under a ceiling fan. The samples were then washed with 0.1% HgCl, wiped the sample with sterile cotton, then washed with 70% ethanol for 1 min and rinsed thoroughly with sterile distilled water. The samples were again washed with 3% Sodium hypochlorite for 5 min and again washed thoroughly with sterile distilled water; this final water wash was collected for sterility test.

The outer layer of the cortex of the sample is peeled using sterilized sharp knife. Small pieces (0.2 cm) were taken from the inner cortical region of the cuttings these pieces were aseptically placed on sterile nutrient agar. The plates were incubated in BOD incubator at 27±2ºC for 72 h in inverted position. To ensure the sterility of the samples, the final wash collected were streaked on nutrient agar plates and incubated.

Study of cultural and morphological characters

The individual colonies grown on nutrient agar were sub-cultured in nutrient media and again transferred to the nutrient agar slants. These were considered as mother culture and used to study cultural and morphological characters. Morphological cha racteristics such as colony colour, size, margin, consistency and

texture of all the isolates were observed. From the total isolates, based on colony morphology, limited number of representative isolates was selected for further investigation. All the selected isolates were sub-cultured in nutrient agar slants and preserved in 4ºC for further investigation. Also, preliminary phenotypic as well as microscopic characterization of bacteria such as Gram reaction, endospore staining, motility, catalase and oxidase activity of all the isolates were performed by adopting standard procedure (Tiwari *et al*., 2009).

Evaluation of bacterial strains for growth promotion

The pure cultures of endophytes were inoculated on nutrient agar plates and left for incubation in BOD incubator at 27±2ºC for 72 h, The bacterial suspension was prepared from these pure cultured by adding 5 mL of sterile distilled water to plates and gently mixed using a camel hair brush. The suspensions were taken in test tubes separately for each isolates and added sterile distilled water. The optical density (OD) of the suspension was checked and adjusted with blank to make OD 600 under spectrophotometer so as to get approximately 8×10^{8} cells/mL.

For bio priming of mulberry seeds, good quality seeds of mulberry var. S799 OP variety were collected from Mulberry Breeding and Genetics Department CSR&TI, Mysore. About 300 seeds were taken in 50 mL test tube containing 10 mL bacterial suspension. These seeds in soaked condition was left for 12 h. The seeds soaked similar way in sterile distilled water served as blank. Thereafter the soaked seeds were spread on Petri plates (15 mm dia.) lined with moistened filter paper and kept for germination. To filter paper was moistened with distilled water to avoid drying using a dropper. Germination was considered when the seeds developed at least 2 mm long radical. The germination percentage was calculated based on the total number of seeds germinated on after seven days. The weight and length of root, shoot and total length of the seedling were recorded from each treatment and control.

Evaluation of antagonistic effect of endophytes

All bacterial isolates were screened for their ability to inhibit fungal growth on PDA plates using dual culture technique (Yoshida *et al.,* 2001). Bacterial inoculations were prepared from cultures grown on NA plates for 72 h whereas fungal cultures were grown on PDA for a weak. These bacteria were then streaked on PDA medium which was inoculated with a plug of 7d old mycelium of each fungus was placed at the center of the plates and single bacteria was streaked along the perimeters of the plates at a distance of 3 cm away from the fungal block. Three replications were kept for each treatment. Plates were incubated at 27±2ºC and the inhibition of fungal growth was assessed by measuring the radial growth of fungus towards and away from the bacterial streak 2^{nd}, 4^{th}, 6^{th} and 8^{th}d after inoculation. The percent of inhibition was calculated following the formula;

$$\text{Inhibition\%} = \frac{\text{Growth away from bacteria - Growth towards bacteria}}{\text{Growth towards bacteria}} \times 100$$

The percentage data were subjected for arcsine transformation before analysis of variance (ANOVA) and mean values were compared for significant difference.

Results and Discussion

Among 10 endophytic bacteria isolated, except two isolates, all were colourless and two isolates were yellow. The colony size of eight isolates was small and two were large. The margin of five isolates was irregular while margin of five isolates were circular. The consistency of three isolates was creamy and for six isolates it was slimy and for one isolate was mucoid. Texture of seven isolates was convex and of the rest was flat (Table 1).

The gram staining showed that the eight isolates as Gram positive and the rest Gram negative. Regarding the shape, five isolates were cocci and others were bacilli (rod shaped). Four isolates were showed mobility and all were catalase positive. However three isolates En-3, En-5 and En-7 were oxidase positive. Spore staining was positive only in isolates En-4 and En-10 (Table 2).

