

Effect of Selected Microenvironmental Factors on Colony Growth and Cross Infectional Ability of Fungal Pathogens Causing Root Rot Disease of Jak (*Artocarpus heterophyllus*)

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ABSTRACT: Root diseases have become an emerging biotic threat to Jak (*A. heterophyllus*), grown in Sri Lanka. Isolation of the causal agents, identification and determination of the effect of selected microenvironmental factors and the cross-infection ability of the causal agents on other perennial crops towards development of effective management measures were the objectives of the present study. Two *Rigidoporous* microspores isolates and a *Fusarium oxysporum* and a *F. solani* isolate were identified as causal agents of root rot disease of Jak. Colony growth of the two *R. microsporus* isolates and the *Fusarium* isolates responded differently to pH, temperature and light intensity. Differences of colony growth responses were identified even between the two isolates of *R. microsporus*. Among the perennial tree species used, *Artocarpus nobilis* was highly susceptible to all four fungal isolates and the lowest infection ability was shown on *Persea americana*. *Artocarpus altilis* (Rata Del), *Hevea brasiliensis* (Rubber), *Cinnamomum zeylanicum* (Cinnamon), *Durio zibethinus* (Durian), *Psidium guajava* (Guvava) and *Nephellium lapaceum* (Rambutan) were equally-susceptible to the infection by the four fungal isolates, used. Findings of the study are informative when developing an integrated management programme against root diseases of Jak.

Keywords: Colony growth response, *Fusarium oxysporum*, *Fusarium solani*, *Rigidoporus microsporus*

INTRODUCTION

Jak (*Artocarpus heterophyllus*), belonging to Family Moraceae, is an economically versatile tropical tree crop, providing food, timber, fuel, fodder and medicinal and industrial products. Despite the multi-purpose advantages of Jak tree, it is an underutilized crop in most of the Asian countries (Nair *et al.*, 2018), and Sri Lanka is not an exception. In Sri Lanka, the tree is grown as a home garden crop along with

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other perennial trees or naturally exists in forests. Although the economic value of the crop has been well-recognized, very limited research has been done on different aspects of the crop including management of diseases. From the recent past, wilting, gradual decline and the death of the tree are frequent complaints made by owners of Jak trees in Sri Lanka. Field observations based on symptomatology have revealed that such trees are infected with root diseases. However, causal agents of the root diseases of Jak and management measures, effective against the causal agents have not been investigated in detail.

Decline of Jak tree has been reported in Vietnam and Philippines and *Phytophthora palmivora* has been identified as the causal organism (Tri et al., 2015; Borines et al., 2014), where chlorosis, wilting, defoliation, trunk cankers and tree death are the symptoms. *Artocarpus nobilis*, an endemic tree grown in homesteads of Sri Lanka, shows leaf discoloration, leaf fall and die back of affected shoots along with the formation of rhizomorphs on roots and on the collar region (Madushani et al., 2013). The disease has been identified as white root disease caused by *Rigidoporus microporus*, the causal agent of the same disease of rubber (*Hevea brasiliensis*). Limited literature available on Jak root diseases shows that taxonomically-diverse fungi (e.g. Basidiomycota, Oomycota, Ascomycota) are responsible for the root diseases of Jak and taxonomically-related tree species. Hence, management measures have to be developed strategically and holistically to solve the issues of Jak diseases in Sri Lanka.

The objectives of the present study were to identify causal agents of root diseases of Jak and determine the effect of selected microenvironmental factors on growth of the causal agents along with its cross infectional ability on several other perennial trees. Findings of the present study can be used to develop measures effective of integrated management of root diseases of Jak.

