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Morphological Identification of *Rigidoporus microporus* associated with White Root Rot of Hevea (*Hevea brasiliensis*) in Cameroon

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ABSTRACT

White root disease caused by *Rigidoporus microporus* is a severe problem that decreases latex productivity and can even cause mortality of rubber trees. This study was conducted with the objective of evaluating the pathogenicity and morphological characteristics of *Rigidoporus microporus* on different media (PDA, CMA and MEA), at different temperatures (25°C, 30°C and 35°C) and pH (4, 5, 6, 7, 8, and 9). The pathogen was isolated from rotten roots of GT1 rubber trees in Nko'olong (South-Cameroon) and pure cultures were obtained in the Phytopathology laboratory at Ekona (South-West Cameroon). *In vitro*, radial growth of the fungus was monitored. The pathogenicity test on young rubber plants was done following inoculation method. Maximum growth was obtained in combinations (MEA and 30°C) and (PDA, 30°C and pH 4). Pathogenicity test showed white mycelia and rhizomorphs on inoculated rubber plants. The pathogen caused the typical symptoms of white root rot on inoculated rubber plants, with a higher disease severity in clone PB 260.

Keywords: *Rigidoporus microporus, Hevea brasiliensis,* White root rot disease, *in vitro*, Radial growth.

1. INTRODUCTION

The phytopathogenic fungus *Rigidoporus microporus,* synonym *Rigidoporus lignosus* of the Basidiomycetes class causes dry white rot of wood [1]. It is present in all rubber-producing countries, the most seriously affected being: Indonesia, Malaysia, Thailand, Sri Lanka, Philippines, Vietnam, China, Myanmar, Gabon, Cameroon, Ivory Coast, Ghana and Nigeria [2]. In Indonesia, 80 000 ha of rubber trees were affected by white rot [2]. In Malaysia, the incidence of this disease in rubber plantations was between 5 and 15% [3]. In West Africa, *R. lignosus* causes more than 50% of rubber losses in old plantations [4]. In Nigeria, *R. microporus* devastates more than five trees per hectare per year and is responsible for 96% of all root diseases in rubber plantations [5].

This pathogen is responsible for the loss of more than 40% mature trees in rainforests in Cameroon and is responsible for the reduction of rubber plantation latex yields [6]. White rot is considered a threat based on the damages it causes. The study of this fungus is necessary for a better understanding of its morphological characteristics under several conditions of culture as

well as its pathogenicity, in order to propose suitable solutions for its control.

2. MATERIALS AND METHODS

2.1 Collection of samples

Samples were collected in five smallholder plantations in Nko'olong (South Cameroon) in April 2016. The samples included five rotten roots of three trees of the GT1 hevea clone collected over an area of 500 m^2 per plantation. These were taken to IRAD rubber research laboratory in Ekona for isolation and purification on the PDA (Potato Dextrose Agar) culture medium.

2.2 Isolation and purification

2.2.1 Isolation

The roots bearing a white mycelium were cut into 9mm^2 fragments using a sterile scalpel blade. These root fragments were washed with tap water, superficially disinfected in a 5% sodium hypochlorite solution for 10 minutes and in 95% alcohol, then rinsed three times with distilled water to remove traces of the disinfectant. They were deposited on blotting paper and allowed to dry completely. The fungus was isolated on a PDA culture medium. The filtrate obtained was poured into an Erlenmeyer flask containing 15 g of Agar and 20 g of Dextrose. After homogenization, the mixture was adjusted to 1000 ml with distilled water. The medium was sterilized in an autoclave (Science Medecine Industry brand) at a temperature of 121 °C for 30 minutes under a pressure of 1 bar. The dried fragments were removed aseptically under a hood (Bassaire model No. KDS2 Enclosure, voltage 230/205 Volts) near the flame of a Bunsen burner and placed in 90 mm diameter glass Petri dishes containing the PDA culture medium supplemented with streptomycin 250 mg, to prevent the development of bacteria. Each Petri dish contained three to four root fragments. These Petri dishes were incubated at 25 °C for three days.

