Occurrence and characterization of *Colletotrichum gloeosporioides* isolated from *Murraya koenigii*

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Abstract: Curry leaf (*Murraya koenigii*) of the family Rutaceae have long been considered a premier leafy vegetable with attractive habit and graceful leaves. It is used in fresh, dried and processed forms for flavoring food stuffs. The species is native to India and at present it is cultivated in Burma, Ceylon, China, Australia and Pacific Islands. It is extensively used in the indigenous system of medicine as an anti-diabetic agent. Further it is known to possess anti-inflammatory, anti-dysenteric, antioxidant and diverse pharmacological properties. The plant is grown presently in the backyards of every South Indian home, but its nutritive value and medicinal properties have not been fully appreciated. A very common disease in curry leaf, the leaf spot hence has gained least attention of researchers. Curry leaf plants with leaf spot disease were collected from Mysore district of Karnataka, India. The pathogen was isolated on potato dextrose agar (PDA) from surface sterilized small pieces of the leaves and twigs, incubated at 25°C, and identified as *Colletotrichum gloeosporioides* according to its morphological and cultural characteristics. A further study on colony characteristics and reproductive propagules of the pathogen was done.
[Midhila Padman and Janardhana G.R. **Occurrence and characterization of *Colletotrichum gloeosporioides* isolated from *Murraya koenigii.*** New York Science Journal 2011;4(8):70-76]. (ISSN: 1554-0200). http://www.sciencepub.net/newyork.

Key words: *Murraya koenigii*, *Colletotrichum gloeosporioides*.

Introduction

Murraya koenigii considered a medicinal plant is susceptible to only a few fungal pathogens. Epidemics of a fungal leaf spot disease with characteristic necrotic spots were evident during the onset of monsoon season every year. Proper identification and characterization of the pathogen is essential as the review of literature suggests not much work has been done on this particular disease. Preliminary diagnosis of the disease revealed the pathogen is fungus with the formation of fruiting bodies visible as black globose bodies in necrotized tissues of the leaves and the twigs, seen often with the presence of setae. The imperfect state as the active pathogen was detected from growth of fungi in media. The classification, nomenclature and identification of the fungi using fungal identification manual revealed the pathogen is *Colletotrichum gloeosporioides*.

As a result of the studies conducted on leaf spot disease of *Murraya koenigii*, *Colletotrichum gloeosporioides* was isolated for the first time in India. Therefore attention to the occurrence of this fungus was directed and macroscopic and microscopic features of the pathogen and host pathogen interaction was carried out in the study. Before they can penetrate and colonize the host, they must first become attached to the host surface. Attachment takes place through the adhesive of spores, bacteria and seeds through the adhesive materials that vary significantly in composition and in the environmental factors they need to become adhesive. The germ tube is a specialized structure distinct from the fungal mycelium often growing for a very short distance before it differentiates into an appressorium. In Colletotrichum, the penetration of host tissues generally relies on formation of these specialized infection structures called appressoria (Perfect et al., 1999). Appressoria allow the fungus to penetrate the host cuticle and epidermal cell wall directly by means of narrow penetration peg that emerges the base of appressorium. Species of *Colletotrichum*, which are commonly associated with quiescent infections, produce particularly distinctive appressoria (Emmet and Parbery, 1975). The formation of appressoria facilitates the direct penetration of the host and these structures are also believed to play a role in the survival of the pathogen (Emmet and Parbery, 1975).

The host infection process of a number of *Colletotrichum* species has been studied, and can be classified as following either an intracellular hemibiotrophic strategy, a subcuticular intramural strategy or a combination of both strategy, the final phase of all three infection strategies is always necrotrophic (Bailey *et al.*, 1992). Many *Colletotrichum* species initially establish infection by means of a brief biotrophic phase, associated with large intracellular primary hyphae. They later switch to a destructive, necrotrophic phase, associated with narrower secondary hyphae, which ramify throughout the host tissue (Wharton and Uribeondo, 2004).

