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DETECTION OF β-1, 3-GLUCANASE ISOFORMS AGAINST CORYNESPORA LEAF DISEASE OF RUBBER (*HEVEA BRASILIENSIS*)

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Corynespora cassiicola causing leaf diseases of *Hevea* is considered to be a serious problem in most of the rubber growing countries. Production of a pathogenesis related [PR]protein β-1,3-glucanase upon infection was tested in four clones of *Hevea*. Considerable variability in the β-1,3-glucanase activity of enzyme was observed among different clones during pathogenesis. Increased enzyme activity was found in the tolerant clone (GT.1), while a decrease was observed in the susceptible (RRII 105). Three prominent β-1,3-glucanase isozyme bands were detected by 17.5 per cent PAGE in the tolerant clone.

Key words : *Hevea brasiliensis*, *Corynespora cassiicola*, β-1,3-glucanase.

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INTRODUCTION

Leaf disease caused by *Corynespora cassiicola* (Berk. & Curt.) Wei. has been reported from various rubber growing countries. Earlier it was considered to be a minor problem confined to rubber nurseries. The first report of *Corynespora cassiicola* on rubber was by Deighton (1936). Later the disease was reported from India (Ramakrishnan and Pillai, 1961), Malaysia (Newsam, 1961), Nigeria (Awoderu, 1969), Indonesia (Situmorang and Budiman, 1984), Brazil (Junqueira *et al.*, 1985), Sri Lanka and Cameroon (Liyanage *et al.*, 1986), Thailand (Pongthep, 1987), Bangladesh (Rahman, 1988), and Vietnam (Dung and Hoan 1999). Recently severe incidence of this disease on mature trees was reported from Karnataka state in India (Rajalakshmy and Kothandaraman, 1996). Chemical control has been adopted for the control of this disease in the field. The high cost and environmental impact of spraying are the undesirable effects of chemical control. Alternative methods involving biochemical or genetic manipulations of host-pathogen interaction have been found to increase the resistance to fungal pathogens in a number

of crop species. (Hain *et al.*, 1993; Jongedijk *et al.*, 1995). Plants respond to environment as well as pathogen attack through the induction of defense mechanisms including the production of antimicrobial compounds like phenolics, phytolexins and pathogenesis related (PR) proteins or by the involvement of hydroxyprotein rich glycoproteins (Vidhyasekaran, 1997; 1998). A number of PR proteins accumulate in the plant following fungal pathogenesis (Vanloon, 1994). The PR proteins like β-1,3-glucanase (PR 2), chitinase (PR 3) and chitin binding protein (PR 4) have been reported to show antifungal activity in *in vitro* assays (Kombrink and Schroder, 1988). PR protein production can therefore be used as a tool in the screening of *Hevea* clones for resistance against *C. cassiicola*.

Only very limited information is available on the expression of PR protein in rubber (Narasimhan *et al.*, 1998). An early study (Breton and d'Auzac, 1996), indicated the elevation of β-1,3 -glucanase activity in certain clones of *Hevea* due to infection of *Corynespora*. In the present paper the detection and estimation of β-1,3-glucanase activity against *Corynespora* leaf disease in four clones of *Hevea* is presented.

MATERIALS AND METHODS

Plants and pathogen

Budded stumps of the four rubber clones *viz.*, GT 1, RRIM 600, PR 107 and RRII 105 were selected for this study and were grown in polybags in a glass house. Ten to twelve day old light green leaves were used for inoculation. An isolate of *C. cassiicola* showing high virulence was selected from the culture collection of Rubber Research Institute of India. The pathogen was multiplied on potato dextrose agar (PDA) medium for abundant sporulation. Spores were harvested after 6 days and (7×10^4 /ml) suspension was prepared with sterile distilled water.

Induction and challenge

Leaves were inoculated under both laboratory as well as glass house conditions. After the inoculation, the plants were covered with transparent polythene bags. Control plants were sprayed with distilled water and maintained under similar conditions.

Preparation of enzyme extracts

Infected leaf samples from the polybag plants were collected at 24, 48, 72 and 96 h after inoculation for enzyme extraction. The mid rib was removed and the hypersensitive lesion areas of leaves were extracted with 0.05M sodium acetate buffer (pH 5) at 4 °C in a pre-cooled mortar and pestle. The extracts were dialysed against two changes of water and two changes of 0.01M sodium acetate buffer (pH 5) overnight and were used as crude enzymes for assays (Pan *et al.*, 1991).

