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MORPHOLOGICAL STUDIES ON YELLOW SIGATOKA DISEASE IN BANANA CULTIVAR KACHKAL (*MUSA PARADISIACA* ABB) AND ITS CAUSAL ORGANISM

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Abstract

Microscopic study of the isolated fungal growth showed branched, septate hyphal mass, conidia and conidiophores and Clamydospores, similar with reported structure of *Cercospora/Pseudocercospora musae*. White mycelia growth turned greyish in colour after 45-60 days due to fungal toxin production because of nutrient stress in the media. Molecular identification of isolated genomic DNA of pathogen conducted through PCR analysis using reported primer pair of Sigatoka pathogen identification (MM137/R635) produced approximately 1200 bp size of single bands revealing the isolated fungus as *Cercospora/Pseudocercospora musae*. The experimental findings revealed that culture media differentially influenced the growth of the colony formation by the Sigatoka pathogen. V8 Juice Agar media was found to develop earlier mycelia growth than PDA media. Radial expansion in V8 Juice Agar media was more profound than PDA media also. Hence the study signified that V8 Juice agar media influence culture of Sigatoka disease pathogen *Pseudocercospora* more profoundly than PDA media. *C.musae* infection causes slow infection in Kachkal and symptoms appeared very late (15-20 dpi) and instead of typical symptoms appeared as small brown necrotic spots across leaf lamina which enlarged initially with progression in time but halts after 60-65 dpi due to desiccation of the tissue around infection point to restrict further spread of the disease to nearby tissue deploying hypersensitive response.

Key words: Sigatoka, Cercospora, Pseudocercospora musae, Mycosphaerella.

Introduction

Sigatoka leaf spot disease is a major foliar disease of bananas (Blomme *et al*., 2011) and it causes significant annual yield loss of banana across the world (Arzanlou *et al*., 2007; Nwauzoma *et al*., 2011). Causal organism of Sigatoka disease is an ascomytes fungi *Mycosphaerella* (anamorph *Cercospora / Pseudocercospora*) (Braun *et al*., 2014). Sigatoka leaf spot disease complex is caused by three significant species *Mycosphaerella fijiensis* (causes Black Sigatoka disease), *M. musicola* (causes Yellow Sigatoka) and *M. eumusae* (causes Eumusae leaf spot disease) (Jones 2000, Carlier *et al*., 2000, Arzanlou *et al*., 2007). Yellow Sigatoka is more prevalent in India in comparison to the other two species (Nwauzoma *et al*., 2011). Anamorph of *M.musicola* causing yellow sigatoka is *Pseudocercospora musicola* (Synonyms *Cercospora musicola* and *Cercospora musae*, Braun *et al*., 2014). Yellow Sigatoka, caused by *Cercospora musae* Zimm. (anamorph of *Mycosphaerella musicola* Leach) was first reported in Assam in the year 1972 and since it was first reported, it became an economically important fungal disease of banana predominantly in the cultivars of the Cavendish subgroup from Assam (Saikia, 1972). Yellow sigatoka is a very destructive foliar disease and without proper management it causes severe defoliation and reduces viable leaves hampering banana production (Arzanlou *et al.*, 2007). In between 1937 and 1941, banana production in Mexico reduces to half and in Honduras production reduces to one third (Meredith, 1970). In Ghana 50-100 percent yield loss due to Sigatoka disease was reported (Perfoura *et al.*, 1993). Yellow sigatoka is reported to cause more than 50 percent of world wide banana production (Burt *et al*., 1997). Maximum Percentage of Disease Index (PDI) of 41.38 percent

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was obtained in banana plot without receiving chemical pesticide spray due to yellow sigatoka disease (Thammaiah *et al*., 2005). Hence chemical fungicides and some cultural practices like de-suckering, drainage, weed control etc. are the only control management of Sigatoka in commercial banana, but extensive use of fungicides also brings collateral issues like pollution, health hazards, fungicide resistance etc (Aguilar-Barragan *et al*., 2014). To develop a green sustainable chemical free control measure such as developing resistant lines information on the pathogen and its interaction with host is very much essential. Therefore this study has been undertaken for better knowledge of the host pathogen interaction.

Methods and Methodology

To study the host-pathogen interaction, one economically important Sigatoka resistant banana cultivar of Assam, Kachkal (Genome ABB) was chosen (Mishra, 2000). Healthy suckers collected from the Horticulture Orchard of Assam Agricultural University, Jorhat, were planted in large pots filled with a soil (double autoclaved soil and vermi compost in 2:1 ratio) and grown inside shade net house till emergence of two to three leaves.

