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RESEARCH ARTICLE

Some factors affecting *in vitro* production, germination and viability of conidia of *Corynespora cassiicola* from *Hevea brasiliensis*

T.H.P.S. Fernando[1*], C.K. Jayasinghe[1], R.L.C. Wijesundera[2] and D. Siriwardane[1]
[1] *Rubber Research Institute, Dartonfield, Agalawatta.*
[2] *Department of Plant Sciences, Faculty of Science,University of Colombo, Colombo.*

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Abstract: The fungus *Corynespora cassiicola* causes the very destructive Corynespora leaf fall disease of *Hevea brasiliensis*. The present population of *C. cassiicola* from Sri Lanka includes highly, moderately and poorly sporulating isolates. The fungus sporulated freely on potato dextrose agar at 10 – 35 ºC with a peak at 30 ºC. Cultures showed maximum conidia production after an incubation period of 12 days. Conidia produced well-branched germ tubes from both apices. The percentage germination reduced significantly at 1.5×10^6 conidia / mL. Germination of conidia occurred at temperatures between 5 – 40 ºC with the optimum between 15 – 35 ºC. Free water was not essential for germination but promoted germination. Conidia remained viable when the relative humidity was above 90 % and the ultraviolet radiation (254 nm) inactivated conidia. The most favourable temperature conditions for germination of *C. cassiicola* conidia described above has been observed to be prevalent in the main rubber growing areas in Sri Lanka, particularly during the period of refoliation, making it favourable for the establishment of the disease.

Keywords: *Corynespora cassiicola*, Corynespora leaf fall disease, environmental conditions, fungal pathogen, pathogenicity, rubber plant.

INTRODUCTION

Corynespora cassiicola (Burk. & Curtis) Wei. is a destructive plant pathogen with a wide geographical distribution across tropical, sub-tropical and temperate countries. It has become increasingly important in Australia (Silva *et al*., 2003), USA (Pernezny *et al*., 2002), Italy (Martini *et al*., 2007), Japan (Oka *et al*., 2006), Argentina (Hong *et al*., 2007), Brazil (Silva *et al*., 2006) and South and South East Asia (Chee, 1988; Darmano *et al*., 1996; Jacob, 1997; Jayasinghe, 2003). This fungus affects more than 500 economically important crop species belonging to 50 families, causing leaf spots and rots in stems, fruits, seeds and flowers. The devastating leaf fall disease of *Hevea brasiliensis* (Willd. ex Juss.) Mull. Arg. is notable among them.

Natural rubber (NR) from *Hevea brasiliensis* is one of the most versatile industrial raw materials. The numerous uses of natural rubber, together with its environmental benefits have made this crop important. Corynespora leaf fall disease has become a threat to the natural rubber plantation industry by limiting its productivity level. There have been several global epidemics of this disease, shattering the confidence of rubber growers in the newly bred rubber clones. Hence, rubber growers are uncertain about the disease tolerance of the clones, recommended for planting. The highly dynamic nature of this pathogen and the existence of different physiological races are important factors in the rapid increase of the severity of the disease (Pernezny *et al*., 2002; Silva *et al*., 2003).

The epidemics of Corynespora leaf fall disease caused by *C. cassiicola* has been reported from several Asian countries (Hashim, 1995; Jayasinghe *et al.,* 1996; Jacob, 1997). The main factors favouring the development of the pathogen are the presence of susceptible clones, new races of the pathogen and favourable weather conditions, i.e. high humidity, temperature of 20 − 30°C and dandy weather with moderate rainfall (Radziah *et al.,* 1996; Situmorang *et al.,*1996).

[*] Corresponding author (thpsfernando@yahoo.com)

Development of disease resistant clones has been identified as the best long-term strategy for the management of the disease. However, in sudden disease outbreaks, which occur commonly, the chemical control methods are vital to rehabilitate mature rubber plantations. To optimize the use of chemicals in disease control, information about the epidemiological factors of the disease is essential. However, such information available for the new population of *C. cassiicola* are not adequate (Chee, 1988); (Review of Plant Pathology, up to Aug. 2010). In this background, the present study was undertaken to investigate the factors affecting conidia production, germination and viability of the rubber isolates of *C. cassiicola*.

