MORPHOLOGICAL CHARACTERIZATION OF HYPOXYLON WOOD ROT FUNGUS IN KENYAN TEA

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ABSTRACT
Hypoxylon fungi species causes Hypoxylon wood rot disease in tea (*Camellia sinensis* (L) O. Kuntze). It exists in both asexual and sexual states. To assess the diversity of the fungi in Kenya, 58 fungus isolates were collected from different tea growing regions in Kenya and characterized based on cultural and morphological features. Radial growth of the different isolates on PDA differed significantly. Based on visual assessment of cultural similarity, 10 groupings were observed. The characteristics were mainly effused pulvinate stromata, composed of mainly spherical perithesia and amyloid asci with brown ellipsoid in-equilateral ascospores. *Hypoxylon* fungi were prevalent (disease index 0.76 to 0.82) in clonal as compared to seedling teas (0.05-0.36). The disease was fairly prevalent at elevated tea growing areas (2100-2250 m asl) than at 1826-1969 m asl. Morphological characteristics of the stromata did not show much variation with respect to tea growing areas; however, treatment of stomatal bodies with 10% KOH released extractable pigments (brown), indicating that *H.* species is infecting tea in Kenya.
Keywords: Hypoxylon, Cultural, Stromata, Characterization, Tea, Kenya

INTRODUCTION
Hypoxylon spp are generally wood colonizers found mostly in the tropics than sub-tropics. The fungus belongs to the family Xylariaceae(Ascomycota) with about 41 genera andover 130 recognized species and varieties (Sanchez-Ballesteros et al. 2000). Hypoxylonspp exists as saprophytes of facultative parasite in the order Xylariales and class Ascomycetes (Rogers, 1979). Like most Xylariaceous members, the fungus exists in both asexual (anamorphic) and sexual (teleomophic) states. The asexual state is known as *Geniculosporium serpens* (Kenerley and Rogers, 1976). Generally, Xylariaceae fungi occur mostly on dicotyledonous angiosperms with few incidences reported on monocotyledons and conifers (Rogers, 1979; Whalley, 1989). In Kenya, the pathogen causes one of the major diseases of tea (Otieno, 1993). The fungus has significant economic and ecological importance as it affects productivity and biomass degradation (Venkata Ram, 1974; Onsando, 1989; Otieno, 1993, 1994; Sánchez-Ballesteros et al. 2000).

Traditionally, Xylariaceae can be identified based on fruiting body morphological characteristics namely stromatal form, stromatal colour, ascospore shape and dimension or ascus bearing sporocarp (perithecia), ascus morphology and ascospore release (Ainsworth et al. 1971; Rogers, 1979; Whalley, 1996). Later, the combination of teleomorphic and anamorphic characters, and chemotaxonomy through the stromatal pigment colours in 10% potassium hydroxide (KOH) led to the recognition of new species (Ju and Rogers, 1996). The general appearance of the ascalapical apparatus is an important characteristic amongst members of the family Xylariaceae (Laessoeet al. 1989; Juand Roggers; 1996; Whalley, 1996). Detailed ascal apparatus such as ascospore wall ornamentation can be distinguished through the use of scanning electron microscope (Suwanasaiet al., 2013). Ascal apparatus in most species stain blue, dark blue (amyloid) or occasionally reddish brown when pre-treated with Melzer's iodine reagentor potassium hydroxide solution after pretreatment with KOH (Eriksson, 1966; Kohn andKorf, 1975;Nannfeldt 1976). Members of Xylareaceaus fungi have characteristic featureof the ascospore; *Hypoxylon* and *Daldinia* have usually inequilaterally ellipsoid, *Biscogniauxia* have more frequently subglobose, *Xylaria* are often broadly crescent-shaped, while in *Rosellinia* are typified by long attenuate ends (Petrini 1992).