Isolation and culture of the pathogen Isolation, purification and culture of the pathogen

Infected leaves of banana identified by their typical symptoms were collected, cut into small pieces, surface sterilized under aseptic hood with 70% alcohol (1min) and 0.1% Mercuric Chloride (1min), each followed by several time rinse with double distilled water and cultured in V8 juice agar media. Culture is then purified by single hyphae method. The pure culture was maintained by periodic sub-culture in readymade specific media V8 juice agar (formulated by blending 8 vegetable juices) at 28°C.

Growth measurement of fungus in artificial media and statistical analysis

Radial growth (from the initial inoculum until the extreme area of mycelia) was measured in 20 culture plates at 5 time points after inoculation.

Mean radial expansion (r) was calculated by measuring radial distance from center to the edge of each of the petriplate.

Mean radial expansion with standard deviation (SD) were calculated for both the culture media viz. V8 Juice Agar and Potato Dextrose Agar (PDA) and for visual demonstration of differential growth pattern in both the media at different time points histogram is plotted in Graph Pad Prism 8.3.1 software. The correlation analysis (one tailed at 95% confidence level) was done in GraphPad Prism 8.3.1 software between radial mycelial expansion obtained in V8 Juice Agar Media vs. PDA Agar Media" to calculate P value, r value and R square value for testing the significance of the data.

Microscopic detection of the pathogen

Microscopic study of lactophenol blue stained fungal culture done to study fungal mycelia growth and spore at 15-20 days old, 30-35 days old and 45-50 days old fungal culture.

Molecular detection of the pathogen

For molecular detection of pathogen, genomic DNA of the fungus was isolated by CTAB method followed by PCR amplification using primers designed from internally transcribed spacer (ITS) region of *M.musicola* (Johanson and Jegar, 1993). PCR was performed with approximately 100 ng of fungal genomic DNA as template in a 10 µl reaction volume in Thermal cycler (Applied Biosystems 2720) using primer pair MMus137 (GGCGCCCCCGG AGGCCGTCTA) as forward primer and R635 (GGTCCGTGTTTCAAGACGG) as reverse, taking annealing temperature 65°C. The final reaction mix consist of 1X PCR buffer, 0.25mM $MgCl_2$, 0.4mM of dNTPs, 0.6 µM of forward and reverse primers each and 1U Taq DNA Polymerase molecular biology grade water used to make up the volume. The reaction profile consisted of 35 cycles of denaturation at 94ºC (1 min), annealing at 65ºC for 1 min and extension at 72ºC (1 min) with initial temperature for denaturation 94ºC (5 min) and final extension was done at 72ºC (7 min).

Artificial inoculation *C. musae* to host plant and morphological studies on disease progression

Artificial inoculation of 15-20 days old cultures of *C.musae* was done in Kachkal plants with 3-4 opened leaves using injection and cotton swab methods (Sinclair and Dhingra, 1985). Progress of the disease in the host plants were observed and noted down at different time points.

Results

Isolation, purification and culture of the pathogen

From the second day of inoculation white mycelia growth became visible in the media Fig. 1 which in 20-25 days almost entirely covered the whole petri plate within 20-25 days of inoculation. 40-45 days old fungal culture turned grayish Fig. 2.

Growth measurement of fungus in artificial media and statistical analysis

The pathogen which was grown in V8 Juice agar media became visible white mycelial growth after two

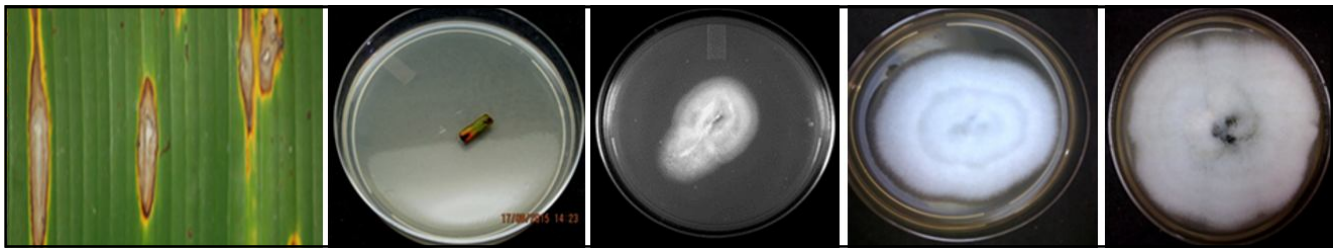

Fig. 1: Isolation and Culture of pathogen inV8 juice agar media.

