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Biocontrol potential of phylloplane bacterial antagonist against chilli anthracnose and powdery mildew disease

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Abstract

Studies on the nature of phylloplane microflora of chilli yielded two new isolates each of *Pseudomonas fluorescens* (CPf1 and CPf2), *Bacillu subtilis* (CBs1 and CBs2), and *Streptomyces roschi*.(CSr1 and CSr2).The efficacy of the newly identified isolates of *P. fluorescens, B. subtilis* and *S. roschi* was very less when compared to the existing strain Pf1 of *P. fluorescens,* Bs1 strain of *B. subtilis* and Sr1 strain of *S. roschi* . Based on the studies on efficacy and antagonism, Pf1 of *P. fluorescens* and Bs1 strain of *B. subtilis* were selected for further studies.

Key words: Anthracnose- Powdery mildew- spore germination-germtube elongation

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Introduction

Chilli (*Capsicum annum*) is the fourth most important vegetable crop of the world and first in Asia, with world production of approximately 122.34 million tonnes of fresh chilli and 2.8 tonnes of dry chilli in 2010. The most important producers and exporters of chilli include China, India, Mexico, Morocco, Pakistan, Thailand and Turkey. Demand for chilli in the world is increasing every year. Chilli is a very remunerative spice crop of the Indian subcontinent (Sharma *et al.,* 2005) and occupies an area of about 0.81 million ha which accounts for 25% of the world production (Chandra Nayaka *et al*., 2009). In Tamil Nadu, chilli is cultivated on 49.0 thousand hectares of land with 31.8 thousand tonnes of production. Chilli not only meets domestic consumption but also helps in earning foreign exchange. One of the great challenges facing the world is to produce adequate food for the growing population. Under these circumstances, one third of the global food production is estimated to be destroyed annually by over 20,000 species of insects, diseases, weeds, mites, nematodes, rodents and other field storage fungi and pests (McEven,1978).

The diseases generally cause economic loss in high plains but it can be severe in warm humid environments. Disease occurs when spores land on the leaves or stems, germinate in the presence of free moisture and directly penetrate and infect the plants. Plants are most susceptible, and infection begins at flowering and continues till maturity.

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Unsuccessful attempt to control the anthracnose and powdery mildew diseases in chilli by using chemical methods, and their dire consequences of environmental pollution, residual toxicity, development of resistance by pathogen, cost ineffectiveness have lead to search for alternate methods. Successful use of biocontrol agents in managing other plant disease impressed to test their efficacy in managing the anthracnose and powdery mildew diseases in chilli under laboratory condition. Application of antagonistic phylloplane bacteria has been probably more successful for the control of plant pathogenic bacteria rather than fungi. Ecofriendly management of disease involves the use of two or more methods to reduce disease incidence as integrated best management practices, which need a good knowledge of disease epidemiology, applicability of control option and the predisposing factors. Biocontrol, can be used to produce an effective ecofriendly disease management strategy, which needs thorough understanding of antagonistic reactions that could suppress the pathogens. The present article deals with the characterization of bacterial strains isolated from the phylloplane of chilli and their antagonistic potentialities against *C. capsic* and *L. taurica.*

MATERIALS AND METHODS

Isolation of phylloplane microflora of chilli leaves

Phylloplane microflora of chilli was estimated by using the modified 'leaf washing technique of Dickinson (1971). Leaf samples were collected from five locations in Coimbatore district. Discs of 4 mm diameter each were cut randomly from five leaves with the help of sterile cork borer. Fifty discs were placed in

250 ml conical flask containing 100 ml sterile distilled water and shaken for 20 minutes to get homogenous suspension of the microbial propagules. One ml of this suspension was pipetted out separately into the Nutrient agar medium, King's B agar and Kenknight's agar and mixed thoroughly. The plates were incubated at room temperature (28 $\pm$ 2 ^{0} C). The plates were observed regularly for the growth of microflora. The bacterial colonies were counted, purified sub cultured and maintained in King's B (*Pseudomonas fluorescens*), Nutrient agar (*Bacillus subtilis*) and Kenknight's agar *Streptomyces roschi*. Different microflora were identified using different confirmative tests and the cultures were maintained for further studies.

