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Identification of *Coniella musaiaensis* as Pathogen Causing Stem Rot Disease of *Hibiscus cannabinus* L. in Terengganu, Malaysia

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Abstract: *Hibiscus cannabinus* (kenaf) plant is claimed as one of the fast-growing herbaceous plants with the high potential as a fiber or lignocelluloses material which is widely planted in Setiu, Terengganu, Malaysia. However, the stem rot disease was observed to be the most problematic in getting the good yields. Microbes associated with*H. cannabinus* that showing typical symptoms of rot-like disease were isolated using direct plating techniques. Koch'spostulates proved that *Coniella musaiaensis*was fungus that caused stem rot disease to kenaf out of four isolated fungi. Plant-pathogen interaction revealedthe mechanism of infection by direct penetration of fungus through the outer surface of stems, since present of appressorium on the surface of host (*H. cannabinus*).

Key words: Hibiscus cannabinus · Stem rot disease · *Coniella musaiaensis* · Koch postulatess · Fungi · Plant-pathogen interaction

INTRODUCTION

Hibiscus cannabinus L. (kenaf) holds a promising potential in the Malaysian biocomposite industry, as its long fibers are suitable in the process of making a number of products such as pulp and paper, fiber and particle boards, as well as fiber reinforced plastic components and chemical absorbent [1]. It is also known as future crops as it is a source of cellulose fiber and is a potential crop for the production of ropes, twines, coarse, burlap and fiberboard [2-4].

One of the most serious problem that limit kenaf production in many plantation areas in Setiu, Terengganu is the stem rot diseases. The most frequent pathogenic fungi on kenaf were *Phytophthora* sp, *Leveillula taurica and Sclerotium rolfsii*. The main diseases of kenafare foot, stem and collar rots and wilting caused by *Phytophthora* sp., while *S. rolfsii* causing collar rot and powdery mildew caused by *L. taurica* [5-7]. *Coniella musaiaensis* found to be a leaf spot and stem canker on *H. sabdariffa* [8] but no report of this fungus on *H. cannabinus* yet in Malaysia. The objectives of this study are isolation and identification of the causal pathogens from stem rot diesease and study the mechanisms of pathogenicity of the isolated pathogens on kenaf.

MATERIALS AND METHODS

Sampling: Kenaf plants showing typical symptom of stem rots were collected from kenaf plantation at Telaga Papan, Setiu, Terengganu, Malaysia and brought to the laboratory for isolation and identification of the pathogenic fungi.

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Koch's Postulates: Direct Plating Method. The infected stems were cut into 1-2 cm^2 pieces, separately soaked in 10% sodium hypochlorite solution for 60 seconds, washed with distilled water for one minute, then dried with sterile filter paper and placed on Potato Dextrose Agar (PDA) [9]. The plates were incubated in the dark at 27-30 °C for 48 hours and observed for sporulating fungi under a light microscope. Pure cultures of recovered fungi were prepared from single conidia and maintained on PDA slant as stock culture at 4 °C in the freezer. The sporulating fungi were picked up using a wire needle, stained with LCB and then observed under a Digital Florescence Microscope (Leica/DM5000B Color Digital Camera. The fungi were confirmed to their genus, based on their conidial morphological characteristics.

Preparation of Target Test Plant: The seeds were planted at 1.25 to 2.5 cm depth in a polybag (16 cm diameter x 23 cm height) containing a mixture of soil (top soil: organic fertilizer: sand) in the ratio of 3:2:1 and left in the field and normally emerges two to four days after planting [10]. They were watered twice daily to soil saturation. A small mycelia plug of pure fungal strain from 14-days old culture was put at the wounded part on the plant stem surface.

Plant Inoculation: *Hibiscus cannabinus* plants at three whorl stage (1 month) were used in these experiments. The plants were inoculated with the inoculums by making a small cut at the plant stem surface. The inoculation was done at four different places on each plant for every type of fungi, with four replicate each. The inoculated parts were then wrapped with soft cotton and sealed. The soft cotton was wet with water to maintain the relative humidity. The disease incidence was recorded at 48-72 hr after inoculation. Disease severity was recorded daily until the disease stopped progressing or severe damage of the host [11, 12].