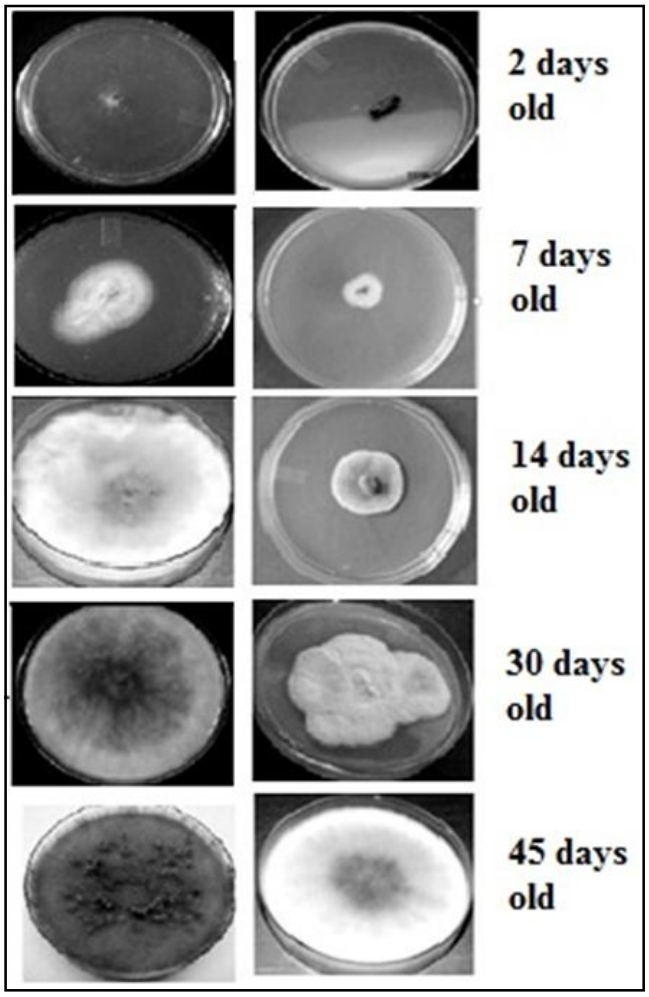

Fig. 2: Fungal growth observed in V8 juice agar media and PDA media in 2, 7, 14, 30 and 45 days old cultures.

days of inoculation, but in PDA plates fungal hyphae was visible only after 4/5 days after inoculation. Growth rate on subsequent time points is faster in V8 Juice Agar plate culture in comparison to PDA plates. After 14 days of inoculation in V8 Juice Agar Plate around 90% of the plate got covered but in PDA plate less than 50% area was covered with mycelial growth. After 30 days mycelia hypae in V8 Juice agar plate covered the whole plate and the center of the plate started turning grey while in PDA growth reached only around 50% of the Petri plate. In 45 days old culture, fruiting body sprouted on the top and mycelia color turned grey in V8 Juice agar plate, while in PDA plates white mycelial growth covered the whole Petri plate and in the center the color started turning a little grey.

Radial measurements was taken from center of culture plate to edge of the mycelial expansion and average radial expansion and standard deviation was calculated table 1. In V8 Juice agar media radial expansion of mycelial growth measured 2.34±0.53 mm in 2 days old, 13±4.29 mm in 7 days old, 38.6±3.86 mm in 14 days old and 45mm in both 30 and 45 days old culture. In PDA media radial expansion of mycelial growth measured measured 0 in 2 days old, 5.75±0.94 mm in 7 days old, 14.04±2.24 mm in 14 days old, 26.33±7.6 mm in 30 days old and 45mm in 45 days old culture.

GraphPad Prism 8.3.1 software plotted histogram as interleaved grouped bar graph. Mean radial expansion of 20 replicates with SD at 2 days, 7 days, 14 days, 30 days and 45 days interval in both the media (V8 Juice agar and PDA) plotted depicted gradual expansion of the mycelia. In V8 Juice agar fungal growth is faster and more than PDA Fig. 3.

