In vitro antifungal efficacies of aqueous extract of *Dumortiera hirsuta* (Swaegr.) Nees against sporulation and growth of postharvest phytopathogenic fungi

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Abstract

The aqueous extract of *Dumortiera hirsuta* (Swaegr.) Nees, a bryophyte, was found to be effective against seven postharvest phytopathogens tested. The fungitoxicity of the extract was measured by percent spore germination inhibition and percent radial growth inhibition using poisoned food technique. The inhibition of spore germination by the *Dumortiera* extract was within the range of 400 to 550 ppm. In poisoned food technique, out of seven postharvest phytopathogenic fungi tested six were completely inhibited by the *Dumortiera* extract with concentration range of 550 to 600 ppm. The mode of action of extract (350 ppm) was examined in the case of *Aspergillus niger* van Tieghem. Distinct morphological changes were observed in treated hyphae in comparison to control . The treatment clearly showed anomalies in the hyphae; cell wall of became flaccid, cytoplasm started leaving the cell wall and became granulated.

Key words: *Dumortiera hirsuta,* Antifungal, Extract, Postharvest Phytopathogens

Introduction

Post-harvest diseases destroy 10-30% of the total yield of crops and the loss is considerable in a few perishable crops especially in developing countries (Agrios, 2005; Kader, 2002). Fungi are the most prevalent pathogens causing destruction and economic loss of most fresh fruits and vegetables during storage and transportation (Sommer, 1985). This necessitates proper post-harvest handling so that there is availability of nutritious food and reduced loss between harvesting and consumption. Fungicide application is the usual practice to control postharvest diseases. The use of synthetic fungicides over past decade has been more restrictive due to carcinogenic effects, residual toxicity problems, environmental pollution, occurrence of microbial resistance and high inputs and has also increased consumer concern (Marín et al., 2003; Rial-Otero et al. 2005).

Phytochemicals, being safe and biodegradable, form better choice asan antimicrobial agent. Various natural plant products are reported by various workers which are gaining importance due to their profuse antifungal activity postharvest pathogens (Sharma and Verma, 1991; Sharma, 1992,; Bishop and Thornton, 1997; Tripathi and Dubey, 2004; Sharma et al., 2006; Sharma and Tripathi, 2006; Sharma et al., 2007; Sharma and Tripathi, 2008a; 2008b; Sharma et al., 2008).

Dumortiera hirsuta (Schwaegr.) Nees is a mat forming, conspicuous monotypic thalloid liverwort. It is available in almost all bryogeogrphical regions of India, found growing in shady humid places by streams and waterfalls, and on moist or wet rocks. Bryophytes are oldest known terrestrial plants. One of the features that helped bryophytes to survive all through the years is their content of biologically active compounds. Even though bryophytes are medicinally important still they are not fully exploited.

The antimicrobial activity of various bryophytes was reviewed in detail by Banerjee (2001). The antimicrobial activity was reported by the products of various bryophytes like *Sphagnum portoricense*, *S. strictum* and *Conocephalum conicum* as early as in 1952 (Madsen and Pates, 1952).

The search of more new antifungal natural plant products could be more useful in controlling postharvest pathogens. The present piece of work reports the antifungal efficacy of *Dumortiera hirsuta* against seven postharvest pathogens tested for spore germination inhibition and radial growth inhibition. The microscopic studies dealing with *Aspergillus niger* van Tieghem also give an idea about the mode of action of *Dumortiera* extract.

Materials and Methods

Plant material

D. hirsuta were collected from the Southern Indian region from Tamil Nadu. The description of collection was as follows Nilgiri hills- Gudulur (Naduvattum), ca. 1900m, 29.03.2001, P.K. Verma and Afroz Alam, 13670-13673/2001 (LWU); Ootacamund (Iduhatty), c.a. 1700-2200m, 02.12.2001, P.K. Verma and Afroz Alam, 14716/2001, 14719/2001 (LWU); Gudulur (Devala), ca., 1100m, 27.09.2002, P.K. Verma and Afroz Alam, 16032/2002 (LWU). Plant specimens were identified from the Lucknow University Hepatic Herbarium (LWU) of Bryophytes, India (Figure 1).

