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A comparative study of *Colletotrichum* species causing anthracnose in *Hevea*

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Abstract

Colletotrichum acutatum *and* C. gloeosporioides *are destructive fungi causing diseases in a wide range of plants. In many instances they are responsible for mixed infections. The present study revealed that the spore productivity is higher in slow growing species* C. acutatum *at all temperatures investigated. Spores of* C. gloeosporioides *germinated earlier but both reached a maximum after 7-8 hrs and in* C. gloeosporioides *the elongation of germ tubes was much faster. The temperature influenced greatly the germination of spores in the two species and self-inhibition of* C. acutatum *occurred at much higher spore concentrations than in* C. gleosporioides. *The germination response of dry smears to temperature change was poor in both the species and drying reduced germination. Free water was not essential but when present promoted germination in both the species. None of the spores germinated below 85% RH at RT. The slower growth rate of* C. acutatum *was also evident in the liquid medium. PG activity of* C. acutatum *was higher than that* of C. gloeosporioides *and molecular weights of the enzyme were significantly different in the two species. The activity of PL was more or less similar in the two species. In laboratory studies* C. acutatum *appeared to be less virulent than* C. gloeosporioides.

Key words: biology, *Colletotrichum acutatum, Colletotrichum gloeosporioides*, pathogenicity

Introduction

Colletotrichum leaf disease (CLD) of *Hevea brasiliensis* is regarded as one of the major causes of declining yields of rubber in the American & Indian Tropics (Mitra & Mehta, 1938), Thailand, (Anon, 1960), Sri Lanka (Samrajeewa *et al*., 1985), China (Kaiming, 1988), Malaysia (Wastie, 1967) and West Africa (Senechal *et al*., 1987). The fungus *Colletotrichum gloeosporioides* was believed to be the causative agent of CLD of *Hevea brasiliensis* in all rubber growing countries since the early 1900's (Petch, 1905). However, study carried out in Sri Lanka in 1997, revealed that the fungus *Colletotrichum acutatum* plays the major role in the development of CLD in Sri Lankan rubber plantations (Jayasinghe *et al*., 1997).

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During the recent past *C. acutatum* has become increasingly important in many parts of the world including Brazil (Henz *et al*., 1992), USA (Howard *et al*., 1992), Italy & Spain (Simpson *et al*., 1994), France (Denoyes & Baudry, 1995) and Japan (Sato *et al*., 1996). Later in the year 2002, *C. acutatum* was reported from India (Thakurdas *et al*., 2002) and Further, it has been shown that both species are responsible in causing mixed infections in a variety of economically important crops including avocado, mango, almond, peach, pecan, straw berry (Freeman *et al*., 1998) and apple (Sutton, 1990).

The biology of both *C. gloeosporioides* and *C. acutatum* has been has been investigated (Mitra & Mehta, 1938; Samarajeewa *et al*., 1985; Sato *et al*., 1996; Jayasinghe & Fernando, 1997; Freeman *et al*., 1998; Fernando *et al*., 2000; 2001). However, no comparative investigation of the two species has been carried out. In view of the importance of the mixed infections, especially in *Hevea*, the present investigation was undertaken to compare the important biological and pathological aspects of the two species.

Materials and Methods

Ten monoconidial cultures of each species *C. acutatum* and *C. gloeosporioides* obtained from diseased rubber leaves were maintained on potato dextrose agar (PDA). In preliminary studies, the colony growth on PDA at RT (28±2ºC), conidial shape, colony colour and the sensitivity to benomyl (Benlate, 50% ai, Du Pont) were tested to confirm the identity of the species (Jayasinghe & Fernando, 1998). Two typical cultures *C. acutatum*; R1 & R2 (IMI 375216) and *C. gloeosporioides*; K1 (IMI 371799) and K2 were used for most of the studies.