2.2.2 Purification

The visible fungal colonies developed at the periphery of the root fragments and those most similar to the fungus *Rigidoporus microporus* (white mycelium, fluffy texture and radial growth) were removed and aseptically transplanted into new Petri dishes containing the culture medium. After four successive subcultures on the PDA culture medium, the pure cultures of *Rigidoporus microporus* were obtained. Pure cultures were observed under the ordinary electron microscope (Labolux 11, Leizt) for identification. The identification of *Rigidoporus microporus* was based on the morphological and microscopic characteristics of the mycelium and basidiospores in comparison with the characteristics of the fungus presented in the literature [7, 2].

2.3 Morphological characterization

2.3.1 Determination of best culture medium and incubation temperature for radial growth

Mycelial discs of 5 mm diameter taken from the periphery of the pure cultures were placed at the center of the Petri dishes containing the PDA, Corn Meal Agar (CMA) (Difco), and Malt Extract Agar (MEA) (Difco) media in order to evaluate the radial growth and morphology of the pathogen after 12 days of incubation. The cultures were incubated at 25, 30 and 35 °C. Measurement of the radial growth (C) of the fungus was done daily and at the same time by a perpendicular trace on the center of the fungus in each Petri dish. The test was repeated three times. The following formula was used [8]:

$$C = [(d1+d2)/2]-d_0$$

Where: C = Mycelial growth; d1 and d2 = culture diameters measured in both perpendicular directions and d_0 = diameter of the cutter (5 mm).

The daily radial growth rate (V) was calculated at the incubation temperature of 25 °C. It refers to the average daily diameter increase of the fungus calculated using the formula:

$$V= [(DMj1-DMj0) + (DMj2-DMj1) +...+ (DMjn-DMjn-1)]/n$$

Where: DMjn is the average growth (diameter) measured on the nth day, DMjn = (d1 + d2) / 2; d1 is the first diameter and d2 is the second diameter measured on the nth day. DMj0 = 5mm (inside diameter of the cutter).

2.3.2 Determination of the best pH of culture media on the mycelial growth of *R. microporus*

The three culture media were supplemented with a strong acid, 1N HCl to decrease the pH and a strong 1N NaOH base to increase it to obtain the desired pH (4, 5, 6, 7, 8, 9 and 10). The electronic pH-meter (pHS-3C of Searchitec brand) was used to measure the different pH values. A magnetic stirrer was used to homogenize the solutions. The solutions obtained were placed in an autoclave at a temperature of 121 °C for 30 minutes under a pressure of 1 bar for sterilization. The cooled media were supplemented with 250 mg streptomycin prior to distribution in the Petri dishes under the hood near a flame from a Bunsen burner. After solidification of the culture medium, each Petri dish received a 5 mm diameter fragment of the five days old fungus from the PDA medium. The explant was removed aseptically using a cutter (sterilized with alcohol and flamed) and placed at the center of each Petri dish. These Petri dishes were incubated in Kottermann 2737 incubators at 25 °C, 30 °C and 35 °C for 12 days (the time required for the first colonies of *Rigidoporus microporus* to completely cover a 70 mm Petri dish). The test was repeated three times. The daily radial growth in diameter of the mycelium was recorded.

2.4 Pathogenicity test on young plants

Five weeks old Hevea plants (clones GT1 and PB 260) were inoculated. The preparation of the inoculum was carried out according to the modified method of Rodesuchit (1998) [9]. It consisted in cultivating isolates of *Rigidoporus microporus* on the PDA culture

medium at 30 °C and at pH 4 on the one hand, to be cultivated in a sterilized medium (containing 100 g of sawdust, 3 g of corn bran and 2 g of sugar) moistened with distilled water on the other hand. The inoculum was introduced into polyethylene bags and placed in an incubator at 30 °C for 20 days. The artificial inoculation was made by depositing the inoculum at the bottom of each polyethylene bag (14 × 24 cm) containing previously sterilized soil. The inoculated plants were kept in a nursery for eight weeks. At the end of the experiment and according to the degree of attack (severity) of the inoculated plants, a score was assigned to each of the plants according to the severity scale of Nandris et al. (1983) [4].