Disease Assessment: Disease assessment was based on the number of plant affected out of the number of plant inoculated, expressed as the percentage of disease plants [13-15] and the disease severity was based on the area of plant tissue showing symptom of the disease [15]. The progress of the disease was assessed based on the disease development on the stems.The plants were scored for their severity of disease affliction on the scale; 0 = healthy; 1 = 10% diseased; 2 = 20% diseased; 3 = 30% diseased; 4 = 40% diseased; 5 = 50% diseased; 6 = 60% diseased; 7 = 70% diseased; 8 = 80% diseased; 9 = 90% diseased; 10 = plant death [9].

Genotypic Identification of Pathogenic Fungi: Fungal strains were cultured in 250 mL Erlenmeyer-flasks containing 100 mL of Potato Dextrose Broth (PDB) medium (2.4 g of PDB) for two to five days depending on species using a rotary shaker (30 °C, 120 rpm). The mycelium was collected by centrifugation (Sigma/Sigma 3-K16) and grinded to fine paste using liquid nitrogen by mortar and pestle. 40 mg of the grinded sample was transferred into a 1.5 mL Eppendorff tube and the DNA was extracted by Plant DNA Isolation kit (Promega, USA). PCR was carried out by using universal primer set; LROR (5'-ACCCGCTGAACTTAAGC-3') and LR7 (5'-TACTACCACCAACA-TCT-3'). PCR amplifications were performed in 25 ìL volumes containing 0.5 unit Taq DNA Polymerase (Fermentas); dNTP mix (10 mM each of dCTP, dGTP, dATP and dTTP); 10X buffer A, 1.5 mM MgCl2, 0.5 mM primer and 2 ìL of genomic DNA. Amplification was performed in a thermocycler (Master Cycler Gradient) with the following temperature profiles: 94 °C for 30 sec (denaturation), annealing at 50 °C for 30 sec and extension at 72 °C for 1.5 min. The PCR product (1.5 kb 28 sRNA) were sent for sequencing and then blasted by nucleotide blast software to determine theiridentity.

Plant-Pathogen Interaction: Light Microscopy and Cross Sectioning. Infected and healthy stems' sections were fixed by soaking in 70% formaldehyde-acetic acid-alcohol (FAA) followed by graded series of dehydration process; 30% ethyl alcohol, 50% ethyl alcohol, 60% TBA, 70% TBA, 85% TBA, 95% TBA and finally transferred to 100% TBA. This was followed by infiltration process; transferred to paraffin oil/TBA, then to paraffin oil/TBA mixture with solidified wax and lastly the sections were transferred into pure wax in an oven at 60°C. The sections were then embedded in 100% wax (Tissue Embedding Center: Leica EG 1160) followed by sectioning by using Semi-Motorized Rotary Microtome (Leica RM 2245). The sample slices were then immersed in water bath (Leica 1210) at 37°C and gently mounted on slide, then transferred to hot plate (Leica HI/ 1220) to dry the sample and finally stained by using Auto Stainer (Leica XL). The samples were then viewed under Digital Florescent Microscope (Leica/DM5000B Color Digital Camera).

Scanning Electron Microscopy: The healthy and infected stems of *H. cannabinus* were used. The samples were cut horizontally and fixed with 2.5% glutaraldehyde in 0.05 M sodium phosphate, then washed with sodium cacodylate buffer. Finally they were fixed in 1 % osmium tetroxide and washed again with 0.1 M sodium cacodylate buffer as

before. The samples were dehydrated through a series of graded ethanol (10%, 20%, 30%, 40%, 50% 70%, 80%, 90% and then 100% acetone). They were soaked for 10 minutes at each concentration except for 100% ethanol in which they were soaked for twice. Finally, they were soaked in 100 % acetone. The samples were dried and mounted on aluminum stubs and coated with Au/Pd using sputter coater, then were viewed under scanning electron microscope (PHENOM G2).

