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TOXIC METABOLITE FROM CLOVE ISOLATE OF *CYLINDROCLADIUM QUINQUESEPTATUM*

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ABSTRACT

Cylindrocladium quinqueseptatum *Boedijn & Reitsma causes seedling blight and extensive defoliation on a wide variety of plants and is widely distributed in the humid tropics. It is a serious pathogen of* Eugenia caryophyllata *in Sri Lanka and has been identified as a potential pathogen on* Hevea brasiliensis *in the Eastern Hemisphere. All clove isolates; IMI 342173 (Rt), 359378 (Aw), 359379 (Rw) and 359380 (Kp) of* C. quinqueseptatum *tested were found to be capable of secreting toxic substances to the growing medium. Toxic culture filtrates proved to be thermostable (up to 100°C) and host specific. The type and the size of the lesions produced by the crude toxin of different isolates varied markedly. Isolate, Rt produced the largest lesions (highest score) indicating its aggressive nature in toxin production while Aw and Rw isolates showed a mild reaction. When the reaction of* Hevea *clones towards the crude toxin was considered three main clusters of clones were distinguished through cluster analysis indicating the marked variation of* Hevea *clones grown in the Eastern Hemisphere in their sensitivity to the crude toxin. The possibility of making use of this toxin as a tool in screening* Hevea *populations for* Cylindrocladium *leaf spot disease has also been discussed.*

Key words: *Cylindrocladium quinqueseptatum, Eugenia caryophyllata, Hevea brasiliensis,* toxin

INTRODUCTION

Cylindrocladium quinqueseptatum Boedijn & Reitsma (telemorph: *Calonectria quinqueseptata* Figueiredo & Namekata) is a common pathogen of *Eucalyptus* spp. in Nothern Australia (Pitkethley, 1976; Bolland *et al.*, 1985), Brazil (Figueiredo & Namekata, 1967), India (Sharma & Mohanan, 1982; Sharma *et al.*, 1984), Indonesia (Peerally, 1974), Mauritious (Peerally, 1974) and Vanuatu (Ivory *et al.*, 1993) and on

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Eugenia caryophyllata (clove) in India (Sarma & Nambiar, 1978), Indonesia (Reitsma & Slooff, 1950) and Sri Lanka (Jayasinghe & Liyanage, 1982). It is also reported to cause leaf spots and defoliation in certain clones of *Hevea brasiliensis* in Malaysia (Anon, 1972) and China (Kaiming, 1987), *Annona squimosa* in Brazil (Figueiredo & Namekata, 1970), *Terminalia paniculata* in India (Mohanan & Sharma, 1985) and *Camellia sinensis* in Mauritius (Peerally, 1974).

In Sri Lanka the fungus *C. quinqueseptatum* was first identified as a pathogen on *Eugenia caryophyllata* in 1982 (Jayasinghe & Liyanage, 1982) and it causes seedling blight and severe defoliation of clove during heavy monsoon periods. Leaf spots caused by *C. quinqueseptatum* on *Hevea brasiliensis* was first reported in Malaysia in 1972 (Anon, 1972). Later it was observed that five rubber clones grown in Malaysian budwood nurseries were severely affected with this pathogen (Anon, 1990) and results of subsequent studies carried out in Sri Lanka using clove isolates showed that certain *Hevea* clones commonly grown in the Eastern Hemisphere are also highly susceptible to *C. quinqueseptatum* infections (Jayasinghe & Wijesundera, 1996).

