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Effect of different bio-agents and botanicals on leaf spot (*Colletotrichum capsici* Syd.) of betelvine plants (*Piper betel* L.) *in vitro*

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Abstract
Betel vine is popularly known as *Pan* in India. Pan is the local vernacular Hindi name used for leaves of betel vine plant (*Piper betel* L.). The present research work was conducted at Department of Plant Pathology, SHUATS, Allahabad, U. P. during 2016-2017. The result revealed that treatments *viz.*, T_0-Control (Inoculated), T_2-*T. harzianum*, T_3-*T. viride*, T_4-*Bacillus subtilis*, through Dual Culture and T_1-Copper oxychloride, T_5-Neem oil and T_6-Tobacco leaf extract leaf extract through poisoned food technique. Evaluated *in vitro* against leaf spot *C. capsici* of betelvine. While, per cent inhibition of the test pathogen ranged from 12.21 to 71.02 per cent. Among fungal bio-control agents maximum inhibition of 71.02 per cent was recorded with T_4-*Bacillus subtilis*, followed by T_3-*T. viride* (70.52%), T_2-*T. harzianum* (68.54%), T_4-*Bacillus subtilis* (28.55%), and T_5-Neem oil (62.19%) found maximum inhibition. However, the minimum inhibition of per cent was recorded on T_6-Tobacco leaf extract (12.21%).

Keywords: Anthracnose, *Bacillus subtilis*, betelvine (*Piper betel* L.), copper oxychloride, neem oil, tobacco leaf extract, *Trichoderma harzianum*, and *Trichoderma viride*

Introduction
The deep green heart shaped leaves of betel vine are popularly known as *Pan* in India. Pan is the local vernacular Hindi name used for leaves of betel vine plant *(Piper betel*.L.*).* It is a perennial creeper belonging to family Piperaceae. On account of its immense medicinal, social, religious and export value, betel vine is a cash crop of economic importance and is extensively grown on large scale in different parts of India. India is the largest producer and exporter of betel leaves in the world (Arulmozhiyan *et al.*, 2005) [1]. In India, Madhya Pradesh is the leading State in betal vine production. Approximately 15 different varieties are cultivated in MP. The vine is a shade loving perennial root climber. There are about 100 varieties of betel vine in the world, of which about 40 are found in India and 30 in West Bengal (Guha, 1997; Maity, 1989; Samanta, 1994). The most probable place of origin of betel vine is Malaysia (Chattopadhyay and Maity, 1967).
Betel leaf is traditionally known to be useful for the treatment of various diseases like bad breath, boils and abscesses, conjunctivitis, constipation, headache, hysteria, itches, mastitis, mastoiditis, leucorrhoea, otorrhoea, ringworm, swelling of gum, rheumatism, abrasion, cuts and injuries etc. as folk medicine while the root is known for its female contraceptive effects (Chopra *et al.*,1956; Khanra, 1997). Not only that, the betel leaves really do not have any match as a cheap, natural and easily available appetizer, digestive, mild stimulant, aphrodisiac and refreshing mastication. Chewing of betel leaves produce a sense of well-being, increased alertness, sweating, salivation, hot sensation and energetic feeling with exhilaration. It also increases the capacity to exercise physical and mental functions more efficiently for a longer duration but it may produce a kind of psychoactive effect causing a condition of mild addiction leading to habituation and withdrawal symptoms (Chu, 2001; Garg and Jain, 1996).
The pan leaves are affected by common agents which introduce microbial pathogens onto leaf surface, they are agency of wind, its direction and presence of microbial load, agency of water and its properties, soil, its properties and during post-harvest stages, factors like packaging material, moisture content and finally water in which the leaves are submerged before they are converted into betel quid. The leaves, which are subjected to these factors, develop leaf diseases like 'Leaf Spot' and 'Leaf Blight' resulting in economic loss to distributors, shop keepers and people involved in this trade (CSIR, 1984).

Out of the diseases, anthracnose caused by *Colletotrichum capsici* (Syd.) Butler and Bisby and bacterial leaf spot caused by X*anthomonas campestris* pv. *betlicola* **(**Patel, Kulkarni and Dhande) Dye, Phytophthora leaf and foot rot caused by *P. palmivora* and basal rot caused by *Sclerotium rolfsii* are the main yield limiting factors of the betelvine cultivation all over India. The pathogen *X. c.* pv. *betlicola* has been renamed as *Xanthomonas axonopodis* pv. *betlicola* (Vauterin *et al.*, 1995). The leaf spot of betelvine has been also reported to be caused by a fungus – bacterium complex (Bhale *et al*., 1985; Deka *et al*., 2005.). In such leaf spot, *C. capsici* is always associated with the bacterium *X. a.* pv. *betlicola*. Since, betel leaves are consumed fresh; considerable emphasis has been given to the less persistent and more eco-friendly means of managing betelvine disease. The diseases complex can be effectively controlled by the chemicals like 0.5% Bordeaux mixture or 0.1% copper – oxychloride (Yadav *et al*., 1993).

