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Antisporulant Activity of Watery Extracts of Plants against *Sclerospora graminicola* Causing Downy Mildew Disease of Pearl Millet

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Abstract: Watery extracts of forty plant species commonly growing in across India have been screened for antisporulant activity against *Sclerospora graminicola* (Sacc.) Schroet., the causative agent of pearl millet downy mildew. The collection represented 38 genera of 30 families. The extracts of thirteen species did not show any effect, whereas the activity of extracts of *Allium sativum*, *Clematis gouriana*, *Evolvulus alsinoides*, *Mimusops elengi*, *Parthenium hysterophorus*, *Piper nigrum* and *Tagetes erecta* were commensurable to that of marketed botanical fungicides and Mikal 70 wp. The crude extracts of 12 species (*Agave americana*, *Aloe vera*, *Artemisia parviflora*, *Citrus limon*, *Citrus sinensis*, *Eucalyptus globosus*, *Euphorbia hirta*, *Leucas aspera*, *Murraya koenigi*, *Ocimum sanctum*, *Santalum album* and *Zingiber offinale*) completely inhibited the zoosprorangium formation while in the case of remaining 8 plants the crude extracts reduced only partially the sporulation. The antisporulant activity of commercialised *Azadirachta* preparation (Nutri-Neem) was more pronounced than that of *Reynutria* based one (Milsana) and *Sabadilla* (veratrin), however, these botanical preparations held off synthetic fungicides and the most active watery extracts.

Keywords: Pearl millet, downy mildew, plant extracts, antisporulant activity

INTRODUCTION

The defence strategies of plants against their pathogens are multitudinous, including the use of antimicrobial chemicals that either can be *de novo* formed during the pathogenesis (phytoalexins) or are constitutive components (phytoanticipins) of cells[1]. On the other hand, pathogens have evolved mechanisms to evade these barriers that demand application of various pesticides in crop production. Present control technologies of downy mildews discouple the pathogen's life cycle mainly in two points of ontogeny. The applied chemicals either destroy spores, preventing the infection or inhibit the biotrophic thallus, anticipating the formation of new infective propagules. The preventive control of *Sclerospora graminicola*, the causative agent of pearl millet downy mildew (PMDM) meets difficulties. The remediative capacity of its host plant is reduced by the short vegetation, and as a consequence of it, pesticides are accumulated in the grain[2] challenging to application of synthetic chemicals after formation of tillers. The pathogen shows high natural variation in agressivity[3], even progenies of the same oospore could be classified into distinct pathotype groups[4]. Although the downy mildew tolerance of pearl millet can be enhanced by diverse methods[5,6] the possibilities of biocontrol measures[7] as well as enhancement of plant resistance with chemical treatment[8] were presented, none of these approaches resulted the economically accepteble level of control. Thus, the vulnerability of the resistance to the disease has been a major cause of concern as even 10 % disease incidence cause economic loss threatening the net return[9,10]. The only tool for creditable control of this endobiotrophic peronospora is the use of systemically acting acylanilide derivatives. However, the calculability of management of pearl millet downy mildew has been threatened by emergence of acquired tolerance to this group of chemilcals in India[11,12]. Looses caused by PMDM urges development of alternative control agents. One approach to discover newer antimildew compounds is to search for their presence in natural sources[13]. Microbial species or strains that do not invade the plant usually are more sensitive to the components of preformed barriers than a viable pathogen of this plant[14]. Consequently, phytoanticipins and precursors of phytoalexins can represent a prospective tool for PMDM management[15].

In the present investigation, we have studied the antisporulant activity of watery extracts of plants growing at almost all locations throughout pearl millet producing areas of India. The inhibitory effect of extracts against *S. graminicola* was compared to that of marketed fungicides and phytochemicals.

