

Induction of Sexual Stage and Colony Morphology of Some Isolates of *Sclerotium rolfsii* Causing Spotted Leaf Rot in Plants

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Twenty-two isolates of *Sclerotium rolfsii* causing spotted leaf rot from Varanasi, India were grown on 6% *Cyperus rotundus* rhizome meal agar (CRMA) medium for the induction of asexual stage (*Athelia rolfsii*). Only one isolate obtained from *Sphaeranthus indicus* formed basidial stage on CRMA medium while the other 21 isolates did not. Basidial stage was also produced in *S. indicus* isolate at different concentrations (5.5, 6.0 and 6.5% w/v) of CRMA medium. Size of basidia, sterigmata and basidiospores of this isolate was measured. Basidia clavate, hyaline and measured $10\text{--}12 \times 4\text{--}5 \mu\text{m}$ in size, basidiospores hyaline, unicellular, subglobose to ellipsoid produced on sterigmata and measured $3\text{--}5 \times 2\text{--}4 \mu\text{m}$ in size, sterigmata hyaline and measured $4\text{--}5 \times 1.5\text{--}2 \mu\text{m}$ in size. The results of the present study revealed wide variation in spotted leaf rot isolates of *S. rolfsii*. A reddish zone around the colony of *S. rolfsii* isolate from *Vernonia* sp. was observed on CRMA medium. HPLC analysis of the zone revealed the presence of gallic and ferulic acid which were also thought to be responsible for reduced mycelial growth of the isolate on CRMA medium.

KEYWORDS: CRMA, HPLC analysis, Phenolic acids, *Sclerotium rolfsii*, Sexual stage induction

Sclerotium rolfsii Sacc. is a devastating soil-borne pathogen in the tropics and subtropics with a staggering host range of more than 500 plant species including both monocots and dicots (Aycock, 1966; Punja, 1985). *S. rolfsii* causes collar rot, foot rot, root rot, fruit rot and spotted leaf rot in several plants (Sarma *et al.*, 2002). Some workers reported leaf spot disease in several plants, viz., *Dioscorea* sp., *Solanum dulcamara*, *Hemidesmus indicus*, etc., caused by *S. rolfsii* (Amusa, 1999; Campbell *et al.*, 2002; Singh and Pavgi, 1965). The mechanisms of sexual stage formation in many plant pathogenic fungi, especially facultative parasites, are not well understood. Tu and Kimbrough (1978) described *Athelia rolfsii* (Curzi) Tu & Kimbrough as the perfect stage of *S. rolfsii* while studying *Rhizoctonia-Sclerotium* complex which is the currently accepted binomial for the sexual stage of *S. rolfsii* (Punja *et al.*, 1982). Basidial stage of *S. rolfsii* has been reported as of infrequent occurrence in nature (West, 1947; Ghosh and George, 1955; Kulkarni and Ahmed, 1967) but can be induced in culture (Goto, 1930; Mundkur, 1934; Misra and Haque, 1960; Ahmed and Kulkarni, 1966; Kulkarni and Ahmed, 1967; Ahmed *et al.*, 1966; Punja *et al.*, 1982; Singh *et al.*, 1996). Singh *et al.* (1996) were able to induce sexual stage of this fungus within a week on *Cyperus rotundus* rhizome powder supplemented water agar medium. The asexual stage induction of *S. rolfsii* on *C. rotundus* rhizome meal agar (CRMA) medium has been confirmed by some other worker also (Singh, 2002). Recently, Sarma *et al.* (2002) observed that

the frequency of sexual stage producing isolates in nature is very low as out of 26 isolates only 4 produced sexual stage. Sarma *et al.* (2003) induced asexual stage in this medium on different concentrations of fresh rhizome powder and leaves paste of *C. rotundus*. The purpose of the present study was to see whether basidial stage is formed in spotted leaf rot causing isolates of *S. rolfsii* also. The isolates were also compared for their morphological characters. The results are presented here.

Materials and Methods

Isolation and maintenance of the fungal culture.