The production of toxins as metabolic by-products in culture filtrates of phytopathogenic fungi and their involvement in the development of a number of destructive diseases in plants have been documented since 1930's (Tanaka, 1933; Meehan & Murphy, 1947; Brian *et al.*, 1949; Hiroe *et al.*, 1958; Nozoe *et al.*, 1965; Luke & Gracen, 1972; Yoder, 1980; Scheffer & Livingston, 1984; Strobel *et al.*, 1988; Scheffer, 1989; Pascual & Raymundo, 1993). Phytotoxins are now known from more than two dozens of genera and toxins from two genera namely *Drechslera* (Meehan & Murphy, 1947; Shukla *et al.*, 1987; Sugwara *et al.*, 1987 & 1988) and *Alternaria* (Tanaka, 1933; Hiroe *et al.*, 1958) are extensively studied. Several uses and potential uses of toxin have been pointed out since 1950's and utilized to screen plant populations for resistance to certain diseases (Wheeler & Luke, 1955; Schertz & Tai, 1969; Steiner & Byther, 1971; Onesirosan *et al.*, 1975).

The first report on the toxic effect of culture filtrates of *Cylindrocladium quinqueseptatum* is from India on the toxin activity of the *Eucalyptus* isolate (Anahosur *et al.*, 1976). Subsequently, Kaushik & Gupta (1991) reafirmed the findings of Anahosur *et al.* (1976). In both these studies only the preliminary work has been reported, such as the best medium for toxin production and effect of autoclaving on the activity of the crude toxin. Apart from these two short communications, there appears to be no systematic study though *C. quinqueseptatum* has a wide host range and distribution in the humid tropics. This paper reports the pattern and variation of toxin production by four clove isolates, some biological and physical properties of the crude toxin and reaction of different *Hevea* clones towards the crude toxin.

METHODS AND MATERIALS

The organism

Four *C. quinqueseptatum* isolates, Aw (IMI 359378), Rt(IMI 342173), Rw(IMI 359379) and Kp(IMI 359380) collected respectively from Avissawella, Ratnapura, Ruwanwella and Kuruwita representing four major rubber growing areas were used in initial experiments while Rt isolate was selected to study the properties of the toxin produced by *C. quinqueseptatum*.

Extraction of the crude toxin

After carrying out a series of initial experiments the following procedure was employed to obtain the crude toxin. Hundred ml Erlenmeyer flasks each containing 25 ml of modified Fries medium (Luck and Wheeler, 1955) was inoculated with three mycelial plugs, 4-mm in diameter, taken from a 6-day old colony of the test fungus grown on CDA at room temperature 28 ± 2°C. The cultures were incubated at room temperature under normal light and dark regime as stationery cultures. Unless otherwise stated cultures were harvested 9-d after incubation by filtering through Whatman No. 1 paper and then through 0.22 μ millipore filter. The resulting culture filtrate was stored at 4°C in small aliquots of 10 ml and used as the source of toxin.

Detection of toxin activity

Detached leaves of uniform maturity (apple green leaves) of *Hevea brasiliensis* were used to detect toxin activity in the culture filtrate. A needle point injury on the leaf tissue was made superficially and 0.02 ml of the culture filtrate was placed on it. Six drops (0.02 ml) were placed on either side of the midrib of the upper surface and inoculated leaves were incubated at approx. 100 % RH in trays lined with moist blotting papers at room temperature for 48 h. Filtrates from uninoculated media served as controls. At the end of the incubation period the inoculated drops were removed by using a blotting paper and the reaction was ranked from class I - V based on the chart given in Table 1. At least six leaves were inoculated per treatment and ratings given to 24 lesions (randomly). Results were analysed using the nonparametric procedure NPARIWAY, available in statistical package SAS (SAS, 1987). The statistical method employed was the Kruskal Wallis test and the results were presented in the form sum of ranks. Subsequent paired comparisons were made using Wilcoxon 2 - sample test.

Time course of toxin production

To determine the variation of toxin production with time the isolates were grown upto 30 days as described previously and the culture filtrates were harvested at 3-d intervals. The toxin activity of all samples were examined as above.

