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Significance of Microsclerotia in the Epidemiology of Black Pepper Anthracnose and an Approach for Disease Management in Nurseries

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Abstract

Anthracnose incited by *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. is a wide spread and economically important disease of black pepper. In the present study, role of microsclerotia (MS) in the trans-seasonal perpetuation of *C. gloeosporioides* was investigated. Microscopical examination of the runner shoots exhibiting necrotic lesions revealed the presence of dark, melanized structures which resembled MS. The excised necrotic regions when subjected to high humidity produced acervulus with setae. Under *in vitro* conditions, *C. gloeosporioides* produced MS predominantly on the aerial surface as inseparable congregations, enmeshed in the mycelial mats in potato dextrose broth and as individual units 7–8 days after incubation on glass slides. Sequential events in the formation of MS included germination of conidia, formation of conidial anastomosis tubes, aggregation of hyphae, and the formation of melanized microsclerotial bodies. Three types of microsclerotial germination were observed under *in vitro* conditions *viz*., sporogenic, myceliogenic and both. PCR confirmation with *Cg*Int species-specific primer and ITS4 resulted in 450-bp amplification. Since, runner shoots are predominantly used as propagating material in black pepper, an approach was devised to manage anthracnose under nursery conditions by treating the 2- to 3-node cuttings (nursery planting material) with carbendazim (12%)—mancozeb (63%) @ 0.1% for 30 min. The results of the study suggests a new facet in the disease cycle of black pepper anthracnose, indicating that the pathogen survives as microsclerotia *in planta* and could act as a potential source of inoculum.

Introduction

Black pepper (*Piper nigrum* L.), popularly known as 'King of Spices' is one of the most important and widely used spice globally. Black pepper originated in the moist evergreen forests of Western Ghats of South India, which is also considered as its primary centre of origin. The spice valued for its dried, mature fruits (berries) is cultivated on a commercial scale in India, Indonesia, Vietnam, Brazil, Malaysia and Sri Lanka. In India, the cultivation is mainly confined to Kerala, Karnataka, and Tamil Nadu (Ravindran 2000). Black pepper suffers from a wide spectrum of diseases of which, anthracnose is economically important and occurs throughout the black pepper growing areas of India. Anthracnose incited by *Colletotrichum gloeosporioides* (Penz.) Penz and Sacc, the ubiquitous, hemi-biotrophic ascomycetous fungus is a major concern to black pepper production. The disease affects all the aerial parts, and foliar as well as spike infections are considered to be the most damaging phase in disease

progression (Anandaraj and Sarma 1995; Sainamole Kurian et al. 2000).

The disease is characterized with manifestation of necrotic spots surrounded by yellow halo developed especially on foliage. The disease exacerbates during the postmonsoon season and attains maximum severity during September–October (Biju et al. 2012, 2013). The disease often attains epiphytotic proportions especially at higher altitudes where misty conditions prevail. Nair et al. (1987) reported that, severity of the disease varied from 28–34% and a resultant crop loss to the extent of 1.9–9.5%.

Earlier studies indicated that allied species of *C. gloeosporioides* perpetuating in the form of various survival structures in soil, crop debris, and collateral hosts serve as primary sources of inoculum for the succeeding season and have the potential to initiate disease under conducive weather conditions (Eastburn and Gubler 1990; Norman and Strandberg 1997; Yoshida and Shirata 1999; Sankar and Kumari 2002; Amusa et al. 2005). Several species belonging to the genus *Colletotrichum* including *Colletotrichum acutatum* (Norman and Strandberg 1997), *Colletotrichum coccodes* (Dillard and Cobb 1998), *Colletotrichum truncatum* (Khan and Sinclair 1992; Jackson and Schisler 1995; Buchwaldt et al. 1996), *C. gloeosporioides* (Nithya and Muthumary 2009) are reported to produce microsclerotia as a mechanism for trans-seasonal perpetuation in the crop debris and soil. The term microsclerotia (MS) is used to describe small (200–400 μm), melanized, compact pseudoparenchymatous hyphal aggregates produced by several fungi for persistence in the soil and decaying plant material as survival structures (Tsuneda et al. 2001). Kendrick and Walker (1948) showed that sclerotia in the soil or in association with plant debris are the primary source of inoculum for tomato anthracnose pathogen. Earlier studies on nutrition demonstrated that MS of *C. truncatum* could be produced in liquid culture under specific nutritional conditions (Boyette 1988; Jackson and Bothast 1990).

Confusion between *Colletotrichum* species is compounded by the broad host range and the occurrence of more than one species on a given host (Freeman et al. 1998). According to Lewis Ivey et al. (2004), molecular tools such as PCR with species-specific primers are useful in distinguishing species of *Colletotrichum* that cannot be distinguished using morphological methods. Maymon et al. (2006) used *CgInt* as species-specific primer coupled with ITS4 for identification of *C. gloeosporioides* from Statice (*Limonium* spp.). It is hypothesized that, annual recurrence of black pepper anthracnose under field conditions and in nurseries could be primarily due to randomly distributed primary foci of infection including infected leaves of preceding season, annual or perennial collateral hosts, plant debris and over-summering survival structures. Based on the hypothesis, the present investigation was formulated to explore the possibilities of *C. gloeosporioides* forming oversummering quiescent survival structures *in planta*, i.e. the infected plant parts in black pepper and to develop a strategy to manage the disease under nursery conditions.