Twenty-two isolates of *S. rolfsii* causing spotted leaf rot in various hosts (Table 1) were collected from plants growing within the campus of Banaras Hindu University, Varanasi, India. The fungus was isolated by inoculating tiny sclerotia or mycelial bits formed on diseased plants or lesions of the plants in Petri dishes containing potato dextrose agar (PDA) medium and the plates were incubated at $25 \pm 2^\circ\text{C}$ for 7–10 days till sclerotia were formed. From each plate single sclerotium was taken out and inoculated into PDA slants. The isolates were thus purified and such slants were stored at 4°C for further use.

Athelial stage induction. Fresh rhizomes of *C. rotundus* were collected from field, washed thoroughly in tap water, air-dried and stored at -4°C . During experiments, the rhizomes were crushed to powder in an electric grinder. The rhizome powder was then incorporated (6% w/v) into distilled water and agar (1.5% w/v) medium

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Table 1. Characteristics of some isolates of *Sclerotium rolfsii* causing spotted leaf rot

Host	Year of collection	Colony type	Growth diameter (mm) after			Sclerotial colour
			24 h	48 h	72 h	
<i>Artrica</i> sp.	2002	Compact	27	61	85	DB
<i>Clerodendron inerme</i>	2002	Compact	19	45	73	DB
<i>Sphaeranthus indicus</i>	2001	Compact	8	20	58	DB
<i>Spathodea companulata</i>	2001	Fluffy	20	55	90	DB
<i>Morus nigra</i>	2002	Fluffy	22	48	89	RB
<i>Solanum melongena</i>	2002	Compact	25	70	90	DB
<i>Ruellia</i> sp.	2002	Compact	12	30	48	DB
<i>Commelina benghalensis</i>	2001	Compact	23	61	78	RB
<i>Ficus infectoria</i>	2002	Compact	22	45	72	RB
<i>Desmodium gangeticum</i>	2001	Compact	15	43	64	DB
<i>Coccinia indica</i>	2001	Compact	27	69	90	DB
<i>Commelina</i> sp.	2002	Compact	9	12	15	DB
<i>Rauvolfia serpentina</i>	2001	Compact	21	41	84	DB
<i>Vernonia</i> sp.	2002	Fluffy	18	44	82	RB
<i>Ficus religiosa</i>	2001	Compact	18	41	84	DB
<i>Ageratum canozoides</i>	2001	Compact	21	55	87	DB
<i>Corchorus acutangulus</i>	2001	Compact	11	24	57	DB
<i>Vigna mungo</i>	2002	Compact	22	51	79	RB
<i>Cajanus cajan</i>	2001	Compact	6	11	26	DB
<i>Blepharis boerhaviaefolia</i>	2001	Compact	12	34	66	DB
<i>Caladium</i> sp.	2002	Compact	10	42	83	RB
<i>Bombax malabaricum</i>	2002	Compact	16	23	45	RB

DB = dark brown; RB = reddish brown; h = hours.

(CRMA: *C. rotundus* rhizome meal agar medium) (Prithiviraj *et al.*, 2000). Five-millimeter (dia) mycelial discs from 4-day-old cultures of *S. rolfsii* (twenty two different isolates) were inoculated onto the center of the Petri plates containing CRMA medium. Similarly, mycelial discs were also inoculated in Petri plates containing PDA medium that served as control. The plates were incubated at $25 \pm 2^\circ\text{C}$ under darkness and constantly observed every day after 4 days of inoculation for sexual stage induction.

Rhizome powder was used at different concentrations (5.5, 6.0 and 6.5% w/v) prepared in distilled water with agar (1.5% w/v) in the medium and sexual stage induction in isolates *S. rolfsii* was observed. Five-millimeter (dia) mycelial discs from 4-day-old cultures of this isolate of *S. rolfsii* was inoculated onto the center of the Petri plates containing different concentrations of CRMA medium. Similarly, Petri plates containing PDA medium were also inoculated as control. The Petri plates were incubated at $25 \pm 2^\circ\text{C}$ under darkness maintained by covering with black paper sheet and constantly observed every day for sexual stage formation. The whole experiments were repeated three times and at least three plates were used in each experiment. Measurements of basidia, basidiospores and sterigmata were also conducted.

Cultural variation. Radial growth (colony diameter in mm after 24 h, 48 h, 72 h), colony morphology and sclerotial colour were examined on PDA. At least three plates

of PDA were inoculated with a mycelial disc (5 mm dia) taken from the margin of an actively growing colony (3-to 4-day-old) of each isolate. The inoculated plates were incubated at $25 \pm 2^\circ\text{C}$. The colony diameter was measured every day upto 3 days. The plates were regularly observed for sclerotium formation and maturation. The data from the replicated plates were averaged. The morphological variation of the colonies on PDA was observed after 4 days of inoculation.