Except three isolates, treatment with all other isolates increased seed germination compared with untreated control. A higher germination (>96%) was observed due to biopriming with bacterial isolates, En-7 (98.45%), En-10 (97.26%), En-5 (97.34%), and En-2 (96.14%). The germination in case of three isolates was less than that of control (Fig, 1).

There was significant difference in root length and seedling

Table 1. Cultural characterization of endophytic bacteria

Bacterial isolate	Colour	Size	Margin	Consistency	Elevation
En-1	Colourless	Small	Irregular	Creamy	Convex
En-2	Colourless	Small	Circular	Slimy	Flat
En-3	Colourless	Small	Irregular	Creamy	Convex
En-4	Colourles	Small	Irregular	Slimy	Flat
En-5	Colourless	Large	Irregular	Slimy	Flat
En-6	Colourless	Small	Circular	Creamy	Convex
En-7	Yellow	Small	Circular	Slimy	Convex
En-8	Yellow	Small	Circular	Slimy	Convex
En-9	Colourless	Large	Irregular	Mucoid	Convex
En-10	Colourless	Small	Circular	Slimy	Convex

Table 2. Microscopic characterization of endophytes

Bacterial isolate	Gram reaction	Shape	Motility	Catalase	Oxidase	Spore staining
En-1	+	Cocci	-	+	-	-
En-2	+	Cocci	-	+	-	-
En-3	-	Rod	+	+	+	-
En-4	+	Rod	-	+	-	+
En-5	-	Rod	+	+	+	-
En-6	+	Cocci	-	+	-	-
En-7	+	Rod	+	+	+	-
En-8	+.	Cocci	-	+	-	-
En-9	+	Cocci	-	+	-	-
En-10	+	Rod	+	+	-	+

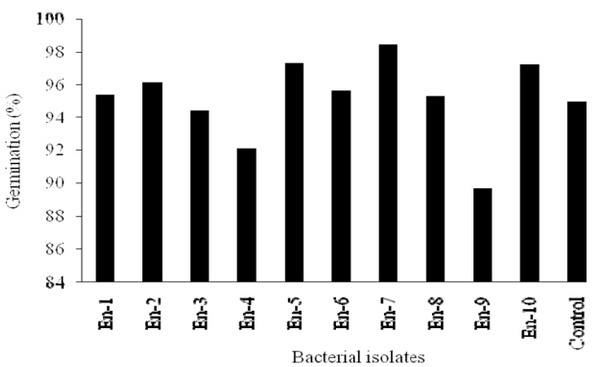

Fig. 1. Influence of endophytic bacteria on seed germination.

length. However, no significant difference observed in shoot length and seedling weight. The seedling weight was found higher (0.013 g) in case of seeds treated with bacterial isolates En-5 and En-7, followed by En-2 and En-10 (0.12). The least weight was observed in case of treatment with En-9 (0.007 g). Similarly, shoot length was more in seeds treated with isolate En-7 (24.50 mm) followed by En-8 (23.9 mm) treated lots and was least in case of isolates En-3 and En-9 (21.18 mm) which were less than that of control (22.17 mm).

The antagonistic effect of the bacterial isolates was tested against *R. bataticola* showed significant (p<0.05) influence of the bacteria days after inoculation and their interaction on the inhibition of fungal growth. All the bacterial isolate showed some degree of inhibition on the growth of *R. bataticola* in the PDA medium. The isolate En-7 completely inhibited *R. bataticola* followed by En-5 (66.67%). The inhibition was less in case of En-3 (14.48 %) En-6 (15.93%) and least in En-2 (12.29%). The inhibition rate was highest in 4th d after inoculation. Though isolates En-5, En-7, En-8 and En-10 showed complete inhibition in the initial stage, only En-7 showed complete inhibition up to

Table 3. Influence of endophytic bacteria on seedling growth of mulberry

Bacterial strain	Seedling weight (g)	Shoot length (mm)	Root length (mm)	Total length (mm)
En-1	0.010	23.033	7.500	30.533
En-2	0.012	23.133	11.233	34.367
En-3	0.010	21.167	7.267	28.433
En-4	0.009	23.233	9.133	32.367
En-5	0.013	22.467	14.800	37.267
En-6	0.008	22,933	13.367	36.300
En-7	0.013	24.500	16.133	40.633
En-8	0.009	23.933	13.167	37.100
En-9	0.007	21.600	9.200	30.800
En-10	0.012	23.300	11.467	34.767
Control	0.010	22.167	13.033	35.200
CD (p<0.05)	NS	NS	0.00511**	2.76**

NS- Not significant, ** significant p<0.01

the last stage. In other cases the fungus grown with the progress of days after inoculation. There was no significant difference in inhibition of *R. bataticola* among bacterial isolates En-1, En-2, En-3, En-6 and En-9. Similarly no significant difference was found between En-8 and En-10 (Table 4, Fig. 1).