Materials and Methods

Survey for black pepper anthracnose incidence and isolation

Surveys were undertaken in the black pepper plantations representing high-altitude and high-rainfall zone of Appangala (12°26′N Latitude and 75°45′E Longitude), Kodagu District, Karnataka, India, during the months of March to July for two consecutive years (2013 and 2014) with the objectives to assess the status of anthracnose disease and explore the possibility of trans-seasonal perpetuation of the pathogen in the form of quiescent structures *in planta*. The samples exhibiting characteristic anthracnose symptoms (on leaves) and with atypical symptoms manifested on runner shoots (necrotic lesions) were collected in polythene bags, sealed, and brought to the laboratory for subsequent examination. The pathogen was isolated following standard procedures described by Faisal et al. (2014).

Examination of runner shoots under high-resolution microscopy

The runner shoots exhibiting necrotic lesions were collected from the field. The necrotic regions were excised under laboratory conditions; thin sections were prepared and observed under the microscope. The necrotic regions excised from the shoot were also placed in the moist chamber and observed periodically.

Induction of MS under *in vitro* conditions

Liquid culture

To produce MS under *in vitro* conditions, 50 ml of potato dextrose (PD) broth was poured into 250-ml flasks and autoclaved for 20 min. The sterilized PD broth was inoculated aseptically with 5-mm discs of 7-day-old culture of *C. gloeosporioides*. The flasks were maintained at room temperature (25 ± 2°C) and

shaken frequently to inhibit mycelial growth on the walls. Microsclerotia formed inside the flasks were separated from *C. gloeosporioides* liquid cultures by sieving the culture broth through filter paper.

Slide culture

Conidial suspension of *C. gloeosporioides* (1×10^6 spores/ml) were prepared in 2% sucrose solution and smeared on glass slides. The slides were incubated in moist chamber under ambient temperature conditions (25 ± 2°C) and observed daily for microsclerotial formation. MS formed on the glass slides were harvested individually, air-dried on two layers of filter paper, and stored at 4°C for further studies.

Effect of temperature and aeration on germinability of MS

Germinability of the air-dried MS was determined on glass slides placed in the moist chamber. Individual MS were placed equidistantly (30 per slide) and observed at different time intervals. Different modes of germination were studied under sealed (with parafilm) and unsealed conditions under 12-h photoperiod and complete darkness at 20° and 30°C. At the end of 5-day period, germinability was assessed by observing 30 MS on each plate. The MS were considered to have germinated, if only conidial masses were present on the sclerotium surface (sporogenic), only hyphal production (myceliogenic) and both sporogenic and myceliogenic. The mean percentage of sporulated sclerotia was recorded for each temperature and photoperiods.

Activation of MS under *in vitro* conditions

In order to study the process of activation of MS leading to the formation of acervuli and conidial mass, the experiment was carried out under *in vitro* conditions. Runner shoots exhibiting necrotic lesions were collected during the month of March. The runner shoots were made into small bits with few necrotic lesions. One part of the sample was placed on PDA to study the viability of the MS which was ascertained by the emergence of hyphae after incubating under ambient conditions. The other part was placed in the moisture chamber and observations were recorded at 24-h interval till the formation of MS.

Molecular confirmation of pathogen

Genomic DNA were extracted from the mycelial mat of *C. gloeosporioides,* runners infected with MS and infected leaves from single plant by cetyltrimethylammonium bromide (CTAB) method as described by Knapp and Chandlee (1996). Mycelium was harvested by filtration through sterile filter paper and stored at −70°C until used for DNA extraction. One gram of frozen mycelium, MS, and infected plant samples was ground to fine powder in liquid nitrogen and incubated in 5 ml, 2% CTAB extraction buffer (10 mM trisbase (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, CTAB (2%), mercaptoethanol (0.1%), and PVP (0.2%) at 65°C for 1 h. The suspension was added with equal volume of phenol–chloroform–isoamylalcohol (25 : 24 : 1) mixture. It was vortexed to mix two phases, followed by centrifugation at 14 498 ***g*** for 5 min. The supernatant was transferred to a clean tube and mixed with equal volume of ice cold isopropanol and incubated at 25°C for DNA precipitation. The precipitate was collected by centrifugation and the pellet was washed with 0.1 M ammonium acetate in 70 per cent ethanol and incubated for 15 min. The pellet was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and the DNA concentration was estimated using spectrophotometer (Genway Genova; Bibby Scientific Ltd., Dunmow, UK) at 620 nm.

