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Response of Mulberry Brown Leaf Spot Fungus *Myrothecium roridum* to Different Plant Extracts

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Anti-fungal potential of 5 plant extracts *viz., Eucalyptus citriodora, Allium sativum, Cassia sophera, Chromolaena odorata* and *Datura metel* on the growth of mulberry brown leaf spot pathogen *Myrothecium roridum* were examined. Except for the aqueous extract of *Allium* bulb, ethanolic leaf extract of all other plants more efficiently reduced the colony growth of the fungus on potato-dextrose-agar. Of which, *Allium* and *Eucalyptus* extracts were more effective. Initiation of radial growth of *M. roridum* on solid media was deferred maximum 6 days by ethanolic *Eucalyptus* extract and 4 days by aqueous *Allium* extract at 0.4 mg · ml^{-1}. In the liquid media amended with *Eucalyptus* extract (0.4 mg · ml^{-1}) complete inhibition of sporulation was noticed upto 8 days, and initial inhibition of mycelial bio-mass generation was considerably diminished with time and reduction was 1.3 fold 14 days after application. While, complete inhibition of mycelial growth for 6 - 14 days was recorded with ≥ 0.1 mg · ml^{-1} commercial eucalyptus oil. However, rejuvenation of growth appeared when fungus was re-inoculated in fresh media. Post-inoculate application of different doses of *Eucalyptus* and *Allium* extracts significantly ($p < 0.05$) reduced the disease severity in potted mulberry. However, persistence of the effect up to 28 days was apparent at ≥ 1.0 mg · ml^{-1} and effectively was on par with carbendazim (1 mg · ml^{-1}). Almost equal control ability of 1.0 mg · ml^{-1} *Eucalyptus* extracts can be achieved by *ca.* 10 times lowered dose of commercial eucalyptus oil. It seems, the toxic principle of *E. citrodora* to *M. roridum* is fungistatic in nature and may have essential oil based origin.

Key words: Disease control, Growth inhibition, *Morus alba*, *Myrothecium roridum*, Plant extracts

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Introduction

Brown leaf spot, caused by *Myrothecium roridum* is one of the major diseases of mulberry in Eastern and North - Eastern India. Under suitable weather condition (30 - 32°C temperature, 80 - 90% relative humidity and > 10 rainy days/month) the disease may cause 10 - 12% loss in leaf yield due to necrotic lesion development and subsequent defoliation (Qadri *et al.*, 1999). Methods for disease control available to growers include application of fungicides carbendazim (0.1% active ingredient) or mancozeb (0.2% active ingredient), despite being non-biodegradable (DeWaard *et al.*,1993; Knight *et al.*,1997). Restriction has already been imposed on the use of different carbamate fungicides in some European countries due to their adverse effect on non-target organisms and environment (Gullino and Kuijpers, 1994). In India also, resistance to carbendazim and mancozeb has been reported in pathogens of various field crop (Gangawane, 1990). Since, mulberry is a perennial plant cultivated in tropics almost round the year (5 - 6 crops) for silkworm rearing as a monoculture (Oka and Ohyama, 1986), chances of emergence of resistant pathogen strains is even greater in mulberry than other crops. Hence, an alternate method of control is urgently being sought. A promising approach is the use of biodegradable, more target specific and less toxic natural products (Aditya Chaudhuri, 1991; Mandaki

et al., 2001). Therefore, the objectives of this study were to a) evaluate the efficacy of five locally available plant extracts (PEs) including commercial eucalyptus oil (EO) against the growth of *M. roridum in vitro* and brown leaf spot severity *in vivo*, b) compare the potential of suitable botanical(s) with recommended dose of fungicide carbendazim, and c) assess the preliminary systemicity of botanical (s) *in vitro*.

Materials and Methods

Plant material

The dehulled bulb of *Allium sativum* L. and fresh leaves of *Eucalyptus citriodora* L., *Cassia sophera* L., *Datura metel* L. and *Chromolaena odorata* King & Robinson, were collected from the local market and field of Central Seicultural Research and Training Institute, Berhampore, West Bengal, respectively. The EO was procured from the West Bengal Forest Development Corporation, Jhargram, West Midnapore, West Bengal. Freshly washed leaves or bulbs (50 g) were weighed, finely chopped and ground in a mortar and pestle with distilled water or 90% (v/v) ethanol. The homogenate was sieved through 4 layers of cheese-cloth and centrifuged at 5,000 g for 15 min. Finally, the supernatants were made up to 50% (w/v) and kept at 0 - 4°C for a fortnight for further use.

