Retraction Notice

Title of retracted article: Management of White Root Rot Disease (Fomes) in Hevea brasiliensis Plantations in Cameroon

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Journal: American Journal of Plant Sciences (AJPS)
Year: 2017
Volume: 8
Number: 7
Pages (from - to): 1646-1658
DOI (to PDF): https://dx.doi.org/10.4236/ajps.2017.87114
Paper ID at SCIRP: 77172
Article page: http://www.scirp.org/Journal/PaperInformation.aspx?PaperID=77172
Retraction date: 2017-6-30

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Date initiative is launched: 2017-6-30

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The paper does not meet the standards of "American Journal of Plant Sciences".

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Editor guiding this retraction: Prof. Sukumar Saha (EiC, AJPS)

American Journal of Plant Sciences, 2017, 8, 1646-1658
http://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

Management of White Root Rot Disease (Fomes) in *Hevea brasiliensis* Plantations in Cameroon

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How to cite this paper: Nam, M.G., Wawa, N.S., Ejolle, E.E. and Nkengafac, N.J. (2017) Management of White Root Rot Disease (Fomes) in *Hevea brasiliensis* Plantations in Cameroon. *American Journal of Plant Sciences*, **8**, 1646-1658.
https://doi.org/10.4236/ajps.2017.87114

Received: May 2, 2017
Accepted: June 20, 2017
Published: June 26, 2017

Abstract

The white root rot disease is a serious menace to rubber plantations causing tree loss and consequent yield losses. Both chemical and biological control measures have been used with different success rates. The aim of this study was to evaluate the efficiency of Onazole, Alto, Rubazole and Comet Plus; locally used fungicides for the treatment of this disease both *in vitro* and on farm. The result of the *in vitro* trial showed that Alto and Rubazole inhibited mycelial growth for three weeks while Onazole and Comet Plus inhibited mycelial growth throughout the study period. All the fungicides inhibited mycelial growth when compared to the control where there was no growth inhibition throughout the experiment. In field trials, the incremental girth for fungicides treated trees were dependent on the initial health situation of the trees with initially healthy trees having a slightly higher girth increment than diseased trees. Tree collar application of the fungicides ensured recovery of most diseased trees, especially those that were not terminally infected (dead) at the time of application of the fungicide. The fungicides are thus promising for the control of white root rot in immature rubber plantations.

Keywords

Fungicide, *Hevea brasiliensis*, *In Vitro*, On-Farm, White Root Rot

1. Introduction

Root rot fungi constitute one of the main disease problems affecting rubber plantations worldwide [1]. Among the root diseases, white root disease caused by the fungus *Rigidoporus lignosus* and commonly known as Fomes, constitutes a major constraint to the growth, health and productivity of rubber trees in Africa [2] and other rubber growing countries [3]. As reported by Guyot and

Flori in 2002, [4], it can result in substantial death of trees and sometimes losses of a whole stand. Such tree loss would greatly reduce the tree stand and as such result in low field yields, increased wind damage facilitated by the clearing created by loss of trees, and increased costs of production due to tapper re-tasking in such fields. In fact, yield losses of up to 50% have been reported in older rubber plantations [5].

Under plantation conditions, the white root rot disease spreads fast and infects nearby trees especially in poor sanitation and poor drainage areas. Its pathogen also survives with fruiting bodies on dead trees, branches/twigs, decomposing tree stumps and even on decaying leaves [6]. The main constrain to the success of control methods for this disease is that treatments are only carried out on diseased trees and their effectiveness depends on reliable and early detection of pathogens [7]. Chemical treatment, which helps to restrain the epidemics [2] [8] is one of the currently recommended control methods. Biological methods [3] [9] have also been employed in the control and treatment of the white root rot disease in *Hevea brasiliensis* plantations.