Materials and Method

Isolation of the Pathogen

A total of 18 *Murraya koenigii* leaf samples from Mysore district of Karnataka were analyzed for *Colletotrichum* incidence and association with leaf spot disease for three consecutive years during the onset of rain. Samples were brought to the laboratory in clean plastic bags and kept at 4^0 C. All the samples were subjected to mycological analysis. Fresh leaf samples showing characteristic necrotic and chlorotic spots were selected, surface sterilized using 70% ethyl alcohol and cut into small pieces of 1mmx1mm and plated on PDA medium and incubated. The plates were incubated in alternating periods of 12h darkness and 12h of light at 25$\pm$2 °C for 7 days. The associated microbial colonies expressed will be isolated and identified using fungal manuals and keys**.**

Cultural isolates and their identification

The *Colletotrichum* isolates were cultured onto plates containing semi-synthetic PDA in order to study cultural characteristics. The colony morphology and cultural characteristics were evaluated on both sides. All the fungal isolates were maintained on PDA slants at 4°C for further studies. The characters such as size, shape of conidia, existence of setae, cultural characters such as colony color, growth rate and colony texture were considered for the identification of species (Barnett and Hunter 1999; Sutton 1980; Sutton 1992).

Morphological and cultural characteristics

Fungal isolates were grown on PDA plates in alternating dark and light cycles at 25°C. For mycelial growth and colony characteristics petriplates containing PDA were inoculated at the centre of each plate with 5mm diameter mycelial disc that was taken from the margin of a 7 day old culture grown on PDA. Colony diameter of each isolate was measured daily for three weeks at 25° C. Colony color of each fungal isolate was also examined about three weeks after inoculation. Three replicates of each isolate were evaluated. To determine morphological characters, conidia formed on PDA for 7 day incubation were harvested with sterile distilled water and observed under compound light microscope. One hundred conidia from each isolate were measured in length and width with 3 replicates. (Kim et al., 2008).

Colony characteristics on different media

Radial growth and sporulation of the isolates were compared on 7 culture media. The media selected for cultural studies include Potato carrot Agar, Carrot Agar, Potato Dextrose Agar (PDA), Czapek Dox Agar (CZA), Malt Extract Agar (MEA) and Potato Dextrose Broth. Cultures maintained on PDA at 4°C were used for cultural studies. 5mm diameter mycelial agar disc of each isolate were placed on the surface of the selected culture medium and incubated at 24° C for 8-12 days in alternate dark and light cycles. Conidial morphology was examined using a compound microscope, photographed and compared.

Pathogenicity test

The pathogenicity of *Colletotrichum gloeosporioides* from leaf spot infected samples were determined. The ability of fungus to produce symptoms characteristic of leaf spot disease was evaluated by inoculating greenhouse grown *Murraya koenigii* plants. After inoculation plants were evaluated weekly for symptoms.

Inoculam preparation

Inoculam was prepared from 6-8 day old cultures grown on PDA. Conidial suspensions used for inoculations were prepared in sterile deionized water, filtered through four layers of cheesecloth and diluted to $5X10^5$ conidia/ml. Inoculations were performed by spraying conidial suspension with a sprayer directly into the crown of plants (Kenzie et al., 2006). The suspensions were sprayed to runoff with handheld sprayer. After inoculation, each plant was enclosed by plastic bag for 48 hours to maintain high relative humidity.

Plant inoculation

Plants were grown in 30cm pot and were maintained in greenhouse at 25°C. Plants were inoculated by spraying conidial suspensions with $1X10^6$ to $1X10^7$ conidia/ml. All treatments contained 0.02% Tween 20 to increase dispersion of conidia to the leaves. After inoculation the plants were maintained at 100% relative humidity by covering with plastic bags for 72 hours. Inoculation experiments consisted of 10 plants with one control plant mock inoculated with 0.02% Tween 20. Fungal germination, appressorial formation and symptoms appearance were recorded at different time points after inoculation (Horowitz et al., 2002).

Reisolation of pathogen

The symptomatic plant showing characteristic necrotic or chlorotic spots were chosen for reisolation of pathogen. Leaf spots regions from diseased plants were surface sterilized, sectioned, and plated onto Potato Dextrose Agar (PDA) for re-isolation and confirmation of the pathogen.