Table 1. *Estimation of infection on leaves inoculated with* Cylindrocladium quinqueseptatum *toxin*

Rating	Nature of lesion
I	minute lesions resulted due to pin point injury
II	lesions with a size of pin head
III	diffused lesions with a diameter of 2-3 mm
IV	diffused lesions with a diameter of 4-5 mm
V	diffused lesions with a diameter of 6-7 mm
VI	diffused lesions with a diameter more than 8 mm

Effect of temperature

For this experiment the toxins from isolate Rt was employed. The toxin (culture filtrate having toxin activity) was exposed to different temperatures, 40°, 50°, 60°, 70°, 80°, 90°, 100°, 110° and 120°C for 10 m and thereafter activity was assessed on two different *Hevea brasiliensis* clones *viz.* RRIC 100 and Tjir 1.

Effect of crude toxin on different hosts

The activity of crude toxin from the isolate Rt was tested on several genera, *Cocos nucifera*, *Desmodium ovalifolium*, *Eucalyptus grandis*, *Eugenia caryophyllata*, *Hevea brasiliensis*, *Ipomea batatas*, *Oriza sativa*, *Polyanthia* sp., *Psopocarpus tetragonolobus*, *Pueraria phaseoloides* and *Saccharum officinarum*. Activity was assessed on different hosts by placing 0.02 ml of the crude toxin on detached young leaves (leaves from different development stages were included) while making a needle puncture as described for *Hevea*. Treated leaves were incubated in humid chambers at room temperature for 72 h. Six leaves were assessed from each plant species.

Variation among isolates in toxin production and reaction of different *Hevea* clones towards the toxin

The method described by Steiner and Byther (1971) was modified slightly and employed. Eighteen *H. brasiliensis* clones grown in the eastern hemisphere or experimentally proved to be potential clones for future use were screened using the crude toxin produced by *C. quinqueseptatum* isolates Aw, Kp, Rt and Rw. Toxins from the isolates Rt, Kp, Aw and Rw were extracted incubating 9,12,15, and 18 days respectively. The clones evaluated were RRIC 36 (Sri Lanka), RRIC 45 (Sri Lanka), RRIC 52 (Sri Lanka), RRIC 100 (Sri Lanka), RRIC 102 (Sri Lanka), RRIC 103 (Sri Lanka), RRIC 105 (Sri Lanka), RRIC 121 (Sri Lanka), RRIC 130 (Sri Lanka), RRIC

131 (Sri Lanka), Tjir 1 (Indonesia), PB 86 (Malaysia), PB 260 (Malaysia), PR 235 (Indonesia), HP 74 - 193 (Sri Lanka), RRIM 712 (Malaysia), RRIM 600 (Malaysia) and IAN 873 (South America). Detached apple green leaves of *Hevea brasiliensis* were placed on inverted petri dishes in humid (approx. 100% RH) chambers and a needle point injury on leaf tissue was made. Subsequently, 0.02 ml drops of the crude toxin were placed on each point where injury was made. Six drops were introduced on to each leaf and there were six replicate leaves from each *Hevea* clone for each isolate. The reaction was assayed after 48 h as described previously.

RESULTS AND DISCUSSION

Time course of toxin production: Crude extracts of all clove isolates (Rt, Kp, Aw and Rw) were found to be toxic on *Hevea* leaves indicating the presence of toxic substances. These results are in agreement with Anahosur *et al.* (1976) and Kaushik & Gupta (1991) who showed the ability of the eucalyptus isolate of *C. quinqueseptatum* to produce a toxin. However, this is the first report of toxin production by the clove isolate of *C. quinqueseptatum.*

Toxin production was initiated after 72 h incubation in all isolates. Isolate Rt had maximum toxin activity on the 9th day after incubation while Kp isolate showed the maximum on 12th day. Isolates Aw and Rw gave the highest amounts on 15th and 18th day respectively (Fig. 1).