MATERIALS AND METHODS

Identification of Fungal Pathogens

Root pieces excised from root rot infected Jak trees were wrapped in moist newspapers till rhizomorphs were developed. Sections of rhizomorphs were transferred aseptically to PDA plates and cultures were purified by dilution plate technique. Fungi isolated onto PDA were purified by subculturing and dilution plate technique. Well separated fungal colonies were cultured for seven days and subjected to genomic DNA extraction. Briefly, a mycelial mass from an area of 1 cm² was mixed with 500 µl of SDS extraction buffer (Arnold and Lutzone, 2007) and ground thoroughly with a sterile pipette tip. An aliquot of 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added and mixed gently by inverting. The mixture was centrifuged at 12000 rpm for 15min and DNA was precipitated with ice-cold isopropanol and centrifuged at 12000 rpm for 3 min. Pellet was washed with 70% ethanol at 12000

rpm for 3 min. The supernatant was discarded and the air-dried DNA pellet was mixed with 25 µl of ultrapure water.

PCR amplification, DNA sequencing and Homology search

Genomic fungal DNA (50 ng) was used for PCR amplification with ITS1 (5'TCC GTA GGT GAA CCT GCG G3') and ITS4 (5'TCC TCC GCT TAT TGA TAT GC3') primers (White *et al.*, 1990) and the PCR conditions were programmed for 35 cycles, each cycle having a denaturation step at 94°C for 30 sec, annealing at 49°C for 30 sec and extension at 72 °C for 1 min and a final elongation step at 72 °C for 5 min. PCR products were subjected to gel electrophoresis. Then they were subjected to DNA sequencing (Macrogen, Korea) and homology search were done (BLAST, NCBI). 492 bp and 500 bp band size were shown in two isolates of *Rigidoporus microporus* 550 bp and 570bp (8F and 9T) were shown in *F.solani* and *F.oxysporum* (K3 and B1) respectively.

Confirmation of Pathogenicity

After identification of the purified fungal isolates (*Rigidoporus microporus*, *Fusarium solani* and *F. oxysporum*) by molecular methods, pathogenicity was confirmed according to standard Koch's postulates. Pathogenicity of *Rigidoporus* isolates was confirmed according to the method followed by Fernando *et al.* (2012). Briefly, autoclaved mature Jak root pieces (10 cm in length) were inoculated with *Rigidoporus* isolates, separately and the root pieces were kept at room temperature in a humid chamber till the development of rhizomorphs. Thereafter, the root pieces were buried in sterilized soil containing plastic containers at a depth of 5 cm. Three months old Jak seedlings, exposed to water deficit conditions for 24 hr were planted in the pots containing the *Rigidoporus*-inoculated root pieces. Collar region of three months old seedlings were rubbed by a sand paper and 0.7cm mycelia discs of both *Fusarium* species were placed on partially wounded area of collar region with moistened cotton wool. Seedlings were kept in a plant propagator for one month. Each fungal pathogen isolate was replicated with 10 seedlings. After observing the wilt symptoms in Jak seedlings, root pieces of the seedlings were used for isolation of the pathogen onto PDA medium. Fungal colonies re-isolated from the infected-Jak seedlings were subjected to PCR using ITS1 and ITS4 primers. PCR products were sent for DNA sequencing (Macrogen, Korea) and DNA homology search was done for confirmation of the identity of the re-isolated fungi.

Effect of Temperature, pH and Light Intensity on Colony Growth of Fungal Isolates

Among the identified fungal isolates, two *R. microporus* isolates and *F. oxysporum* and *F.solani* isolates were used to determine the effect of several selected micro-environmental parameters (*i.e.* temperature, pH and light intensity) on the colony growth of fungi. Initially, a suitable medium for culturing all above four fungal isolates was selected using potato dextrose agar (PDA), Lima bean agar (LBA) and

malt extract agar (MEA) as an observational study. Based on colony growth rate, LBA was determined to be the most suitable medium for culturing the four fungal isolates *in vitro*. To determine the effect of pH, LBA was adjusted from pH 4 – 11 using diluted NaOH or HCl and eight separate experiments were conducted for the four fungal isolates to check the effect of each pH level with 10 replicates per isolate. Temperature effect was determined by exposing the fungal isolates grown on LBA to 15 °C, 20 °C, 25 °C and 30 °C using a temperature controlled incubator. Separate experiments were conducted to check each temperature effect using 10 replicates per fungal isolate. Fungal cultures were exposed to three light intensities (normal light conditions (0.6 lux), diffused light conditions (0.05 lux) and dark conditions (0 lux) as three separate experiments with 10 replicates per each isolate. In all experiments, treatments were arranged according to a complete randomized design. Colony growth was measured at two day intervals.