In Cameroon, root rot disease control is commonly carried out by chemical treatment of infected trees. Prophylactic treatment in immature plantings could produce better results. However, results from such treatments have not always been fully satisfactory as the costs of fungicides for full treatment are often beyond small farmers' reach. Furthermore, regular and continuous application of the same formulation could generate resistance of some fungal races to the applied treatments [10].

In this study, four formulations (Onazole, Alto, Rubazole and Comet Plus) were evaluated for their bio-efficacy in the protection of young (immature) rubber trees against the growth and development of the *Hevea* white root rot disease (WRRD).

2. Materials and Methods

The study was conducted *in vitro;* evaluating the effect of the formulations on fungal colony and an on-farm trial was carried out on the effect of the formulations on the growth of the tree as well as mycelia at the collar of immature rubber trees.

2.1. *In Vitro* Evaluation

For the *in vitro* evaluation, diseased trees were identified, marked and specimens of infected roots were cut, placed in labeled paper bags, and transported to the laboratory.

The fungicide was tested by impregnation into potato dextrose agar (PDA) and infestation of the later with purified isolates of the fungus.

2.2. Preparation of Culture Media

Potato dextrose agar (PDA) was used as culture medium to study the growth and development of pure isolates of *R. lignosus* from roots of infected *Hevea*

trees. Potato was bought from the local market, while dextrose and agar were obtained from an imported formula.

About 200 g of potato were weighed, boiled for 30 minutes in 800 ml of distilled water, filtered through a clean muslin cloth and the filtrate made up to 1000 ml using distilled water. Thereafter, 2.5 g of dextrose were placed in a 500 ml flat-bottom flask to which 5 g of agar were then added. About 250 ml of potato filtrate were then added to the flask and the latter was stopped with cotton wool and sealed using an aluminum foil. The flask was gently shaken to mix up its contents and then autoclaved for 20 minutes at 120˚C and at a pressure of 1 bar. The culture medium was allowed to cool to 40˚C and 15 ml of it poured into sterilized Petri dishes, allowed to solidify and kept for 1 day before plating with pure isolates.

The eventual composition (in 1000 ml) of the medium contained potato (200 g), monosaccharide dextrose (10 g), agar (20 g) and distilled water to make 1 litre of suspension. Throughout, precaution was taken to avoid contamination through preparation in an incubating room on a table previously sterilized with conc. ethanol (95%).

2.3. Isolation of Fungal Isolates

Fomes isolates were collected from roots of diseased trees and cultured on PDA at 28˚C. After at least 2 days, the cultures were observed under a microscope (100×) and Fomes isolates identified based on a comparison of their morphology with those of different fungi present. Fomes isolates where then extracted and re-cultured on PDA and the process re-conducted (at least 4 cycles) until pure cultures were obtained.

2.4. Impregnation and Growth of the Fungus

In order to evaluate the effect of the different fungicide treatments, the culture media (PDA) was impregnated with the different treatments:

- Treatment I (Control): The prepared PDA was poured on Petri dishes and used as such without any impregnation of fungicide,
- Treatment II: Here, 10 ml Onazole 100EC was poured onto 2 litres of PDA and the whole stirred (using a magnetic stirrer) for 15 minutes before pouring on Petri dishes
- Treatment III: Here, 10 ml Alto100 SL was poured onto 2 litres of PDA and the whole stirred (using a magnetic stirrer) for 15 minutes before pouring on Petri dishes
- Treatment IV: Here, 30 g Rubazole granules was crush, sieved and poured onto 2 litres of PDA and the whole stirred for 15 minutes before pouring on Petri dishes
- Treatment V: Here, 2 ml of Comet® Plus was poured onto 2 litres of PDA and the whole stirred (using a magnetic stirrer) for 15 minutes before pouring on Petri dishes

Pure Fomes isolates were placed at the centre on each of the culture media in

a Petri dish, sealed and stored at 28˚C. To help in the measurement of mycelia growth, two lines intersecting at the centre of each Petri dish were drawn as shown in Figure 1(a) and Figure 1(b). The lines were drawn so as to divide each Petri dish into four equal parts. The radial growth was measured (in mm) daily (every 24 hours), with a meter ruler. The ruler was placed at the centre of the dish to the periphery of the colony along the lines. The measurements were taken four times for each Petri dish following the four lines drawn on the dish. The average of the four readings per Petri dish was used for data analysis and interpretation. Five Petri dishes in three replicates constituted each treatment, making a total of 15 Petri dishes per treatment.