Effect of culture age, media and temperature on sporulation

To evaluate the effect of age on sporulation the two isolates from each species were grown separately at 28±2°C (RT) under on PDA and spore production was estimated at 2 d intervals. To determine the best medium for sporulation six different media; PDA, lima bean agar (LBA), Czapek dox agar (CDA), malt agar (MA), corn meal agar (CMA) and water agar (WA) were tested. Spore production was also estimated for the two isolates at 9 different temperatures ranging from 5-40°C after incubating the cultures at thermostatically controlled incubators in the dark for 6 days.

To determine the number of spores produced, the cultures were flooded with 10 ml distilled water and the colony surface mechanically disturbed with a scalpel to suspend conidia. The solution after dilution to 100ml was shaken for 5min in an orbital shaker at 30 rpm. The resulting suspension was filtered through muslin cloth and the spore concentration measured using a haemocytometer after appropriate dilution. Diameters of the

cultures were measured and colony areas determined and the number of spores produced cm^{-2} and per culture calculated. Four replicates were used in all experiments.

Germination and viability of spores

Spore suspensions from 7-d old cultures of isolates R2 and K1 grown on PDA at RT were used to investigate the effect of incubation period, spore concentration, temperature, humidity and UV radiation on germination and the viability of spores. The spore suspensions were prepared as described above. The required amount of the spores, 0.02 ml drops of a spore suspension were pipetted on to clean glass slides and they were incubated in moist chambers and incubated at RT. After incubation for 10 and 12 h, spores were stained with lacto-phenol cotton blue (Difco) and examined under microscope. Fifty spores were counted per drop and means of at least three drops used to calculate per cent germination. A spore was considered germinated when its germ tube was longer than half the length of the spore. Four slides were sampled per treatment.

Effect of spore concentration and incubation period

Germination was examined at spore concentrations ranging from 5×10^4 to 20×10^6 spores ml^{-1}. Drops (0.02 ml) were placed on glass slides and germination was assessed hourly up to 12 h.

Effect of temperature

Spore germination was observed at eight temperatures ranging from 10-40 ºC. The spore drops placed on glass slides served as wet smears while on another set of slides dry smears were obtained by carefully blotting the water off, 3 min. after the drops were applied (Fernando *et al*., 2000). Samples were withdrawn after 8 h and the germination assessed.

To assess the effect of temperature on spore viability, dry smears of spores were incubated in moist chambers at temperatures ranging from 10º - 40 ºC. Samples were withdrawn after 2, 3, 6, 12 h and transferred to a moist chamber at RT and incubated for 8h and % germination was taken to assess the viability.

Effect of humidity

Dry smears of spores on glass slides prepared as above were incubated at RT at different humidities (CMI, 1984). Saturated aqueous solutions of potassium sulphate, potassium nitrate, potassium chloride, sodium chloride, sodium nitrate and ammonium sulphate in sealed Kilner jars were used to obtain 96, 91, 85, 80, 75 and 63% RH respectively. Approx. 100% RH was obtained by replacing the salt solutions in Kilner jars with distill water and the wet smears incubated in it were used as the control.

Effect of UV radiation

Germination was assessed after exposing spores, as either wet or dry smears on glass slides to UV light from two sources (254 nm Hanovia lamp and 366 nm Camag lamp: 0.3m above the slides) for up to 3 h. Thereafter, dry smears were re-wetted and samples incubated under 100% rh at RT and germination assessed after 10 and 12 h.

Enzyme production and growth in liquid media

The two isolates (R2 & K1) were grown in ammonium tartrate liquid medium (Byrde & Fielding, 1968) with either citrus pectin or carboxy methyl cellulose (CMC) (Sigma) as the main carbon source. Twenty ml of the liquid medium was dispensed in 250 ml Erlenmayer flasks which were inoculated with a 1 cm^2 agar block having the fungus obtained from 7-d old cultures grown on PDA.

