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In vitro SPORULATION, CULTURAL CHARACTERIZATION, IDENTIFICATION AND PHYLOGENY OF GRAY MOLD FUNGUS [*Botryotinia ricini* (Godfrey) Whetzel.)] OF CASTOR (*Ricinus communis* L.)

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ABSTRACT

Control of *Botryotinia ricini* (Godfrey) Whetzel., gray mold pathogen of castor, is a challenge due to the lack of known sources of complete resistance and effective disease management practices. Isolation of pure culture of causal pathogen and its profuse sporulation are imperative for any pathological or molecular understanding of infection or resistance mechanisms and host-pathogen interactions. Season-dependent disease occurrence coupled with sparse conidial production *in vitro* has been a serious constraint. A culture medium (BRS) based on V8 juice broth supplemented with salts and minerals was developed and culture method and conditions optimized for profuse *in vitro* sporulation of gray mold fungus. ITS barcoding identified the isolate from Hyderabad as *Amphobotyrs ricini* (*A. ricini* isolate IRHT-S1) which was closely related to *A. ricini* strain Cop-Ar5. Phylogenetic analysis of the isolate with 20 related fungal species revealed that *A. ricini* forms a separate clade. Studies on growth response, culture characteristics and sporulation of *A. ricini* in different media ingredients revealed that the culture morphology differed with medium composition, especially in medium with 60 g L^{-1} dextrose, while capsule/ epicarp extract was dispensable. The addition of salts such as $MgSO_4$ (1.0 g L^{1}), KCl (1.0 g L^{-1}) and $FeSO_4.7H_2O$ (0.02 g L^{-1}) favoured sporulation.

Key words: *Amphobotrys ricini*, anamorph, culture medium, sporulation, ITS sequence

INTRODUCTION

India is the major producer of the economically important oilseed crop castor (*Ricinus communis* L.) and ranks first in its global production (Anonymous, 2017). Further, it is the largest supplier of castor seed oil, meeting about 80% of the global demand. However, castor production in India has shown a steep decline in 2016-17, with estimated production of 14.21 lakh tonnes, mainly attributed to the decrease in net sown area, due to farmer's shift to other remunerative crops (Shah and Anish, 2017). Pests and diseases are serious constraints in improving castor production and productivity. Gray mold of castor caused by a necrotrophic fungus *Botryotinia ricini* (Godfrey) Whetzel, is a major disease, causing severe yield losses which may be as high as 100%, when continuous rainfall and low temperature prevails during capsule formation. Gray mold disease occurs world-wide and has been reported in epidemic form in USA in 1958 and in Andhra Pradesh (India) in 1987 (Dange *et al*., 2005; Soares, 2012). In India, the disease poses serious threat to the castor crop mainly in Andhra Pradesh and Tamilnadu (Soares, 2012). The gray mold disease is season-dependent and mostly occurs during October-November months when the conditions of temperature <28°C, poor sunshine and high

humidity due to short continuous drizzles prevail. The necrotrophic pathogen infects seed-bearing spikes directly, sporulates therein and turns the whole spike into a mass of spores, thereby devastates the crop in a short span of 2-3 days.

Gray mold fungus *Botryotinia ricini* belongs to the family Sclerotiniaceae (Helotiales, Ascomycota) and is characterized by dark, plane-convex and elongated sclerotia (Soares, 2012). The anamorphic state (asexual reproductive phase) of *Botryotinia ricini* was named as *Botrytis ricini* N.F. Buchw (Buchwald, 1949). However, in 1973 the genus *Amphobotrys* was erected based on the distinctive pattern of conidiophore ramification and since then, the anamorphic state was known as *Amphobotrys ricini* (N.F. Buchw.) Hennebert, which is responsible for disease epidemics (Hennebert, 1973). The fungus survives in soil as sclerotial mass during off-season and infects reproductive parts of castor under favourable environmental conditions. Gray mold is generally confined to spikes/ racemes and seen on other plant parts during severe infection. Gray mold in Korea was observed on castor leaves as blight, the sporulation occurred in marginal and central part of leaves and the disease was reportedly found due to *B. cinerea* and *A. ricini* (Hong *et al*., 2001). The disease initially appears on capsules or flowers as small blackish spots, exuding a drop of yellow liquid, followed by whitish mycelial growth and the infection from these spots spread to the whole raceme. During profuse growth and sporulation, olive gray coloured woolly growth is noticed and the infected flowers/ capsules appear soft, covered with dusty powder, which results in rotting of capsules. Presently neither effective control measures to gray mold nor completely resistant cultivars are available.

Although gray mold causes severe yield losses in castor world-wide, yet not much information is available about the processes or molecular mechanisms involved in the infection of *A. ricini*. Resistance mechanism and host-pathogen interactions are not well elucidated in this highly host-specific necrotroph**.** Mechanism of infection is known in a related species *B. cinerea* (Lalève *et al*., 2014). An understanding of molecular mechanisms underlying host-pathogen interactions not only provides information on infection strategy of a pathogen but also enables researchers to devise effective disease-management strategies (Parvathy *et al*., 2016). Sufficient conidial inoculum must be available in order to conduct molecular or pathological studies throughout the year. The gray mold fungus sporulates in castor spikes only during favourable conditions in September-November months and hence any study on screening for resistance to the pathogen is a season-dependent process. To ensure the availability of conidial inoculum throughout the year, cut-spikes of field-grown plants were infected with spores at weekly intervals, under artificial conditions (75% humidity and 28°C temperature) in glasshouse, and spores collected from infected capsules. Hence plants had to be maintained in field throughout the year as a source of cut-spikes. Optimization of culture medium and method for profuse sporulation of pathogen, isolation of pure culture and species characterization are pre-requisites for undertaking a detailed study on host-pathogen interaction of gray mold in castor. Profuse mycelial growth and sclerotia formation under *in vitro* culture conditions have previously been reported (Raoof and Prasad, unpublished). Sporulation was not achieved in PDA under normal conditions. The present study was, therefore, aimed to optimize the culture medium and method for profuse sporulation of gray mold fungus and also to identify gray mold fungus by sequencing the ITS region and elucidate the genetic relatedness with other gray mold/rot causing fungal species.