2.5. Field Location and Experimental Design

Following an initial survey in five estates of the Cameroon Development Corporation-CDC (Malende, Meanja, Mukonje, Sonne and Missellele), an infested immature monoclonal rubber plantation was identified at the Meanja Rubber Estate. This was in the Mile 29 Section; Block 1; Clone PB 260, planted in 2010. The block covered an area of about 40 ha and had been established from a previous rubber plantation.

Trees on the plot were marked out in a randomized complete block design consisting of three (03) blocks; with each block containing five treatments, notably:

- Treatment I: Control with no fungicide application
- Treatment II: Application of Onazole 100EC® at the rate of 10ml/tree
- Treatment III: Application of a 10 ml Alto® (1.0 a.i.)/tree, reference treatment
- Treatment IV: Application of 30 g Rubazole granules
- Treatment V: Application of 2 ml Comet® Plus (0.95 a.i.) /tree

The Onazole 100 CE® and Alto 100SL® formulations are both systemic fungicides of the triazole family, contain the same active ingredient, Cyproconazole (at 100 g/L) and have the chemical formula, 2-(4-chlorophynyl)-3-cyclopropyl-(1H-1, 2, 4-triazole-yl)-butan-2-ol.

Rubazole 10 g/kg; GR at the rate of 30 g/tree is granular.

(a) (b)

Figure 1. Fungal growth measured on petri dishes with no application (a) and with fungicide application (b)

The Comet® Plus formulation, is a combination of two active ingredients: Pyraclostrobine - F500® (at 100 g/L) and Fenpropimorphe (at 375g/L).

Each experimental block had a total of 20 theoretical trees per treatment (2 lines of 10 trees per line) with adjacent unit separated by a guard row of 1 line, replicated 3 times. The entire trial involved a total of 300 trees.

2.6. Disease Identification Procedure

A census was conducted at the start of the trial, after which each tree was separately numbered, its girth measured at 150 cm above the union, and the presence of the disease ascertained. To ensure this, the soil at the collar of each tree was excavated to a depth of 10 - 15 cm and a width of 15 - 20 cm. The presence of Fomes mycelia was ascertained and each tree labeled using one of the following notations:

- W: for healthy trees and whose adjacent neighbours were equally protected;
- B: for trees infested with Fomes;
- R: for heavily infested and dying trees;
- D: for damaged trees; and
- X: for missing points.

Omorusi and others in 2013 [11] affirmed that for disease control, collar inspection presentation is a more useful means than foliar inspection as infected trees could be detected at a much earlier stage. Many infected trees detected by foliar inspection are usually beyond protection.

2.7. Preparation and Application of Treatment Solutions

The respective treatments were applied by pouring the required quantities of diluted fungicidal solutions on each tree or applying the granules on the base of each tree.

As concerns Treatment II to V which involved application of some quantity of the fungicide, the procedure involved preparation of the fungicidal solution following dilution of the initial stock and eventual application to the rubber trees. The procedures for preparation of the various solutions were as follows:

- Treatment II which involved the application of Onazole 100EC ® at the rate of 10 ml/tree, 1000 ml Onazole 100EC ® stock solution was poured in 200 litres of water and the whole stirred to produce a 10 ml Alto® product in 1 litre of solution.
- Treatment III which involved application of 10 ml/tree Alto® (1 a.i., the recommended dose), 1000 ml Alto® 100 SL stock solution was poured in 200 litres of water and the whole stirred to produce a 10 ml Alto® product in 1 litre of solution.
- Treatment IV-Involved application of 30 g Rubazole granules by evenly encircling this at the base of each tree (producer recommendation).
- Treatment V involved diluting 200 ml of Comet® Plus stock solution in 200 litres of solution (using water) and stirring the whole to produce a 1 ml Comet® Plus product in 1 litre of solution. The treatment then involved application of

2 ml/tree Comet® Plus (0.95 a.i.) per tree;

After identification of diseased trees, a funnel-shaped furrow (10 - 15 cm deep and 15 - 20 cm wide) was dug out at the base of each tree. Two litres each of 10ml Onazole, 10 ml Alto® and 2 ml Comet® Plus solutions were applied at the collar of the trees for Treatments II, III and V respectively. Using these solutions, the first litre was poured at one half of the trunk's side at a height of 20 - 30 cm above the ground and the second on the opposite half, ensuring that the collar was fully covered with fungicide solution during each application. For 30 g Rubazole, 15 g was spread on the ground on either half of the trunk, ensuring the collar was fully covered with the granules during each application. During this study, fungicide application was done once in the month of June and monitoring began in July.

2.8. Field Monitoring

On a monthly basis and for a period of six months (July 2016 to December 2016), regular visits were made to the field during which information was collected on:

- Newly infested trees: This involved inspection of the tree's collar to check for the presence of the fungal mycelium (Figure 2 and Figure 3) on the tree collar which had been previously mulched with grass for a period of one month to encourage mycelial development [4].

Figure 2. Tree stem dug up to expose the collar

Figure 3. Tree stem mulched to encourage mycelia growth

- Recovery of diseased trees: This involved inspection of the tree's collar to check for the disappearance of the fungal mycelium after mulching trees using the procedure previously described;
- Trunk girth measurement: measurements were made at 150 cm above the union, using a tape, at the beginning and at the end of the experiment to determine each tree's incremental girth over the period of the study.

2.9. Data Analysis

Data collected was analysed using analysis of variance (ANOVA) and means separated with Student's t test at $P < 0.05$. The software used was the JMP 5 SAS software [12].

3. Results and Discussions

The results obtained highlighted the effects of the applied fungicide treatments on the growth and development (*in vitro*) of fungal colonies, girth of rubber trees and health of trees.

3.1. Effect of Fungicide on *In Vitro* Fungal Colony Growth

Growth of Fomes mycelia on PDA culture media was monitored and measured periodically to determine the effects of the various treatments on the growth of the fungus.

As presented in Figure 4, mycelial growth on media without impregnated fungicide (Treatment 1) was spontaneous, increasing at a fast rate to attain a maximum growth over a period of about three (03) weeks, at which sporulation started. Impregnation of the culture media with Onazole and Comet® PlusF500 ensured complete inhibition of fungal growth throughout the period of observation. This was not the case for fungus infested agar to which Alto100 SL ® and Rubazole were applied as the mycelial growth seemed to, after an initial inhibition for about two (02) weeks, increased progressively with time. Sporulation was delayed till after a period of about eight (08) weeks after culturing for mycelia that had been treated to Alto100 SL ® and Rubazole.

