Application of a robust microplate assay to determine induced β -1,3-glucanase and chitinase activity in the cotton plant

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ABSTRACT

Resistance is induced in cotton plants as the result of either viral infection or exogenous application of elicitors. Induced resistance can be evaluated by determining the production of β -1,3-glucanase and chitinase in plants as a biochemical parameter. The assays being used for the determination of chitinase and β -1,3-glucanase activity are laborious and not cost-effective, as the reducing sugars produced by the substrates colloidal chitin and laminarin are very expensive. The concentration of both substrates was standardized and reduced to 0.25% from 4% in a modified microplate assay, which appeared to be more effective. The amount of β -1,3-glucanase and chitinase produced was significant and determined by the new modified assay. The sensitivity of the microplate assay was significantly raised approximately one- to twofold.

METHOD SUMMARY

The existing procedure for determining resistance induced in cotton plants by viruses and elicitors is time-consuming and expensive. However, this resistance can be determined by β -1,3-glucanase and chitinase activity through a modified, cost-effective and robust technique. Here the authors present a 96-well polystyrene microplate assay, instead of a quartz cuvette assay, in which a decreased amount of costly substrates was used and a large number of samples were processed in a short time.

KEYWORDS:

β -1,3-glucanase • chitinase • cotton • elicitors • microplate assay

Plants have specific mechanisms against infection through their defense responses. Chitinases, polyphenol oxidases, glucanases, proteinase inhibitors and other pathogenesis-related (PR) proteins are produced as a result of induced defense responses and act as a physical barrier to pathogens [1]. A wide range of phytopathogenic organisms attack plants, and these enzymes and proteins play a significant role in enhancing the defense-related mechanisms against them [2]. Viruses, fungi, bacteria, animals and plants are inhibited by chitinases, whereas β -1,3-glucanase has a vital role in a variety of developmental processes and defensive behaviors of plants against pathogens. The inhibitory action of plant chitinases is achieved either by direct hydrolysis of the chitin in fungal cell walls or by releasing polysaccharide oligomers, stimulating plant defense reactions [1,2]. Based on amino acid sequence similarity, chitinases are classified into two glycosyl hydrolase families: 18 and 19. Family 19 chitinases are generally found in plants and some bacteria, whereas family 18 chitinases are found in a broad range of organisms, including viruses, bacteria, fungi, animals and plants [3]. There have been only a few reports of bacterial chitinase expression in plants [4,5]. The β -1,3-glucanase gene family is involved in a wide variety of plant physiological developmental processes as well as pathogen defense mechanisms. The hydrolysis of 1,3- β -D-glucosidic linkages in β -1,3-glucans is catalyzed by the enzymes of β -1,3-glucanases and is widely found in bacteria, fungi and viruses. However, plant β -1,3-glucanases, classified as the PR-2 family of PR proteins, have been proposed to defend against pathogen infection in two ways: by hydrolyzing β -1,3-glucan, a major structural component of fungal cell walls, and by promoting the release of cell wall-associated elicitors that further stimulate defense reactions [6]. Infection of resistant plants with viruses triggers a local hypersensitive reaction in the host [7], which is thought to block the systemic spread of the virus and result in a local lesion response. Callose, a (1 $\rightarrow$ 3)- β -D-glucan [8], is deposited between the plasma membrane and cell wall as part of the hypersensitive reaction to infection by fungi [9] and viruses as well as in response to abiotic stresses [10]. Therefore, β -1,3-glucanases play a role in affecting the pathogenicity of viruses [7].

The enzymatic activity of β -1,3-glucanase and chitinase can be deployed as a biochemical marker for evaluating resistance. This is determined not only for plants [11,12] but also for microbial preparations [13,14]. To determine β -1,3-glucanase activity, a technique developed by Dygert *et al.* has been used widely [15]. To overcome the problem of copper oxidation during measurement, Zheng and Wozniak developed a robust microplate assay capable of measuring β -1,3-glucanase activity in 20 tobacco plant samples within 1 h [16]. In addition,

tion, as a substitute for an expensive cuvette assay, a microplate assay was developed to estimate β -1,3-glucanase activity in microbial preparations using 3,5-dinitrosalicylic acid (DNS) reagent as an oxidant [17]. DNS reacts with the carbohydrates in an alkaline solution with ketone or aldehyde groups. The concentration of the reductant directly affects the depth of color within a specific concentration range, that is why reducing sugars in plants and animal feed are widely determined by DNS [18].