Fungal material

The pathogen (Myrothecium roridum Tode) was isolated from an infected mulberry field of Central Seicultural Research and Training Institute, Berhampore, West Bengal. Pure culture was maintained on potato dextrose agar (PDA) in the dark at 4°C and sub-cultured at an interval of 20 days. Assays were carried out either with potato-dextrose broth or solidified with 20 mg · ml^{-1} agar and 20 $\mu g \cdot ml^{-1}$ streptomycin sulphate, amended by different PEs or EO.

In vitro assay

Initially, effect of different PEs on the mycelial growth of *M. roridum* was tested by agar well method (Dhingra and Sinclair, 1985). Conidial suspensions (1.6×10^6 conidia · ml^{-1}; 2 ml · $plate^{-1}$) were uniformly spread over PDA (20 ml) in 90 mm sterile Petriplates. Different PEs (0.025 - 0.1 mg · ml^{-1}), sterilized by membrane filtration (Millipore, pore size 22 μm), were added in the newly made central well separately. The well filled with distilled water or 90% (v/v) ethanol was kept as control. Plates were incubated at 28 ± 2°C in dark for 5 days and then the inhibition zone was measured.

Time course of fungal growth inhibition was conducted by poison agar assay (Grover and Moore, 1962). The sterilized PEs and EO were amended in 20 ml of cooled (45 - 50°C) molten PDA with a final concentration of 0.05 to 0.4 mg · ml^{-1}. Mycelial plugs of 5 mm diameter, from a 7 days old culture, were inoculated on center of the plates. Colony diameter was determined on every alternate day upto 18 days.

For mycelia biomass and sporulation rate estimation, *Eucalyptus* extracts or EO were amended in 25 ml Erlenmeyer flask. The medium was inoculated with 1×10^7 conidia · ml^{-1} in active growth phase (7 - 10 days old culture stock) and incubated over a rotory shaker at 28 ± 2°C in dark. Three flasks from each treatment were removed at an interval of 48 hrs, vortexed thoroughly and the conidial concentration was recorded from an aliquot of 100 μl by microscopic (Leica, Wild MPS 52) observation using haemocytometer (Aneja, 1993). The rest of the mycelia mat was harvested on Whatman 42 filter paper by vacuum filtration (Millipore Vac 1) and oven dried (70°C for 72 hrs) for recording the biomass.

In vivo assay

Mulberry plants (*Morus alba* cv S_1) were grown in earthen pots (diameter 36 cm, depth 25 cm) containing 15 kg mixture of soil and farm yard manure (1:1) and kept under natural light/dark cycle and temperature (21 - 34°C) conditions. One month old plants (after basal pruning) were inoculated with *M. roridum* spore suspension (1×10^6 conidia · ml^{-1}; 15 ml · $plant^{-1}$). Different PEs and EO were sprayed to run-off (30 ml · $plant^{-1}$) at 5 days after inoculation and kept in completely randomized manner. Control plants were similarly sprayed with distilled water or 5% (v/v) ethanol. Disease severity (percent disease index) was recorded by randomly selected 3 branches of each plant on a 0 5 scale (Maji *et al.*, 2000) either after 21 days (for initial screening) or at an interval of 7 day up to 28 days using standard formula (Govindaiah *et al.*, 1989).

Results

In vitro growth

Initial experiment was designed to estimate the anti-fungal efficacy of selected PEs on the colony growth of *M. roridum* 5 days after treatment. In general, *Allium* and *Eucalyptus* extracts more effectively retarded the colony growth compared to others (Fig. 1a, b). At the lowest dose (0.02 mg · ml^{-1}), inhibition zone was 10.6 mm by aqueous *Allium* extract and 6.7 mm by ethanolic *Eucalyptus* extract. However, concentrations ≤ 0.06 mg · ml^{-1} of aqueous and ≤ 0.04 mg · ml^{-1} of ethanolic extracts of *Cassia,*