Leaf clearing

Pieces of leaves containing visible infection sites were placed in lactophenol with 0.05% (w/v) trypan

blue and heated over flame for approximately ten seconds until boiling. Leaf pieces were examined by light microscopy and photographed (Shen et al., 2001).

Germination studies

Young conidia from 10-30 day old colonies are allowed to settle into microscopic slide coverslip. The coverslips were then placed in glass humidifiers and placed in an incubator maintained at a constant temperature of 15, 25 and 35°C. At intervals of 6, 12, 24, 48, 72 and 96 hours, the chambers are opened briefly to remove coverslips. The inoculated coverslips removed at given temperature is fixed immediately in lactophenol cotton blue to arrest further development. Germination percentage was determined by microscopic observation of at least 300 spores per coverslip. Conidia are scored as germinated when germ tube exceeded their lateral radius (Miller and Gubler2003).

Results

Isolation of pathogen and Identification of pathogen

In general, leaf lesions are dark brown to black which initially appear as minute black circular spots. A diagnostic feature of Colletotrichum infection, the production of dark tufts of setae which appear as hair-like structures within the lesion was observed often associated with leaves. Colletotrichum produces spores within an acervulus. The disk or cushion shaped acervuli break through the surface of host tissue. Conidia are short, ovoid to cylindrical, and single celled. The pathogen was isolated in pure cultures on PDA media, subcultured and maintained on PDA slants at 4° C for further studies.

Morphological characterization of Isolated Pathogen

Colony on PDA were grey colored, with abundant production of acervuli and conidia. The conidiogenous cells were hyaline, cylindrical, or tapered and measured up to 20 by 3 to 4 µm. Setae were produced by most isolates. They were septate, dark brown, thick walled, acicular, and up to 200 µm long. Both Cultural and morphological characteristics of the isolates were examined. The bottom of PDA cultures for the all the isolates was grey.

Colony characteristics on different media

The colony on PDA was compared with other six media for sporulation, acervular formation and other morphological features. All the strains of Colletotrichum tested grew quickly, usually covering the whole surface of the petriplate in 10-15 days, except CZA and showed profuse sporulation on potato carrot agar compared to other media used. This medium was better than PDA for observing the main microscopic features of the fungi. Acervular conidomata were present in all the media with the production of conidiogenous cells directly on the agar surface and/or throughout the aerial mycelium of the colony.

Potato Dextrose Agar (PDA)

Colonies on PDA grew very quickly, occupying the whole surface of the Petri dish in 10-15 days. They were greenish gray with pinkish to salmon patches, powdery to velutine, profusely sporulated, and with abundant production of conidiomata; the reverse was grayish. The conidia were straight, cylindrical to slightly clavate, hyaline, obtuse at the apex, extremely variable in length, and measured 14 to 16 by 3.75 µm. Acervuli black, ranges in size from 200 to 640 by 225-615 µm.

Malt Extract Agar (MEA)

Colonies on MEA grew moderately fast, occupying the whole surface of the Petri dish in 15-20 days. They were greenish gray, powdery to velutine, profusely sporulated, and with less abundant production of conidiomata; the reverse was grayish. The conidia were borne on elongated phialides in acervular conidiomata, straight, cylindrical to slightly clavate, hyaline and septated, extremely variable in length, and measured 12 to 16 µm by 3.75 µm. Large number of acervuli were formed in groups at the centre, either immersed in agar or distributed throughout the media. Acervuli black, ranges in size from 325 to 545 µm. by 213 to 587 µm..

Czapek Dox Agar (CZA)

Colonies on CZA grew very slowly, occupying the whole surface of the Petri dish in 20-25 days. They were white with pinkish to salmon patches, powdery to velutine, profusely sporulated, and with abundant production of conidiomata; the reverse was uncolored with large number of acervuli seen as minute black spots throughout the media. The conidia were straight, cylindrical to slightly clavate, hyaline, obtuse at the apex, extremely variable in length, and measured 12 to 16 by 3.75 µm. Acervuli black, ranges in size from 200 to 640 µm by 225 to 615 µm.