MATERIALS AND METHODS

Plant materials and reference compounds: This survey was carried out during vegetation period of the pearl millet in 2003 to identify the easily available

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plants in pearl millet producing regions of India. A total of 40 species representing 38 genera of 30 families were collected from districts Mysore (12.18 N - 76.42 E, 770m above sea level (ASL)), Mandya (12.33 N – 76.54 E, 695 m ASL), Mercara (12.26 N - 75.47 E, 1145 m ASL) and Hassan (13.01 N - 76.10 E, 957 m ASL), and Gopalaswamy Hills, Chamarajanagar district (11.56 N - 77.00 E, 780 to 1455 m ASL). The plants were identified taxonomically and authenticated at the Herbarium, Department of Botany, Mysore (Table 1). Fungicide dimethomorph (Acrobat 50 wp, Shell, UK) was supplied by the manufacturer whereas metalaxyl (Ridomil 25 wp, Ciba-Geigy, Swiss) was extracted from marketed product. Digitonin, podophyllotoxin and veratrine mixture were purchased from Sigma-Aldrich (USA). The commercial preparations Mikal 70 wp (Rhone Poulenc, France), Nutri Neem (Nutri-Tech Solutions, Pty Ltd., Australia) and Milsana® Bioprotectant Concentrate (KHH BioSci, Raleigh, USA) were also used as a reference.

Test organisms: The downy mildew pathogen *S. graminicola* (pathotype 1) was isolated from naturally infested pearl millet (*Pennisetum glaucum* (L.) R. Br., hybrid HB3) in Bogadi village of Mysore district (Karnataka state, India) during 1970 by Shetty. The strain was maintained on greenhouse grown pearl millet plants (hybrid HB3) before being used as inoculum for the experiments. The method was described in detail by Safeulla[16].

Preparation of extracts: The collected plant material in fresh conditions was weighted *in situ*, than cooled and stored to be prevented loosing of water. Tissues were cut into pieces and triturated with distilled water (50 g with 50 ml). The homogenate was allowed to settle for 1 hour than it was filtered through the muslin cloth and centrifuged at 10000 rpm for 15 min. The supernatant was stored at 0-4 °C in a closed container and used as a crude extract. Dilutions (1:9, 1:99 and 1:999) have been made with distilled water before application. Tween 20 was addedd (0.02 %) as wetting agent.

Determination of antiperonospora activity: Leaves with disease symptoms were collected from artficially infected plants grown in the field and washed in distilled water, excess water was then removed. The leaves were cut into ≈1 cm^2 pieces which were subsequently immerged for 30 minutes into test solution (crude extract and its ten-fold dilutions as well as 5, 0.5, 0.05, 0.005 and 0.0005 percent w/v of reference substances). Treated pieces were then incubated 12-14 hours in moist chambers (plastic trays lined with wet filter paper) at 22±1 ^oC in the dark. To determine the intensity of sporulation, a 0-2 scale was used where the proportion of leaf area covered by zoosporangia was graded as follows: 0, no sporulation; 1, the sporulation was inhibited partially; and 2, the intensity of sporulation was indistinguishable of that on untreated control pieces, respectively. All tests were carried out in quadruplicates.

RESULTS AND DISCUSSION

Most of extracts inhibited the zoosporangium formation of *S. graminicola*. Scores of the degrees of inhibition caused by these extracts are presented in Table 1. Only extracts of thirteen plants did not show any activity (species 28-40), whereas the extracts of *Allium sativum*, *Clematis gouriana*, *Evolvulus alsinoides*, *Mimusops elengi*, *Parthenium hysterophorus*, *Piper nigrum* and *Tagetes erecta* proved to be active on the level of Mikal 70 wp that is widely used as a fungicide of curative activity (Table 2). Extracts of the leaves of twelve other plants (species 8-19) also strongly inhibited the sporulation, however, these were significantly less efficient then the reference compounds. The crude extracts of eight plants (species 20-27) reduced the zoosporangium formation partially. Contrary to glycosteroid digitonin, the activity of marketed botanical pesticides (Nutri-Neem, Milsana and Sabadilla) and podophyllotoxin was significantly lower that of the synthetic fungicides dimethomorph and metalaxyl.