Generally, the distribution of Xylariaceous fungi is affected by temperature as was evidenced in Thailand and Philippines where the fungi exhibit high diversity due to the tropical climate (Okaneet al. 2008;Osonoet al. 2009; Seehananand Petcharat, 2011;Vasilyeva et al. 2012). *In-vitro* studies have reported that growth temperature of the fungus range been 9ºC to 30ºC with optimum growth at 25ºC (Onsando, 1985). In Kenya, the disease was first reported in Kericho, Nandi and Limuru tea farms and the prevalence increases with the age of the tea plant (Onsando, 1985). The disease manifests as gradual rot of branches that form the tea frame, beginning from the pruning cuts on the primary branches, resulting in sectorial death which ultimately culminate in the death of the whole bush (Otieno, 1997). On dead host wood tissue it is recognized as fructification of dark grey or black irregular and raised patches (Venkata Ram, 1970). The transmission agents of the pathogen propagules (ascospores) onto the exposed wood (resulting from pruning cuts or damaged bark), include windborne ascospores, windblown rain splashes and rain drop splashes (Otieno, 1997). There is limited literature on the diversity of *Hypoxylon* fungus in tea growing regions and its disease prevalence. In this study, morphological characteristics of vegetative (asexual) and stromata (sexual) states of *Hypoxylon* fungus prevalence in different tea growing regions (counties), genotypes and altitude were assessed.

MATERIALS AND METHODS

Wood Rot Specimens Collection

Due to asymptomatic inversion of living host tissue, Xylariaceae are collected from dead wood of angiosperms (Petrini and Petrini, 1985). The wood sampling was accompanied by the assessment of disease incidence in different tea growing counties. Naturally infected tea wood bearing the fruiting body was sampled from both East (Kirinyaga, Nyeri, Kiambu, and Meru North sampled in August 2015) and West (Nyamira, Nandi and Kericho sampled in October 2015) of Rift Valley. However, disease incidence for Kericho was not assessed because the pruned tea fields had fully recovered hindering satisfactory examination of the tea frame. In fields where the disease incidence was high, random representative plants were taken as compared to low disease incidence field where all infected plants were sampled. The samples were put in a brown khaki paper bag, labeled accordingly and transported to the laboratory where they were air-dried and stored at room temperature.

Fungal Isolation and Vegetative Comparisons

The fungi were isolated following a modified method used by Kenerley and Rogers (1976) which involves taking a colony and maintaining iton potato dextrose agar (PDA). In total, 65 samples were used with 58 new isolates and 7 stock cultures previously collected from Kirinyaga County. All the cultures were coded with respect to the county and location from which they were collected. These were used to study vegetative growth rates and cultural morphological comparisons on PDA. The cultures were made by transferring 2 mm x2 mm block of inoculum taken from periphery of seven days old cultures on PDA. Three replicates of each isolate were inoculated, and incubated at room temperature on a laboratory bench. Radial growth of fungi isolates were measure in millimeter at two day interval for 10 days, and the data subjected to ANOVA using SAS 9.1 statistical software. Cultural morphology were compared based on visual similarities/differences on the tenth day.

The fungal stromata were analyzed for extractable pigments (chemotaxonomy) using 10% potassium hydroxide (KOH) in three replications (Kohn and Korf, 1975;Nannfedt, 1976). The morphology of the fruiting bodies (stromatal surface and vertical section) was examined under a dissecting microscope. Squash preparations from the stromata for ascii and ascospores measurements was done using distilled water. Apical ascal apparatus was examined using (Kohn and Korf, 1975; Nannfedt, 1976) methods.

Assessment of Hypoxylon Wood Rot Disease Incidence

Disease incidence was assessed in triplicateby randomly selecting a row of tea plants within a pruned field of tea. In each row 25 tea busheswere cleaned by removing litter to allow thorough examination of the entire tea frame. The tea bushes were successively assessed for the symptoms of the wood rot disease. A dead tea plant with symptoms of the disease was regarded that the death was due to the wood rot.The genotype of tea in each site was noted as mono-clonal, mixed clonal or seedling tea, and respective altitudes were taken.The disease incidence was recorded as 1 to indicate presence and 0 for absence of the disease and expressed as (Otieno 2003):

$$\text{Disease incidence} = \frac{\text{Total number of plants with disease}}{\text{Total number of plants observed}} \times 100$$

The calculated disease incidence in different regions were subjected to ANOVA analysis using SAS statistical software and correlated with the altitude.

