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Betel Vine Leaf Extract Inhibits Mildew Fungus of *Nyctanthes arbor-tristis* Growth under *in vitro* Conditions

[1]K. Sumangala Bhat, [1]Jose Thomas, [2]Vivek Kempraj, [3]A. Danesh and [3]B.Y. Sridhara
[1]Biowave Resources LLP, SMV Layout, Bangalore, 560110, India
[2]Division of Entomology and Nematology, Indian Institute of Horticultural Research, Bangalore, 560089, India
[3]Probiosys Center of Excellence, Automed Systems Pvt Ltd., KIADB Industrial Area 1st Phase, Kumbalagodu, Bangalore, 560074, India

Corresponding Author: K. Sumangala Bhat, Biowave Resources LLP, SMV Layout, Bangalore, 560110, India

ABSTRACT

Powdery mildews are a group of common pathogenic fungi infecting wide range of host plants, often causing serious damage and crop loss. Considering the indiscriminate application of synthetic fungicides and their hazardous environmental impact, development of safer alternatives became crucial. In an attempt to investigate the efficacy of betel vine leaf extract on powdery mildew of *Nyctanthes arbor-tristis*, *in vitro* culture method for the fungus has been developed and comparative efficacy of the extract on the pathogen has been evaluated with commercial fungicide, Bavistin. Biochemical profiling of the hexane extract of betel vine leaf by GC/MS has indicated the presence of 29 different compounds, with Safrole and eugenol as the major components. Successful propagation and confirmation of viability of the mildew under *in vitro* conditions have been established. Bioassay on mycostatic efficacy of the hexane extract of betel vine leaf on mildew has yielded MIC of 6%. The study has confirmed potential of betel vine leaf extract for development of safer alternative fungicide to regulate mildew disease of plants.

Key words: Powdery mildew, GC/MS, safrole, eugenol, MIC

INTRODUCTION

Powdery mildew is one of the serious diseases of plants caused by a group of fungi belonging to the phylum Ascomycota. They are known to infect wide range of plants including cultivated, wild and forest plants (Pathak *et al*., 1992). Some species of powdery mildew fungi grow on surfaces of all parts of the plant superficially or epiphytically whereas some are limited only on leaf surface. These fungi produce hyphae on upper and lower surfaces of host leaves. A few genera produce endophytic mycelium, while few others produce epiphytic mycelium. They infect all aerial parts of the host including fruits, flowers, stems and leaves (Heffer *et al*., 2006). Infection by powdery mildew results in mottled and yellowed leaves and retardation of growth and vigor of the plant because of the nutritional deficiency (Smith, 1994).

The possible way to resist the infections caused by mildew fungi include use of chemical fungicides like triazoles, pyramidines, polyoxins, phenylpyrolles, etc. and biological fungicides like ecoguard, Bio-Trek 22G, etc. (Rouabhi, 2010). Some of the common plants infected by powdery mildew include deciduous azaleas, buckeye, catalpa, cherry, dogwood, euonymus, honeysuckle,

lilac, privet, roses, serviceberry, maples, herbaceous plants like chrysanthemums, dahlias, delphiniums, kalanchoes, phlox, snapdragons and zinnias. Mildews often infect several horticultural crops such as grapes, vegetables and ornamentals causing huge commercial loss.

Modern fungicides are not capable to kill the fungi but inhibit their growth. Some of these fungicides cause harmful effects to the environment (Fairbrother *et al.*, 2007). Fungicidal residues accumulate in the soil and water causing toxic effects to other organisms (Harmsen, 2007). Entry and accumulation of fungicidal residues in the food chain is a possibility leading to health hazards (Brooks and Roberts, 1999). Fungicides like vinclozolin banned from use due to their mutagenic property (Hrelia *et al.*, 1996). Plant derived essential oils and extracts have been identified as suitable alternatives to resist the pests as well as for disease control (Brent and Hollomon, 1998; El-Zemity and Ahmed, 2005). Plants produce phytochemicals, a potential source of bioactive compounds. Essential oils and extracts of plants are major sources of useful phytochemicals (Bajpai and Kang, 2012). Several reports are available on effectiveness of phytochemical compounds on plant pathogenic fungi (Bajpai and Kang, 2010), soyabean oil against dogwood and so on (Bajpai and Kang, 2010; Deyton *et al.*, 2011).