Identification of bacterial isolates

Characterization of the different cultures of bacteria was done according to the methods recommended in the laboratory manual for the identification of plant pathogenic bacteria, published by the American Phytopathological Society (Schaad, 1988) and for each test 24 to 48h old cultures were used.

Biochemical tests for *Pseudomonas* sp

Gram staining

Twenty four hour old culture of the bacterium was used for Gram staining.

KOH test

A loopful of bacterial culture was put on a clean glass slide. One drop of 3 % KOH solution was placed over it and thoroughly mixed with the help of a needle. Bacterial chromosomes separated out as thin threads, indicated Gram negative bacteria.

Oxidase test

Twenty four hour old bacterial cultures were spot inoculated on oxidase disc and change in colour of the disc from white to purple or blue was observed.

Starch hydrolysis

Nutrient agar containing 0.2 per cent soluble starch was used. The test cultures were spotted on the Petri plates. Starch hydrolysis was tested after 48 h of incubation by flooding the agar surface with Lugol's iodine solution. Formation of a colourless zone around the bacterial growth in contrast to the blue background of the medium indicated positive reaction.

Pyocyanin and Fluorescein test (King *et al*., 1954)

Pseudomonas agar F (casein enzymic hydrolysate, 10 g; protease peptone, 10 g; K_2HPO_4, 1.5 g; $MgSo_4$, 1.5 g; agar, 15 g; distilled water, 1000 ml) favours the formation of fluorescein whereas *Pseudomonas* agar P (peptone, 20 g; $MgCl_2$, 1.4 g; K_2So_4, 10 g; agar, 15 g; distilled water, 1 l) stimulates the pyocyanin production and reduces fluorescein formation (King *et al.,* 1954). All the isolates of fluorescent pseudomonads were tested for the production of fluorescein and pyocyanin.

Biochemical tests for *Bacillus* sp

Gram staining

Twenty four hour old culture of the bacterium was used for the staining. Gram staining was employed to study the Gram reaction.

Catalase test

Smears of 24 h old bacterial cultures were prepared on clean slide and covered with a few drops of three per cent hydrogen peroxide. Effervescence indicated the presence of catalase in the culture.

Anaerobic growth

Bacterial cultures were inoculated into the tube containing glucose broth and incubated in an anaerobic jar. Alternatively the broth was overlaid with sterile mineral oil and incubated at 24°C for observing the bacterial growth.

Voges- Proskaeur test

Forty eight hour old cultures were inoculated into five ml of the nutrient agar (NA) broth dispersed in test tubes. After an incubation period of seven days, 0.6 ml alpha naphthol solution (5% in 95% alcohol) and 0.2 ml of 40 % aqueous solution of KOH were added to one ml of the culture. The mixture was shaken for few minutes and allowed to stand for two hours. A crimson colour indicated positive test.

Utilization of citrate

The bacterial culture was streaked on the surface of Simmon's citrate agar slant. Blue colour indicated the utilization of citrate. Original green colour indicated non-utilization of citrate.

Growth in NaCl

The culture was inoculated into the tube containing nutrient broth supplemented with 3, 5 and 17 % concentration of sodium chloride and observed daily for the growth up to seven days.

Starch hydrolysis

Nutrient agar containing 0.2 per cent soluble starch was used. The test cultures were spotted on the Petri plates. Starch hydrolysis was tested after 48 h of incubation by flooding the agar surface with Lugol's iodine solution. Formation of a colourless zone around the bacterial growth in contrast to the blue background of the medium indicated positive reaction.

Biochemical characterization of *Streptomyces* sp

Biochemical characteristics of isolated actinomycetes was determined by the methods described by Shirling and Gottlieb (1966). All tests were performed at room temperature (28 ± 2°C).

Gelatin hydrolysis (Collins *et al.*, 1995)

The isolate was tested for the production of gelatinase, which is a proteolytic exo enzyme and capable of hydrolyzing gelatin. Solidified gelatine agar plates were streaked with the actinomycetes isolates and incubated for 5 days at 30°C. Hydrolysis was confirmed by flooding the plates with mercuric chloride solution.