RESULTS

Vegetative Growth of Hypoxylon Fungi

The radial growth of the different isolates from different location varied significantly ($P \leq 0.05$) throughout the period of growth. At the 10^{th}, day growth ranged from 24.0 to 57.0mm diameter (Figure 1). Isolate NAND30, had the lowest radial growth (24.0mm) while both isolate KERC5 and NAND13 had the highest (57.0mm) (Figure 1). Colony growth and visual colony comparison did not show any trend nor associate to the geographical locations (Figure 1 and 2).

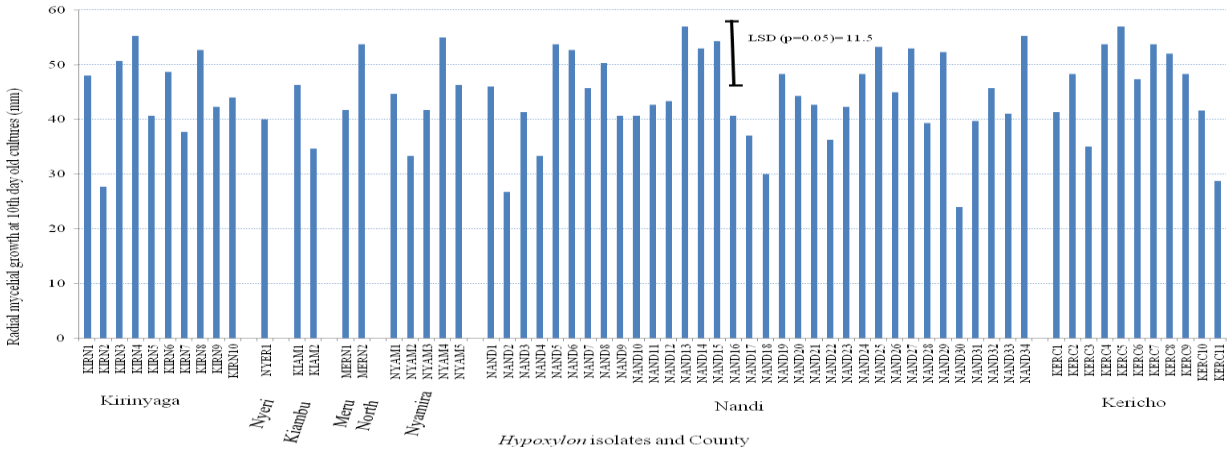


Figure 1. Radial mycelia growth of 10 days old cultures of different *Hypoxylon* isolates grouped based ontea growing counties. (*Bar:* LSD between any two mean radial mycelia growth)

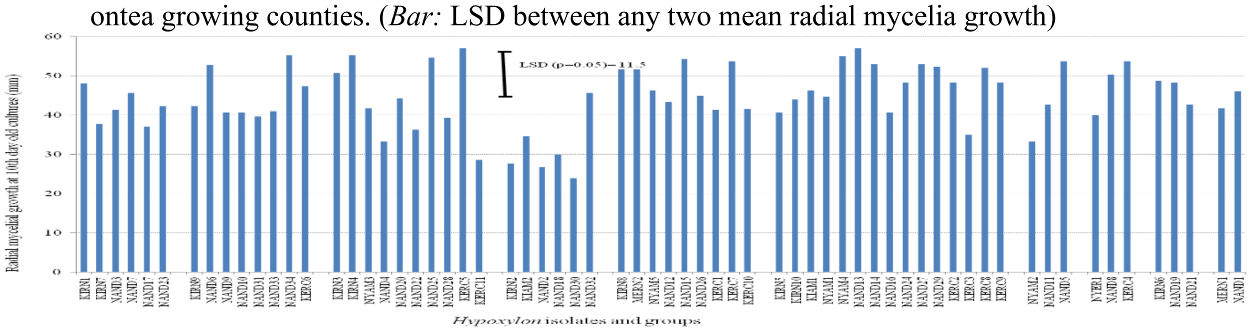


Figure 2. Radial mycelia growth of different *Hypoxylon* isolates grouped based on cultural characteristics (nature of mycelia, colony form, margins, zonated, wriggled pigmented exudates and colony size. (*Insert:* LSD bar between any two mean radial mycelia growth)

