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IN VITRO ANTAGONISM CAUSED BY SOME SPECIES OF FUNGI ON *RIGIDOPORUS LIGNOSUS*.

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ABSTRACT

Fungi potentially antagonistic to Rigidoporus lignosus were isolated from a) by placing soil samples on agar plates previously colonized by R. lignosus, or on Trichoderma selective agar and b) from basidiomycete fruit bodies in rubber plantations. Modes of action of potential antagonists against R. lignosus were evaluated by in vitro methods viz: production of volatile or non-volatile inhibitory compounds and ability to overgrow colonies of R. lignosus on agar. Some Trichoderma spp. were strongly antagonistic to R. lignosus in most of the tests. T. longibrachiatum (isolate DZE10) completely inhibited R. lignosus on agar by producing volatile inhibitory metabolites. Trametes sp. (isolates ISO16 & KIR8) antagonized R. lignosus on dual membered plates.

Key words: Biocontrol, antagonism, rubber, mycelial cords, *Rigidoporus lignosus, Hevea brasiliensis.*

INTRODUCTION

White root disease of *Hevea brasiliensis* (Willd. ex Adr. & Juss) is notoriously difficult to manage in most rubber growing countries. In regions such as West Africa, the white root rot is responsible for yield losses up to 50% in old plantations (Nandris, Nicole & Geiger, 1987). In 1970s, heavy casualties due to white root disease in rubber plantations were recorded in Sri Lanka (Liyanage, Liyanage, Peiris & Halangoda, 1977).

Fungal infection occurs through root contact with infected roots of neighboring trees or with inocula in soil. Similarly, mycelial cords generating from these sources may also grow through soil and cause infections. New infections can be prevented by proper cultural and management practices, but control of the disease using fungicide is difficult as the operation involves removal of infected tissues of the roots, subsequently applying a fungicide. Peries (1965) described the possibility of reducing the disease incidence by adding 114 g of sulphur to the soil in planting holes in replantings. This practice causes acidification of the soil, which enhanced the growth of *Trichoderma* spp. that inhibited *R. lignosus* by producing antibiotics.

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The fungicides recommended for treatments are expensive and highly toxic to users and the environment. Therefore, the increasing awareness of major problems such as pollution and the accumilation of toxic chemicals in our food chains, forces us to use non-chemical methods for disease management in agricultural crops.

In this paper we describe methods of isolating fungi from different substrates and evaluation of their modes of antagonism against *R. lignosus* with a scope of using them as biocontrol agents.

MATERIALS AND METHODS

Fungal cultures

The *R. lignosus* (isolate RT1) used throughout the study was obtained from *Rigidoporus* infected rubber roots collected from Ratnapura, a rubber growing area in the wet zone of Sri Lanka. The fungus was maintained on 2% (Difco) Malt Extract Agar at 27 °C (MEA: 15 ml per 9 cm diameter Petri plate), or in elm wood blocks (2 cm^3) which were autoclaved at 15 p.s.i. for 30 min.

Isolation of fungi potentially antagonistic to *R. lignosus* from soils on *Rigidoporus*-pre-colonized agar

To isolate fungi antagonistic to *R. lignosus*, the pre-colonized agar plate method described by Foley & Deacon (1985) was followed. Soils collected from rubber lands of Sri Lanka (top soil) were air-dried for 5-6 days on the indoor bench and sieved through a 2 mm mesh. This soils are subsequently referred to prepared soils in this manuscript. Petri plates (9 cm in diameter) containing 15 ml MEA, were centrally inoculated with 5 mm plugs of *R. lignosus* and incubated at 27°C in darkness, until margins of the colonies reached the edge. Then the agar was cut into six equal sectors and transferred to sterile empty petri plates. Two agar sectors were employed on each plate. Aliquots (0.4 g) of prepared soil samples were carefully placed on the youngest margin of agar sectors. Ten sectors were used for each sample. After 7 and 14 days of incubation in darkness at 27 °C, plates were microscopically examined. Mycoparasitic or Antagonistic fungi were detected by their reproductive structures produced on mycelia of *Rigidoporus*. Using a sterile fine needle, any spores or mycelia suspected as being antagonists were transferred to plates of Potato-Dextrose Agar [PDA, (Difco) 15 ml per 9 cm diameter petri plate]. The isolated fungi were subsequently purified and tested against *R. lignosus* in dual-membered plates as described by Dennis & Webster (1971c), placing plugs (5 mm) removed from colonies of *R. lignosus* and the test fungi on MEA, 3 cm apart from each other. Those fungi which overgrew *R. lignosus* colonies or produced a zone of inhibition between colonies of *R. lignosus* were considered as antagonists to *R. lignosus*. They were retained for further studies. Identification of *Trichoderma* was based on reproductive structures (Rifai, 1969) and the hyphomycetes were identified using keys of Barron (1968) and Ellis (1976).