Cultures were harvested at 2 d intervals by filtering through Whatman No. 1 filter papers. The resulting culture filtrates were stored at 0 ˚C and used to detect enzyme activity. To assess growth, the dry weight of mycelium obtained by the residual mycelium was dried to a constant weight at 80°C and was measured at 2 d intervals.

In each experiment, all samples had three replicates and the results were analysed using PROC ANOVA in statistical package, SAS (SAS, 1987) for each day using least significant difference LSD.

Determination of enzyme activity

Polygalacturonase (PG): The agar plate method (Dingle *et al*., 1953) and the viscosity reduction method (Nema, 1992) were used to determine PG activity. In the viscosity reduction method, Ubbelhode (Technico) viscosity meter was used.

Pectin lyase (PL): Thiobarbituric acid method (Ayers *et al*., 1966; Fernando *et al*., 2001) was used to determine PL activity. Enzyme activity is expressed as arbitrary units (AU).

Cellulolytic enzymes: Activity of ß-glucosidase and cellobiase was determined by the hydrolysis of the chromatogenic substances p-nitrophenyl - ß D- glucopyranoside and p-nitro phenyl ß -D- cellobioside respectively (Bydre & Fielding, 1968). The colour of the phenate iron was estimated by measuring absorbance at 403 nm. The enzyme activity is expressed as AU.

Pathogenicity

Young (copper - brown stage) detached leaves of *Hevea* clones RRIC 100, RRIC 121, IAN 873, RRIC 52, RRIC 130, RRIC 104, Tjir 1, PB 86 were inoculated separately with the two isolates from each species. Six drops of an aqueous conidial suspension (drops of 0.02 ml, $1X10^5$ spores ml^{-1}, prepared from 7 d old cultures grown on PDA) from each isolate were placed on either side of the mid rib (3 drops on each side) on the abaxial surface of the leaves. They were incubated upto 4

days at RT under 100% RH. Leaves inoculated with sterilized distilled water served as controls. There were six replicate leaves for each clone and for each isolate. Depending on the sizes of the lesions produced, they were grouped in to five categories and finally the mean lesion score was obtained (Fernando, 2000).

Results and Discussion

Culture age, media and temperature on sporulation

The sporulation pattern of the two species, was similar, both starting to sporulate on the 2nd day after incubation. Though the growth rate of *C. gloeosporioides* (K1 & K2) was much faster than *C. acutatum* (R1 & R2), after six days of incubation period *C. acutatum* (R1 & R2) showed profuse sporulation producing 13.3 X 10^6 (± 1153.25) and 18.24 X 10^6 (± 2086.05) spores cm^{-2} respectively. Sporulation of *C. gloeosporioides* (K1 & K2) was comparatively poor producing only 20.5 X 10^4 (±419.3) and 27.8 X 10^4 (±125.8) spores cm^{-2} respectively.

Though there were four media *viz.* PDA, LBA, MA and CDA in which the sporulation was high, PDA was the best for sporulation.

Spore production occurred between 10 - 35°C. No spores were observed at temperatures 5 and 40°C. At all temperatures *C. acutatum* maintained its higher spore productivity than *C. gloeosporioides*. The sporulation of isolates *C. acutatum* (R1 & R 2) was about eight times greater than *C. gloeosporioides* (K1 & K2) at 30°C where optimum sporulation was observed (Fig. 1).

