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Assessment of root zone mycoflora of three *Hevea brasiliensis* (Rubber) clones at Akwete plantations and their *in vitro* growth inhibition of *Rigidoporus lignosus*

Monday Ubogu

Department of Biological Sciences, University of Agriculture, Makurdi, Benue State, Nigeria

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ABSTRACT

The rhizosphere and rhizoplane mycoflora of three rubber clones, PB 5/51, GT1 and PB28/59, were determined using the soil dilution plate method and serial washing of root lengths respectively. These were plated on PDA plates. Five genera of fungi, Aspergillus, Trichoderma, Penicillium, Botryodiplodia and Mucor, were isolated from the root zones of all three rubber clones. In both the rhizosphere and rhizoplane, the preponderance of Aspergillus among the various rubber clones was in the following order, PB 28/59 > GT1 > PB5/51. While for Penicillium, PB 5/51> GT1 > PB 28/59. The occurrence of Trichoderma in the rhizoplane was in the following order, PB 5/51 > GT1 > PB 28/59, while for the rhizosphere, GT1 > PB28/59 > PB 5/51. Botryodiplodia and Mucor were only isolated from the rhizoplane of all three rubber clones with relatively low level of occurrence. Isolates of A. niger, Trichoderma spp. and Penicillium spp. from the root zone of clone PB 5/51inhibited mycelia growth extension of R. lignosus by 17.4, 32.5 and 21.0 % respectively which were significantly higher than those from GT1 and PB28/59, which did not differ statistically ($p = 0.05$). Clear zone of inhibition against the pathogen were only produced by isolates of Penicillium spp. and A. niger in the range of 10.1 to 13.0mm, which did not differ significantly among the three rubber clones ($p = 0.05$).

Keywords: Rhizoplane, Rhizosphere, Clone, Antagonism, Fungi.

INTRODUCTION

Most rubber plantations in Nigeria are planted with varieties of rubber referred to as clonal rubber. Thus there are certain well-defined characters that are constant with a clone. Some rubber clones are susceptible to certain diseases of rubber. PRIM 600 Malaysia, for instance, is a high yielding clone but it is susceptible to Phytophthora and pink disease [1].

The rubber tree, *Hevea brasiliensis* (Mull arg.) is prone to many diseases. However, *R. lignosus*, the causal agent of white root rot disease of rubber, is the pathogen most feared by planters throughout the rubber growing regions of the world [2]. In Nigeria, the white root rot disease of rubber is the most serious. It accounts for about 94% of incidences of all root diseases and kills up to five *Hevea* trees/ha [3].

The Rubber Research Institute of Nigeria (RRIN), which maintains two groups of plantations of rubber, one in Iyanomo near Benin, Midwestern Nigeria, and the other in Akwete in Abia State, eastern Nigeria, reported that the white root rot disease continued to be the most serious problem of rubber on the plantations in Iyanomo, where it

accounts for 99% of diseased trees representing nearly 47% of trees inspected, despite the regular round of inspections and treatments [4, 5]. On the other hand, Dr. I. K Ugwa and Dr. T. Esekahade, both of the RRIN regard the disease as of no serious concern in the Akwete plantations (Personal Communication). One possible reason for the differential response of rubber to the white root rot disease at the two stations of the RRIN, have been attributed to the inherent quantitative difference in the proportion of antagonists of *R. lignosus* in the root zone of rubber at the two locations [6].

The study of root-associated microorganisms and their antagonistic potentials is important not only for understanding their ecological role in the rhizosphere and their interaction with plants but also for any biotechnological application [7]. Establishing the composition of antagonistic microorganisms towards soil-borne phytopathogens is especially important from the point of view of biological protection of plants [8]. It is on this basis of assessing the various degree of protection among different rubber clones against *R. lignosus* based on the composition of their respective antagonistic root zone mycoflora that investigations were carried out at the Akwete plantations.

