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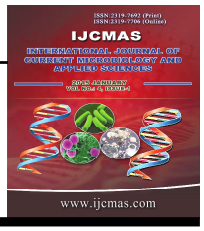

Original Research Article

Isolation, characterization and ultra structure of *Mycosphaerella musicola* – etiological agent of yellow sigatoka isolated from banana (*Musa spp*)

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A B S T R A C T

In the present study *Mycosphaerella musicola* – etiological agent of yellow sigatoka was detected and isolated from Grand Nain cultivar of Ghogaon, Maharashtra banana cultivating area. *M. musicola* was isolated on potato dextrose agar, which showed yellow colonies. Sporodochia and Conidia were observed by microscopic study under 10 x and 40 x lens. Pathogenicity of *M. musicola* as the causal agent of yellow sigatoka disease on banana was proved and Koch's postulates were fulfilled. SEM studies were conducted for more objective examination of the *M. musicola.* Masses of conidia were produced in sporodochia on upper surface of the leaf. Conidia were smooth, cylindrical and varied in shape from straight to curve observed at X-3000, X-10000 and 30000 and length at 5µm, 1 µm and 0.5 µm. on the Scanning Electron Microscope (SEM). This result suggested that *M. musicola* a causative agent of yellow sigatoka on Grand Nain cultivars of banana in Maharashtra was confirmed which would help in planning to control it.

Keywords

Banana, *Mycosphaerella musicola*, Microscopic studies, pathogenesis and SEM

Introduction

Banana is one of the commercial crop plants in India produced in different states including Maharashtra. Cultivated edible banana and plantains are derived from the progenitor species *Musa acuminata* (A genome) and *Musa balbisiana* (B genome), which are both members of the section Eumusa. Banana (*Musa* spp.), the most fascinating and popular fruit crop being commercially grown in many tropical and subtropical countries for its utilization as dessert and as staple food. Being fourth most important food after rice, wheat and milk products tolls up as major fruits crop of India (Noorulla *et al.,* 2013). These commodity fruit crops are amongst the most important across tropical and sub-tropical regions, contributing to food security, nutrition and poverty alleviation (Passos *et al.,* 2013). Productivity of banana in India in the year 2012-13 was 34.2MT/HA. In India Production of banana is highest in state of Tamil Nadu (19.4%), followed by Gujarat (17.1%). The other leading banana producing states are Maharashtra (13.6%), Andhra Pradesh (12.2%), Karnataka (9.5%),

Bihar (6.4%), Madhya Pradesh (6.4%), West Bengal (4.1%) Assam (3.2%), Odissa (2.0%), and (6.2%) other states. Banana is cultivated in all over the Maharashtra. (Database of National Horticulture Board, Ministry of Agriculture, Govt. of India, 2013). Pawar *et al.* (2010) reported banana (*Musa paradisica* L.) is one of the leading tropical fruit crops. It ranks next to mango in both area and production in India. Nanded district of Maharashtra has favorable climate to grow banana varieties like Basrai and Ardhapuri. The Sigatoka disease complex of banana involves three related ascomycetous Fungi, *Mycosphaerella fijiensis*, *M. musicola*, and *M. eumusae* the exact distribution of these three species and their disease epidemiology remain unclear, because their symptoms and life cycles are rather similar reported by Arzanlou *et al* (2007). Three species of Mycosphaerella are known to cause Sigatoka leaf diseases in banana. *M. musicola* causes yellow Sigatoka, *M. fijiensis* causes black Sigatoka and *M. eumusae* leaf spot as per as these three species yellow sigatoka has the widest distribution though black Sigatoka is rapidly replacing it in many tropical coastal regions suggested Surridge *et al*. (2003).