Table 1: Severity scale of white root rot of rubber roots [4]

Note	Assessment of the importance of the attack
0	No mycelial traces
1	Non-aggregated mycelial filaments
2	Rhizomorphs
3	Rhizomorphs + pin holes
4	Rhizomorphs + Localized necrosis visible with the naked eye
5	Rhizomorphs + Partial pivot rot <20%
6	Rhizomorphs + Partial rottening of the pivot from 20 to 50%
7	Rhizomorphs + pivot rot > 50 %
8	Foliar Symptoms of the Disease
9	Plant Death

2.5 Experimental design and data analysis

The experimental design for testing the effect of the culture medium and the incubation temperature and pH effects as a function of time was a factorial test. The data collected on the radial growth were subjected to repeated-variance analysis using the SAS version 9 software. The different combinations were compared using the least squares method.

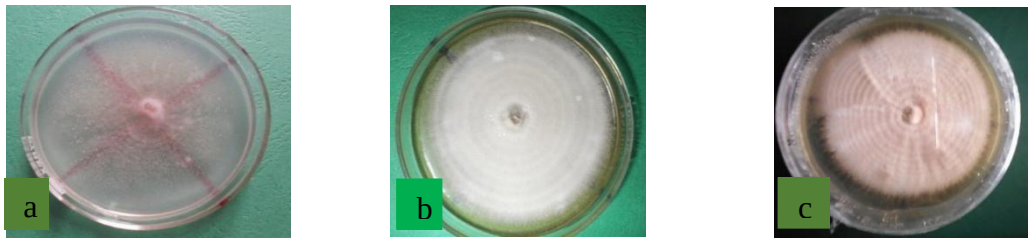
3. RESULTS AND DISCUSSION

3.1. Description of symptoms *in vivo*

Rigidoporus microporus was found on the surface of the roots forming numerous mycelial or rhizomorphic cordons (Figure 1a). The latter were rather thin and had a color that varied from purple-white to orange-yellow. The pathogen formed several (1-2mm) thick rhizomorphs that grew and attached strongly to form a dense, highly branched network on the surface of the root bark. The pathogen produced white mycelial filaments (Figure 1b) near the collar. *Rigidoporus microporus* was also found in the form of carpophores which were yellowish-white on the upper side and orange-yellow on the underside at the collar of dead or live rubber trees. These fructifications had a semicircular shape with a diameter of about 20 cm and a perpendicular growth with respect to the collar (Figure 1c).

Figure 1: Rhizomorphs, (a) mycelial filaments (b) and carpophores (c) of *R. microporus* at rubber collar (clone GT1)

3.2. Identification of the fungus on the PDA, CMA and MEA culture media at 25 ± 2 °C



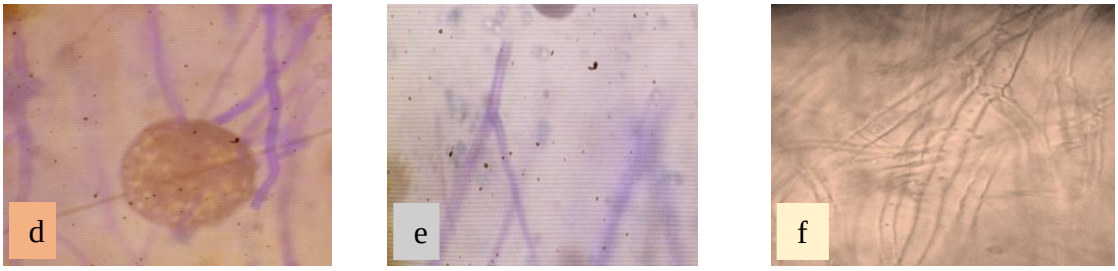


Figure 2: *Rigidoporus microporus* pure cultures on CMA, MEA and PDA culture media (a, b, c respectively); Hyphae and globose spores of the fungus on CMA, MEA and PDA (d, e, f respectively) incubated at 25 ± 2 °C for 12, 13 and 14 days, respectively.

Table 2: Macroscopic and microscopic description of *Rigidoporus microporus*

Parameters	Culture media		
	PDA	**CMA**	**MEA**
1	White and brownish as they grow old	Colourless	White and brownish as they grow old
2	Cream white	Colourless	Yellowish
3	Duvet feeder	Duvet feeder	Duvet feeder
4	Flat surface with regular outlines	Flat surface with regular outlines	Flat surface with regular outlines
5	1.18 mm/d	5.22 mm/d	5.01 mm/d
6	Six	Five	Six
7	After 14 days	After 12 days	After 13 days
8	Translucent	Transparent	Translucent
9	Septate and colourless	Septate and colourless	Septate and colourless
10	Globules and hyalines	Globules and hyalines	Globules and hyalines