METHODS AND MATERIALS

Fungus

Fifteen *Corynespora cassiicola* isolates were obtained from diseased leaves of different clones of *Hevea brasiliensis* (Fernando *et al.*, 2009). All isolates were purified and single conidia cultures were raised on potato dextrose agar (PDA) at room temperature (RT: 28 ± 2 ˚C). Based on cultural, reproductive, physiological characters and pathogenicity, which have been studied earlier (Fernando *et al.,* 2009), three isolates (E, L, A) were selected for this study. Cultures were maintained on PDA at RT after establishing their pathogenicity on rubber plants.

Inoculation of the fungus: In the experiments described below, 7 mm diameter mycelial plugs obtained from actively growing 8 d old cultures of the relevant isolate of the fungus on PDA were used for inoclulation. To prepare conidial suspensions, 10 d old cultures of the fungus were flooded with 10 mL sterilized distilled water and the colony surface was mechanically disturbed with a paint brush. The resulting suspension was filtered through muslin cloth and the conidia concentration was adjusted as necessary, using a haemocytometer.

Estimation of conidia production: To determine the number of conidia produced, 10 d old cultures on PDA were flooded with 10 mL of sterile distilled water and the colony surfaces were mechanically disturbed with a paint brush to suspend all conidia. The suspension, after dilution to 50 mL with sterile water, was shaken for 5 min using an orbital shaker (GFL, Germany) at 30 rpm. The resulting decoction was filtered through a muslin cloth and the conidia concentration was measured using a haemocytometer after appropriate dilution with sterile distilled water. Diameters of the individual cultures were measured to determine growth and colony area. Each experiment was replicated 4 times.

Effect of culture age, media, and temperature on conidia production

Culture age: The three isolates (E, A, L) were grown for 12 d at RT under normal light and dark regimes, on 10 mL PDA in Petri dishes. Five replicates were used for each treatment. The conidia production was determined as described above at 2 d intervals.

Culture media: To identify the best medium for sporulation, six commercially prepared media (Difco) namely PDA, Lima Bean Agar (LBA), Czapek Dox Agar (CDA), Malt Agar (MA), Corn Meal Agar (CMA), Water Agar (WA) and laboratory prepared leaf extract agar (described below) were used. Each medium (10 mL) was inoculated with the fungus and incubated at RT under normal light and dark regimes. Five replicates were used for each treatment. At the end of the incubation period, conidia production was estimated as described above.

***Preparation of leaf extract agar*:** Semi-mature leaves (10 g) from the clone RRISL 202 (CLFD susceptible clone) were ground in 100 mL sterile water and filtered through a muslin cloth. Four grams of commercial agar powder (Difco) was dissolved in 50 mL of water and mixed with 50 mL of the leaf extract and sterilized at 120 °C for 20 min.

Temperature: Conidia production on PDA was estimated at 5, 10, 15, 20, 25, 30, 35, 40 ˚C temperatures for the three isolates (E, A, L). The media were inoculated and incubated in thermostatically controlled incubators (ASTELL-12) under continuous darkness for 12 d.

Factors affecting conidia germination and viability:

The high sporulating isolate, E was used for all the experiments on conidia germination and viability. Test conidial suspensions were obtained using 10 d old cultures grown on PDA.

Percentage germination: To determine the percentage germination of conidia, 0.02 mL of a conidial suspension (5 × 10^4 conidia / mL) was placed on a clean glass slide with the aid of a micropipette. The slides were placed on inverted Petri dishes, which were kept in plastic trays lined with moist blotting papers (approx. 100 % RH) and incubated at RT for 10 and 12 h in moist chambers. At the end of the incubation period, conidia were stained with

a commercial preparation of lacto phenol cotton blue (Difco) and examined under the microscope.

A conidium was considered as germinated when the germ tube was longer than half the length of the conidium. Fifty conidia were counted per drop and the means of at least 3 drops were taken per replicate to calculate percentage germination. Four slides were sampled per treatment.

***Conidia concentration*:** Percentage germination of conidia were also tested at seven different conidial concentrations ranging from 1×10^4 to 1.5×10^6 conidia / mL on glass slides. Droplets of 0.02 mL were placed on slides and incubated as described by Sharma and Mohanan (1991). Percentage germination of conidia was recorded as described above after 12 h of incubation period.