Determination of the Cross Infection Ability of Fungal Pathogens of Jak

Artocarpus nobilis (Wal Del), *Artocarpus altilis* (Rata Del), *Hevea brasiliensis* (Rubber), *Cinnamomum zeylanicum* (Cinnamon), *Durio zibethinus* (Durian), *Psidium guajava* (Guvava), *Nephellium lappaceum* (Rambutan) and *Persea americana* (Avocado) were selected as the host crops to check the cross infection ability of the four fungal isolates. Root pieces (approximately 15cm in length and 5cm of diameter) from above tree species were surfaced sterilized with teepol, following 20% sodium hypochloride aqueous solution and rinsed with running tap water for 30 min. Then the root pieces were aseptically inoculated with 1cm discs of each fungal isolate. Separate experiments were carried out according to a complete randomized design with 5 replicates for each host species. These artificially-inoculated root pieces were placed in a humid chamber for a period of one week and the lesion length was measured at two day intervals. From inoculated root pieces of each host plant, pathogen was re-isolated. Confirmation of *F. solani* and *F. oxysporum* cultures were done based on colony and spore morphology. Colonies of the fungal isolates were subjected to DNA extraction, PCR amplification, DNA sequencing and homology search for confirmation of identity.

RESULTS

Identification of Fungal Pathogens

Four purified fungal cultures which showed distinct colony morphology were identified by molecular methods and the details are given in Table 1.

Table 1. Identity of fungal isolates based on DNA homology search.

Code of fungal isolate	Description of the best homologue	Query cover (%)	E-value	Identity (%)	Accession number
R1	<i>Rigidoporus microporus</i> SEG	88	0	99	MG199553.1
R8	<i>Rigidoporus microporus</i> PM52	30	0	99	KX090082.1
B1	<i>Fusarium oxysporum</i> F102	85	0	95	KJ512160.1
K3	<i>Fusarium solani</i> 21019	28	0	97	KY348699.1

Isolate R1, can be identified as *R. microporus*, as it gave the best match with *R. microporus* SEG isolate with a 88% query cover, 0.00 E-value and 99% identity (Table 1). Similarly, isolate B1 can be identified as *Fusarium oxysporum* as it gave 85% query cover, 0.00 E-value and 95% identity with *F. oxysporum* F102 isolate. Though, isolate K3 gave a 28% query cover with a 0.00 E-value and a 97% identity, spore morphology clearly showed the presence of macro and micro conidia unique to *Fusarium* spp. (Figure 1), hence the isolate K3 can be considered as *F. solani*. R8 isolate gave the best match with *R. microporus*, but with a 0.00 E-value and a 99% identity, but with a 30% query cover.

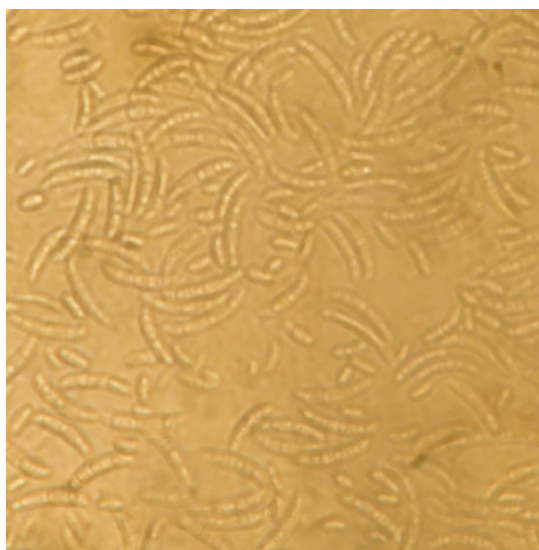


Figure 1. Macro (sickle-shaped conidia) and micro (small and oval shaped) conidia of fungal isolate K3 (magnification x 400).

