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Growth promotion and mildew suppressive effect of phylloplane bacteria of mulberry (*Morus* spp.)

■ **P. M. Pratheesh Kumar**

Central Sericultural Research and Training Institute, Srirampuram, **Mysuru (Karnataka) India**

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ABSTRACT

Studies were conducted to evaluate growth promotion and mildew (*Phyllactinia corylea*) suppressive effect of phylloplane bacteria of mulberry (*Morus* spp.) *in vitro* and *in vivo* and the effective bacteria were identified. Among 18 bacteria, 4 isolates showed highly significant (P<0.01) reduction with >70 per cent suppression of conidial germination. The highest reduction was showed by the isolate Pb-5 (13.87%) by suppressing 86.13 per cent conidial germination followed by Pb-4 (17.23%) with 82.17 per cent. Similarly, six isolates significantly increased the seed germination. Significantly high (P<0.01) seed germination was obtained (93.44%) with treatment of the isolate Pb-6 followed by Pb-3 (86.22%), Pb-7 (86.22%) and Pb-4 (85.47%). Most effective bacterial isolates were identified as *Bacillus megaterium* (Pb-1) *Bacillus subtilis* (Pb-1) *Bacillus cereus* (Pb-1) and *Pseudomonas aeruginosae* (Pb-1). *In vivo* studies revealed highly significant (P<0.01) with >60% reduction of disease severity with the application of *B.megaterium* (63.42%) and *B.cereus* (60.73%). The study suggests exploration of either of these bacteria for biological control of mildew in mulberry.

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Email : pratheesh.pm@gmail.com

INTRODUCTION

Nutritive value of mulberry leaf fed to silkworm is the key determinant of quality of silk produced. Physiobio-chemical alteration due to foliar diseases of mulberry leaves is reflected in the quality and quantity of silk produced and hence, the virtual loss due to diseases is greater than that of visual loss (Pratheesh Kumar *et al*., 2003). Huge loss was reported (Qadri *et al.*, 1999) due to powdery mildew (*Phyllactinia corylea)* in mulberry. Therefore, reliable methods for mildew control were always a matter of concern among farmers. Cost of plant chemical protectants, harmful effect on environment, development of resistant pathogen strains and sensitive nature of silkworm avert farmers to use chemicals for mulberry crop protection. This situation thus, warrants search for eco-compatible alternate methods.

The leaf surface has long been considered a hostile environment which constitutes a large microbial habitat

(Bulgarelli *et al*., 2013). These microbial communities are diverse are important for plant health and growth (Vorholt, 2012). Phylloplane bacterial communities have potential to influence plant biogeography and ecosystem function through their influence on the fitness and function of their hosts. This microbiota offers indirect protection against pathogens (Arnold *et al*., 2003) by production of antibiotic compounds and showing competition for resources (Braun *et al*., 2010). Thus, phylloplane bacteria for foliar diseases control was explored in agricultural systems such as rice (Akter *et al*., 2014) beans (Patro *et al*.,2002),Tea (Sowndhararajan *et al*., 2012) sunflower (Kong *et al*., 1997), groundnut (Kishore *et al*., 2005). Phyllosphere microbes also promote plant growth through the production of hormones (Ryu *et al*., 2006 and Fatima *et al*., 2016).

In mulberry, attempts were made against bacterial (Maji *et al*., 2003a) and fungal (Maji *et al*., 2003b) pathogens with phylloplane bacteria. However, these studies were limited only on antagonistic effect of the bacterial isolates against the pathogen. Positive effect on growth by introducing such organism on host plant is vital to establish a beneficial relation for their exploration. The present study was conducted to explore the mildew suppressive and growth promotion effect of bacterial isolates of mulberry phylloplane.

MATERIAL AND METHODS

Isolation of phylloplane bacteria from mulberry leaf:

Healthy mulberry leaves (variety V1) were collected in sterilized polythene bags from the experimental gardens maintained with recommended package of practice (Dandin *et al*., 2003) at Central Research and Training Institute, Mysuru. Sterilized nutrient agar (Hi-media) at 121°C at 15psi for 20 minutes and poured in 15ml sterilized petri-plates and kept for solidification. The lower side of the mulberry leaves was gently touched on the solidified media and the plates were incubated in a BOD incubator at 28± 2°C for 48 hours for formation of bacteria colonies. After incubation the colony characteristics were recorded and colonies differing in their morphological characters were individually isolated in Nutrient Agar (Hi-Media) media and pure cultures were made and kept for further studies.