Estimation of β-1,3- glucanase activity

β-1,3-glucanase activity in the extract was totally assayed colorimetrically through laminarin-dionitrosalicylate method (Abels and Forrence, 1970; Pan *et al.*, 1991). The enzyme extract (62.5 µl) was added to 62.5 µl of 4 per cent laminarin and then incubated at 40° C in a water bath for ten minutes. The biochemical reaction was stopped by adding 375µl of dinitro salicylic reagent and heating the sample for ten minutes in a boiling water bath. The coloured solution obtained was diluted with 4.5ml of double distilled sterile water, vortexed and its absorbance at 500nm determined. The crude enzyme extracts

mixed with laminarin under no incubation was considered as blank. One unit of enzyme activity is defined as the amount of enzyme that produce, reducing sugars equivalent to one micromole of glucose equivalent per ten minutes under the above conditions.

Native polyacrylamide gel electrophoresis (PAGE) and detection of β-1, 3- glucanase isomers

Electrophoresis under native conditions was performed on slab gels by the method of Davis (1964) with a 17.5 per cent separating and stacking gels. The gel was run with the constant current 30mA for 4 h at 4 – 6 °C by an anodic electrode buffer system.

Enzyme staining

After electrophoresis, the PAGE gel (containing two replications of treatment) was cut into two halves, washed with double distilled water thrice and incubated in 0.05M potassium acetate buffer (pH 5.0) for five minutes with slow shaking. One half of the gel was incubated at 40°C for 30 minutes in a mixture containing 75ml of 0.05M potassium acetate buffer (pH 5.0) and 1g laminarin dissolved in 75ml of water by heating in a boiling water bath. The other half of the gel was incubated in the same conditions without laminarin inorder to detect the proteins other than β-1,3-glucanase. The PAGE gel was then transferred to a glass tray containing 0.4g of 2,3,5-triphenyl tetrazolium chloride in 200 ml of 0.1M NaOH. The tray was kept in boiling water bath until the bands appeared.

RESULTS AND DISCUSSION

Variation in symptom expression was observed between the clones. In RRII 105 and PR 107 the initial symptom appeared within 24 h and clearly visible symptom was observed within 48 h. In GT 1 hypersensitive reaction appeared 48 h after inoculation. In RRIM 600, the leison-development was slower and was observed in 72 h. Leison development was slow and the size of the leisons were smaller in resistant reactions. While susceptible reaction was recorded in PR 107, RRII 105 and RRIM 600 very limited leison formation was observed in GT 1 (Fig 1).

β-1, 3 -glucanase activity

The β-1,3-glucanase activity was estimated from the inoculated leaves of all the four clones and the results are given in Figs. 2(a-d) . In 24 h after inoculation, 18 units of β-1,3 -glucanase activity was estimated in GT 1 (Fig. 2a) and 12 units in RRIM 600 (Fig. 2b). The other two clones (RRII 105 and PR 107) showed lower enzyme activity (Figs. 2 c&d) than the controls. In the case of RRIM 600, the β-1,3 -glucanase activity in inoculated plants remained constant throughout the experiment. But in GT 1, there was progressive increase in the enzyme activity up to 96 h. Inoculation caused reduction of enzyme activity in RRII 105 and PR 107 at the later stages of the experiment.

The β-1,3-glucanase appeared as bright red bands on native PAGE gels. Three major β-1,3-glucanase isozyme bands were detected in induced leaves of GT 1 at 96 h, *viz.*, G1, G2 and G3 (Fig. 3a). No positive reaction was detected in other clones, both in induced and control leaves. The other half of PAGE gels containing the same enzyme preparations when incubated without laminarin in the buffer and stained with triphenyl tetrazolium reagent showed only one red band in the crude extracts (indicated by arrow) of induced GT 1 at 96 h

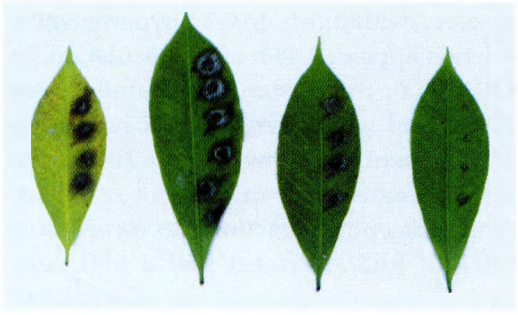

Fig. 1. Variation in the symptom expression of *C. cassiicola* on the leaves of different rubber clones (after 96 h incubation)

(Fig. 3b). This observed band was not β-1,3-glucanase but was formed by staining of the reducing sugars, which were released from the protein by the reagent suggesting that control without substrate should be included in the enzyme assays.