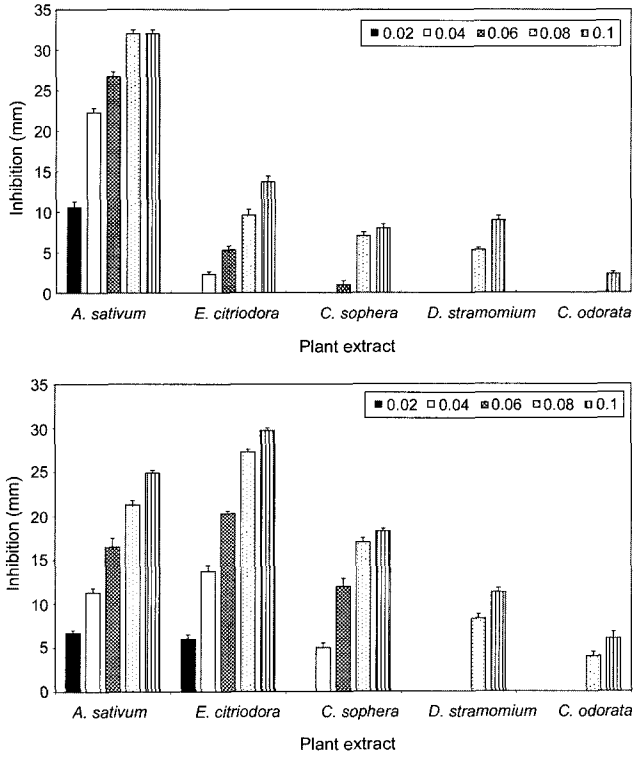

Fig. 1a. Effect of different aqueous plant extracts on the colony growth of *Myrothecium roridum* on solid media after 5 days of inoculation. Vertical bar represent the standard error of the mean (n = 3). Plant extract values expressed as mg · ml^{-1}.

Fig. 1b. Effect of different ethanolic plant extracts on the colony growth of *Myrothecium roridum* on solid media after 5 days of inoculation. Vertical bar represent the standard error of the mean (n = 3). Plant extract application values expressed as mg · ml^{-1}.

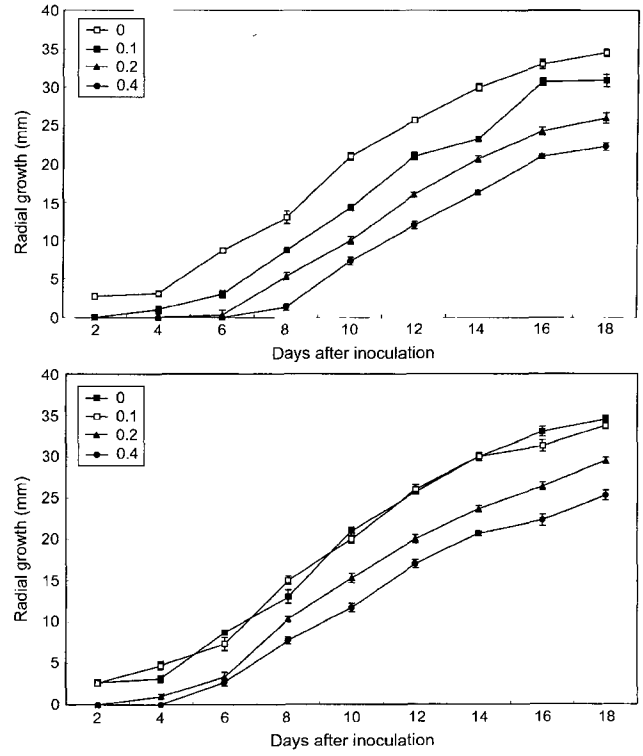

Fig. 2a. Time course of *Myrothecium roridum* growth under the influence of ethanolic extracts of Eucalyptus citriodora on solid media. Vertical bar represent the standard error of the mean (n = 3). Plant extract values expressed as mg · ml^{-1}.

Fig. 2b. Time course of *Myrothecium roridum* growth under the influence of aqueous extracts of *Allium sativum* on solid media. Vertical bar represent the standard error of the mean (n = 3). Plant extract values expressed as mg · ml^{-1}.

Datura and *Chromolaena* were almost ineffective on the pathogen growth. Therefore, further time course of radial growth was conducted with aqueous *Allium* and ehtanolic *Eucalyptus* extracts amended solid media. Growth was measured in terms of radial increase of colony over a period of 18 days. It increased gradually (but not linearly) between 4 - 14 days (Fig. 2a, b), then declined and reached the edge of the petriplate after 19 – 21 days. While, no apparent growth was observed in carbendazim amended media at recommended dose of 1 mg · ml^{-1} (data not shown). In the *Eucalyptus* extract (0.4 mg · ml^{-1}) amended media, complete inhibition of radial growth was noticed up to 6 days and overall growth was inhibited 35% at 18 days (Fig. 2a). While, *Allium* extract, at the same dose, fully checked the radial growth only up to 4 days and overall reduction was 26% (Fig. 2b). No other alteration in gross mycelial morphology was apparent in both the PEs treatments.