***In vitro* evaluation of phylloplane bacteria against powdery mildew:**

In vitro evaluation of phylloplane bacteria against *P. corylea* was conducted in the laboratory through conidial germination approsoria formation. Healthy mulberry leaves were collected in sterile polythene bags. These leaves were washed with sterile distilled water. The bacterial solutions were prepared from 3 days old culture of phylloplane bacteria. The bacteria were grown in NA medium and loop of bacteria was added in sterile distilled water and adjusted 5 x 10^7 Cfu/ ml. The leaves were dipped individually in the bacterial solution for 5 minutes and then air dried in the room condition (28±2°C). These leaves individually inoculated with phylloplane bacteria were kept in petri plates measuring 15 mm diameter separately with adaxial side up. One control set was kept without inoculating with phylloplane bacteria. The inoculation of powdery mildew was done by gently tapping severely mildewed mulberry leaves on the treated leaves in such a way that each leaf receives 200-300 conidia/ cm^2. Three replications were kept against each treatment and control.

The Petri plates were kept in room temperature (28±2°C) overnight under fluorescent light. The harvesting of conidia and assessment of germination was done following the method of Biswas *et al.* (1993). The conidia were then harvested using cellophane tape by gently adhering on the treated leaves. These cellophane tape were then adhered on the microscopic slides and viewed under light microscopic for germination. The number of conidia germinated, total number of conidia and number of appressorium formed were enumerated. The per cent of germination and approsoria formed were calculated by following formula :

$$\text{Germination (\%)} = \frac{\text{Number of conidia germinated}}{\text{Total number of conidia}} \text{x} 100$$

$$\text{Appressorium formed (\%)} = \frac{\text{Number of appressoria}}{\text{Total number of approsoria}} \text{x } 100$$

Bio priming mulberry seeds with phylloplane bacteria:

Three days old culture was used for preparing bacterial suspension. About 5ml of distilled water was added to plates with bacterial colony and gently mixed and poured to test tube and diluted using sterile distilled water and adjusted the OD at 600 nm so as to get 1x 10^8 cell per ml under spectrophotometer. About 5 ml of the

suspension was taken in a test tube. The mulberry seeds of variety (K2) were soaked in bacterial solution for 24 hrs separately for each bacterium. Seed soaked in same amount of sterilized distilled water served as control. Three replications with 100 seeds each were kept against each treatment and control. After 24 hrs, the soaked seeds were spread separately on moistened filter paper in a petriplate measuring 15 mm diameter. The filter paper was moistened frequently dropping same volume of sterilized distilled water to maintain moisture for each treatment and control.

Germination was considered to have occurred when the seeds developed at least 2 mm long radical. In order to evaluate the germination rate, the germinated seeds were counted for every 24 hrs. The final germination percentage was calculated on 14th day after seed germination and measured length of root, shoot and total length in centimeters. The germination per cent and vigour index (VI) were calculated following international rules for seed testing (ISTA, 1999).

$$\text{Germination (\%)} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds taken}} \times 100$$

Vigour index= (Mean root length + mean shoot length) x % of germination

Biochemical test of bacterial isolates:

The effective isolates were primarily identified based on their cultural characters, morphology, pigmentation, motility and Gram staining. Further identification was done by performing several biochemical test such as catalase, oxidase, methyl red, Voges Proskauer, citrate utilization, indole production, nitrate reduction, casein hydrolysis, gelatin hydrolysis and substrate utilization test following standard procedures (Collins and Lyne, 1984; Cappuccino and Sherman, 2005 and Dubey and Maheshwary, 2005).

***In vivo* testing of bacteria against powdery mildew:**

The efficacy of bacteria for control of powdery mildew was conducted using four selected bacterial isolates through *in vivo* studies. The experiment was conducted using mulberry plants (variety V1) of shoot age 45 days grown in earthen pots naturally infected with powdery mildew. About 200 ml of bacterial suspension were prepared separately with selected bacterial isolates as mentioned earlier for *in vitro* studies adjusting to get approximately 1x10^8 cell per ml. These bacterial suspensions were sprayed separately on the mulberry plants using a hand held sprayer. Control plants were sprayed with equal volume of sterilized distilled water. Five replications were maintained against each bacteria and control. The severity of the disease was assessed using a 0-7 scale (Krishna *et al*., 1987) where 0= No disease;1= traces of mildew specks; 2=1-10 per cent leaf area mildewed; 3=11-20 per cent leaf area mildewed 4= 21-30 per cent leaf area mildewed; 5=31-50 per cent leaf area infected; 6=51-75 per cent leaf area mildewed and 7=>76 per cent leaf area mildewed. The disease severity was computed by following formulae (Biswas *et al*., 1993).