Mycosphaerella musicola is a fungal plant pathogen, which is the causal agent of Yellow Sigatoka leaf spot disease on banana plants. The ascomycete fungus *Mycosphaerella musicola* (anamorph *Pseudocercospora musae*), the causal agent of Sigatoka (yellow Sigatoka) disease, was first described as a pathogen of banana in Java in 1902 (Zimmerman, 1902). The first major disease epidemic of yellow Sigatoka was reported in Fiji in 1913 (Massee, 1914). It is spread over short distances by conidia and ascospores, over long distances it is the movement of infected germplasm such as diseased leaves and suckers that is likely to be responsible. Sigatoka leaf spot (*Mycosphaerella musicola*) disease is a limiting factor in banana production in India and other place (Nwauzoma *et al*., 2011). Noorulla *et al.* (2013) reported that the survey conducted in the seven major banana growing districts of Karnataka implied occurrence of yellow disease in all growing areas. Yellow Sigatoka, which is caused by *Mycosphaerella musicola* / *Pseudocercospora musae*, is the primary biotic problem for the Brazilian banana crop, with the causal agent presenting high pathogenic variability among the pathogen isolates (Gomes de Araujo *et al.*, 2014). Blomme *et al.* (2011) stated that the impact of diseases and pests, especially Sigatoka leaf spots has been recognized as a serious constraint to *Musa* production in different parts of the world. Annual average of lesions development of yellow sigatoka caused by *Mycosphaerella musicola* were calculated at 14 days intervals for 57 banana sites in North Queensland production region situated in the wet tropics between Cardwell and Innisfail reported by Gerald *et al*. (2003). Two types of spores are involved in the propagation of yellow Sigatoka, namely, ascospores and conidia (Cordeiro, 1997). Conidia (asexual spores) are usually produced continuously in environments of high relative humidity. They are disseminated by the washing of the leaf surface by rain or dew, which explains the severe infections sometimes observed in the tiller under more mature plants. However, ascospores (sexual spores), although produced at the same lesions from which conidia were released previously, appear later and are forcibly ejected from pseudothecia, also owing to high relative humidity, and even in dry climates, but owing to greater leaf wetness periods **(**Simmonds, 1966). Thus, the density of conidia in the air is related to the intensity of yellow Sigatoka, the decrease in the incubation periods and symptom generation

always associated with variable temperature and relative humidity (Guyot and Cuille 1958). The global population of *Mycosphaerella musicola* is the cause of Sigatoka (yellow Sigatoka) disease of banana. The isolates of *M. musicola* examined were grouped into four geographic populations representing Africa, Latin America and the Caribbean, Australia and Indonesia (Hayden *et al.,* 2003). *M. musicola* attacks almost all the commercially cultivated varieties in the country and the most severely affected cultivars include Rasthali, Poovan group, Karpooravalli, Red banana, Cavendish group and Robusta while Nendran is relatively tolerant to the fungus (Chandra, 1991). The pattern of spread of *M. musicola* around the world has been well documented (Stover, 1962; Mourichon and Fullerton, 1990) and dispersal mechanisms for the pathogen on a regional scale are well understood. Yellow Sigatoka (*Mycosphaerella musicola*) disease is limiting factor for production of banana in all over the world so that their characterization is most important factor to control its spread.

Materials and Methods

Collection of Samples: Banana leaf samples showing disease symptoms of yellow sigatoka (yellow spots) were randomly collected from village Ghogaon of Sangli district, Maharashtra (India).

Detection, isolation and characterization of etiological agent of Yellow Siagtoka (*Mycosphaerella musicola*): Microtome sections were done as described by Purvis *et al.* (1964). Infected lesion of leaves was observed under microscope (10 and 40X) with lacto phenol cotton blue staining method. Banana leaves were surface-sterilized with 95% ethanol, and lesions were cut from the leaves and lesions were crushed in mortar and pestle with sterile saline water then suspension was streak on potato dextrose agar plates and kept for incubation for 3-5 days. After incubation, the profuse productions of sporodochia were observed in most lesions, and the sporodochia and conidia identified as *M. Musicola* by microscopic examination with lactophenol cotton blue stain (Brun, 1958).