Carrot Agar

Growth on Carrot agar was very quick, occupying the whole surface of the Petri dish in 4-5 days. They were greenish gray with pinkish to salmon patches, powdery to cottony or velutine, profusely sporulated, and with abundant production of conidiomata; the reverse was uncolored. The conidia were straight, cylindrical to slightly clavate,

hyaline, obtuse at the apex, extremely variable in length, and measured 12 to 16 by 3.75 µm. Acervuli formed were slimy and smooth compared to that formed on PDA or MEA, formed within 6 to 8 days of incubation, black and ranges in size from 240 to 450 by 300 to 475 µm.

Carrot Potato Agar

Growth on potato carrot agar was very quick, occupying the whole surface of the Petri dish in 4-5 days. They were white with pinkish to salmon patches, powdery to cottony or velutine, profusely sporulated, and with abundant production of conidiomata; the reverse was uncolored. The conidia were straight, cylindrical to slightly clavate, hyaline, obtuse at the apex, extremely variable in length, and measured 12 to 16 by 3.75 µm. Acervuli formed were slimy and smooth, formed within 7 to 10 days of incubation, black and ranges in size from 240 to 450 by 300 to 475 µm. Formation of setae was clearly visible under stereo microscope and compound microscope.

Plant Inoculation

The isolate was infective and was detected in infected tissues. Large number of leaves with necrotic spots was observed 25 days post inoculation. The fungus could be detected inside the leaves as bright mycelium. A large number of primary mycelium with many branches developed on the leaves that grew to secondary branches as the infection proceeds further. Mycelia were also seen grown inter or intracellularly during later stages of the infection. Symptoms were first observed as minute black spots. Necrosis began in small sizes in later stages and gradually turned larger in size. The presence of acervuli was observed at this stage of infection with the presence of setae characteristic to *Colletotrichum* infection. No disease symptoms were apparent in five of the infected plants.

Leaf clearing

By 24-48 hours post-inoculation on Murraya koenigii, the conidia had germinated to and clavate appressoria had formed followed by formation of infection vesicle. The primary hyphae grew progressively longer and septa were visible. At 78 hours narrower secondary hyphae began to emerge, penetrated the host cell walls further, and then radiated outwards at 96 hour. The secondary hyphae showed further extensive intercellular growth. The infected epidermal cells during the growth of fungi within the cells appeared to be alive suggesting the that the fungus was undergoing a biotrophic phase.

Germination studies

Isolates usually had conidia with one septa. Conidial germ tube emerged from both directions of the conidia. The percent germination data at each point of time showed conidial germ tubes emerged within 6 hours and become highly significant by 96 hours with the germ tube length measuring 115 to 180 µm.

Pathogen development

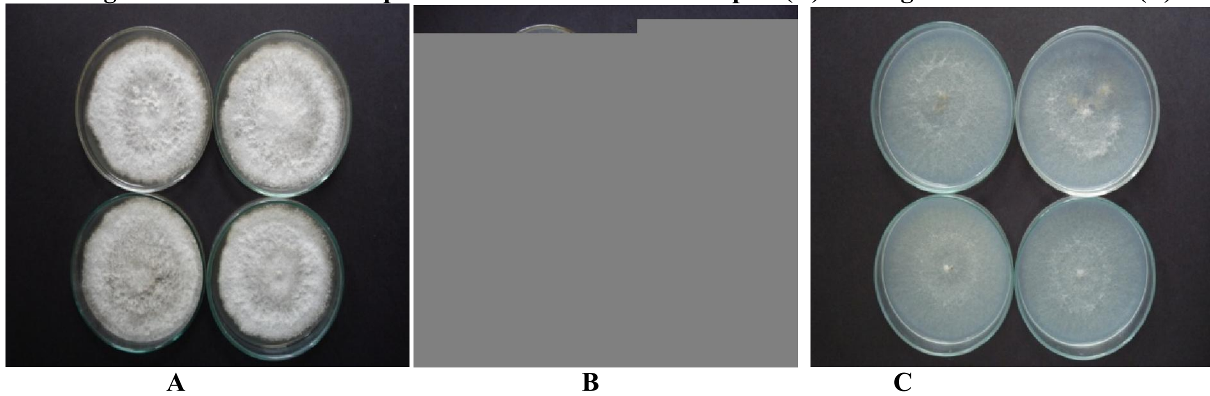
Germinating conidia formed appressoria, which further developed infection peg that caused the penetration into the host cell. The pathogen developed and established itself with the stages germ tube formation, appressorium, vesicles and primary and secondary hyphae in the tissues of artificially infected plants.