Casein hydrolysis (Brown, 2009)

The isolate was streaked on skim milk agar plates and incubated at room temperature for 5 days. Hydrolysis of casein was confirmed by flooding the agar with mercuric chloride solution and the plates were observed for the presence of clear zone surrounding the colonies and considered for positive reaction**.**

Hydrogen sulfide production test

Sulfide indole motility (SIM) agar deep tubes were stab inoculated with actinomycetes isolates and incubated at 35°C for 4-5 days. Formation of black colour along the line of stab inoculation indicated H_2S production. Hydrogen sulphide production was carried out according to (Cowan, 1974).

Indole production test

The actinomycetes isolates were inoculated into glucose tryptone broth and incubated for 5 days. About 0.3 ml of Kovac's reagent was added and mixed well. After incubation, the reddening of the alcohol layer within a few minutes indicated indole production by the culture.

Methyl red Voges Proskauer test

MR-VP tests were performed to differentiate bacteria that produce acid from those that produce acetoin, a neutral product. The actinomycetes isolates were inoculated into MR-VP broth and incubated at 35°C for 4-5 days. The positive Methyl red test was indicated by change in colour of broth from yellow to red by the addition of methyl red indicator after incubation. Positive response of Voges Proskauer test was indicated by the development of red colour in MR-VP broth by addition of Baritts' reagent.

Urease test

Urease test of actinomycetes isolates was performed on urea agar containing the pH indicator phenol red. The actinomycetes isolates were inoculated and incubated for 5 days. The development of red colour in the broth indicated the positive reaction for the test

***In vitro* screening of bacterial antagonists against *C. capsici* (Vidhyasekaran *et al.*, 1997)**

Two native isolates each of *P. fluorescens, B. subtilis and S. roschi* were screened along with *P. fluorescens* - Pf1 (TNAU strain) *and B. subtilis* - Bs1(TNAU strain) and *S. roschi*- Sr1 (TNAU strain) received from the Department of Plant Pathology, TNAU, Coimbatore. The bacterial cultures were streaked 1cm away from the edge of the plate on each PDA medium. A nine mm mycelial disc of *C. apsici* was placed to the most distal point of the Petri plate perpendicular to the bacterial streak. The plates were incubated at room temperature (28 ± 2°C) for four days and the mycelial growth of the pathogen and inhibition zone were measured.

Effect of bacterial antagonists on the conidial germination and germ tube growth of *L. taurica*

The culture filtrates of the bacterial antagonists were used for assessing their antagonistic activity against the conidial germination and germ tube growth of

RESULTS AND DISCUSSION

Table.1. Biochemical characterization of *Pseudomonas fluorescens* and *Bacillus subtilis* isolated from phylloplane of chilli plant

Bacterial isolate	KOH test	Pigment production	Anaerobic growth	Oxidase test	Starch hydrolysis	Pyocyanin test	Fluorescein test	Catalase	Voges-Proskaeur test	Utilization of citrate	Growth in Nacl
Pseudomonas fluorescens											
CPf 1	+	+	+	+	+	+	+	-	-	-	-
CPf 2	+	+	+	+	+	+	+	-	-	-	-
Bacillus subtilis											
CBs 1	-	-	+	-	+	-	-	+	+	+	+
CBs 2	-	-	+	-	+	-	-	+	+	+	+

L.taurica. The culture filtrates of *P. fluorescens* isolates, *B. subtilis* and *S. roschi* were obtained by growing these organisms in King's B , Nutrient broth and Kenknight's broth respectively at room temperature, 28 ± 2°C, for 48 hours. The culture filtrates were centrifuged at 5000 rpm for 20 minutes at 4°C for clarification. The efficacy of culture filtrates was tested by using the cavity slide technique.