***Incubation period*:** To find the optimum incubation period for germination, conidia droplets (0.02 mL) obtained from a 5×10^4 conidia / mL suspension were placed on glass slides and incubated inside polystyrene boxes lined with moistened blotting papers at 28 ± 2 ºC. The slides were examined for germination at 2 h intervals for 14 h and the percentage germination was assessed.

***Temperature*:** Germination of conidia was observed at seven different temperatures ranging from 10 – 40 ˚C. Conidia droplets (5×10^4 conidia / mL) on glass slides served as wet smears, while on another set of slides dry smears were obtained by carefully blotting off the water of the droplets, 2 min after the droplets were placed on slides. Slides having both dry and wet smears were placed on inverted Petri dishes, which were inside polystyrene boxes lined with moist blotting papers. These boxes were incubated at 10, 15, 20, 25, 30, 35 and 40 ˚C in thermostatically controlled incubators. Samples were withdrawn at 10 and 12 h intervals and percentage germination was assessed as described earlier. Four slides were used for each treatment.

To study the effect of different temperatures on the viability of conidia, wet and dry smears (0.02 mL drops of a 5×10^4 conidia / mL, suspension) of conidia on glass slides were incubated in moist chambers at temperatures of 10 and 40 ºC in thermostatically controlled incubators. The samples were withdrawn 2, 3, 6, 12 and 24 h after incubation and kept at RT for 10 and 12 h in moist chambers to assess percentage germination.

***Free water*:** In another experiment, slides having both dry and wet smears as described above were placed on inverted Petri dishes, which were inside polystyrene boxes lined with moist blotting papers. These boxes were incubated at RT under 100 % RH (approx.). Percentage germination was assessed after 12 h of incubation.

Dry smears (0.02 mL drops of a 5×10^4 conidia / mL, suspension) of conidia on glass slides were incubated at RT for 2, 3, 6, and 12 h and a drop of water was added. The smears were kept at room temperature for 12 h in moist chambers to assess percentage germination.

***Relative humidity*:** Conidia droplets (5×10^4 conidia / mL) kept on glass slides as described earlier were incubated at RT under humidities of 100, 96, 91, 85, 80, 75 and 63 % for 12 h. Saturated aqueous solutions of potassium sulphate, potassium nitrate, potassium chloride, and sodium nitrate in sealed Kilner jars were used to obtain the required relative humidity (CMI, 1984). Hundred percent relative humidity (RH) (approx.) was obtained by replacing the salt solutions in Kelner jars with distilled water. Samples were withdrawn after 12 h and the percentage germination was assessed. Wet smears incubated at 100 % RH served as controls.

Dry smears of *C. cassiicola* on glass slides were incubated under different humidities at RT. The samples were withdrawn after 2, 3, 6, 12 h and kept at RT for 10 and 12 h in moist chambers to assess percentage germination.

***UV radiation*:** Germination of conidia was assessed after exposing conidia either as wet or dry smears on glass slides to UV light from two sources (253.7 nm, "Hanovia" lamp placed, 0.33 m above conidia and 366 nm "Camag" lamp placed 10 m above conidia). To study the effect of UV radiation on the viability of conidia, dry smears were placed for 20, 40, 60, 80, 100, 120 min under normal light (control) or under the respective UV source. The treated dry smears were re-wetted and all the samples were incubated in moist chambers at RT and percentage germination was assessed after 12 h.

RESULTS

In an earlier study (Fernando *et al.,* 2009), 15 isolates of *C. cassiicola* were obtained from different clones of *H. brasiliensis* and based on cultural, reproductive and pathogenic characters, the isolates were grouped into three cluster groups. Three isolates (E, L and A) were selected (one isolate representing each cluster group). For studies on germination and viability, only isolate E (a good sporulator) was employed in the present study.

Factors affecting conidia production of *C. cassiicola*

***Culture media*:** Conidia production of the two isolates (E and A) occurred on four media. On CMA and WA the isolates E and A did not produce conidia and the isolate L did not produce conidia on any of the media tested. Isolate E was the most prolific conidia producer. A significant variation in the production of conidia among different isolates was observed (Table 1).

