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The biotic effect of the surface microflora on *Phytophthora meadii* infection of rubber petioles

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Abstract

Microbes in petiole surface of Hevea brasiliensis *Muell. Arg (rubber) resistant (RRIC100, BPM24) and susceptible (PB86, RRIM600) clones to* Phytophthora *leaf disease were isolated. The abundance of microorganisms and their antagonism towards* Phytophthora meadii in vitro *were investigated.* Penicillium *and* Pestalotiopsis *spp. were isolated from all the clones, while highly antagonistic* Trichoderma spp. *were found only on petioles of resistant clones. Surface sterilisation of petioles of resistant clones caused a significant increase in* P. meadii *infection showing the possible contribution of petiole surface inhabiting fungi in resistance. Antagonistic bacteria were associated with petioles of susceptible clones possibly increasing its susceptibility to* P. meadii. *Results suggest a possibility of increasing the resistance towards* Phytophthora *leaf disease in susceptible rubber clones by introducing* Trichoderma *as a biological control agent.*

Key words: antagonists, biocontrol, leaf disease, *Trichoderma*

Introduction

Hevea brasiliensis (Willd. Ex Adr. De Juss.) Muell. Arg. (rubber tree) is considered as a major plantation crop in many south Asian, African and Latin American countries which produce natural rubber for the world market. The economies of this tree crop mainly depend on the latex yield potential and disease resistance. Many high yielding rubber clones have succumbed to devastating leaf disease caused by *Corynespora cassiicola*, forcing their withdrawal from plantations. Some *Phytophthora*, *Colletotrichum* and *Oidium* species also cause leaf diseases resulting in seasonal crop losses.

Diseases caused by *Phytophthora* on rubber are petiole infection, pod rot, bark rot, abnormal leaf fall and shoot die-back. At present, *P. meadii* McRae (the most common sp.), *P. botryosa* Chee, *P. citrophthora* (R.E. Sm. & E.H. Sm.) Leonian, *P. nicotianae* Van Breda de Haan var. *parasitica* (Dastur) Waterhouse and *P. phaseoli* Thaxter are causal agents of rubber diseases in Sri Lanka (Liyanage & Wheeler, 1989). However, *P. Palmivora* (Butl.) Butl. (Danthanarayana *et al.*, 1984) and *P. citricola* (Liyanage, 1989) occurred rarely on rubber in Sri Lanka.

The most distinct symptom of the disease is seen on the petiole, where

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characteristic dark brown lesions appear with white globules of coagulated latex causing leaves fall along with the petiole, while they are still green, or some times after assuming reddish color. Severe infection can cause a complete defoliation resulting in retardation of the growth and the yield. In bark rot conditions vertical depressions are seen just above the tapping cut area during the initial stages. At later stages when the bark is removed, black stripes can be seen on the woody area.

The leaf fall caused by *Phytophthora* spp. occurs during the southwest monsoon in the wet regions. The fungus has a wide range of alternate hosts and usually survives on mummified rubber pods, stalks, shoots or in their debris in the soil or, as thick walled oospores or chlamydospores. With the onset of rain, the fungus spreads rapidly and attacks mature green rubber pods, which contain ideal substrates for growth and sporulation of the fungus.

At present, a number of clones, which can withstand *Phytophthora* infection at least moderately, are available. Normally, the clones are recommended only after thorough screening for resistance to the disease. RRIC100 is one of the most resistant clones to the *Phytophthora* leaf fall and bark rot disease in Sri Lanka. However, the resistance level of the clones can vary according to the geographic locality.

Although, application of fungicides controls leaf diseases in the field as well as in nurseries, the cost involved is relatively high, reducing the crop's economy. Exploiting resistant clones and adoption of bio-control measures would therefore be more acceptable.