Ethyl acetate fractionation for HPLC analysis. Out of twenty two, one isolate from *Vernonia* sp. was highly sensitive to CRMA medium. This isolate showed reddish pigment around the fungal colony on CRMA medium after 4 days of incubation at $25 \pm 2^\circ\text{C}$ under darkness. After 7 days of inoculation, three samples of equal amount (5 g), one from *S. rolfsii* uninoculated CRMA medium and the other two samples from reddish pigment zone of CRMA medium around the *S. rolfsii* colony as well as CRMA medium beyond the reddish pigment zone of the same plate were taken out and homogenized in 10 ml of ethyl acetate in a pestle-mortar. Samples were kept for 24 h in screw-capped sample bottles. The ethyl acetate fraction was re-extracted twice, the pooled fractions of the samples were then filtered through Whatman No. 1 filter paper, and the filtrate was evaporated under vacuum (Buchi Rotavapor Re Type). Dried samples were re-suspended in 1.0 ml of HPLC grade methanol by vortexing

and stored at 4°C for further analysis.

HPLC analysis. High performance liquid chromatography (HPLC) of the samples was performed with HPLC system (Shimadzu Corporation, LC 10 ATVP, Kyoto, Japan). Reverse phase chromatographic analysis was carried out in isocratic conditions using RP C-18 HPLC column at room temperature. Running conditions included injection volume: 5 μ l, mobile phase: methanol-0.4% acetic acid (80 : 20, v/v), flow rate: 1 ml/min, detection at 290 nm and attenuation response 0.03 AUFS. Samples were filtered through membrane filters (Pore size 0.20 μ m, Millipore) prior to injection. Tannic, gallic, oxalic, caffeic, vanillic, ferulic, chlorogenic, o-coumeric, gentisic and cinnamic acids were used as internal and external standards. Phenolic compounds present in the samples were identified by comparing retention times (R.T.) of the standards and by the co-chromatography. Contents of phenolic acids were calculated by comparing peak areas of standards with those in the samples. Concentrations of phenolic compounds were statistically analyzed.

Results and Discussion

One isolate of *S. rolfsii* causing spotted leaf rot in *S. indicus* produced athelial stage on CRMA medium whereas, the other twenty-one did not (Table 1). Athelial stage induction in *S. indicus* isolate occurred after 9 days of inoculation. Colonies were slow growing with profuse, fluffy mycelium on CRMA medium while, white, smooth, fast-growing colonies were observed in control. The teleomorph was not found in PDA except *S. melongena* isolate where athelial stage developed infrequently after 20~25 days of inoculation. The number of sclerotia produced on CRMA medium was less than PDA.

The basidial stage formed in all concentrations (5.5, 6.0 and 6.5%) of *C. rotundus* rhizome meal (CRM) in the CRMA medium. Colony was smooth, spreading with hyphal strands from centre to towards periphery. Vigorous mycelial growth with less number of sclerotia were formed in 6% of CRM compared to the two other concentrations viz., 5.5 and 6.5% of CRM in the CRMA medium.

Basidia formed abundantly in clusters which were clavate, mostly 4-spored, 10~12 \times 4~5 μ m; basidiospores hyaline, unicellular, thin-walled, 3~5 \times 2~4 μ m, subglobose to ellipsoid, produced on sterigmata, which were sub-cylindrical, tapering and pointed at tip, 4~5 \times 1.5~2 μ m (Fig. 1a, 1b).