***Culture age*:** The two isolates E and A commenced production of conidia on PDA, 4 days after inoculation under normal dark and light regimes at RT. The cultures showed maximum conidia production after an incubation period of 12 days (isolate E – 21.7×10^5 and isolate A - 19×10^4 conidia / cm ^{2}).

***Temperature*:** Conidia production occurred between 10° – 35° C. No sporulation or growth of the colonies of the isolates was observed at temperatures below 5° C and above 35° C. The highest conidia production was at 30° C in both isolates E and A. Isolate L did not produce conidia at the range of test temperatures (Figure 1).

Factors affecting conidia germination and viability

Conidia of the isolates produced germ tubes from both apices. The germ tubes were well branched and elongated rapidly.

***Conidia concentration*:** At conidia concentrations of 1×10^4 conidia / mL, 100 % germination was observed. With the increase of concentration, the percentage of germination decreased. The percentage germination reduced significantly at concentrations 1×10^6 and 1.5×10^6 conidia / mL (Table 2).

Table 1: Production of conidia by *C. cassiicola*, isolates A and E on different media

Media	Conidia production × 10^4 / cm^2		
	Isolate A	Isolate E	Isolate L
PDA	19.33^A	216.67^m	0
LBA	8.0^B	47.67^0	0
CDA	7.0^B	123.0^n	0
MA	2.33^B	14.33^p	0
CMA	0^C	0^q	0
WA	0^C	0^q	0

* Means with the same letter are not significantly different based on Duncan's multiple range test

Table 2: Effect of conidia concentration on germination in *C. cassiicola*

Conidia concentration (No of conidia / ml)	% Germination
1.0×10^4	100
5.0×10^4	98 (± 1.155)
1.0×10^5	95 (± 2.887)
2.5×10^5	90 (± 5.0)
5.0×10^5	90 (± 4.041)
1.0×10^6	68 (± 3.606)
1.5×10^6	30 (± 7.638)

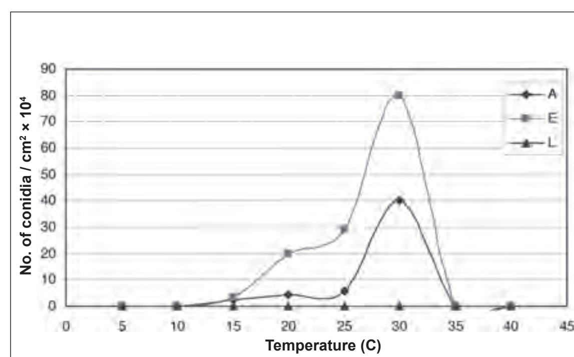

Figure 1: Conidia production of *C. cassiicola* isolation after 12 days on PDA at different temperatures

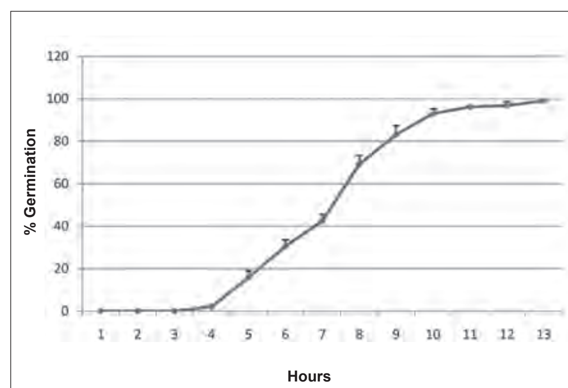

Figure 2: Effect of incubation time on germination of *C. cassiicola* isolate E conidia at RT

Table 3: Percentage germination of *C. cassiicola* conidia after incubation for 10 hours and 12 hours at 100 % RH as dry smears following exposure to different temperatures