1: Color of the upper surface of the mycelium; 2: Color of the underside of the mycelium; 3: Mycelial texture; 4: Relief and contours of the mycelium; 5: Radial growth rate; 6: Number of concentric circles; 7: Number of days required for complete coverage of the 70 mm Petri dish; 8: Opacity; 9: Microscopic nature of hyphae; 10: Microscopic nature of Spores

3.4. Effect of culture medium and incubation temperature on the radial growth of *R. microporus*

The interaction between incubation temperatures (25, 30 and 35 °C) and culture media (PDA, CMA and MEA) was highly significant ($p < 0.05$). The growth of *R. microporus* varied in each culture medium with the incubation temperature as a function of incubation time. The temperature and the culture medium each had a significant influence on mycelial growth. Incubation time exerted a highly significant effect on mycelial growth; the incubation times are not independent (Table 3).

Table 3: Repeated-measure Variance Analysis of Effect of Culture Medium and Incubation Temperature on Radial Growth of *R. microporus* versus Incubation Time

SV	dl	SCE	CME	F	P > F	Adj P> F	
						G - G	H-F-L
Temps	11	930.132	84.557	1735.04	<0.0001	<0.0001	<0.0001
temps*MC	22	12.835	0.583	11.97	<0.0001	<0.0001	<0.0001
temps*Temp	22	2.392	0.109	2.23	0.0020	0.0661	0.0538
temps*MC*Temp	44	26.342	0.598	12.28	<0.0001	<0.0001	<0.0001
Erreur (temps)	198	9.649	0.048				

*SV: Source of variation; MC: Culture medium; temps*MC : interaction between time and culture medium; temps*Temp :interaction between time and temperature; temps*MC*Temp: interaction between time, culture medium and temperature; dl : Degree of freedom; SCE: Sum of squares of deviations to mean; CME: Average square of deviations to mean; F: Calculated Fisher value; P = Probability; Adj P: Adjusted Probability; GG: Greenhouse-Geisser correction factor of sphericity; HFL: Huynh-Feldt correction factor of sphericity.*

The interaction between culture medium and incubation temperature which allowed maximum growth of *Rigidoporus microporus* after twelve days of incubation was MEA at 30 °C. This interaction is not significantly different from the other interactions: (CMA, 35 °C), (CMA, 25 °C) and (PDA, 30 °C). On the other hand, the interaction PDA and 35 °C minimized mycelial growth of the fungus (Table 4). At temperature 30 °C, *Rigidoporus microporus* completely covered the 65 mm Petri dish on the 12th, 13th and 14th days in MEA, PDA and CMA culture media respectively (Figure 3).

Table 4: Comparison of culture medium and temperature interactions for mycelial growth using the least squares method twelve days after incubation (X12)

Culture medium	Temperature	Avg. MC (mm)
MEA	30°C	65 a*
CMA	35°C	64.33 a
CMA	25°C	62.7 a
PDA	30°C	62.33 a
MEA	25°C	56.83 ab
MEA	35°C	51.33 b
PDA	25°C	50.17 b
CMA	30°C	47.67 b
PDA	35°C	44 bc

** The averages with the same letter in the same column are not significantly different at P ≤ 0.05. Avg: Average; MC: Least squares; X12: Observation of mycelial growth after 12 days of incubation*

