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Identification of the Causal Agent of Leaf Spot of Betelnut and *in vitro* Evaluation of Fungicides and Plant Extracts Against it

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Abstract: Naturally infected leaf samples of betelnut having characteristic symptoms of spots were collected from the campus of Khulna University, Bangladesh. The pathogen of this disease was identified on the basis of growth characters, acervuli production and conidial features on PDA medium as *Pestalotia palmarum*. The species was found pathogenic on excised leaves of the betelnut. Among the six fungicides tested *in vitro* Bavistin of three doses (100, 200 and 300 ppm) and Tilt 250 EC (100 and 200 ppm) were most effective in inhibiting radial growth of *Pestalotia palmarum*. Among the seven indigenous plant extracts tested *in vitro*, two doses (4 and 5%) of garlic (*Allium sativum*) extracts were found most effective in inhibiting the radial growth of the fungus.

Key words: Leaf spot, betelnut, fungicide, plant extract

INTRODUCTION

Betelnut (*Areca catachu*) is a perennial fruit plant belonging to the family Palmaceae and it is an important cash crop in Bangladesh. It is a part and parcel for the hospitality of most of the villagers and some cases for city people. It is mainly grown in the districts of Noakhali, Barisal, Khulna, Rangpur, Patuakhali, Bhola, Chittagong, Comilla, Sylhet and Jessore[1]. It is the most important crop for coastal areas of Bangladesh. Large gardens of betelnut may be found in those areas[1]. Every year leaf spot disease attacks the gardens and decreases the growth and development of the tree as well as the yield of the fruit. This disease is a serious problem in all over the betelnut growing regions of Bangladesh.

Tilt 250 EC, Cupravit-50 WP, Dithane M-45, Bavistin, Macuprax etc. have been recommended to use against control of gray leaf spot of coconut caused by *Pestaliopsis palmarum* in Bangladesh. Bavistin was the most effective fungicide against this pathogen[2]. But different plant extracts like Neem (*Azadirachata indica*), Tulshi (*Ocimum sanctum*), Garlic (*Allium sativum*) etc. have been recommended to use against *Pestalotia* spp.[3]. Because existing practice of chemical control is considered as costly and dangerous to public health. A large number of plants are known to possess anthelmintic properties[4]. In Bangladesh there is no considerable work on fungicides and plant extracts to control *Pestalotia palmarum* on betelnut. To avoid the environmental pollution as well as to minimize higher production cost and considering the facts mentioned earlier the research program was under taken to identify the pathogen of leaf spot of betelnut and to evaluate the response of fungicides and plant extracts against this pathogen.

MATERIALS AND METHODS

The experiments were conducted at Plant Protection Laboratory of Khulna University, Bangladesh in the year of 2001-2002.

Collection of sample: Diseased plant parts of betelnut (*Areca catechu*) showing typical leaf spot symptoms were collected from Khulna University campus, Bangladesh.

Isolation of the fungus: The fungus was isolated from the infected leaf of betelnut following standard procedures[5,6]. The infected diseased samples along with healthy tissues were cut into small pieces and surface sterilized by dipping in 0.1% sodium hypochloride (NaOCl) solution for two minutes. The treated plant tissues were washed three times with sterilized distilled water. Excess water was decanted by soaking with sterilized blotting paper. The cut pieces were then placed onto sterilized potato dextrose agar (PDA) in glass petridishes (20 mL/petridish) and incubated in a incubator at 28±1°C until mycelium formation. The hyphal tips were transferred onto PDA plate after growing the mycelium. The new plates were incubated at 28±1°C for conidia production.

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Purification and preservation: To obtain pure culture of the pathogen, the hyphal tips were transferred aseptically onto PDA plate by using the flame sterilized tip of an inoculation needle. The plate was incubated at room temperature for seven days. Advance hyphae were collected and transferred into the test tube slants containing PDA and incubated at room temperature for seven days. After incubation, the slants were carefully checked for contamination and then preserved at 4°C in a refrigerator for further use.

Identification of fungus isolates upto species: The fungus was identified on the basis of morphological characteristics suggested by Sutton[7], Gubap[8] and Singh[9].

Pathogenicity test of *Pestalotia* sp.: Healthy leaves of betelnut samples were cut into 4 cm^2 pieces and surface sterilized with 70% ethanol for ten second. Excess water was decanted by soaking with sterilized blotting paper. They were transferred onto sterilized water agar in sterilized petridishes. Sample pieces were injured softly by flame sterilized pointed needles. Advanced hyphae and acervuli were cut from the margin of pure cultures carefully with the help of flame burned cork borer and placed at three places (two at both margins and one at the center) onto the injured excised leaf of the host and incubated at 28±1°C for ten days. After seven days of incubation the samples were checked for appearance of characteristics symptoms.