The bacterial isolates and interaction between bacterial isolates and days after inoculation significantly (p<0.05) influenced the inhibition of *F. oxysporum* in PDA. The mean inhibition was higher (70.45%) in case of En-7 followed by En-8 (68.65%) and En-10 (66.44%). The mean inhibition was least (<10%) in case of isolates En-1 (8.24%), En-4 (8.43%), En-2 (8.45) and En-9 (9.71%). There was no significant difference in inhibition of the fungus between the bacterial isolates En-3, En-7, En-8 and En-10. Maximum inhibition was found on 16th day after inoculation by the bacterial isolate En-8 (75.47%) followed by En-10 (74.07%) and En-7 (72.97%). The days after inoculation did not significantly influence the inhibition of the fungus (Table 5, Fig. 1).

Bacterial endophytes are present in most of the plants (McInroy and Kloepper, 1995; Sturz, 1995). The beneficial effects of bacterial endophytes on their host plant appears to occur through similar mechanisms as described for rhizosphere-associated bacteria. These mechanisms have been reviewed in great detail by Kloepper *et al*. (1999) or, thereafter by Gray & Smith (2005) and Compant *et al*. (2005). The host endophyte interaction has been defined as altruism, commensalisms, symbiosis or passivity to pathogenicity. Whatever the specific relationship involved, internal plant colonization by bacteria constitutes a vast and as yet little mapped ecological niche. The diversity of ten putative endophytic bacteria isolated from host tissue was assessed. Difference in colony morphology gave an indication of the variation among the endophytes. The isolates studied were chosen for their dominance, uniqueness or differences with others in colony morphology.

Table 4. Inhibition of mycelia growth of *R. bataticola* by endophytic bacteria

Bacterial isolates	Inhibition (%) days after inoculation								Mean	
	Day-2		Day-4		Day-6		Day-8			
En-1	29.80	(32.78)	26.48	(30.91)	14.68	(22.40)	3.84	(11.16)	18.70	(24.31)
En-2	21.21	(27.17)	19.99	(25.87)	6.04	(14.08)	1.93	(6.49)	12.29	(18.40)
En-3	22.84	(28.48)	20.20	(26.59)	10.97	(19.24)	3.92	(11.26)	14.48	(21.39)
En-4	61.36	(51.74)	15.83	(22.54)	14.46	(21.90)	10.20	(17.81)	25.46	(28.50)
En-5	100.0	(88.20)	100.0	(88.20)	33.33	(29.40)	33.33	(29.40)	66.67	(58.80)
En-6	32.32	(34.56)	17.56	(24.69)	8.97	(16.64)	4.85	(12.54)	15.93	(22.11)
En-7	100.0	(88.20)	100.0	(88.20)	100.0	(88.20)	100.0	(88.20)	100.0	(88.20)
En-8	100.0	(88.20)	100.0	(88.20)	0.00	(0.00)	0.00	(0.00)	50.00	(44.10)
En-9	35.61	(36.36)	17.11	(24.32)	15.73	(23.00)	8.30	(16.17)	19.19	(24.96)
En-10	100.0	(88.20)	100	(88.20)	0.00	(0.00)	0.00	(0.00)	50.00	(44.10)
Mean	60.21	(56.39)	51.72	(50.77)	20.41	(23.49)	16.34	(19.30)		

CD (p<0.05): Bacteria=13.83, Days = 7.98, Bacteria x days =19.55
The figures in parenthesis are arc sin transformed values