Considering the maximum delayed response of *M. roridum* by *Eucalyptus* extract, further investigation on mycelial biomass and sporulation pattern were conducted with the same along with eucalyptus oil, a potent source of anti-fungal essential oils (Shahi *et al.*, 1999). The mycelial biomass was restricted to 35% of control at 8 day of inoculation of ethanolic *Eucalyptus* extract (0.4 mg · ml^{-1}), but the inhibition was diminished to 18% at 14 days (Table 1). The sporulation initiated after 8 days and rate was significantly low throughout the test period (73% of control).

On the other hand, eucalyptus oil more effectively reduced the colony growth of the pathogen at lower doses (0.025 – 0.1 mg · ml^{-1}) in solid media (Table 2). However, at concentration > 0.1 mg · ml^{-1}, *in vitro* growth was completely inhibited up to 6 – 14 days. The nature of toxicity was determined by re-inoculated the inhibited mycelial plug on fresh PDA plate. Fungal growth was rejuvenated in all the tested concentrations of EO (0.1 – 0.4 mg · ml^{-1}), only the time of rejuvenation was delayed 2 – 6 days. The

Table 1. Time course of *Myrothecium roridum* mycelial bio-mass and sporulation under the influence of ethanolic extract of *Eucalyptus* (0.4 mg · ml^{-1}) in liquid broth

Incubation (day)	Mycelial bio-mass (mg DW)		Sporulation (conidia × 10^6)	
	Control	Treatment	Control	Treatment
2	55 ± 2.6	0 ± 0	0 ± 0	0 ±0
4	104 ± 3.3	16 ± 2.5	0.05 ± 0.01	0 ± 0
6	171 ± 2.4	62 ± 1.9	4.9 ± 0.4	0 ± 0
8	205 ± 4.5	132 ± 2.1	19.1 ± 1.5	0 ± 0
10	231 ± 3.9	160 ± 6.1	53.8 ± 2.6	0.04 ± 0.001
12	226 ± 3.3	177 ± 2.4	64.4 ± 1.6	4.3 ± 0.03
14	222 ± 3.3	182 ± 3.1	66.6 ± 1.1	17.4 ± 1.9

Data are mean ± SE of three individual observations.

fungus could not grow from the carbendazim (1 mg · ml^{-1}) treated plates.

In vivo growth

All the *in vitro* tested concentrations of different PEs were quite ineffective to control leaf spot severity *in vivo* (data not shown). The *Eucalyptus* and *Allium* extracts at 50 mg · ml^{-1}, inhibited brown leaf spot severity significantly ($p < 0.05$) at 14 days of treatment, but other three PEs had little effect on *in vivo* disease control (Table 3). Moreover, both the aqueous and ethanolic extracts of *Eucalyptus* had equal potential to control the disease severity. While, the effectiveness of aqueous *Allium* was 1.4 fold more than ethanolic extracts. Moreover, the effect of these two extracts on leaf spot severity was dose dependant (Table 4). The inhibitory effect, evident from 7 days onwards, was greatest at the highest concentration tested. Contrary to carbendazim, maximum inhibition was observed at 14 days of treatment in both the PEs. Generally, the persistence of disease control ability increased with the increment of concentrations. The commercial EO at 5 mg · ml^{-1}, showed almost same control efficacy of 50 mg · ml^{-1} *Eucalyptus* extracts.

Table 3. Effect of different aqueous and ethanolic plant extracts (50 mg · ml^{-1}) on the brown leaf spot severity *in vivo*

Treatment	Aqueous		Ethanolic	
	PDI*	Reduction (%)	PDI	Reduction (%)
Control	13.7 ± 0.7 a	--	12.9 ± 0.7 a	--
A. sativum	6.9 ± 0.7 e	49	8.5 ± 0.1 d	34
E. citriodora	6.2 ± 0.3 f	54	6.2 ± 0.2 f	52
C. sophera	11.6 ± 0.6 d	15	11.4 ± 1.1 c	11
D. stramomium	12.3 ± 0.3 c	10	11.9 ± 0.7 b	08
C. odorata	13.0 ± 1.1 b	05	11.8 ± 0.4 b	08
Carbendazim	7.3 ± 0.4 e	47	7.0 ± 0.3 e	46

Data are mean ± SE (n = 3). Percent disease index at 14 days of treatment. Values in the column followed by the different letter are significantly different ($p < 0.05$) by LSD test. Interaction between aqueous and ethanolic PEs is non significant ($F = 0.57$).