Isolation of *Trichoderma* spp. antagonistic against *R. lignosus* on *Trichoderma* selective agar

The medium was prepared similarly to that of Papavizas (1982) with a slight modification. It contained (per liter of liquid) 200 ml V-8 juice, 1 g glucose, 20 g agar, 100 mg each of neomycin sulphate, bacitracin, and penicillin G, 25 mg chlortetracycline HCl, 20 mg nystatin and 500 mg sodium propionate. Agar was autoclaved separately and supplemented with solutions of the above ingredients. Petri plates (9 cm diameter) containing 15 ml of supplemented agar were employed for isolation. Micro-volumes of prepared soils were introduced on to the agar by using a sterile moistened tip of a metal rod. Plates were incubated at 27 °C in darkness. Mycelia or spores suspected as of *Trichoderma* spp. were transferred to fresh PDA. At least one *Trichoderma* isolate was selected from each soil sample for the investigation. Identification was based on the reproductive structures (Rifai, 1969). Antagonism of *Trichoderma* spp. against *R. lignosus* was studied using the dual membered plate method as described above.

Isolation of basidiomycetes antagonistic against *R. lignosus* from basidiocarps

Fruiting bodies of wood decaying fungi from different rubber growing sites were intensively air-dried using a fan for about 24 hours and thereafter on the indoor bench for 3-4 days at 27 °C. Cap surface of basidiocarp was sterilized with 70% ethanol in sterile distilled water. Then the top layer of cap was peeled using a sterile scalpel, and pieces of filamentous tissues were transferred to MEA supplemented with benomyl (10 g ml^{-1}), streptomycin and chlortetracycline (each 30 g ml^{-1}). Plates were then incubated at 27 °C in darkness for 3-4 days until fungal colonies appeared on the agar. White fungal colonies suspected as basidiomycetes were transferred to fresh MEA and observed under the microscope either for availability of clamp connections or what appeared to be dolipore septa. Identification of the fungi was based on fruit body structures (Fergus, 1960; Watling, 1973).

Effect of volatile secondary metabolites of antagonistic fungi on *R. lignosus*

The method employed was similar to that used by Dennis & Webster (1971b). Test fungi were centrally inoculated and grown on plates contained 15 ml of PDA for 2-3 days (depending on their growth rate) until the colony margin just reached the edge. *R. lignosus* was centrally inoculated with 5 mm diameter inoculum disks onto separate plates containing 15 ml of MEA. The bases of plates with *R. lignosus* were placed inverted over the bases of plates colonized by test fungi, or uncolonised PDA plates as controls and sealed with parafilm. There were six replicates for each fungal isolate. Linear growth of *R. lignosus* was recorded 4, 5 and 6 days after incubation at 27 °C.