Postharvest phytopathogenic fungi

The postharvest phytopathogenic fungi were obtained from the culture collection of Mycology and Plant Pathology Division, Botany Department, University of Lucknow. The fungal

species used in the experiments were *Alternaria alternata* MPPLU 01 (Aa), *Aspergillus niger* MPPLU 05 (An), *Botrytis cinerea* MPPLU 09 (Bc), *Botryodiploidia theobromae* MPPLU 07 (Bt), *Fusarium oxysporum* f. sp. *gladioli* MPPLU 33 (Fog), *Penicillium expansum* MPPLU 24 (Pe), and *Penicillium chrysogenum* MPPLU 27 (Pc). The cultures of the phytopathogenic organisms were maintained on the Potato Dextrose Agar (PDA) at 4^{o} C.

Preparation of Plant extracts

D. hirsuta plants were initially washed with tap water and then with 2% aqueous sodium hypochlorite solution and finally with sterile distilled water. It was then grinded with sterile distilled water at rate of 1ml g^{-1} of tissues (1:1 w/v) with the pestle and mortar and filtered through muslin cloth and the liquid was centrifuged at 5000 rpm for 20 minutes. The clear supernatant was decanted and filtered through sterilized bacteria free Millipore filter (0.22µm pore size) using Swinnex filter adapter attached to a syringe. This solution served as standard plant extract solution (100%), which was stored in the refrigerator at 4 ± 1^{0}C for further use. The solution was used within 24 hrs.

Antifungal efficacy

Spore germination assay

In the spore germination assay, thirteen concentrations (50 to 700 ppm) of *Dumortiera* extract were tested against each test fungi. The fungal spores obtained from 10-day-old cultures of the fungi were taken and placed on the cavity glass slides in triplicate. The slides containing the spores were incubated in a moist chamber at 25 ± 2^{o} C for 24 hrs. Each slide was then fixed in lactophenol-cotton blue and examined under high power (400×) microscope for recording number of spore/conidia germinated using haemocytometer. About 200 spores were counted and the number of spores germinated was scored to calculate the percentage of the spore germination (Surender, 1987).

Poisoned food technique

The antifungal efficacy of the *Dumortiera* extract was also evaluated against the test fungi by the poisoned food technique of Grover and Moore (1962). PDA (20 ml) was poured into the sterilized Petri dishes and various concentrations (50-700 ppm) of sterilized plant extract were added. The medium was supplemented with the same amount of distilled water instead of extract for the control sets. The test fungi were incubated at 25 ± 2^{o}C. On the 7^{th} day, the growth of the test fungi was recorded and the percentage inhibition and Minimum Inhibitory Concentration (MIC) was computed after comparison with the control. Growth inhibition of the treatment against the control was measured by mycelial growth inhibition and calculated as per formula of Pandey et al.,1982.

Percentage of mycelial growth inhibition = (dc-dt)/dc×100; where dc = Average diameter (in mm) of fungal colony in control, dt = Average diameter (in mm) of fungal colony in treatment.

Light Microscopy

Light microscopy was done only in the case of *Aspergillus niger* to observe the effect of *Dumortiera* extract on growth and morphogenesis of the test fungus and to understand the mode of action of the extract. Treated (with 350 ppm of plant extract) and control sample of mycelia were taken from the periphery of the fungus colony grown on PDA after four days of incubation. The samples were fixed in lacto-phenol-cotton blue and examined under the microscope (Nikon

ECLIPSE E200, Japan) at 400× to examine structural abnormalities. Photographs were taken with the help of computer attached Samsung COLOR CAMERA SAC-410PA.

