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A rapid and simple chitinase assay to screen tea genotypes for resistance against blister blight

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Abstract

Plant naturally responds to fungal attack by a complex network of defense mechanisms. Among these, production of pathogenesis related (PR) proteins is an important mode of resistance against pathogen invasion. Chitinase is one such PR protein and a potent biomarker for the identification of promising resistant germplasm. In this study, Gram's iodine based detection assay for chitinase production in tea cultivars has been attempted to screen and validate the data available on the resistance level of the cultivars against blister blight disease. Small wells in the glycol-chitin-agarose/agar plates were loaded with enzyme extract and flooded with Gram's iodine after 24h. The glycol-chitin formed a bluish complex with iodine, whereas the areas around the wells showed a sharp and distinct clearance zone, against the blue background. The diameter of the zone increased with increased amount of chitinase present in the sample. These observations were validated with field data on blister blight disease incidence in these cultivars and discussed. The new approach is an easy, reproducible, cost effective, user and environmental friendly qualitative method for rapid screening of the tea cultivars in breeding program.

Keywords: Chitin, chitinase, cultivar, pathogenesis, tea

Introduction

Tea (*Camellia sinensis* (L.) O. Kuntze) plants, grown for their leaves, are being invaded by fungal pathogens like *Exobasidium vexans,* causing blister-blight infection and *Pestalotiopsis theae* causing grey-blight disease, which deteriorate its foliage, thus incurring qualitative and quantitative loss to the tea industry. Enhancing the resistance of tea clones to fungal pathogens is desirable to minimize the incidence of these diseases. Plants naturally respond to fungal attack by a complex network of defense mechanisms *viz*. synthesis of (1) polymers forming physical barriers, (2) anti-microbial metabolites, and (3) pathogenesis related (PR) proteins (Iseli *et al.,* 1996). Of the above, the production of PR proteins is considered to be an important mode of resistance against pathogen invasion as their accumulation results in the quantitative increase of major soluble proteins of the host (Stintzi *et al.,* 1993).

Plant chitinases are considered to be PR proteins since their activity is greatly induced by fungal, bacterial and viral infections as well as by general signals of stress, both chemical and environmental (Kasprzewska, 2003). The enzyme is involved in the catalytic hydrolysis of chitin, the insoluble linear β-1,4 linked polymer of N-acetylglucosamine found in the cell wall of fungi and the exoskeleton of insects (Muzzarelli, 1977). Although extensive research has been carried out to understand this enzyme on different aspects in various dicotyledonous plants and few important monocotyledonous crops like rice, barley, maize, *etc*., very little studies have been taken up in tea

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with regard to its characterization during disease infestation. Screening of tea cultivars for PR proteins is of immense importance, keeping in view the demand for new enzymes and the improvement of their biotechnological applications. Previous studies on other plant species demonstrated the potential use of chitinase as a biochemical marker for identifying, resistant germplasm and promising cultivars for breeding (Roberts *et al.,*1994; Vleeshouwers *et al.,* 2000).

Currently, many detection methods *viz*., colorimetric, fluorescent based, radioactive, reflectance spectroscopy, and direct detection of activity after polyacrylamide gel electrophoresis are available (Howard *et al.,* 2003). These methods, albeit satisfactory, have the following difficulties *viz*., large sample size requirement, use of hazardous chemicals, expensive equipment and time consuming. Therefore, attempt was made to develop a rapid and reproducible assay for estimation of chitinase activity in tea cultivars which can be used as a biochemical marker to screen tea genotypes for resistance to blister blight. The gel diffusion and Gram's iodine based detection assay developed in the present study is an easy, reproducible, cost effective, and user and environment-friendly qualitative method for screening the tea cultivars for resistance against blister blight pathogen *E. vexans.*

Materials and methods

Plant material

Tea clones, from the UPASI- Tea Research Institute, Valparai (latitude 10º 30´N, longitude 27º 0'S and altitude 1,050 m above mean sea level) were selected for the present study. The first leaves of the actively growing shoots were used for crude enzyme extraction. These were harvested between 9 and 10 a.m., frozen and transferred immediately in liquid nitrogen and stored at -80 ºC till further use.

Protein extraction

Frozen leaf tissues were ground using a mortar and pestle and homogenized in 0.01 M sodium acetate (pH 5.0) in a ratio of 3 mL of buffer for 1 g of fresh tissue. The homogenate was filtered through two layers of cheesecloth, centrifuged at 12000 rpm for 15 min at 4 °C and the supernatant was used in the present study. The protein content of the supernatant was measured using Coomassie plus protein assay reagent with bovine serum albumin as the standard (Bradford, 1976) and was used as crude enzyme extract.

Chitinase assay

Chitinase activity was assayed quantitatively by spectro-photometry using glycol chitin or colloidal chitin as substrate according to Sun *et al.,* (2006).