Confirmation (Koch Postulates studies) of etiological agent of Yellow Siagtoka (*Mycosphaerella musicola*): Conidial suspensions were prepared by growing isolates for 1-2 weeks until sporulation occurred in potato dextrose broth and the conidia were then scraped off into a 1.5 ml sterile plastic tube containing sterile distilled water. Conidial suspensions were standardized to 5 × 106 conidia per ml by diluting culture filtrates with sterile distilled water (Brown *et al*., 1998). Conidial suspensions (20 µl) were sprayed/ injected on to healthy detached banana leaves. One subset of leaves was wounded by puncturing with a sterile needle before inoculation. Disks containing mycelia from the actively growing edge were cut using a sterile cork borer and placed on a subset of healthy wounded and unwounded detached banana leaves. After few days disease spot of *Mycosphaerella musicola* were developed on banana leaf. Necrotic lesions were sectioned. To re-isolate pathogens at the end of the trials, tissue pieces were cut aseptically from symptomatic leaves, surface sterilized in 1% NaOCl for 1 minute and placed onto Potato dextrose agar. Plates were incubated for 3-5 days, and colonies were subcultured and identified with morphologically by staining method. The lactophenol cotton blue (LPCB) wet mount preparation is the most widely used method of staining and observing fungi - A drop of 70% alcohol placed on the clean grease free

microscope glass slide. Mount the fungus in the drop of alcohol .Added one or two drops of the lactophenol/ cotton blue mountant/ stain before the alcohol dries out. Holding the coverslip between forefinger and thumb, touch one edge of the drop of mountant with the coverslip edge, and lower gently, avoiding air bubbles. Observed the slide under 10x and 45x lense (Olympus – CX21i) for identification reported by Leck (1999).

Scanning Electron Microscopic study of etiological agent of Yellow Siagtoka (*Mycosphaerella musicola*)

Infected plant tissues for SEM analysis were prepared as described by Vigil *et al.*, (1984). The samples were washed with 25mM phosphate buffer and immersed through a series of ethanol concentrations, in the end leaving in 100 per cent ethanol overnight. The samples were then viewed using JEOL - SEM (JSM – 6360A) and photographed in different angles at the Department of Physics, Savitribai Phule Pune University, (MS), India.

Results and Discussion

Detection, Isolation and characterization of etiological agent of Yellow Siagtoka (*Mycosphaerella musicola*): Banana leaf samples showing disease symptoms of yellow sigatoka was randomly collected from village Ghogaon of district Maharashtra (India).

Results of thin section studies conducted on the infection process of yellow sigatoka fungus in Grand Nain cultivar of banana indicated that the symptoms of the disease varied in their intensity, size and area of spread among different cultivars. The differences in the patterns and the severity of the symptoms among the cultivars indicated their level of susceptibility or tolerance to the fungal invasion. This was evident from the observations of lots of spots visually seen on the banana leaf. In the microscopic study of *Mycosphaerella musicola* with fungal stain (Lacto phenol cotton blue) Sporodochia and Conidia were observed (10X and 40X lens) on the leaf samples (Fig.a and b).

In the isolation of fungus after incubation, yellowish colony of fungus were observed on potato dextrose agar plate's (Fig. e) morphological characterization of fungus as follows:

The profuse production of sporodochia was observed in most lesions of the infected leaf samples. Sporodochia (mass of tightly aligned conidiophores on a dark stoma) develop in the substomatal air chamber and the conidiophores grow through the stoma pore in a tuft-like fashion. As more conidiophores emerge (predominantly on the upper surface), sporodochia become erumpent, guard cells become disrupted and the adjacent epidermis is pushed back. Brown coloured, paler towards the apex, slightly curved, rarely branched, aseptate, geniculated, narrow towards the apex, without conidial scars, bottle-shaped and with rounded apices conidiophores were observed.

Conidia were borne terminally and singly on the conidiophore. Their appearance was pale to very pale olivaceous blue, smooth, straight or variously curved, cylindrical to obclavate-cylindrical observed under 10x and 40x microscopic lens (Fig c and d). Banana fields were surveyed in 34 villages of seven districts of Tamil Nadu to record the incidence of yellow Sigatoka disease (YSD) caused by *Mycosphaerella musicola. Mycosphaerella musicola* were identified based on symptom appearance and was confirmed by morphological characteristics

(Shanthiyaa *et al.*, 2013).