Results and Discussion

The aqueous extract of *D. hirsuta* was found effective against the seven postharvest pathogens tested. The fungitoxicity of the extract was measured by percent spore germination inhibition and percent radial growth inhibition using poisoned food technique. As it is evident from the Table 1 that all seven postharvest pathogen's spore germination was found completely inhibited by the *Dumortiera* extract within range of 400 to 550 ppm concentrations. In spore germination assay it was found that the complete inhibition of spore germination was found at concentration of 400 ppm for *A. niger*, at 450 ppm for *A. alternata* and *P. expansum*, at 500 ppm for *B. cinerea*, *F. oxysporum* f. sp. *gladioli*, *P. chrysogenum*, at 550 ppm for *B. theobromae* (Table 1). In poisoned food technique it was found that out of seven postharvest phytopathogenic fungi tested, six was found completely inhibited by the *Dumortiera* extract with concentration range of 550 and 600 ppm. The minimum inhibitory concentration (MIC) was found to be 550 ppm for *A. niger* and *P. expansum*, 600 ppm for *A. alternata*, *B. cinerea*, *F. oxysporum* f. sp. *gladioli* and *P. chrysogenum*. The fungus *B. theobromae* could not be completely inhibited at the maximum concentration tested i.e. 700 ppm (Table 2). The similar type of spore germination inhibition and mycelia growth inhibition was previously reported by various workers by using different natural antifungal products against the postharvest phytopathogens at various differing concentrations of the antifungal products (Fallik and Grinberg, 1992; Saks et al., 1995; Zambonelli et al., 1996; Beg and Ahmad, 2002; Daferera et al., 2003 ; Nguefack et al., 2004). A number of workers reported the inhibition of spore germination using different testing methods (Begum et al., 1993; Pattnaik et al., 1996). The concentration of the extract in the present study is less than many other antifungal natural products previously tested by various workers (Beg and Ahmad, 2002; Antonov et al., 1997).

The observations of *A. niger* hyphae examined under light microscope at 400 × magnification, after treatment with 350 ppm concentration of *Dumortiera* extract is presented in Figure 2. In addition to inhibited growth, mycelial colony grown in the presence of extract seemed to consistently exhibit distinct morphological changes when compared to control. In microscopic examination of untreated mycelium, hyphae had homogenous and clear cytoplasm (Figure, 2a). Mycelium treated with 350 ppm *Dumortiera* extract showed anomalies in the hyphae, cell wall of hyphae became flaccid, cytoplasm started leaving the cell wall and cytoplasm become granulated. At some places highly reduced cytoplasm in the hyphae showing clear retraction of cytoplasm from hyphae and large portion of hyphae was found without cytoplasm (Figure, 2b). The similar type of results showing degeneration of fungal hyphae was reported after treatment with *Thymus vulgaris* essential oil (Zambonelli et al., 1996). Loss of cytoplasm from the *A. niger* hyphae after treatment of essential oil of *Citrus sinensis* was also reported (Sharma and Tripathi, 2008a). The present study showed agreement with these results reported.

The present study presents the antifungal potentiality of *Dumortiera* extract against the postharvest phytopathogens. The present study clearly shows the mode of action of extract against the test pathogen *A. niger*. The potential antifungal compound can be isolated in mass volume by various techniques like tissue culture so that the antifungal compound can be of economic value in controlling the phytopathogens in postharvest commodities in eco-friendly way.

Table 1: Efficacy of different concentrations of *Dumortiera* extract on percent spore germination inhibition of seven postharvest pathogens after 7 days of incubation at 25±1°C.

Conc. (ppm)	Percent inhibition of sporulation of seven postharvest pathogens						
	Aa	**An**	**Bc**	**Bt**	**Fog**	**Pe**	**Pc**
50	5.9	4.7	4.2	2.4	7.4	3.7	6.3
150	15.1	13.8	12.2	10.1	19.4	16.9	17.8
200	27.7	29.3	26.2	19.7	27.4	29.8	31.4
250	39.1	47.8	34.1	31.4	40.2	47.1	40.7
300	58.6	64.3	47.9	46.3	54.9	63.6	52.7
350	78.3	80.1	68.3	60.4	71.2	77.2	67.9
400	92.1	100.0	80.3	71.8	84.9	93.1	80.7
450	100.0	100.0	93.4	84.3	99.2	100.0	94.6
500	100.0	100.0	100.0	97.1	100.0	100.0	100.0
550	100.0	100.0	100.0	100.0	100.0	100.0	100.0
600	100.0	100.0	100.0	100.0	100.0	100.0	100.0
650	100.0	100.0	100.0	100.0	100.0	100.0	100.0
700	100.0	100.0	100.0	100.0	100.0	100.0	100.0

All values are mean of three replicates

Table 2: Efficacy of different concentrations *of Dumortiera* extract on percent radial growth inhibition of seven postharvest pathogens after 7 days of incubation at 25±1°C.