Colonies were generally white throughout the growth period except isolate MERN1 and NAND32 which had light brown-orange centers at the end of the growth period. Other differences visible included colony texture, size, shape, elevation and zoning in concentric ring/rings. Using these characteristics, the isolates were grouped in 10 groups (Plate1).

Fruiting Body Analysis

All stomatal surfaces were made up of dark-grey to black carbonaceous matter with conspicuous ostioles opening above the general surface of stromatal. Three main stomata forms were observed with corresponding shapes of perithesia (Plate 2). All were effuses-puvinate with different surface topology with respective shapes of the perithesia borne. Six wood samples had obovois shaped perithesia,45 samples had obovoid to spherical shaped perithesia and 7 had obovoidand smallperithesia (Plate 2). Ascal apparatus when treated with iodine solution gave amyloid reaction (bluing of ascal apparatus) (Plate 3).

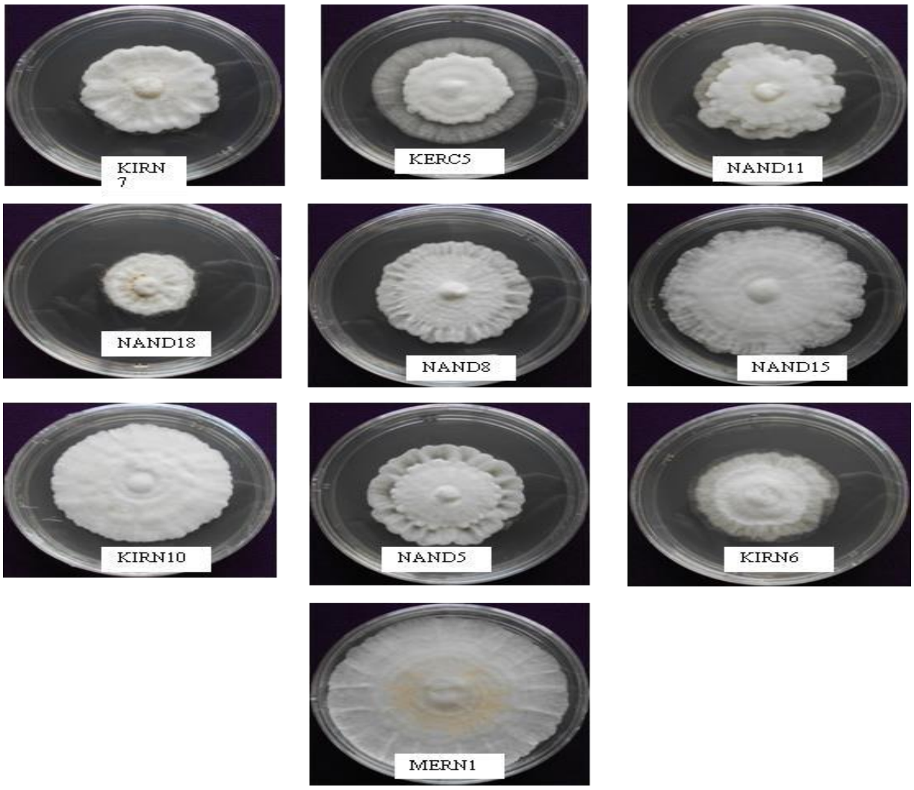
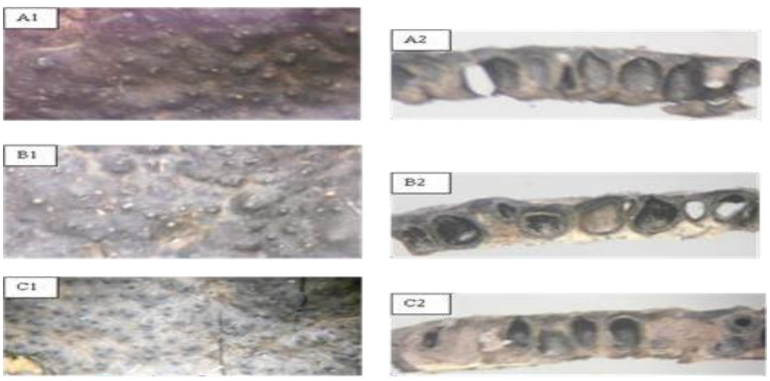