Yellow sigatoka disease found on leaves of Grand Nain cultivar of banana (*musa spp*) in the Maharashtra dist. Sangli banana cultivating areas. This disease caused by ascomycetous fungi *Mycosphaerella musicola* . It was successfully isolated from infected necrotic lesion of banana leaf. The observation of sporodochia and conidia same fungus was morphologically identified as *Mycosphaerella musicola* by microscopic examination with lacto phenol cotton blue stain. The morphological study reported here confirmed that the yellow sigatoka disease on banana in Maharashtra, India is caused by *Mycosphaerella musicola*, hence verifying the identification by Van den Boom and Kuhne (1969) of the disease as yellow sigatoka.

Although the teleomorph was not present, conidial and conidiophore morphology of the fungus present in the sigatoka lesions confirmed to the description of the anamorph of the yellow sigatoka pathogen, *Mycosphaerella musicola*. According to Simmonds (1966), conidia are produced continuously throughout the rainy season and disseminated through a film of free water, resulting from either rainwater or dew dripping on the leaves. Furthermore, among all the cultivars traditionally planted in Brazil, cv. Saquarema, which belongs to the Cavendish (AAA) subgroup, has a high degree of susceptibility to yellow Sigatoka (Gasparotto *et al.*, 2006). Leach (1941) reported that the rate of ascospore production per leaf injury is considerably lower than that for conidia. However, the discharge of ascospores can happen owing to increased relative humidity, in a manner independent of a water film over the leaf lesion. Ascospores can be released even from the lowest leaves of the plant, which are not affected by dew, but this is not true for conidia. The author also mentioned that the ideal temperature range for this release is between 21.1 and 28.91_C but gave no details regarding the duration of the incubation period or latency. In both in Queensland, Australia and Fiji, the climatological factors most commonly associated with yellow Sigatoka were relative humidity and temperature, and the disease generally reached its maximum activity during the periods of lower temperature and maximum relative humidity (Wardlaw, 1961). In the present investigation, it was observed that the cultivar of Grand Nain is highly susceptible to this yellow sigatoka (*Mycosphaerella musicola*).

Koch Postulates studies: After injection of 20 µl conidial suspensions in to healthy banana leaf with the help of sterile needle. Yellow spots of fungus (*Mycosphaerella musicola*) were observed on banana leaf after four days (Fig. f). Necrotic lesions were sectioned. To re-isolate pathogens at the end of the trials, tissue pieces was cut aseptically from symptomatic leaf, surface sterilized in 1% NaOCl for 1 minute and placed onto Potato dextrose agar (Fig. g). After five days incubation yellow coloured colonies were observed on potato dextrose agar media. Isolated colony was selected and after staining with lactophenol cotton blue (LPCB) wet mount sporodochia and conidia were observed under microscope to confirmation of fungus (Fig. h and i). The detailed result of Koch postulates studies are presented in Table 2.

It is clear that from Table 2 that the causative agent of yellow sigatoka disease is confirmed as *Mycosphaerella musicola* .

According to Wardlaw (1961), the highest incidence of small, striped lesions visible to the naked eye on the second, third or fourth leaf depends on the banana variety and the environmental conditions. The pathogen

associated with the leaf spot diseases on the two respective hosts, and secondly to establish pathogenicity, thereby confirming by Koch's postulates (Koike *et al.,* 2011). Pathogenicity of *Mycosphaerella musicola* as the causal agent in banana yellow sigatoka disease was proved and Koch's postulates were fulfilled in this study.