Overgrowth of test fungi on colonies of *R. lignosus*

Plates (9 cm in diameter) contained 15 ml of MEA were inoculated at the margins with 9 mm agar disks removed from growing colonies of *R. lignosus*. They were incubated at 27 °C in darkness for 8 days until the colony margin reached the opposite edge. Inoculum (9mm) disks cut from growing cultures of test fungi on water agar (WA) were placed on the youngest margin of *R. lignosus* colonies. Similarly, instead of mycelial plugs, 10^4 air-dried spores of test fungi or 10^4 spores in 0.1 ml of 3% sterilized malt extract (ME) also employed as different types of inocula to be placed on the youngest margin of *R. lignosus* colonies. For obtaining spores, 7-14 days old cultures on PDA were scraped with a sterile spatula after addition of 2 ml sterile distilled water. Then the mycelial-spore suspension was transferred to small sterile plastic tubes, 0.1 ml of Tween 80 was added, and the contents were homogenized using a T25 (IKA-Ultra-Turrax Labortechnik) homogenizer at 13500 rev. min^{-1} for 90 sec. After settling, the suspension was filtered through sterile Whatman filter paper (No. 54) to remove mycelial fragments. The concentration of spores in the suspension was determined using a haemocytometer. To obtain dry spore, a volume of the spore suspension containing 10^4 spores was blotted on sterile Whatman filter paper (No. 3). The area which contained the spores was demarcated and carefully cut and dried for 1 hour at 35 °C in an oven and aseptically placed inverted on the youngest margin of *R. lignosus*. In an alternative method, a spore suspension contained 10^4 spores was mixed with 0.1 ml 3% sterile ME and placed on to the youngest margin of *R. lignosus* colonies. Overgrowth of the test fungi on *Rigidoporus* pre-colonized agar was measured after 6 and 12 days. For this, two adjacent strips of agar, ran accross test fungal mycelial plug towards *R. lignosus* mycelial plug (5 mm wide), were marked right across the bases of plates. After 6 days, pieces of agar (5 mm^2) from one strip were removed successively begining from the one adjoined to the mycelial plug of the test fungus and placed inverted on plates contained 10 ml PDA. On each plate the consecutive number of the agar piece were marked (for eg. 1, 2 or 3...so on) and then the plates were incubated at 27 °C. After 2-3 days plates were examined and outgrowth of test fungi or *R. lignosus* from agar pieces was observed. If the test fungus was outgrown only from the first three agar pieces, it was clear that the test fungus had overgrown for approximately 15mm on *R. lignosus* colony. Difference between test fungi and *R. lignosus* was easily distinguishable by mycelial characters and perhaps by color and sporulating charactors.

RESULTS

Using a range of methods, several species of fungi antagonistic against *R. lignosus* were isolated from Sri Lankan soils (Table 1). Among the isolates obtained from the pre-colonized agar plate method, *Penicillium*, *Aspergillus*, *Fusarium* and *Trichoderma* species were abundant (data not shown). In addition, a black toruloid mycelial fungus was also isolated. Selected isolates were tested for their mode of antagonism to *R. lignosus viz*: production of volatile antibiotics and ability to overgrow mycelia of *R. lignosus* on MEA, and the results are shown in Table 1.

Effect of volatile metabolites of antagonistic fungi on *R. lignosus*

Few of the fungi isolated by the pre-colonized agar plate method which inhibited *R. lignosus* on agar did so by producing volatile antibiotics (Table 2). Some isolates had the potential to inhibit *R. lignosus* upto 32%, whereas *Trichoderma longibrachiatum* (isolate DZE10) isolated on TSM completely stopped its growth. The growth of *R. lignosus* did not reduce (but some times increased) when suspended over colonies of *Phlebiopsis gigantea*, *Trichoderma harzianum* (strain TV 12b), *T. polysporum* (strain T.12) and *T. koningii* (strain R5,24) (Table 2 column 3).

Overgrowth of antagonistic fungi on colonies of *R. lignosus*

To evaluate the potential of isolates to displace the fungal pathogen already established in a food base, their ability to overgrow mycelia of *R. lignosus* was studied and the results are summarized in Table 3. Few antagonists obtained from soils by the pre-colonized agar plate method overgrew *Rigidoporus* colonies. Some of the *Trichoderma* isolates [e.g. *T. koningii* (isolate G10P1) and *Gliocladium virens* (isolate G10P)] completely over grew *R. lignosus* colonies. None of the antagonistic basidiomycetes overgrew *Rigidoporus* colonies.