Piper betle (betel vine) a perennial creeper contains varying amount of volatile oils (Sharma *et al.*, 1983). The plant has been used traditionally as anti oxidant, anti inflammatory, antiseptic, pancreatic lipase stimulant and wound healer (Saxena *et al.*, 2014). Reports from northern plains of Uttar Pradesh have confirmed antimicrobial activity of betel vine extracts against certain strains of bacteria and fungi (Sharma *et al.*, 1983). Therefore, this plant has been selected for the study. *Nyctanthes arbor-tristis* is a common flowering plant growing in gardens and temple premises (Sharma *et al.*, 2012) and infected by powdery mildew *Oidium braunii* in South India (Hosagoudar, 1984). Present study was aimed to investigate the inhibitory effect of hexane extract of betel vine leaf on mildew of *N. arbor-tristis* under *in vitro* condition.

MATERIALS AND METHODS

Isolation and *in vitro* culturing of mildew: Powdery mildew affected leaves of *N. arbor-tristis* were collected from the field and the spores were brushed and transferred to 10 mL of sterile distilled water for preparing spore suspension. Potato Dextrose Agar (PDA) medium was prepared and incorporated with grilled uninfected fresh leaves of the host plant, *N. arbor-tristis* at a concentration of 4% and gentamycin at a concentration of 1.3%. Mildew was inoculated by adding 1 mL of spore suspension with a concentration of 4×10^5 spores mL^{-1} to the petri plate containing modified PDA medium mentioned above. The inoculated media were kept for incubation at room temperature and kept under regular observation. On sporulation, the organism was stained using lacto phenol blue and identified as powdery mildew. Pure culture of the species was raised through standard protocol (Lennette *et al.*, 1985).

Validation of *in vitro* culture of mildew: Sub culturing of the mildew fungus was made through inoculating the spores on fresh culture medium for monitoring its growth. In addition, spores collected from pure culture were inoculated on lower surface of fresh uninfected leaves of *N. arbor-tristis*, surface sterilized and kept on sterile moist cotton pads in petri plates. The inoculated leaves were incubated at room temperature for confirming the *in vitro* growth and virulence of the fungus.

Phytochemical profiling of the betel vine leaf extract: The hexane extract of betel vine leaf was subjected to GC-MS analysis using a HP 6890 system (Agilent Technologies, USA) coupled with a mass selective detector (HP 5973; Agilent Technologies, USA) operated in electron impact mode (source temperature at 230°C; transfer line temperature at 250°C). HP 5 MS phenyl methyl siloxane non-polar capillary column (Length: 30 m; ID: 0.25 μm) was used. The mobile phase was Helium (99.9% pure; Praxair, India) with a flow rate of 1 mL min^{-1}. Split inlet with a split ratio of 50:1 and temperature of 280°C was set before injecting the samples. Oven temperature program was set to 70°C min^{-1} with 2 min hold and a ramp of 10°C min^{-1} till 260°C. The MS detector was maintained at 280°C. Mass spectra of detected compounds were compared using spectral libraries (Wiley 2012 and NIST 2012 versions).

***In vitro* bioassay for mycostatic activity of hexane extract of betel vine leaf:** Growth inhibitory activity of the betel vine leaf hexane extract on the mildew fungus under *in vitro* condition was evaluated by plate assay. Six sets of petri plates in triplicate containing modified PDA medium as described above were prepared and added with 1, 2, 4, 6, 8 and 10% leaf extract. Pure culture of the mildew was inoculated in to each plate and kept for incubation at room temperature. The fungicide bavistin was used as positive control at same concentration as that of the hexane extract. The solvent, isopropanol was used as negative control at same concentration.

RESULTS

***In vitro* culturing and validation of viability of mildew:** Mildew spore suspension inoculated in modified PDA medium exhibited appearance of colonies by 24 h of incubation. Initially small colonies of the fungus could be observed in the culture medium (Fig. 1a). After 48 h of incubation, cotton like appearance of white mycelia were found on culture media (Fig. 1b). On 72 h of incubation, the colonies turned light green in color with spores (Fig. 1c). Sub culturing of the mildew fungus in fresh culture medium showed growth of the fungus in same pattern as observed in original culture (Fig. 2a-b). Inoculation of the spores produced under *in vitro* conditions on fresh uninfected leaves of the host plant, *N. arbor-tristis* has resulted in appearance of infectious patches on under surface of the leaves kept on wet cotton pads. Cotton like cushion of mycelia are observed on the leaf surface (Fig. 2c).