Table 5. Inhibition of mycelia growth of *F. oxysporum* by endophytic bacteria

Bacterial isolates	Inhibition (%) days after inoculation								Mean	
	Day-2		Day-4		Day-6		Day-8			
En-1	13.96	(17.69)	9.00	(17.36)	8.13	(15.95)	1.85	(4.54)	8.24	(13.88)
En-2	18.03	(24.27)	8.17	(15.97)	3.82	(8.96)	3.77	(9.10)	8.45	(14.58)
En-3	59.12	(50.26)	63.79	(53.01)	67.79	(55.43)	67.83	(55.45)	64.63	(53.54)
En-4	19.15	(25.09)	5.45	(10.58)	4.29	(11.95)	4.85	(12.44)	8.43	(15.02)
En-5	41.90	(40.34)	47.10	(43.15)	54.64	(47.67)	60.20	(50.89)	50.96	(45.51)
En-6	21.44	(26.90)	10.07	(17.53)	8.45	(16.56)	7.98	(16.11)	11.99	(19.27)
En-7	65.50	(54.03)	71.28	(57.59)	72.07	(58.13)	72.97	(58.71)	70.45	(57.12)
En-8	60.76	(51.22)	66.03	(54.37)	72.35	(58.31)	75.47	(60.35)	68.65	(56.07)
En-9	5.05	(12.50)	5.59	(11.13)	11.36	(15.85)	16.82	(23.69)	9.71	(15.79)
En-10	58.06	(49.64)	63.56	(52.88)	70.07	(56.88)	74.07	(59.39)	66.44	(54.70)
Mean	36.29	(35.19)	35.00	(33.36)	37.29	(34.57)	38.58	(35.06)		

CD (p<0.05): Bacteria = 6.90, Days = NS, Bacteria x days = 9.76
The figures in parenthesis are arc sin transformed values

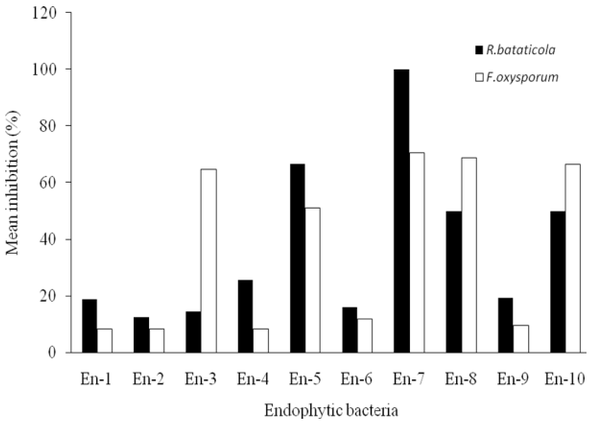

Fig. 2. Inhibition of growth of two fungi by various endophytic bacteria

As an endophyte, motility is an advantage for moving inside the plant system. The present study showed that 40% of the bacteria were highly motile. Interestingly, the proportion of Gram positive isolates was found predominant Endophytic bacteria residing within plant tissues have been reported to be promoting the plant growth directly or indirectly. The growth stimulation due to endophyte association can be as a consequence of nitrogen fixation (Hurek *et al.,* 2002; Iniguez *et al.,* 2004; Sevilla *et al.,* 2001) or the production of phytohormones or by enhancing availability of minerals (Sessitsch *et al.,* 2002; Sturz *et al.,* 2000). Further, volatile substances such as 2-3 butanediol and aceotin produced by bacteria seem to be a newly discovered mechanism responsible for plant growth promotion (Ryu *et al.,* 2003). Also, endophytes produce adenine ribosides that stimulate growth and mitigate browning of pine tissues (Pirttilä *et al.,* 2004). The growth stimulation observed in the present study could be attributed to any one or combination of these factors.

Endophytic bacteria are able to lessen or prevent the deleterious effects of certain pathogenic organisms. Endophytes from potato plants showed antagonistic activity against fungi (Berg *et al.,* 2005; Sessitsch *et al.,* 2004) and also inhibited bacterial pathogens belonging to the genera *Erwinia* and *Xanthomonas* (Sessitsch *et al.,* 2004). Some of the endophytic isolates are reported to produce antibiotics and siderophores (Sessitsch *et al.,* 2004). In the study, some bacterial isolates showed significant inhibition of *R. bataticola* and *F. oxysporum.* Diseases of fungal, bacterial, viral origin and in some instances even damage caused by insects and nematodes can be reduced following prior inoculation with endophytes (Kerry, 2000; Sturz *et al*., 2000; Ping & Boland, 2004; Berg & Hallmann, 2006). Endophytic bacteria interact more closely with the host plant and therefore, could be efficient biological control agent in sustainable crop production.

The study reveals that there are many endophytic bacteria in mulberry which are beneficial to the plant by helping in growth promotion and also antagonistic to the pathogens associated with root rot disease of mulberry. These endophytic bacterial isolates could be explored for improving mulberry growth and protection from soil borne diseases.

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