Growth diameters of the isolates obtained from *Artrica* sp. and *C. indica* were found to be maximum (27 mm) whereas growth of *C. cajan* isolate was found to be minimum (6 mm) after 24 h of inoculation. Growth diameter of *S. melongena* isolate was maximum, i. e., 70 and 90 mm after 48 and 72 h of inoculation, respectively. Mini-

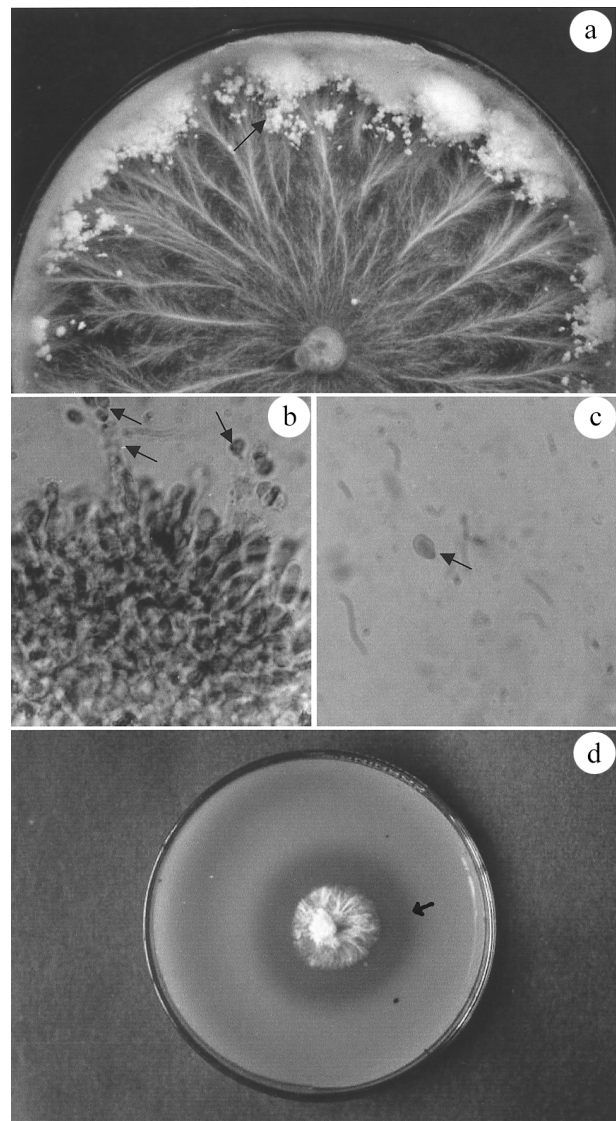


Fig. 1. (a) Athelial stage (arrow) of *Sclerotium rolfsii* isolated from *Sphaeranthus indicus* on CRMA medium, $\times 1.5$. (b) Basidia bearing basidiospores (arrow), $\times 1000$. (c) Single basidiospore, $\times 1000$. (d) Reddish pigmented zone (arrow) around the colony of *S. rolfsii* isolated from *Vernonia* sp. on CRMA medium, $\times 0.75$.

mum growth diameter (11 and 15 mm) was observed in *C. cajan* and *Commelina* sp. after 48 and 72 h of inoculation, respectively. Mycelial growth was fluffy in 3 isolates while, compact in rest 19. Sclerotia were 1~3 mm in diameter, reddish brown to dark brown with smooth or pitted surface (Table 1).

HPLC analysis revealed 2.17 μ g/g gallic acid and 8.22 μ g/g ferulic acid in uninoculated CRMA medium. While, in case of reddish pigmented zone of CRMA medium and CRMA medium around reddish pigmented zone after inoculation contained only gallic acid (0.03 μ g/g) (Figs. 1c, 2).