Species-specific PCR amplification

In order to confirm the species, PCR amplification with ITS4 primer coupled with specific primer *Cg*Int (5′ GGCCTCCCGCCTCCGGGCGG 3′) was carried out (Mills et al. 1992). Amplification was conducted with total reaction volume of 20 μl in Eppendorf master cycle gradient thermal cycler. The PCR programme consisted of one cycle at 95°C for 5 min followed by 30 cycles of reaction profile involving denaturation at 95°C for 30 s, annealing at 65°C for 30 s and extension at 72°C for 90 s and a final extension for 10 min at 72°C (Lima et al. 2012).

Agarose gel electrophoresis

The PCR products were resolved in 1.2 per cent (w/v) agarose gel in 1× TAE buffer (0.4 M Tris, 0.2 M acetic acid, 10 mM EDTA; pH 8.4) containing 0.5 μg/ml ethidium bromide. The PCR product along with gel loading buffer (6× containing 0.25 per cent bromophenol blue, 0.25 per cent xylene, cyndol FF and 3 per cent glycerol) was loaded. Electrophoresis was carried out at 100 V, and the gel was documented using an Alpha Imager (Alpha Innotech Corporation, San Leandro, CA, USA). The sizes of the PCR products were determined by comparing with standard 100-bp

or 1-kb molecular marker (Bangalore Genei Pvt. Ltd., Bangalore, India). The PCR product was purified using QIA quick PCR Purification Kit and sequenced.

Evaluation for fungicidal sensitivity under *in vitro* conditions

Sensitivity of *C. gloeosporioides* was tested against ten fungicides *viz*., benomyl, propiconazole, hexaconazole, metalaxyl-mancozeb, Bordeaux mixture, chlorothalonil, triadimefon, carbendazim, carbendazim-mancozeb, and mancozeb under *in vitro* conditions employing poisoned food technique. The fungicides were tested at six different concentrations *viz*., 0.05%, 0.125%, 0.25%, 0.5%, 1%, and recommended dose according to the manufacturer. Fungicidal suspensions were prepared by dissolving requisite quantities of each fungicide in warm PDA. A total of 20 ml of the medium were poured into Petriplates and medium without fungicide served as control. Mycelial discs (5 mm diameter) from the advanced margin of 5-day-old culture of the isolates were placed at the centre of each Petriplate, and each treatment was replicated thrice. The plates were incubated at 25°C, and observations on radial growth of the colony were recorded 7 days after the incubation period. The per cent inhibition of the colony growth was calculated using formula:

$$\frac{C - T}{C} \times 100$$

where C = growth of culture in control plate, T = growth of culture in fungicide-treated plate.

Evaluation for fungicide treatment of planting materials under nursery conditions

Black pepper cuttings with three nodes were prepared from the runner shoots with linear necrotic lesions. The cuttings were dipped in different fungicides *viz*., hexaconazole, propiconazole, metalaxyl-mancozeb, Bordeaux mixture, copper oxychloride, carbendazim, and carbendazim-mancozeb at recommended concentration for 30 min. The treated cuttings were planted in polybags and observations on disease incidence were recorded 6 weeks after planting and repeated at 7-day interval for a period of 1 month.

Data analysis

The *in vitro* bioassay experiments were laid out in completely randomized design (CRD), and the data recorded in per cent were transformed to $\sin^{-1}$ percentage transformation. The transformed data were statistically analysed using the software package AGRES version 7.01 @ 1994 (Pascal Intl Software Solutions, New York, USA).

Results

Survey for anthracnose incidence in black pepper

The black pepper plantations in and adjoining areas of Appangala, Kodagu, Karnataka, were surveyed during March 2013 to July 2014 for recording the incidence of anthracnose disease. During the surveys, it was noticed that symptoms of disease started appearing during the months of May–July. In most of the plantations, delayed infection during the preceding season resulted in the formation of randomly distributed discrete necrotic lesions either with grey centre or with shot holes and dark brown margin on the older leaves of lateral branches (plagiotrophs), with infection on the adjacent newly emerged leaves (Fig. 1). Necrotic lesions were also observed on the runner shoots in majority of the plantations during April–July (Fig. 2). During the surveys, it was observed that manifestation of symptoms on the young foliage of plagiotrophs adjacent to basins of the vines was comparatively lesser where the runner shoots were removed for producing planting material. In contrast, disease symptoms were more on the newly emerged foliage of plagiotrophs near ground level where runner shoots with necrotic lesions were retained. However, survival structures were not found associated with symptomatic lesions derived from the leaves. Microscopical examination of the peeled tissue from the necrotic zone of the runner shoots revealed the presence of dark, melanized globose structures embedded in the tissue. Further, isolation made from the necrotic lesion yielded typical colony of *C. gloeosporioides* on PDA medium The necrotic lesions when subjected to high humidity in a moist chamber for activation of structures resulted in the production of acervulus with dark setae (Fig. 3).