Response of the identified *Pestalotia* spp. to six different fungicides: Six fungicides namely Dithane M-45 (Manganese ethylenebisdithiocarbamate), Vitavax-200 (5, 6-dihydro-2-Methyl-1, 4-oxathiin-3-carboxanilide), Bavistin DF (2-benzimidazolecarbamic acid, methyl ester), Rovral 50WP (3-(3,5-dichlorophenyl)-N-(1-methylethyl)-2, 4-dioxo-1-imidazolidinecarboxamide), Tilt 250EC (1-[2(2,4-dichlorophenyl)-4-propyl-1,3-dioxolam-2yl-methyl]-IH-1, 2, 4,-triazole) and Ridomil gold MZ 68WP (Manganous ethylenebisdithiocarbamate +N-(2,6-Dimethylphenyl)-N-(Methoxyacetyl)-alaninemethyl ester) were evaluated in *in vitro* against *Pestalotia* sp. following poison food technique[10]. Concentrations used for fungicide were 100, 200 and 300 ppm except Tilt 250 EC. Concentrations for Tilt 250 EC were 50, 100 and 200 ppm. Fungicidal suspensions were prepared by dissolving requisite quantities of each fungicide in warm PDA to have required concentrations. The fungicides were thoroughly mixed with the medium after autoclaving. About 20 mL of the medium was poured in each 9 cm sterilized petridishes. The medium without fungicide served as control. Mycelial discs were prepared using a cork borer (6 mm diameter) from the tip of five days old cultures of the isolates. One disc of the isolate was placed at the center of a petridish after solidification of PDA. Each treatment was replicated three times. The plates were incubated in an incubator at 28±1°C for five days. Fungal growth was measured by averaging the two diameters taken at right angles for each colony after five days of inoculation.

Percentage inhibition of the fungal growth of was recorded using the following formula:

$$\%\text{inhibition} = \frac{x-y}{x} \times 100$$

Where:
x = Growth of control plate
y = Growth of fungicide treated plate

Response of the identified *Pestalotia* sp. to seven different plant extracts: Plant extracts were prepared using leaves of neem (*Azadirachta indica*), bulb of onion (*Allium cepa*) and garlic (*A. sativum*), leaves of tulshi (*Ochimum sanctum*), rhizome of zinger (*Zingiber officinale*), leaves of betel vine (*Piper betel*) and marigold (*Tagetes patula*) were collected and extracts were prepared at 3, 4 and 5% for each case. To prepare the extracts from the different plant parts were macerated in distilled water and sap was collected with the help of mortar and pestle. Sap were centrifuged and filtered through filter papers. The filtrate was designated as standard suspension. Plant extract suspensions were prepared by dissolving requisite quantities of each plant extract in warm PDA to have required concentrations. The plant extracts were thoroughly mixed with the medium after autoclaving. After autoclaving about 20 mL of the medium was poured in each 9 cm sterilized petridishes. The medium without plant extract served as control. Inoculation, incubation and measurement of radial growth of the fungus were done using the same procedure as fungicides.

Experimental design and data analysis: This experiment was conducted in a laboratory with three replications following Completely Randomized Design. Data were analyzed by MSTAT-C computer program. Before analysis data of inhibition percentage was transformed by using arc sine transformation method. The significant difference, if any, among the means were compared by DMRT.

RESULTS AND DISCUSSION

Collection and identification of causal fungi: Characteristic symptoms of leaf spot of betelnut were recorded from the infected leaf samples as yellowish or

brownish or dark brownish spot surrounded by yellow halo. The centers of the spots were brown to black. Spots were more or less circular. In case of heavy infection blighting occurred and wither of the infected leaves. All the samples, upon incubation on PDA produced acervuli. The acervuli produced four celled conidia containing setulae. Both the ends of the conidia were pointed with hyaline lower end. On the basis of the characteristics studied in the experiment and by using standard keys, the fungus was identified as *Pestalotia palmarum*.

Pathogenicity test of *Pestalotia palmarum*: The species was found pathogenic on excised leaves of betelnut. The isolate was found pathogenic and produced characteristic symptoms of leaf spots on betelnut.

Response of *Pestalotia palmarum* to fungicides: Effect of fungicides against *Pestalotia palmarum* was significantly differed from the control in response of radial growth irrespective of concentrations at 1% level of significance (Table 1). All doses of Bavistin and 100 and 200 ppm of Tilt 250 EC inhibited 100% of radial growth of *P. palmarum*. These treatment combinations were significantly differed with other treatment combinations.

Second highest inhibition of radial growth (95.89%) was found in case of Rovral at 300 ppm and it was significantly different with other treatment combinations.