Phytochemical profiling of the betel vine leaf extract: Phytochemical profile of the hexane extract of betel vine leaf has revealed a total of 29 different compounds as its components (Table 1). Safrole and eugenol have emerged as the abundant compounds.

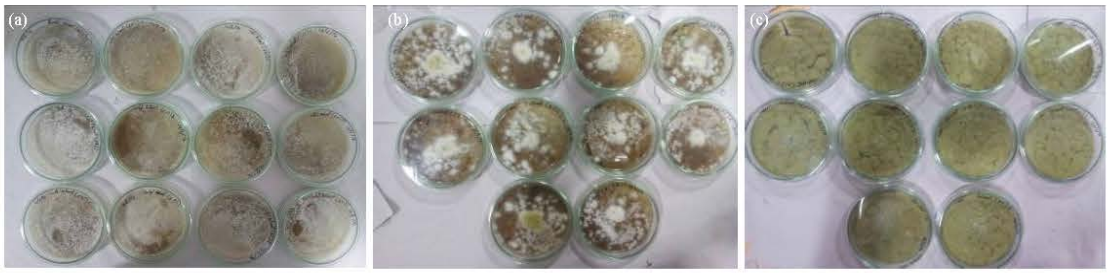

Fig. 1(a-c): Growth of mildew in culture medium at (a) 24, (b) 48 and (c) 72 h of incubation

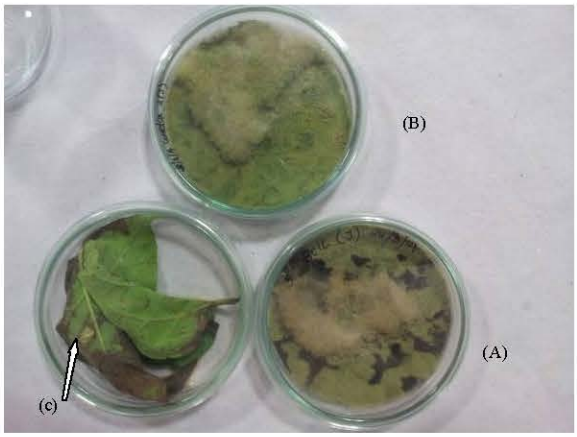

Fig. 2: Validation of mildew culture under *in vitro* conditions. A and B: Growth of fungus in subculture and C: Infection on leaf of *Nyctanthes arbor-tristis*

Table 1: Phytochemical components identified from hexane extract of betelvine leaf

Compounds/Chemicals	Area (%)	CAS No.	RT
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	0.01	28564-83-2	4.99
Phellandrene	0.13	99-83-2	5.45
Alpha-Terpinene	0.69	99-86-5	5.62
p-Cymene	0.62	99-87-6	5.74
Sabinene	1.33	3387-41-5	5.83
Gamma-Terpinene	1.14	99-85-4	6.41
o-Guaiacol	0.47	90-05-1	6.73
Linalool	0.95	78-70-60	6.87
Tujene	0.52	998109-42-7	7.23
Terpine-1-ol	0.23	586-82-3	7.54
Terpine-4-ol	2.52	562-74-3	8.14
Alpha-Terpineol	0.33	98-55-5	8.31
Safrole	17.22	94-59-7	9.88
Eugenol	15.36	97-53-0	10.80
Isoeugenol	0.11	5932-68-3	10.90
Alpha-Copaene	2.28	3856-25-5	11.03
Beta-Bourbonene	0.73	5208-59-3	11.17
Methyleugenol	1.96	93-15-2	11.27
Beta-Caryophyllene	2.20	87-44-5	11.63
Beta-Cubebene	0.59	13744-15-5	11.72
Gamma-Cadinene	0.37	39029-41-9	11.92
Alpha-Humulene	2.38	6753-98-6	12.06
Beta-Selinene	4.48	17066-67-0	12.52
Alpha-Selinene	5.06	473-13-2	12.63
Caryophyllene oxide	0.85	1139-30-6	13.73
Camphene	0.20	79-92-5	13.89
Germacrene B	0.15	15423-57-1	14.09
Longifolene	0.22	475-20-7	14.79
Phytol	0.52	150-86-7	18.97
Total	63.62		