In vitro production of MS

Liquid culture

In order to produce MS under laboratory conditions, the pathogen culture was inoculated on PD broth. The MS were predominantly formed on the aerial surface as congregations (Fig. 4) and entangled in the mycelial mat. Initially, growth was characterized by the formation of thick mycelia mat. As compact

Fig. 1 Symptoms of anthracnose on black pepper leaves. [Colour figure can be viewed at wileyonlinelibrary.com]

Fig. 2 Necrotic lesions on runner shoots. [Colour figure can be viewed at wileyonlinelibrary.com]

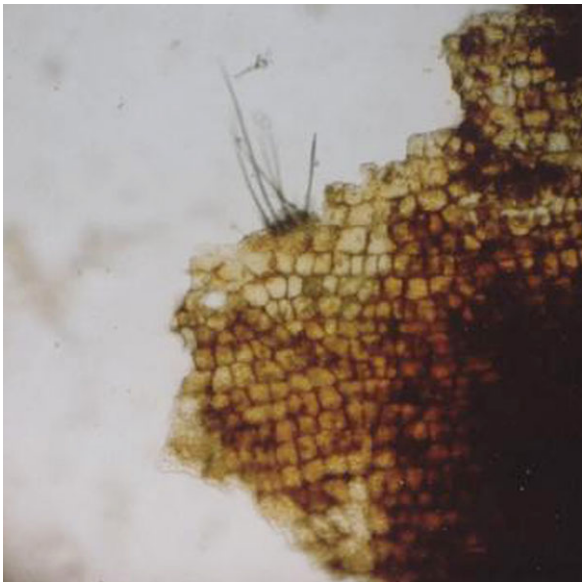


Fig. 3 Activation of microsclerotia under moist condition. [Colour figure can be viewed at wileyonlinelibrary.com]

hyphal aggregates/MS initials began to form, the culture broth became less viscous. However, the MS could not be separated into single units by following the standard protocols as they were enmeshed in the fungal hyphae.

Fig. 4 Congregation of microsclerotia on aerial surface of broth. [Colour figure can be viewed at wileyonlinelibrary.com]

Slide culture

For the production of MS under *in vitro* conditions and for their easy separation into single units, a simple method was devised. The conidial suspension prepared in 2% sucrose solution when spread uniformly on glass slides and incubated in moist chamber under ambient temperature conditions led to the production of MS 7–8 days after the incubation period (Fig. 5).

Sequential events in the formation of MS *in vitro*

The process leading to the formation of MS comprised of five major sequential events which were recorded upto 140 h *viz*., (1) germination of conidia (2) formation of conidial anastomosis tubes (CAT) and subsequent hyphal interaction (3) aggregation of hyphae (4) formation of unmelanized, multicellular globose structures, and (5) formation of melanized, darkly pigmented globose MS (Fig. 6a–e, Table 1).

Effect of temperature and aeration on germinability of MS

Three types of germinations *viz*., sporogenic (production of conidial mass) (Fig. 7a), myceliogenic (production of hyphae) (Fig. 7b) and both sporogenic and myceliogenic germinations (Fig. 7c) were exhibited by *C. gloeosporioides* under laboratory conditions when the MS were subjected to sealed or unsealed conditions at 20–30°C under 12-h photoperiod and

complete darkness. Under sealed condition, germination was primarily sporogenic (66%) at 20°C and myceliogenic germination was higher (56.6%) at 30°C, whereas under complete dark condition, myceliogenic germination was observed to be the maximum (80%) both at 20° and 30°C. In unsealed condition, at 12-h photoperiod and complete darkness, myceliogenic germination was found to be the maximum (60–76.6% and 46.6–66.6%, respectively) both at 20° and 30°C (Table 2). In sporogenic germination, slightly creamish conidial masses were profusely produced on MS surface and in myceliogenic germination, hyphae were produced. Creamish mass of conidia with profuse hyphae were produced on MS which exhibited both sporogenic and myceliogenic germination. In general, under both sealed and unsealed conditions, most of the MS germinated by producing mycelia (myceliogenic) at both the temperature regimes followed by sporogenic-myceliogenic and sporogenic.

Fig. 5 Production of microsclerotia on glass slide. [Colour figure can be viewed at wileyonlinelibrary.com]