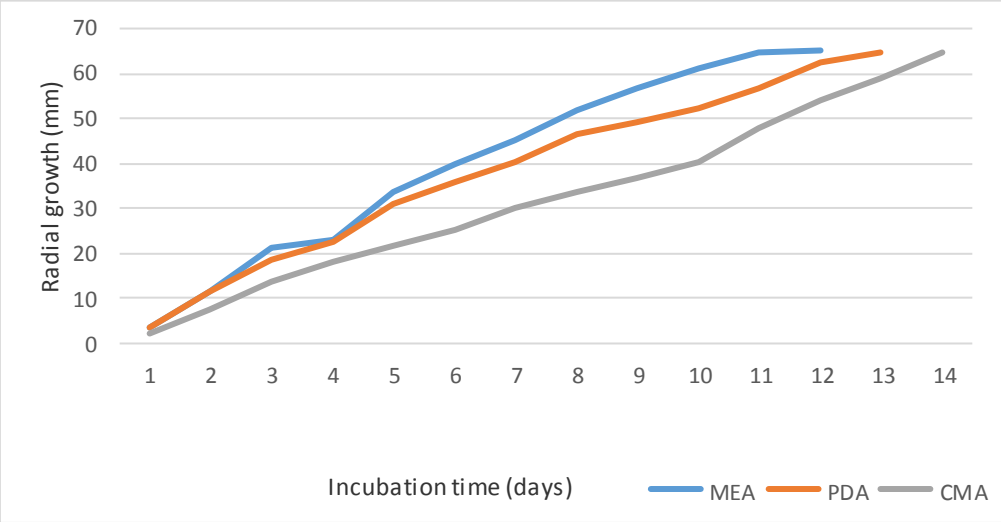


Figure 3: Evolution of the radial growth of *Rigidoporus microporus* on MEA, PDA and CMA culture media after 12, 13 and 14 days of incubation at 30 °C.

3.5. Effect of the pH of culture media and incubation temperature on the radial growth of *R. microporus*

The interaction between pH (4 to 9) of the culture media (PDA, CMA and MEA) and incubation temperature (25, 30 and 35 °C) was highly significant ($p < 0.05$). The growth of *R. microporus* varied with each pH of the culture medium and each incubation temperature as a function of the incubation time. The interactions between pH-culture medium, and culture medium-incubation temperature were highly significant. Incubation time had a highly significant influence on mycelial growth; incubation times were not independent (Table 5).

Table 5: Repeated-Variance Analysis of Effect of pH of Culture Medium and Incubation Temperature on Radial Growth of *Rigidoporus microporus* as a Function of Incubation Time

SV	dl	SCE	CME	F	Pr > F	Adj P> F	
						G - G	H-F-L
Time	11	4504.417	409.492	5607.79	<0.0001	<0.0001	<0.0001
Time*pH	55	41.115	0.747	10.24	<0.0001	<0.0001	<0.0001
Time*MC	22	48.215	2.192	30.01	<0.0001	<0.0001	<0.0001
Time*Temp	22	32.634	1.483	20.31	<0.0001	<0.0001	<0.0001
Time*pH*MC	110	122.284	1.112	15.22	<0.0001	<0.0001	<0.0001
Time*pH*Temp	110	12.268	0.111	1.53	0.0006	0.0798	0.0782
Time*MC*Temp	44	45.463	1.033	14.15	<0.0001	<0.0001	<0.0001
Time*pH*MC*Temp	220	61.423	0.279	3.82	<0.0001	<0.0001	<0.0001
Erreur (Time)	1188	86.750	0.0730				

*SV: Source of variation; pH: Hydrogen potential; MC: Culture medium; Time*pH: interaction between time and pH;Time*MC : interaction between time and culture medium; Time*Temp: interaction between time and temperature;Time*pH*MC : interaction between time, pH and culture medium; Time * pH * Temp: interaction between time, pH and temperature; Time * MC * Temp: interaction between time, culture medium and temperature; Time * pH * MC * Temp: interaction between time, pH, culture medium and temperature; dl: degree of freedom; SCE: Sum of the squares of the deviations; CME: Average square of deviations; F: Calculated Fisher value; P = Probability; Adj P: Adjusted Probability; GG: Greenhouse-Geisser correction factor of sphericity; HFL: Huynh-Feldt correction factor of sphericity.*

