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GROWTH AND SPORULATION OF *COLLETOTRICHUM ACUTATUM* AND C. *GLOEOSPORIOIDES* AND BIOCHEMICAL CHANGES DUE TO INFECTION IN *HEVEA BRASILIENSIS*

Sadanand K. Mushrif, Shaji Philip, Annakutty Joseph, E. Edwin Prem and C. Kuruvilla Jacob

Rubber Research Institute of India, Kottayam – 686 009, Kerala, India

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The *Colletotrichum* leaf disease caused by *Colletotrichum acutatum* and *C. gloeosporioides* is an important disease during the immature phase of rubber (*Hevea brasiliensis*). The aggressiveness of the pathogen varies between the species and therefore a study was undertaken to understand the physiological requirement of both the species and the biochemical changes consequent to infection. Both species preferred fructose as carbon source and aspargine as nitrogen source. They also responded similarly to pH and temperature levels, with pH 6.0 to 7.5 and temperature 25 ^{0}C being optimum for growth and sporulation. The changes in the activity of peroxidase, phenylalanine ammonia lyase and total protein content were studied by inoculating the two species of *Colletotrichum* on the leaves of clones RRII 105 and RRIM 600. Significantly higher activities of peroxidase and phenylalanine ammonia lyase were noticed in the clone RRIM 600 inoculated with *C. acutatum* at 24 and 48 h after induction, respectively. Steady increase in total protein content was also observed up to 72 h in this clone when inoculated with *C. acutatum*. The tolerance in the clone RRIM 600 to *Colletotrichum* is attributed to this higher enzyme activity.

Keywords: Carbon, *Colletotrichum acutatum*, *C. gloeosporioides*, *Hevea brasiliensis*, Nitrogen, Oxidative enzymes

INTRODUCTION

Colletotrichum leaf disease of rubber [*Hevea brasiliensis* (Willd. ex Adr. de Juss.) Muell. Arg.] is widespread in the traditional rubber growing areas in India and causes significant damage to plants in nurseries and young plantations (Deka, *et al.*, 1996; Manju, *et al.*, 1999). Economic losses due to the disease have also been reported from other rubber growing countries. In Indonesia, the persistence of this disease over a long period resulted in loss of yield up to 50 per cent and delay in maturity of rubber trees up to three years (Basuki, 1992). The disease generally occurs from April to May and August to October in traditional regions, whereas in North East India, it prevails throughout the year except in the winter months (December-February).

Tender leaves are highly susceptible to the disease. Initially the symptoms develop as minute brown circular lesions on the

Correspondence: Sadanand K. Mushrif (Email: skmushrif@gmail.com)

leaves, which later develop into thick lesions and then rise above the leaf surface as conical projections. In severe cases, the leaves become distorted, turn black, shrivel and fall off, leaving the petioles on the stem for a short period. Die-back is common under severe conditions. Two species *viz.* *Colletotrichum acutatum* and *C. gloeosporioides* are reported to cause this disease in rubber, the former being more aggressive. Jayasinghe *et al.* (1997) reported that *C. acutatum* is the major cause of *Colletotrichum* leaf disease in Sri Lanka. In India also, similar observations were made by Saha *et al.* (2002). Based on the morphological characters of the pathogen, Kumar *et al.* (2002) identified *C. acutatum* as the major species in India.

A study was undertaken to elucidate the differences between these two species in their physiological requirements and in the biochemical changes they cause in two popular clones of *Hevea brasiliensis*.

MATERIALS AND METHODS

The two species *viz.* *C. acutatum* and *C. gloeosporioides* isolated from infected rubber leaves were maintained on potato dextrose agar (PDA) for carrying out physiological studies. Modified Czapek's broth was used as a standard medium for various studies.

Different sources of carbon like glucose, dextrose, galactose, fructose, sucrose, lactose and mannitol were tried. The amount of carbon present in 1 per cent glucose was taken as the standard to maintain the carbon level in all the sources. Aspargine, peptone and urea as sources of organic nitrogen and potassium nitrate, ammonium nitrate, ammonium sulphate, sodium nitrate and calcium nitrate as inorganic sources were tried. The amount of nitrogen present in 0.2 per cent aspargine was retained as standard to maintain the nitrogen level in all the sources. Czapek's broth (50 ml) with 1 per cent carbon and 0.2 per cent nitrogen of different sources was dispensed in 100 ml conical flasks and sterilised. These flasks were inoculated with 7 mm diameter mycelial plugs from seven-day-old cultures of the pathogens and incubated for 10 days.