Plate 1. Representative isolates of *Hypoxylon*fungi on PDA, from the ten groups. (Note the Compact mycelia, raised central plateau-, wavy or sooth margins, wriggled centre (NAND18), high convex colony (KIRN10) flat, pigmented center with exudates, varying colony size).

2. 

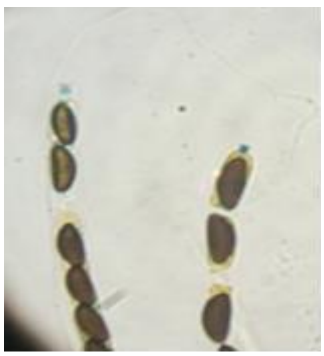
3. 

Plate 2. Different stromatal forms of Hypoxylon species in tea; A1and2- Closs-up of effused-pulvinatestromatal surface with obovoid shaped perithesia vertical section; B1and2- Closs-up of effused-pulvinatestromatal surface with obovoid to spherical shaped perithesia and B1and2- Closs-up of effused-pulvinatestromatal surface with small obovoid shaped perithesia

Plate 3. Amyloid ascal apparatus at the tips, stained blue with iodine solution

On treatment with 10% KOH, all stromata produced brown/amber extractable pigmentsm (Plate 4).

Plate 4. Chemotaxonomy test of the *Hypoxylon* fruit body 10% potassium hydroxide (KOH) showing the release of extractable brown/amber pigment

Hypoxylon Wood Rot Disease Incidence

Generally, Hypoxylon wood rot disease of tea varied significantly ($p \leq 0.05$) in the different tea growing countiesand locations (Table 1).Clonal teas andmixed clones had significantly ($p \leq 0.05$) higher disease incidence than seedling teas. The disease incidence was4.0%and81.30%in seedling andclonal teas,respectively (Table 1). The relationship between the disease incidence and altitude increased with altitude however the relationship was not significant.

Table 1: Hypoxylon wood rot incidence in different tea growing counties

County and Location	Altitude (M)	Tea Genotype	Incidence of *Hypoxylon* wood rot (%)
Kirinyaga Loc. A	2231	Mono-clonal	81.0 a
Loc. B	2047	Mixed clones	74.0 a
Nyeri	1969	Seedling	8.3 f
Meru North	1848	Seedling	6.7 f
Kiambu	2241	Seedling	16.7 def
Nyamira Loc. A	1658	Seedling	4.0 f
Loc. B	1794	Seedling	30.7 bcd
Nandi Loc. A	2134	Seedling	46.3 b
Loc. A	2197	Seedling	7.7 f
Loc. B	2087	Seedling	29.3 cde
Loc. B	2171	Seedling	41.7 bc
Loc. C	2117	Seedling	14.3 ef
C.V. (%)			21.8
LSD (p=0.05)			16.2

DISCUSSION

Hypoxylon fungi may affect an individual tea bush or a group of adjacent bushes as is often the case for susceptible hosts. The affected primary branch breaks off reducingthe tea frameeventually affecting productivity.Wood rot disease is characteristic with typical irregularly shaped, dark-grey to black superficial and raised structures on the dead (rotten) host wood (Otieno, 1993). *Hypoxylon*wood rot disease most frequently start from the pruning cut, at the upper part of the tea frame, and progress downwards to the primary branches if not controlled. This nonetheless often extends to the collar region killing the entire bush. The disease is further reported to be influenced by nitrogen and potassium nutrition in tea. In a study by Otieno (2003), the disease development was slower where the potassium was applied as straight fertilizer at different time from the application of nitrogenous fertilizer and increased with increase in nitrogen. The plant genotype also influences the severity of the disease as realized in this study (Table 1). During the assessment and collection of the samples, one of the tea fields with long history persistent low potassium (K), had high disease incidence.