Cotton plants were used in this experiment for the optimization of an assay to determine β -1,3-glucanase and chitinase. Enzymatic activity of β -1,3-glucanase and chitinase in cotton plants was induced through the inoculation of *Cotton leaf curl virus* (CLCuV) and exogenous application of elicitors like salicylic acid (SA) and benzothiadiazole (BTH).

CLCuV is a geminivirus transmitted by the whitefly (*Bemisia tabaci* Genn.) [19–22] and is well known for causing major losses to cotton production in Pakistan. The virus contains ssDNA with two geminate particles. The symptoms of CLCuV are upward and downward curling of the leaves, with small and large vein thickening. A leaflet structure known as enation, appearing on the underside of the leaf, is a characteristic symptom of the CLCuV Multan strain [23]. CLCuV acts as a "helper virus" for two dissimilar types of DNA satellite molecules of non-viral origin: beta satellites and alpha satellites. These satellites play a vital role in the suppression of host defense mechanisms, which leads to enhanced virulence of the virus. Enhanced virulence results in systemic infection and severe symptoms of disease [23].

Salicylic acid (SA) acts as a signaling hormone and provides a pathway for the activation of a defense reaction [24]. SA is an endogenous regulator that has a definite role in defense mechanisms against biotic and abiotic stresses [25]. The expression of PR genes is closely related to the exogenous application of SA and also confers resistance against various pathogens, such as viruses, bacteria and fungi [26,27].

Benzothiadiazole (BTH) is a chemical analog of SA. It has been used effectively to induce resistance to an extensive variety of diseases affecting field crops [28]. BTH is a powerful systemic acquired resistance activator downstream from SA signaling. It increases crop resistance by activating systemic acquired resistance in many plant species, including bean, cauliflower, cucumber, tobacco, apple and pear [29]. BTH also induces resistance against geminivirus in pepper plants [30].

The resultant induction of the aforementioned enzymatic activity was determined through an easily modified microplate assay in which laminarin and colloidal chitin were used as substrates and reducing sugars were quantified by DNS reagent. This method would work as an alternative to cuvette-based macroassay, which is expensive and time-consuming.

Methods

An experiment was planned to evaluate the effect of treatments over 15 days by quantifying chitinase and β -1,3-glucanase enzymes in cotton plant leaves. The highly susceptible cotton variety CIM-496 was grown in a screen house measuring 600 sq ft in earthen pots measuring 12 × 9 × 6 in. At four-leaf stage treatments, BTH (Sigma-Aldrich, MO, USA) and SA (Sigma-Aldrich) were exogenously applied with an atomizer at concentrations of 2.20 mM (equal to 300 mg/l) and 10 mM, respectively. The CLCuV Multan strain was inoculated using whiteflies (*B. tabaci*). Aviruliferous whiteflies were reared on CLCuV Multan strain-infected plants in BugDorm (MegaView Science Co., Ltd., TXG, TW) cages measuring 75 × 75 × 115 cm with nylon 150 mesh. There were three biological replicates of each treatment as well as three technical replicates of each treated sample. The samples were collected for analysis after 72 h of elicitor application and from virus-inoculated plants when disease symptoms appeared. The experiment was carried out twice with the same number of plants and treatments, and the data were compiled by taking the average of two independent experiments.

Enzyme extraction

Freshly detached cotton leaves (1 g) from treatments were vigorously homogenized using liquid nitrogen in a precooled pestle and mortar, followed by the immediate addition of 2 ml of chilled 0.05-M sodium acetate buffer (pH 5.0), and transferred to a 1.5-ml centrifuge tube for centrifugation at 13,500 × g for 15 min at 4°C. The supernatant was used directly as an enzyme in the assay. For protein determination, leaves were extracted separately in sodium phosphate buffer (pH 7.2). Total proteins were estimated using Bradford reagent and bovine serum albumin as the standard [31].

