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Biochemical changes in cotton plants due to infestation by cotton mealybug *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae)

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Abstract: The study on biochemical changes in cotton plants (*Gossypium hirsutum* L.) due to infestation by cotton mealybug *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae) was conducted at CICR Nagpur during 2014-15. Total protein contents estimated from the shoots of the healthy plants (4.29 mg/g) indicated 50.5% increase over the healthy plants (2.85 mg/g). Total phenol content increased by 185.7% in the mealybug infested plants (0.20µg/g) over the healthy plants (0.07µg/g). Insignificant difference in the level of total soluble sugar was observed in mealybug infested plants (1.00µg/g) as compared to healthy plants (0.90µg/g). Total reducing sugar was found to be unaffected with the mealybug infestation. Although there was depletion in all the photosynthetic pigments *viz.*, chlorophyll a (19.1%), chlorophyll b (23.7%), total chlorophyll (21.2%) and carotenoids (20.8%) due to the mealybug infestation, these values were not statistically different in the healthy plants. This is the first report on biochemical changes in cotton plant due to infestation of *P. solenopsis*.

Keywords: Biochemical changes, Cotton, Mealybug, *Phenacoccus solenopsis*

INTRODUCTION

Cotton is an important cash crop, grown extensively in different parts of the world. In India cotton is grown on 11.88 million ha with 35.2 million bales production (1bale=170kg) (CCI, 2016). Pest attack is the major biotic stress factor limiting plant growth and crop production in all the cotton growing areas of the world. The yield losses due to attack by insect pests can goes up to 60-70% of the potential yield of cotton if proper control measures are not taken up at appropriate time. In India, genetically modified cotton called 'Bt-cotton' occupies 95% (=11.28 m ha) of total cultivated area which offers good protection against three cotton bollworms, *viz.* American bollworm *Helicoverpa armigera* (Hubner), Spotted bollworm *Earias insulana* (Boisduvel), *E. vittella* (Fab.) and Pink bollworm *Pectinophora gossypiella* (Saunders). However, a sizable number of regular sucking pests including Jassid *Amrasca biguttula biguttula* (Ishida), Aphid *Aphis gossypii* Glover, Thrips *Thrips tabaci* Lind., Whitefly *Bemisia tabaci* (Gennadius) etc are dominant insect species damaging cotton crop. In the recent past 7 mealybug species of Hemiptera order infesting cotton have been identified. These are cotton mealybug *Phenacoccus solenopsis* Tinsley, papaya mealybug *Paracoccus marginatus* Williams and Granara de Willink, pink hibiscus mealybug *Maconellicoccus hirsutus* (Green), spherical mealybug *Nipaecoccus viridis* (Newstead), striped mealybug *Ferrisia virgata* Cockrell, mango mealybug *Rastrococcus iceryoides* (Green) and ber mealybug *Perissopneumon tamarindus* (Green). *P. solenopsis* is the predominant species in north and central India while *P. marginatus* in south India (Nagrare *et al.*, 2013). Remaining species are of minor in nature and observed in traces distributed in three cotton growing zones of India.

Cotton mealybug, *P. solenopsis* is widespread and polyphagous pest, feeds on variety of crop plants worldwide and cause economic losses. Infestation of mealybugs on the cotton plant varies from different host plants. Mealybug nymphs and adults affect the aerial plant parts; mainly the shoots and leaves and suck sap from it. Cotton plants infested with *P. solenopsis* in the vegetative stage shows stem distortion, twisting and bushiness of the affected portion whereas in mature plants the infestation can be seen on the squares and bolls which greatly cause qualitative as well as quantitative losses. Mealybug infested cotton plants shows symptoms of distorted and bushy shoots, crinkled or twisted leaves, and in severe cases infested plants dries up completely resulting very low or no yield (Nagrare *et al.*, 2013).

Plants are exposed to variety of biotic and abiotic stresses throughout their life among which biotic stress due to attack by insect pests is one of the greatest threats to plants that result in to substantial reduction in the potential yield (Sharma *et al.,* 2003). The feeding of insects on plants induces biochemical and physiological changes in the host plants, affecting the life processes of host plants (Gomez *et al.*, 2004). Plants

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respond to changes associated with insect feeding through various morphological, biochemical and molecular mechanisms. Accumulation of various compounds in accordance with the kind and degree of damage in the plant is to counter insect attack as defense mechanism (War *et al.*, 2012; Punitahvalli *et al.*, 2013). The defensive compounds are either produced constitutively or in response to plant damage and affect feeding, growth and survival of insects (War *et al.*, 2012). The evolution of chemical defence in plants is linked to the emergence of chemical substances that are not involved in the essential photosynthetic and metabolic activities. These substances are secondary metabolites which are organic compounds that are not directly involved in the normal growth development or reproduction of organisms (Fraenkel, 1959) and often produced as by-products during the synthesis of primary metabolic products (Whittaker, 1970). Major known defence chemicals include plant secondary metabolites, protein inhibitors of insect digestive enzymes, proteases, lectins, amino acid deaminases and oxidases (Chen, 2008; Punithavalli *et al.*, 2013).