Table 2: Determination of MIC of betel vine leaf extract on mildew

	1 (%)		2 (%)		4 (%)		6 (%)		8 (%)		10 (%)	
Sample	1	2	1	2	1	2	1	2	1	2	1	2
Betel vine extract	63.5	63.5	56.7	56.7	44.1	-	-	-	-	-	-	-
- Control	63.5	63.5	63.5	63.5	63.5	63.5	63.5	50.24	50.24	63.50	63.5	-
+ Control (bavistin)	63.5	63.5	63.5	63.5	50.2	50.2	12.5	15.80	9.61	7.06	4.9	8.03

Evaluation of mycostatic activity of betel vine extract: *In vitro* bioassay on mycostatic activity of the test sample of betel vine extract exhibited concentration dependent inhibitory effect on growth of the mildew fungus in culture medium. Results of the extent of inhibition observed at different concentrations of the test sample on fungal growth are presented in Table 2. Minimal inhibitory concentration of the test sample was found to be 6%. Bioassay result has further confirmed that positive control, Bavistin and negative control could not prevent growth of the mildew even at the concentration of 10%. Therefore, effectiveness of the leaf extract against mildew is comparatively much higher than the commercial fungicide Bavistin.

DISCUSSION

Powdery mildews are obligate plant pathogens and considered to be difficult to grow under *in vitro* conditions. However, very few reports are available on establishing successful cultures of mildews in culture media in the laboratory (Arabi and Jawhar, 2002; Tu *et al*., 2012; Cai *et al*., 2013) (Patent No. CN 102002464). Current study has demonstrated and confirmed the *in vitro* culturing of the powdery mildew of *N. arbor-tristis* in PDA medium supplemented with fresh uninfected leaf tissue of the plant and confirmed *in vitro* propagation of the fungus through sub culturing the pathogen. Validation of the *in vitro* propagation of the fungus has been demonstrated through successful re-infection of the fresh un-infected leaves of the host in laboratory, confirming viability and virulence of the cultured mildew.

Mildew disease is one among the chronic plant diseases warranting effective control measures in agricultural systems, grapes being the most severely affected crop. The control strategies employed for phytopathogenic fungi are dominated by application of chemical fungicides. Bavistin represents an extensively used broad-spectrum commercial fungicide on fruits and vegetables. Considering the high levels of health risk associated with application of chemical fungicides on fruits and vegetables, development of safe and eco-friendly products for the protection of such crops becomes crucial. Antimicrobial property of two varieties of betel vine leaf oil has been reported based on comparative analysis of the chemical profile and *in vitro* bioassay on bacteria and fungi by Saxena *et al*. (2014), where eugenol has been identified as the abundant and common compound responsible for the activity. Current study has confirmed safrole and eugenol as the abundant compounds in betel vine leaf extract. These compounds have been known to exhibit antimicrobial and insecticidal activities (Pineda *et al*., 2012; Bhat and Kempraj, 2009). Current study has established the mycostatic role of these compounds in synergy with other compounds found in the leaf extract. Investigations on *in vitro* culturing and inhibitory effect of commercial fungicides or natural products are much limited. Bajpai and Kang (2012) have demonstrated *in vitro* and *in vivo* antifungal activity of *Magnolia liliflora*, on a spectrum of common plant pathogenic fungi. However, mildew fungi were not included in the study. Inhibitory effect of chitosan on powdery mildew of cucumber seedlings was reported by Moret *et al*. (2009). The

investigation of Wurms and Chee (2011) has established control of powdery mildew of apple seedlings using emulsion of anhydrous milk fat and soybean oil. Current study has demonstrated *in vitro* propagation of the powdery mildew fungus infecting *N. arbor-tristis* and confirmed inhibitory effect of betel vine leaf extract on the pathogen at a level better than the commercial fungicide Bavistin. The study can be further extended to field conditions focusing towards product development for commercialization.

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