Confirmation of Pathogenicity

Table 2. Percentage of Jak seedlings showed symptoms of above ground parts one month after inoculation of the four fungal isolates.

Treatment	<i>rhizomorphs</i> on root surface	Symptom observed	Symptoms in above ground parts
Control	0	No symptoms in above ground parts	0
Isolate R1	100	Wilting and discoloured roots when split opened	1
Isolate R8	100	Wilting and discoloured roots when split opened	1
Isolate B1	-	Leaf yellowing and discoloration of collar region in longitudinal section of stem, Penetration of lesion in to outer layer of the seedlings, discoloured roots when split opened	2
K3	-	Leaf yellowing and discoloration of collar region in longitudinal section of stem, penetration of lesion in to outer layer of the seedlings	2

Rhizomorphs could be observed in the roots of *Rigidoporous*-inoculated seedlings. Above ground symptom development was very slow in Jak seedlings, even after one-month period.

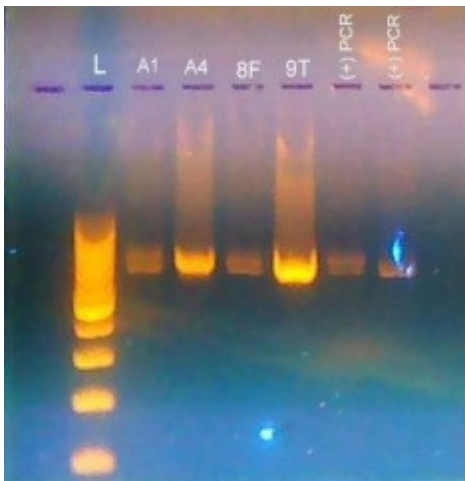


Plate 1. Visualization of PCR products of *Rigidoporus* isolates performed on 2% agarose gel. L= 100bp ladder, 8F and 9T= *Rigidoporus microporus* isolated from jack trees, A1- *Rigidoporus microporus* isolated from *H. brasiliensis*, A4- *Rigidoporus microporus* isolated from *C. zeylanicum* (+)PCR= positive control.

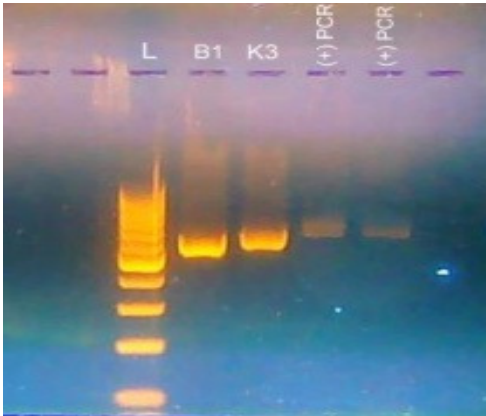


Plate 2. Visualization of PCR products of *Fusarium* isolates performed on 2% agarose gel. Notes: L= 100bp ladder, B1= *Fusarium oxysporum*, K3 = *Fusarium solani*, (+)PCR= positive control.

Effect of pH, Temperature and Light Intensity on Growth of Fungal Isolates

pH 6 has resulted in the highest colony diameter for *Rigidoporus microporus* both isolates and *Fusarium oxysporum* isolate. *F. solani* isolate has grown best when the pH of the medium was 4.0. All four fungal isolates showed the best colony growth at normal light and diffused light conditions, and there was no significant difference on the colony diameter between the two light intensity levels (Table 4). However, *R. microporus* SEG1 isolate showed equal colony growth under all light intensities (even under dark conditions).