Discussion

Apart from chemical control, little information is available on the applicability of botanicals for the control of different fungal diseases of mulberry (Sarvamangala *et al.*,

Table 2. Time course of *Myrothecium roridum* growth under the influence of eucalyptus oil on solid media

Dose (mg · ml^{-1})	Colony growth (mm) days after inoculation				
	2	6	10	14	18
0.000	2.7 ± 0.3	8.7 ± 0.4	21.0 ± 0.5	30.0 ± 0.5	34.5 ± 0.8
0.025	0.0 ± 0.0	6.0 ± 0.2	17.3 ± 0.7	28.3 ± 0.4	31.2 ± 0.7
0.050	0.0 ± 0.0	3.3 ± 0.3	13.6 ± 0.6	25.3 ± 0.5	28.4 ± 0.6
0.075	0.0 ± 0.0	1.3 ± 0.2	10.3 ± 0.3	18.3 ± 0.7	23.9 ± 0.9
0.100	0.0 ± 0.0	0.0 ± 0.0	5.6 ± 0.5	11.7 ± 0.6	18.6 ± 0.8
0.200	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	5.0 ± 0.6	10.7 ± 0.3
0.400	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	6.4 ± 0.3
Rejuvenation of growth at fresh media:					
0.100	0.0	2.2	8.3	15.4	24.6
0.200	0.0	0.9	6.4	13.4	20.4
0.400	0.0	0.0	3.3	9.4	15.6

Data are mean ± SE (n = 3). For rejuvenation study 6 days old mycelial plug from different treatments were re-inoculated in the fresh PDA. Values are mean of two individual observations.

Table 4. Time course of brown leaf spot severity under the influence of ethanolic *Eucalyptus citriodora* extract (a) and eucalyptus oil *Allium sativum* extracts *in vivo* (b)

(a)

Treatment	Dose ($mg \cdot ml^{-1}$)	Percent disease index			
		7 DAA*	14 DAA	21 DAA	28 DAA
Control	0	3.7 ± 0.4 a (0)	7.9 ± 0.2 a (0)	13.4 ± 0.5 a (0)	18.2 ± 0.6 a (0)
E. citrodora	25	2.8 ± 0.3 b (24)	4.9 ± 0.3 b (38)	11.5 ± 1.3 b (15)	17.6 ± 0.4 b (04)
	50	2.8 ± 0.5 b (24)	4.7 ± 0.2 c (41)	9.2 ± 0.5 e (31)	10.8 ± 0.5 d (41)
	75	2.4 ± 0.5 c (35)	2.9 ± 0.3 g (63)	7.5 ± 0.3 d (44)	10.0 ± 0.5 f (45)
Eucalyptus oil	2.5	2.4 ± 0.4 c (35)	4.4 ± 0.4 d (44)	8.5 ± 0.5 e (37)	12.1 ± 1.1 c (33)
	5.0	2.0 ± 0.5 d (46)	4.0 ± 0.3 e (49)	7.5 ± 0.8 e (44)	10.5 ± 0.8 e (42)
	7.5	1.9 ± 0.1 d (48)	3.4 ± 0.5 f (56)	7.4 ± 0.5 e (45)	10.9 ± 0.6 d (40)
Carbendazim	0.2	2.3 ± 0.2 c (38)	4.5 ± 0.3 d (43)	6.7 ± 0.3 f (50)	10.1 ± 0.3 f (44)

(b)

Treatment	Dose ($mg \cdot ml^{-1}$)	Percent disease index			
		7 DAA*	14 DAA	21 DAA	28 DAA
Control	0	5.3 ± 0.1 a (0)	8.5 ± 0.8 a (0)	13.5 ± 1.4 a (0)	17.3 ± 0.9 a (0)
A. sativum	25	3.4 ± 0.6 c (35)	5.1 ± 0.5 b (40)	9.1 ± 1.4 b (33)	14.5 ± 0.4 b (16)
	50	3.0 ± 0.3 d (43)	4.9 ± 0.4 c (42)	8.5 ± 0.4 d (37)	7.5 ± 0.3 e (56)
	75	2.8 ± 0.3 e (47)	3.7 ± 0.2 d (56)	8.1 ± 1.2 e (40)	8.8 ± 0.6 d (49)
Carbendazim	02	4.1 ± 0.1 b (23)	5.1 ± 0.2 b (40)	8.9 ± 0.4 c (34)	9.2 ± 0.5 c (47)

*Days after application of PE.