MATERIALS AND METHODS

Root Sample Collection

Samples of roots of one-year old rubber plants were collected in June, 2004, from three mature plantations comprising of three different rubber clones designated as PB5/51, GT1and PB/28/59 (with no history of fertilizer application) at the RRIN, Akwete, for microbiological analysis. The age of the sample plants was selected for ease of identification in the field, and of uprooting. The young plants were randomly uprooted from about ten metres radius of the centre of each rubber clone plantation to minimize possible mingling with seedlings arising from neighbouring clones.

The roots were shaken lightly to detach loosely adhering soil particles, before being taken to the laboratory in polythene bags surface sterilized with 70 % ethanol. The root samples were subjected to microbial analysis, either soon on arrival in laboratory, or within 24 h of storage in refrigerator at 4^0C.

Isolation of rhizosphere mycoflora

Adopting the method of Abdel-Rahim *et al*.[9], soil particles released following more vigorous shaking of the roots of a batch of 20 rubber plants for each clone were collected as the rhizosphere soil. After thoroughly mixing of the soil on sterile filter paper, aliquots of 1.0 g of soil were suspended in distilled sterile water, to prepare dilutions of 10^{-3} which from preliminary experiment yielded the best plates for fungal colony counting. One milliliter of 10^{-3} dilutions were plated out in 20 ml molten PDA and swirled to ensure even distribution of inoculum. The PDA plates were amended with a mixture of streptomycin and ampicillin for the isolation of the fungal flora. Ten replicate plates were incubated at room temperature and fungal counts taken between 48-72 h. The fungi were identified, and occurrence determine per gram of soil.

Isolation of rhizoplane mycoflora

The method for isolating root-surface mycoflora was essentially that of Harley and Waid [10], as adapted by Ikediugwu and Ejale [11]. For each rubber clone, 120 root segments, each 5 mm in length were excised from both the tap and secondary roots of the batch of 20 rubber plants, and serially washed together twenty times in 100 ml of sterile water, contained in 250 ml conical flask. The flask containing the root segments was shaken vigorously by hand for 2 mins at each wash. Both flask and water were changed up to the fifth wash, but thereafter, only the water was changed. This was carried out up to 15^{th} wash as preliminary studies revealed that washing root length of rubber plant up to 10^{th} time is enough to detached loosely adhering propagule from root surface, and so appropriate for isolating rhizoplane bacteria and fungi [6]

Root segments washed for up to 15 times were dried between sheets of sterilized tissue paper and plated out on PDA, six root segments per plate, giving a total of 20 replicate plates for each clone. The plates were incubated at room temperature (28-30^0C) for up to seven days, during which fungal colonies growing out of the root segments were identified their frequency of occurrence among the root segments determined.

Antagonism of R. lignosus by root zone fungal isolates from the different rubber clones

Fungi isolates from the root zone of each of the rubber clones, PB 5/51, GT1 and PB 28/59, were respectively tested for their *in vitro* antagonism performance against the white root rot fungus, *R. lignosus*.

Estimation of the degree of antagonism of the fungi isolated from the respective rubber clones against *R. lignosus* (obtained as pure culture from RRIN, Iyanomo) was based largely on percentage mycelia extension growth inhibition of the pathogen, adopting the method of Ferreira *et al*.,[12]. A 24 h old culture of the pathogen at room temperature was opposed with individual isolates on PDA plates. Agar disc inoculum of each of the isolates of *Trichoderma* spp. and *B. theobromae* were inoculated 6.5 cm away from the growing edge of the colonies of the pathogen in 12 cm Petri dishes, while that of *Penicillium* spp., *A. niger*, and *Mucor* sp. were placed 3.5 cm away from the growing edge of the pathogen in 9 cm Petri dishes. The spacing of the inocula of the isolates from the pathogens on the plate is related to the extension growth of the individual colonies. Four replicate plates of each of the pairing were incubated at room temperature.