Table-1: Table showing percent germination and germ tube length at different stages of development

Incubation (hours)	No of conidia examined	No of conidia germinated	Percentage germination (%)	Germ tube lenth (µm)
06	2000	1437	72	33-56
12	2000	1531	77	66-91
24	2000	1861	93	115-180
48	2000	1981	99	›180
72	2000	1996	100	›180
96	2000	2000	100	›180

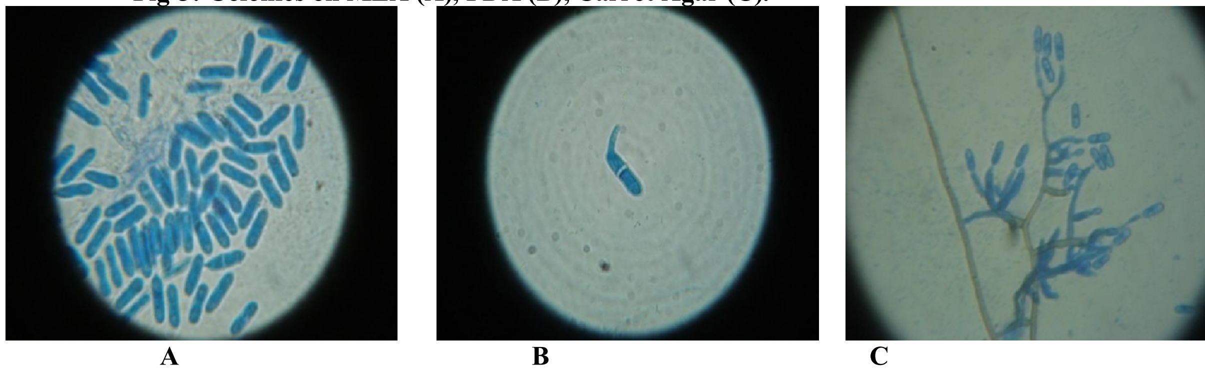
A B

Fig-1: Characteristic leaf spot infection from various samples (A). Pathogen isolated on PDA (B).



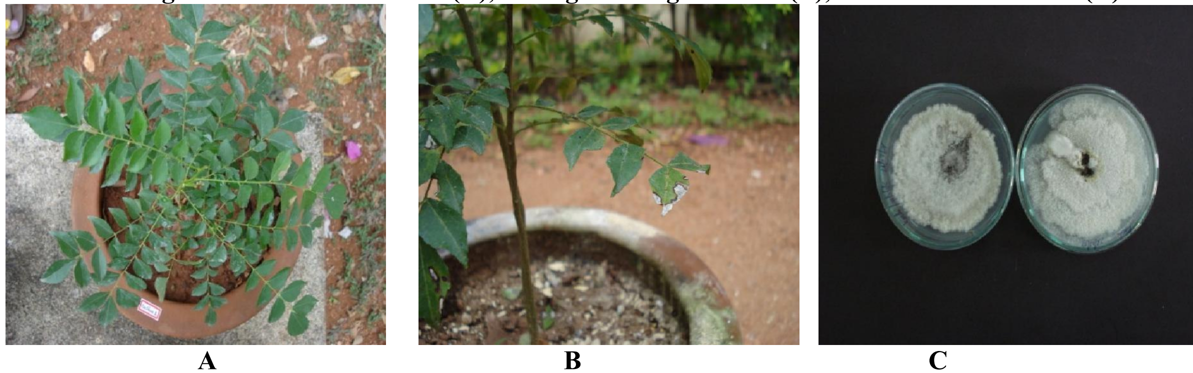
A B C

Fig-3: Colonies on MEA (A), PDA (B), Carrot Agar (C).



A B C

Fig-4: Conidia under 100X (A), Emergance of germ tube (B), Formation of conidia (C)



A B C

Fig-5. Healthy leaves before inoculation (A), Infected leaves after artificial inoculation and incubation under high relative humidity (B), Pathogen re isolated on PDA (C).