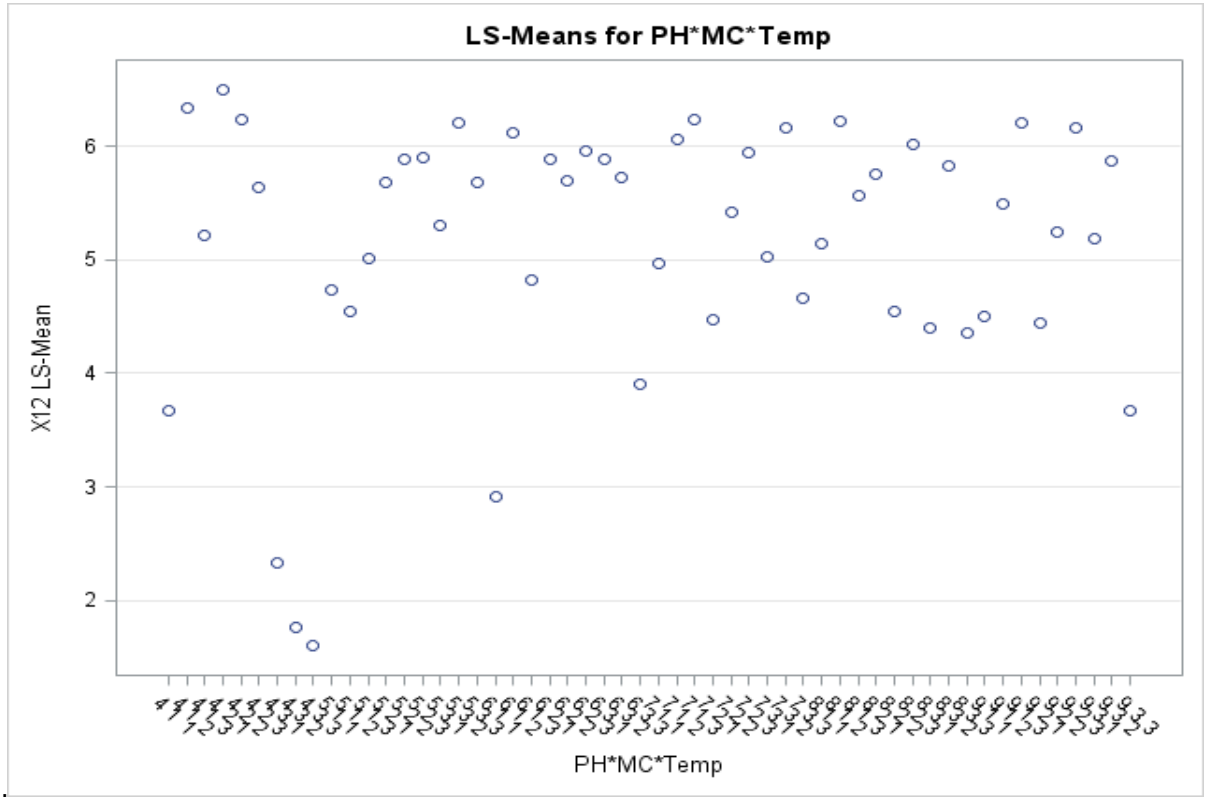
The interaction between culture medium and incubation temperature, which allowed maximum mycelial growth of *Rigidoporus microporus* after twelve days of incubation, was the PDA culture medium at pH 4 and at a temperature of 30 °C. It was not significantly different from the interactions between the CMA culture medium at pH 4 and at 25 °C as well as the MEA culture medium at pH 5 and at 30 °C. The interaction between the MEA culture medium at pH 4 and temperature 35 °C minimized mycelial growth of the fungus on the same day (Table 6).

Figure 4 shows all the interactions between the pH of the culture media and the temperature for mycelial growth twelve days after incubation according to the least squares method.

Table 6: Comparison of the pH-culture medium interactions and mycelial growth temperature using the least squares method twelve days after incubation (X12)

pH	Culture media	Temperature (°C)	Avg. MC (mm)
4	PDA	30	65 a*
4	CMA	25	63.33 a
4	CMA	30	62.33 a
7	PDA	35	62.33 a
8	PDA	30	62.17 a
5	MEA	30	62 a
9	PDA	35	62 a
6	PDA	30	61.67 a
7	MEA	30	61.67 a
9	CMA	35	61.67 a

** The averages with the same letter in the same column are not significantly different at P ≤ 0.05. Avg. MC: Average of Least Squares; X12: Observation of mycelial growth after 12 days of incubation. Only the first ten combinations out of 54 are shown in Table 6.*



*pH * MC * Temp: interaction between the pH of the culture medium and the temperature; Culture medium 1: PDA, Culture medium 2: CMA, Culture medium 3: MEA, Temperature 1: 25 °C, Temperature 2: 30 °C, Temperature 3: 35 °C; X12 LS-Mean: Least squares mean on the twelfth day after incubation*

Figure 4: Scatter plots of least squares means of all interactions between pH of culture media and incubation temperature

3.6. Test of Pathogenicity

The pathogenicity test of *R. microporus* on clones GT1 and PB 260 showed that isolates of *Rigidoporus microporus* caused symptoms of white root rot in the inoculated plants. After eight weeks, white and rhizomorphic mycelial filaments with point penetrations (pin holes) were observed on the pivots of the inoculated rubber plants (Figure 5). The fungus *Rigidoporus lignosus* was successfully re-isolated on PDA medium.

