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Research Article

In vitro and *in vivo* synthetic fungicides control of *Rigidoporus microporus* on Para rubber in Nigeria

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Abstract: *Rigidoporus microporus* is a major threat to *Hevea brasiliensis* in the rubber growing regions of the world. *In vitro* synthetic fungicide sensitivity assays of Tridemorph, Benomyl and Bayfidan at seven different concentrations, and *in vivo* effectiveness of the three fungicides on *R. microporus* inoculated on rubber seedlings were evaluated. The three chemical fungicides used in this study were effective on *R. microporus*, with Tridemorph being the most effective. The minimum inhibitory concentration value for Bayfidan was between fungicide concentrations of 0.001µg/ml and 0.005µg/ml. Percentage of plant death and presence of rhizomorph recorded at two months after inoculation were higher than that recorded at the termination of five months experimental period after inoculation. Tridemorph treatment had the highest plant health both at two months and at five months after inoculation. Decline in plant death from the third months onward suggested a reduction in fungal activity of *R. microporus*. Tridemorph was most effective among the three fungicides tested as it exhibited higher mycelial percentage inhibition in the management of *R. microporus in vitro* and higher plant health of rubber seedlings *in vivo.*

Keywords: *Hevea brasiliensis*, chemical control, *Rigidoporus lignosus*

Introduction

Hevea brasiliensis (Willd. ex A. Juss.) Muell. Arg. commonly called Para rubber is a perennial tree crops where the individual plant is an economic entity, and whose healthy existence is significant to the productivity of the crop (Rao, 1975). White root rot is a major threat of *H. brasiliensis* in the rubber growing regions of the world (Rao, 1975; Nandris *et al*., 1983) except in India (Jayasinghe, 2010). *Rigidoporus microporus* (Sw.) Overeem is the causal agent of white root rot. It is a silent killer, where the above ground symptoms are produced once the roots are fully damaged. Foliar symptoms are initiated subsequently with the destruction of the root system (Jayasinghe, 2010). Its above ground symptoms indicate that the trees are mostly beyond treatment and recovery, as rapid progress of infection makes death imminent (Ismail and Azaldine, 1985). White root rot is an important economic factor in the rubber industry since it kills the trees irrespective of age or vigour.

In Nigeria, approximately 65% of rubber diseases are caused by fungal pathogens (Begho, 1995). In West Africa, the white root

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rot is responsible for yield losses of up to 50% in old rubber plantations (Nandris *et al.,* 1983; Jayasuriya *et al.,* 1996). The success of fungicide application is higher when infection is mild; as such the most effective control is to identify infections at the early stage for effective treatment. In the management of *R. microporus* the initial approach is disease avoidance and in situations where disease avoidance fails, farmers resort to chemical control. However there are risks involved with pesticide use which include; danger to people, effect on the environment, residual build up, pesticide resistance and effect on non-target organisms such as beneficial insects, birds, domestic animals, and sometimes the crop itself. The successful use of chemicals in the control of rubber diseases has been extensively reported by various scientists (Jayasinghe *et al.,* 1995; Jayasuriya and Deacon, 1996, Jayasuriya *et al.,* 1996). Tridemorph is one chemical that has generally been recommended for the treatment of white root rot (Jayasinghe *et al.,* 1995). However different active ingredients have been recommended in the different regions of the world. In most instances, collar protectant containing fungicides and sulphur amendments (Peries, 1965), systemic active ingredients such as propiconazole, hexaconazole (Lam and Chiu 1993), and other triazoles (Gohet *et al*., 1991), triadimenol, pentachloronitrobenzene (PCNB), triadimefon (Ng and Yap 1990), pentachlorophenol (PCP) (Jayasinghe *et al*., 1995) and phenol (Jayaratne *et al.,* 1997) were found to be effective against *R. microporus*. This research work is aimed at evaluating the use of three synthetic fungicides (Tridemorph, Benomyl and Bayfidan) *in vitro* and *in vivo* in the management of *R. microporus.*

Materials and Methods

Isolation of *Rigidoporus microporus* culture

Rigidoporus microporus isolate in this study was obtained from infected rubber roots collected from rubber plantation in RRIN, Iyanomo, and stored on Potato Dextrose Agar (PDA) medium**.** Cultures were maintained at 4 °C and sub-culturing done monthly to maintain fresh culture for the experiments.