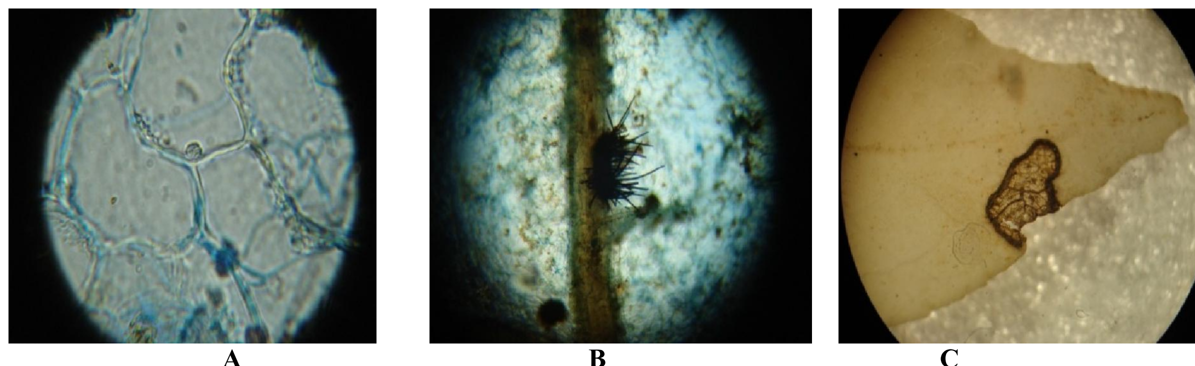


Fig-6. Inter and intracellular growth of hyphae within the cells (A), Formation of acervuli with characteristic setae on infected tissues (B), Region of necrotised tissues in the later stages of infection (C).

Discussion

The main aim of this objective was to identify the causative agent responsible for leaf spot disease of *Murraya koenigii* which appear to be a destructive epidemic in many regions of Karnataka and Kerala. In a morphotaxonomic comparison, isolates from necrotic and chlorotic spots of the leaf and twig samples of leaf spot affected plants were uniform in appearance and identified as *Colletotrichum gloeosporioides*. *Colletotrichum* species is one of the most economically important groups of fungi. Because of its economic importance as a plant pathogen *Colletotrichum* has received a lot of attention from numerous authors, and several molecular methods have been developed to detect it in plant tissue (Braithwaite et al., 1990, Mills et al., 1992) or to determine phylogenetic relationship (Freeman et al., 1993, Sreenivasaprasad et al., 1996). However the taxonomy of *Colletotrichum* is still unclear. Atleast11 generic synonyms have been reported for *Colletotrichum*, and ~900 species have been included in the genus (Sutton, 1992).

Colletotrichum gloeosporioides Penz. is a facultative parasite which belongs to the order *Melanconiales.* The fungus produced hyaline, one-celled, ovoid to oblong, slightly curved or dumbbell shaped conidia, 12-16 µm in length and 3-4 µm in width. Masses of conidia appear pink or salmon colored. The waxy acervuli that are produced in infected tissue were subepidermal, typically with setae, and simple, short, erect conidiophores. These identification marks were helpful in preliminary diagnosis of the disease to detect the pathogen isolated from leaf materials of infected plants on PDA.

It was necessary to prove the pathogenicity aspect of pathogen, hence kochs postulated was confirmed. Small discolored necrotic spots appeared three weeks after fungus inoculation. These spots were similar to those observed on plants from outdoor environments. Within six weeks after inoculation, the leaves of *M. koenigii* developed more necrotic spots that led to small necrotic regions. *C. gloeosporioides* was consistently reisolated from the symptomatic tissues of both fern species. No symptoms appeared on the water-inoculated plants. The fungus was re-isolated from diseased plant material authenticating the role of *Colletotrichum gloeosporioides* as the causative agent of leaf spot disease in *Murraya koenigii*.