Table. 2. Biochemical characterization of *Steptomyces roschi* isolated from Phylloplane of chilli plant

Bacterial isolate	H_2S production	Nitrate reduction test	Urease test	Casein hydrolysis	Gelatin hydrolysis	Starch hydrolysis	Methyl red test	Voges proskauer test	Indole test
Steptomyces roschi									
C Sr 1	+	+	+	+	-	+	-	-	-
C Sr 2	+	+	+	+	-	+	-	-	-

Table.3. Phylloplane microflora of chilli leaves collected from different areas of Coimbatore district

Location	*Pseudomonas fluorescens*	*Bacillus subtilis*	*Streptomyces roschi*
Coimbatore (TNAU)	-	-	-
Coimbatore (Perur)	CPf 1	-	-
Coimbatore(Othakal mandabam)	-	-	
Coimbatore (Mathampatti)	-	C Bs 1	C Sr 1
Coimbatore (Lakshmipuram)	CPf 2	C Bs 2	C Sr 2

Studies on the antagonistic effect of phylloplane microflora of chilli

In order to find out the nature of micro flora present in chilli leaves, samples were collected from five locations and observations were recorded on the nature of the microflora. The study resulted in identification of 2 strains of *P. fluorescens* (CPf1 and CPf2), Two strains of *B. subtilis* (CBs1 and CBs2) and two strains of *Streptomyces roschi*.(CSr1 and CSr2).). In order to study the efficacy of bacterial antagonists obtained from chilli, a comparative study was conducted along with Pf 1 of *P.fluorescens,* BS1 of *B.subtilis* and Sr1 of *S.roschi,* maintained in the Department of Plant Pathology, Tamil Nadu Agricultural university, Coimbatore. The results indicated that the strains Pf1 and BS1 were found to be more inhibitory to the mycelial growth of *C.capsici* and spore germination of *L. taurica* when compared to the other new strains. As the newer strains were comparatively less effective *P.fluorescens* strain (Pf1) and *B.subtilis* strain (Bs1) were selected.

Table. 4. Antagonistic activities of different bacterial strains against the growth of *C. capsici*

Biocontrol agent	Radial mycelial growth (mm)	Per cent inhibition over control
Pf 1	39	59.17 [a]
		-17.56
CPf 1	58.33	34.40 [c]
		-35.91
CPf 2	65	26.96 [d]
		-31.24
Bs 1	43.12	51.55 [b]
		-45.86
CBs 1	44.14	50.40 [b]
		-45.23
CBs 2	70	21.34 [e]
		-27.49
Sr 1	63	29.21 [d]
		-32.71
CSr 1	71	20.22 [f]
		-26.71
CSr 2	65.67	26.21 [de]
		-30.79
Control	89	

*Mean of three replications
Values in parentheses are arcsine-transformed values
In the column, means followed by a common letter are not significantly different at the 5% level by DMRT

Similar reports on the inhibition of growth of *C.capsici* and *L.taurica* have been made. The mycoparasitic potential of *Pseudomonas* spp is well documented (Keel and Defago, 1997; Whipps, 1997). This phenomenon has often been used as means for *in vitro* screening of biocontrol agents (Hadar *et al*., 1979; Elad *et al*., 1980). Blakeman (1982) reported that when the bacteria *P. cepacia* and *B. mycoides* sprayed on the foliage at 7-14 days interval in 3 successive seasons reduced the *Alternaria* leaf spot of tobacco. The previous reports were made on the basis of studies on fluorescent *Pseudomonas* spp. EM 85 showed strong antagonistic effect against *R.solani*, causal agent of damping off, and promoted the plant growth of cotton. The inhibition of pathogen might be due to the production of antifungal metabolites. Production of antibiotics such as iturin, bacillomycin, zwittermycin A and surfactin is responsible for their antifungal action (Constantinescu, 2001; Hiradate *et al*., 2002; Yu *et al.,* 2002).