In nurseries, leaf blades and stalks of rubber clones succumb to leaf diseases during humid weather, which provide extremely favourable habitat for fungi. In Sri Lanka, pathogenic fungi such as *Alternaria*, *Botryodiplodia* spp., *Colletotrichum acutatum*, *C. gloeosporioides*, *Fusarium*, *Phomopsis*, *Pestalotiopsis* spp., and non-pathogenic fungi such as *Aspergillus*, *Beltrania*, *Cladosporium*, *Curvularia*, *Mucor*, *Penicillium*, *Periconia*, and *Trichoderma* spp. were reported as phylloplane fungi on rubber (Jayasinghe, 1997). They may affect important pathogens such as *Phytophthora meadii* McRae on petioles. However, any relationship between surface inhabiting fungi and rubber leaf pathogens particularly *Phytophthora* in resistant or susceptible clones has not been reported. This paper presents the results of a comparative study conducted on fungi from petiole surface of susceptible and resistant clones of rubber *in vitro*. The study may contribute to develop a model for the biological control of *Phytophthora* leaf disease (*P*ld) of rubber, as an alternative for control.

Materials and Methods

Phytophthora culture

Phytophthora meadii (IMI 385259) used has been isolated from an infected petiole of rubber clone PB86 (Jayasuriya *et al.*, 1999), and

maintained on Lima Bean Agar (LBA) (Difco).

Isolation of phylloplane microorganisms from rubber petioles

Twenty fully matured petioles were excised from ten 4-year-old trees of each of four clones (ensuring the middle part of petioles is untouched) in the early hours of the day. The clones, RRIC100, BPM24, are resistant and the clones, RRIM600, PB86, are susceptible to *P*ld (Jayasinghe, 1996) and grew in the same location. Petiole pieces (*ca* 2 cm) cut with a sterile blade, were aseptically transferred to Potato Dextrose Agar (PDA) (Difco) plates and the plates were incubated for 3 days at room temperature (27 ± 2° C) under natural day/night light cycle. Fungi and bacteria grown were transferred onto PDA or nutrient agar (Difco) respectively. Fungal spp. were identified to the genus according to Barron (1968) and Rifai (1969) and bacteria were gram stained.

Determination the interactions between the isolated petiole surface fungi and *P. meadii*

Fungal isolates were paired with *P. meadii*, in 9 cm diam. agar plates containing 15 ml of PDA as described by Jayasuriya (1997) with modifications. Mycelial plugs (5 mm diam.) cut from the growing edge of 3 day old colonies of *P. meadii* and each test fungus (on PDA) were placed to opposite each other *ca* 5 cm apart. Cultures were incubated in a glass culture chamber for 3 days at room temperature. The interaction of both fungi was assessed according to following scale (Jayasuriya, 1997):

0= no visible signs of inhibition of *P. meadii*, the mycelium of *P. meadii* overgrows the colony of the test fungus; 1= the inhibition zone between two organisms > 2 cm in width, *P. meadii* grows across the centre line of the agar dish; 2= both organisms stop to grow on the contact line at the centre of the agar, 3= the inhibition zone between the two organisms < 1 cm in width, test fungus grows across the centre line of the agar; 4= the inhibition zone > 1 cm in width, test fungus grows across the centre line of the agar; 5= test fungus overgrows or displaces *P. meadii*.

Determination of the interaction between petiole surface inhabiting bacteria on *P. meadii*

A mycelial plug (5 mm diam.) of *P. meadii* was placed at the centre of an agar plate containing 15 ml of nutrient agar as described above. A bacterium was streaked opposite *ca* 5 cm from the *P. meadii* plug and the plates were incubated for 3 days as described above. The interactions between bacteria and *P. meadii* was assessed according to following rating: 1= *P. meadii* colony diameter is inhibited to 2/3 compared to the control, with a distinct inhibition zone; 2= *P. meadii* colony diameter is inhibited to 1/2 compared to the control, with a distinct inhibition zone; 3= *P. meadii* colony diameter is inhibited to 1/3 compared to the control, with a distinct inhibition zone; 4= no inhibition of *P. meadii* colony diameter compared to the control. The degree of the inhibition or

stimulation was calculated by summing all the scale points and made average values by dividing with available number of isolates.