Conc. (ppm)	Percent radial growth inhibition of seven postharvest pathogens						
	Aa	**An**	**Bc**	**Bt**	**Fog**	**Pe**	**Pc**
50	0.0	1.2	0.0	0.0	2.8	0.0	0.0
150	6.0	7.8	5.7	0.0	10.4	7.8	6.2
200	14.0	15.7	12.8	10.3	19.8	13.9	12.8
250	31.3	33.9	28.2	16.9	32.4	21.9	20.9
300	45.2	46.8	39.5	26.5	45.6	30.1	31.9
350	57.9	60.1	50.2	33.2	58.3	43.7	40.6
400	69.4	74.2	63.7	49.7	71.3	59.3	54.1
450	77.4	88.3	76.9	57.3	84.9	74.1	69.3
500	86.5	99.4	85.5	65.3	96.4	87.3	84.1
550	98.5	100.0	97.2	74.2	100.0	100.0	97.5
600	100.0	100.0	100.0	83.7	100.0	100.0	100.0
650	100.0	100.0	100.0	90.1	100.0	100.0	100.0
700	100.0	100.0	100.0	96.8	100.0	100.0	100.0

All values are means of three replicates

Figure 1: Habit of *Dumortiera hirsuta* (Sw.) Nees,

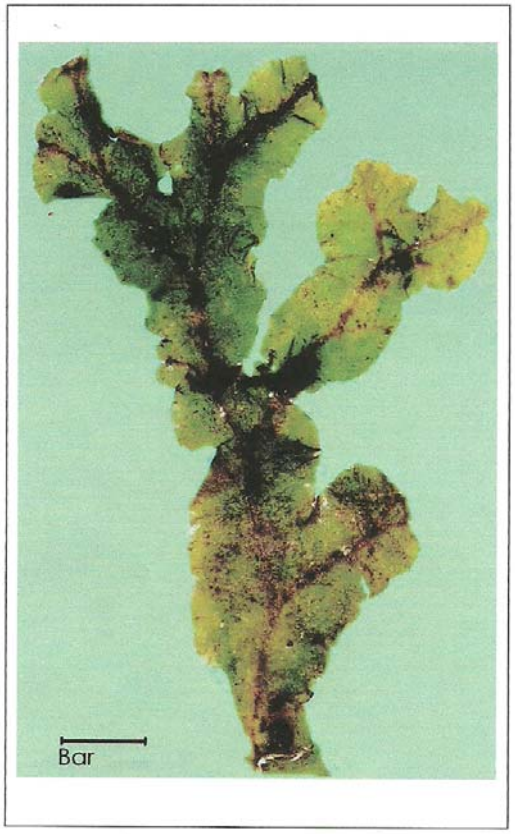
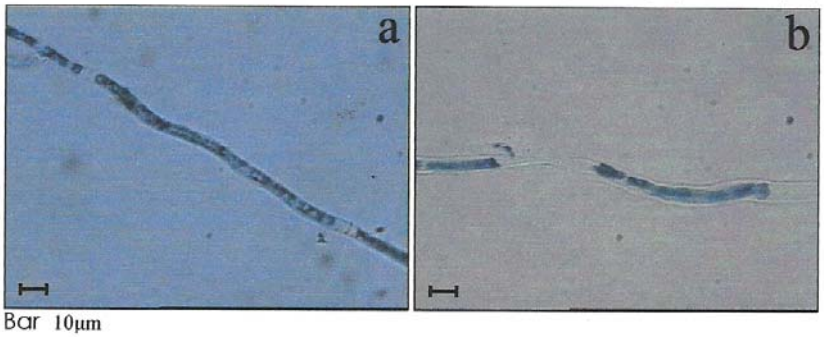


Figure 2: Microphotographs of *Aspergillus niger* mycelium grown on PDA with or without *Dumortiera hirsuta* extract after 4 days of incubation at 25±1°C. **(a)** *A. niger* control (untreated) mycelium, structure is homogenous, Bar 10µm. **(b)** Mycelium treated with 350 ppm of extract showing anomalies in the hyphae, cell wall of hyphae became flaccid, cytoplasm started leaving the cell wall and at some places highly reduced cytoplasm in the hyphae,



Bar 10µm

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