MATERIALS AND METHODS

Media composition and culture conditions

For the isolation of pure culture, the culture medium with capsule extract was prepared from gray mold-susceptible castor cv. DCS 9. The pericarps from individual capsules were separated from seeds, weighed and 100 g material L^{-1} (10% w/v) crushed in liquid nitrogen. The powdered material was boiled in 600 mL distilled water. The extract was sieved through muslin cloth and the process repeated 2-3 times. For preparing the culture medium (BRS), 20 g V8 juice broth powder (HiMedia),

5 g yeast extract (HiMedia), 10 g peptone (HiMedia) and 20 mM potassium phosphate buffer (pH 7.0) were dissolved in capsule extract. The pH was adjusted to 5.5 and the volume made up to 1 L. Then 15 g agar was added and medium autoclaved at 15 psi for 20 min. Streptomycin @ 50 mg L^{-1} was added to the culture medium after autoclaving to prevent any bacterial contamination. The medium used for sporulation was referred to as BRS (*B. ricini* sporulation) medium.

The infected spikes of highly susceptible variety of castor DCS 9, with profuse sporulation of gray mold pathogen, were collected from the experimental field of ICAR-Indian Institute of Oilseeds Research Farm, Rajendranagar (India) during 2012. Scrapings from infected capsules without staining were observed under light microscope to ensure the presence of round conidia of gray mold fungus. The infected capsules, cut to 0.5-1.0 cm^2, were placed at the centre of culture plate, firmly touching the medium and a drop of sterile distilled water added on the top. The petri-plates were placed in a tray with moistened blotting sheets. The trays were covered with aluminum foils and incubated in a BOD incubator (Caltan® seed germinator) at 22±1°C and with 75% relative humidity. The blotting sheets in tray were moistened at 24 h interval. The first sub-culturing in fresh medium was done after 10 days growth. From primary culture, the agar discs (0.5-1.0 cm^2) with fungal growth (mycelia or spores) were cut and inoculated into fresh culture plates and incubated. Sub-culturing was continued after 8-10 days. Microbial inoculation and culturing were carried out under sterile conditions.

Microscopic observation

The culture plates were observed under a stereo-microscope (Wild Heerbrugg, Switzerland) for gray mold spores. For morphological characterization, the spores and mycelium were observed without stain under a compound microscope (Nikon H600L, Japan) using 200 and 400X magnification, respectively, and images captured by Nikon Digital Sight DS-FI1C camera. Cultural characteristics such as colony colour (mycelium and spores), growth pattern and sclerotia production on the media were studied and recorded when mycelial growth was complete by about 9 days.

Preparation of glycerol stocks

The spores of gray mold fungus were scraped off the culture plate, collected in distilled water and mixed with glycerol to a fine suspension for preparing 75% glycerol stock. The conidial glycerol stocks were stored in -80°C.

Culture characteristics in different media

The effect of dextrose, sucrose and capsule extract on the growth and sporulation of gray mold fungus was assessed. BRS culture medium [composition per litre: 20 g V8 juice broth, 5 g yeast extract, 20 mM potassium phosphate buffer (pH 7.0), 10% w/v capsule extract, 1.5% agar, pH 5.5] was used as control. Another control taken was modified BRS medium [BRS medium without 20 mM potassium phosphate buffer + 2 g L^{-1} K_2HPO_4, 1.0 g L^{-1} $MgSO_4$, 1.0 g L^{-1} KCl and 0.02 g L^{-1} $FeSO_4.7H_2O$]. The composition of other media tested were CE (BRS medium without capsule extract), DX (BRS + 60 g L^{-1} dextrose (4 mL sterile 30% w/v dextrose added after autoclaving the medium)) and MIIS (BRS + 10 mM sucrose). The conidial suspension from 75% glycerol stock was streaked with sterile water in 1: 1 ratio, spread on media plates containing 50 mg L^{-1} streptomycin and incubated at 22±1°C with 75% humidity. The experiment was conducted in a completely randomized design with each treatment replicated three times. The observations on sporulation were monitored at 3-4 days intervals. The spores from a plate were harvested and counted with the help of a hemocytometer (Superior, Germany).

Pathogenicity test

B. ricini culture was revived in BRS (*Botryotinia ricini* sporulation) culture medium from glycerol stock of spore suspension. Fresh spores were harvested from the plates in 1 mL sterile distilled water, spore clumps broken in an ultrasonic bath sonicator (3.5L100H, Ultrasonic, S.V. Scientific) to form a fine suspension and spore count taken using hemocytometer (Superior, Germany) with Nikon

H600L compound microscope. The spore suspension, diluted with sterile distilled water to a final concentration of 10^6 spores mL^{-1} with Tween 20 (0.2 mL L^{-1}), was sprayed on immature capsules of susceptible castor cv. DCS 9 taken in Petri-plates and incubated at 22±1°C with 75% humidity. The plates with blotting sheets were moistened daily with sterile water. Control plates were sprayed daily with sterile water alone. Symptom development was monitored regularly at 3-4 days interval and observations recorded.