RESULTS AND DISCUSSION

Koch's Postulates: There are four types of pure fungal strain obtained after undergoing several series of subculture by direct plating technique. Those samples were labeled as FT3, FS7, FS2 and FS1.The morphology characteristics of the posibble pathogenic fungiwere observed and recorded (Table 1 and Figure 1). The shape and the number of septa within conidia can be used in comparing with the experimental results. Nevertheless, Nelson *et al.* [16] had stated that species identification by morphological traits is problematic because characteristics like mycelial pigmentation, formation, shape and size of conidia are unstable and highly dependent on composition of media and environmental conditions. Phenotypic variation is abundant and many expertises are required to distinguish between closely related species and to recognize variation within species.

The inoculation was done at four different places on each plant for every type of fungi, with four replicates each. Out of four, the fungus FT3shows typical symptom of rot (Figure 2(a)) and Figure 2(b)) highly similar to the natural infection (Figure 2(c)) in the field. The stem showed small 'rot-like' black spot symptom that started to appear about 2 weeks after inoculation was done.

The disease assessment can be done by determining the disease incidence and also disease severity of the plant. The disease incidence can be obtained by calculating the number of plant affected out of the number of plant inoculated [13, 15]. In this experiment, four replicates of plants out of four plants inoculated had been affected and showed the typical symptom of 'rot-like' disease. The disease incidence was determined as follows:

$$\text{Disease incidence} = \frac{\text{Number of plant affected}}{\text{Number of plant inoculated}} \text{ x } 100\%$$

On the other hand, the disease severity can be determined based on the area of plant stem showing symptom of the disease. In this experiment, the inoculated stems became infected with 0-10% severity in the first two weeks, 10-20% severity in week 3 and death in week 7. The affected plant showed stunted growth compared to healthy plant (Figure 3).

Genotypic Identification: The genotypic identification was done with the DNA extracted from sample FT3. The LROR and LR7 primers were used to amplify 28S large subunit ribosomal RNA gene and the product sizeobtained was 1.5 kb.DNA sequencing was then carried out to further confirm the identity of these fungi. The pathogenic strains showed 99% similarities with 28S ribosomal RNA of *C. musaiaensis* strain AR3534.Reffering to previous study on the *C. musaiaensis*, which is found to cause a disease of a serious leaf spot and stem canker on *H. sabdariffa* var. sabdariffa, commonly known as Roselle, which is in the same family to H. cannabinus [8] and there is no report or study been done about these fungus on H. cannabinus stem rot disease. After all H. cannabinus is still new in Malaysia especially in Terengganu which is the plantation was done on briss (sandy) soil. It is an annual crop related to cotton, is of economic importance in the Caribbean and it is commonly known as sorrel in Trinidad and Tobago [17]. From those studies, the possibility of *C. musaiaensis* infection on *H. cannabinus* are high since that disease infected H. sabdariffa which is a member of Malvaceae family and genus Hibiscus. This evidence was strengthened further by Adeoti *et al*. [18], who showed that *C. musaiaensis* infected members of Malvaceae family comprising of *Gossypium hirsutum* L., *Abelmoschus esculentus* L. and *H. sabdariffa* L. in experimental studies.

Plant-Pathogen Interaction: Further investigation of fungi can be done through cross-sectioning of healthy and infected stem. The fresh stem cell showed clearness of the cell without any infection, while the infected stem cell (Figure 4) showed interference within the cell with the existence of the blackish-brown cell. Then, the infection was further confirmed and detected by observation of the infected sample under scanning electron microscope. The infected stem cell also showed the structure of fruiting body of fungus on the stem surface where it penetrated into the inner cell (Figures 5(a) and (b)) while, the healthy stem was free from any interference and fungal infection (Figures 5(c) and (d)). Fungal plant pathogens have evolved diverse mechanisms for penetrating into host plant tissue, ranging from entry through natural openings to various mechanisms of direct penetration through the