Enhanced activity of β-1,3-glucanase upon fungal disease development in resistant/tolerant crop varieties is a common phenomenon. The enhanced level of β-1, 3-glucanase upon *C. cassiicola* infection in GT1 indicates tolerant reaction. The observation on Corynespora disease incidence in the field (Rajalakshmy and Kothandaraman, 1996) as well as the reaction of this clone under artificial inoculation (present study) indicated the tolerant nature of GT 1. Pan *et al.*, (1991) reported a similar

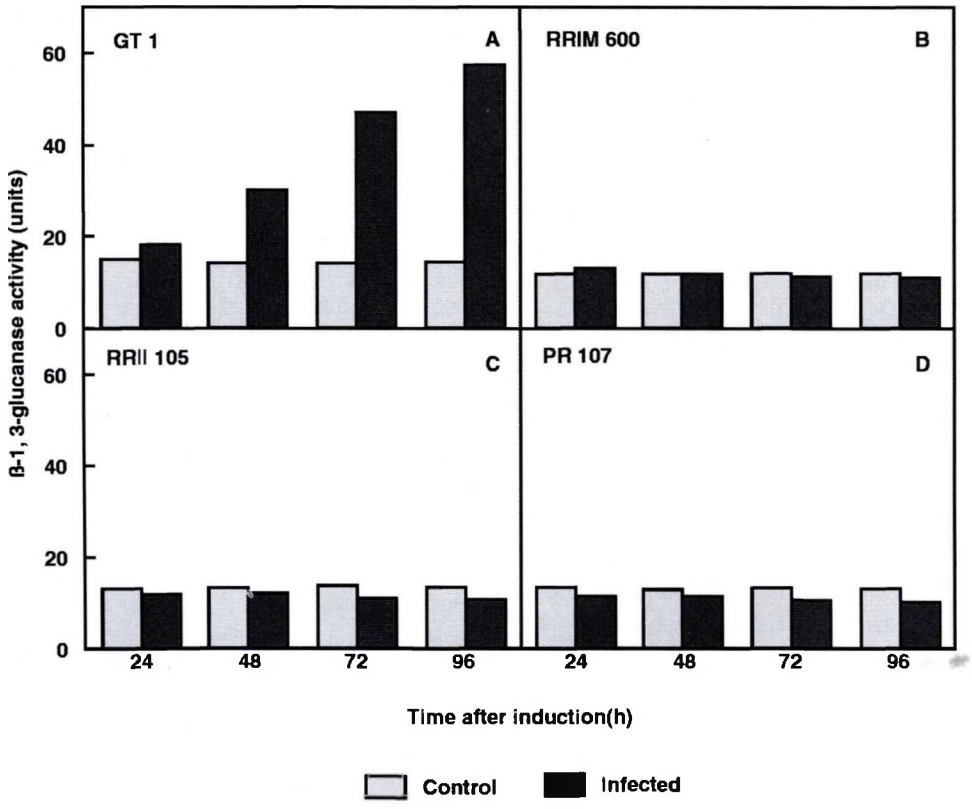

Fig. 2. β-1,3-glucanase activity in *Hevea* clones on infection by *C. cassiicola*

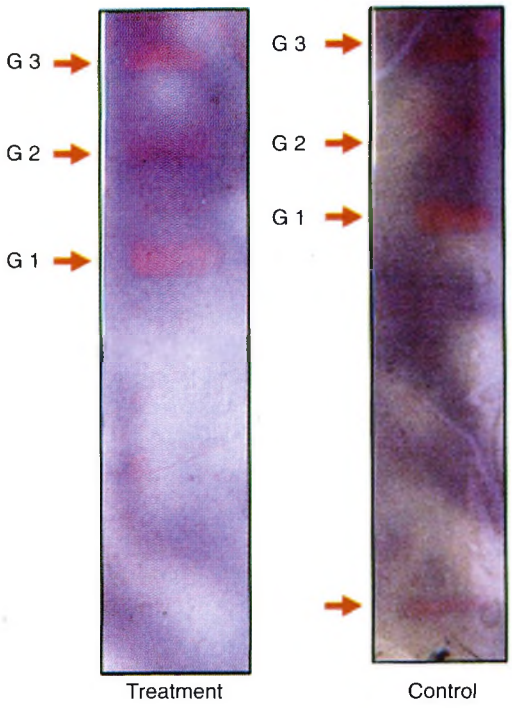

Fig. 3. β–1, 3–glucanase isoforms indced by *C. cassiicola* on leaves of clone GT1 (96 h after inoculation)

enhanced level of β-1,3-glucanase upon fungal infection in tobacco and tomato and established the role of this enzyme in resistant reactions. While studying the resistant reaction in tomato against *Phytophthora infestens* Christ and Mosinger (1989) also confirmed the role of β-1,3-glucanase in resistant reactions.

The level of the resistance was positively correlated to the elevation of β-1,3-glucanase activity with hypersensitive reaction giving higher enzyme activity. The enzyme activity was maximum in GT 1 where a typical hypersensitive reaction to *Corynespora* was observed. This suggests that β-1,3 -glucanase may play a marked role in the resistance mechanism.

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