Molecular characterization of gray mold fungus and its relation with other fungal species

ITS (internal transcribed spacer) sequencing of mycelium of *B. ricini* was carried out at Amnion Bio-sciences (Bangalore, India) using standard procedures for DNA isolation from mycelium, PCR of genomic DNA with universal primer pairs (White *et al*., 1990) ITS1: 5' TCCGTAGGTGAACCTG CGG 3' and ITS4: 5' TCCTCCGCTTATTGATATGC 3' and sequencing of cloned amplicon by Sanger's di-deoxy chain termination method. To identify the isolated species grown *in vitro*, the homology of sequence spanning the ITS region was analyzed using BLAST of NCBI (http://*www.ncbi.nlm.nih.gov/BLAST/*). The complete sequences of ITS1 and ITS2, either alone or along with 5.8S rRNA, or partial sequences of 28S rRNA or partial sequences of both 18S and 28S rRNA were analyzed using BLAST to retrieve the organisms of greater homology in each case. The ITS sequences of 20 fungal species/strains showing maximum homology to ITS sequence of isolated pure culture of gray mold fungus were extracted, aligned by multiple alignment programme MUSCLE (*www.phylogeny.fr*) (Dereeper *et al*., 2008) and a cladogram/phylogram developed using phylogeny analyses "Advanced" with Gblocks program to eliminate poorly aligned positions (*www.phylogeny.fr*), PhyML for tree building and TreeDyn for tree rendering. The default mode of maximum likelihood (ML) was used for phylogram/cladogram construction.

RESULTS AND DISCUSSION

In vitro sporulation and isolation of pure culture of gray mold fungus

Culturing of pathogen in best suitable medium is the first important step for any pathological research. The BRS medium **[**composition: 20 g L^{-1} V8 juice broth powder, 5 g L^{-1} yeast extract, 10 g L^{-1} peptone, 20 mM potassium phosphate buffer (pH 7.0), 10% w/v capsule extract, 15 g L^{-1} agar and pH: 5.5] was found effective in inducing *in vitro* sporulation of gray mold fungus. In primary culture in BRS medium, contamination with other fungal species like *Aspergillus* and occasionally dark gray spores of *B. ricini* were observed along the fringes of plates. Mycelial agar disc taken from the fringe of primary culture plate and/or spores alone when used as inoculum for sub-culturing gave good sporulation (Fig. 1a), while mycelial inoculum taken from the centre of plate resulted in white mycelial growth. For 2nd sub-culturing, when the medium piece with spores was inoculated, profuse sporulation was observed by 4th day. Sporulation is not spontaneously induced but requires a certain period of mycelial growth. As mycelium became aged, it turned creamy or off white and developed sclerotial mass by 8th day. Sporulation was achieved in BRS broth as well. Pure culture of gray rot pathogen was isolated by sub-culturing (Ibrahim and Rahma, 2009) which also avoided any contamination. Blowing of dry spores for single conidia culture (Godfrey, 1923) or direct inoculation with dry spores (Duarte *et al*., 2013) was not adopted since sporulation under field/ natural conditions was season-dependent (occurring only by September-November), hence unsuitable for culture studies throughout the year. Further, the spores may dry under sunny bright conditions. When spores from glycerol stock were streaked on BRS medium, mycelial growth was either absent or minimum; however profuse sporulation covered the entire plate by 6th day of incubation (Fig. 1b). Thus, the conidia as inoculum has better potential for sporulation than vegetative hyphae or mycelium, since spores retain their viability in glycerol stocks at -80°C than vegetative hyphae. Though the organism outgrows all pathogens or contaminating fungi (Godfrey, 1923), spore suspension was not directly

prepared from dry spores, but from *in vitro*-raised spores. Dilution of glycerol stock with sterile water in 1:1 ratio during plating resulted in faster and better mycelial growth before sporulation. The glycerol stock could be used for nearly 2-3 months at -80°C, for culture retrieval. Sporulation started by 3-8 days depending upon the age or period of storage of glycerol stock.

Modified BRS medium with additional ingredients K_2HPO_4 and salts ($MgSO_4$, $FeSO_4.7H_2O$ and KCl), was better in inducing sporulation than BRS medium (Fig. 1c). Conidial inoculum with spore load of 10^6-10^7 mL^{-1} was harvested from BRS medium while 10^7 conidia mL^{-1} were harvested from modified BRS medium. Sporulation was sparse and mycelial growth predominant in PDA (Fig. 1d). Though no visible sporulation was observed in PDA plates, occasionally few spores were observed in the margin and middle region of culture, along with hyaline white mycelium. Spores or conidial mass appeared as dark spots in a branching hyphal mass in culture plate, when observed under microscope (Fig. 1e). The mycelium was septate, branched and hyaline to light brown. The hyphal mass produced dichotomously branching conidiophores with perfect round unicellular hyaline to light brown conidia at the tip of sterigmata (Fig. 1f-g). The growth rate of gray rot fungus in BRS medium is depicted in Fig. 1h. Mycelial growth started by 2nd day and sporulation by 3rd day but gray spores were visible by 4th day in BRS medium.