The correlation analysis among mean radial mycelial expansions in V8 Juice Agar Media vs. PDA was done in GraphPad Prism 8.3.1 software. The correlation study revealed significant (*) one tailed P value 0.0354 (P<0.05),

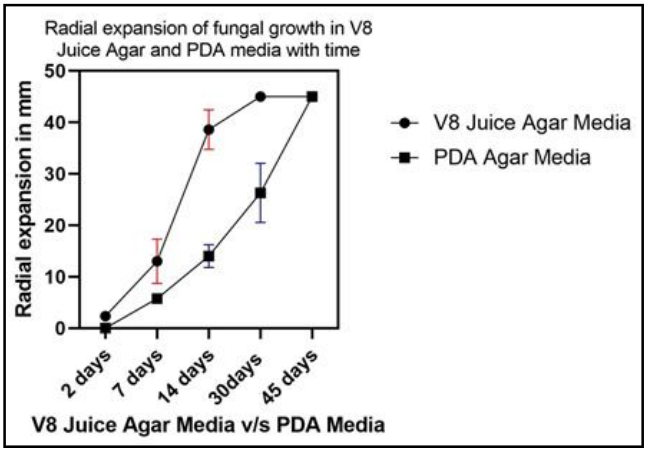

Fig. 3: Plotting Mean and error with SD in XY graph connecting points and error bar with line graphically represents the growth rate of the fungus in V8 Juice Agar v/s PDA media with progressing time.

Table 1: Radial growth/ expansion from the center monitored in culture of the pathogen in V8 Juice agar media and PDA media in 2 days, 7 days, 14 days, 30 days and 45 days old cultures.

Sl. No.	2 days old culture		7 days old culture		14 days old culture		30 days old culture		45 days old culture	
	V8	PDA	V8	PDA	V8	PDA	V8	PDA	V8	PDA
1	2.5	0	18.75	6.75	40.25	14.75	45	30.5	45	45
2	2	0	19.25	6	38	15	45	30	45	45
3	3	0	15.25	6	45	14	45	30	45	45
4	2.25	0	12.5	7	36.75	13.75	45	32.5	45	45
5	2.75	0	14.75	7.25	42	12	45	21.75	45	45
6	2.5	0	15.25	6.5	38.25	14.75	45	27.5	45	45
7	2.25	0	13.5	6.75	37	14.5	45	25	45	45
8	3	0	14.75	5	44	15	45	35	45	45
9	1	0	5.5	6.75	34.25	16.5	45	32.5	45	45
10	3	0	17.25	4.75	41.5	10.5	45	27.5	45	45
11	3.25	0	18.75	5.25	45	10	45	19	45	45
12	2.75	0	19.25	6	45	15	45	25	45	45
13	2.25	0	7.5	4.25	36	12	45	17.5	45	45
14	2.25	0	7.25	5	34.75	13.5	45	20	45	45
15	2	0	10	4.25	36.25	11	45	14.75	45	45
16	2	0	9.75	6.5	37	20	45	25	45	45
17	2	0	10.5	6	36	15	45	30	45	45
18	1.75	0	10.5	4.75	34.75	13.5	45	20.5	45	45
19	2	0	9.5	5.25	33.25	15	45	32.5	45	45
20	2.25	0	10.25	5	37	15	45	30	45	45
Average	2.3375	0	13	5.75	38.6	14.038	45	26.325	45	45
SD	0.5273	0	4.295	0.942	3.856	2.2408	0	5.7560	0	0

r value 0.8460 (Pearson r) at 95% confidence interval (-0.1429 to 0.9896) and R squared value 0.7158. Taking mean radial expansion of 20 replicates with SD at Y axis and time points (2 days, 7 days, 14 days, 30 days and 45 days) in X-axis for both the media (V8 Juice agar and PDA) the XY graph was plotted in GraphPad Prism 8.3.1 software. The XY graph showed gradual increase of the mycelia growth in both the media but V8 Juice agar it grows sooner than PDA and finally both the data merged at 45 days old culture Fig. 4. Scattered plot graph

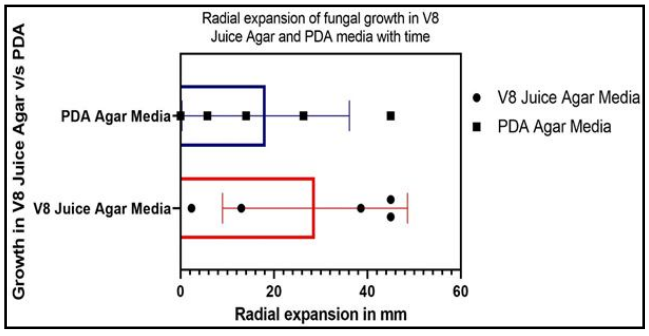

Fig. 4: Scattered plot with bar (horizontal column graph) plotting mean with SD graphically depicting the growth pattern of the fungus with progressing time in both V8 Juice Agar and PDA media.

constructed in GraphPad Prism 8.3.1 software graphically located the position of radial expansion in each culture at different time points in relation to average growth with standard deviation. Here also average growth rate in more in V8 Juice agar than PDA Fig. 5.