Colonisation of rubber petioles by *P. meadii*

The test was carried out on sterile or un-sterile rubber petioles. Fifty petioles from 10 trees of each clone were collected from 4-year-old trees of clones resistant (RRIC100) or susceptible (PB86) to *Phytophthora.* The middle parts of petioles retained untouched, and about 25 petioles were surface sterilised by dipping in 70 % (v/v) ethanol for 1 min followed by gently wiping with a sterile absorbent cotton wool, rinsing in sterilised distilled water (sdw) followed again by mopping with sterile absorbent cotton wool, and air drying on a laminar flow for 10 min. The effect of sterilisation was checked by aseptically placing few of petiole segments (*ca* 2 cm each, cut with a sterile blade) on PDA and incubating the plates for 7 days in a glass culture chamber at room temperature. The test showed 100 % sterility of petioles. The sterilisation procedure did not damage the petioles epidermis, when examined under a stereo binocular microscope (×300). Another 25 petioles were used directly after collection without surface sterilisation and were considered as controls.

Both sets of petioles were arranged horizontally on glass rods placed parallel on two separate plastic trays. They were inoculated with a zoospore suspension (10^4 spores ml^{-1}) of a 6-day old *P. meadii* as previously described (Jayasuriya *et al.*, 1999). Inocula were applied at four to eight points (*ca* 2 cm apart from each other) on each petiole depending on their lengths. A total of 35-40 inoculation points were employed on five petioles in each case. The inoculated petioles were incubated as described early. The number of progressive lesions, which appeared as tiny black spots on petioles of RRIC100 and as 1-3 cm lesions on petioles of PB86, out of the total number of inoculation points on petioles was counted after 3 days of incubation at room temperature. Mean scores of % infections out of the total inoculated points of each clone were subjected to arcsine transformation and analysed using Wilcoxon-2-sample test (normal approximation) employing NPAR1-WAY procedure in SAS.

Effect of petiole washings on germination of *P. meadii* zoospores

Thirty grams of untouched petioles obtained from RRIC100, RRIM 600 or PB 86 clones (from 5-8 trees of each clone grown in the same location, with the edges were sealed with melted paraffin to prevent leaking any substances from petiole tissues to the suspension) were placed in separate glass containers containing 50 ml of sdw and were shaken at 50 rpm for 15 min on an orbital shaker. The petiole washings were collected and filtered through Millipore filters (NALGEN® PES 0.2 μm), freeze-dried immediately and the residue was dissolved in 1 ml of sdw. One μl of the suspension placed on sterilised glass slide was added to 29 μl

of *P. meadii* zoospore suspension having 3×10^4 zoospores ml^{-1} and after 1 h, a drop of cotton blue in lactophenol was added to the solution to terminate germination before counting. One μl of sterilised distilled water added to 29 μl of *P. meadii* zoospore suspension having 3×10^4 zoospores ml^{-1} served as control. The germinating zoospores were counted under 100× magnification. Zoospores were considered to have germinated when the germ tube was longer than the breadth of the spore. Approx. 250 randomly selected zoospores were assessed using five different slides prepared using each petiole-washing from each clone and the results were analysed by ANOVA.

Results

Antagonistic micro-flora on petiole surface

The effect of fungi and bacteria isolated from petiole surface on the growth of *P. meadii* is shown in Table 1. *Penicillium* and *Pestalotiopsis* spp. were common inhabitants on petioles of both clones. However, highly antagonistic *Trichoderma* spp. (degree of antagonism = 5) were isolated only from the petioles of resistant clones, while BPM24 petioles possessed all fungal spp. that were isolated from susceptible petioles as well. Other fungi were not consistently isolated from 4 clones, while bacteria, which caused

Table 1. *Degrees of interactions of petiole surface inhabiting fungi or bacteria on* P. meadii

Fungi or bacteria	*H. brasiliensis* clones			
	PB86	RRIM600	RRIC100	BPM24
Penicillium spp.	2	**2**	**2,3**	**2**
Fusarium spp.	**1, 3**	**3**	-	**3**
Aspergillus niger	-	**3**	-	3
Aspergillus sp.	-	-	-	2
Pestalotiopsis spp.	1	1	2, 3	**3**
Botriodiplodia spp.	2, 3	2	-	2, 3
Colletotrichum sp	-	2	2	2
Trichoderma sp.	-	-	5	5
Ca rating average	2	2.16	2.8	2.7
Bacterial spp.				
($Gram^+$) cocus	+3	+3	-	+2
($Gram^-$) rod shape	+3	+3	-	-
Ca rating average	3	3	0	2

Values or plus marks indicate the degrees of surface fungal or bacterial antagonism on *P. meadii* as referred in the text, Bold-face-values indicate abundance or more frequent presence of the particular organism in the petiole surface. Absence of data indicates no isolation of the organism from particular clone.

antagonism on agar were abundantly isolated from petioles of susceptible clones.