Measurement of the mycelial extension growth of *R. Lignosus*, towards and away from the test antagonist, was made daily. General observations were also made on the growth of the pathogen and the test antagonists on a daily basis. Any zone of inhibition occurring between the organisms was also measured. Percentage mycelial extension growth inhibition of *R. lignosus* was calculated by subtracting distance of mycelial growth towards antagonist, from distance of growth away from antagonist, dividing by mycelial growth away, multiplying this by 100 [12].

Statistical analysis

Data obtained from replicate plates were calculated using the measure of central tendency (mean) and dispersion (standard deviation). The effect of rubber clone on the occurrence of fungal isolates in the root zone of the rubber plant, and their performance on the growth inhibition of *R. lignosus* were analyzed using Analysis of Variance (ANOVA) and the Student's t test at ($p = 0.05$).

RESULTS

Total fungal counts in the rhizosphere of each of the three rubber clones

The results of the total fungal counts in the rhizosphere soil in each of the three rubber clones in Akwete plantations (Table 1), showed that the total fungal populations were essentially the same irrespective of the clone examined ($p = 0.05$). The total fungal counts for rubber clone, PB 5/51, GT1 and PB28/59 in the rhizosphere soil were found to be 5.5×10^4, 6.0×10^4 and 5.3×10^4 cfu/g respectively.

Occurrence of fungi in the rhizosphere and rhizoplane in each of the three rubber clones

Qualitatively, the three rubber clones examined at Akwete plantations haboured the same mycoflora in their respective root zones. The following five genera of fungi were isolated, *A. niger*. *Trichoderma* spp., *Penicillium* spp., *Botryodiplodia theobromae* and *Mucor* sp. However, *B. theobromae* and *Mucor* sp. were only isolated from the rhizoplane in all three clones (Table 2 and 3).

Analysis of variance showed that the preponderance of a fungal genera in both the rhizosphere and rhizoplane is dependent on the rubber clone ($p = 0.05$). Among the three rubber clones, *A. niger* occurred more in the following order, PB 28/59 > GT1 > PB5/51in both the rhizosphere and rhizoplane ($p = 0.05$). While the reverse was the case for *Penicillium* spp., PB 5/51> GT1 > PB 28/59 ($p = 0.05$). The preponderance of *Trichoderma* spp. did not follow a similar trend at rhizoplane and rhizosphere among rubber clones. While the occurrence of *Trichoderma* spp. assumed the following order, PB 5/51 > GT1 > PB 28/59 in the rhizoplane, that of the rhizosphere followed a different pattern, GT1 > PB28/59 > PB 5/51 ($p = 0.05$). The frequency of occurrence of *B. theobromae* and *Mucor* sp. were though found to be small at the rhizoplane, their occurrence among rubber clones were statistically different, with the exception of GT1 and PB28/59 were the occurrence of *B. theobromae* were statistically the same.

Antagonism of R. lignosus by root zone fungal isolates from the different rubber clones

Results of the *in vitro* mycelial extension growth inhibition of *R. lignosus* test by fungal isolates from each of the three rubber clones showed that all isolates inhibited the growth of *R. lignosus* with the exception of *Mucor* sp. (Table 4).

Isolates of *A. niger*, *Trichoderma* spp. and *Penicillium* spp. from the root zone of clone PB 5/51inhibited mycelia growth extension of *R. lignosus* by 17.4, 32.5 and 21.0 % respectively. These were found to be significantly higher than their corresponding genera of fungal isolates from the two other rubber clones, GT1 and PB 28/59, which were statistically not different in their mycelial extension growth inhibition pattern (p = 0.05). Isolates of *B. theobromae* from clone PB 28/59, produced the highest percentage mycelia extension growth inhibition of the pathogen (42.3 %) among the five genera of fungi, this was also found to be significantly higher than *B. theobromae* from the two other rubber clones which did not differ significantly (p = 0.05).