Microscopic identification of yellow sigatoka

Lectophenol blue stained microscopic Slides prepared from 15 days old culture showed only branched, septate hyphal growth. In 30 days old culture conidiophore fascicles, Conidiophore tips and Conidia were observed in 45 days old plates. Microscopic structure resembles with reported structure of *Pseudocercospora musicola* (BPI 438701) by Crous and Braun (2003).

Molecular identification of yellow sigatoka pathogen

PCR analysis conducted with isolated genomic DNA using MM137/R635 primer pair produced approximately 1200 bp size of single bands Fig. 6.

Artificial inoculation *C. musae* to host plant, morphological studies on disease progression

Symptom development is relatively slow, late and less severe in Kachkal. Around 15-20 dpi as symptoms appear as tiny brown necrotic spots which increase in size and

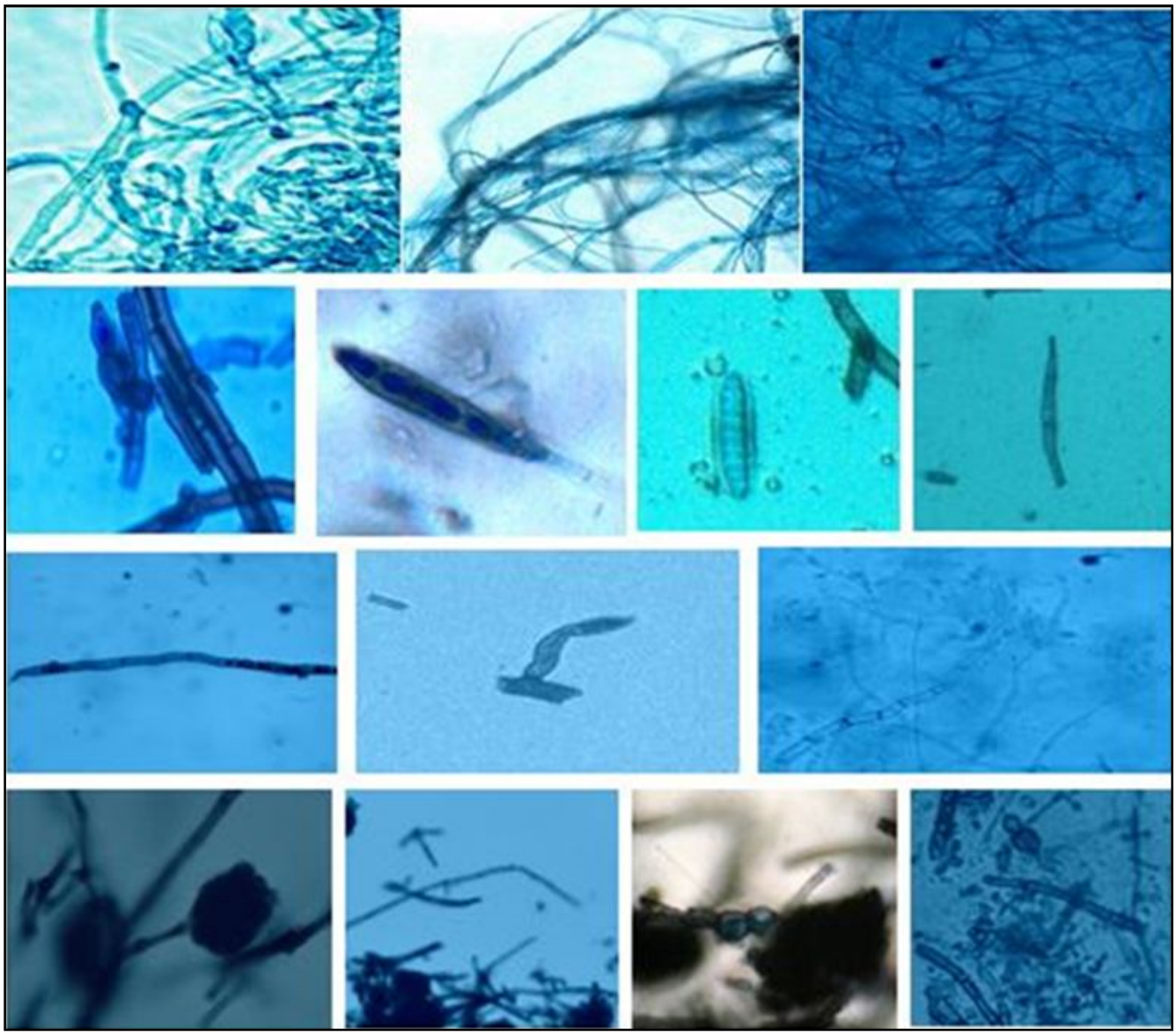

Fig. 5: Microscopic identification of the fungus. Fungal hyphae stained in Lactophenol blue and observed under microscope: septate hyphae, conidia and chlamydospores (under 40x) i-iii fungal hyphae, iv-vii conidia; vii-x conidiophore fescicles; xi-xii conidial vescicle; xiii-xiv chlamydospore.