Effect of pathogenicity

On surface-sterilised or undisturbed petioles from susceptible clones, all infections after application of *P. meadii* zoospore suspension appeared as large lesions of 1-3 cm, while those of on surface-sterilised or undisturbed petioles from resistant clone, appeared as tiny lesions of 0.1-0.3 cm diameter. The infection rate on petioles of susceptible clones was 40.5% (Table 2). The infection was significantly ($P<0.0005$) lower (6.9 %) on sterilised petioles. On petioles of resistant clone, the infection rate was 2% and 7.5 % on the undisturbed and surface sterilised petioles respectively.

Effect of petiole washings on germination of *P. meadii* zoospores

Significantly ($P<0.05$) lesser zoospores were germinated in petiole washings of resistant clone (RRIC100) than the washings from petioles of susceptible clones (PB 86, RRIM 600) (Table 3).

Table 2. *Effect of surface sterilisation of petioles of* Hevea brasiliensis *on the colonisation of* Phytophthora meadii

Clones	Response to *P*ld	Disease severity index (%)		(P > | Z |)
		Unsterilised (control)	Surface sterilised	
RRIC100	*Resistant*	2.0 ± 2.0	7.5 ± 2.7	0.0925
PB86	*Susceptible*	40.5 ± 7.0	6.9 ± 2.9	0.0005

Values are means of *ca* 40 inoculated points ± SEM, *P*ld = *Phytophthora* leaf disease. Non surface sterilised petioles were inoculated without surface sterilisation, P= absolute probability, Disease severity index was calculated out of *ca* 40 inoculated points.

Table 3. *Effect of petiole washings on* Phytopthora meadii *(MAD86) zoospore germination*

Rubber clones	Response to *P*ld	Germination (%)	% germination compared to the control
Control (sdw)		89.55^{a}	100
RRIC100	Resistant	69.33^{c}	77.42
PB86	Susceptible	77.33^{b}	86.35
RRIM600	Susceptible	79.77^{b}	89.07
		LSD = 0.03967	

Values (means of 10 replicates) with same letters are not significantly different ($P<0.05$). *P*ld = *Phytophthora* leaf disease.

Discussion

Microorganisms co-exist in the phylloplane in a well-balanced system which is controlled by antagonism or competition for nutrients and/or space through production of antibiotics (Deacon, 1984), mycoparasitism (Lumsden, 1981) and 'hyphal interference' (Ikediugwu & Webster, 1970). The results of the present study show the presence of a few microorganisms, on petiole surface of rubber clones grown in the field located in the same area, which are antagonistic to *P. meadii in vitro*. *Penicillium* spp., were common on all types of clones, and less antagonistic than *Trichoderma* spp., which were present on petioles of resistant clones of rubber. The test on dual cultures in which *Trichoderma* and *P. meadii* were paired, confirmed its antagonism on *P. meadii*. Although *Aspergillus* or *Fusarium* spp. were strongly antagonistic to *P. meadii* (Table 1), *Trichoderma* spp. were reported as antagonists on *Aspergillus* and *Fusarium* spp. (Calistru *et al.*, 1997). Thus *Trichoderma* on RRIC100 and BPM24 would be considered as a major significance to the resistance perhaps by production of water-soluble antibiotics. *Trichoderma* is also strongly antagonistic towards some leaf pathogens of rubber such as *Colletotrichum gloeosporioides* (Penz.) Sacc, *Corynespora cassiicola* (Berk. & Curt.) Wei, or *Bipolaris heveae* (Petch) Von Arx (Jayasuriya, 1997) and they are detrimental to several other plant pathogens too (Dumas & Boyonoski, 1992; Bell *et al.*, 1982; Kelley, 1997). Therefore, it is of interest to investigate the effect of an artificial inoculation of *Trichoderma* spores with adequate nutrients on to the susceptible petiole surface and explore the possibility of *Trichoderma* to suppress *Phytophthora* infections providing the favourable environmental factors for survival of the introduced inocula of the antagonist prevail.