Figure 5: Mycelial (a) and rhizomorphic filaments (b) on clone PB 260 and mycelial filaments on clone GT1 (c) eight weeks after inoculation

Table 7: Pathogenicity of *Rigidoporus microporus* on Hevea plants PB 260 and GT1

Repetition 1			Repetition 2		
Number	PB 260	GT1	Number	PB 260	GT1
1	0	0	1	3	1
2	3	0	2	2	0
3	1	0	3	0	0
4	1	1	4	3	0
5	1	0	5	1	0
6	0	0	6	1	1
Control	0	0	Control	0	0

0: severity of white root rot corresponding to no mycelial traces; 1: Severity of white root rot corresponding to the presence of non-aggregated mycelial filaments; 2: Severity of white root rot corresponding to the presence of rhizomorphs; 3: presence of rhizomorphs with point penetrations following the Scale of severity of white root rot of rubber roots byNandris et al. (1983).

According to the classification scale of Nandris *et al*. (1983), the severity of the disease was higher on clone PB 260 compared to that of clone GT1 whose maximum level of severity was 1[4]. Table 7 shows the severity of the disease for each plant species.

Rigidoporus microporus is a saprophytic pathogenic fungus of the class Basidiomycetes. It is manifested by the presence of mycelial filaments, rhizomorphs and carpophores. The symptoms described *in vivo* in this study corroborate those described by Jayasinghe (2011) in Malaysia [2]. The morphological characteristics described *in vitro* are similar to those described by Nandris (1985) and Jayasinghe (2011) [10, 2]. In this study, its mycelial growth varied according to the culture media tested, the incubation temperature and the pH of the medium. At 25 °C, the optimum mycelial size obtained on the seventh-day (40 mm) on MEA culture medium is less than that obtained by Jayasinghe (2011) on the same medium (50 mm) after the same incubation time [2]. This could be due to the fact that some fungi grow better on low- or high-temperature organic media (poor or rich in mineral elements), others on more selective media [11, 12]. Fungi generally require different temperature and pH conditions during their development. These two factors influence the stages of their life cycle. Moreover, the temperature which favored the optimal growth of *Rigidoporus microporus* is 30 °C. This is in agreement with the mycelial growth results obtained by Shim *et al*., (2005) on another Basidiomycete *Macrolepiota procera* [13].

The pH of the medium affects the metabolism of fungi: at low pH, the protoplasmic membrane is saturated with hydrogen ions, thus limiting the passage of essential cations, while at high pH it is saturated with hydroxyl ions and the entry of essential cations is limited. All the pH tested promoted the mycelial growth of the fungus studied. The mycelial growth optimum was observed at pH 4 (very acid) and at a temperature of 30 °C on a PDA culture medium after 12 days of incubation. These results are not similar to those obtained by other authors on other basidiomycetes notably *M. procera* and *Lignosus rhinoceros* with their optimum mycelial growth between pH 5 and pH 8 [13, 14, 15]. Moreover, the works of Sandhu and Arora (1985) and Galhaup *et al.* (2002) are similar to the results found [16, 17]. They showed that the optimum growth of other Basidiomycetes such as *Polyporus sanguineus* and *Trametes pubescens* was around pH 4.5. This may be due to the fact that acidic pH would enhance the activity of lytic enzymes [18].

4. CONCLUSION

The results obtained show that the culture medium, the incubation temperature and the pH of the medium influence the radial growth of *Rigidoporus microporus in vitro* as a function of the incubation time. The culture conditions which allowed maximum growth of the fungus were: the PDA culture medium at a temperature of 30 °C and pH 4. The results confirmed that in the absence of a pH variation *Rigidoporus microporus* grew better on the MEA culture medium at a temperature of 30 °C. *In vivo*, this causative agent was responsible for

the presence of mycelia, rhizomorphs and carpophores present at the collar of rubber GT1 clones. *Rigidoporus microporus* was isolated from the infected roots of the rubber clone GT1. The pathogen caused the typical symptoms of white root rot on inoculated rubber plants, with a higher disease severity in clone PB 260.

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