Table 1: Range of septa isolated from infected part of *Hibiscus cannabinus*

		Morphology		
Sample of fungal pathogen	Range of septa (mm)	Spore shape	Color	Texture
FT3	5-10	spherical	brownish	dry-like
FS7	2-4	oval	reddish-white	fur-like
FS2	3-5	oval	pinkish-white	fur-like
FS1	3-5	oval	brownish	fur-like

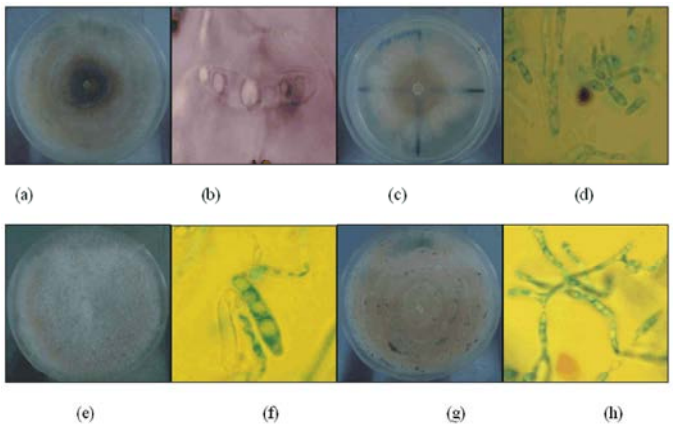

Fig. 1: Macroscopic and microscopic (1000X magnification) views of isolated fungi from infected *H. cannabinus*: (a) nd (b) Fungus strain FT3. (c) and (d) Fungus strain FS7. (e) and (f) Fungus strain FS2. (g) and (h) Fungus strain FS1

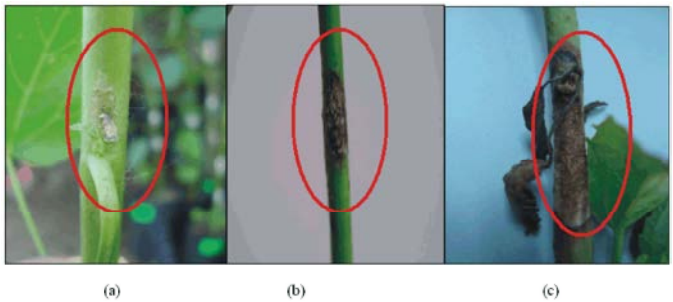

Fig. 2: *Hibiscus cannabinus* stem showing typical symptom of 'rot-like' disease; (a) affected plant at early stage, (b) affected plant at final stage, (c) natural infection in the field

Fig. 3: *Hibiscus cannabinus* (A) healthy plant, (B) affected plant after 3 weeks of inoculation

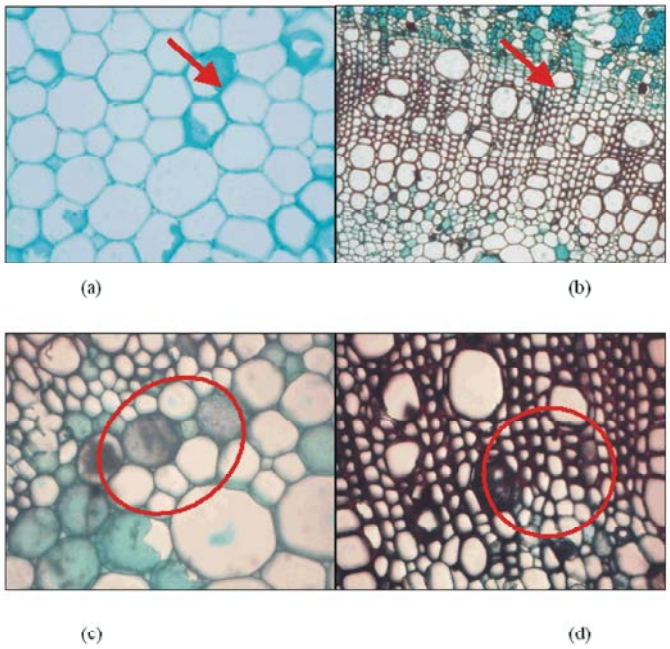

Fig. 4: Cross sectional view of stem sample; (a and b) fresh stem, (c and d) infected stem (viewed under a digital florescence microscope at 40X magnification)