Some Gram-positive bacteria such as *Bacillus subtilis* 205, were used as biocontrol agents (Fiddaman & Rossall, 1995). Although several bacterial species were associated with the petiole surface of susceptible PB86, they do not seem to produce enough antibiotics on petiole surface to inhibit germinating *P. meadii* zoospores as nutrients are minimum on the petiole surface. Instead, bacteria spp. (Gram-positive or -negative) on susceptible petiole surface may enhance the germination of zoospores as the elimination of bacteria on PB86 by surface sterilisation, caused a significant drop of % infection rate. This could probably be described as a positive contribution of bacteria spp. towards the susceptibility to *P*ld. Bacterial cell wall polysaccharides or lipopolysaccharides retain Ca^{+2} (Davis *et al.*, 1968; Gubler *et al.*, 1989), amino acids and sugars (Donaldson & Deacon, 1993b) which are known to be necessary for *Phytophthora* zoospore germination could be attributed to this response. In addition, gum Arabic, sodium alginate and polygalacturonate also known to induce encystment of *Pythium* spp. *in vitro* (Donaldson & Deacon, 1993b). Furthermore, compounds rich in uronic acids (a constituent in bacterial capsules

(Davis *et al*, 1968)}, such as poly-*D*-galacturonic acid, poly-*D*-glucuronic acid, pectin or alginates induced encystment of *Pythium* and *Phytophthora* zoospores (Irving & Grant, 1984; Grant *et al.*, 1985; Zhang *et al.*, 1990; Jones *et al.*, 1991; Donaldson & Deacon, 1993a).

The increase of *P. meadii* infection rate on RRIC100 as a result of surface sterilisation can probably be attributed to the elimination of petiole surface inhabiting antagonistic microbes, while the 9 % reduction of same compared to the non surface sterilised on PB86 clone, most probably be due to the elimination of surface associated bacteria which may be favourable for germination of zoospores.

Though the organisms in the non-sterile petiole-washings were not isolated on agar, results indicated the possible availability of antibiotics that are inhibitory to germinating zoospores in sterile petiole-washings of RRIC100. As petioles were properly sealed before shaking and not shaken in sdw for a longer period, it could be assumed that no phenolics has been leached from the cut ends of petioles to the washing. Therefore, the antibiotics in the washing of resistant petioles could mostly be of fungal origin as bacteria were sparsely associated, while the washing of susceptible petioles may contained antibiotics of either fungal or bacterial or both. However, *Trichoderma* or bacteria need enough nutrients for secretion of antibiotic, which might be a crucial factor on petioles as the only nutrient sources virtually available may be the dead mycelial walls of fungi or dead bacterial cells.

Therefore, the antagonistic microbial activities on petiole surface of different rubber clones may have a relationship on pathogen invasions especially of *P. meadii*, the zoospores of which are more sensitive to environmental factors. Therefore, it would be possible to note that an application of germinating antagonistic *Trichoderma* spores to susceptible rubber plant canopies, would control some leaf diseases by increasing resistance.

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Articles from Journals:

1. Seneviratne, P., Flegman, A.W. and Wijesekera, G.A.S. (1995). The problem of surface sterilization of shoot materials of *Hevea*. *Journal of the Rubber Research Institute of Sri Lanka* 75, 51-60.
2. Saha, S., Singh, J.P., Verma, J.P. and Jayaraman, J. (2001). Population dynamics of cotton phylloplane bacteria antagonistic towards *Xanthomonas campestris* pv. *Malvacearum*. *Indian Phytopathology* 54, 409-413.

Books:

1. Domsch, K.H., Gams, W. and Anderson, T.H. (1980). *Compendium of Soil Fungi*. Vol.1. Academic Press, New York. 89 pp.
2. Kimball, J.W. (1970). *Cell Biology*. Addison Wesley Publishing Co., California. 199 pp.