For pH studies, the pH of the basal medium (Czapek's broth) was adjusted to 2.0 to 8.0 (at 0.5 intervals) with 0.1 N NaOH or 0.1 N HCl solutions. The temperature studies were carried out using different temperatures ranging from 5^0 to 40^0 C at an interval of 5^0 using BOD incubator. Three replications were maintained for each treatment in a completely randomised design. At the end of the incubation period, mycelial mats were harvested by filtering through Whatman No. 41 filter paper, washed thoroughly with sterile water and dried at a temperature of 60^0C to constant weight. The dry weight of the mycelial mats was recorded. The number of spores in the culture per microscopic field (2.04 mm^2) was counted and assigned to following sporulation grades.

(-) = Nil (No spores); (+) = Poor (1-50 spores per microscopic field); (++) = Fair (51-100 spores); (+++) = Good (101-200 spores) and (++++) = Excellent (> 200 spores).

Budded stumps of two rubber clones *viz.* RRII 105 (susceptible) and RRIM 600 (tolerant) were raised in polybags for the biochemical studies. The two species of *Colletotrichum* were grown on PDA medium and the spore suspension in sterile water (2×10^4 spores/ml) was sprayed on the plants uniformly using an atomiser. The plants were

covered with polythene bags for 24 h to maintain high humidity. Control plants were sprayed with sterile water. Five replications were maintained for each treatment. Leaf samples were collected at 24, 48, 72 and 96 h after inoculation and changes in biochemical parameters *viz.* peroxidase, phenylalanine ammonia lyase and total protein were studied.

Peroxidase activity was estimated spectrophotometrically (Shimadzu, UV-1601, Japan) at 436 nm using guaiacol as substrate (Putter, 1974). The enzyme activity was expressed as units per gram of fresh weight of the sample. Phenylalanine ammonia lyase (PAL) activity was studied (Brueske, 1980) using L-phenylalanine as substrate and expressed in µmol trans-cinnamic acid /mg protein/min. Total protein was estimated and expressed in mg/g of leaf sample (Lowry *et al.*, 1951).

RESULTS AND DISCUSSION

Both species showed differences in their response to different carbon sources, though both showed maximum preference for fructose and least for mannitol (Table 1). Between the two species, the growth was better for *C. acutatum* irrespective of the carbon sources. The growth of *C. acutatum* was significantly better than that of *C. gloeosporioides* when dextrose, fructose and sucrose were incorporated in the medium.

Significant differences in response to various nitrogen sources were obtained for both the species. However, the species-level variation was not significant (Table 2). Better growth was noticed in aspargine-amended medium for both the species. Potassium nitrate was least preferred by *C. acutatum* whereas *C. gloeosporioides* had poor growth in potassium nitrate, sodium nitrate and calcium nitrate-amended media.

Table 1. **Effect of carbon source on the growth of *Colletotrichum* spp.**

Carbon source	Dry weight of mycelium (mg)		
	C. acutatum	*C. gloeosporioides*	Mean
Glucose	148.4	147.1	147.8
Dextrose	121.5	84.9	103.2
Galactose	139.9	158.7	149.3
Fructose	198.3	167.9	183.0
Sucrose	175.9	84.8	130.4
Lactose	75.7	85.9	80.8
Mannitol	48.4	68.7	58.6
Mean	129.7	93.0	

CD (P = 0.05) Carbon source -14.01; Species -26.21; Carbon source x species - 37.01

Table 2. **Effect of nitrogen source on the growth of *Colletotrichum* spp.**

Nitrogen source	Dry weight of mycelium (mg)		
	C. acutatum	*C. gloeosporioides*	Mean
Ammonium nitrate	297.9	287.7	292.8
Potassium nitrate	233.3	264.0	248.7
Sodium nitrate	354.8	241.1	297.9
Calcium nitrate	291.4	229.4	260.4
Ammonium sulphate	263.8	326.5	295.2
Aspargine	409.6	344.7	377.2
Peptone	296.5	320.8	308.6
Urea	258.5	315.5	287.0
Mean	300.7	291.2	