Table 2: Antisporulant activity of commercial fungicides and reference substances

Treatment		Concentration (%) of substances				
Substances	Form[1]	0.0005	0.005	0.05	0.5	5
Dimethomorph	A	-	+	+	++	++
Metalaxyl	A	-	+	+	++	++
Mikal	B	-	-	+	+	++
Digitonin	A	-	-	+	+	++
Podophyllotoxin	A	-	-	-	+	++
Veratrin	A	-	-	-	+	+
Nutri-Neem	B	-	-	-	+	+
Milsana	B	-	-	-	+	++

The antisporulant activity was evaluated by following scale; full inhibition (++), partial inhibition (+) and no inhibition (-).

[1]A = 25 % methanolous stock solution of active ingredients containing 1 % of Tween 20 was used for preparing dilution series. The methanol and Tween 20 did not exhibited any inhibitory effect alone when applied at maximum doses (5 and 0.2 %, respectively). B = Commercial preparations were used.

Excepting few species (3, 7, 11, 20, 33 and 34) large number of data was reported on the antimicrobial activities of tested plants, although the published data refer mainly on responses of human associated microbes (*Candida*, *Trychophyton*, *Esherichia coli* and various other bacteria) and minor part relates to phytopathogenic fungi (Table 1). Moreover, *S. graminicola* is taxonomically distant from majority of pathogens tested earlier. Concerning to the antioomycete effects few data were available on the activity of plants examined

Table 1: Inhibitory effect of plant extracts on zoosporangium formation of *Sclerospora graminicola*.

Examined plants			Activity of extracted plant material			
			Part	Extracts		
No.	Species (Family)[1]	Origin[2]	used[3]	Crude	1:9	Reported against fungi[5]
	Strong					
1	*Allium sativum* L. (Liliaceae)[a,b]	MY	B	++	+	F18, F25
2	*Clematis gouriana* Roxb. (Ranunculaceae)	MY	T	++	+	F38, F45, F46
3	*Evolvulus alsinoides* L. (Convolvulaceae)[a,b]	HA	T	++	+	--
4	*Parthenium hysterophorus* L. (Asteraceae)	MY	L	++	+	--
5	*Tagetes erecta* L. (Asteraceae)	MY	L	++	+	--
6	*Piper nigrum* L. (Piperaceae)[a,b]	MY	S	++	+	F6, F18, F6, F10, F11, F22, F29, F32, F38, F40, F45, F46
7	*Mimusops elengi* L. (Sapotaceae)	MY	F	++	+	--
	Remarkable					
8	*Agave americana* L. (Agavaceae)	MY	L	++	-	F5
9	*Aloe vera* L. (Liliaceae)[a,b]	MY	L	++	-	F12, F21, F40
10	*Euphorbia hirta* L. (Euphorbiaceae)[a,b]	MY	L	++	-	--
11	*Artemisia parviflora* Wight. (Asteraceae)	CH	T	++	-	--
12	*Leucas aspera* Spreng. (Lamiaceae)	MY	L	++	-	F9
13	*Ocimum sanctum* L. (Lamiaceae)[a,b]	MY	L	++	-	F9, F10, F11, F23, F38, F40
14	*Citrus sinensis* (L.) Oesbeck. (Rutaceae)[b]	MY	P	++	-	F33, F35
15	*Citrus limon* L. (Rutaceae)[a,b]	MY	L	++	-	F6, F10, F18, F38, F44, F47
16	*Murraya koenigi* Spreng. (Rutaceae)	MY	L	++	-	F39
17	*Zingiber officinale* Roscoe (Zingiberaceae)[b]	MY	R	++	-	F6, F14, F21, F25, F40
18	*Santalum album* L. (Santalaceae)[a,b]	MY	L	++	-	--
19	*Eucalyptus globosus* Labill. (Myrtaceae)	MY	L	++	-	F2, F4, F11, F16, F21, F28, F30, F40
	Weak					
20	*Thuja occidentalis* L. (Cupressaceae)	MY	L	+	-	--
21	*Artemisia pallens* Wall. (Asteraceae)	CH	L	+	-	--
22	*Helianthus annuus* L. (Asteraceae)[a]	MY	S	+	-	F42
23	*Dalbergia latifolia* Roxb. (Papilonaceae)[a]	ME	L	+	-	--
24	*Ocimum basilicum* L. (Lamiaceae)[a]	MY	L	+	-	F5, F7, F14, F23, F27, F29, F44, F47
25	*Datura metel* L. (Solanaceae)[b]	MY	L	+	-	F5, F6, F37
26	*Azardirachta indica* A. Juss (Meliaceae)[a,b]	MY	L	+	-	F3, F5, F6, F13, F15, F17, F19, F20, F21, F23, F24, F26, F29, F34, F37, F43
27	*Calotropis gigantea* (L.) W. T. Aiton (Asclepiadaceae)[b]	MY	L	+	-	F9, F13, F23, F29
	Inactive					
28	*Salix tetrasperma* Roxb. (Salicaceae)	MA	L	-	-	F1, F31
29	*Artemisia vulgaris* L. (Asteraceae)	CH	L	-	-	--
30	*Chrysanthemum indicum* L. (Asteraceae)	MY	L	-	-	--
31	*Strobilanthes heynenus* Ness. (Acanthaceae)	CH	F+L	-	-	--
32	*Cassine glauca* (Rottb.) Kuntze (Celastraceae)	MY	L	-	-	--
33	*Cucurbita maxima* Duchesne (Cucurbitaceae)a	MY	L	-	-	--
34	*Mimosa pudica* L. (Mimosaceae)	MY	L	-	-	--
35	*Mirabalis jalapa* L. (Nyctganiceae)	MY	L	-	-	--
36	*Achyranthes aspera* L. (Amaranthacea)[a,b]	MY	L	-	-	F14, F23
37	*Bixa orellana* L. (Bixaceae)	MY	L	-	-	F44
38	*Ixora coccinea* L. (Rubiaceae)	MY	L	-	-	F41
39	*Rosa indica* L. (Rosaceae)[a,b]	MY	L	-	-	F7
40	*Zizypus rugosa* Lam. (Rhamnaceae)	MY	L	-	-	F38