Articles from Books/Collective Publications:

1. Yogaratnam, N. (1983). Weeds and weed control. In: *Handbook of Rubber Culture and Processing*, pp. 99-102 (Eds. O.S. Peries and D.M. Fernando). Rubber Research Institute of Sri Lanka, Agalawatta, Sri Lanka.

2. Butcher, D.N. (1983). The culture of isolated roots. In: Tissue Culture Methods for Plant Pathologists. pp.13-17 (Eds. D.S. Ingram and J.P. Helgeson), Blackwell Scientific Publications. London.

Thesis/Dissertations

1. Mendis, M.H. (1981). Growth requirements of *Hevea* stem callus. MSc Thesis. University of Sri Jayawardenapura, Sri Lanka.
2. Samaraweera, M.K.S.A. (1979). A study of the growth regulator N-dimethylaminosuccinamic acid. PhD Thesis. Long Ashton Research Station.UK.

Tables: Tables should be typed on separate sheets, numbered consecutively in arabic numerals and be designed to fit the page dimensions and occupy no more than one printed page. Vertical rules should not be used. The only lines appearing on a table should be horizontal. Headings should be provided for columns and the first letter should be in capitals. The approximate location of the tables should be indicated on the right hand side margin of the main text.

Photographs/Figures/Illustrations: These should be as concise as possible and not larger than a Journal page. Size of the Journal text page is 6" x 5" (17 x 13.5 cm). Illustrations should be drawn in Indian ink on white bristol board, oil (tracing) paper or graph paper. Folded sheets of illustrations in the printed book are not desirable. Roneoed or photostat copies of illustrations are not acceptable. Photographs should be glossy prints of not more than 6"x 4" in size. Colour pictures are accepted only in case where colour is essential. It should be clearly understood that illustrations are not absolutely essential in a paper, but may be provided only when the descriptions provided in the text are inadequate, and should be kept to the minimum.

Graphs done on Excel are acceptable.

Titles for all figures and graphs should be typed separately and attached to the figures. If you have more than one graph to be included in one page, submit each graph separately with a sample giving details of placement of graphs and any text to be included.

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Scientific names: When first cited, genus, species and authority must be given; later the genetic name may be abbreviated to its initial letter, e.g. *F. lignosus, H. brasiliensis* and so on. It is preferable if cultivars are cited preceded by the abbreviation cv. As botanical names are usually printed in italics, these should be underlined. **Units:** SI (metric) should be used. Authors are advised to consult the relevant British standard (also the Sri Lanka Standard). **Abbreviations:** Ad hoc abbreviations should be avoided and authors are advised to consult the B.S. on "Letter symbols, signs and abbreviations". It should please be remembered that abbreviations are alike in singular and plural. The placing of a dot (stop) after a standard abbreviation should be avoided unless the abbreviated form is likely to be taken for another word, e.g. in. for inch, where a full stop would be necessary. **Headings:** The pattern of headings should be as simple and brief as possible. Usually not more than three classes are needed, including the main class of headings such as INTRODUCTION, MATERIALS AND METHODS and so on. The latter class is usually in bold typed block letters, placed in the centre of the page. The second order headings are bold, left justified and 3rd order headings should be italic bold and always run on headings. **Numerals:** Numerals should be used for specific units of measurements, e.g. 4 cm. 10 gal. 5h, 2 ft and so on. Other quantities, up to and including ten, must be spelt, (except in tables and figures), e.g. two leaves, six plots, eight trees, five larvae, ten days and so on. Although measurements such as days, weeks, months, years, miles are specific, it is preferable to fit them into the latter group by spelling the quantities (up to ten) preceding such measurements. Sentences should never start or end with a numeral. **Capitalization:** Generally initial capitalizing should be limited to names or designations; common names of organic compounds, chemicals and minerals are usually not capitalized except in the case of proprietary products. For examples, aluminum, oxalic acid, boron, potassium, urea *etc*, are not capitalized whereas Antimycin, Epikote, Dutrex, Vaseline *etc* are capitalized.

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