Penicillium spp. and *A. niger* were the only genera of fungi that produced clear zone of inhibition against the pathogen. This ranged from 10.1 to 13.0 mm (Table 5). The zones of inhibition produced against the pathogen among the two genera of fungi and the three rubber clones were statistically the same (p = 0.05).

Table 1: Total fungal counts (cfu/g) in the rhizosphere of three different rubber clones

Rubber Clone	Mean fungal count (cfu/g) ± SD
PB 5/51	$5.5 \times 10^4 \pm 1.5 \times 10^3$
GT1	$6.0 \times 10^4 \pm 2.2 \times 10^3$
PB 28/59	$5.3 \times 10^4 \pm 1.5 \times 10^3$

Table 2: Occurrence of fungi (cfu/g) in the rhizosphere of three different rubber clones in Akwete plantations.

	Fungal counts (cfu/g) Rubber clone		
Isolate	PB 5/51	GT1	PB28/59
A. niger	2.4×10^4	3.8×10^4	4.4×10^4
Trichoderma spp.	5.0×10^2	1.3×10^3	6.9×10^2
Penicillium spp.	3.0×10^4	2.0×10^4	8.4×10^3
B. theobromae	0.0	0.0	0.0
Mucor sp.	0.0	0.0	0.0

Table 3: Percentage frequency of occurrence of fungi in the root segments of three different rubber clones in Akwete plantations

	% frequency of occurrence Rubber clone		
Isolate	PB 5/51	GT1	PB 28/59
A. niger	44.3 ± 4.6	52.0 ± 5.5	56.9 ± 3.3
Trichoderma spp.	51.1 ± 3.6	44.5 ± 5.9	37.8 ± 2.1
Penicillium spp.	2.2 ± 0.5	1.0 ± 0.7	0.0
B. theobromae	0.9 ± 0.3	1.8 ± 0.5^a	2.1 ± 0.9^a
Mucor sp.	1.5 ± 0.5	0.7 ± 0.3	3.2 ± 0.8

**Values followed by the same letter did not differ significantly (P = 0.05).*

Table 4: *In vitro* mycelia extension growth inhibition by root zone fungi isolates from three different rubber clones.

	% mycelia inhibition Rubber clone		
Fungal Isolate	PB 5/51	GT1	PB 28/59
A. niger	17.4 ± 1.5	10.7 ± 0.2^a	12.7 ± 0.3^a
Trichoderma spp.	32.5 ± 3.4	27.8 ± 3.4^b	26.9 ± 7.7^b
Penicillium spp.	21.0 ± 2.4	11.7 ± 4.6^a	14.8 ± 1.3^a
B. theobromae	36.7 ± 1.4^c	33.25 ± 1.5^c	42.3 ± 2.0
Mucor sp.	0.0	0.0	0.0

**Values followed by the same letter did not differ significantly (P = 0.05).*

Table 5: *In vitro* production of clear zone of inhibition by root zone fungal isolates from three different rubber clones.

	Zone of inhibition (mm) Rubber Clone		
Fungal Isolate	PB5/51	GT1	PB 28/59
A. niger	11.0 ± 1.2^a	10.9 ± 0.9^a	13.0 ± 0.5^a
Trichoderma spp.	0	0	0
Penicillium spp.	11.0 ± 1.6^a	10.6 ± 0.7^a	10.1 ± 1.0^a
B. theobromae	0	0	0
Mucor sp.	0	0	0

**Values followed by the same letter did not differ significantly (P = 0.05).*

DISCUSSION

The total fungal populations among the three rubber clones did not differ significantly. Total fungal populations in the rhizosphere soil of rubber clones examined were in the range of 10^4 cfu/g of soil. Although, culturable fungal counts from a fertile soil have been reported to be around 10^6 fungal "propagule" cfu/g [13]. The population of fungi in the rhizosphere soil of spring wheat was reported to be 3.6 x 10^4 cfu/g [14]. This is in agreement with the findings of this study .Variation in the fungal population in soil may be attributed to the complex nature of soil which varies with location and period of isolation.