Determination of minimum inhibitory concentration

Three synthetic fungicides were used in the study namely-Tridemorph with the trade name Calixil (a.i. *N*-tridecyl-2, 6-dimethylmorpholine), Benomyl (also marketed as Benlate) (a.i. Methyl 1-[(butylamino) carbonyl]-H-benzimidazol-2-yl carbamate) and Bayfidan (a.i. triadimenol 250 g/l) (Merkle *et al*., 1984). *In vitro* fungicide sensitivity assays according to Jo *et al.* (2006) was employed. Tridemorph, Benomyl and Bayfidan were added at concentrations of 0.001, 0.005, 0.01, 0.05, 0.1, 0.5 and 1 µg/ml. The effects of the synthetic fungicides were evaluated on the mycelial growth of the *R. microporus* using the poisoned food techniques (Rajani *et al.,* 2012, Ogbebor *et al.,* 2015a).

Two millilitre of each concentration of the fungicides were introduced into the Petri dishes to which 15ml of PDA was added for the test experiment; the control experiment was devoid of fungicide. Each plate was fortified with a mixture of antibiotic solution consisting of streptomycin 300 mg and ampicillin 125 mg suspended in 10 ml of sterile distilled water (Agotini and Timmer, 1992) and allowed to gel for a period of 6-10 hr before inoculation. Each plate was inoculated at the side, 10mm from the edge of the Petri dish with a mycelial disc of 5mm in diameter of the pathogen, taken from the periphery of actively growing 3 day old *R. microporus* culture. Petri dishes were incubated at 28 ± 2 °C. Effects of synthetic fungicides were assessed by recording the daily radial growth of the mycelia of *R. microporus* taken from the edge of the inoculum to the Petri dish.

The Percent Inhibition (PI) of growth in each of the treatments was calculated with respect to the control (Kaiser *et al*., 2005; Ogbebor *et al.,* 2007). The Percent Inhibitions were rated for their inhibitory effects using the scale described by Sagoyomi (2004); 0%

inhibition-not effective, > 0-20% inhibition-slightly effective, > 20-50% inhibition-moderately effective, > 50- < 100% inhibition-effective, and 100% inhibition-highly effective.

Minimum inhibitory concentration, defined as the lowest concentration of fungicide that allows no more than 20% *R. lignosus* mycelial growth compared to the growth in the control plate at day one (Savitha and Rathnavijaya, 2011) was used in assessing the minimum inhibitory concentration of the synthetic fungicides.

In vivo evaluation

Inoculation of rubber seedlings and evaluation of the three synthetic fungicides on *R. microporus* were carried out as follows. Healthy rubber seeds from rubber tree were collected and planted in designated plots in the nursery; the seedlings were not transferred thereafter. Two Polyvinyl Chloride (PVC) pipes each of 45 cm in length were cut and buried with 20 cm of their length into the soil around which the rubber seeds were placed per plot. The open ends of the PVC pipes were covered with polytene bags held in place with rubber band to prevent soil and other materials from falling into them. After germination, young seedlings were watered regularly. At one month after planting, each replicate plot was thinned to contain 40 stands of rubber seedlings of uniform growth size.

Rigidoporus microporus culture was prepared by culturing in sterilized inoculum medium (100g rubber wood sawdust, 3g rice bran and 2g sugar, moistened with 20 ml of water) contained in inoculation bottles and incubated at 28 ± 2 °C for 30 days. Base carrier (100g rubber wood sawdust and 3g rice bran, moistened with 20 ml synthetic fungicides) was used for the synthetic fungicides.

Inoculation of the seedlings was carried out by removing the pipes and filling the holes with the different treatments (Tridemorph + *R. microporus*; Benomyl + *R. microporus*; Bayfidan R. *microporus*; *R. microporus* control and negative control). *R. microporus* inoculum and base carrier of synthetic fungicides were applied close to the taproot of the one month old seedlings at a depth of 20 cm in the soil through pre-bored holes protected with PVC pipes inserted during planting of seedlings to avoid wounding of the roots. Any plant that died during the course of the experiment was uprooted and disease assessment carried out. Early stage of infection was evaluated two months after inoculation by choosing ten plants at random. At the time of termination of the experiment, five months after inoculation, twenty five surviving plants selected randomly were removed from the soil and assessed for infection.