Table. 5. Effect of culture filtrates of different bacterial strains on conidial germination of *L. taurica*

Bacterial strains	Per cent germination *				Mean	Per cent reduction over control
	12h	24h	48h	72h		
Pf 1	12.24^{c}	42.00 ^{ab}	42.14 ^{c}	36.00 ^{b}	23.59	69.03
	-20.44	-40.4	-40.15	-36.87		
CPf 1	12.20^{c}	40.00 ^{b}	40.77 ^{b}	40.02 ^{c}	33.24	56.37
	-20.44	-39.23	-39.64	-39.23		
CPf 2	10.43^{b}	47.00 ^{c}	46.12 ^{d}	48.12 ^{d}	37.91	49.77
	-18.81	-43.28	-42.76	-43.91		
Bs 1	9.82^{a}	36.80 ^{a}	30.14 ^{a}	34.42 ^{a}	27.79	63.52
	-18.24	-37.35	-33.27	-35.91		
CBs 1	13.40^{d}	47.00 ^{c}	48.11 ^{c}	43.50 ^{d}	38	50.12
	-21.39	-43.28	-43.91	-41.27		
CBs2	15.69^{e}	53.34 ^{d}	50.76 ^{e}	40.14 ^{c}	39.98	47.52
	-23.26	-46.89	-45.4	-39.29		
Sr 1	16.80 ^{e}	53.60 ^{d}	54.83 ^{e}	50.29 ^{e}	43.75	42.57
	-24.2	-47.06	-47.75	-45.11		
CSr 1	15.57 ^{d}	50.54 ^{f}	53.12 ^{e}	50.12 ^{e}	42.33	44.44
	-23.18	-45.29	-46.78	-45		
CSr 2	15.40 ^{d}	52.00 ^{g}	56.12 ^{f}	50.10 ^{e}	43.4	43.03
	-23.11	-46.14	-48.5	-45.06		
Control	30.54 ^{g}	85.00 ^{h}	96.20 ^{g}	93.03 ^{f}	76.19	-
(Distilled	-33.52	-67.21	-78.76	-74.66		
water)						

*Mean of three replications
Values in parentheses represent arc sine transformed values.In the column, means followed by a common letter are not significantly different at 5% level by DMRT

Table. 6. Effect of culture filtrates of different bacterial strains on germ tube growth of *L. taurica*

Bacterial strains	Germ tube growth (8m)*			Mean	Percent inhibition over control
	24h	48h	72h		
Pf1	30.16 ^{c}	42.18 ^{c}	46.17 ^{c}	14.03	90.94
	-33.58	-40.45	-42.76		
CPf1	34.62 ^{b}	30.66 ^{b}	34.18 ^{b}	31.15	79.88
	-36.03	-33.58	-35.73		
CPf 2	30.12 ^{b}	79.12 ^{d}	80.00 ^{d}	63.08	59.27
	-33.27	-62.8	-63.43		
Bs1	24.12 ^{a}	20.48 ^{a}	30.14 ^{a}	24.91	83.91
	-29.4	-26.85	-33.27		
CBs 1	50.68 ^{d}	58.14 ^{cd}	60.18 ^{c}	56.33	63.62
	-45.34	-49.66	-50.83		
CBs2	57.00 ^{e}	80.18 ^{f}	83.12 ^{d}	73.43	52.58
	-49.02	-63.51	-65.73		
Sr1	42.00 ^{g}	70.88 ^{g}	79.18 ^{d}	63.99	58.74
	-40.4	-62.03	-62.8		
CSr 1	52.00 ^{f}	81.22 e	84.18 ^{d}	72.46	53.23
	-46.14	-64.3	-66.5		
CSr 2	47.34^{e}	76.12 d	76.00 ^{cd}	66.48	57.07
	-43.45	-60.73	-60.67		
Control (Distilled water)	90.66	`174.22	200	154.88	-

Figures in the parenthesis are arc sine transformed values.
The data are mean of three replications
In the column, means followed by a common letter are not significantly different at 5% level by DMRT

REFERENCE

Anthracnose disease of chilli pepper.*Asian Seed Health Centre Technical Bulletin.* P. 1-13.

Blakeman, J.P. 1982. Chemical environment of leaf surfaces with special reference to spore germination of pathogenic fungi. *Pestic.Sci.,*4: 575-588. https://doi.org/10.1002/ps.2780040415

Brown, A.E. 2009. Benson's Microbiological Applications, Laboratory Manual in General Microbiology, 11th ed. McGraw- Hill companies, New York, USA.