Cuvette assay for β -1,3-glucanase

Laminarin (Sigma-Aldrich) and dinitrosalicylic acid methods [32] were used to determine β -1,3-glucanase activity. The reaction was prepared using a 4% laminarin and enzyme extract mixed in an equal volume of 62 μ l, followed by incubation at 40°C for 10 min. The reaction was terminated by adding 375 μ l of DNS reagent. The reaction mixture was heated for 5 min in boiling water, and optical density was measured at 550 nm with a SmartSpec 3000 UV/visible spectrophotometer (Bio-Rad Laboratories, Inc., CA, USA). Enzymatic activity was designated as μ g glucose produced per min per mg of protein.

Cuvette assay for chitinase

Chitinase activity was measured using chitin from shrimp shells (Sigma-Aldrich) as substrate. Colloidal chitin was prepared according to the described method [33]. Sodium acetate buffer 50 mM (pH 6.0) was used to prepare a 1% solution of colloidal chitin, and 300 μ l was mixed in 1 ml of enzyme sample, followed by an incubation of 30 min at 40°C [34]. The reaction was terminated by adding 600 μ l of DNS [35]. Insoluble chitin was removed by keeping the mixture in hot water for 20 min. After boiling, the resultant mixture was chilled

Figure 1. DNS standard curves for calculating released reducing sugars for (A) microplate assay and (B) cuvette assay. DNS: 3,5-dinitrosalicylic acid.

and centrifuged. The subsequent reducing sugars were calculated at 550 nm using a spectrophotometer [36]. Chitinase activity was designated as U mg^{-1} of protein.

Microplate-based assay

Enzymatic production of chitinase and β -1,3-glucanase was estimated separately using 0.25% colloidal chitin and laminarin, respectively, as substrates. The reaction was prepared by mixing 20 μl of each substrate solution with 10 μl of enzyme extract. The mixture was added to a Multiplate 96-well non-skirted PCR plate (Bio-Rad Laboratories, Inc.) and sealed with Axygen® sealing film (Corning Life Sciences, AZ, USA). The PCR plate was incubated for 15 min at 40°C in a 96-well MyCycler (Bio-Rad Laboratories, Inc.). After incubation, 1.3% phenol (Sigma-Aldrich), 20% Na-K-tartrate, 100 μl of DNS reagent (Sigma-Aldrich) and 1.25% NaOH were mixed and heated for 5 min at 95°C. The prepared mixture was cooled at room temperature, and 100 μl was added to a 96-well microtiter ELISA plate. The absorbance of reduced sugars in the microplate was calculated at 550 nm using an ELx800 ELISA reader (BioTek Instruments, Inc., VT, USA). Enzymatic activity was calculated by the quantity of enzyme, which produced μg of reducing sugar per min per mg of protein under both sets of conditions.

Glucose standard curve

For the standard curve, scientific-grade glucose (Sigma-Aldrich) at a concentration of 750 $\mu\text{g/ml}$ was added to non-skirted PCR plates in a quantity of 0, 20, 40, 60 and 80 μl each in three replicates. Water was added to make a volume of 100 μl , followed by the addition of 100 μl DNS reagent in reaction. Each plate was heated at 95°C for 5 min in a thermocycler. Each reaction was cooled to room temperature and transferred to a microtiter ELISA plate, and absorbance was measured at 550 nm in a spectrophotometer. The regression equation was derived from the standard curve (Figure 1).

The regression equation for microassay under the standard condition:

$$Y = 0.0479X - 0.0192 \quad (\text{Eq. 1})$$

$$X = Y + 0.0192 \div 0.0479 \quad (\text{Eq. 2})$$

Table 1. Comparison of enzymatic activity measured by microplate- and cuvette-based assays using 0.25% laminarin and colloidal chitin as substrates.