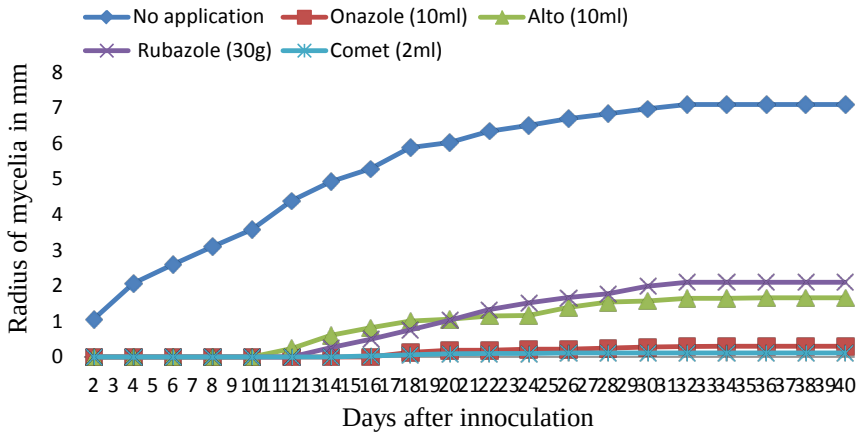

Figure 4. Effect of different fungicides on the growth of Fomes mycelia on PDA.

The results on Table 1 showed that there were significant differences between the inhibitory properties of the various fungicides. With the most effective being Onazole and Comet which fell under the same group (with the lowest mean mycelia radius), followed by Rubazole and Alto which were under the same group and lastly by the control (no application) where there was no inhibition.

Most of these fungicides are Sulphur based and the results from this study are similar to those of Chang et al, 1991 [8] who reported that the systemic fungicides reduced fomes but the success rate is fungicide type specific. The results of this study are in agreement with a study by Sujeewa and others in 2013 [13] who used fungicides to effectively control the root rot pathogen *in-vitro*. Similar results on the inhibition of mycelial growth in *Phytothora infestans* using cyazofamid were observed by Mitani *et al*., [14]. The results of this study suggest that Alto100 SL ® and Rubazole should be used in combination with other fungicides to ensure the complete suppression of mycelia growth. Azole fungicides such as prochloraz, propiconazole and cyproconazole have been widely used in disease control. The mode of action of these fungicides is based on inhibition of cytochrome P450 sterol14 a-demethylase (P45014DM), a key enzyme of the sterol biosynthetic pathway [15]. However, triazoles have no effect against spore germination because spores contain enough sterol for the formation of germ tubes. Some spores even have enough sterol to produce infection structures so, in some cases, triazoles may not be effective against infection of the host tissue [16]. This is evident from the results obtained with Rubazole and Alto which did not prevent sporulation.

Comet plus contains two active ingredients which are collectively known as strobilurins which work by inhibiting mitochondrial respiration that prevents spore germination and mycelial growth in plant pathogens [17]. This could account for its effectiveness in inhibiting fungal growth.

3.2. Effect on the Incremental Girth of *Hevea* Trees

The incremental girth of trees at six (6) months after collar application of treatments was used as a parameter to evaluate separately the effects of different fungicide applications on the vigour of healthy and diseased immature rubber trees. Results obtained showed some differences in girth increment that could be attributed to the health status of the trees and the applied treatments (Figure 5).

Table 1. Mean mycelial growth as influenced by fungicide treatment.

Fungicide Treatment	Radius (mm)
No application	5.39[a]
Rubazole (30 g)	1.07[b]
Alto (10 ml	0.94[b]
Onazole (10 ml)	0.15[c]
Comet (2 ml)	0.0 6[c]

Means with the same letter in a column are not significantly different ($p < 0.05$), LSD = 0.63.

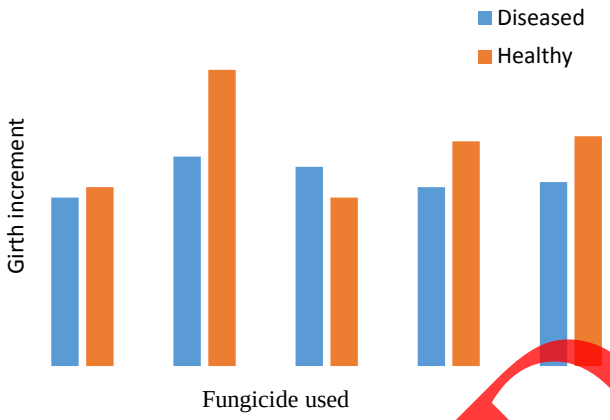

Figure 5. Incremental girth of Hevea trees after application of different fungicide formulations.