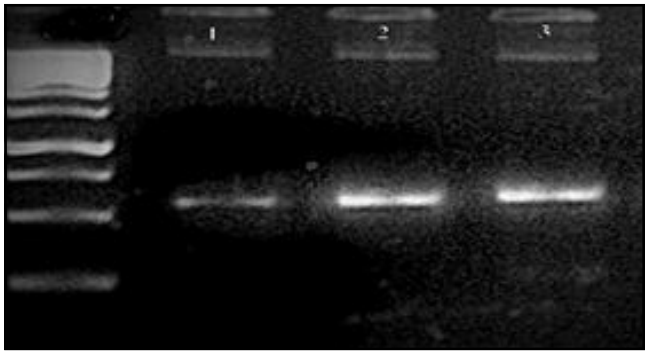

Fig. 6: Electrophoresis result of PCR products run in 1% Agarose gel gave the bands at 1200 bp size on amplifying the fungal genomic DNA with reported primer pairsMM137/R635.

central tissue dried surrounded by brown ring at 25 to 30 dpi. At 60-65 dpi the spread of the lesions halted and new young leaves appeared in the plants Fig. 7.

Discussion

Artificial media has significance impact in colony morphology and hence selecting the most efficient media is very much important. The *C. musae* growth was obtained within 2-3 days after inoculation in the media where V8 juice agar medium was more efficient media than PDA for fungus isolation in terms of mycelia growth, moreover it is reported to be favourable for sporulation in *Mycosphaerella musae* (Henderson *et al*., 2008). 15 days old culture showed only branched and septate hyphal growth. Conidia and conidiophores were found in 20-25 days old cultures. Clamydospores are found in 45-60 days

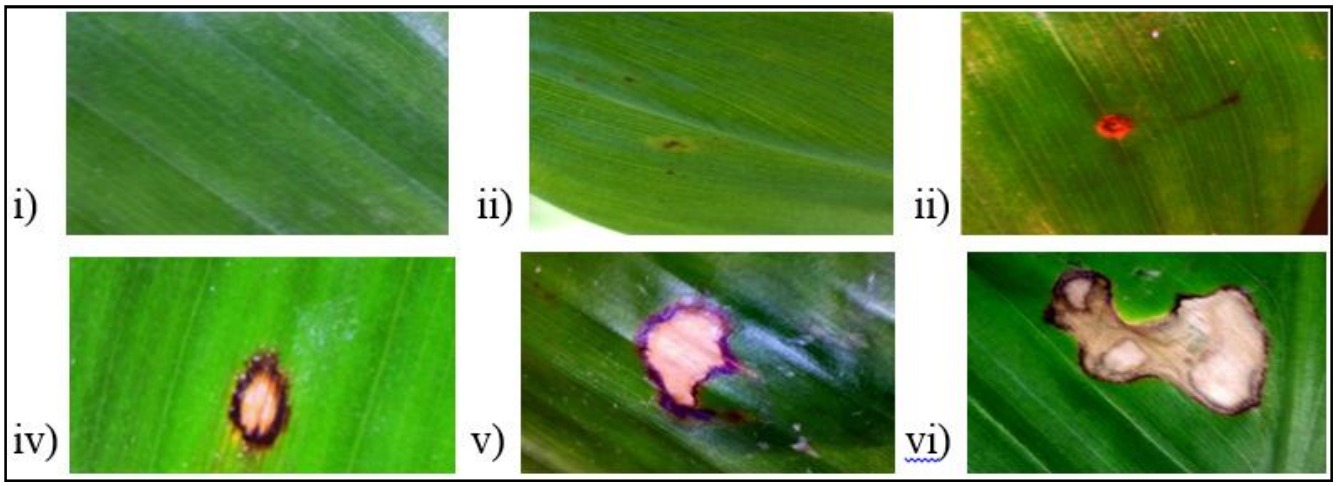

Fig. 7: Different stages of symptom development after inoculation in Kachkal after i) 10 dpi, ii) 20dpi, iii) 30dpi, iv) 40 dpi, v) 50 dpi, vi) 60dpi.