Chandra Nayaka, S., Udaya, A.C., Shankar, Niranjana, S.R., Prakash, H.S. and Mortensen,C.N.2009.

Collins, C.H., Lyne, P.M., Grange, J.M. and Falkinham, J.O. Microbiological methods. 8th Edition: Arnold Publication.

Constantinescu, F. 2001. Extraction and identification of antifungal metabolites produced by some *Bacillus*

subtilis strains.*Analele Institutului de Cercet ri pentru Cereale Protectia Plantelor,* 31:17-23.

Cowan, S.T. 1974. Cowan and Steel's Manual for the Identification of Medical Bacteria, 2nd edn. Cambridge: Cambridge University Press.

Dickinson, C.H. 1971. Cultural studies of leaf saprophytes. In:*Ecology of Leaf surface Microorganisms.*(Eds.). T.F. Preece and C.H. Dickinson Academic Press, London. P.129-137.

Elad, Y., Chet, I. and Katan, J. 1980. *Trichoderma harzianum*: A biocontrol agent effective against *Sclerotium rolfsii* and *Rhizoctonia solani*. *Phytopathology,* 70: 119-121 https://doi.org/10.1094/Phyto-70-119

Hadar, Y., Chet, I. and Hewis, Y. 1979. Biological control of*Rhizoctonia solani* damping off with wheat bran culture of *Trichoderma harzianum. Phytopathology,*69: 64-68 https://doi.org/10.1094/Phyto-69-64

Hiradate, S., Yoshida, S., Sugie, H., Yada, H. and Fujii, Y. 2002. Mulberry anthracnose antagonists (iturins) produced by *Bacillus amyloliquefaciens* RC-2. *Phytochemistry,*61: 693-698. https://doi.org/10.1016/S0031-9422(02)00365-5

Keel, C. and Defage, G. 1997. Interaction between beneficial soil bacteria and root pathogens.Mechanisms and ecological impact.In: *Multitrophic Interactions in Terrestrial systems.* (Eds.) A.C. Grange and U.K. Brown. Black Well Scientific Company, Oxford, P. 27-46.

King, E.O., Ward, M.K. and Raney, D.E. 1954.Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.,*44: 301-307.

Mc Even, F.L.1978. In: *Food Production-The challenges of pesticides*. *Bioscience*, 28: 773. https://doi.org/10.2307/1307252

Schaad, N.V. 1988. Laboratory guide for identification of plant pathogenic bacteria. 2nd ed. American Phytopathological Society, St. paul, MN, P. 158.

Sharma, P.N., Kaur, M., Sharma, O.P., Sharma, P. and Pathania, A. 2005. Morphological, pathological and molecular variability in *Colletotrichum capsici*, the cause of fruit rot of chillies in the subtropical region of north-western India. *J. Phytopathol*., 153: 232–237. https://doi.org/10.1111/j.1439-0434.2005.00959.x

Shirling, E.B. and Gottlieb, D. 1996. Methods for characterization of streptomyces species. 16(3); 313-340. https://doi.org/10.1099/00207713-16-3-313

Vidhyasekaran, P., Rabindran, R., Muthamilan, M., Nayar, K., Rajappan, K., Subramanian, N. and Vasumathi, K. 1997.Development of powder formulation of *Pseudomonas fluorescens* for control of rice blast.*Plant Pathol*., 46: 291-297. https://doi.org/10.1046/j.1365-3059.1997.d01-27.x

Whipps, J.M. 1997. Developments in the biological control of soil-borne plant pathogens.*Adv. Bot. Res.,* 26: 1–134. https://doi.org/10.1016/S0065-2296(08)60119-6

Yu, G.Y., Sinclair, J.B., Hartman, G.L. and Bertagnolli, B.L. 2002. Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*.*Soil Biol. Biochem.,* 34: 955-963. https://doi.org/10.1016/S0038-0717(02)00027-5