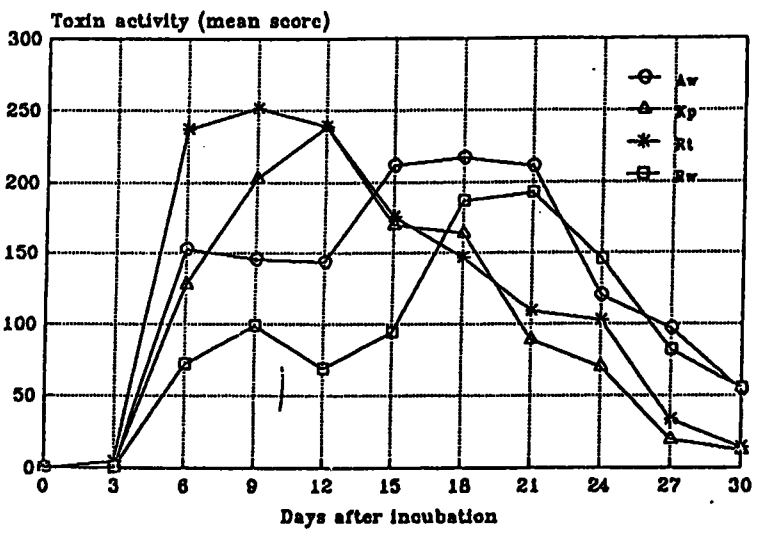

Fig. 1. Time course of toxin production by four *C. quinqueseptatum* isolates

Effect of temperature. Temperatures upto 100°C had no effect on the crude toxin when activity was assessed on detached *Hevea* leaves of two clones, Tjir 1 and RRIC 100. However, the activity decreased significantly when the toxin was heated to 110°C and above for 10 minutes (Fig. 2). These observations are contrary to findings of Anahosur *et al.* (1976) and Kaushik & Gupta (1990) who reported that crude toxin produced by Eucalyptus isolate of *C. quinqueseptatum* is autoclavable (15 lbs pressure for 15m). This contradiction may be due to the difference in isolates used and/or different assay methods employed.

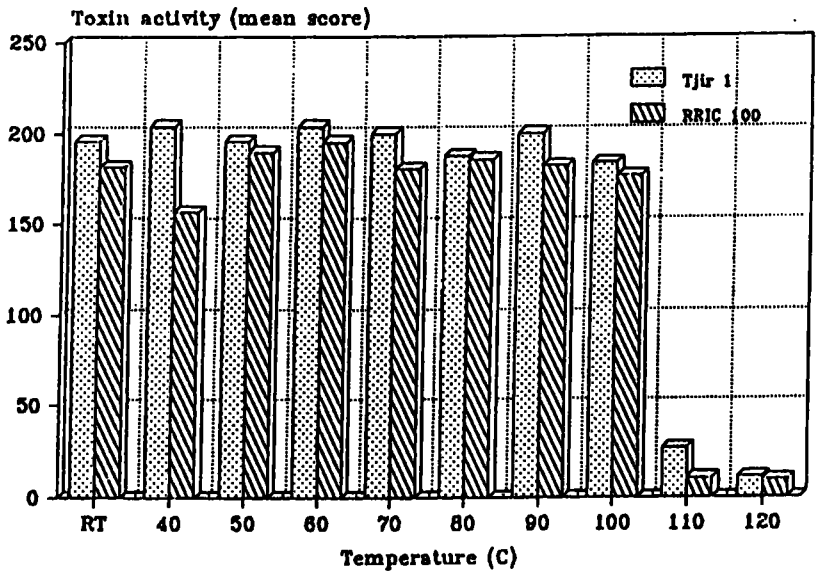

Fig. 2. Effect of temperature on the activity of crude toxin produced *by C. quinqueseptatum*. Observations on two *Hevea brasiliensis* clones, Tjir 1 and RRIC

Reaction of plant species to toxic culture filtrates: Intense lesions were observed on the leaves of *Eucalyptus grandis*, *Eugenia caryophyllata*, *Hevea brasiliensis* and *Polyanthia* sp. within 72 hours while no lesions were produced on *Cocos nucifera*, *Desmodium ovalifolium*, *Ipomea batatas*, *Oryza sativa*, *Psophocarpus tetragonolobus*, *Pueraria phaseoloides* and *Saccharum officinarum* even with incubation periods upto five days. *E. grandis* (Peerally, 1974c & Pitkethley, 1976), *E. caryophyllata* (Reitsma & Slooff, 1950; Sarma & Nambiar, 1978; Jayasinghe & Liyanage, 1982) and *H. brasiliensis* (Anon, 1972) which showed positive reactions are all natural hosts of the fungus *C. quinqueseptatum*. Hence, this toxin might be considered as a host selective toxin. The lesions produced after the introduction of crude toxin (on 5th day) were comparable with the symptoms produced on leaves infected by the fungus.