[1]The species marked with *a* is Ayurvedic plant while that with *b* is a common bazar medicine[21]. [2] Specimens originate from Mysore (MY), Mandaya (MA), Chamarajanagar (CH), Mercara (ME) and Hassan (HA) districts, respectively. [3] Leaves (L), twigs (T), flowers (F), bulbs (B), rhizome (R), seeds (S) or peel (P) of the plant were used for extraction. [4]The plant material was extracted with equivalent to mass of distilled water and the resulted solution was applied in the screening process directly or diluted with distilled water 1:9 and 1:99 ratios, respectively. The antisporulant activity was evaluated by following scale; full inhibition (++), partial inhibition (+) and no inhibition (-). The hundred fold diluted extracts did not exhibited activity.

[5] The fungal species with reported sensitivity to extracts of the given plants are as follows: F1-*Alternaria alternata*[25,26], F2-*A. solani*[27], F3-*A. tenuis*[28], F4-*A. triticina*[27], F5-*Aspergillus sp.*[29–32], F6-*A. niger*[26,31,33–38], F7-*Botrytis fabae*[39], F8-*Botrytis cinerea*[40–42], F9-*Ceratocystis paradoxa*[43], F10-*Cochliobolus miyabeanus*[44], F11-*Colletotrichum capsici*[26], F12 - *C. coccodes*[45], F13-*C. lindemuthianum*[27], F14-*C. musae*[46–49], F15-*Glomerella cingulata*[42], F16-*Didymella bryoniae*[50], F17-*Drechslera oryzae*[28], F18-*Fusarium spp.*[35,51–53], F19-*F. moniliforme*[54], F20-*F. nivale*[55], F21-*F. oxysporum*[27,28,36,45,48,49], F22-*F. pallidoroseum*[26], F23-*F. proliferatum*[42,47], F24-*F. solani*[56], F25-*F. udum*[17], F26-*Gaumanomyces graminis*[55], F27-*Geotrichum candidum*[57], F28-*Helminthosporium oryzae*[27], F29-*Botryosphaeria rhodina*[26,46,58], F30-*Macrophomina phaseolina*[27], F31-*Melampsora rust*[59], F32-*Penicillium citrinum*[26], F33-*P. digitatum*[58,60,61], F34-*P. expansum*[42], F35-*P. italicum*[60], F36-*Phomopsis caricae-papayae*[26], F37-*Puccinia arachidis*[62,63], F38-*Pyricularia oryzae*[64–66], F39-*Pythium species*[18], F40-*Rhizoctonia solani*[27,45,66,67], F41-*Saccharomyces cerevisiae*[68], F42-*Sclerotinia sclerotiorum*[69], F43-*Sphaerotheca fuliginea*[55], F44-*Trichoderma*[30,70], F45-*Ustilago maydis*[71], F46-*U. nuda*[71], F47-*Verticillium fungicola*[70].