old culture plates where mycelia growth turns greyish in colour due to fungal toxin production because of nutrient stress in the media. Similar observation of fungal hyphae, ascospores, conidia and fruiting bodies were found in morphological studies on *Mycosphaerella* spp. done by Henderson *et al.*, (2008). Similar morphology of *Mycosphaerella* spp. with septate (0-6 in number) hyphae, ascospores, pycnidia and perithecia. was observed by Selvarajan *et al*., (2001) also. Genomic DNA of the fungus was extracted from 20-25 days' old mycelia mass using cetyl methyl ammonium bromide (CTAB) method. CTAB method was also used by Nakyanzi (2002) for extraction of *M. fijiensis* genomic DNA. Modified Sodium dodecyl sulphate (SDS)-CTAB method was used by Umesha *et al*., (2016). A rapid and efficient modified CTAB method was used by Aamir *et al*., (2015) for isolation of genomic DNA from food-borne fungal pathogens. Identification of the Sigatoka pathogen is very much difficult as the three pathogens *M. musicola, M. fijiensis* and *M. eumusae* can co-exist in the same lesion and they have almost identical morphology and epidemiology (Crous, 1998; Nakyanzi, 2002; Maxwell *et al*., 2005; Arzanlou *et al*., 2007). Johanson and Jeger (1993) developed a molecular identification technique using specific primers (MMus137 as forward) and R635 as reverse primer) to overcomes these problems. In the present investigation same primer pairs was used for PCR based identification of the pathogen as described by Johanson and Jeger (1993) and same sized intact single 1200 bp band was obtained as described by.Johanson and Jeger (1993). Surridge *et al*., (2003) also used the same primer pairs and confirmed involvement of *M. musicola* with Yellow Sigatoka symptoms in South Africa.

Symptom development in Kachkal due to *C.musae* infection was slow and symptoms appeared very late (15-20 dpi) and instead of typical Yellow Sigatoka leaf spots small brown necrotic spots appeared across the leaf lamina. The size of the necrotic spots enlarged in 30 dpi, however spread of the spots was restricted after 60-65 dpi. Kachkal is reported as a Yellow Sigatoka resistant banana cultivar of Assam (Mishra, 2000) Therefore Kachkal probably responds to infection by a hypersensitive response to restrict the pathogen growth by desiccating tissues at that point of infection to stop further spread. In field condition also, similar disease progression in banana and symptom development due to yellow Sigatoka disease were observed by other workers (Meredith, 1970; Henderson *et al*., 2006). Hence, time of recognition of the invading pathogen and the faster activation of host defense mechanisms is a key difference between the resistant and susceptible plants. To prevent pathogen colonization, resistant cultivar can rapidly deploy a wide variety of defense responses but susceptible plant deploy weaker and slower responses which fails to restrict pathogen spread (Manickavelu *et al*., 2010).

Conclusion

In the present study, microscopic and molecular identification of the pathogen isolated from banana leaf with typical symptoms of Yellow Sigatoka confirmed the fungus to be *Cercospora/Pseudocercospora musae*. The microscopic structural details and molecular identification technique can be further use as reference to identify the yellow sigatoka isolates. The fungus showed faster growth in V8 juice Agar media in comparison to PDA. Hence V8 juice can be used as specific media for isolation of yellow sigatoka pathogen. The disease showed slow progression in cultivar Kachkal. Kachkal being resistant (reported by Mishra, 2000) seems to deploy hypersensitive reaction to restrict pathogen

spread in tissue by subjecting tissue dryness around the infection.

References

Aamir, S., S. Sutar, S.K. Singh and A. Baghela (2015). A rapid and efficient method of fungal genomic DNA extraction, suitable for PCR based molecular methods. A rapid and efficient method of fungal genomic DNA extraction, suitable for PCR based molecular methods. *Plant Pathology & Quarantine*, **5(2):** 74-81.