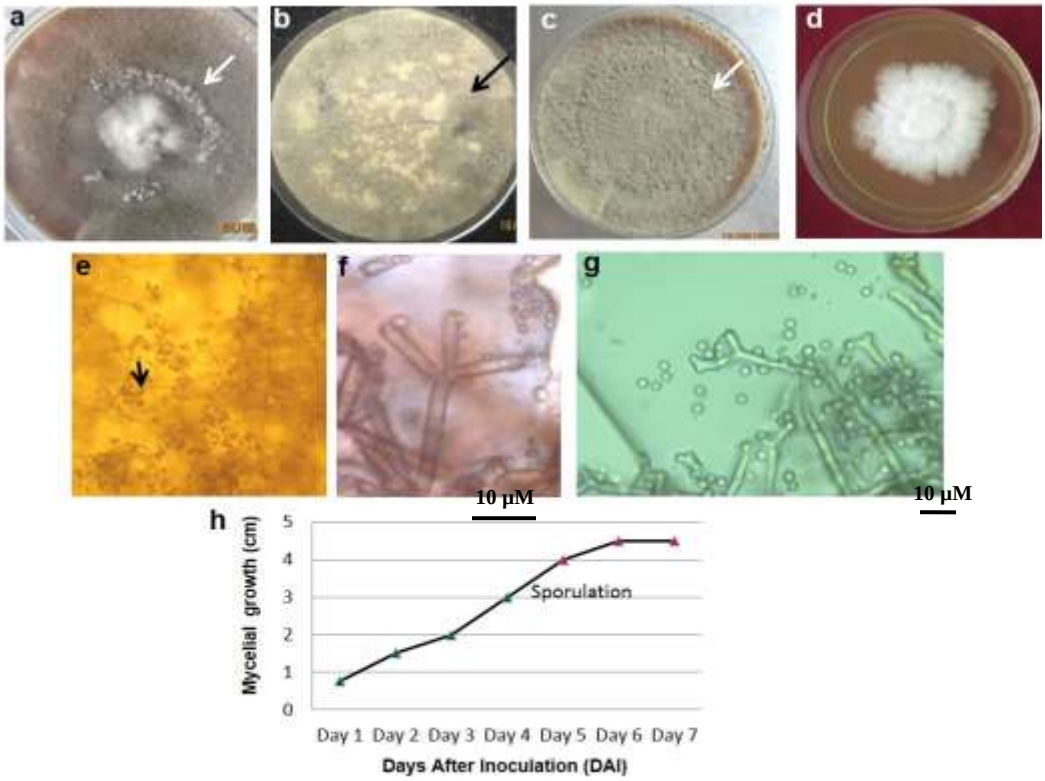

Fig. 1: *B. ricini*, the causal pathogen of gray mold disease in castor, and its *in vitro* sporulation

a) Sporulation in 1st sub-culturing plate (90 cm dia.) using agar piece with mycelium alone taken from the fringe of primary culture plate; b) Sporulation in BRS medium using 75% glycerol stock of conidial suspension of pure culture as inoculum showing mycelium and gray spores; c) Profuse sporulation in modified BRS medium using 75% glycerol stock of conidial suspension of pure culture as inoculum; d) Mycelial growth on 9th day on PDA; e) Dark spores or conidial mass in hyphal network in culture plate observed under stereomicroscope (10X) captured using canon digital camera; f) Conidiophores of *B. ricini* producing spores (400X magnification, scale bar: 10 µM); g) Conidiophores with hyaline round conidia of *B. ricini* (200X magnification, scale bar: 10 µM); h) Mycelial growth of gray mold fungus in BRS medium; the sporulation starts by 3rd day and became visible as gray spores by 4th day after inoculation. Arrows indicate spores or regions of sporulation.

Sporulation in most fungi is dependent on C:N ratio of the medium; and the optimal C:N ratio for sporulation varies with species and method used (Engelkes *et al*., 1997; Elson *et al*., 1998; Mo *et al*., 2005; Gao and Liu, 2010). A culture of *Colletotrichum truncatum* grown in a medium with C:N ratio of 15:1 produced more conidia than with C:N ratio of 40:1 or 5:1, while glucose exhaustion was often coincident with conidia formation (Jackson and Bothast, 1990). The N sources like yeast extract and peptone were added to BRS medium to alter C:N ratio. V8 juice broth contains vegetable extracts, L-asparagine, calcium carbonate, glucose and yeast extract; and V8 juice agar was chosen as it has C:N ratio of nearly 10:1, almost optimal for sporulation (Kadir *et al*., 2007). The BRS medium contains C and N sources and also potassium phosphate buffer with KH_2PO_4 and K_2HPO_4. Potassium phosphate buffer can be replaced with K_2HPO_4. Modified BRS medium contains salts $MgSO_4$, KCl, $FeSO_4$ and K_2HPO_4 which are also present in other sporulation media. Acidic pH of 5.5 favours the fungal growth.

Growing a fungus on long series of culture media reportedly is not profitable since its growth is equally vigorous on most media (Godfrey, 1923). Different media tested varied in inducing conidia formation, with some proving more effective than others in producing a heavy mat of conidiophores and conidia, without any sclerotia. Conidia production was heavy on corn meal agar with little white mycelium and occasional sclerotia; while on PDA more mycelium was evident. Oatmeal agar at first produced profuse conidia but was later covered by white mycelium and abundant sclerotia. Transfers made from media producing profuse conidia to new conidia-producing media (oatmeal agar) resulted in profuse sclerotia formation in new medium and was not due to strain variation (Godfrey, 1923). This indicated that after conidia production, sclerotia formation occurs irrespective of media. In present study, sclerotia formation occurred when culture was aged or stored for a long period at low temperature or under stressed nutritional/environmental conditions. PDA promoted profuse mycelial growth of gray-mold pathogen (*Botrytis cinerea*) of chickpea and hence desi-chickpea seeds were used to develop medium for sporulation (Sheila and Nene, 1987). PDA supported sclerotia production, while malt-extract agar and oatmeal agar enriched with gallic acid and L-asparagine promoted sporulation in *A. ricini* (Hong *et al*., 2010; Duarte *et al*., 2013; Prasad and Bhuvaneshwari, 2014). The American form of gray mold pathogen was suspected to be from India, perhaps carried along with seed (Godfrey, 1923). A culture retrieved from India in 1921 which appeared identical in form and size, behaved differently than American form. It produced a few conidia, more white mycelium, less sclerotia and not so definitely dichotomous conidiophores in culture (Godfrey, 1923). The pathogenic isolate from India (Hyderabad) reportedly had difficulty in sporulation in common media and produced only sclerotia. Hence, it was imperative to optimize the culture medium/culture conditions for effective conidia formation or sporulation. Thus, we report here the isolation of pure culture of gray mold fungus by sub-culturing and subsequent plating of the glycerol stocks of spore suspension in modified BRS medium (without capsule extract) for profuse sporulation, that can be used for infecting castor throughout the year.

