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Defensive role of *Gossypium hirsutum* L. anti-oxidative enzymes and phenolic acids in response to *Spodoptera litura* F. feeding

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ABSTRACT

The responses of the cotton plant, *Gossypium hirsutum* L. to herbivory by *Spodoptera litura* F. was studied in various laboratory experiments as a measure to understand the defense strategies of certain plant metabolites. Insect feeding damage enhanced the concentration of total phenol content and proteins, whereas amount of carbohydrates and amino acids were reduced. The experiments on estimation of anti-oxidative enzymes revealed stimulation in peroxidase, catalase, and superoxide dismutase levels and reduction in polyphenol oxidase and phenylanine ammonia lyase levels, signifying their defensive role in the plant. Specific phenolic acid changes were further carried out using High Performance Liquid Chromatography (HPLC), and distinct elevations in the gallic acid, catechin, and caffeic acid levels were observed in the infested cotton plant. Feeding assays towards *S. litura* with these phenolic compounds revealed activated detoxifying enzymes including β-glucosidase, carboxyl esterase, and glutathione-S-transferase in the insect gut indicating the toxicity. The aim of present study is to aid in further use of these specific phenolic acids towards effective management of the cotton pest, *S. litura*.

Introduction

Plants respond to herbivore damage from the single leaf to whole tree. The induced resistance developed in the plant decreases the herbivore performance through localized or systematic response. Many studies indicated induced resistance in the plants due to the attack of pest (Bi et al., 1997). These plants can defend themselves from the attacking pests by producing defensive chemicals, constitutive in nature as metabolites and self-protective proteins, and these plant defense strategies are generally activated in the presence of insect attack. This facilitates the specific plant–insect interactions (Karban and Baldwin, 1997) which lead to a new array of defense strategies that aid in effective pest management.

Primary metabolites such as proteins, carbohydrates, and amino acids, as well as secondary metabolites, such as phenolic acids and enzymes, are most likely to be influenced by the insect herbivory. Insect infestation will trigger the plants' anti-oxidative enzymes and biochemicals that play a vital role in plant protection (Usha Rani and Jyothsna, 2009; Abdul et al., 2012). Studies have supported that enhanced anti-oxidative enzymes and defense proteins in plants act as toxic chemicals to the attacking pest, thereby deterring the pest feeding. The detoxifying enzymes of the insects then aim to neutralize these toxins in the detoxification process. We studied the defense responses of cotton plant to the herbivory by *Spodoptera litura*, mostly in the form of altered metabolites (mainly phenolic acids) and attempted to correlate their role in cotton plant defense mechanisms towards *S. litura*.

Cotton, *Gossypium hirsutum*, is a natural plant fiber that estimates for about 25 million tonnes of world production, accounting for 2.5% use of world's arable land. Cotton is of great economic importance due to its role as a principal raw material in the textile industry. As such, effective practices must be maintained to meet the demand, but there are observed large losses due to economically destructive pests of cotton crop. The cotton leaf worm, *Spodoptera litura*, is one of the major pests of the cotton plant that accounts for severe leaf damage to the plant. In our earlier studies, we found that *S. litura* feeding induces chemical changes in various plants (Usha Rani and Jyothsna, 2009, 2010). Hence, we aimed at studying the responses of cotton plants towards the feeding of this pest, with antifeedant nature of enhanced plant phenolic acids and their toxic nature to the insect.

Materials and methods

Plants

Cotton, *Gossypium hirsutum* seeds (wild variety) were soaked in water for 10 h and incubated at 28 °C for 24 h. The germinated seeds were planted in 2 L pots in a greenhouse. The plants were watered

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every two days; and fertilized (N: K: P = 20: 20: 20) once a week. Plants at 45 days old having 8 leaves were used for the experiments.

Insects

The culture of *Spodoptera litura* (Fab) were collected from the fields in Hyderabad and grown in the laboratory. The diet consisted of bouquets of fresh cotton leaves, with their petioles immersed in glass conical flasks (100 ml capacity) containing tap water, which kept the leaves fresh for longer periods. The larvae were reared in big plastic tubs (15 L capacity) covered with muslin cloth, and a bouquet of cotton leaves were the ovipositional substrate. Larvae pupated at the bottom of the plastic tub or on the leaves. Pupae were collected in glass Petri dishes (7.6 cm diameter) and placed inside a big nylon mesh cage (30 × 30 × 45 cm) with a Zinc bottom. A bouquet of cotton leaves was offered to the emerging adults for oviposition.

Pest feeding

Cotton plants of 45 day old were brought to the laboratory. A single 3rd instar pre-starved (for 24 h) larva was released on the terminal leaf of each plant and confined to the point of release by enclosing it in a muslin bag. They were released into the plant during the early hours of the day. Insects were allowed to feed on the leaves for 24 h, and the remaining leaf portions after feeding by caterpillars were used for the biochemical and enzyme analysis. The leaves lateral to the infested leaf were also taken to analyze the effect of larval feeding on other parts of the plant. Larvae usually consumed approximately 30–50% of the leaf during 24 h of feeding. Fresh leaves from the normal and healthy cotton plants were the controls for comparison.