Treatment	β -1,3-glucanase assay U mg ⁻¹		Chitinase assay U mg ⁻¹	
	Microassay	Cuvette assay	Microassay	Cuvette assay
Control	17.98 ± 0.03d	9.27 ± 0.81c	108.67 ± 0.40c	97.67 ± 1.67d
Virus-inoculated	30.75 ± 0.71c	16.25 ± 2.29b	148.67 ± 1.02b	122 ± 2.19c
SA (10 mM)	37.83 ± 0.40b	21.39 ± 1.86ab	193 ± 1.79b	145 ± 2.90b
BTH (2.2 mM)	42.99 ± 0.57a	27.10 ± 1.85a	194 ± 1.81a	154 ± 2.13a
Statistics				
LSD value	5.61	5.83	4.21	7.47
CV	2.67	16.74	1.39	3.05
Chi-square	8.8	1.56	3.93	0.49
t-value	11.67	8.72	15.03	19.29

Values significant at p < 0.05. Means in each column with the same letter are not significantly different at <0.05. BTH: Benzothiadiazole; CV: Coefficient of variation; LSD: Least significant difference; SA: Salicylic acid.

$$Y = OD_{\text{Sample}} - OD_{\text{Control}} \quad (\text{Eq. 3})$$

$$\beta\text{-1,3-glucanase/Chitinase (U/mg)} = X \times SV \div VSE \times PC \quad (\text{Eq. 4})$$

The value of X from Equation (2) was put in Equation (4) and solved.

$$\begin{aligned} &= (Y + 0.0192) \div 0.0479 \times SV \div (VSE \times PC) \\ &= 20.876 \times (Y + 0.0192) \times SV \div (VSE \times PC) \end{aligned} \quad (\text{Eq. 5})$$

Equation (5) was used for the calculation of β -1,3-glucanase and chitinase activity in microassay. The regression equation for cuvette assay under standard condition: $Y = 0.0445X - 0.0303$

$$X = Y + 0.0303 \div 0.0445 \quad (\text{Eq. 6})$$

Putting the values in Equation (4).

$$= 22.47 \times (Y + 0.0303) \times SV \div (VSE \times PC) \quad (\text{Eq. 7})$$

Equation (7) was used for the calculation of β -1,3-glucanase and chitinase activity in the macroassay. PC represents protein concentration, SV represents sample volume and VSE represents volume of sample enzyme..

Statistical analysis

The collected data set was statistically analyzed and subjected to analysis of variance and Duncan's multiple range test for the comparison of treatments, and least significant difference values were also calculated for minimum significant difference among treatments. Chi-square test and t-test were employed for the determination of statistically significant differences between the two procedures. All statistical analyses were performed using Statistix 8.1 software (Informer Technologies, Inc., FL, USA).

Results & discussion

Enzymatic activity of β -1,3-glucanases and chitinases plays a crucial role in the defense mechanisms of plants. The quantification of this enzymatic activity requires an accurate and robust technique that produces reliable and efficient results. Many scientists have developed assays for the determination of these enzymes within plant and microbial preparations [16,37]. Here the authors optimized the modified microplate assay for the determination of β -1,3-glucanase and chitinase activity in cotton plants as a result of CLCuV infection and exogenous application of elicitors like BTH and SA. Therefore, the microplate-based method was optimized for only β -1,3-glucanase activity in *Trichoderma* spp. [37].

The authors' results showed a substantial increase in the enzymatic activity of β -1,3-glucanase and chitinase in elicitor-treated as well as virus-inoculated cotton plants compared with control (Table 1). To estimate the enzymatic activity of β -1,3-glucanase, the substrate laminarin was used at a concentration of 0.25% in a modified assay, which produced consistent results, whereas at higher concentrations

Figure 2. Comparison of β -1,3-glucanase activity of microassay (uppercase lettering) and macroassay (lowercase lettering). Different letters show a significant ($p = 0.05$) difference between the means.

Figure 3. Comparison of chitinase activity of microassay (uppercase lettering) and macroassay (lowercase lettering). Different letters show a significant ($p = 0.05$) difference between the means.