Materials and Methods

Experiments were laid out in Randomized block design with seven treatments including treated check, T_1-Copper oxychloride, T_2-*T. harzianum*, T_3-*T. viride*, T_4-*Bacillus subtilis*, T_5-Neem oil and T_6-Tobacco leaf extract and untreated T_0-Control (Inoculated) with three replicates during two consecutive years of 2016-2017 and 2017-2018 at laboratory, Department of Plant Pathology, Sam Higginbottom University of Agriculture Technology and Sciences, Allahabad.

For Pathogenicity test surface sterilized (0.1% $HgCl_2$) seeds of anthracnose susceptible betelvine Cv. JS-335 were sown (@ 10 seeds /pot) in the earthen pots (25 cm dia) filled with steam sterilized potting mixture of soil: sand: FYM (2:1:1). Five healthy growing betelvine plants per pot were maintained, watered regularly and kept in the screen house for further growth. The mass multiplication of test pathogen (*Colletotrichum capsici*) was done on the PDA in petri-plates. Spore suspension of the test pathogen was prepared by harvesting freshly sporulating 7-8 days old culture in plates by flooding with 5-10 ml sterile distilled water. The resultant spore-cum-mycelial suspension was filtered through double-layered muslin cloth and filtrate obtained was suitably diluted with sterile distilled water to get inoculum concentration of 3-5 x 10^{-6} spores/ml. Thirty days old seedlings of betelvine were artificially inoculated by spraying the conidial suspension (3-5 x 10-6 conidial/ ml) of the test pathogen with automizer. Seedlings sprayed with sterile water (without inoculum) were also maintained as suitable control. Inoculated plants were incubated in the screen house where high humidity (>80%) and optimum temperature (24±2 °C) were maintained for further development of anthracnose symptoms. Subsequently reisolation of the pathogen was done and identification and symptomatology were studied.

Poisoned Food technique

The fungicides and plant extracts amended PDA was poured (15 - 20 ml/plates) in sterilized Petri plates (90 mm dia.) under aseptic conditions. Each treatment with respective concentration was replicated thrice. On solidification of PDA in Petri plates, all treatment plates were inoculated / seeded aseptically by placing in the center with 5.0 mm uniform, mycelial disc obtained from 7 days old culture of *Colletotrichum capsici* multiplied on agar plates (Nene and Thapliyal, 1993) [15].

Dual culture technique

Disc (5 mm) of *Colletotrichum capsici* was placed at the center on a petri-plates containing solidified PDA medium and disc (5 mm) of *Trichoderma harzianum* was placed at opposite from center. A loopful of 24 hour old culture of *Pseudomonas fluorescens* was inoculated at 2 cm just opposite to the pathogen on each plate (Dennis and Webster, 1971) [14].

All the treatment (inoculated) and control petri plates where then incubated at 24 ± 2 °C in BOD incubator till the control plates were fully covered with mycelial growth of the test pathogen.

Observations on radial mycelial growth of *Colletotrichum capsici* were recorded in each treatment and replication and per cent growth inhibition of the test pathogen over control was worked out by following formula (Vincent, 1927).

$$\text{Percent inhibition (I)} = \frac{C - T}{C} \times 100$$

Where

C = Growth of test fungus (mm) in control plate,

T = Growth of test fungus (mm) in treatment plates

Results

The results obtained are being presented as follows. Identification of pathogen is done through a piece of sporulating mycelium which was mounted with lactophenol cotton blue and observed under the light microscope. Based on typical symptoms on foliage and pods, cutural characteristic of the fungus on PDA and microscopic observations recorded such as, mycelium - hyaline, septate and branched. Acervuli - the acervuli were oval to conical and appeared single. It was dark brown to black in colour and measured 181.0 X 275.5µ in size, with numerous black, needles like intermixed long and short setae. Conidia - single celled, smooth, hyaline, curved and measured 21 to 23.5 X 3.8 to 4.1 µ in size. Conidiophores were simple and elongated. The measurements were recorded with the help of stage and occular micrometer. The fungus was identified and confirmed with the help of available literature (Anisworth 1973) as *Colletotrichum capsici* Syd, causing anthracnose of betelvine.

Symptomatology

Initial symptoms of anthracnose on foliage were noticed at 125-130 DAT on betelvine crop. The most prominent symptoms occurred on foliage were brown coloured patches with gray coloured centre on upper surface and scorched appearance on the lower surface. In advance stage necrosis of leaf vein, leaf rolling, petiole canker, and defoliation occurred. Acervulli on infected leaf resembled small pinkish coloured patches surrounded by the minute blackish brown setae. Infected leaves finally dried out prematurely with shriveled.

In vitro evaluation of fungicides, botanicals and bioagents

Present study was undertaken for eco-friendly management of leaf spot (*Colletotrichum capsici* Syd.) of betelvine (*Piper betel* L.). Seven treatments *viz*., T_0-Control (Inoculated), T_2-*T. harzianum*, T_3-*T. viride*, T_4-*Bacillus subtilis*, through Dual Culture (Dennis and Webster, 1971) [14], and T_1-Copper oxychloride, T_5-Neem oil and T_6-Tobacco leaf extract by Poisoned food Technique were evaluated *in vitro* against *C.*

capsici, the results of which are presented in Table No.1 and 2 respectively. The result revealed that all species of bio-agents exhibited antagonistic effect against *C. capsici* and were found to inhibit significantly the radial growth of the pathogen over check control and T_1-Copper oxychloride (67.17%) and control (0.00%).