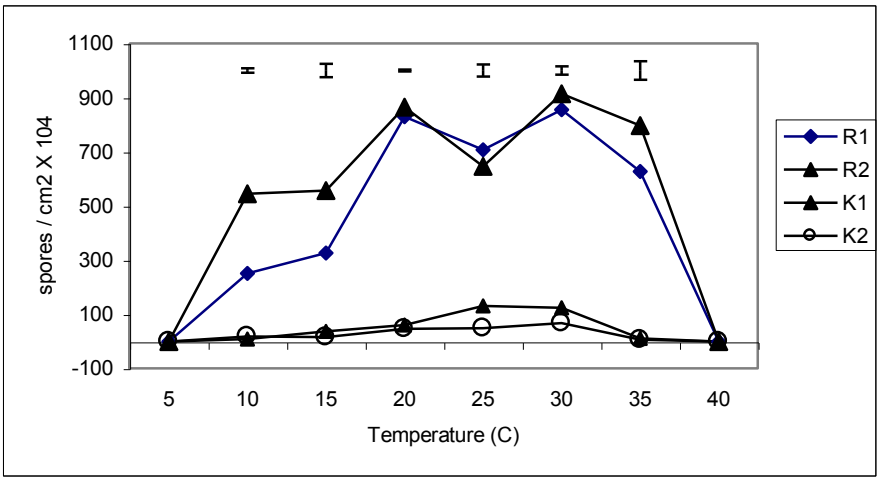

Fig. 1. Effect of temperature on spore productivity of *C. acutatum* isolates (R1 & R2) and *C. gloeosporioides* isolates (K1 & K2). Bar represents LSD 0.05 I, high/low

Germination and viability of spores

Germination commenced in *C. gloeosporioides* (after 2h) a little earlier than *C. acutaum* (after 3h). Both species showed maximum germination 7-8 hrs after incubation (Table 1). The elongation of the germ tube was also more rapid in *C. gloeosporioides* but in *C. acutatum* formation of appressoria was seen in shorter germ tubes.

Both isolates showed self-inhibition at high concentrations. Though there was a reduction, *C. acutatum* (R2) spores germinated at much higher concentrations than *C. gloeosporioides* (K1). Germination of R2 and K1 was totally inhibited at concentrations 10 X 10^6 and 50 X 10^5 spores per ml respectively.

Temperature greatly influenced germination in both species. Germination occurred above 10°C and below 40°C with an optimum range of 20 - 30°C (Fig. 2). The ability of *C. acutatum* and *C. gloeosporioides* to germinate at a wider range of temperatures probably facilitates its spread in both temperate and tropical countries. Results of experiments carried out to find the critical effect of exposing spores from the two species to various temperatures revealed that a considerable number of spores can remain viable, provided that the RH is high.

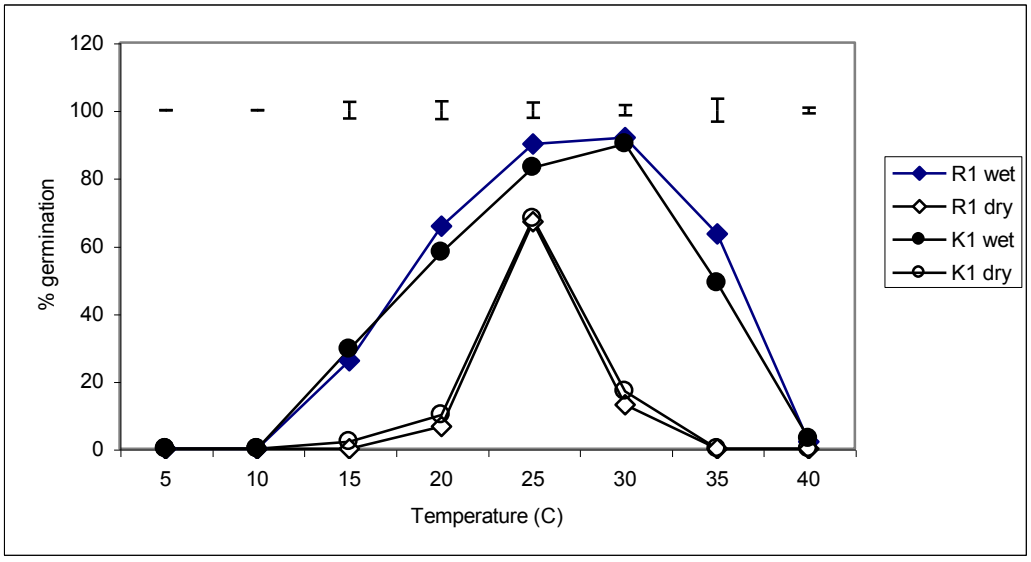

Fig. 2. Effect of temperature on spore germination under wet and dry conditions *C. acutatum* (R2) and *C. gloeosporioides* (K1). Bar represents LSD 0.05 I, high/low