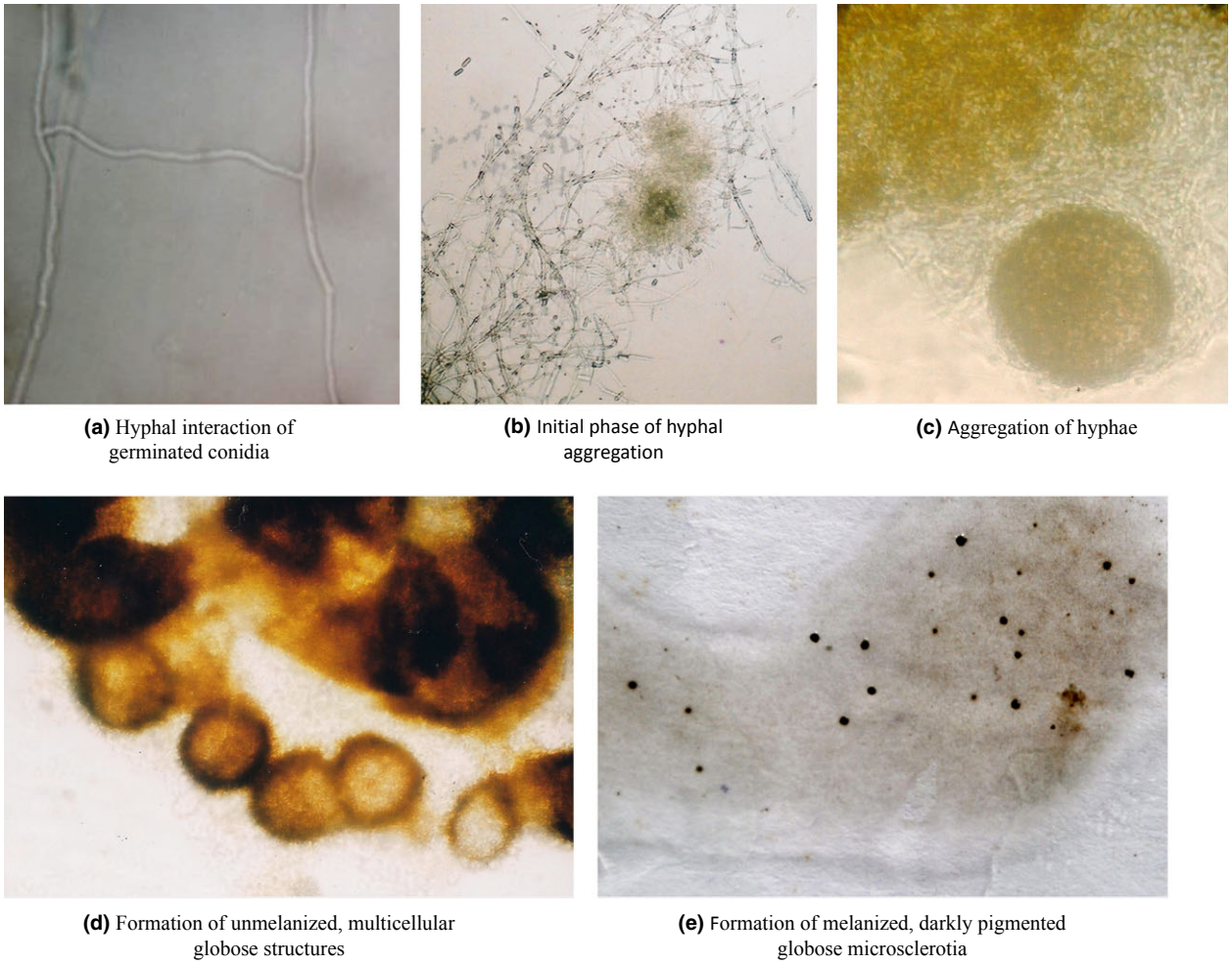


Fig. 6 Sequential events in the formation of microsclerotia *in vitro*. (a) Hyphal interaction of germinated conidia; (b) Initial phase of hyphal aggregation; (c) Aggregation of hyphae; (d) Formation of unmelanized, multicellular globose structures; (e) Formation of melanized, darkly pigmented globose microsclerotia. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1 Sequential events in the formation of microsclerotia *in vitro*

Chronological events	Hours
Conidial germination	12–24
CAT formation, hyphal interaction	20–48
Hyphal aggregation	40–72
Formation of unmelanized, multicellular globose structures	80–110
Formation of melanized microsclerotia	130–140

Activation of MS under *in vitro* conditions

Studies on activation of MS of *C. gloeosporioides* in the runner shoots of black pepper showed that, the MS were activated within 7 days when subjected to high humid conditions, by producing acervuli with setae and subsequent production of conidia embedded in a matrix under *in vitro* conditions (Fig. 8a,b,c,d).

PCR amplification with species-specific primers

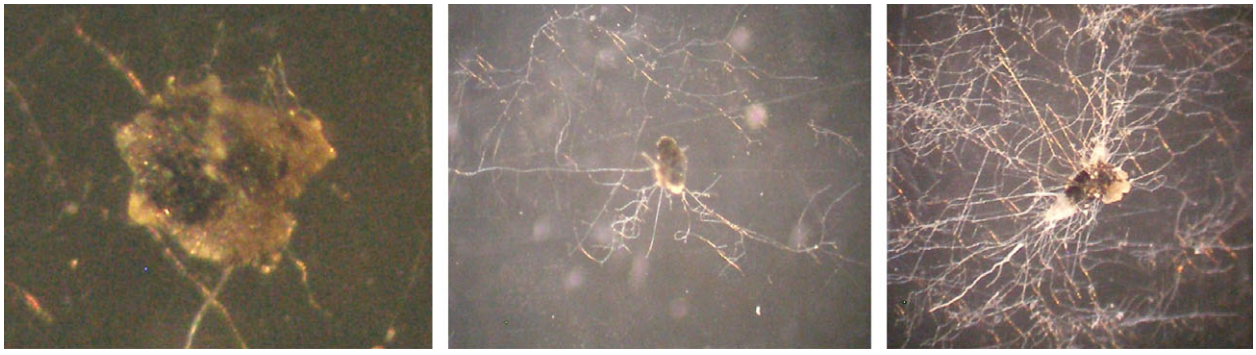
DNA was isolated from MS, infected runners and leaves, pure culture of *C. gloeosporioides* and subjected to PCR amplification with *Cg*Int and ITS 4 species-specific primers which produced the expected 450-bp amplicon. The results confirmed identity of the pathogen as *C. gloeosporioides* associated with infected plant parts, MS and pure culture. The sequence was blasted in nucleotide blast search, NCBI, and found to have 100% similarity with *C. gloeosporioides,* which further confirmed identity of the pathogen. The sequence was submitted to NCBI with accession number KY236319.