T. harzianum (strain TV 12b) rapidly grew across the pre-colonized agar from all types of inocula. In contrast, *T. longibrachiatum* (isolate DZE 11P) very poorly colonized from any type of inocula. A third category of behaviour was shown by *T. koningii* (isolate G10P1) which grew well only from agar disk inocula and not from spores. *G. virens* (isolate G10P) grew from agar inocula but relatively poorly from spores unless these were supplemented with nutrients.

Table 1. *Fungi antagonistic to* R. lignosus *obtained from different sources, their modes of antagonism and descriptions of sources from where fungi were obtained.*

Antagonistic fungi (isolate)	Mode of antagonism	Soil type	Soil pH	Agro-climatic zone
Isolated by the pre-colonized agar plate method				
Penicillium spp. (isolates DFS1, DFS3, DFS7)	Ab	RYP	3.9-4.3	Wet
Aspergillus spp. (isolates DFS12, DZE1, KTS1)	Ab	RYP	3.9-4.3	Wet
Fusarium sp. (isolate DFS4)	Ab/HI	RYP	3.9-4.3	Wet
Black toruloid mycelial fungi (isolates DFS14, 15, 17, 18, 10)	OG	RYP	3.9-4.3	Wet
Gliocladium virens (isolate G10P)	Ab	RYP	3.1	Wet
Trichoderma koningii (isolate G10P1)	Ab	RYP	3.1	Wet
Trichoderma longibrachiatum (isolate DZE11P)	Ab	RBE	6.8	Dry
On *Trichoderma* selective medium				
Trichoderma spp.(13 isolates)	Ab	RYP/RBE	3.1-7.3	Dry/Wet
Trichoderma longibrachiatum (isolate DZE10)	Ab	RYP	5.3	Dry
Isolated from fructifications				
Schizophyllum commune (isolate ISO12)	OG	\		Wet
Trametes spp. (isolates ISO10, ISO16, KIR8)	OG/HI			Wet

Ab= antibiosis, HI= hyphal interference (Jayasuriya & Deacon, 1995), OG= overgrow, RYP= red-yellow podsolic soils, RBE= reddish-brown earth.

Table 2. *Effect of volatile metabolites of test fungi on* R. lignosus*. *Colony radial growth of* R.lignosus *suspended over the colonies of test fungi.*

Antagonistic fungi (isolate)	colony radial growth rate cm/day	% of control growth rate
Control	0.95±0.14	100
Aspergillus niger (isolate KGS3)	0.98±0.16	103**
Fusarium sp. (isolate DFS4)	0.89±0.12	93.7**
Phlebiopsis gigantea	1.31±0.06	137.8**
T. harzianum (strain TV12b)	1.03±0.1	108.4**
T. viride (strain TV62)	0.72±0.14	75.7**
T. polysporum (strain T.12)	1.02±0.1	107.3**
T. koningii (strain R5.24)	1.07±0.23	112.6**
Trichoderma sp. (isolate KGRAO)	0.09±0.04	9.9**
T. longibrachiatum (isolate DZE10)	0	0**
T. koningii (isolate G10P1)	0.26±0.01	50.0**
T. longibrachiatum (isolate DZE11P)	0.33±0.01	63.4**
Gliocladium virens(isolate G10P))	0.28±0.02	48**

*means of 24 measurements ± SEM. **values of growth rates are significantly (P>0.05) different from the Student's T test.

Table 3. *Overgrowth (cm) of test fungi employed as different types of inocula on* R. lignosus *(after 12 days)**.

Antagonistic fungi	agar disc	10^4 dry spores	10^4 spores in 3% malt extract
Isolated from soils by the pre-colonized agar plate method			
Fusarium sp.(isolate DFS4)	32±1.4	NP	NP
Aspergillus sp. (isolate DFS12)	0	NP	NP
Aspergillus niger (isolate KGS3)	5±5	NP	NP
Gliocladium virens (isolate G10P)	60±0	25±5.2	60±0
Trichoderma koningii (isolate G10P1)	60±0	0	0
Trichoderma longibrachiatum (isolate DZE11P)	3.3±0	9±3.6	16±2.9
Isolated from soils by placing on TSM			
Trichoderma longibrachiatum (isolate DZE10)	60±0	NP*	NP*
Trichoderma sp. (isolate KGRAO)	60±0	NP*	NP*
Obtained from stock cultures			
Trichoderma viride (strain TV62)	5±0	60±0	60±0
Trichoderma polysporum (strain T.12))	5±0	NP*	NP*
Trichoderma koningii (strain R.5.24)	5±0	NP*	NP*
Trichoderma harzianum (strain TV 12b)	60±0	60±0	60±0
Isolated from basidiomycetous fructifications			
Schizophylum commune (isolate ISO 12)	0	NP	NP
Trametes spp. (isolates ISO16, ISO10, KIR8)	0	NP	NP