Table 1. *Effect of incubation period on spore germination of* C. acutatum *(R2)* C. gloeosporioides *(K1)*

Incubation period (hours)	% germination	
	***C. acutaum* (R2)**	***C. gloeossporioides* (K1)**
1	0	0
2	0	0.73 (±0.4)
3	2.5 (±0.5)	1.23 (±0.25)
4	8.83 (±4.96)	23.7 (±1.53)
5	45 (±3.0)	40.7 (±3.1)
6	73.0 (±2.6)	92.0 (±1.0)
7	86.0 (±5.3)	92.7 (±1.53)
8	94.3 (±4.04)	95.0 (±1.0)

Four - hour exposure of *C. gloeosporioides* to 35°C as dry smears caused the total loss of viability while in *C. acutatum* the germination was reduced to 10% even after an exposure period of 8h to 40°C as dry smears. Long periods of exposure (>12h) to lower temperatures (<10°C) significantly reduced % germination in both species.

Nearly all spores (> 95%) from both the species germinated in the presence of free water and around 90% germinated in the absence of free water provided that they were exposed to humidities above 96% (Table 2). However, germination reduced significantly (<3%) in both species when incubated at 91% RH and none germinated at 85% RH (Table 2). Free water was not essential for germination but promoted germination in both *C. acutatum* and *C. gloeosporioides*.

Table 2. *Effect of relative humidity on spore germination* C. acutatum *(R2) and* C. gloeosporioides (K1)

Relative humidity (%)	% germination	
	***C. acutatum* (R2)**	***C. gloeosporioides* (K1)**
100 (wet)	97.33(±2.52)	95.3 (±4.16)
100 (dry)	77.83 (±1.26)	85.0 (±3.0)
96	75 (±1.0)	75.0 (±1.0)
91	5.67 (±2.08)	3.33 (±1.52)
85	0	0

As shown earlier the growth of *C. gloeosporioides* is faster than *C. acutatum* (Jayasinghe *et al*., 1997). Similarly, in the liquid medium with the carbon sources; pectin (Fig. 3a) and carboxy methyl cellulose (Fig. 3b), the growth of *C. acutatum* was slow.

When grown in liquid medium with citrus pectin as the main source of carbon, both species produced PG very early, on the 2nd day after inoculation. However, *C. acutatum* (R2) showed significantly high PG activity than in *C. gloeosporioides* (K1) (Fig. 4). Past literature shows that the molecular weights of PG from *C. acutatum* isolate of rubber were around 32000 (Fernando *et al*., 2001) while for *C. gloeosporioides* it was (Senaratne *et al*., 1991). The production of PL was more or less similar between the two species (Fig. 5). But the molecular weight of PL