Per cent inhibition of the test pathogen ranged from 12.21 to 71.02 per cent. Among fungal bio-control agents, maximum inhibition of 71.02 per cent was recorded with T_4-*Bacillus subtilis*, followed by T_3-*T. viride*, (70.52%), T_2-*T. harzianum* (68.54%), T_5-Neem oil (62.19%), found maximum inhibition. The minimum inhibition of per cent was recorded on T_6-Tobacco leaf extract (12.21%). Similar results were reported by (Jayalakshmi *et al.* 1998), (Patel and Joshi 2001) [13], who found the maximum inhibition of *Colletotrichum gloeosporioides* by *Trichoderma viride*. Also the maximum per cent inhibition of C. gloeosporioides was achieved due to *T.viride* as earlier observed by (Haralpatil, 2005) for anthracnose of *Piper betle* (Patel and Joshi, 2001) [13] for leaf spot of turmeric and (Bhave, 2005) [4] for leaf spot of black pepper.

These results thus support the present findings. Recent works have shown that common plant disease such as root rot disease, wilt, fruit rot and other plant diseases can be managed by *Trichoderma* spp. (Begum *et al.*, 2010; El Komy *et al.*, 2015; Howell, 2002; Mbarga *et al.*, 2012) [3, 6, 8, 11]. The secondary metabolites secreted by *Trichoderma* spp. have proven its role in suppressing the growth of pathogenic microorganisms and stimulating the plant growth (Contreras-Cornejo *et al.*, 2015a, Contreras-Cornejo *et al.*, 2015b; Kubicek *et al.*, 2001; Kullnig *et al.*, 2000) [9, 10]. Besides, the interaction between plant and *Trichoderma* spp. successfully regulate root architecture, increase the length of lateral and primary root that result in the effectiveness of nutrient uptake by the plant (Cai *et al.*, 2013; Naseby *et al.*, 2000; Yedidia *et al.*, 2001) [5, 12].

Table 1: Antagonistic activity of bio-agent on *Colletotrichum capsici* by dual culture

Treatments	2nd day (24hr)		3rd day (48hr)		4th day (72hr)		5th day (96hr)		6th day (120hr)		Mean	
	Radial growth (mm)	Inhibition (%)	Radial growth (mm)	Inhibition (%)	Radial growth (mm)	Inhibition (%)	Radial growth (mm)	Inhibition (%)	Radial growth (mm)	Inhibition (%)	Radial growth (mm)	Inhibition (%)
Control (Inoculated)	8.33	00.00	19.17	00.00	28.50	00.00	37.33	00.00	44.67	00.00	28.07	0.00
T. harzianum	7.5	9.96	11.00a	42.62	0.00	100.00	00.00	100.00	00.00	100.00	4.03a	68.54
T. viride	7.00	7.92	11.67a	34.79	0.00	100.00	00.00	100.00	00.00	100.00	3.70a	70.52
Bacillus subtilis	6.33	24.01	15.83	17.42	00.00	100.00	00.00	100.00	00.00	100.00	3.73a	71.02
S. Ed. (±)			0.760		0.767		0.777		0.803		0.823	
C.D. (P = 0.05)			2.363		2.386		2.415		1.702		2.561	

Table 2: Antifungal activity of fungicide and botanicals on *Colletotrichum capsici* by food poisoned technique

Treatments	2nd day (24hr)		3rd day (48hr)		4th day (72hr)		5th day (96hr)		6th day (120hr)		Mean	
	Radial growth (mm)	Inhibition (%)	Radial growth (mm)	Inhibition (%)	Radial growth (mm)	Inhibition (%)	Radial growth (mm)	Inhibition (%)	Radial growth (mm)	Inhibition (%)	Radial growth (mm)	Inhibition (%)
Control (Inoculated)	8.33a	00.00	19.17a	00.00	28.50a	00.00	37.33a	00.00	44.67a	00.00	28.07	0.00
Copper oxychloride	7.67a	15.97	12.50a	39.12	0.00	100.00	0.00	100.00	0.00	93.15	4.68	67.17
Neem oil	5.83b	1.92	8.00c	11.32	8.83c	15.79	9.50c	12.48	9.83c	19.54	8.40	62.19
Tobacco leaf extract	8.17a	30.01	17.00a	58.27	24.00b	69.02	32.67b	74.55	37.83b	79.09	23.93	12.21
S.Ed. (±)	0.589		0.770		0.789		0.896		0.867			
C.D. (P = 0.05)	1.831		2.394		2.453		2.787		2.696			

Conclusions

Present research work concluded that the pathogen *Colletotrichum capsici* was found to be associated with anthracnose of betelvine and *in vitro* results were revealed that *Bacillus subtilis* grew quickly and inhibited pathogen mycelial growth by 71.02%, followed by *T. harzianum* (70.52%), but botanical Neem oil inhibited pathogen mycelial growth by 62.19%.

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