mean extents of overgrowth (mm) ± SEM for 4 replicates. NP= tests not performed. NP= tests not performed due to unavailability of conidiospores. TSM= *Trichoderma* selective medium

DISCUSSION

Among the fungi that colonized on *R. lignosus* pre-colonized agar, species of *Penicillium*, *Aspergillus* and *Trichoderma* produced colonies on 90% of the pre-colonized agar sectors (data not shown). In few cases *Fusarium* spp. were also isolated. Another frequently observed fungus was a black toruloid mycelial fungus, which did not produce reproductive structures; it rapidly overgrew on *R. lignosus* pre-colonized agar or on agar pre-colonized by any other fungi which were available on plates. Due to the availability of a large number of test fungi isolated by the pre-colonized agar plate method, only few of them were selected for further investigation. *Fusarium* and *Penicillium* spp. produced volatile antibiotics which inhibited *R. lignosus* on agar. However, only *Fusarium* sp. (isolate DFS4) overgrew *Rigidoporus* colonies for a distance of 32 mm. It also caused damage to *R. lignosus* hyphae in studies of Jayasuriya & Deacon (1995), where hyphal interactions of fungi were studied on water agar films using the video microscopy.

Trichoderma spp. isolated from either methods were highly antagonistic against *R. lignosus* on MEA. Some of them rapidly overgrew colonies of *R. lignosus*. It thus seems that different *Trichoderma* isolates having different antagonistic properties, could be selected from rubber plantation soils by employing different methods of isolations.

When compared the isolates obtained from pre-colonized agar plate method *G. virens* (isolate G10P) and *T. koningii* (isolate G10P1) were equally overgrew *R. lignosus* colonies and produced volatile inhibitory metabolites (Table 1). However, *T. koningii* was slightly differed from *G. virens* in its ability to overgrow *Rigidoporus* colonies when applied on to the colonies as dry spores. However, studies of Jayasuriya & Deacon (1995) indicate the outcomes of hyphal interactions between *R. lignosus* with *G. virens* (isolate G10P) and *T. koningii* (isolate G10P1), where *T. koningii* caused damage to *R. lignosus* hyphae, while *G. virens* did not damage *Rigidoporus* hyphae at all. Similarly, results of the Table 2 indicate that these two particular isolates caused a 50% inhibition of *R. lignosus* on agar by producing volatile antibiotics.

Trichoderma spp. have been successfully used in controlling wood decaying basidiomycetes (Sirinivasan, Staines & Bruce, 1992; Smith, Blanchard & Shortle, 1981) as well as *Sclerotium rolfsii* (Wells, Bell & Jaworski, 1972), *Rhizoctonia solani* (Hader, Chet & Henis, 1979) and Ascomycetes such as *Sclerotinia sclerotiorum* (Lib) de Bary (Singh, 1991). The mechanisms of control by *Trichoderma* spp. have been categorized as competition for nutrients (Hulme & Shields, 1970), production of inhibitory soluble metabolites (Dennis & Webster, 1971a; Bruce, Austin & King, 1984), production of volatile metabolites (Dennis & Webster, 1971b) and mycoparasitism involving the production of lytic enzymes (Sandhu & Kadra, 1982; Elad, Chet & Henis, 1982; Chet, 1990; Sivan & Chet, 1988).