To quantify the rate of infection on inoculated plants dead or living, several criteria were used: time between inoculation and plant death, presence of rhizomorphs on roots, length of stem and tap root, occurrence of reactional rhizogenesis i.e. neo-formation of lateral roots that replaced the original decayed tap root, colonization rate of root tissues and foliar symptoms. Root penetration by rhizomorph was measured by presence of neo-formation of lateral roots that replaced the original decayed tap root of the rubber seedlings. Foliar symptom was assessed by using the disease score–rating chart from which infection indexes were calculated according to Adekunle and Ogbebor (2005).

The *in vitro* experiment was conducted in a Completely Randomized Design with four replications; while the *in vivo* experiment was conducted in a Randomized Complete Block Design with four replications. The experiments were carried out twice and data from the two experiments were combined for statistical analysis using Genstat (8.1) software.

Results

Fungicidal control of *R. microporus*

Concentration effects of Bayfidan, Benomyl and Tridemorph on mycelial growth of *R. microporus* after 4 day incubation (28 ± 2 °C) are presented in Table 1. The interaction of the three fungicides at seven different concentrations at day 4 after inoculation was significant ($P < 0.01$). The highest percent Inhibition (100%-highly effective) was recorded for Tridemorph

(concentration of 0.05 µg/ml), while the lowest percent Inhibition (73.33%) was recorded for Bayfidan at concentration of 0.001µg/ml. Percentage inhibition recorded with Tridemorph (0.050, 0.100, 0.500, 1.000µg/ml) and Benomyl (0.5, 1.0 µg/ml) were rated highly effective ($p < 0.05$). The percent Inhibition in the 7 concentrations of Bayfidan; 4 concentrations of Benomyl (0.001, 0.005, 0.010, 0.050µg/ml) and three concentrations of Tridemorph (0.001, 0.005, 0.010µg/ml) were rated effective.

The percent Inhibition in the 3 fungicides increased with increase in fungicides concentrations (Bayfidan at concentration of 0.001µg/ml increased from 73.33% to 93.33% at concentration of 1.000µg/ml; Benomyl increased from 75.33% to 100%; and Tridemorph increased from 87.33% to 100%). The percent Inhibition recorded at fungicide concentration of 0.001 µg/ml with Bayfidan and Benomyl were not significantly different ($p < 0.05$).

Table 1 Percentage inhibition of *Rigidoporus microporus* by three synthetic fungicides after 4 days incubation at 28 ± 2 °C.

Concentration (µg/ml)	Mycelial growth inhibition (%)/Effectiveness level					
	Bayfidan		Benomyl		Tridemorph	
0.001	73.33^{a}	(E)	75.33^{a}	(E)	87.33^{cd}	(E)
0.005	83.67^{bc}	(E)	77.33^{a}	(E)	93.67^{e}	(E)
0.01	85.67^{bcd}	(E)	82.00^{b}	(E)	94.00^{e}	(E)
0.05	85.67^{bcd}	(E)	82.67^{b}	(E)	100.00^{f}	(HE)
0.1	88.67^{d}	(E)	90.00^{de}	(E)	100.00^{f}	(HE)
0.5	88.33^{d}	(E)	100.00^{f}	(HE)	100.00^{f}	(HE)
1	93.33^{e}	(E)	100.00^{f}	(HE)	100.00^{f}	(HE)

Means with the same letters in table are not significantly different from each other **(**Tukey's range test, $\alpha = 0.05$). All values are mean of 4 replicates. E = effective**;** HE = highly effective.

Minimum inhibitory concentration of fungicides

Minimum inhibitory concentration of the mycelial growth of *R. microporus* by the three synthetic fungicides (Tridemorph, Benomyl and Bayfidan) is presented in Table 2. The minimum inhibitory concentrations were detected within the range tested at the various concentrations of Tridemorph, Benomyl and Bayfidan, with the exception of Bayfidan at concentration of 0.001µg/ml.

Table 2 Minimum inhibitory concentration of the three synthetic fungicides against *Rigidoporus microporus*.