A wide range of cultural variations in radial growth rate, colony texture, shape exudation and elevationwas observed. All fungal growth were initially white with few isolates showing yellowish brown pigmentation. Previous studies have reporteddistinct variation in growth parameters among different species of *Xylariahypoxylon*, a fungus related to the *Hypoxylon* spp. (Chacko and Rogers, 1981). More distinctive morphological characteristics of anamorphic (asexual) states are observed under high resolutionmicroscopy (i.e. diffractive interference-DIF and scanning electron microscopy – SEM). In Europe, species of*Hypoxylon*werefirst described based on growth rate, colour, texture, zonation,margin forms,and anamorphic structures like conidiophores and conidia (Hsichet al. 2005; Faurnier, 2010; Hai-Xia et al. 2012). In addition, detailed characterization of fruiting body has been used extensively to distinguish *H.* species (Faurnier, 2010, Hai-Xia, et al. 2012). With advances in technology, molecular tools such asrDNA, β-tubulin and α-actin sequencingareused to identify new collections of *Hypoxylon* spp. (Mazzagliaet al., 2001, Hsichet al. 2005, Suwannasaiet al. 2013).

The fruit body of the *Hypoxylon* fungi in tea showed less diversity on its surface (topology) morphology and form (Plate 2). Generally, all stromata were effused-pulpinate, with either obovoid, obovoid to spherical and ovoid perithecia, bearing amylodascii (Plate 2 and 3. All fruiting bodies released brown/amber extractable pigment with 10% KOH. The later is a reliable characteristic for separation when other related species of *Hypoxylon*are involved (Ju and Rogers, 1996). The ascal apparatus were amyloid when treated with iodine solution test and dark brown ellipsoid in-equilateral ascospores.Faurnier (2010), Hai-Xia (2012) carried out detailed morphological characterization of fruit body to distinguish new species of *Hypoxylon* collected (Faurnier, 2010, Hai-Xia, et al. 2012).

Hypoxylon wood rot disease incidence varied significantly ($P \leq 0.05$) in the different tea growing counties. However, the incidencewas found prevalent tomonoclonal andmixed clonalteas (81 and 74%, respectively) as compared to the seedling teas (ranging between 4.0 to 46.3%) (Table 1). It was also found to be more common in higher altitude than in lower altitude. Similarly, *in vitro* studies by Onsando (1985) reported that the fungus has a wide growth temperatures range(9°C to 30.5°C) with 25°C being optimum.

Generally, temperature is one of the most important factors that influence disease occurrence and the development of most plant disease because it can affect either the host or the pathogen itself.In the present study, the weak relationship between the fungus and the altitude for the different tea growing regions, imply that the fungus is well adapted to the different Agro-ecological zones where tea is grown. This indicate that the host(tea genotype) is keyin disease severity. However, adequate nutritional regimes can also be used to minimize the disease severity particularly in field with low incidence (Otieno, 2003).

RECOMMENDATION

Since Hypoxylon wood rot is a disease that occurs in older tea, above 15 to 20 years old from planting, it is vital to have a clonal screening and selection criteria to avoid unwarranted loss of plants that may result due to the pathogen inversion. In this study, it was found that the disease incidence is influenced mainly by the teagenotype. Therefore, aselection criteria for tea needs to be sought for screening of tea clones before release and probably breed for resistance to the disease.

CONCLUSION

Based on cultural characteristics,the *Hypoxylon* isolates are diverse which should be confirmed using alternative techniques. Fruit body chemotaxonoxy confirm that the wood rot pathogen in tea is by the *Hypoxylon* species. The disease incidence is mainly influenced by the host genotype.

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