Variation in isolates in crude toxin production: Lesions were produced on detached leaves of all *Hevea* clones when culture filtrates from isolates Rt and Kp were asayed. Aw and Rw isolates did not produce lesions on some inoculation sites in a few *Hevea* clones. However, the size of the lesions produced by different isolates varied markedly (Fig. 3 & Table 2) suggesting marked variation in toxin production in different isolates.

Rt isolate had the highest score (Table 2) when mean lesion score on all *Hevea* clones was considered indicating a higher aggressiveness. A mild reaction was shown by the Aw and Rw isolates (Table 2).

Table 2. *Lesion score of four isolates of* C. quinqueseptatum *on* Hevea *leaves*

Isolate	Aggressiveness of isolate*
Rt	1657.8 a
Kp	1544.2 b
Aw	854.2 c
Rw	523.1 d

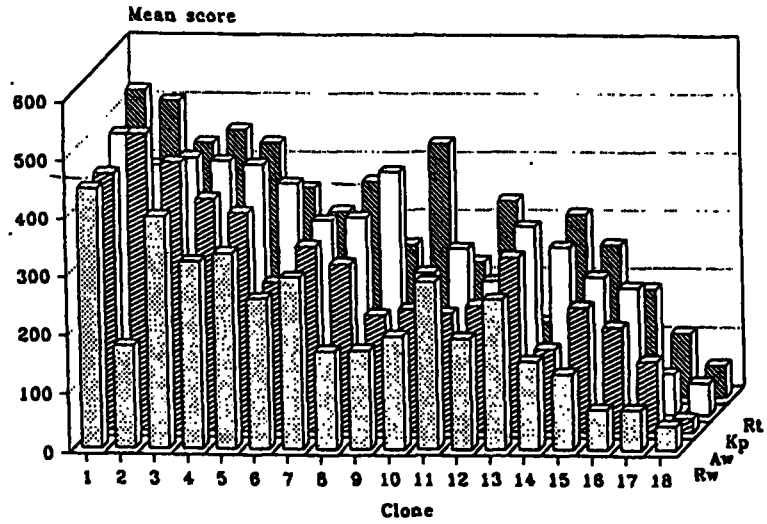

Fig. 3. Variation in lesion score resulted on eighteen *Hevea brasiliensis* clones after placing 0.02 ml of crude toxin produced by four *C. quinqueseptatum* isolates *viz.* Awissawella (AW), Ratnapura (Rt), Kuruwita (Kp) and Ruwanwella (Rw)

1. RRIC 105	2. Tjir 1	3. RRIC 36	4. RRIC 121	5. RRIC 100
6. PB 86	7. RRIC 45	8. RRIC 52	9. PR 235	10. HP 74-193
11. RRIC 103	12. PB 260	13. RRIM 712	14. IAN 873	15. RRIM 600
16. RRIC 130	17. RRIC 131	18. RRIC 102		

Multvariate analysis on reaction of* Hevea brasiliensis *populations towards crude toxin: Three main clusters of clones were distinguished (Fig. 4) through cluster analysis. The clones which showed an extremely severe reaction namely RRIC 105, RRIC 36, Tjir 1, RRIC 121 and RRIC 100 represent cluster 1. Eleven clones is found in cluster 2 while RRIC 131 and RRIC 102 formed cluster 3 which is the least sensitive to the toxin (Fig. 3 & 4). These observations confirmed that a marked variation exist among the *Hevea* clones grown in Eastern Hemisphere in sensitivity to the crude toxin produced by *C. quinqueseptatum.*