Preparation of the substrate and qualitative activity assay

Agarose solution (1% w/v) was prepared in sodium phosphate (0.01 M pH 5.5) and heated to boiling point. One mL of a 1 per cent glycol chitin solution was added to 100 mL of the agarose solution. The resulting suspension was stirred to ensure homogeneous distribution of the substrate and 30-mL aliquots were poured into polypropylene petri dishes (15 cm diameter). The agarose was allowed to cool and solidify for 20-25 min. Small wells (3 mm diameter) were carved in the agarose gels at a distance of 1.5 cm from each other to form a grid. The protein concentration in the crude enzyme extracts of tea clones were equalized among all samples and 100 µL of each sample (10 mg mL^{-1}) was loaded in the wells. After incubation at 37 °C for 24 hours, the plate was flooded with Gram's iodine (2 g KI and 1 g iodine in 300 mL distilled water) for 3 to 5 minutes. After incubation, plates were observed for the zone of clearance around the well. The assay was performed at 37 °C for different lengths of time to optimize the procedure as described above.

Chitinase detection and quantification

The standard chitinase from *Aspergillus niger* (Sigma-Aldrich) was used for validation of assay results and quantification of activity in enzyme extract of tea clones. Dilutions of standard chitinase were prepared to contain 25, 50, 75, 100 and 125 U mL^{-1} in sodium phosphate buffer (0.01 M pH 7.0). Hundred microliters of each concentration were placed in the wells of the reaction plate to get 2.5, 5.0, 7.5, 10 and 12.5U concentrations. Each concentration was assessed five times per experiment.

After the assay for standard and samples, the plates were stained using Gram's iodine as described above. After staining, the iodine solution was discarded and the gel plates were gently washed with distilled water for 5 min at room temperature in an orbital shaker. The assay plates were photographed using GENSCAN gel documentation system under white light. The diameter of the sharply discernible, clear, hydrolyzed zone in bluish black background was marked on the bottom of each plate. Chitinase activities in samples from tea clones were calculated from a regression line correlating the hydrolyzed zone diameter from standard chitinase on a logarithmic scale (Fig. 2B).

Results and discussion

Qualitative chitinase assay

The glycol chitin-agarose plates used for the assay were observed for the zone of clearance around the wells loaded with the crude enzyme extracts. The glycol chitin in the plates had formed a bluish complex with iodine, whereas the areas around the wells showed a sharp and distinct clearance zone, against the blue background. Glycol chitin present in the zone was digested by the chitinase present in the extract and thus the formation of the bluish complex was not observed (Figs. 1 & 2A). The diameter of the zone increased with increased amount of chitinase in the sample and thus the area of the clear zone is a direct measure of the amount of chitinase produced by the respective sample. Thus, by naked-eye observation, larger the area of the clear zone around a given well, higher is the chitinase in the sample, which makes the assay an easy method for ascertaining the amount of the enzyme present in the sample.

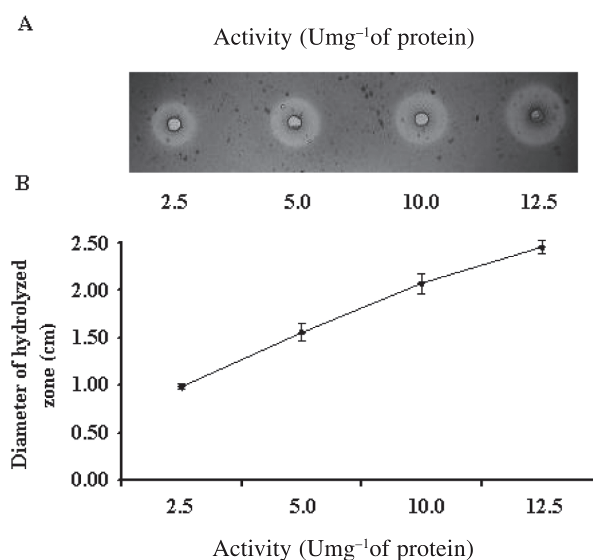

Fig. 2(A). Representative gel-diffusion assays of chitinase activity from standard chitinase of *Aspergillus niger*. Gram's iodine was used to stain unhydrolyzed glycol chitin, and the diameters of hydrolyzed zones were measured on agarose plates. (B) A representative regression line correlating the diameter of the hydrolyzed zone was determined on a logarithmic scale. The regression equation for predicting enzyme activity was $\ln E = -0.9327 + (0.9488 \times D)$ at the assay conditions of 28ºC and pH 5.5, where E = the unit of enzyme activity and D = the diameter of the hydrolyzed zone in centimeters. The coefficient of determination for the regression line was 0.98. Error bars represent standard deviation calculated from the three independent assays