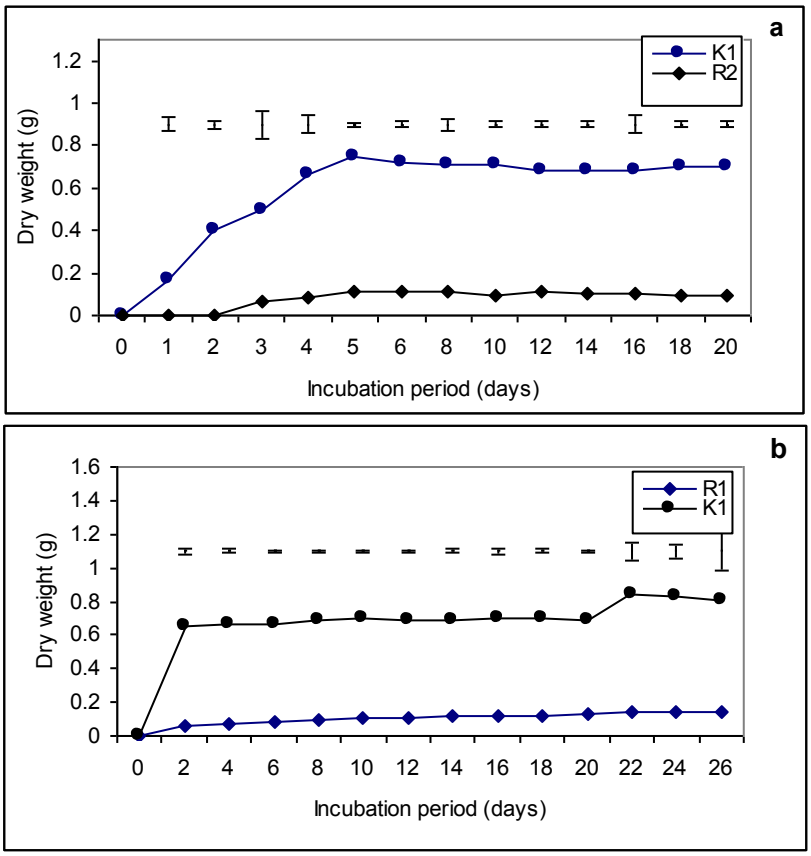

Fig. 3. Growth pattern of *C. acutatum* (R2) and *C. gloeoesporioides* (K1) in liquid culture (a) citrus pectin & (b) carboxy methyl cellulose as the main carbon sources. Bar represents LSD 0.05 I, high/low.

from *C. acutatum* isolates of rubber was 24000 (Fernando *et al*., 2001) and *C. gloeosporioides* obtained from the same host showed a molecular weight of 4150 (Senaratne *et al*., 1991). These results clearly show that physiological differences existing between the two species. When the isolates were grown in liquid medium with CMC as the main source of carbon both isolates produced B- glucosidase and cellobiase. Similar patterns of secretion were shown by both isolates.