The mycoflora of the root zone of the three rubber clones were qualitatively the same. This comprise of *Aspergillus niger, Penicillium* spp., *Trichoderma* spp. *B. theobromae* and *Mucor* sp. The composition of the root zone mycoflora however, differs quantitatively. The preponderance of each of the fungi genera isolated from the root zone was dependent on the particular rubber clone. It has been reported that the abundance and composition of *Verticillium* antagonists in the rhizosphere was plant species dependent [7]. The composition of microorganisms in the root zone is influenced by root exudates [15]; the chemical composition of the substances exudated by root is related to the genus, species, cultivar and age of plant [16, 17]. The degree of tolerance of the microorganisms to excretory substances, as well as competition between the colonizing species, is likely to play important roles in determining the pattern of occurrence of microorganisms on the roots of rubber tree [6].

The root zone of rubber plants has been reported to habour antagonistic genera of fungi belonging to *Aspergillus, Penicillium, Trichoderma* and *Botryodiplodia* [18, 6]. *Trichoderma* and *Penicillium* species are important biocontrol agents of many plant pathogens. Antibiotic producing species of *Penicillium* have been employed in the control of plant pathogenic fungi [19, 20, 21].

Trichoderma spp. currently consists up to one third of all fungal biocontrol preparations produced and sold for the control of diseases on agricultural crops during cultivation and storage period [22]. *Trichoderma* spp. antagonizes plant pathogens directly through antibiosis, by virtue of more than 100 metabolites that have antibiotic activities, mycoparasitism and hyphal disruption, or through competition for nutrients/space with the pathogen [8, 23, 24]. Some strains establish robust and long-lasting colonization of root surfaces and penetrate into the epidermis and a few cells below this level. They produce or release a variety of compounds that induce a localized resistance response, and this explains their lack of pathogenicity to plants [25]. *Trichoderma* spp. also inhibit or degrade pectinases and other enzymes that are essential for plant pathogenic fungi [26].

Although *A. niger* has been shown to be effective in inducing resistance in rice to sheath blight [27], and in the control of the pathogen, *Rhizoctonia solani*, through antibiosis, overgrowth and hyperparasitism [28], it has been reported to have limited capacity as a reliable control agent for *R. lignosus*, since the later subsequently, overgrew the colony of the former in an *in vitro* study [6].

Clone PB 55/51 appears to habour consistently more of the *Penicillium* spp. and *Trichoderma* spp., which were the most dominant fungi in this study (with the exception of *A. niger*) and efficient antagonists of *R. lignosus* based on *in vitro* antagonism test, than clone GT1 and PB 28/59. Furthermore, fungal isolates of *A. niger, Penicillium* spp., and *Trichoderma* spp. from the root zone of clone PB 5/51 were more potent than those of the other two clones of rubber in the inhibition of the pathogen. Although, the mycelia extension growth inhibition potency of fungi isolated from the root zone of clone GT1 and PB28/59 against the pathogen were statistically the same, clone GT1 haboured more of the potent antagonists of *R. lignosus* than PB 28/59.

Disease incidence in rubber plants have been shown to vary with rubber clones [29] .The results of this study shows that all three clones of rubber at Akwete plantations have different, inherent degree of protection against *R. lignosus* based on their root zone mycoflora. However, clone PB5/51seems to have the highest degree of protection than clone GT1 and PB 28/59. Comparatively, clone PB 28/59 appears to be the least protected among the rubber clones examined. The dominance of *A. niger* both at the rhizosphere and rhizoplane of clone PB 28/59, together with the highest mycelial growth inhibitory performance of its isolates of *B. theobromae*, is likely to produce an overall low influence on *R. lignosus* due to the limited antagonistic capacity of *A. niger* and the low level of occurrence of *B. theobromae*.

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