CD (P = 0.05) Nitrogen - 38.63; Species – NS; Nitrogen source x species - 54.63

Table 3. **Effect of pH on the growth and sporulation of *Colletotrichum* spp.**

pH	*C. acutatum*		*C. gloeosporioides*		Mean
	Dry weight of mycelium (mg)	Sporulation	Dry weight of mycelium (mg)	Sporulation	
2.0	50.0	+	50.5	+	50.3
2.5	64.0	+	60.4	+	62.2
3.0	111.1	+	82.7	+	96.9
3.5	107.6	++	104.3	++	106.0
4.0	127.1	++	115.4	++	121.3
4.5	133.5	++	128.7	++	131.1
5.0	209.7	+++	177.4	+++	193.6
5.5	224.3	+++	239.5	+++	231.9
6.0	263.7	++++	252.0	++++	257.9
6.5	263.8	++++	266.0	++++	264.9
7.0	260.9	++++	270.2	+++	265.6
7.5	226.3	+++	261.9	+++	244.1
8.0	183.0	++	229.0	+++	206.0
Mean	171.2		172.2		
CD (P = 0.05)	pH - 22.71; Species – NS; pH x species - NS				

Both the species of *Colletotrichum* responded similarly to the different levels of pH and maximum growth was observed at pH levels of 6.0 to 7.0 (Table 3). The least growth was obtained at pH levels of 2.0 to 3.5. Excellent sporulation was obtained at pH levels of 6.0 to 6.5 for both the species, which remained high even at pH 7.0 for *C. acutatum*.

The two species of *Colletotrichum* responded similarly to the different levels of temperature (Table 4). Maximum growth was noticed at a temperature of 25 °C and negligible growth was recorded at temperature levels of 5 °C and 40 °C. Excellent sporulation was recorded at 25 °C and 30 °C for *C. acutatum* and at 25 °C for *C. gloeosporioides*.

Purkayastha and Gupta (1975) found that *C. gloeosporioides* gave maximum yield on fructose followed by dextrose and sucrose as carbon sources and nitrogen in the form of aspargine. Rajak (1985) observed maximum growth of *C. falcatum* in glucose followed by fructose, maltose and galactose, while the preferred temperature and pH were 25 °C and 6.5, respectively. Maximum growth was observed with sorbitol followed by sucrose as carbon sources while peptone and ammonium nitrate as nitrogen sources for *Colletotrichum corchorum*, the causal agent of anthracnose of jute (Ray and Purkayastha, 1977). Jayasinghe and Fernando (1998) reported significantly slower growth of *C. acutatum* compared to *C. gloeosporioides* at room temperature and they also found growth differences of the two species at a wide range of temperature ranging from 15 °- 32.5 °C with an optimum between 25 °C and 30 °C.

Table 4. **Effect of temperature on the growth and sporulation of *Colletotrichum* spp.**

Temperature (°C)	*C. acutatum*		*C. gloeosporioides*		Mean
	Dry weight of mycelium (mg)	Sporulation	Dry weight of mycelium (mg)	Sporulation	
5.0	5.3	-	5.3	-	5.3
10.0	76.7	+	57.4	+	67.1
15.0	133.7	++	131.4	++	132.6
20.0	267.0	+++	241.6	+++	254.3
25.0	325.6	++++	302.7	++++	314.2
30.0	212.1	++++	180.1	+++	196.1
35.0	74.4	++	93.7	++	84.1
40.0	8.4	-	6.7	-	7.5
Mean	137.9		127.4		
CD (P = 0.05)	Temperature - 30.0;	Species - NS;	Temperature x species - NS		

From the present study, it is clear that the two species of *Colletotrichum* differed significantly in their response to different carbon and nitrogen sources. However, their response was similar to the different levels of temperature and pH. Carlile *et al.* (1994) reported that different sugars can serve as substrate sources for fungi, which is likely to reflect on their availability in the usual habitat of the organism.