Scanning Electron Microscopic study of etiological agent of Yellow Siagtoka (*Mycosphaerella musicola*)

The morphology of all isolates from banana leaves in South Africa appeared to be consistent with that of *Pseudocercospora musae* (Jones, 2000), *anamorph of Mycosphaerella musicola* .Scanning electron microscopic studies were conducted for a more objective examination of the infection process. SEM examinations of the infected tissues at five stages of infection revealed the direct relation between the symptoms and the fungal population in the host tissues. Scanning images of leaf surface near stomata showed that there are very fewer amounts of mycelia in the tolerant cultivar Grand Nain (Fig. j) widely scattered and profusely branched mycelia were noticed.

This explains our observation on the time taken for the development of a single spot from initiation to maturity, wherein the development of a single spot from 0 to maturity stage took a longer period (29 days) in the tolerant cultivar Nendran when compared to susceptible cultivar Robusta (17 days) (Kannan and Prakasam, 2012). Masses of conidia were produced in sporodochia on upper surface of the leaf. Conidia were smooth, cylindrical and varied in shape from straight to curved observed at X-3000, X-10000 and 30000 and length at 5µm, 1 µm and 0.5 µm. on the Scanning Electron Microscope (SEM) (Fig j,k and l). Sporodochia developed in sub-stomatal chambers and emerged through the stomatal pore.

Conidiospores were straight, sometime slightly curved and bottle shape. They lacked septa and were unbranched. The conidiospore apex was rounded with no significant scarring. Conidia were pale yellow, smooth, cylindrical and varied in shaped from straight to curved, ranging from 50-120 µm in length and 2-6 µm in width. Conidial apices were obtuse and the base without a thickened hilum. No conidia were produced in culture. Although the teleomorph was not present in the sigatoka lesion conformed to the description of the yellow sigatoka pathogen, *Mycosphaerella musicola* Surridge *et al*. (2003).

On the basis of Morphological, Pathogenesis and Scanning Electron Microscopic studies the occurrence, prevalence and confirmation of *Mycosphaerella musicola* – etiological agent of yellow sigatoka on Grand Nain cultivar of banana in Maharashtra banana cultivating areas was studied.

Thus the above investigation as a result of the studies on the infection of *Mycosphaerella musicola* on Grand Nain cultivars of banana in Maharashtra has given an in-depth knowledge to the establishment of the pathogen and the resulting progress of the disease which would help in planning to control and the spray schedules and other management aspects of the disease for sustainable agriculture of banana in Indian environment.

Table.1 Properties of fungus colony

S. No.	Morphological characteristics	Observation
1	Size	2mm
2	Shape	Circular
3	Colour	Yellowish
4	Margin	Irregular
5	Opacity	Opaque
6	Consistency	Moist
7	Elevation	Convex
8	Sporodochia	Present
9	Conidiophores	Present
10	Conidia	Present

Table.2 Results of Koch Postulates studies

S. R.	Steps Used	Observations	Conclusion
1.	Grand Nain Variety	Symptoms yellow spots on leaves	Sigatoka disease was observed
2.	Isolation of etiological agent of sigatoka (Grand Nain Variety)	Sporodochia and conidia were observed	*Mycosphaerella musicola* was isolated and identified
3.	Inoculated into healthy plant (Grand Nain Variety)	Typical sigatoka spots were observed	Sigatoka disease produced
4.	Isolated from inoculated and diseased plant leaves (Grand Nain Variety)	Sporodochia and conidia were observed	*Mycosphaerella musicola* isolated and identified