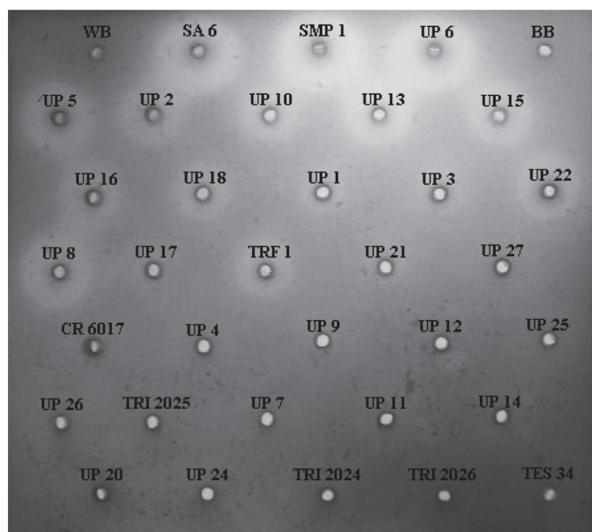

Fig. 1. Representative gel-diffusion assays for chitinase activity from protein extracts of tea clones. Each well contained 100 µL of crude protein extracts. Wells labeled as WB and BB contained sterile distilled water and the incubation buffer (0.01 M sodium acetate, pH 5.0), respectively as negative assay control. The experiment was repeated thrice

Based on the plate assay results, the chitinase activites in each clone was calculated by regression analyses using the data obtained from three independent experiments of standard chitinase (Fig. 2A & 2B). The regression equation for

Table 1. Chitinase activities and grouping of tea clones

Tea clone	Diameter of hydrolyzed zone[a]	Quantitative chitinase activity[b]	Disease resistance index[c]	Groups[d]
SMP 1	3.9	23.45 ± 2.46	0.5	Highly resistant
SA6	3.0	16.77 ± 3.53	3.0	Highly resistant
U6	2.9	14.70 ± 0.71	6.6	Resistant
U5	2.5	11.10 ± 0.15	9.0	Resistant
U2	2.4	12.13 ± 1.09	11	Tolerant
U16	2.4	11.10 ± 1.13	11	Tolerant
U13	2.2	11.41 ± 1.39	13	Tolerant
U15	2.1	12.07 ± 1.24	13	Tolerant
U18	2.0	11.13 ± 0.43	13	Tolerant
U10	2.0	9.99 ± 0.52	15	Tolerant
TRF1	1.8	10.07 ± 1.02	15	Mostly susceptible
U22	1.8	10.09 ± 1.08	15	Mostly susceptible
U27	1.7	9.36 ± 1.03	16	Mostly susceptible
U1	1.7	8.14 ± 0.94	16	Mostly susceptible
U3	-	9.77 ± 0.08	16	Mostly susceptible
CR6017	1.4	7.69 ± 0.84	17	Mostly susceptible
U8	0.9	11.05 ± 0.17	18	Mostly susceptible
U17	-	8.41 ± 0.45	18	Mostly susceptible
U21	-	5.03 ± 0.88	19	Mostly susceptible
U25	-	3.45 ± 0.13	23	Susceptible
TRI2025	-	1.62 ± 0.15	24	Susceptible
U9	-	1.11 ± 0.17	26	Susceptible
U12	-	1.05 ± 0.15	27	Susceptible
U4	-	0.68 ± 0.06	28	Susceptible
U26	-	0.32 ± 0.13	30	Susceptible
TRI2024	-	0.23 ± 0.15	31	Highly susceptible
U11	-	0.09 ± 0.06	32	Highly susceptible
U24	-	0.25 ± 0.16	32	Highly susceptible
U14	-	0.31 ± 0.10	34	Highly susceptible
TRI2026	-	0	38	Highly susceptible
U20	-	0	40	Highly susceptible
U7	-	0	46	Highly susceptible
TES34	-	0	48	Highly susceptible

[a] Diameter of hydrolyzed zone as estimated from plate assay in centimeter.
[b] Chitinase activity of tea clones determined by quantitative assay of three independent estimations as described in Materials and methods (Umg^{-1} of protein ± SD).
[c] Blister blight disease resistance index in field condition (Annual report, UPASI, 2006).
[d] Grouping of tea clones based on the plate assay

predicting enzyme activity at 28 ºC and pH 5.5 was $\ln E = -0.9327 + (0.9488 \times D)$, where E and D represents the unit of enzyme activity and the diameter of the hydrolyzed zone in centimeters respectively. The coefficient of determination for the regression line was 0.98 (Fig. 2B).

Based on the qualitative analysis of chitinase activity, the tea clones were grouped into highly resistant, resistant, tolerant, mostly susceptible, susceptible and highly susceptible clones (Table 1). The grouping and values thus obtained for each clone coincide with the estimated quantitative values (Table 1) and their disease incidence index values as observed in field conditions. The clones SA-6 and SMP-1 showed highest chitinase activity (23.45 and 16.77 U mg^{-1} of protein), than other clones tested, which may contribute to their highly resistant nature against blister blight pathogen infection. On the other hand, the highly susceptible clones like UPASI-14 and TES-34 have least and no chitinase production, respectively.

In conclusion, the assay and screening method developed in this study avoids the use of hazardous chemicals, saves time and materials and can be performed with small sample quantities. Therefore, plant breeders could potentially screen the tea germplasm for promising resistant varieties using this method which can also be utilized in the studies of the enzyme induction in plant–pathogen interactions, acquired resistance and plant defense systems.

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