	% Germination of conidia									
Exposure time (h)	2h		3h		6h		12h		24h	
Incubation time (h)	10h	12h	10h	12h	10h	12h	10h	12h	10h	12h
Temperature exposed (°C)										
10	100 (± 0)	100 (± 0)	100 (± 0)	100 (± 0)	38 (± 3.06)	49 (± 5.51)	0 (± 0)	10 (± 3.61)	0 (± 0)	0 (± 0)
15	100 (± 0)	100 (± 0)	100 (± 0)	100 (± 0)	25 (± 22.5)	40	0 (± 0)	10 (± 0)	0 (± 0)	0 (± 0)
20	100 (± 0)	100 (± 0)	100 (± 0)	100 (± 0)	50 (± 5.29)	40 (± 7.64)	10 (± 7.50)	20 (± 5.51)	0 (± 0)	0 (± 0)
25	100 (± 0)	100 (± 0)	100 (± 0)	100 (± 0)	15 (± 2.52)	20 (± 4.58)	12 (± 3.0)	20 (± 4.16)	0 (± 0)	0 (± 0)
30	100 (± 0)	100 (± 0)	100 (± 0)	100 (± 0)	28 (± 6.43)	38 (± 4.73)	25 (± 5.0)	40 (± 3.6)	0 (± 0)	10 (± 4.73)
35	40 (± 3.61)	40 (± 7.94)	15 (± 3.06)	25 (± 3.61)	0 (± 0)	0 (± 0)	-	-	-	-
40	10 (± 3.61)	10 (± 3.06)	0 (± 0)	0 (± 0)	-	-	-	-	-	-

***Incubation period*:** Conidia germination on glass slides commenced after 3 hours of incubation. The percentage germination rapidly increased 5 hours after incubation and reached the maximum after 12 hours of incubation (Figure 2).

***Temperature*:** Germination occurred at temperatures between 5 – 40 °C. The optimum temperature range was between 15 – 35° C when incubated as wet smears on glass slides (Figure 3). The elongation of the germ tube was very rapid between 25 – 35 °C. When incubated as dry smears on glass slides, the dryness reduced percentage germination at all temperatures and the germ tubes were comparatively weak. The germ tubes were very thin compared to the controls.

Three or more hours exposure to 40 ºC totally inhibited conidia germination (Table 3). At 35 ºC exposure for

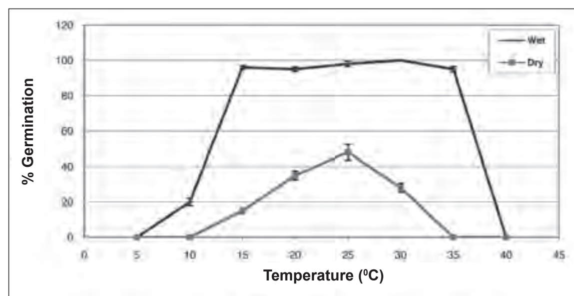

Figure 3: Effect of temperature on germination of *C. cassiicola* isolate E in wet and dry smears

Table 4: Percentage germination of *C. cassiicola* conidia at different relative humidities for an incubation period of 12 hours at RT

Relative humidity (%)	Percent germination (%) (12 h of incubation)
100 (Wet)	100 (± 0)
100 (Dry)	55 (± 7.64)
98	40 (± 3.06)
95	20 (± 8.74)
92	14 (± 7.02)
90	10 (± 2.52)
88	0 (± 0)

2 hours and 3 hours drastically reduced percentage germination, while exposure for 6 hours caused total inhibition. Germination was 100 % after exposure for 2 hours and 3 hours at all temperatures except 35 and 40 ºC. Exposure for 6 hours or more reduced germination significantly at all temperatures, while 24 hours incubation caused total inhibition (Table 3).

Relative humidity

Conidia germinated in the presence of free water at RT. In the absence of free water, only 55 % conidia germinated when exposed to 100 % RH. Germination reduced significantly when conidia were incubated at RH less than 100 %. Percentage germination reduced to 40 % when exposed to 98 % RH, and to 20 % when exposed to 95 % RH. The percentage germination reduced to 10 % when incubated at 90 % RH, and at 88 % RH conidia showed no germination (Table 4). Exposure to 92 % RH totally inhibited conidial germination. Exposure to 95 % RH also drastically reduced percentage germination and an exposure for 6 hours caused a total loss of viability (Table 5).