The ability of a plant pathogen to cause a biotrophic or necrotrophic interaction appears to reflect a fundamental difference in its biology and the biology of interaction. The requirement to feed on living or dead cells will influence much more than just the nature of nutrients used by the micro organism. This will affect many aspect of the physiology and morphology of the pathogen and host (Goodwin, 2001). The infected cells of *Murraya koenigii* did not appear to be damaged until the end of biotrophic phase, but during the end of necrotrophic phase, there is extensive tissue damage and necrosis. The infection process began with conidia germinating to produce germ tube with an appressorium. Following penetration there was growth of primary hyphae which grew for two or three days, invading adjacent epidermal cells. The host cells remain intact during all these phases of mycelia growth. Thin secondary hyphae arise as side branches of large primary hyphae. As these hyphae spread through host tissues as intra or intercellular hyphae, extensive host cell degradation occurs. The symptoms of leaf spot then became visible often with the appearance of Acervuli within the damaged necrotic spots. Evaluation of conidial germination results showed optimal germination temperature at 25°C by 48 hours.

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References:

1. Perfect, S.E., Hughes, H.B., O'Connel, R.J., and Green, J. 1999. *Colletotrichum*: A model genus for studies on pathology and fungal plant interactions. Fungal Genetics. Biology. 27: 186-198.
2. Emmet, R.W., and Parbery, D.B. 1975. Appressoria. Annu. Rev. Phytopathol. 13: 147-167.
3. Bailey, J.A, O'Connel, R.J, Pring, RJ, Nash C. 1992, Infection strategies of *Colletotrichum* species. In : *Colletotrichum*: biology, Pathology and Control. J.A. Bailey and M. J. Jeger eds. CAB International, Wallingford, UK, pp. 88-120.
4. Kim, J.T., Park, S., Choi, W., Lee, Y., Kim, H.T. 2008. Characterization of Colletotrichum isolates causing anthracnose of pepper in Korea. Plant Pathol J. 24(1): 17-23.
5. Dauch, A.L., Ahn, B., Watson, A.K., Seguin, P., and Tabaji-Hare, S.H. 2006. Molecular monitoring of wild type and genetically engineered *Colletotrichum coccodes* biocontrol strains in planta. Plant Dis. 90: 1504-1510.
6. Horowitz, S., Freeman, S., and Sharon, A. 2002. Use of green flourescent protein-transgenic strains to study pathogenic and nonpathogenic lifestyles in *Colletotrichum acutatum*. Phytopathology. 92(7): 743-749.
7. Braithwaite, K.S., Irwin, J.A.G., and Manners, J.M. 1990. Ribosomal DNA as a molecular taxonomic marker for the group species Colletotrichum gloeosporioides. Aust Syst Bot. 3: 733–738.
8. Mills, P.R., Hodson, A., and Brown, A.E. 1992. Molecular differentiation of *Colletotrichum gloeosporioides* infecting tropical crops. In: *Colletotrichum*: Biology, Pathology and Control. In : J.A Bailey and M.J. Jeger, eds. CAB Int., Wallingford, UK, pp. 269-288.
9. Freeman, S., Pham, M., and Rodriguez, R.J. 1993. Molecular genotyping of *Colletotrichum* species based on arbitrarily primed PCR, A+T rich DNA, and nuclear DNA analyses. Exp. Mycol. 17: 309-322.
10. Sreenivasaprasd, S., Mills, P., Meechan, B.M., Brown, A. 1996. Phylogeny and systematics of 18 *Colletotrichum* species based on ribosomal DNA spacer sequences. Genome. 89(3): 499-512.
11. Goodwin, P.H. 2001. A molecular weed-mycoherbicide interaction: *Colletotrichum gloeosporioidesb* f. sp. *Malvae* and round-leaved mallow, *Malva pusilla*. Can. J. Plant Pathol. 23: 28-35.
12. Wharton, P.S., and Uribeondo, J.D. 2004. The biology of *Colletotrichum acutatum*. Anales del Jardin Botanico de Madrid. 61(1): 3-22.
13. Mac Kenzie, S.J., Legard, D.E., Timmer, L.W., Chandler, C.K., and Peres, N.A. 2006. Resistance of strawberry cultivars to crown rot caused by *Colletotrichum gloeosporioides* from Florida is nonspecific. Plant Dis. 90: 1091-1097.

7/1/2011