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Determination of Molecular and Biochemical Changes in Cotton Plants Mediated by Mealybug

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ABSTRACT

Phenococcus solenopsis (cotton mealybug) is a devastating insect pest of many countries of the world including Pakistan. Due to its piercing sucking type feeding behavior, it injures cotton plants mechanically and induces several cytological and physiological changes in the host. These changes have been studied after subjecting healthy plants with mealybug under controlled conditions and it was recorded that mealybug attack enhanced lignin, cellulose and hemicellulose contents remarkably. It was also observed that defensive biochemicals of cotton i.e. phenolics and terpenoids were also significantly increased (up to 7 times) with progressing time (0-3 hr) in injured plants. Defensive enzymes i.e. phenyl ammonia lyase (PAL), polyphenol oxidase (PPO) and peroxidase (POD) also showed direct relationship with the passing time (0-3 hr) after mealybug feeding. Similarly, expression of thaumatin-like, metallothionein and profilin genes was enhanced with the elicitation of plant defenses due to insect herbivory. There was no connection found between pathogenesis related Pseml gene and plant defense against herbivory. Study concluded that mealybug did not modulate all plant defenses. There were specific biochemicals and defense related genes influenced by the attack of mealybug.

1. Introduction

Nature has armed plants with defense weapons to ensure their survival against harsh conditions; and which are generally categorized as constitutive and induced responses of plants due to external factors. Those external factors which can modulate plant responses may be chemical, physical or biological in nature, among which the insect herbivory is also an external stimulus [1]. Herbivory induces plant defense panel against biotic and abiotic factors [2]. Frequently reported changes in plant tissues due to insect feeding are modulation of defense chemicals [3,4]. Moreover, production of enhanced transcriptional rate of plant defense genes with concomitant increase in pathogenesis related proteins has also been reported in some insect plant interactions [5].

Cotton mealybug is an economically important pest of many ornamentals, medicinals, food crops and forest plants, with wide host spectrum [6]. Both the nymphs and adults of mealybug can harm crop plants by feeding upon phloem sap and egesting honeydews [7]. Cotton is one of the preferred hosts of mealybug without any specification of crop variety [8]. Massive population, colossal host spectrum and huge economic losses give mealybug an identical position among agriculturally important insect pests. It is necessarily important to study its impact on physiology and defense systems of plants. Therefore, physiological effects of mealybug on innate defense system of cotton plants have been studied. It will develop better understandings among researchers about the plant behavior against this insect.

2. Methodology

2.1. Collection of samples

Cotton plants were grown in green house of 'Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan' and randomly divided into two equal sets (each set containing 10

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plants) at the age of 45 days. One set was subjected to mealybug attack at the rate of 10 mealybug adults per plant and second set was left as negative control. Both the plant sets were covered with floating net cover of propylene and placed side by side in the greenhouse under controlled conditions (26 ± 2 °C). Time course studies of both plant sets were conducted regarding physiological and histological changes induced in cotton plants. All chemical reagents and lab equipment used in this study had been purchased from Sigma Aldrich.

2.2. Biochemical studies

Lignin was stained using phloroglucinol solution (0.1%) which was prepared in aqueous HCl solution (20%). Immersing plant material for 1-2 min in this solution provided the cytostaining of lignin in plant tissue. Then microtome was used to prepare transverse sections of the tissue and the sections were examined under microscope [9]. The sections were cut at different time intervals for evaluating the rate of accumulation of lignin contents.

Two percent of potassium iodide solution was prepared in water and 0.2 g of iodine was dissolved in it. Transverse sections of cotton stem were placed in it for 15 min and then covered with glass coverslips on glass slides. Drops of H_2SO_4 were placed along the edges of glass coverslips (on the microscope slide) and allowed for passive penetration under them. After incubation of 5 minutes at room temperature, stained cellulose contents of plant tissue sections were observed under microscope [10].

Hemicellulose was quantified by Sulfuric acid method. Plant tissue was immersed in 0.5% aqueous solution of H_2SO_4 and percentage mass recovery provided the amount of hemicellulosic contents of cotton tissue which had been dissolved in acidic solution [11]. Cytostaining of hemicellulose contents were also carried out by adopting phenol sulphuric acid method [12], which also stained phenolics accumulations as dark grey masses inside plant tissue.

Time course studies for evaluating phenolic contents in plant tissue were determined by adopting the recommended method of Mujica et al. [13] with small modifications. Ten grams dried plant powder was extracted with aqueous methanol solution (80%). Extracts were re-extracted with a solvent mixture of ethyle acetate/diethyle ether (1:1).