Fig.1 Samples collected from dist. Sangli, Maharashtra

Fig. (a) *Mycosphaerella musicola* under 10x lens

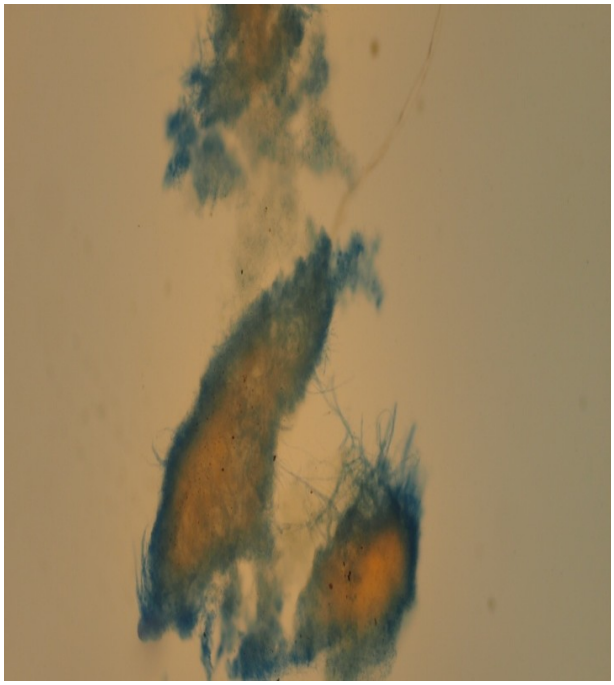


Fig. (b) *Mycosphaerella musicola* under 40x lens

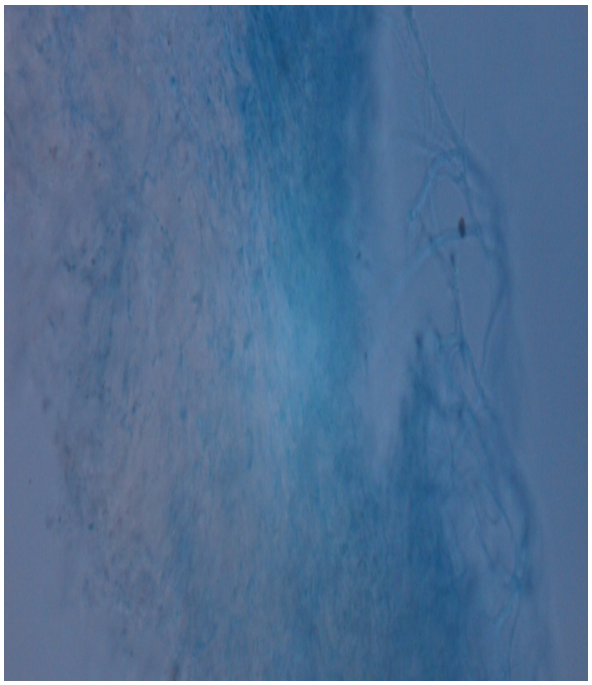


Fig. (c) *Mycosphaerella musicola* under 10x lens.