In most cases, the girth increment was higher in healthy trees than in diseased trees. This is obvious for the diseased trees had dual functions of growth and defense. Defense reactions of the tree to fungal attack consist mainly of cellular hypertrophy and hyperplasia, stimulation of cambial activity, lignification and suberization of walls, callose deposition on pores of the sieve tubes, and formation of tyloses [18]. Geiger *et al.* 1986 [19] reported that these reactions are characterized by changes in the enzymatic metabolism of the host, particularly peroxydases.

Onazole had the highest incremental girth both in the diseased and healthy trees while the control treatment (no fungicide) had the lowest for the both cases. This is similar to the observation of Wahengbam *et al*., 2013 [20] who found out that some fungicides improved the germination and growth of seeds/ seedlings of some crop plants. The improvement in growth parameters could have been because fungicide application suppressed and /or eliminated pathogenic populations or due to the increase in the growth promoting factors i.e. increase in cytokinin or gibberellins production etc. The lowest girth increment for the control plot was obvious for there were no fungicides to stimulate girth growth.

In *Hevea brasiliensis* plantations the girth of the trees is highly correlated to the yield with trees with larger girths producing more latex than smaller trees. This observation ties with observations of other researchers who found lower dry rubber contents DRC in white root rot infected trees compared to healthy trees [21].

3.3. Effect of the Application of Different Fungicide Formulations on Proportion of Diseased and Healthy Trees

Application of the various formulations decreased the proportion of diseased trees irrespective of the fungicide used (**Figure 6(a)**) while increasing the proportion of healthy trees, or better still, recovered diseased trees (**Figure 6(b)**). Some fungicides of the triazole family were recommended for controlling white root rot disease of *Hevea* [2]. As explained by Porntip *et al*., 2016 [22] recovery of plants could be attributed to more synthesis of Phenylalanine ammonia lyase

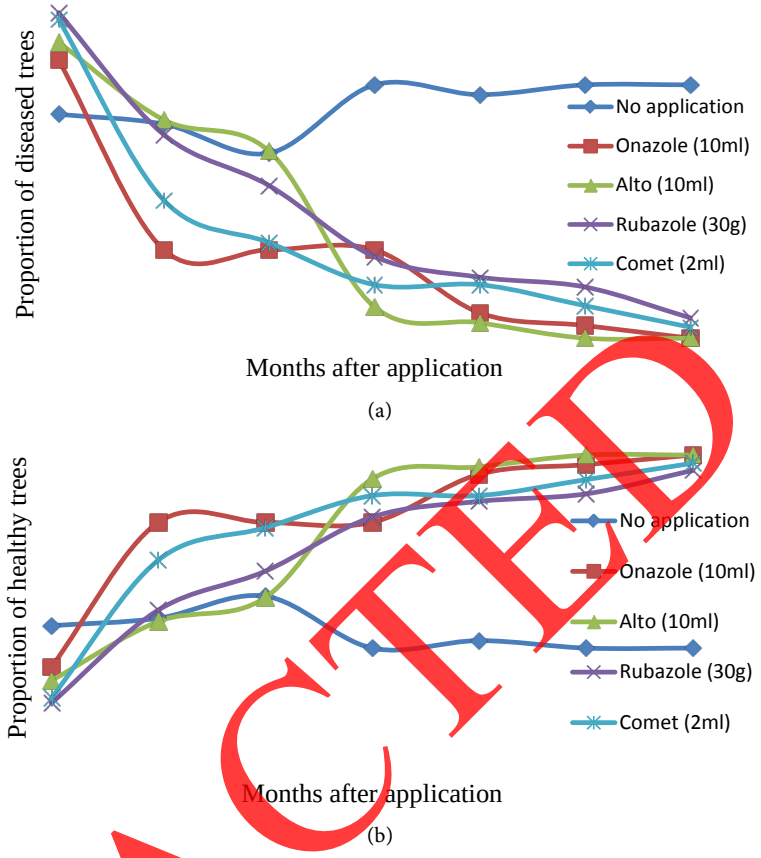

Figure 6. Effect of the application of different fungicide formulations on the evolution in the proportion of (a) diseased and (b) healthy *Hevea* trees.