$$\text{Disease severity (\%)} = \frac{\text{Sun of all numerical values}}{\text{Number of leaves scored x maximum disease category}} \times 100$$

Statistical analysis:

The data were subjected for analysis of variance (ANOVA) using INDOSTAT and the means were compared for significance difference.

RESULTS AND DISCUSSION

The *in vitro* conidial germination study showed significant influence of phylloplane bacteria and the hours after inoculation on suppression conidial germination (Table 1). Among eighteen bacterial isolates ten bacterial isolates significantly (P<0.05) reduced conidial germination. Among these, four isolates highly (P<0.01) reduced the conidial germination suppressing conidial germination more than 70 per cent. The highest reduction was showed by the isolate Pb-5 (13.87%) by suppressing 86.13 per cent followed by Pb-4 with conidial germination of 17.23 per cent suppressing 82.17 per cent. Similarly in presence of two isolate Pb-12 (21.06%) and Pb-15 (25.28%) also the conidial germination was lesser suppressing 78.94 per cent and 74.72 per cent, respectively. Higher conidial suppression was found 72hr after inoculation (74.94%).

Various phylloplane bacterial isolates influenced the seed germination of mulberry (Table 2). Among the bacterial isolates, six isolates significantly increased the seed germination. Highly significant (P<0.01) seed germination (93.44%) was obtained with the treatment of Pb-6 followed by Pb-7 (86.22%), Pb-3 (86.22%) and Pb-4 (85.47%). However, biopriming with twelve isolates resulted less germination compared to control (82.46%) with least germination in Pb-9 (70.22%).

Table 1: Influence of phylloplane bacteria on conidial germination of *P. corylea*

Bacterial isolates	Conidial germination (%)							
	24-hr		48-hr		72-hr		Total	
Pb-1	37.00	(37.46)	64.03	(53.15)	76.39	(60.93)	59.56	(50.51)*
Pb-2	74.05	(59.37)	78.91	(62.66)	90.62	(72.17)	81.78	(64.73)
Pb-3	62.72	(52.37)	71.24	(57.57)	75.25	(60.17)	69.86	(56.70)*
Pb-4	11.50	(19.82)	17.10	(24.42)	23.99	(29.33)	17.23	(24.52)**
Pb-5	7.44	(15.83)	15.47	(23.16)	20.05	(26.60)	13.87	(21.86)**
Pb-6	69.39	(56.41)	77.49	(61.67)	81.77	(64.73)	76.40	(60.94)*
Pb-7	70.98	(57.40)	78.02	(62.04)	85.83	(67.89)	78.60	(62.44)
Pb-8	76.14	(60.76)	82.95	(65.61)	88.68	(70.33)	82.89	(65.57)
Pb-9	80.26	(63.62)	85.99	(68.02)	95.08	(77.18)	87.86	(69.61)
Pb-10	75.01	(60.00)	82.25	(65.08)	89.34	(70.94)	82.60	(65.34)
Pb-11	68.23	(55.69)	77.51	(61.69)	80.69	(63.93)	75.66	(60.44)*
Pb-12	13.94	(21.92)	23.56	(29.04)	26.50	(30.98)	21.06	(27.31)**
Pb-13	79.33	(62.96)	86.18	(68.18)	93.21	(74.89)	86.77	(68.67)
Pb-14	61.90	(51.89)	71.16	(57.52)	74.29	(59.54)	69.23	(56.31)*
Pb-15	18.55	(25.51)	26.98	(31.29)	30.87	(33.75)	25.28	(30.18)**
Pb-16	68.81	(56.05)	76.99	(61.34)	81.13	(64.25)	75.82	(60.54)*
Pb-17	72.43	(58.33)	82.29	(65.12)	84.78	(67.04)	80.08	(63.49)
Pb-18	76.60	(61.07)	84.88	(67.12)	90.70	(72.25)	84.50	(66.81)
Control	76.74	(61.16)	84.07	(66.47)	90.82	(72.36)	84.31	(66.67)
Mean	57.56	(49.35)*	67.63	(55.32)	74.94	(59.96)*		