Ten grams of cotton plant tissue was extracted with ethyle acetate for 15 min at 40 °C for terpenoids extraction. Organic materials were removed through filtration and acidic substances through adding 5% KOH, 300 ml. Basic compounds were isolated by adding 300 ml, 5% HCl solution. The remnants of extracts were washed with water in a separating funnel. Washed extracts were concentrated up to 100 ml and centrifuged for 15 min at 5000 rpm. After removing the sediments, remaining solution was rotary evaporated at 40 °C and quantification was carried out through spectrophotometer by dissolving it in $CHCl_3$ [14].

2.3. Histological studies

Defense related enzymes are the most effective weapons of plants to control invading pathogens. These are included polyphenol oxidase (PPO), phenyl ammonia lyase (PAL) and peroxidase (POD). For detection of the enzymes, protein extract of plant tissue was prepared by crushing unit mass of tissue in a pre-chilled pestle and mortar in the presence of ice cold 0.1 M sodium phosphate buffer of pH 6.8. After sufficient stirring, liquid was centrifuged at 10000 rpm for 20 min. The supernatant was taken and used in further experiments of individual enzyme detection.

Activity of PAL was studied by using the method of Burrell and Rees [15]. The enzyme extract (0.2 ml) along with sodium borate buffer (2.5 ml, 8.8 pH) was added to 0.03 ml phenylalanine and let it undisturbed at 37 °C on water bath. Then 0.5 ml of 1 M Trichloroacetic acid was added to reaction mixture and light absorbance was recorded at 290 nm in spectrophotometer for formation of Trans-cinnamic acid.

PPO activity was determined by adopting the method of Mayer et al. [16]. The enzyme extract (0.2 ml) was accompanied with 1.5 ml, 0.01 M Catechol and spectroscopy was carried out at 495 nm.

For determination of POD activity, method of Polle et al. [17] was adopted. Substrate solution was prepared by adding 5 mM Potassium Phosphate (pH 5.25), 10 mM H_2O_2 and 40 mM Guaiacol and 50 μl enzyme extract was added to it assuming the extinction coefficient 25.5 $mM^{-1}cm^{-1}$ [18]. Spectrophotometric results were recorded at 436 nm.

Table 1
Detailed primer sequences used to target pathogenesis related specific genes in cotton plants. Primers were used to amplify specific genes from both treatments of cotton plants through reverse transcriptase PCR.

Genes	Accession Numbers	Primer Sequences (5′----3′)
Metallothionein	HM137170	F-GTACATGGGACACACCGACCA
		R-GTACAAAACTGTTTTCTGGACACGTAA
Pathogenesis related Pseml	AF211850	F-ATGGTGTCAGGGACTTCATCAAC
		R-CTAGCAGTATAAGTTGGGATTGGA
Thaumatin-like	HM202602	F-GCTTTGGGAGTTTCGTTGATATTCAT
		R-ATGGTGGTGCCGCAGAAGAC
Profilin	JQ697838	F-ATGAGTTGGCAGCAATTCGTGGA
		R-TCAGCCGTTACCTGCACCCTT

2.4. Expression of defense genes

Expression of four different types of plant defense genes i.e. metallothionein, thaumatin-like, pathogenesis related Pseml and profilin was studied in cotton plants by performing Multiplex RT-PCR. For the multiplication of cDNA templates of above mentioned genes, primer sets were designed from NCBI database (Table 1) with the accession numbers of HM137170, HM202602, AF211850 and JQ697838; respectively. RNA (1-2 μg) was subjected to reverse transcriptase at 25 °C for 6 minutes, 40 °C for 60 minutes and 70 °C for 9 minutes. Moreover, the reaction of 35 cycles was preceded with the 95 °C for 10 sec and annealing temperature for 15 sec (for Multiplex reaction annealing temperature was adjusted to 60 °C); and elongation at 72 °C for 15 sec. Reaction product was electrophorated on agarose gel and analyzed through GELANALYZER (Lazar, Hungary) to give a precise view of bands properties.

3. Results

Lignin, cellulose and hemicellulose contents were induced in plant tissue with mealybug attack. The darker color stem sections in Fig. 1 A, C, E indicates increased amounts of lignin, cellulose and hemicellulose, respectively. Whereas, the B, D, F sections of Fig. 1 indicate lower contents of lignin, cellulose and hemicellulose in plants without mealybug attack. The induced phenolics rich inclusion bodies are also visible in Fig. 1E. Increased strength of structural barriers make the direct penetration of plant pathogens more difficult but provide an easy way to pathogen searching for injured susceptible surfaces.

An increasing trend was observed in phenolics deposition under the plant surface punctured by mealybug, as time passed (Fig. 2 A, B). After 45 minutes of mealybug attack, low phenolics accumulation was visualized, while the identical plant tissue exhibited higher phenolic contents after 90 minutes of herbivory. Deposition of the phenolic contents can be estimated by comparing tissue sections of variable injury period (Figure 2).