Cultural characterization and pathogenicity of gray mold fungus

During primary culture in dextrose medium, distinct white cottony mycelial growth was observed. In 1st subculture, the mycelium turned thick, hard and creamy, with corrugations resembling the convulsions of human brain. Profuse sporulation, characterized by dense mass of grayish black spores, was visible by 9th day at the corrugations and from the fringe of culture plates. In 1st subculture, transfer from control (BRS) to dextrose medium or vice versa resulted in sporulation, but not when transferred from dextrose to again dextrose medium or sucrose to dextrose medium. Sucrose did not favour sporulation at any stage. Sub-culturing from sucrose to dextrose medium resulted in hard corrugated mycelium but no sporulation even by 9th day (Fig. 2a). Presence of dextrose in media throughout during primary culture and subsequent subcultures inhibited sporulation. Sporulation was achieved by including dextrose only once, either in primary or in sub-culture media. Monosaccharides thus appeared to favour sporulation than disaccharides. The results were verified with glycerol stock suspension using same culture in all the media composition simultaneously to avoid culture variations

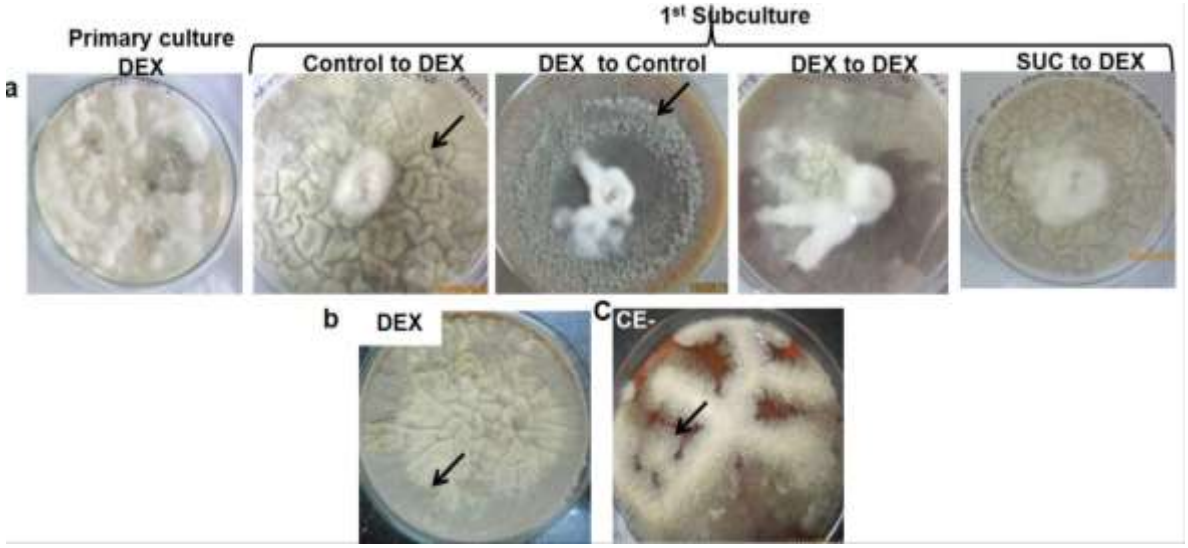

Fig. 2: Cultural characteristics of gray mold pathogen in different media

a) Effect of dextrose and sucrose (10 mM) on culture morphology and sporulation in primary culture and 1st subculture; Control: BRS medium, DEX: BRS medium with 60 g L^{-1} dextrose, SUC: BRS medium with 10 mM sucrose; b) Gray mold fungus culture (from glycerol stock) in dextrose medium showing sporulation along mycelial corrugations and fringes of culture plate; c) Sporulation in absence of capsule extract CE-: without capsule extract, Arrows show spores or regions of sporulation.

(Fig. 2b). Sporulation was achieved without including capsule extract in medium (CE-) (Fig. 2c).

Dextrose medium (higher C:N ratio than BRS) promoted mycelial growth in primary culture and induced sporulation in subsequent cultures. But sporulation was not promoted in medium with sucrose with a C:N ratio lower than dextrose medium, thereby indicating that not only C:N ratio or concentration, but also carbon source influences sporulation. Delay in sporulation and variation in culture morphology in dextrose-containing medium is unlikely due to strain variations, since the same conidial inoculum was used simultaneously in different media and a positive control (BRS medium) was maintained in every experiment. Preliminary analysis with respect to the effect of media composition on morphology or cultural characteristics showed that culture morphology and spore induction varied in different culture media (Parvathy *et al*. unpublished).