Induction of sexual stage in *S. rolfsii* is infrequent in

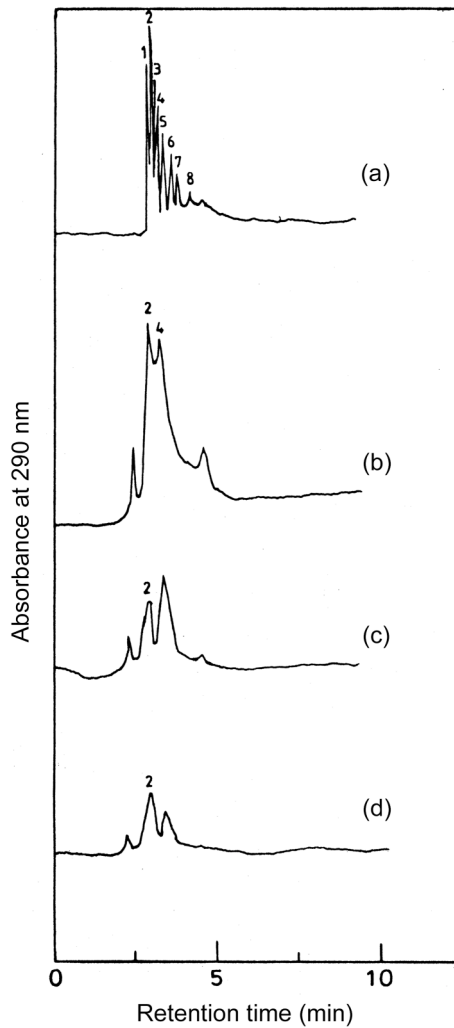


Fig. 2. HPLC analysis of ethyl acetate fraction of CRMA medium after 7 days of inoculation (a) Peaks of standards, (b) Uninoculated CRMA medium, (c) Reddish pigmented zone of CRMA medium, (d) CRMA medium around reddish pigmented zone after inoculation of *S. rolfsii* (*Vernonia* sp. isolate) (peak nos. 1 = tannic acid, 2 = gallic acid, 3 = vanillic acid, 4 = ferulic acid, 5 = o-coumeric acid, 6 = chlorogenic acid, 7 = cinnamic acid).

nature (West, 1947) but has been induced in culture (Mundkur, 1934; Mishra and Haque, 1960; Punja, 1985; Singh *et al.*, 1996). The extent of hymenial development was influenced by the medium on which isolates were grown, the conditions of inoculation, the isolate, diameter of mycelial plugs and light intensity (Punja *et al.*, 1982; Prithiviraj *et al.*, 2000). These authors reported that, in general, nutritional or environmental conditions that restricted optimal growth and sclerotium production by the fungus favoured hymenial development and they also reported that there was no apparent correlation between host or area of isolation and ability to form hymenia. Induction of aethelial stage of *S. rolfsii* on different concentrations of *C. rotundus* rhizome powder and leaf paste was reported by

Sarma *et al.* (2003). They found prominent effect of the concentration on sexual stage induction. They also found fresh rhizome and leaves were more effective for sexual stage induction than dried rhizome powder and leaves.

The results of the present study reveal wide variation in colony characters, growth and sclerotium formation in leaf spot causing isolates of *S. rolfsii* on CRMA medium. Since the sexual stage of this fungus is rare in nature and its role in the life cycle is unknown, Nalim *et al.* (1995) advocate that the genetic exchange in mycelia of *S. rolfsii* isolates is limited to mycelial compatibility. Production of the teleomorph in leaf spot isolate of *S. rolfsii* on CRMA medium may strengthen the claim that genetic exchange may occur through normal genetic recombination during basidiospore formation. The absence of the teleomorph in most of the isolates may either be due to the loss of the ability to produce basidiospores during the evolution process or they require specific nutritional/ecological conditions (Sarma *et al.*, 2002). Production of lesser number of sclerotia on CRMA medium than PDA is confirmed by earlier reports (Prithiviraj *et al.*, 2000). This may be due to less availability of simple sugars in this medium (Henis *et al.*, 1965). Different isolates of *S. rolfsii* may have different requirements for induction of basidial stage; in *Rhizoctonia solani*, for example, there are marked differences among isolates in the conditions required for fruiting (Stretton *et al.*, 1964). Similarly, Kulkarni and Ahmed (1967) reported differences among isolates of *S. rolfsii* in their ability to form sexual stage on selected media. Many workers (Punja, 1985, 1988; Sarma *et al.*, 2003) reported sexual stage induction in collar rot causing isolates of *S. rolfsii*. Whereas, induction of sexual stage in leaf spot isolates is rare. Punja *et al.* (1982) induced sexual stage in the isolate from leaves of *Ficus pumila* on PDA containing 2% activated charcoal (C-PDA). Our report will help in study of biology of *S. rolfsii* and disease-cycle of spotted leaf rot causing isolates.

Sexual stage was induced mostly in darkness. The size of basidia and basidiospores are identical to those reported by Goto (1930), Mundkur (1934), Kulkarni and Ahmed (1967), and Punja *et al.* (1982). We revealed that the presence of gallic and ferulic acid in CRMA medium and gallic acid in the reddish zone by HPLC. Therefore, the combination of gallic and ferulic acid which are reported to be antifungal may be suspected the cause of growth inhibition of *S. rolfsii* on CRMA medium.

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