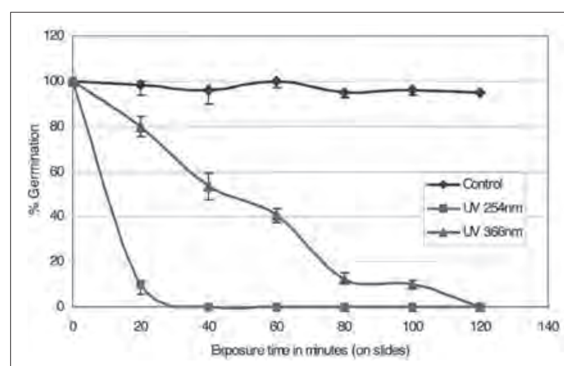

Figure 4: The viability of detached conidia after exposure to ultra violet radiation as dry smears on glass slides - *C. cassiicola* – isolate E

Table 5: Percentage germination of *C. cassiicola* conidia after incubation for 10 hours and 12 hours at 100 % RH as dry smears following exposure to different relative humidities

	% Germination of conidia									
Exposure time (h)	2 h		3 h		6 h		12 h		24 h	
Incubation time (h)	10h	12h	10h	12h	10h	12h	10h	12h	10h	12h
Relative humidity (%)										
100 (aq)	100 (± 0)									
100 (dry)	87.6 (± 2.52)	92.3 (± 2.52)	91.3 (± 1.15)	90 (± 5)	73.33 (± 10.4)	77.66 (± 2.51)	39.33 (± 1.5)	45.66 (± 8.14)	29 (± 3.6)	37.66 (± 2.51)
98	71 (± 3.6)	76.33 (± 3.21)	64.33 (± 4.04)	66 (± 5.29)	47.33 (± 6.42)	50.66 (± 9.01)	38.33 (± 7.63)	42.66 (± 6.42)	8 (± 5.29)	10.66 (± 8.32)
95	22.66 (± 6.42)	15.66 (± 9.29)	21.66 (± 10.4)	12.33 (± 2.51)	0	0	0	0	0	0
92	0	0	0	0	0	0	0	0	0	0

UV radiation

Under both UV sources conidia showed 100 % germination in the presence of free water. No germinated

Table 6: Percentage germination of *C. cassiicola* conidia after incubation for 12 hours at 100 % RH as wet and dry smears

UV Source	% germination of conidia	
	Wet	Dry
254 nm	*100 (± 0)	0 (± 0)
366 nm	100 (± 0)	28 (± 6.11)

* Germ tubes had unusual shapes

conidia were observed in dry smears (Table 6). Twenty minute exposure of conidia to 254 nm as wet smears inactivated the conidia significantly and 45 min exposure was detrimental. UV radiation of 366 nm was not totally effective even with an exposure time of 60 min. An exposure time of 2 hours to 366 nm UV source was detrimental (Figure 4).

DISCUSSION

Many factors are known to govern the pathogenicity of *C. cassiicola* on *H. brasiliensis*, i.e. conidia concentration, availability of free water, temperature, relative humidity and the presence of the susceptible leaf stage (Fernando *et al*., 2011). In this study *C. cassiicola* isolates showed significant variations in the production of conidia on artificial media. There were high sporulating, moderately sporulating and also very low sporulating isolates. The results also revealed that free water was not essential but promoted germination and a considerable percentage of conidia germinated, even under dry conditions if high humidity levels were present. Conidial germination was observed within a wide range of temperatures and the conidia had the ability to withstand exposure to temperatures as high as 35 ºC in free water. Results of experiments carried out to determine the effect of exposing conidia to different humidity levels revealed that conidia can remain viable, provided the relative humidity is high (above 92 %). The conidia of *C. cassiicola* as conidia of many other fungi like *Colletotrichum* (Fernando *et al*., 2000) showed self inhibition at high conidia concentrations.