Treatment	Minimum inhibitory concentration	
Bayfidan	0.001	ND
	0.005	+
	0.010	+
	0.050	+
	0.100	+
	0.500	+
	1.000	+
Benomyl	0.001	+
	0.005	+
	0.010	+
	0.050	+
	0.100	+
	0.500	+
	1.000	+
Tridemorph	0.001	+
	0.005	+
	0.010	+
	0.050	+
	0.100	+
	0.500	+
	1.000	+

+: Minimum inhibitory concentration detected within the range tested.
ND: Minimum inhibitory concentration not detected within the range tested.

Effect of synthetic fungicides

***In vivo* evaluation at 2 months after inoculation**

The *in vivo* evaluation of the effects of synthetic fungicides on *R. microporus* at 2 months after inoculation is presented in Table

3. Significant differences were observed between length of stem, length of tap root, plant death, presence of rhizomorph, reactional rhizogenesis, root penetration, and foliar symptoms respectively with the different treatments ($p < 001$). At two months after inoculation the seedlings in Tridemorph treatment had no death (0.00%). *Rigidoporus microporus* control treatment recorded the highest number (0.94%) of plant death. Plant death recorded in the *R microporus* control treatment was significantly ($p > 0.05$) different from those recorded in the other treatments.

In all the treatments rhizomorph developed at the surface of the tap roots. Presence of rhizomorph was comparable among the treatments ($p < 0.05$), except in the negative control ($P > 0.05$). Tridemorph treatments recorded the highest length of stem (36.57 cm) compared to the negative control. Seedling in Benomyl treatment had the lowest length of stem (31.96 cm) and length of tap root (21.68 cm). Length of tap root recorded in with the different synthetic fungicides was not significantly different ($p < 0.05$). Root penetration by rhizomorph of *R microporus* was highest (0.56%) in the *R. microporus* control treatment seedlings and was significantly ($p > 0.05$) different from those recorded in the other treatments including the negative control.

Reactional rhizogenesis was nil in all the different treatments except in *R. microporus* control treatment ($P > 0.05$). Varied percentages of foliar symptoms were recorded with the different treatments on the rubber seedlings. Foliar symptoms of leaf spot were highest in *R. microporus* control treatment and were comparable ($p < 0.05$) to that with Benomyl treatment. The lowest (3.44%) percentage of foliar symptom was recorded in the negative control.

***In vivo* evaluation at 5 months after inoculation**

The *in vivo* evaluation of the effects of synthetic fungicides on *R. microporus* at 5 months after inoculation is presented in Table 4. Significant differences were observed between stem length, length of tap root, presence of rhizomorph, reactional rhizogenesis, root penetration and foliar symptoms of leaf spot respectively with the different treatments (F pr. < 001), except plant death ($p < 0.045$). At 5 months after inoculation of seedlings in the field, the percentage of plant death ranged from 0.11% to 0.61%. Seedlings in *R. microporus* control treatment recorded the highest (0.61%) plant death; while the lowest (0.11%) percentage of plant death was recorded with Tridemorph and in the negative control respectively. Plant death in all the treatments were not significant ($p < 0.05$) except with *R. microporus* control treatment. The highest percentage of the presence of rhizomorph was recorded in the *R. microporus* control treatment; while the lowest was recorded in the negative control.

The length of stem varied with the different treatments, with the highest (69.11 cm) recorded in the negative control. *Rigidoporus microporus* control treatment recorded the lowest (51.89 cm) length of stem, and was followed by Benomyl (56.06 cm). Length of stem observed in the *R. microporus* control treatment, Benomyl and Tridemorph treatments were comparable ($p < 0.05$). Length of tap root recorded in the different treatments was comparable ($p < 0.05$) except in the negative control. Seedlings in the negative control had the highest (59.17 cm) length of tap root, while Benomyl recorded the lowest (45.11 cm) length of tap root.

Seedlings in the *R. microporus* control treatment had the highest percentage of root penetration (0.61%), and percentage reactional rhizogenesis (0.67%); while their negative controls recorded the lowest percentage of root penetration (0.06%) and percentage reactional rhizogenesis (0.00%). The percentage of foliar symptoms recorded varied among the different treatments. Seedlings in *R. microporus* control treatment had the highest (23.06%) percentage of foliar symptom, while the lowest (12.06%) was recorded in the negative control.