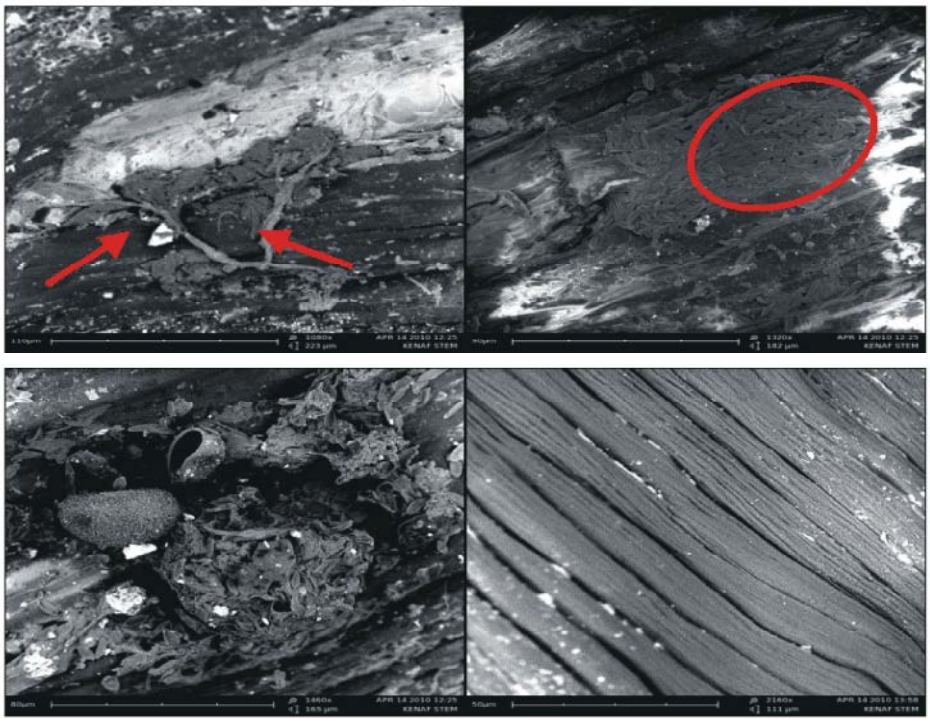

Fig. 5: Electron micrograph of *Hibiscus cannabinus*. (a) The germ tubes of the fungus protruded towards the infected stem cell (viewed under 1080X magnification). (b) Spore colonization on the surface entered the inner cell through the cracked and damaged stem fractures on infected stem cell (viewed under 1320X magnification). (c) The fruiting body of the fungus on the stem surface penetrates into the inner cell on healthy plant (viewed under 2740X magnification). (d) The fiber was clean from any interference and infection of fungi on healthy plant (viewed under 2160X magnification)

outer surface [19]. The result shows the direct penetration of the fungus which occurs through the *H. cannabinus* stem surface. The germ tubes of the fungi protruded towards the stem cells surface into the intracellular cell and the colonization of the spore on the surface cause them to enter the inner cell surface through the cracked and damaged stem fractures.