Table 5. **Changes in peroxidase activity in *Hevea* leaves inoculated with *Colletotrichum* spp.**

Treatment	Peroxidase activity in units			
	Time (h)			
	24	48	72	96
Ca + RRII 105	114	118	115	112
Cg + RRII 105	110	114	109	105
RRII 105 (control)	98	100	99	95
Ca + RRIM 600	139	171	175	156
Cg + RRIM 600	128	141	137	136
RRIM 600 (control)	114	116	114	113

CD (P = 0.05) Species x time - 09.89
Ca - *C. acutatum*, Cg- *C. gloeosporioides*

Increase in peroxidase activity was noticed in the leaves of both the clones infected with *Colletotrichum* compared to the healthy leaves (Table 5). However, a significantly higher peroxidase activity was noticed in the tolerant clone RRIM 600 infected with *C. acutatum* after 24 h. There was no significant variation between 48 and 72 h but the activity was reduced after 72 h although it remained significantly higher than in the other pathogen-clone combination. High peroxidase activity was also noticed in the clone RRIM 600 infected with *C. gloeosporioides* within 48 h after infection, which remained almost steady up to 96 h with no significant reduction. In RRII 105, significant increase in peroxidase activity over control was observed initially, however the levels were significantly lower than that of RRIM 600 throughout the period of observation.

Increase in PAL activity was higher in the inoculated leaves of both the clones compared to the corresponding healthy

Table 6. **Changes in PAL activity in *Hevea* leaves inoculated with *Colletotrichum* spp.**

Treatment combination	PAL activity (µmole- trans-cinnamic acid /mg protein/min) Time (h)			
	24	48	72	96
Ca + RRII 105	85.4	89.8	92.3	91.2
Cg + RRII 105	82.4	86.8	90.3	89.4
RRII 105 (control)	65.6	67.7	66.8	65.4
Ca + RRIM 600	138.8	153.6	161.1	167.4
Cg + RRIM 600	110.4	121.9	127.3	122.6
RRIM 600 (control)	70.2	75.3	75.1	73.6

CD (P = 0.05) Species x time - 08.10
Ca - *C. acutatum* , Cg- *C. gloeosporioides*

Table 7. **Changes in total protein content in the leaves of *Hevea* inoculated with *Colletotrichum* spp.**

Treatment combination	Total protein (mg/ g of fresh weight) Time (h)			
	24	48	72	96
Ca + RRII 105	20.5	24.3	26.7	29.8
Cg + RRII 105	20.1	23.4	24.7	24.1
RRII 105 (control)	22.4	28.9	36.3	32.9
Ca + RRIM 600	19.4	24.3	26.1	25.8
Cg + RRIM 600	17.5	18.2	18.9	19.2
RRIM 600 (control)	18.5	19.2	19.4	18.9

CD (P = 0.05) Species x time - 02.88
Ca - *C. acutatum* , Cg- *C. gloeosporioides*

leaves (Table 6). The infected leaves of RRIM 600 inoculated with *C. acutatum* showed significantly higher activity from 24 h which continued to increase up to 96 h of inoculation. The increase in activity was significantly higher up to 48 h and thereafter it remained steady. The increase in activity when infected with *C. gloeosporioides* was up to 48 h after which it remained constant. The increase in PAL activity was significantly higher in the *C. acutatum* inoculated leaves of RRIM 600 than in the *C. gloeosporioides* inoculated leaves. For RRII 105, though there was an increase in the activity, it was not as pronounced as in RRIM 600 inoculated plants.

A rise in total protein content was observed for both the clones from 24 h of inoculation with both the species of the pathogen compared to the healthy leaves (Table 7). The increase in protein levels was significantly more in the infected leaves of the clone RRIM 600 inoculated with *C. acutatum* up to 72 h after infection. The protein levels in the treatments involving the clone RRII 105 inoculated with both the species and clone RRIM 600 with *C. gloeosporioides* were on par up to 72 h after the inoculation.