Figures in parenthesis are arcsin transformed values C.D.: *P<0.05; **P<0.01

Table 2: Effect of Phylloplane bacteria on seeding growth of mulberry

Bacterial isolates	Germina-tion (%)	Root length	Shoot length	Total length	Vigour index
Pb-1	82.41	2.467*	2.189*	4.656**	383.70**
Pb-2	79.62	1.033	1.533	2.567	204.38
Pb-3	86.22**	2.022*	2.078*	4.100**	315.91*
Pb-4	85.47**	2.089*	1.189	3.278	299.83
Pb-5	84.25*	1.489	2.333**	3.822*	322.00*
Pb-6	93.44**	1.878	1.511	3.389*	316.67*
Pb-7	86.22**	1.622	1.700	3.322	286.42
Pb-8	74.21	2.140*	1.524	3.664*	158.81
Pb-9	70.22	1.860	1.648	3.508*	130.61
Pb-10	80.24	1.356	1.367	2.722	218.41
Pb-11	82.14	0.844	1.922	2.767	227.28
Pb-12	85.22*	1.600	2.622**	4.222**	359.80**
Pb-13	80.24	2.000	1.189	3.189	255.89
Pb-14	76.39	1.967	1.411	3.378*	258.05
Pb-15	74.12	1.956	2.511**	4.467**	331.09**
Pb-16	78.44	1.556	1.976*	3.522	276.27
Pb-17	74.29	1.480	1.522	3.004	109.95
Pb-18	82.14	2.111*	1.767	3.878*	318.54*
Control	82.46	1.833	1.436	3.269	269.56

C.D.: *P<0.05; ** P<0.01

Highest root length (2.47mm) was obtained due to biopriming with isolates Pb-1 followed by Pb-18 (2.11mm) and Pb-4 (2.089mm) and least (0.844mm) in case of seedlings obtained from Pb-11 bioprimed seeds. Likewise, highly significant (P<0.01) elongation of shoot was found in the seedlings obtained after biopriming with the isolates Pb-12 (2.622 mm), Pb-15 (2.51 mm) an Pb-5 (2.33 mm). The shoot length was found least (1.18 mm) in case of the seeds treated with isolates Pb-4 and Pb-13. The seedling length was significantly high (P<0.01) in case of seedlings obtained from seeds bioprimed with isolate Pb-1 (4.66 mm) followed by Pb-15(4.47 mm), Pb-12 (4.22 mm) and Pb-3(4.10 mm). The total seedling length was found less than that of control (3.27 mm) in case of seedlings obtained from three isolates with least (2.57 mm) due to isolate Pb-2. The bacterial biopriming influenced the vigour index of mulberry seedlings. Biopriming with three bacterial isolates Pb-1 (383.70), Pb-12 (359.80) and Pb-15 (331.09) resulted highly significant (P<0.01) seedling vigour (Table 2).

The four efficient bacterial isolates selected were further characterized morphologically and based on biochemical reactions showed (Table 3) that these bacterial isolates are *Bacillus megaterium* (Pb-1) *Bacillus subtilis* (Pb-1) *Bacillus cereus* (Pb-1) and *Pseudomonas aeruginosae* (Pb-1). Highly significant (P<0.01) reduction in disease severity was observed due to application of all four bacterial isolates. The severity was less (20.67%) due to the application of *B.megaterium* (Fig.1) and *B.cereus* (22.19%) and high in control plants (56.54%). Bacterial isolates *B.megaterium* (63.42%) and *B.cereus* (60.73%) controlled >60% disease compared with the un treated control (Fig. 2).

Table 3: Biochemical characterization of antagonistic bacterial isolates

Biochemical tests	*B. megaterium* (Pb-1)	*B. subtilis* (Pb-5)	*B.cereus* (Pb-12)	*P. aeruginosae* (Pb-15)
Basic characters				
Gram	+	+	+	-
Shape	Rod	Rod	Rod	Rod
Pigment	-	-	-	+
Motility	+	+	+	+
Catalase	+	+	+	+
Oxidase	+	-	-	+
Methyl Red	-	-	-	-
Voges Proskauer	-	+	+	-
Citrate utilization	+	+	+	+
Indole production	-	-	-	-
Nitrate reduction	+	+	+	+
Casein hydrolysis	+	+	+	
Gelatin hydrolysis	+	+	+	+
Substrate utilization				
Starch	+	+	+	-
D-Glucose	+	+	+	-
D-Xylose	+	+	-	-
D-Arabinose	+	+	-	-
Lactose	+	+	-	-
Sucrose	+	+	+	-
Fructose	+	+	+	-
Galactose	+	+	-	-
Maltose	+	+	+	-
D-Mannose	-	+	-	-
D-Mannitol	+	+	-	+