Pathogenicity of *in vitro* raised spores or isolated pure culture of fungus was tested. Separated immature capsules of susceptible cv. DCS 9 in Petri-plate, sprayed with spores of gray mold fungus, exhibited disease symptoms similar to spikes in whole plants in field (Fig. 3). A brownish ooze/ exudate was visible on immature capsules and the tissues exhibited browning by 3rd day. Sporulation

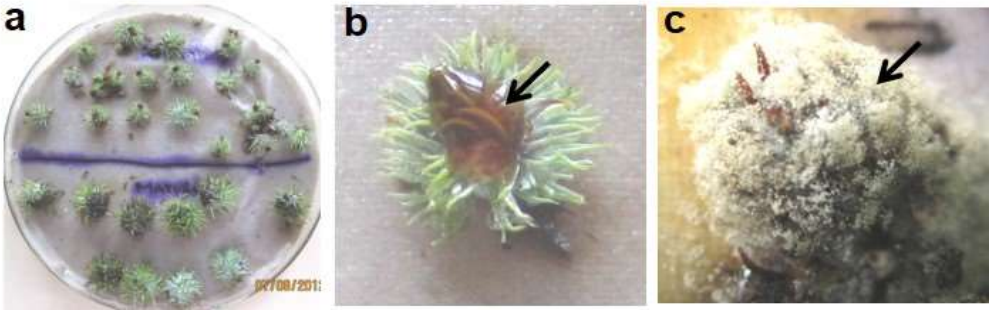

Fig. 3: Pathogenicity of isolated pure culture of gray mold fungus; a) Separated capsules of susceptible castor cv. DCS 9 sprayed with *in vitro*-raised spores 3 days post treatment b) Brownish ooze from immature capsules 3 day after treatment c) Completely infected immature capsule with fungal sporulation seen as woolly mass of conidia

was visible on 4th day as white mass which turned gray on 5th day. Profuse sporulation was seen by 7th day when the whole capsules were covered by woolly mass of grayish conidia confirming the pathogenicity of isolated culture. Similar symptoms were observed in cut-spike experiments as well. The spores isolated from dextrose media were virulent, retained pathogenicity and infected capsules of susceptible castor.

Molecular characterization of isolated fungus

Cultural morphology of gray mold fungus varied with media composition such as in dextrose. Conventional diagnostic tests based on morphology and growth on selective media is time-consuming laborious process and requires considerable skill and taxonomic expertise for correct identification within the closely-related species. Molecular characterization using ITS sequence is more reliable than cultural characteristics and widely used; and is accepted as a standard molecular marker for fungal barcoding as well as for molecular phylogeny (Sharma *et al*., 2015). The ITS region conventionally includes the entire ITS1, 5.8S rRNA gene and ITS2 portion of nuclear rDNA cistron (Schoch *et al*., 2012). Due to greater sequence variation, the ITS1/ ITS2 domains are more suited for species and strain identification than 18S (small subunit), 5.8 S and 28S region (large subunit) (Iwen *et al*., 2002). The sequence of 474 bp amplified using universal primers, from mycelium of gray mold fungus includes partial sequence of 18S rRNA gene, ITS region (complete sequences of ITS1, 5.8S rRNA gene and ITS2) and partial sequence of 28S rRNA gene. Sequencing and BLAST analysis of ITS region of gray mold fungal isolate showed close homology (99% identity) to *Amphobotrys*