Ridomil and Dithane M-45 were poor performing fungicides *in vitro*. Lowest inhibition was found in case of Dithane M-45 at 100 ppm (26.0%) and it was statistically similar to Ridomil at 100 ppm but statistically different from the rest treatment combinations.

The results of this experiment support with the findings of other scientists. Islam[11], Dutta and Begum[12], Saw and Raut[13], Joshi and Raut[14], Kundalkar *et al.*[15], Selvan *et al.*[16] and Khalequzzaman *et al.*[2] reported that Bavistin (Carbendazin), Tilt 250 EC Cupravit and Dithane M-45 (Mancozeb) performed best against *Pestalotia palmarum in vitro*. In the present experiment the performance of Dithane M-45 was not good but Bavistin was effective in all the concentrations (100, 200 and 300 ppm) and Tilt 250 EC (200 and 300 ppm) completely inhibited the fungal growth. Therefore Bavistin and Tilt may be recommended in field trail for management of the disease caused by *P. palmarum*.

Response of *Pestalotia palmarum* to plant extracts: Effect of plant extracts against *Pestalotia palmarum* was significantly different from the control in response of radial growth irrespective of concentrations at 1% level of significance (Table 2). Garlic extract at 4 and 5% concentrations were found the best plant extracts, which inhibited 100% of radial growth of *P. palmarum*. They were significantly different from other treatment combinations.

Table 1: Effect of fungicides on radial growth of *Pestalotia palmarum*

	Inhibition percentage at different concentration		
Fungicide	100 ppm	200 ppm	300 ppm
Vitavax-200	42.56g	59.45e	62.55de
	(45.71)	(74.11)	(78.71)
Rovral	55.40f	64.67d	78.84b
	(67.79)	(81.42)	(95.89)
Bavistin	89.05a	89.05a	89.05a
	(100)	(100)	(100)
Ridomil	32.30ij	35.15i	40.44gh
	(28.53)	(33.12)	(42.04)
Dithane M-45	30.68j	38.57h	39.25gh
	(26.0)	(38.84)	(39.99)
*Tilt-250 EC	74.84c	89.05a	89.05a
	(93.13)	(100)	(100)

Figures in the parenthesis indicate original mean
* Indicates concentration of 50, 100 and 150 ppm
Values with same letter were not significantly different at the 1% level
Values without parenthesis is arcsine transformed value of percent data

Table 2: Effect of plant extract on radial growth of *Pestalotia palmarum*

	Inhibition percentage at different concentration		
Plant extract	3%	4%	5%
Garlic	40.59b	88.76a	88.76a
	(42.30)	(100)	(100)
Neem	14.03c	20.97c	20.97c
	(6.38)	(12.81)	(12.82)
Tulshi	14.63c	20.33c	15.09c
	(7.02)	(12.14)	(13.40)
Onion	1.259d	1.258d	1.256d
	(0)	(0)	(0)
Ginger	1.256d	1.256d	1.256d
	(0)	(0)	(0)
Betelvine	1.256d	1.256d	1.256d
	(0)	(0)	(0)
Marigold	1.256d	1.256d	1.256d
	(0)	(0)	(0)

Figures in the parenthesis indicate original mean
Values with same letter were not significantly different at the 1% level
Values without parenthesis is arcsine transformed value of percent data

The neem and tulshi 5%, inhibited 12.82 and 13.40% of radial growth, respectively. No inhibition of radial growth were found incase of all doses of Onion, Marigold, Betelvine and Ginger plant extracts. They were not statistically similar but were different from the rest treatment combinations. Neem (*Azadirachata indica*) and tulshi (*Ocimum sanctum*) inhibited germination of *Pestalotia psidii in vitro*[3]. In this experiment the performance of neem and tulshi was not good.

Garlic (*Allium sativum*) performed best against root knot nematode (*Meloidogyne javanica*) *in vitro*[17] and blight of sunflower (*Alternaria alternate*)[18].

Onoin (*Allium cepa*) accelerated the growth of (*Alternaria alternata*) *in vitro*[19]. In this experiment onion, marigold, betelvine and ginger have no inhibiting activity of fungus but increased the mycelial growth of fungus.

Garlic extract was found as an effective botanical to control the fungus. It will avoid environmental pollution as well as will minimize higher production cost. Garlic extract may be recommended for seed treatment of betelnut and for field trial.

The *Pestalotia palmarum* was identified from the naturally infected sample of betelnut and the pathogen was pathogenic. Bavistin and Tilt were effective fungicides against *P. palmarum* of betelnut. Garlic was effective plant extract against *P. palmaru* of betelnut. To maintain sound environment, garlic extracts can be used as biological control tool against *P. palmarum* of betelnut.

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