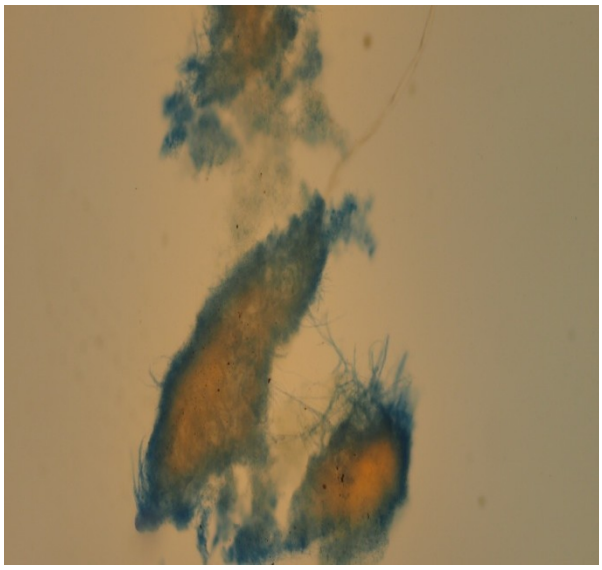


Fig. (d) *Mycosphaerella musicola* under 40x lens

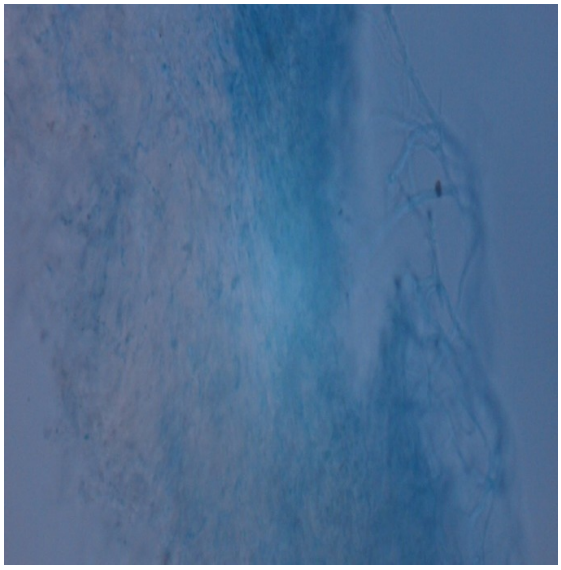


Fig.(e) Isolation of *Mycosphaerella musicola* from Grand Nain banana cultivar

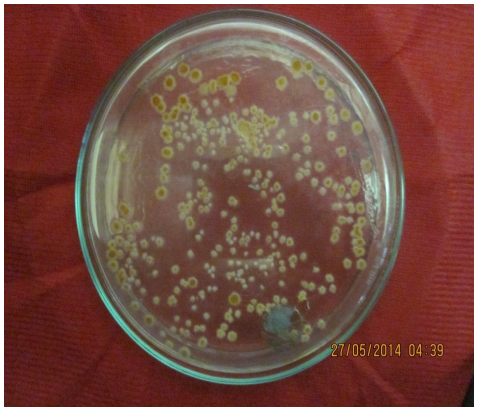


Fig.(f) Spots of yellow sigatoka (*Mycosphaerella musicola*) on Banana leaf

Fig.(g) Isolation of *Mycosphaerella musicola* from Grand Nain banana cultivar

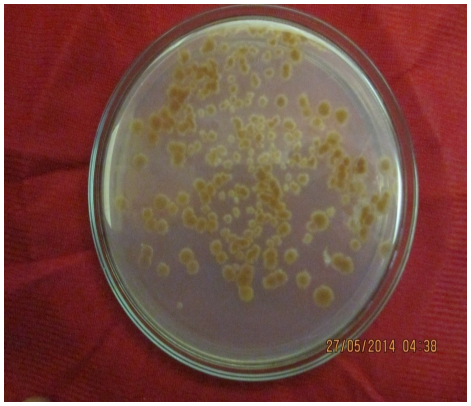


Fig. (h) *Mycosphaerella musicola* (Grand Nain banana cultivar) under 10x lens

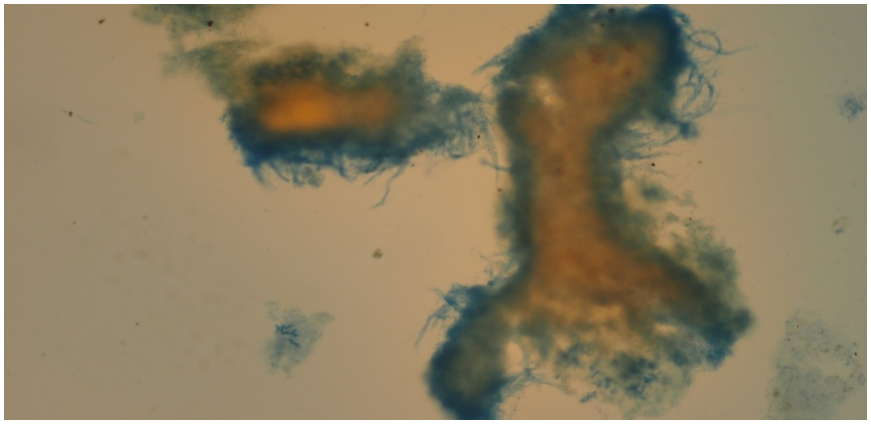


Fig. (i)*Mycosphaerella musicola* (Grand Nain banana cultivar) under 40x lens

Fig.(j) SEM of *Mycosphaerella musicola* at X-3000(5 µm)