Chemicals

All the chemicals used for biochemical, enzyme, and HPLC analysis were of the analytical grade (Sigma–Aldrich, Germany). Rotenone, gallic acid, catechin, caffeic acid, and methanol were used for feeding bioassays.

Biochemical and enzymatic estimations

Biochemical estimations were carried out to analyze the herbivore wound induced changes in the plant. Standard methods were carried to estimate the total phenolic content (Singleton and Rossi, 1965), amino acids (Moore and Stein, 1954), total carbohydrates (Dubois et al., 1956), and proteins (Lowry et al., 1951). All experiments were carried out in 4 sets, with 5 replicates in each set. The biochemicals were expressed in mg/g FW (Fresh Weight).

The enzyme activities of the pest-fed and normal healthy plants were analyzed and compared. Standard methods were followed for measuring the activities of peroxidase (POD) (Kar and Mishra, 1976), polyphenol oxidase (PPO) (Ozturk and Demir, 2003), catalase (CAT) (Aebi, 1984), superoxide dismutase (SOD) (Beyer and Fridovich, 1987), and phenylalanine ammonia lyase (PAL) (Dickerson et al., 1984). All experiments were carried out in 4 sets, with 5 replicates in each set. The enzymatic estimations were expressed in Units/g FW (Fresh Weight).

Analysis of individual phenolic acids

Sample preparation

The phenolic extractions were done as per the method described earlier by Usha Rani and Jyothsna (2010) with minor modifications. One gram leaf was weighed and extracted in 95% methanol for 3 days under continuous shaking condition. The solution was filtered and evaporated into dryness by rotavapor. The dried material was re-suspended in 2 ml of HPLC grade methanol. It was filtered through 0.2 μm membrane, and the filtered sample was injected into the HPLC column for analysis.

HPLC analysis

Phenolic acids were analyzed using HPLC according to the method described by Tuzen (2003). The separation of phenolic compounds was accomplished on a Gilson GX-271 semi preparative HPLC system. The column was C_{18} (2.5 × 30 cm Gilson apparatus), and a liquid handler with auto injector was employed. For phenolic acid analysis, a gradient elution program was applied; and elution was done with solvent A (acetic acid/water (2:98 v/v)) and solvent B (acetic acid: acetonitrile: water (2:30:68 v/v)) as mobile phase. Initial condition was programmed as 100% A; 0–5 min, changed to 100% B; 25–35 min., with a flow rate of 1.0 ml/min. The sample injection volume was 100 μl. The signals were detected at 254 nm. Retention times for the standard compounds, and the major peaks in the extract were recorded. Identification and determination of the separated compounds were made by comparing retention time and structure of UV spectra of the separated compounds with standards. All the experiments were performed in five independent replicates.

Feeding bioassay

To understand how the individual phenolic acids may affect the relationship between the foliar quality and herbivore performance as well as to explain the leaf phenolic role in larval feeding, feeding bioassay was conducted. No-choice leaf disk assay described earlier by Akhtar and Isman (2004) with slight modifications was employed to determine the antifeedant activity of pure phenolic compounds. Clean glass petri dishes (15 cm diameter) were used as test arenas. Using a cork borer, fresh leaf disks (10.6 cm^2 area) were cut from foliage of greenhouse-grown cotton plants. Two doses (50, 100 μg/10 cm^2 of leaf) of the test material (phenolic acids) were sprayed uniformly on the surface of leaf disks at 50 μl per disk. Rotenone was used as a standard to compare the antifeedant index. A pre-starved (for approximately 3 h), actively-feeding early 3rd instar larvae of *S. litura* was placed individually into the petri dishes containing the treated and pre-weighed cotton leaf disks. The control leaf disk was sprayed with 50 μl of absolute methanol. Moistened filter paper lined the bottom of the petri dish and prevented the drying of the leaf. The experimental containers were kept in an illuminated growth chamber at 28 ± 2 °C. There were 10 replicates for each treatment, and all the treatments were repeated on 3 different days to avoid day to day variation, if any. Uneaten leaf material and consumed leaf by each larva after 12 h and 24 h of treatment were recorded by measuring the leaf area with a leaf area meter (Area meter AM 300, ADC Bioscientific Ltd).

The percentage antifeedant index was calculated following the formula reported by Lewis and Van Emden (1986):

$$\text{Antifeedant index}(\%) = [(C-T)/(C+T)] \times 100$$

where, C: Area of leaf disks consumed in control; and T: Area of leaf disks consumed in treatment.

Phenolic acids effect on insect detoxification enzymes

To find the effects of ingested phenolic acids on larval performance and growth β-glucosidase, carboxyl esterase, and glutathione-S-transferase, the enzymes were analyzed using standard experimental procedures. The feeding assays were similar to that of antifeedant assays as described above. The third instar *S. litura* larvae, starved for 24 h prior to experiments, were fed with 50 and 100 μg/10 cm^2 leaf, treated with phenolic acids (Gallic acid, caffeic acid, and catechin). Rotenone was taken as a standard for comparison. Surface treated host leaves (treated) and normal, untreated leaves (control) were used in the experiment.

Three detoxifying enzymes β-glucosidase, carboxyl esterase, and glutathione-S-transferase are analyzed to study the toxic effect of phenolic compounds on the insect.