CONCLUSION

Several methods had been carried out in this study to identify the possible pathogenic fungi that cause stem rot of *H. cannabinus*. According to genotypic identification had been done, the identified pathogenic fungus that causes the disease was *C. musaiaensis*. It had been further confirmed as a pathogen to the *H. cannabinus*by pathogenecity testing and proved by Koch's postulates. Besides, plant-pathogen interaction also proved the appearance of the fungus by the formation of spore colonization in the cell that blocked the nutrient and water transmission where it will cause the plant stunted in growth and die. So, the direct penetration infection shows the high virulence of *C. musaiaensis.*

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REFERENCES

1. Nor Aini, A.S., M.H. Basri, A.H. Hazandy, S. Ghizan and M.M.N. Fadzhel, 2009. Growth and phenology of kenaf (*Hibiscus cannabinus* L.) varieties. Pertanika Journal of Tropical Agriculture Science, 32(1): 29-33.
2. Webber, C.L. and R.E. Bledsoe, 1993. Kenaf: Production, harvesting and products. InJanick, J. and Simon, J. E., (eds.) New crops. Wiley, New York Press.
3. Petrini, C., R. Bazzocchi and P. Montalti, 1994. Yield potential and adaptation of kenaf (*Hibiscus cannabinus*) in north-central Italy. Industrial Crops Production, 3: 11-15.
4. Yu, H. and C. Yu, 2007. Study on microbe retting of kenaf fiber. Industrial Crops Production, 40: 1806-1809.
5. Agrios, G.N., 2003. Plant Pathology. Elsevier Academic Press.
6. Prota 2 Article, 1759. Vegetables/Legumes. Record Display. Systemic of National Educational, 10(2): 1149.
7. Swart, W.J., 2001. First report of powdery mildew of kenaf *by Laveillula taurica* in South Africa. International Journal of Applied Plant Pathology, 85(80): 923.
8. Persad, C. and M. Fortune, 1989. A new disease of sorrel (*Hibiscus sabdariffa* var. *sabdarifa*) caused by *Coniella musaiaensis* var. *hibisci* from Trinidad and Tobago. Plant Pathology, 38: 615-617.
9. Kadir, J.B., R. Charudattan, W.M. Stalland B.J. Brecke, 2000. Field efficacy of *Dactlaria hingginsii* as a bioherbicide for the control of purple nutsedge (*Cyperus rotundusa*). Weed Technology, 14: 1-6.
10. Webber, C.L., H.L. Bhardwaj and V.K. Bledsoe, 2002. Kenaf production: Fiber, feed and seed. In Trends in new crops and new uses, eds J. Janick and A. Whipkey, 327-339. Alexandria, VA: ASHS Press.
11. Hailmi, M. S., 2011. Potential of *Exserohilium monoceras* as bioherbicides for controlling barnyard grass (*Echinochloa crus-galli*). Journal of Agrobiotechnology, 2: 1-15.
12. Ng, S.C., J. Kadir, M.S. Hailmi and A.A. Rahim, 2011. Efficacy of *Exserohilum longirostratum* on barnyard grass (*Echinochloa crus-galli* spp. *crusgalli*) under field conditions. Biocontrol Science and Technology, 21(4): 449-460.
13 James, W.C., 1974. Assessment of plant disease and losses. Annual Review of Phytopatholog, 12: 27-48.
14. Horsfall, J.G. and E.B. Cowling, 1978. Pathometry: The measurement of plant disease. In Horsfall, J.G. and Cowling, E. B., (eds.) Plant Disease. An Advance Treatise' Academic Press, New York.
15. Kranz, J., 1988. Measuring plant disease *In* Kranz, J. and Rotems, J., (eds.) Experimental Technique in Plant Disease Epidemiology. Berlin: Springler.
16. Nelson, P.E., T.A. Tossoun and W.F.O. Marasas, 1983. *Fusarium* species, an illustrated manual for identification. The Pennsylvania State University Press, University Park and London.
17. Grubben, G.J.H., 1977. Tropical vegetables and their genetic resource. Internaional board for plant genetic resource, FAO, Rome.
18. Adeoti, A.A. and A.M. Emechebe, 1992. Alternative hosts of *Corniella musaiensis* B. Sutton Nig. Journal of Botany, 5: 173-176.
19. Richard, J. and V. Barbara, 1996. Breaking and Entering: Host Penetration by the Fungal Rice Blast Pathogen *Magnaporthe grisea*. Annual Reviews of Microbiology, 50: 491-512.