Arzanlou, M., E. Abeln, G. Kema, C. Waalwijk, J. Carlier, I. de Vries, M. Guzmán and P. Crous (2007). Molecular Diagnostics for the Sigatoka Disease Complex of Banana, **97:** 1112-1118.

Arzanlou, M., G.H.J. Kema, C. Waalwijk, J. Carlier, I. de Vries, M. Guzmán, M. Araya Vargas, J. Helder and P.W. Crous (2009). Molecular diagnostics in the mycosphaerella leaf spot disease complex of banana and for *radopholussimilis*. *Acta Hort.*, **828:** 237-243.

Blomme, G., P. Asten and B. van Vanlauwe (2011). Banana systems in humid highlands of sub-Saharian Africa: *Enhancing resilience and productivity*, **22:** 175-190.

Carlier, J., M.F. Zapater, F. Lapeyari, D.R. Jones and X. Mourichon (2000). Septoria leaf spot of banana: a newly discovered disease caused by *Mycosphaerella musae* (anamorph *Septoriaeumuase*). *Phytopathology*, **90:** 884-890.

Churchill, A.C.L. (2011). Mycosphaerella fijiensis, the black leaf streak pathogen of banana: progress towards understanding pathogen biology and detection, disease development and the challenges of control. *Molecular Plant Pathology*, **12(4):** 307-28.

Crous, P.W. (1998). *Mycosphaerella* spp. and their anamorph associated with leaf spot disease of Eucalyptus. *Mycol. Mem.*, **21:** 1-170.

Crous, P.W. and U. Broun (2003). *Mycosphaerella* and its anamorphs: 1. Names published in *Cercospora* and Passalora. *CBS Biodiversity Series*, **1:** pp. 1-569.

Henderson, J., K. Grice, M.F. Zapater and F. Careel (2008). *Mycosphaerella* spp. Causing leafspot disease on banana. Advanced methods supplement, Plant Health Australia.

Henderson, J., J.A. Pattemore, S.C. Porchun, H.L. Hayden, S. Van Brunschot, K.R.E. Grice, R.A. Peterson, S.R. Thomas-Hall and E.A.B. Aitken (2006). Black Sigatoka disease: new technology to strengthen eradication strategies in Australia. *Australas Plant Pathology*, **35:** 181-193.

Johanson, A. and M.J. Jeger (1993). Use of PCR for detection of Mycosphaerella fijiensis and M. musicola, the causal agents of Sigatoka leaf spots in banana and plantain. *Mycological Research*, **96:** 670–674.

Jones, D.R. (2000). Disease of banana abeca and enset. CAB International, Wallingford, Oxon, UK. CABI Publications.

Mustaffa, M.M. and S. Sathiamoorthy (2004). Banana and plantain R&D in India. *Advancing banana and plantain R&D in Asia and the Pacific*, **13:** 94-102.

Manickavelu, A., K. Kawaura, K. Oishi, T. Shin-I, Y. Kohara, N. Yahiaoui, B. Keller, A. Suzuki, K. Yano and Y. Ogihara (2010). Competitive gene expression analysis of susceptible and resistant near isogenic lines in common wheat infected by Pucciniatriticina. *DNA Res.*, **17:** 221-222.

Meredith (1970). Banana leaf spot disease (Sigatoka) caused by *Mycosphaerella musicola*. Leach. (Commonwealth Mycological Institute, Kew, Surrey, England).

Mishra, A.K. (2000). Epidemiology and management of Sigatoka disease of banana. M.Sc, Thesis, Assam Agricultural University, Jorhat.

Nakyanzi, M. (2002). Molecular and pathogenic diversity of isolates of *Mycosphaerella fijiensis* that cause black sigatoka disease of banana in East Africa. Ph.D. thesis. University Greenwich, East Africa.

Saikia, P.N. (1972). Notes on banana diseases and their control. *Assam Agricultural University*, **3:** 23-28.

Selvarajan, R., S. Uma and S. Sathiamoorthy (2001). Etiology and survey of banana leaf spot diseases in India. *Advancing banana and plantain R & D in Asia and the Pacific*, **10:** 94-104.

Sinclair J.B. and O.D. Dhingra (1985). Basic Plant Pathlogy. Methods.CRC Press, Boca Raton, FL.

Surridge, A.K.J., A. Viljoen, P.W. Crous and F.C. Wehner (2003). Identification of the pathogen associated with Sigatoka disease of banana in South Africa. *Australasian Plant Pathology*, **32:** 27–31.