Table 3. Effect of the pH of culture medium on colony growth of *Rigidoporus* and *Fusarium* isolates.

pH	Mean colony diameter (cm)			
	<i>R.microporus</i> SEG1	<i>R.microporus</i> PM52	<i>F. solani</i>	<i>F.oxysporum</i>
pH 4	4.22 ^{ba}	4.52 ^b	4.48 ^a	4.27 ^b
pH 5	4.4 ^{ba}	4.37 ^b	4.04 ^b	5.38 ^a
pH 6	4.58 ^a	4.77 ^a	4.02 ^b	5.5 ^a
pH 7	3.17 ^b	3.32 ^d	3.31 ^c	3.7 ^c
pH 8	3.27 ^b	3.41 ^d	3.2 ^c	3.94 ^c
pH 9	2.84 ^c	3.71 ^c	3.37 ^c	3.55 ^c
pH 10	3.22 ^b	3.73 ^c	3.16 ^d	2.84 ^d
pH 11	2.08 ^e	2.51 ^e	2.54 ^e	1.39 ^e
CV%	2.03	2.03	2.03	2.03
LSD	1.78	0.24	0.88	0.31

Means with the same letter along the column are not significantly different at P=0.05

Table 4. The effect of different light intensities on colony growth of *R. microporus* isolates and *Fusarium* isolates.

Light intensity (lux)	Mean colony diameter (cm)			
	<i>R. microporus</i> SEG1	<i>R. microporus</i> P M52	<i>F. solani</i>	<i>F. oxysporum</i>
Normal light (0.6)	4.97 ^a	5.77 ^a	4.92 ^a	5.63 ^a
Diffuse light (0.05)	4.9 ^a	5.63 ^a	4.87 ^a	5.43 ^a
Fully dark condition (0)	4.78 ^a	5.01 ^b	4.36 ^b	4.82 ^b
CV%	2.17	2.17	2.17	2.17
LSD	0.16	0.4	0.33	0.42

Means with the same letter along the column are not significantly different at $P = 0.05$

The two *R. microporus* isolates showed the highest colony growth at 25 °C and the *Fusarium* isolates had the best growth at 30 °C (Table 5) six days after incubation. In general, all four fungal isolates had the highest colony growth between 25 – 30 °C. When the temperature increased to 35 °C, all isolates except *Fusarium oxysporum* showed a drastic reduction of the colony growth.

Table 5. The effect of different temperature on colony growth of *R. microporus* and *Fusarium* isolates

Temperature (°C)	Mean colony diameter (cm)			
	<i>R. microporus</i> SEG1	<i>R. microporus</i> PM52	<i>F. solani</i>	<i>F. oxysporum</i>
15	1.54 ^d	1.18 ^c	1.59 ^e	0.7 ^c
20	3.31 ^c	3.34 ^b	2.97 ^c	1.3 ^c
25	4.73 ^a	4.53 ^a	4.13 ^b	3.96 ^{ba}
30	4.02 ^b	4.38 ^a	4.63 ^a	4.18 ^a
35	1.69 ^d	1.39 ^c	2.2 ^d	3.36 ^b
CV%	2.08	2.08	2.08	2.08
LSD	0.42	0.57	0.38	0.79

Means with the same letter along the column are not significantly different at $P=0.05$ *Rigidoporus* isolates showed the best colony growth at 25 °C and *Fusarium* isolates were best grown at 30 °C. Lower temperatures like 15 °C and higher temperatures as 35 °C were not favourable for the growth of both *R. microporus* and *F. solani* isolates.

Determination of Cross Infection Ability of The Fungal Pathogens Isolated from Jak

Lesion length appeared along the inoculated root pieces of different host plants by the four different fungal isolates are given in Table 6. When the root pieces were split longitudinally, discolored area was observed and it was measured as the lesion length.

Table 6. Lesion length shown by *R.microporus* isolates and *Fusarium* isolates on root pieces of different host plants, five days after inoculation.