(PAL) which is a specific branch point enzyme of primary and secondary metabolism and plays a key role in plant development and defense mechanisms.

The proportion of healthy trees increased considerably after fungicide application, irrespective of the fungicide, to attain a maximum level after about one (01) month, with no further recordings of new infections. It could be noted, nonetheless, that on both diseased and healthy trees, the performance of the different fungicides applied, was comparatively slightly better, indicating better efficiency in the control of the WRRD on the *Hevea brasiliensis*. The results of this study are similar to those of other workers who found rubber trees recovering with fungicide treatment [2] [8]. The recovery of some diseased *Hevea* trees was described by John in 1966 [23] as spontaneous healing which depended on the vigour of the tree, the activity of the cork cambium, wound barrier formation and callus growth. Spontaneous healing was common where the inoculum potential was low and this could explain why the infected untreated trees did not all die.

Considerable differences were observed between the four fungicide treatments, which clearly marked themselves from the control with no fungicide as most initially infested trees under this treatment did not recover (**Table 2(a)** and **Table 2(b)**). Thus the highest number of diseased trees (**Table 2(b)**) or on the other hand the smallest number of healthy trees (**Table 2(a)**).

Table 2. (a) Mean number of healthy trees as influenced by fungicide treatment; (b) mean number of diseased trees as influenced by fungicide treatment.

(a)

Fungicide Treatment	Number of trees
Comet (2 ml)	38.0[a]
Rubazole (30 g)	36.7[a]
Onazole (10 ml)	33.0[a]
Alto (10 ml	25.2[b]
No application	24.0[b]

Means with the same letter in a column are not significantly different ($p < 0.05$), LSD = 6.0.

(b)

Fungicide Treatment	Number of trees
No application	24.0[a]
Rubazole (30 g)	9.3[b]
Comet (2 ml)	6.0[b]
Alto (10 ml	4.8[b]
Onazole (10 ml)	4.0[b]

Means with the same letter in a column are not significantly different ($p < 0.05$), LSD = 6.0.

4. Conclusions

This study confirms the role of Sulphur and Sulphur containing compounds in the management of white root rot disease in *Hevea brasiliensis* [24] [25]. Results obtained during a first phase *in vitro* trial highlighted the differential effects of the applied fungicidal treatments on the growth and development of the fungal colonies. Some can inhibit for short while others can inhibit for a long period of time. In field trials, the incremental girth for fungicides treated trees were dependent on the initial health situation of the trees with initially healthy trees having a slightly higher girth increment than diseased trees. Indeed, Fomes infection slightly reduced the growth rate of the young trees.

Tree collar application of the fungicides ensured recovery of most diseased trees, especially those that were not terminally infected (dead) at the time of application of the fungicide. The proportion of trees that remained healthy or had recovered from disease increased over time. As a management strategy to avoid fungicide resistance, it is advised that repeated use of fungicides of the same family alone for example the triazoles or the strobilurins or pesticides with the same mode of action alone should be avoided. When multiple applications are required, it is advisable to alternate fungicides of different families or different mode of action. Some research has shown that there is a clonal variation in the susceptibility to root rot disease in *Hevea brasiliensis* [26], thus additional studies using more clones and increasing the length of the study will give more understanding to root rot disease management in *Hevea brasiliensis*.

Acknowledgements

This study was financed by the 2015 Public Investment Budget (BIP) from the government of Cameroon. The collaboration from the management of the Cameroon Development Cooperation (CDC) is greatly acknowledged.

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