throughout of this study. The extracts from leaves or bulbs of various *Allium* species are know to act against large number of pathogens[17], among them peronosporas which is in accordance with our data. Leaf extracts of *M. koenigi* could control *Pythium* damping off at 67 % when applied via soil in tomato[18] as well as the efficacy of bark-debris of *Eucalyptus* against *Phytophtora* sp. was demonstrated[19]. Broad spectrum of antifungal activity was reported in the case of several test plants (*1, 2, 6, 13, 17*, 19, 25, 26, 27). However, among them only *Z. officinale* and *O. basilicum* exhibited strong antisporulant effect in our tests while *E. globosus*, *A. indica* and *O. sanctum* acted weakly. Analyzing the activity scores of different plant extracts against *S. graminicola* (Table 1) in relation to their effects reported various phytopathogenic fungi, there was clear that the sensitivity spectrum of *S. graminicola* is entirely different. Moreover, no relationship was revealed between taxonomic position of plants and antiperonospora activity of their extracts.

The role of phytoalexins in defence mechanisms was intensively studied meanwhile to constitutive compounds has been paid less attention[15]. The term "phytoanticipin" was proposed for description of the latter group in 1994 and include all types of low molecular weight antimicrobial metabolites other than phytoalexins that are supposed to play role in disease resistance of plants[1]. In our case tissues of healthy plants were collected. It can be presumed that the extracted constitutive compounds of plants were responsible for the antisporulant effect that was manifested in our experiments. Nevertheless, it is often difficult to determine whether a molecule is constitutive or induced, as same of them may normally be presented in hardly detectable quantities, but dramatically increase in concentration after infection. Moreover, the same compound may be performed antifungal substance in one species and a phytoalexin in another[15].

The results of the present work indicate that some of tested plants are promising candidates for PMDM management. These species (*A. sativum*, *P. nigrum*, *C. gouriana*, *E. alsinoides* and *M. elengi*) are found in pearl millet growing areas and and their utilization makes possible the efficient pest management exploiting the local natural resource base. The use of watery extracts possessing with broad spectrum of activity (species 1, 2 and 6) can inhibit whole pathogen complex[20] associated to pearl millet. These plants are not harmful, they are well know sources of drugs sold as bazar medicines[21]. Moreover, the costs of treatment are low and the contamination with residual amounts of pesticides can be avoided[22].

The use of watery extracts of plants in agricultural practices has advantages. The technology of preparation of watery extracts easy to transfer to the farmers and thus to promote sustained millet production[23]. They can be formulated on-farm like herbal tea, moreover, the extracts do not need further bioremediation and are immediately suitable as a foliar spray. Applying such preparations the transmission of *S. graminicola* to new areas by wind can be successfully stopped as zoosporangium formation the key step of pathogens ontogeny[24] can be fully inhibited. The secondary benefit of this technology will be a supply of soluble nutrients, which can be used as a liquid fertilizer enhancing the crop fertility.

For herbal preparations, it may not be essential to pinpoint the active principle, if a product is too complex, it can be standardized in terms of biologic activity parameters. Nevertheless, the analysis of chemical composition of the extracts with remarkable effect can direct to discovery of new antimildew substances which might be promising lead compounds for development of new synthetic molecules with antiperonospora activity.

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