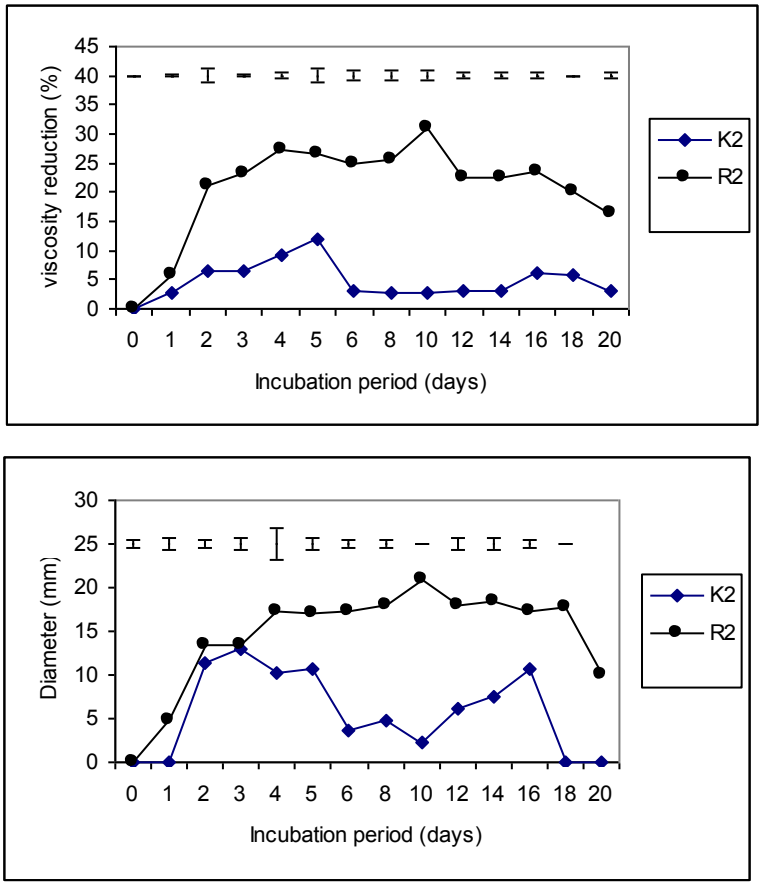

Fig.4. Production of polygalacturonase by *C. acutatum* (R2) and *C. gloeosporioides* (K1), (a) assayed by cup-plate method & (b) by viscosity reduction method. Bar represents LSD 0.05 I, high/low.

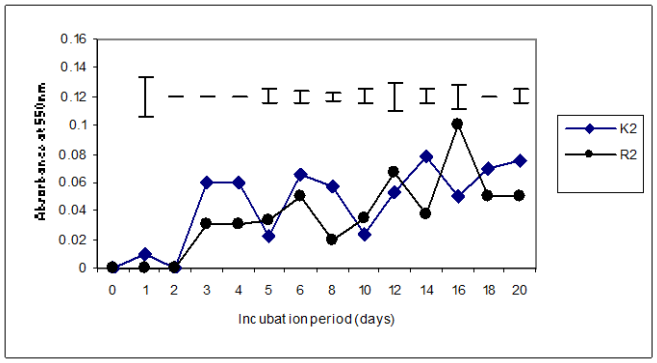

Fig. 5. Production of pectin lyase (PL) by *C. acutatum* (R2) and *C. gloeosporioides* (K1). Bar represents LSD 0.05 I, high/low

Results on the pathogenicity ratings obtained by the detached leaf assay showed two significantly different pathogenicity levels for the two species (Fig. 6). *C. acutatum* showed a lower average pathogenicity level than *C. gloeosporioides* when tested with eight different *Hevea* clones. However, isolations from naturally infected tissues demonstrated that the species *C. acutatum* plays the major role in causing CLD of rubber (Jayasinghe *et al*., 1997). Hence, additional population studies giving special reference to the climatic conditions are needed to substantiate their behaviour in the conditions of mixed infection.

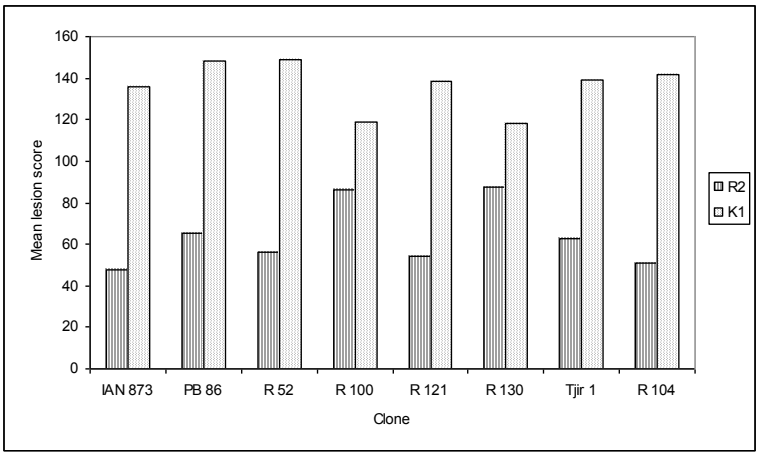

Fig. 6. The mean lesion score obtained by detached leaf method after inoculating artificially with *C. acutatum* (R2) and *C. gloeosporioides* (K1)

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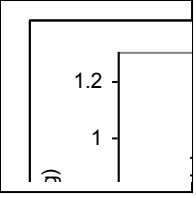

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