Increase in peroxidase, phenylalanine ammonia lyase and total protein was noticed in the infected leaves compared to healthy leaves. Babu and Reddy (1988) noticed increased activity of peroxidase and phenylalanine ammonia lyase in the infected lemon fruits compared to the healthy ones when inoculated with *C. gloeosporioides*. Increased activity of peroxidase and phenylalanine ammonia lyase was reported in the infected *Coccinia* fruits with *C. gloeosporioides* by Reddy and Reddy (1987). Tofazal *et al.* (1999) reported increased activity of peroxidase in leaves of mango infected with *Colletotrichum gloeosporioides*. Das *et al.* (2003) observed increased activity of phenylalanine ammonia lyase and peroxidase at 12 h after inoculation, which reached a maximum two days after inoculation in the spot blotch of wheat caused by *Bipolaris sorokiniana*. Accumulation of phenylalanine ammonia lyase and peroxidase upon challenge

inoculation of groundnut leaves with *Cercosporidium personatum* has also been reported (Kaur and Dhillon, 1989).

The role of peroxidase and phenylalanine ammonia lyase in the process of host-pathogen interaction has been well studied. Vidhyasekaran (1988) reported that the enzyme peroxidase oxidises phenolics to highly toxic quinones and hence it has been assigned a role in disease resistance. Phenylalanine ammonia lyase is the first enzyme of phenyl propanoid metabolism in higher plants and has been suggested to play a significant role in regulating the accumulation of phenolics (Massala *et al.*, 1980), phytoalexins and lignins, the key factors involved in disease resistance (Vidhyasekaran, *et al.*, 1973). In the present investigation, it was noticed that the activity of peroxidase and phenylalanine ammonia lyase was more in inoculated plants of RRIM 600. Chandrasekaran *et al.* (2000) observed the increased peroxidase activity in the resistant cultivar of soyabean than the susceptible cultivar in the pods infected with *C. truncatum*. Similarly, Das *et al.* (2003) reported that the resistant genotype of wheat showed increased peroxidase activity and phenylalanine ammonia lyase than the susceptible genotype when inoculated with *Bipolaris sorokiniana*, the casual agent of spot blotch of wheat. Jebakumar *et al.* (2001) noticed increased activity of phenylalanine lyase in the tolerant variety of pepper to *Phytophthora capsici*. Breton *et al.* (1996) observed significantly higher peroxidase activity in the *H. brasiliensis* clone GT 1, resistant to *Corynespora cassiicola*. The higher activity of peroxidase and phenylalanine ammonia lyase in the clone RRIM 600 observed in the present study might be due to the tolerant reaction of the clone to the *Colletotrichum* leaf disease.

The present study also showed that the increase in peroxidase and phenylalanine ammonia lyase acitivity in the clone RRIM 600, was higher in the *C. acutatum* inoculated plants than in the *C. gloeosporioides* inoculated plants. Uritani (1963) reported that the largest change in peroxidase activity was induced by the race to which the host is resistant and little change by the susceptible race. Thus the higher activity of peroxidase and phenylalanine ammonia lyase in *C. acutatum*-infected RRIM 600 plants can be due to its higher tolerance to *C. acutatum* compared to *C. gloeosporioides*.

Kaur and Dhillon (1989) reported increase in protein content of groundnut leaves inoculated with *Cercosporidium pesonatum*. They observed less alteration in the protein content in the resistant than in the susceptible cultivars of groundnut leaves inoculated with *C. personatum*. Von-Broembsen and Hadwiger (1972) noticed initial increase in protein content of flax infected with *Melampsora lini*. The increase in protein content in the infected leaves of both the clones may be due to (i) enhanced amino acid synthesis (ii) increased protein synthesis either by host or pathogen or their cumulative synthesis (Tomiyama, 1963; Farkas and Stahmann, 1966) and (iii) increased synthesis of more enzymatic proteins (Stahmann, 1967; Uritani *et al.*, 1967).

CONCLUSION

From the present investigation it is concluded that there was variation in the two species of *Colletotrichum* with respect to their utilisation of various carbon and nitrogen

sources. The two species did not vary in their response to different temperature and pH levels. Increased enzyme activity was noticed in tolerant clone RRIM 600 inoculated with *C. acutatum* and *C. gloeosporioides*. The activity was more pronounced with *C. acutatum* than with *C. gloeosporioides* inoculated plants of clone RRIM 600. The higher enzyme activity, being the indication of tolerant reaction, can explain the higher field tolerance of the clone RRIM 600 to *Colletotrichum* spp. than the more popular clone RRII 105.

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