Table 3 *In vivo* evaluation of three synthetic fungicides on *Rigidoporus microporus* at 2 months after inoculation.

Treatment	LS (cm)	LTR (cm)	Rate of infection (%)				
			PD	PR	RR	RP	FS
Bay + *R. microporus*	27.57 [a]	21.17[a]	1.70[a]	100[b]	0.00[a]	0.00[a]	24.33b
Ben + *R. microporus*	31.96[ab]	21.68[a]	1.70[a]	100[b]	0.00[a]	0.00[a]	23.89b
Tri + *R. microporus*	36.57[c]	23.73[a]	0.00[a]	100[b]	0.00[a]	0.00[a]	14.61a
R. microporus control	34.26[bc]	28.24[b]	9.40[b]	100[b]	0.61[b]	0.56[b]	25.17b
Negative Control	46.09[d]	38.64[c]	1.20[a]	16.67[a]	0.00[a]	0.00[a]	3.44a

Means with the same letters along the same column are not significantly different from each other (Tukey's range test, α=0.05). Bay: Bayfidan, Ben: Benomyl, Tri: Tridemorph, LS: Stem length, LTR: Length of tap root, PD: Plant death, PR: Presence of rhizomorph, RR: Reactional rhizogenesis, RP: Root penetration, FS: Foliar symptoms, all readings are mean of 10 plants.

Table 4 *In vivo* evaluation of three synthetic fungicides on *Rigidoporus microporus* at 5 months after inoculation.

Treatment	LS (cm)	LTR (cm)	Rate of infection (%)				
			PD	PR	RR	RP	FS
Bay + *R. microporus*	56.06 [a]	45.11[a]	2.80[a]	61.11[b]	0.06[a]	0.17[a]	18.28bc
Ben + *R. microporus*	56.33[a]	50.11[a]	2.20[a]	55.56[b]	0.06[a]	0.11[a]	18.00bc
Tri + *R. microporus*	58.94[a]	47.67[a]	1.10[a]	55.56[b]	0.06[a]	0.11[a]	15.89ab
R. microporus control	51.89a	46.78[a]	6.10[b]	94.44[c]	0.67[b]	0.61[b]	23.06c
Negative Control	69.11[b]	59.17[b]	1.10[a]	16.67[a]	0.00[a]	0.06[a]	12.06a

Means with the same letters along the same column are not significantly different from each other (Tukey's range test, α = 0.05). Bay: Bayfidan, Ben: Benomyl, Tri: Tridemorph, LS: Stem length, LTR: Length of tap root, PD: Plant death, PR: Presence of rhizomorph, RR: Reactional rhizogenesis, RP: Root penetration, FS: Foliar symptoms, all readings are mean of 10 plants.

Discussion

The use of fungicide in the management of fungal pathogens had been well documented (Chan *et al.,* 1991, Gohet *et al.,* 1991, Donald *et al.,* 2001, Omorusi, 2012). The three synthetic fungicides used in the study were effective against *R. microporus*. Tridemorph was most effective on *R. microporus* while Bayfidan was least effective. Gohet *et al.* (1991) used two applications of triadimenol spaced 6 months apart in liquid form (Alto, Sandoz) or triadimenol applications in granular form (Bayfidan, Bayer) at 0.5 g a.i. per tree, and had a good result in field trials. According to Chan *et al.* (1991), incidence of white root rot in already infected rubber trees declined markedly with progressive rounds for monthly and bimonthly application of Bayfidan than trimonthly; and concluded that growth rates of treated tress which recovered returned to normal about nine months later. Investigation by Omorusi (2012) revealed significant reduction of white root rot disease following treatment by Calixil fungicide (a.i. tridemorph).

Minimum inhibitory concentration was detected at all the concentrations of the three fungicides tested, except with Bayfidan at fungicide concentration of 0.010µg/ml. The minimum inhibitory concentration value for Bayfidan was between fungicide concentrations of 0.001µg/ml and 0.005µg/ml. The result with the minimum inhibitory concentration obtained with Bayfidan indicated that Bayfidan is least effective among the three fungicides tested.