Host plant	Mean length of the lesion (cm)			
	<i>R.microporus</i> EG1	<i>R.microporus</i> PM52	<i>F. solani</i>	<i>F.oxysporum</i>
<i>Artocarpus nobilis</i>	4.42 ^a	5.22 ^a	5.28 ^a	4.2 ^a
<i>Artocarpus altilis</i>	3.42 ^b	3.64 ^b	3.54 ^b	3.68 ^{ba}
<i>Hevea brasiliensis</i>	3.46 ^b	3.86 ^b	3.96 ^b	3.34 ^b
<i>Cinnamomum zeylanicum</i>	3.38 ^b	3.62 ^b	3.7 ^b	3.3 ^b
<i>Durio zibethinus</i>	3.51 ^b	3.68 ^b	2.94 ^{cb}	4.14 ^a
<i>Psidium guajava</i>	2.76 ^{cb}	2.58 ^{cd}	3.67 ^b	3.16 ^b
<i>Nephellium lapaecum</i>	2.56 ^c	2.02 ^{ed}	2.36 ^{cd}	3.54 ^{ba}
<i>Persea americana</i>	1.48 ^d	1.32 ^e	1.64 ^d	1.87 ^c
CV%	2.03	2.03	2.03	2.03
LSD	0.73	0.93	1.02	0.83

Means with the same letter along the column are not significantly different at P=0.05

A.nobilis (Wal del) showed the highest mycelia growth of all four isolates, indicating it as a highly susceptible host for all the fungal pathogen isolates tested. In addition, *D.zibethinus* was highly infectious for *F.oxysporum* (Table 6). In comparison to *A. nobilis*, *A. altilis*, *H.brasiliensis* and *C. zeylanicum* are less susceptible to all four fungal pathogen isolates. Among all the tested host plants, *P.americana* was the least susceptible to all four fungal isolates. Except for *F.oxysporum*, *N.lapaceum* showed less susceptibility to *R. microporus* isolates and *F. solani*.

DISCUSSION

Present study identified four soil borne fungal pathogens (*i.e.* two isolates of *R. microporus*, *F. solani* and *F. oxysporum*) in root rot-infected Jak trees and determined the effect of selected microenvironmental factors on the colony growth of the pathogens and their cross inflectional ability on several other perennial fruit trees. *R. microporus* has been reported as the white root disease causal agent of a wide range of perennial crops, namely *H. brasiliensis*, *A. nobilis*, *Mucuna bracteata*, *Camellia sinensis*, *Cinnamomum zeylanicum*, *Alstonia macrophylla*, *Murraya koenigii*, Coconut, Jak, Mango, Cashew nuts, Carambola, Avocado, Cassava, Cocoa, Yams, Weeping willows, Teak, *Ficus religiosa* and *Mesua ferrea* (Madushani *et al.*, 2013; Madushani *et al.*, 2014; Fernando *et al.*, 2012; Fernando *et al.*, 2016). However, involvement of *F. solani* and *F. oxysporum* as the pathogens of root rot diseases of Jak has not been reported earlier. Further, the present study revealed the presence of *R. microporus* isolates with genetic variations. Isolate SEGI gave the homology to a Malaysian *R. microporus* isolate with a 88 % query cover, 0.00 E-value and 99 % identity. The other isolate, has shown 0.00 E-value and a 99 % identity to a Thailand