Evaluation of fungicides against *C. gloeosporioides* under *in vitro* and nursery conditions

Under *in vitro* conditions, both propiconazole and carbendazim-mancozeb were found effective over other fungicides resulting in 100% inhibition of colony growth at all the concentrations evaluated, followed by carbendazim with mycelial inhibition of 100% in the range of 0.125–1% concentration. Among the fungicides, chlorothalonil was found to be the least effective with a range of 32.5–59% growth inhibition at 0.05–1% concentration, whereas under nursery conditions, carbendazim-mancozeb was superior in which disease development was delayed up to 8 weeks after planting (WAP) with no disease incidence (Fig. 9). Propiconazole and carbendazim though found promising under *in vitro* conditions were not effective in delaying disease development and had 40.4 and 34.5% incidence at 8 WAP. Among the treatments, Bordeaux mixture was found to be least effective with 30.8% incidence at 6 WAP, which reached 45.8% at 8 WAP. Even though the disease incidence in carbendazim-mancozeb treatment attained 14.4% at 10 WAP, the delay did not result in early defoliation which resulted in better establishment of the plants.

Discussion

Several reports have highlighted the role of MS in the oversummering/overwintering of foliar and soilborne pathogens infecting economically important crops and their epidemiological implications. In nature, *Colletotrichum* produce dormant MS as an overwintering structure in the infected moribund tissues



(a) Sporogenic **(b)** Myceliogenic **(c)** Sporogenic + Myceliogenic

Fig. 7 Different modes of microsclerotial germination in *Colletotrichum gloeosporioides*. (a) Sporogenic; (b) Myceliogenic; (c) Sporogenic + Myceliogenic. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 2 Effect of temperature and aeration on germinability of microsclerotia

Sealed								Unsealed							
12 h light -12 h dark				Complete dark				12 h light -12 dark				Complete dark			
20°C		30°C		20°C		30°C		20°C		30°C		20°C		30°C	
Type	%	Type	%	Type	%	Type	%	Type	%	Type	%	Type	%	Type	%
S	66	S	0	S	6.6	S	0	S	3.3	S	0	S	0	S	0
M	60	M	56.6	M	80	M	80	M	60	M	76.6	M	66.6	M	46.6
S + M	33.3	S + M	3.3	S + M	13.3	S + M	0	S + M	36.6	S + M	0	S + M	33.3	S + M	3.3

S, Sporogenic; M, Myceliogenic; S + M, Sporogenic + Myceliogenic.

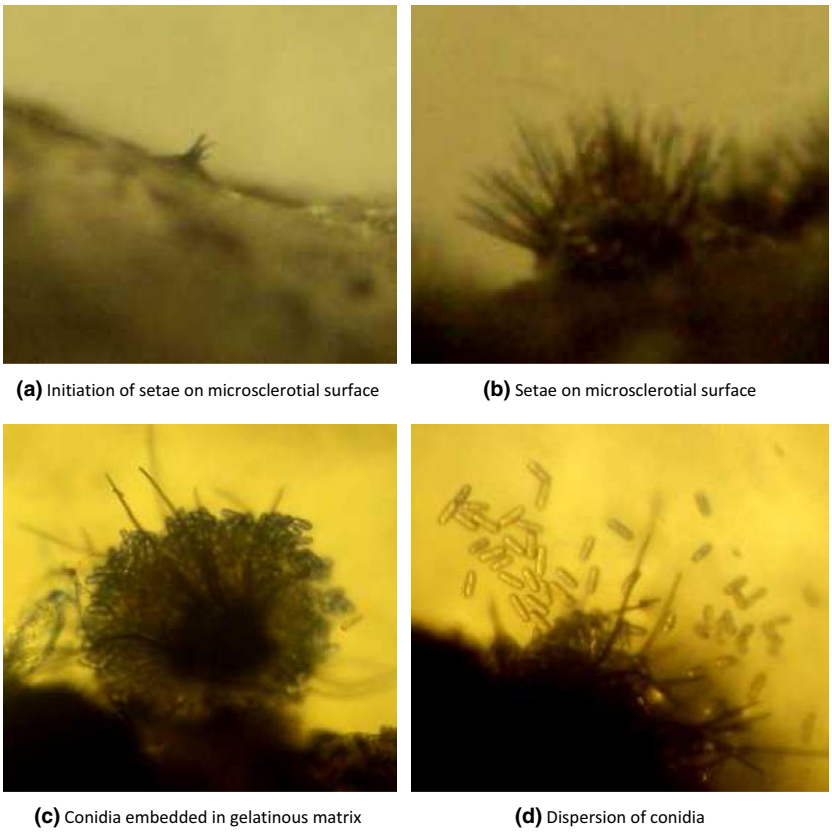


Fig. 8 Activation of microsclerotia of *Colletotrichum gloeosporioides* under *in vitro* conditions. (a) Initiation of setae on microsclerotial surface; (b) Setae on microsclerotial surface; (c) Conidia embedded in gelatinous matrix (d) Dispersion of conidia. [Colour figure can be viewed at wileyonlinelibrary.com]