Data are mean ± SE (n = 3). Values in the column followed by the different letter are significantly different ($p < 0.05$) by LSD test. Data given in the parentheses are percent inhibition of control.

1993; Biswas *et al.*, 1995). Results presented herein provide the first direct evidence, to our knowledge, on the *Myrothecium roridum* induced brown leaf spot control potential of different botanicals. We measured the efficacy by *in vitro* colony growth, biomass production, sporulation and growth rejuvenation in different PEs, as well as *in vivo* control of disease severity by the post inoculation application of PEs. The said parameters are widely used as a standard for the assessment of antifungal potential (Nane and Thapliyal, 1993). Initially, we used one each of polar and non-polar solvents to ensure the full extraction of anti-fungal principle(s) from plants.

All the PEs, either in aqueous or ethanol extracted forms, substantially reduced the mycelia growth of *M. roridum* at varying degree in solid media (Fig. 1a, b). Among these ethanolic extract of *Eucalyptus* and aqueous *Allium* recorded maximum inhibition. Extracts of garlic and eucalyptus have already proved anti-microbial, particularly against Phycomycetes (Mahadevan, 1982; Shukla *et al.*, 1997). Our results are in conformity with those and additionally indicates the degree of inhibition over respective controls diminished considerably beyond 10 days *in vitro* (Fig. 2a, b). These are in corollary with the previous observations on the fungistatic nature of garlic extract against fruit rot pathogen *Aspergillus niger* (Sinha and Saxena, 1999) and *C. citriodora* against dermatophyte *Trichophyton rubrum* (Shahi *et al.*, 1999). While, carbendazim at recommended dose ($1 mg \cdot ml^{-1}$) provided a complete control throughout the test period. Furthermore, *Eucalyptus* extract, at least in liquid media, more efficiently reduced the sporulation (73%) than mycelia biomass production (18%) at 18 days of treatment (Table 1). Even greater pathogen control was achieved by lower dose of EO ($0.25 mg \cdot ml^{-1}$). At higher doses ($\geq 0.1 mg \cdot ml^{-1}$), however, radial growth was completely stopped for 6 - 14 days in solid media (Table 2). Over all the, data reflects a fungistatic and anti-sporulant, but non-curative essential oil based anti *M. roridum* active principle(s) of *E. citriodora*.

Post-inoculation application of $50 mg \cdot ml^{-1}$ *Eucalyptus* and *Allium* extracts gave significant control of brown leaf spot intensity *in vivo*, but other tested PEs were ineffective. The variation in activities among the PEs may be due to the difference in concentration and composition of anti-

fungal compounds in different plants (Dhar *et al.*, 1988). Our *in vivo* findings (Table 3 and 4), not exactly, but grossly supported the *in vitro* results (Fig. 2a, b), only the effectiveness noticed at higher doses. Presence of PEs was seems to be affecting normal growth of the fungus. In case of garlic extract, an increase in vacuoles and subsequent reduction of cytoplasam volume has been reported from different phytopathogenic fungi (Branchi *et al.*, 1997). Furthermore, the control potential of both PEs substantially reduced on prolonged incubation (Table 4), which may indicate a better bio-degradability of the fungitoxic compound(s) in the system. Though the effective disease protection dose of *Eucalyptus* extracts was comparatively higher, but EO at 10 times lower concentration showed better efficacy, even *in vivo* (Table 4).

In a cash crop like mulberry, where leaves are generally utilized at 60 - 70 days interval as silkworm food, use of biodegradable fungistatic plant product for leaf spot control has a better applicability for future sustainable sericulture. Our findings on *in vitro* and *in vivo* protection ability suggests, *E. citriodora* has a potential for such use in brown leaf spot control and proper isolation of essential oil will give better performance.

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