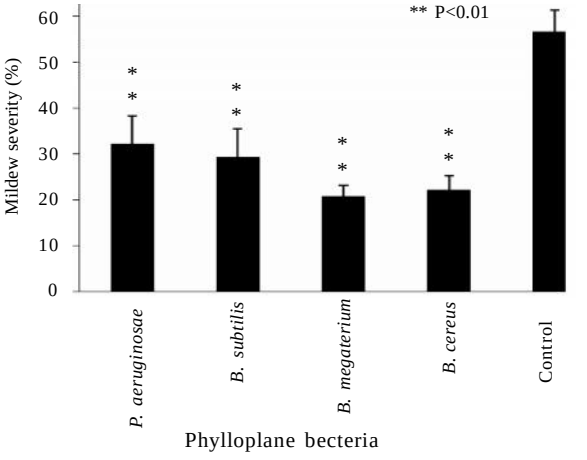

Fig. 1: Effect of selected bacterial isolates on severity of powdery mildew

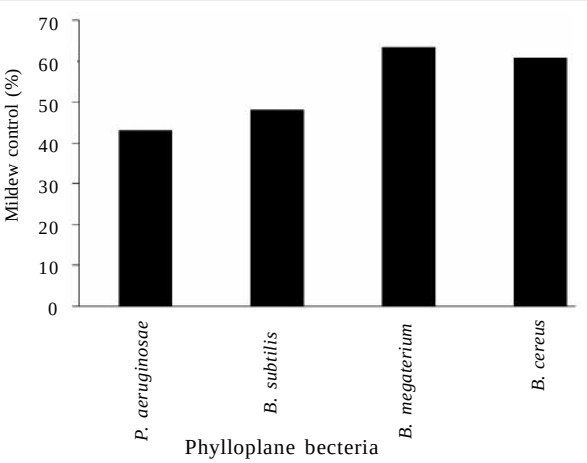

Fig. 2: Effect of selected bacterial isolates on control of powdery mildew

Phylloplane bacteria have ability and protective action as leaf surface organism against foliar pathogens (Saikaia and Chowdhury, 1993). These microbial communities may produce antibiotic compounds and show competition for resources (Braun *et al*., 2010). Also, an indirect mechanism that was found to involve in plant protection by beneficial bacteria is plant mediated induced systemic resistance (Deepika *et al*., 2017). In the present study, among four effective isolates, three were identified as bacillus. Antagonistic activity of several bacillus species against plant pathogenic fungi was reported mainly due to production of different antibiotics (Baker and Cook, 1982). Similarly, species of pseudomonas also reported to have antagonistic to plethora of fungal pathogens and growth promotion activity (Ananthi *et al*., 2014 and Sandhya *et al.,* 2018). In the present study, mulberry seed germination was varied and biopriming with few isolates resulted significantly higher seed germination. The pre germination metabolites get activated on priming the seeds (Farooq *et al*., 2009) and help in the seeds to emerge faster than the unprimed seeds. It was reported that seeds bio primed with gram positive cocci gave the highest germination rate and improved seedling emergence in response to external stress factor (Lautenberg and Dekkers, 2002). It has been reported that the biopriming agent may multiply substantially on seed during biopriming and the effect on seed germination and seedling growth depends on the type of bacterial strain used for the seed treatment (Bhatt *et al*., 2015). This may be the reason for less germination in few cases. The potential of the result of present study is in corroboration with observation made by Maji *et al*. (2003b) wherein the suppressive effect of several phylloplane bacterial isolates were reported against powdery mildew. Further Pseudomonads are reported to induce systemic resistance in mulberry against *P. coryle* (Pratheesh Kumar and Sivaprasad, 2017).

The present findings highlight the holistic approach which emphasizes the significant use of potential phylloplane bacteria to control the powdery mildew. These phylloplane bacteria are eco-friendly, influence the growth and improve vigour could be exploited as alternative to chemical plant protectants.

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