under stressful environmental conditions, which have the potential to survive for several years. It is reported that different species of *Colletotrichum* survive in the form of MS and acervuli in and on the seeds in chilli. During humid, warm, or wet conditions, conidial propagules produced on acervuli or MS are subjected to splash dispersal with the aid of wind-driven rain or irrigation water subsequently leading to rapid spread of the pathogen from infected to healthy foliage and fruits in chilli (Pring et al. 1995; Than et al. 2008). In sugarcane affected with red rot disease, germinated conidia, appressoria, and brownish thick-walled compactly arranged hyphal structures could be detected in the peeled waxy tissues derived from shrivelled rind portions of the infected canes. These dried mass of propagules embedded in the waxy tissues retained viability under *in vitro* conditions and had the potentiality to initiate infection even after 10 months of storage (Singh and Lal 1996).

Electron micrographs of the early stages in microsclerotial development in *Verticillium dahlia* showed that hyphae become swollen and vacuolated

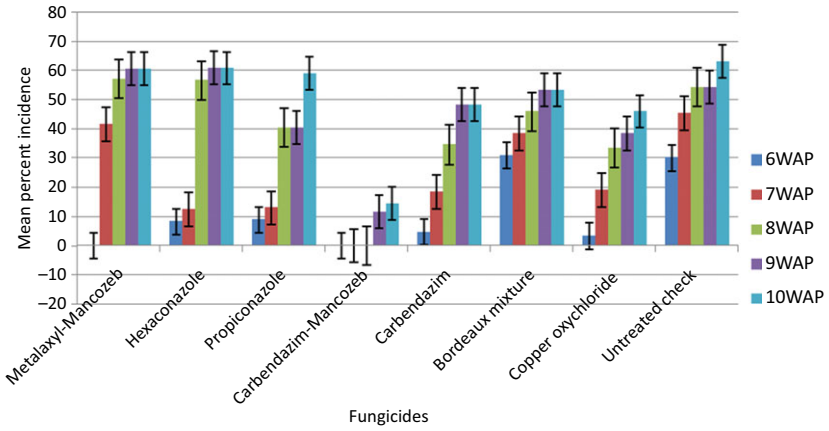


Fig. 9 Evaluation of fungicides against anthracnose disease of black pepper in nursery. [Colour figure can be viewed at wileyonlinelibrary.com]

and extruded melanizing particles into the interhyphal spaces of the MS. Variations in the thickness of melanized material surrounding cells gave the superficial appearance of variations in cell wall. (Griffiths 1970). Gordee and Porter (1961) observed that MS were composed of two morphologically dissimilar cell types; thick-walled melanized cells and thin-walled hyaline cells. They also demonstrated a conversion of carbohydrate to lipid material during the ageing of MS. Cells of mature MS contained large lipid bodies and poorly defined organelles. In culture, MS often became conidiomata by conversion of the surface cell layer to conidiogenous cells (Tsuneda et al. 2001). In the present study, it is noticed that the MS when subjected to high humidity produced abundant conidia on surface indicating that these structures under conducive weather conditions are capable of transforming from dormant to an active phase.

A cascade of metamorphic events modify the fungal tissue to a higher level of differentiation mediated *via* an array of physiological signals leading to translation-level controls to direct fungal tissues to commence explicit differentiation processes. Studies in various fungi have shown a significant correlation between differentiation/no-differentiation processes and the up-regulation of specific antioxidant enzymes, such as SODs, catalases, bifunctional catalase peroxidises, and peroxiredoxins. Several genes and gene products such as SOD gene in *C. graminicola* (Allen 1991), a glutamic acid-rich protein 1 (*VdGARP1*), two genes *viz.*, *VMK1* (encoding mitogen-activated protein kinase) and *VDH1* (a hydrophobin gene) in *V. dahlia* (Feng et al. 2010), and differentially expressed stress-responsive genes in *Nomuraea rileyi* (Song et al. 2013) have been identified to play vital roles in microsclerotial biogenesis during low carbohydrate (nutrient depletion) and high-oxidative-stress conditions in conjunction with a broad shift in metabolic activities. It is inferred from the present study that, in black pepper-*Colletotrichum* pathosystem, abiotic stress (lack of adequate moisture and high temperature) and depletion of essential nutrients in the necrotic tissues would have acted as signals for inducing the pathogen to shift from an active proliferating to a dormant quiescent phase.