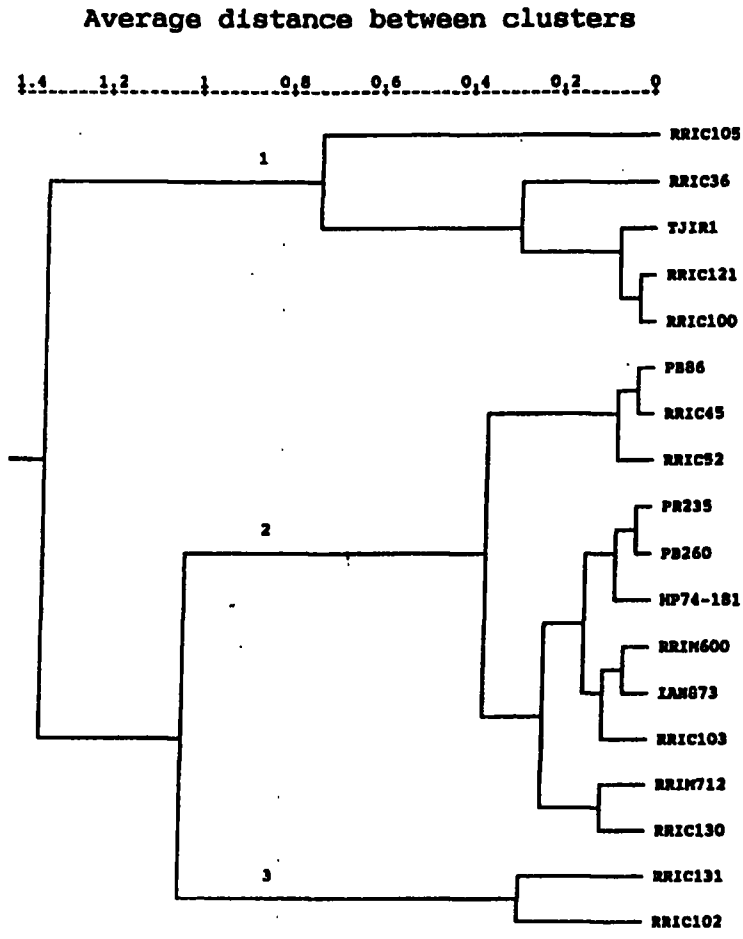

Fig. 4. Dendrogram showing the grouping of 18 *Hevea* clones grown in Eastern Hemisphere based on sensitivity to crude toxin produced by *C. quinque*eseptatum. The average linkage method of clustering was applied to Euclidean distances

In another series of experiments, immature leaves of different rubber clones were inoculated with spore suspensions (0.02 ml of a 1 x 10^5 spores ml^{-1} suspension) of the same isolates of *Cylindrocladium quinqueseptatum* (Jayasinghe & Wijesundera, 1996). The diameter of lesions produced after incubation at room temperature under 100% RH for 48h were measured. The observations revealed that all the clones except RRIC 105 representing cluster 1 in present experiment had comparatively high mean lesion diameters indicating their extreme susceptibility. No comment could be made on RRIC 105 as it was not included in the previous inoculation experiments. Clone RRIC 102, the most resistant genetic material against the crude toxin had only a mean lesion diameter of 7.64 mm when inoculated with spore suspensions. It was the second highest resistant clone among the 21 clones tested in the inoculation experiment (Jayasinghe & Wijesundera, 1996).

Previous researchers have pointed out that toxins produced by pathogens are useful tools in screening cultivars in oat (Wheeler & Luke, 1955), sorghum (Schertz & Tai, 1969), sugarcane (Steiner & Byther, 1971) and tomato (Onesirosan *et al.*, 1975). With regard to rubber the first such attempt comes from Sri Lanka where crude toxin from *Corynespora cassiicola* has been utilized to screen nursery lines of *Hevea brasiliensis* in 1988 (Silva, 1988). The results of our study show that toxin from *C. quinqueseptatum* also can be a tool in *Hevea* breeding programmes.

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