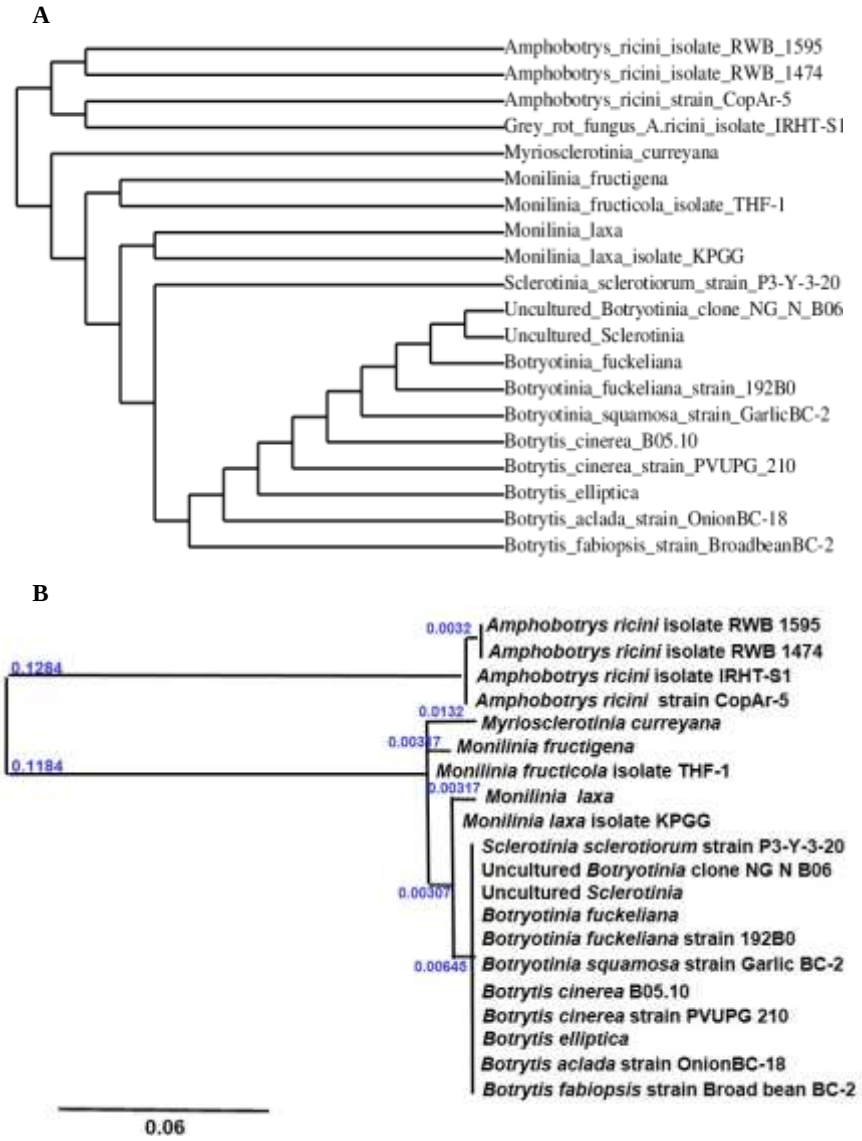

Fig. 4: Relation of *A. ricini* isolate IRHT-S1 with other fungal species: Cladogram showing genetic relation of *A. ricini* isolate IRHT-S1 to 20 fungal species. Phylogram showing evolutionary relation of *A. ricini* isolate IRHT-S1 to other fungal species. The branch length is denoted by numbers above the branch nodes indicating evolutionary distance. Scale bar is shown at the bottom

ricini strain CopAr-5 (Genbank accession; JF433374). The sequence was submitted to Genbank with accession number KT595419. The isolated fungus related to *Amphobotrys ricini* was named as *A. ricini* isolate IRHT-S1 for indicating the isolation from ICAR-IIOR, Rajendranagar, Hyderabad, Telangana (India). *A. ricini* isolate IRHT-S1 showed close homology with *A. ricini* strain CopAr-5 (Genbank accession JF433374), 99 and 100% homology, respectively, using 474 bp sequence (ITS region and partial sequence of 18S and 28SrRNA gene) and with ITS2 sequence alone. On the other hand, gray mold pathogen isolates causing blight on copper leaf in Brazil, *A. ricini* isolate RWB 1474 (Genbank Accession JX961613) and *A. ricini* isolate RWB 1595 (Genbank Accession JX961614) showed only 97% homology to *A. ricini* strain CopAr-5 (Coutinho *et al.*, 2014), indicating possibilities of strain variations in *A. ricini*.

Outbreak of blight of inflorescence of castor bean (*Ricinus communis*) in 1918 in the southern United States was due to a new species of *Botrytis,* suspected to be carried to America from India through seed (Godfrey, 1923). The organism was hence thought to be *Botrytis cinerea* or *Botrytis.* sp. or *B. ricini*. This was first report of the molecular identification of anamorph of gray mold fungus of castor from Hyderabad using ITS sequence, as *Amphobotrys ricini* (Parvathy *et al*., 2013) The nomenclature of the fungus itself had changed in the past 95 years, the disease was reported as *Botrytis* blight and the pathogen as *Sclerotinia ricini*, then *Botryotinia ricini*, *Botrytis ricini* and *Amphobotrys ricini*, the sexual stage being *Scelrotinia/ Botryotinia* (Godfrey, 1923; Whetzel, 1945; Buchwald, 1949; Hennebert, 1973). A wide degree of morphological and cultural variability was observed with *A. ricini* isolates from the same plant, while isolates obtained from different plants separated by 3000 km were identical (Duarte *et al*., 2013).

Phylogenetic relation of Amphobotrys ricini isolate IRHT-S1 with other fungal species

It is difficult to deduce the phylogenetic position of a fungal isolate from ITS region or sequence alone, as they may be more diverse than 28S rRNA gene regions or may not show sufficient sequence variation for reliable species separation. A method of fungal identification using partial DNA sequences of 26S rRNA (D1/D2 26S rRNA) was found to be superior to a method solely based on ITS1 region (Sugita and Nishikawa, 2003). Hence whole 474 bp with ITS region and partial sequences of 18S and 28S were used for BLAST analysis. The complete sequences of ITS1 and ITS2 either alone or along with 5.8S rRNA and or partial sequences of 28S rRNA, or complete 474 bp with ITS region and partial sequences of 18S and 28S rRNA, analyzed by using BLAST, retrieved the organisms of greater homology in each case (Table 1). When ITS2 alone was used, the specificity and precision was maximum (100%) for the hit. The next level of identity was only 75% for the related species, *Monilinia fructicola*. Moreover, not many organisms had homology for use in the construction of phylogenetic tree/ phylogram. The ITS2 (sub-region of ITS of less than 200 bp) or full length ITS1-5.8S-ITS2 sequence based phylogenetics has been reported to clearly distinguish isolates with greater precision (Gokulraj *et al*., 2014; Iotti *et al*., 2005). ITS1 was less specific since 88% homology was seen with animal species such as *Ovis, Macaca* etc., but only 78-79% identity with *Trichopeziza* sp. Use of whole ITS region (ITS1-5.8S rRNA- ITS2) covered more organisms with a wider range of identity such as the fungi belonging to Leotiomycetes (86-87%), Helotiales (86-88%) and *Trichopeziza* sp. The percent identity was higher with ITS1-5.8S rRNA-ITS2 when compared with ITS sequences including partial sequences of 28S alone or of both 18S and 28S. The specificity was almost similar, 99-97% identity being maximum for ITS1-5.8S rRNA-ITS2, ITS1-5.8S rRNA-ITS2 with partial sequence of 28S rRNA and ITS1-5.8S rRNA-ITS2 with partial sequences of both 18S and 28S rRNA. When the whole ITS region with partial sequences of 18S and 28S rRNA was used for BLAST, more organisms were included when compared to that of ITS region alone. Inclusion of more number of organisms which are related or unrelated, improves the usefulness of analysis of phylogenetic relations between organisms. The fungi retrieved by BLAST analysis using whole sequence of 474 bp were different isolates, strains or species of *A. ricini, Monilinia, Botryotinia, Botrytis* and *Sclerotinia*. BLAST analysis revealed gaps due to indels at same position in *Botryotinia, Botrytis and Monilinia* (2 gaps), except *M. fructigena* with one gap at a different position and *Monilinia laxa* with zero gap or close alignment, *Myriosclerotinia curreyana* with 1 gap,