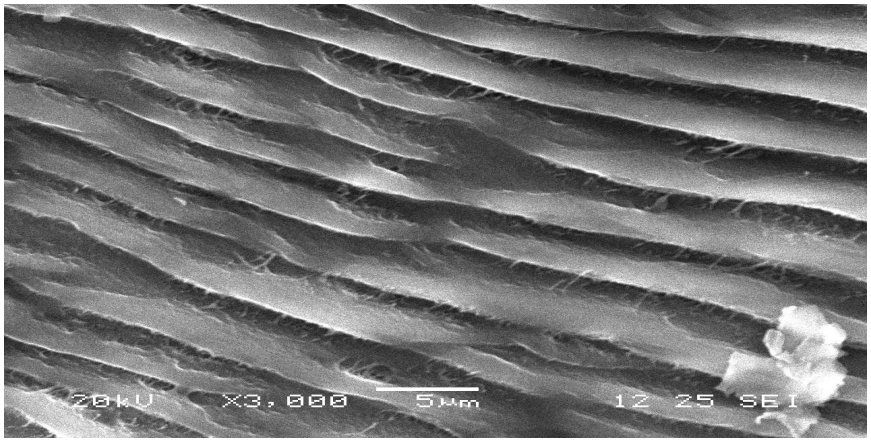


Fig.(k) SEM of *Mycosphaerella musicola* at X-10000(1 µm)

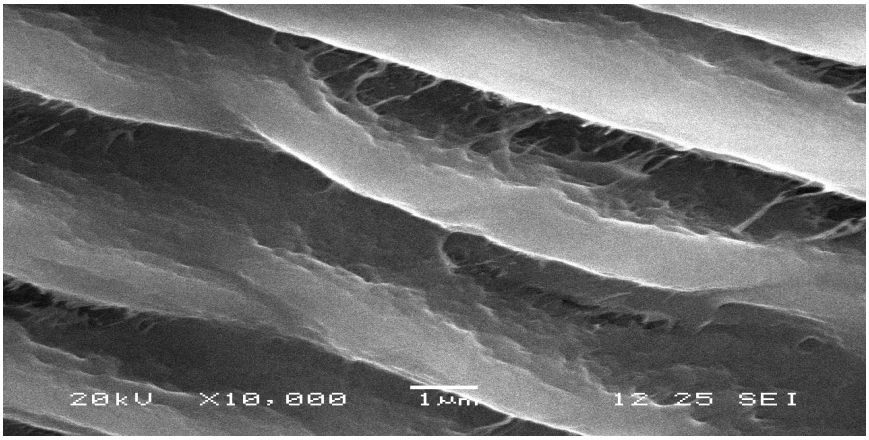
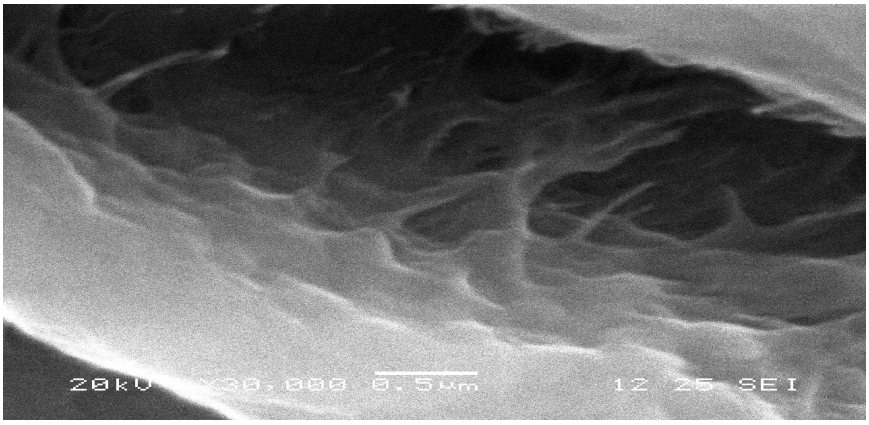


Fig. (l) SEM of *Mycosphaerella musicola* at X-30000(0.5 µm)



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