To distinguish differences in the effects of synthetic fungicides on *R. microporus*, the study was undertaken to approach natural conditions. Insertion of inoculum created artificial system and greatly enhanced infection (Merrill and Shigo, 1979, Nandris *et al*., 1987, Ogbebor *et al.*, 2015a & b).

Seedlings in Tridemorph treatment had the highest plant health (lowest plant death, highest length of stem and length of tap root, and least foliar symptom) both at 2 months and at 5 months after inoculation compared to seedlings in the control experiment. Plant health in seedlings in the control and Tridemorph treatment were not significantly different ($p < 0.05$), except difference observed in the length of stem and length of tap root.

The *in vivo* study showed that the percentage of plant death and presence of rhizomorph recorded at two months after inoculation were higher than that recorded at the termination of five months experimental period. All the treatments developed rhizomorph at the surface of their tap roots with significant reduction recorded in the negative controls at both two months after inoculation and at the termination of the experiment. This demonstrated that relationship existed between pathogenicity and the capacity to produce rhizomorph. Previous works have demonstrated that rhizomorphogenesis and initial penetration of *R. microporus* occur during the first two months after inoculation (Nandris *et al*., 1987).

Decline in plant death from third months onward suggested a reduction in fungal activity of *R. microporus*. This contributed to the reduction in the presence of rhizomorph observed at the termination of the experiment. Similar results had been described in earlier work with rubber seedlings artificially inoculated by *R. microporus* (Nandris *et al.,* 1983). Nandris *et al.* (1983) reported that decrease of the fungal activity observed was due to exhaustion of trophic reserves in the substrate of the inoculum.

In nature however, if a pest population is already at or above economically damaging levels, chemicals are frequently the only suitable answer. In conclusion, Tridemorph was most effective among the three fungicides tested as it exhibited higher mycelial percentage inhibition in the management of *R. microporus in vitro*, and higher growth parameters measured (length of stem and length of tap rot) and lower percentage of rate of infection (plant death, presence of rhizomorph, reactional rhizogenesis, root penetration and foliar symptom) *in vivo.* This study therefore demonstrated that Tridemorph is best among the three synthetic fungicides in the management of *R. microporus* in rubber. However, when using pesticides, we must weigh the benefits against the risks and only use pesticides when necessary.

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کنترل قارچ *Rigidoporus microporus* توسط قارچ‌کش‌های شیمیایی روی درخت کائوچو در نیجریه در شرایط درون زیوه ای و درون شیشه ای

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چکیده: قارچ *Rigidoporus microporus* یکی از عوامل مهم بیماری‌زای درخت کائوچو *Hevea brasiliensis* در مناطق کاشت این درختان در دنیا محسوب می‌شود. در شرایط درون شیشه ای (*in vitro*) اثر قارچ‌کش‌های تریدمورف، بنومیل و بایفیدان در هفت غلظت مورد بررسی قرار گرفت. در شرایط درون زیوه‌ای (*in vivo*) اثر قارچ‌کش‌ها روی قارچ از طریق مایه‌زنی روی گیاهچه‌های کائوچو ارزیابی شدند. هر سه قارچ‌کش مؤثر بودند اما قارچ‌کش تریدمورف مؤثرتر از بقیه بود. حداقل بازدارندگی بایفیدان در غلظت ۰/۰۰۱ تا ۰/۰۰۵ میکروگرم در میلی‌لیتر بود. درصد مرگ گیاه و حضور ریزومورف‌های ثبت شده در دو ماه بعد از مایه‌زنی خیلی بیشتر از ۵ ماه پس از مایه‌زنی بود. قارچ‌کش‌تریدمورف باعث تولید بالاترین گیاهان سالم ۲ تا ۵ ماه پس از مایه‌زنی شد. کاهش مرگ گیاهچه در ماه سوم حاکی از توقف رشد قارچ می‌باشد. براین اساس قارچ‌کش تریدمورف مؤثرترین قارچ‌کش بود و درصد بالایی از رشد میسلیوم‌ها در محیط کشت متوقف نمود. هم‌چنین در شرایط درون زیوه‌ای گیاهان بیشتری از حمله قارچ *R. microporus* در امان ماندند.

واژگان کلیدی: درخت کائوچو *Hevea brasiliensis*، کنترل شیمیایی، قارچ *Rigidoporus microporus*