isolate (PM52), but with a 30 % query cover. Oghenekaro *et al.* (2014) have analyzed molecular variation of *R. microporus* isolates using ITS, LSU, β -tubulin and translation elongation factor 1- α (tef1) gene sequences and identified three distinctive clades corresponding to isolates from Africa, Asia and South/Central America. Therefore, Oghenekaro *et al.* (2014) suggest the possibility of having at least three different species of *Rigidoporus* causing white root disease in rubber. Kumari *et al.* (2014) have determined genetic variation, through RAPD-PCR using 15 random primers using 11 *R. microporus* isolates infecting rubber which were collected from different locations of Sri Lanka. Molecular cluster analysis has identified two distinct clusters with many sub clusters, though there was no correlation between the 11 isolates and their geographic origin (Kumari *et al.*, 2014). Based on the above background, further investigations at molecular level are needed to confirm the identity at species level of isolate R8 used in this study. Growth and differentiation of *Rigidoporus* depend on suitable combination of pH of the culture medium and the nature and the nature of nitrogen and carbon source (Richard and Botton, 1996). Liyanage *et al.* (1977) reported the effect of pH, temperature and light intensity of several *R. lignosus* (*syn. of. R. microporus*) collected from white root-infected rubber trees from different locations. According to Liyanage *et al.* (1977), the optimum temperature for the maximum growth of all 11 isolates has been reported as 30 °C. However, the *R. microporus* isolates obtained from Jak trees showed a different scenario, where the PM52 isolate had the highest colony growth at 25 and 30 °C and SEG1 isolate had the highest colony growth rate at 25 °C. Therefore, the optimum temperature of all *R. microporus* isolates cannot be generalized. It can vary among the isolates obtained from different hosts as well as among isolates obtained from same host.

In the present study the two *R. microporus* isolates from Jak showed different growth responses to pH. SEG1 isolate had the best growth at a range of pH 4 – 8 and PM52 isolate was best grown at pH 6. In contrast to the findings of the present study Liyanage *et al.* (1977) have reported, pH 9 as the best for growth of 11 *R. microporus* isolates, However, one isolate could tolerate low pH levels. With reference to the effect of light intensity Liyanage *et al.* (1977) has reported that *R. microporus* isolates from rubber had a better growth under dark. However, the two *R. microporus* isolates of Jak, used in the present study behaved differently, where SEG1 grew equally well at all light intensities (dark, normal and diffused) but PM52 grew best under normal and diffused light. According to Fayzalla *et al.* (2008), an isolate of *F. oxysporum* f.sp. *lycopersici* and *F. solani* causing wilt and root rot of solanaceous crops have grown best under continuous light, though one isolate of *F. oxysporum* f. sp. *lycopersici* induced growth under continuous darkness. In contrast to the *Fusarium* isolates used in our study, which had the best colony growth around pH 4-6, *F. oxysporum* and *F. solani* causing wilt and root rot of solanaceous crops have grown best at pH 8-9. Effect of pH on sporulation, production of macro and micro conidia and chlamydospores of *Fusarium oxysporum* has been reported by Tyagi and Paudel (2014). Findings of the study revealed valuable information on cross-infectious ability of the four fungal pathogens on perennial trees. All four fungal isolates of Jak are highly infectious on *A. nobilis* and least infectious on *P.americana*. The rest of the tested perennial trees

are equally susceptible to the four fungal isolates of Jak. In addition to that cultures obtained from symptomatic roots of rubber and cinnamon were confirmed as *Rigidoporus microporus* in gel plate 1. Previous studies by Madushani *et al.* (2013) have shown that *R. microporus* isolates obtained from several trees such as *Mucuna bracteata*, *Camellia sinensis*, *Cinnamomum zeylanicum*, *Artocarpus nobilis*, *Alstonia macrophylla* are infectious to rubber. Therefore, cultivating the perennial trees used in the study, along with Jak poses a threat and leaving the stumps of those trees provide inocula for Jak. Based on this study *Artocarpus nobilis*, had a high cross infection rate with white root pathogen in Jak and moderate infection rate with *Artocarpus altilis*, *Cinnamom zeylanicum* and *Durio zibethinus*. Therefore it should not to be advised to cultivate Jak with stumps remaining soils of white root infectious trees of bread fruit, rubber, cinnamon and durian.

CONCLUSIONS

Two isolates of *R. microporus*, *F. oxysporum* and *F. solani* were identified as pathogens responsible for root rot diseases of jak. Colony growth responses of the above fungal isolates to pH, temperature and light intensity were different among the isolates belonging to the same genera and/or species, indicating inter- and intra species variations of the causal pathogens to the selected microenvironmental factors, hence and influence on fungal colonization. All four fungi were infectious on the tested perennial crops being *A. nobilis* the most susceptible and *P.americana* the least susceptible.

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