Table 1: Identity of various combination of ITS sequences of *A. ricini* isolate IRHT-S1 with other fungal species using BLAST

Fungal species/ isolates	Percentage identity with sequences				
	ITS1	ITS2	ITS1-5.8S rRNA-ITS2	ITS1-5.8S rRNA-ITS2-28S rRNA (partial)	18S rRNA (partial) - ITS1 -5.8S rRNA-ITS2-28S rRNA (partial)
Amphobotrys ricini strain CopAr-5	98	100	99	99	99
A. ricini isolate RWB 1595	91	99	97	97	97
A. ricini isolate RWB 1474	91	99	97	97	97
Uncultured fungus clone MOTU_4206_	81	-	-	-	-
Uncultured Leotiomycetes clone 636	-	-	88	-	-
Fungal sp R27	-	-	88	-	-
Helotiales sp.6 KO-2013	-	-	88	-	-
Trichopeziza sulphurea	79	-	-	-	-
T. mollissima	78	-	-	-	-
Uncultured Leotiomycetes clone K563	-	-	87	-	-
Uncultured Helotiales clone 283 MID33 CATMU	-	-	86	-	-
Monilinia fructigena	-	74	-	83	83
M. fructicola isolate THF-1	-	74	Isolate LHF-1 84	83	83
M. laxa	-	72	-	82	82
M. laxa isolate KPGG	-	72	Isolate STT2 82	82	82
Myriosclerotinia curreyana	-	-	-	83	83
Sclerotinia sclerotiorum strain P3-Y-3-20		-	84	83	83
Chlorociboria argentinensis strain PRJ D1471	-	-	84	-	-
Uncultured *Botryotinia* clone NG_N_B06	-	72	85	83	83
Botryotinia fuckeliana	-	-	84	83	83
B. fuckeliana strain 192B0	-	-	-	83	83
B. squamosa strain Garlic BC-2	-	-	-	83	83
Botrytis cinerea B05.10	-	-	84	83	83
B. cinerea strain PVUPG 210	-	-	84	83	83
B. elliptica		-	-	83	83
B. aclada strain Onion BC18	-	-	-	83	83
B. fabiopsis strain Broadbean BC-2	-	-	-	83	83
Uncultured *Sclerotinia*	-	-	82	81	81

Amphobotrys ricini with 3 gaps and uncultured *Sclerotinia* with 5 gaps. The gaps representing indels may have an evolutionary significance and provide necessary information in phylogeny. Since phylogenetic relationships had to be established between closely related organisms, 20 different organisms belonging to different genus or species but closely related to gray mold fungus were chosen. The sequences from fungal species showing 83% or more sequence homology with 474 bp were aligned and grouped using cladogram/ phylogram. Clustering analysis reveals that a 97% similarity cutoff represents a reasonable threshold for estimating number of known species in data sets for both ITS1 and ITS2 (Blaalid *et al.*, 2013).

The gray mold fungal isolate IRHT-S1 was clustered in group with *Amphobotrys ricini* strain CopAr-5 (Fig. 3a). The other two isolates, *A. ricini* isolate RWB 1474 and *A. ricini* isolate RWB 1595 reported to cause gray mold on copper leaf (Coutinho *et al.*, 2014) formed another closely related cluster/group in the cladogram. The second group consisted of *Monilinia, Botryotinia*, *Botrytis* and *Sclerotinia*. *A. ricini* isolate IRHT-S1 was found closely related to *Monilinia* than *Botrytis/*

Botryotinia/Sclerotinia. *A. ricini* fell in a separate clade, while *Botrytis*, *Botryotinia* and *Sclerotinia* cluster in another group, as also reported using three nuclear gene sequences of *G3PDH, HSP60*, and *RPB2* (Yu *et al*., 2012). The evolutionary relationship between the fungal species selected is indicated in phylogram (Fig. 3b). The evolutionary distance as indicated by branch length is different for *A. ricini* group.

A comprehensive phylogenetic study of the entire genus *Botrytis* revealed that species, parasitizing hosts belonging to a single plant family, do not often cluster together and conversely, the related species in a single cluster were pathogens of monocot and eudicot hosts (Staats *et al*., 2005). Analysis of phylogenetic relationships as shown by phylogram indicates that *Amphobotrys ricini* has evolved separately from other fungal species to which it is closely related.

A. ricini, the asexual stage of *Botryotinia ricini* infects thorns in Thailand, *Acalypha australis* in central China, red fire tail/ strawberry foxtail (*Acalypha herzogiana*) and copper leaf (*Acalypha wilkesiana*) in Brazil, strawberry in United States, indicating its wide plant host range mostly belonging to *Euphorbiacea* family (Sanoamuang, 1996; Yu *et al*., 2012; Duarte *et al*., 2013; Coutinho *et al*., 2014; Amiri *et al*., 2016). Based on ITS sequences, possibility of cryptic taxa within *A. ricini* was suggested but the genus remains monotypic (Duarte *et al*., 2013). Molecular characterization using the ITS barcoding of *A. ricini* isolates from different geographical regions over a period of time is necessary to unravel the species or strain variation if any, and also to have a better understanding of the evolution of this plant pathogenic fungi, so as to devise effective management strategies.

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