In studies of Sirinivasan *et al.* (1992) *Trichoderma* isolates antagonized wood decaying fungi by a complex of mechanisms, i.e. inhibitory soluble metabolites, inhibitory volatiles, lytic enzymes. Similarly, iron competition by sideropore production was reported by Anke, Kinn, Bergquist & Sterner (1991). Due to very low concentration of iron in wood, biocontrol of wood decay fungi by producing sideropores could conceivably be a contributory to mechanism, so that siderophore competition for iron between *Trichoderma* and decay fungi deserves further attention (Siriniwasan *et al.*, 1992).

Most of *Trichoderma* isolates investigated herein produced volatile metabolites which suppressed *R. lignosus* on MEA. *T. viride* (strain TV 62) suppressed *R. lignosus* by about 24% but *T. harzianum* (strain TV 12b), *T. polysporum* (strain T.12) and *T. koningii* (strain R.5.24) instead stimulated the growth. This paralleled the results obtained by Dennis & Webster (1971a) where some *Trichoderma* species possibly produced diffusible growth factors which caused a stimulation of growth of the test fungi. The *Trichoderma* isolates obtained from soil on pre-colonized agar plates and TSM, variably inhibited *R. lignosus*. Notably *T. longibrachiatum* (isolate DZE 10) completely inhibited *R. lignosus*, while in studies of Jayasuriya & Deacon (1995) hyphal contacts of the two fungi did not lead to any damage to *R. lignosus* hyphae. *Trichoderma* spp. related to all the categories of isolations were investigated for overgrowing ability on *R. lignosus* colonies. Most of the *Trichoderma* isolates colonized *R. lignosus* colonies in 6-12 days when introduced on agar discs. In some cases, marked difference on overgrowth of *Trichoderma* on *R. lignosus* was not observed when ME was applied as additional nutrients. However, *G. virens* (isolate G10P), behaved in other way when introduced as dry spores. The growth across the colonies of *R. lignosus* was not indicated when it was introduced as dry spores. The interpretation of this phenomenon may be difficult as *G. virens* (isolate G10P) did not differ from other isolates in other properties studied.

Non of the antagonistic basidiomycete overgrew *Rigidoporus* colonies, presumably due to the fact that they may require a substantial nutrient trigger to initiate their competence of antagonism against *R. lignosus*. However, it was evident from studies of Jayasuriya & Deacon (1995) that these antagonistic basidiomycetes have different modes of antagonism such as hyphal interference as described by Ikediugwu, Dennis & Webster (1970), Ikediugwu & Webster (1970a,b), or competition by overgrowing. Therefore, it might be desirable to weaken the pathogen mycelium and investigate the potentiality of antagonists on initiating a displacement of the established pathogen.

In vitro work demonstrated marked difference in antagonistic activity of *Trichoderma* strains obtained from soils of rubber plantations in Sri Lanka. This parallels many other studies that have shown variation in antagonism by *Trichoderma* strains (Jayasuriya & Deacon, 1995; Tong-Kwee & Keng, 1990; Sirinivasan *et. al.*, 1992). Scarselletti & Faull (1994) recently suggested a strong relationship between antifungal activity of *T. harzianum* strain and its production of 6-pentyl μ-pyrone, an antibiotic that is a major component of the coconut aroma of *Trichoderma* (Collins & Halim, 1972). However, Dennis & Webster (1971b) in their studies described that this coconut smell may have come from a secondary product without antibiotic properties.

CONCLUSIONS

In soils of rubber plantations in Sri Lanka, fungi antagonistic against *Rigidoporus lignosus* are naturally existing. From those naturally existing antagonistic fungi, it seems possible to select fungi antagonistic to *R. lignosus in vitro*. Their modes of antagonism against *R. lignosus* differ by their nature of existence in the rhizosphere. Species of *Trichoderma* being strong antagonists in a rhizosphere such as soil, exhibited similar *in vitro* antagonism against *R. lignosus*. However, this antagonism varied among different isolates. Therefore, in selecting *Trichoderma* isolates as biocontrol agents it would seem preferable to use a strain that can colonise from its spores to displace *R. lignosus* from colonized substrate if all other properties of *Trichoderma* are similar. Some antagonistic basidiomycetes may be preferably used *in vitro* as established inocula to displace *R. lignosus* from similar substrates.

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