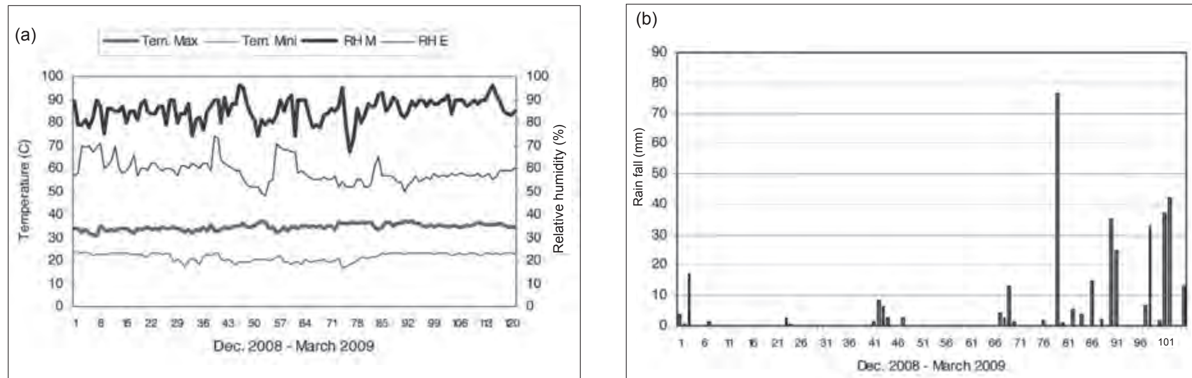

Figure 5: (a) Average daily temperature maxima and minima, relative humidity (morning and evening) and (b) average daily rain fall at Ratnapura, Sri Lanka during defoliation - refoliation period (December 2008 – March 2009).
Source: Tea Research Institute Weather Station, Karapincha, Ratnapura, Sri Lanka.

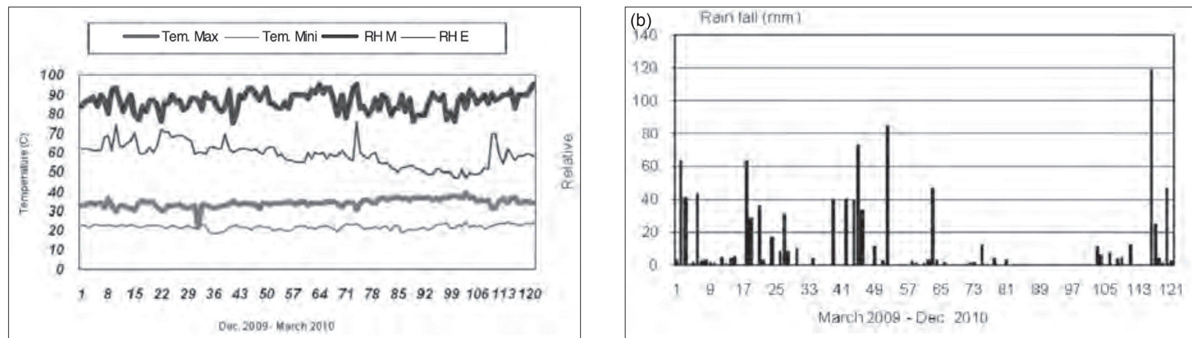

Figure 6: (a) Average daily temperature maxima and minima, relative humidity (morning and evening) and (b) average daily rain fall at Ratnapura, Sri Lanka during the defoliation - refoliation period (December 2009 – March 2010).
Source: Tea Research Institute Weather Station, Karapincha, Ratnapura, Sri Lanka.

C. cassiicola is becoming increasingly destructive in both temperate and tropical countries (Kajornchaiakul, 1987; Chee, 1988; Sinullingga *et al*., 1996; Jacob, 1997; Begho, 2000; Jean, 2000; Pernezny *et al*., 2002; Jayasinghe, 2003; Silva *et al*., 2003; Oka *et al*., 2006; Silva *et al*., 2006; Hong *et al*., 2007; Jinji *et al*., 2007; Martini *et al*., 2007). This can be related to the ability of the fungus to produce conidia within a wide temperature range (from 5 – 40 °C) and the ability of conidia to germinate easily in free water or when high humid conditions are present, as shown in this investigation.

In the main rubber growing areas in Sri Lanka, relative humidity in the morning is reported to be very high (90 – 100 %) and is often associated with showery overcast conditions (Liyanage *et al*., 1986). The minimum temperature is usually above 20 ºC and the maximum below 35 ºC. These environmental conditions are very prominent during the refoliation period (December to March) when immature leaves are present (Figures 5 and 6). Hence, it is clear that the climatic conditions prevailing in the main rubber growing areas, especially during the refoliation period are highly favourable for the establishment and the growth of the fungus. Experiments have shown that lesion development occurs on leaves of all immature stages but mature leaves are resistant (Fernando *et al*., 2010). This strongly suggests that the establishment of the pathogen can occur freely during the period of refoliation.

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