(i.e., 0.75, 1 and 2%), the results were not substantial enough to validate the increase in substrate concentration. To determine chitinase activity, the substrate colloidal chitin was also used at a concentration of 0.25% in this modified assay. Cuvette-based macroassay has been optimized with 4% laminarin for β -1,3-glucanase [32] and 1% colloidal chitin for chitinase [34], and any decrease in concentration has been shown to affect the results of enzymatic activity. Further, the comparison of microplate assay with cuvette-based macroassay in the current study revealed a significant difference in enzymatic activity (Figures 2 & 3). Lower least significant difference and coefficient of variation values reflected the consistency of the microplate assay compared with the cuvette-based assay. Enzymatic assay sensitivity in the cotton plant was almost twofold greater when determined by microplate assay compared with macroassay (Table 1). Ramada *et al.* also found that the sensitivity of the microplate assay was twofold higher than that seen with macroassay when determining β -1,3-glucanase activity in *Trichoderma* spp. [17]. Although the amount of chitinase produced was significantly higher in the microplate assay, the sensitivity was less than onefold higher than that seen with the cuvette-based macroassay.

Zheng and Wozniak devised a microplate assay to estimate β -1,3-glucanase activity in the tobacco plant using 0.4% laminarin as substrate, and reducing sugars were subsequently quantified by copper reagent and neocuproine. Oxidation of reduced Cu^+ due to interaction with air resulted in precipitation that altered the optical density [16]. In the authors' modified microplate assay, an aromatic compound (DNS reagent) was used to measure the reduced sugar without any precipitation problem when dealing with a large number of samples. Even 0.1% laminarin was used as substrate, but to estimate reducing sugars, aniline blue, HCl and NaOH were added to the reaction; after 1 h, a stable sirofluor–laminarin complex was formed and determined by fluorimeter. However, in the authors' optimized assay, only DNS was used to measure the reducing sugars, and no complex was formed [38]. Similarly, for the estimation of chitinase, trichloroacetic acid was used to stop the precipitation formation, followed by centrifugation, which made the process lengthy [39].

The precipitation problem also persists with other substrates (i.e., dye-labeled polysaccharide derivatives like carboxymethyl-chitin-Remazol Brilliant Violet 5R (CM-chitin-RBV) and CM pachyman-Remazole Brilliant Blue (CM-pachyman-RBB), which are used for chitin and 1,3- β -glucanase activity assays, respectively). The precipitation problem is overcome by the addition of 20% HCl, which separates non-degraded polymeric substrate from the reaction mixture before spectrophotometry [40]. This procedure is time-consuming and less sensitive because of precipitation.

The authors' optimized assay for the estimation of chitinase and β -1,3-glucanase is inexpensive, rapid, robust, time-saving, sensitive and accurate when measuring a large number of samples. Along with the cost of laminarin and colloidal chitin, approximately 100 samples can be assayed for only US\$2. In other assays, one has to deal with precipitation problems, larger quantities of substrates and long incubation and centrifugation times, which makes them time-consuming, expensive and difficult when handling a large number of samples. Moreover, the authors' modified assay can be used for both microbial preparations and leaf samples that produce chitinase and β -1,3-glucanase.

Conclusion

Our modified and optimized microplate assay appears to be better, faster and more robust and economical than cuvette assay for the determination of β -1,3-glucanase and chitinase enzymatic activity induced in the cotton plant by viral infection or exogenous application of elicitors.

Future perspective

The enzymes β -1,3-glucanase and chitinase are classified as PR proteins and appear to play a crucial role in the defense mechanisms of plants against pathogens. When evaluating crop varieties for resistance against diseases based on the production of these enzymes, our technique could be utilized for the screening of large germplasms in a short period. Plant varieties with elevated activity of these enzymes could be incorporated into a breeding program for resistance against diseases.

Author contributions

M F Khan worked under the supervision of U Umar. U Umar contributed by conceiving the study, designing the experiments, analyzing the data and proofreading the manuscript. M F Khan contributed by performing all the experiments and drafting the manuscript. Both authors have read and approved the final manuscript.

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