Understanding the relationship between environmental factors and survival of a pathogen is important to clarifying specific eco-physiological bases for the establishment and persistence of infection. Physical factors like aeration, temperature, and light exerted a significant effect on sclerotial germination of *C. coccodes* (Sanogo and Pennypacker 1997). Conidial and mycelial production on sclerotia appeared to be more sensitive to aeration and light compared to temperature. Kendrick and Walker (1948) showed that sclerotia in the soil or in association with plant debris are the source of primary inoculum of *C. coccodes* in epidemics of tomato anthracnose. Frost (1964) attributed these variations in biological responses to differences in the atmospheric humidity between closed and opened Petriplates. Complete darkness under sealed (partially aerobic) more or less simulates the conditions experienced by the pathogen confined to the necrotic lesions on the runner shoots. Inadequate availability of light and complete or partial aerobic conditions prevailing in the necrotic lesions coupled with non-availability of nutrients would have signalled the pathogen to shift from an active vegetative phase to a dormant stage.

Low light interception in the plantations, due to cloudy weather (particularly during June-July) in conjunction with reduced filtered light (due to dense canopy of support/standard trees and other component crop species) and self-shading would induce the MS to germinate by means of hyphae. The weather variables, especially the temperature prevailing

during May–July (17.8–29°C) with sufficient moisture in the form of rainfall, would have activated the quiescent structure and triggered the infection process. Magnitude of conidial production on sclerotia on soil surfaces exposed to sunlight may be greater than that on sclerotia under dense plant canopies. The epidemiological significance of sporogenic germination in the field depends on the location of sclerotia. Sclerotia on a soil surface exposed to sunlight would be expected to play a significant role in the dispersal of *C. coccodes* as they represent a source of conidial inoculum readily available for dissemination by rain splash. However, sclerotia on a soil surface under dense plant canopies or within the soil would produce conidia that may infect plant parts in contact with the soil.

In the present investigation, a PCR-based diagnostic assay was appliedwith the aim to detect *C. gloeosporioides* of black pepper anthracnose pathogen using species-specific designed primer pair of *Cg*Int and ITS 4 which amplified the DNA at 450 bp. Present results were supported by the earlier findings that species-specific PCR primers, designed for the taxonomic identification of *Colletotrichum* species, have been used to distinguish between *C. acutatum* and *C. gloeosporioides* (Forster and Adaskaveg 1999). The size of the amplification product (450 bp) obtained with species-specific primers were similar to previous reports of *C. gloeosporioides* (Talhinhas et al. 2002; Chowdappa et al. 2012).

Vegetative propagation of black pepper is principally effected through 2- to 3-node cuttings prepared from the runner shoots. The infected runner shoots may act as the source of survival and spread from which the hibernating pathogen is transmitted from field in the form of incipient infections to the nurseries. Under field conditions, especially favoured by warm and wet environment, conidia exuded from acervuli (developed on microsclerotial surface) could be disseminated by rain splashes to adjacent young foliage. In nurseries, the infected planting material acts as potential sources of primary inoculum from which the disease initiates and spreads resulting in severe defoliation. Dormant MS in the planting material may play a pivotal role in the biology (disease cycle) and epidemiology of the disease. Due to the trans-boundary movement of planting material, the pathogen may eventually introduced into new regions where recombination with native strains/biotypes results in the evolution of novel recombinants with unpredictable potentialities. Hence, it is highly imperative to inactivate the quiescent pathogen from proliferation at the planting material production phase before nursery establishment with an approach including pre-plant treatment of planting material with effective fungicides. A broad spectra of fungicide with single or multiple site inhibition activity and the molecules possessing systemic activity, have been extensively used to manage diseases incited by *C. gloeosporioides* and other related species in a wide range of crops. Among the recommended fungicides, chlorothalonil, benzimidazoles, triazoles, carbamates, and copper-based fungicides have been reported to be effective under *in vitro* as well as field conditions (Dillard 1988; La Mondida 2001; Kumar et al. 2007). In the present investigation, among the fungicides possessing contact, systemic and combined action evaluated for bio-efficacy, the combination product of carbendazim-mancozeb was found to be promising, under *in vitro* and nursery conditions. Benzimidazole compounds possessing systemic mode of action in combination with protectant molecules (dithiocarbamates) are widely used, usually alternating with to reduce the risk of resistance development. The combination of systemic and contact fungicides would be effective in inactivating the deep seated MS in the affected tissues.

Conclusion

Anthracnose caused by *C. gloeosporioides* (Penz.) Penz. and Sacc. is one among the most prevalent and economically important disease of black pepper. It is inferred from the present study that *C. gloeosporioides* survives in the form of MS which remain quiescent in the runner shoots which is the most commonly used propagation material of black pepper. The MS activated with the commencement of monsoon under field and also under nursery conditions and have the potential to trigger the disease development. Confirmation of identity of pathogen was through specific primers and sequencing of the 450-bp band. An approach was devised to manage the anthracnose disease under nursery conditions by treating the 2- to 3-node cuttings (nursery planting material) with carbendazim-mancozeb (0.1%) for 30 min, by inactivating the MS.

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