Photosynthetic pigment in plant tissue is also one of the most important factors involve in the plant insect interaction. Insect feeding affect the process of photosynthesis. (Gomez *et al.,* 2004) due to the alteration in the photosynthetic pigments. Thus, photosynthetic pigments like leaf chlorophyll content and carotenoids are the key parameters in the photosynthetic productivity gets altered during defensive responses against the attacking insect pest (Mao *et al*., 2007). However, there are scanty reports on biochemical changes in the cotton plants due to infestation by cotton mealybug *P. solenopsis*. Hence the present study was undertaken to understand the different levels of biochemical changes that occurs in the cotton plants (*Gossypium hirsutum* L.) infested with cotton mealybug *P. solenopsis*.

MATERIALS AND METHODS

Cotton plants (*Gossypium hirsutum* L.): Untreated seeds of cotton genotype RCH 2 (Bollgard II) were obtained from Rasi Seeds (P) Ltd. Coimbatore, Tamil Nadu. Cotton plants were raised in the polythene bags containing soil and kept under natural climatic conditions. Care was taken to prevent from insect attack other than *P. solenopsis* on inoculation. Forty to 60 days plants were used for the study.

Culture of mealybug *P. solenopsis*: The culture of *P. solenopsis* mass reared at temperature 27±2 ^{0}C and relative humidity 60±10% at Insectary and biocontrol laboratory of CICR Nagpur was released on the grown up seedlings. Two weeks after the release of mealybugs, shoots were taken for estimation of total soluble proteins (mg/g), total reducing sugars (mg/g), total soluble sugars (µg/g), total phenols (µg/g) while leaves were taken for chlorophyll estimation from both mealybug infested and healthy cotton.

Sample size: A replicated sample of shoots from different five cotton plants of infested and healthy plants blocks were harvested for the estimation of total soluble protein, total reducing sugars, total soluble sugars and total phenols. Similarly, leaves from top portion were harvested for estimation of photosynthetic pigments from each combination from the same location on the plant and with a similar orientation towards the light. The estimations of biochemicals in five replicates were done for each parameter.

Biochemical estimation

Total soluble proteins: Total soluble proteins were estimated according to the method of Lowery *et al*. (1951) with slight modification. Shoots (500 mg each) of mealybug infested and healthy cotton plants were taken and macerated in enzyme assay buffer and centrifuged at 10000 rpm for 10 minutes. Supernatant (0.4 ml) was mixed with alkaline copper sulphate solution (4 ml) and then allowed to stand at room temperature for 10 minutes. Folins-ciocalteau phenol reagent (0.4 ml) was added to the above reaction mixture and further allowed to incubate at room temperature for 30 mins; absorbance was measured at 660 nm. A standard curve was prepared by using known concentration of bovine serum albumin. Protein content in both the types of samples were calculated through the standard calibrated curve of defined concentration of bovine

Table 1. Biochemical changes due to infestation of cotton mealybug *P.solenopsis* and healthy cotton plants *G. hirsutum* (Values are means of 5 observations of each parameter).

Cotton genotype RCH 2 Bollgard II	Plant biochemicals			
	Total soluble protein (mg/g)	Total reducing sugar (mg/g)	Total soluble sugar (µg/g)	Total phenol contents (µg/g)
Infested plant	4.29 ± 0.24	3.36 ± 0.14	1.00 ± 0.35	0.20 ± 0.03
Healthy plant	2.85 ± 0.43	3.37 ± 0.09	0.90 ± 0.28	0.07 ± 0.01
Student T test (0.05)	2.36	NS	NS	2.31
Increase or decrease due to mealybug infestation	+1.44	-0.01	+0.1	+ 0.13
Increase or Decrease (%) over healthy plant	+50.5%	-0.3%	+ 11.1%	185.7%

(+: increase, - : decrease in the content); ± SEM; NS-Non significant

Table 2. Alterations in photosynthetic pigments of the healthy and mealybug (*P. solenopsis* Tinsley) infested cotton plant *G. hirsutum* (Values are means of 5 observations of each parameter).

Cotton genotype RCH 2 Bollgard II	Photosynthetic pigments			
	Chlorophyll a mg/g	Chlorophyll b mg/g	Total Chlorophyll mg/g	Carotenoids mg/g
Infested plant	0.76 ± 0.06	0.29 ±0.02	1.04 ± 0.080	0.28 ± 0.025
Healthy plant	0.94 ± 0.11	0.38 ± 0.06	1.32 ± 0.171	0.34 ± 0.05
Student T test (0.05)	NS	NS	NS	NS
Increase or decrease due to mealybug infestation	-0.18	0.09	0.28	0.07
Increase or decrease (%) over healthy plant	-19.1%	-23.7%	-21.2%	-20.8%

± SEM, (+: increase, - : decrease in the content), NS-Non significant

serum albumin protein (10 – 100 µg) and the total soluble protein content were calculated by the equation (1):

$$\text{Total soluble protein (mg/g)} = \frac{\text{Protein in sample assessed}}{\text{Volume of sample aliquot}} \times \frac{\text{Total volume of extract}}{\text{Weight of plant tissue sample used for assay}} \times 100$$

……..(1)

Total reducing sugars: Total reducing sugars were determined by following the method of Miller et al. (1972) with few modifications. Fresh shoots (0.5 mg each) of mealybug infested and healthy cotton plants were extracted using 5 ml of hot 80% ethanol twice and the supernatant were collected and evaporated on water bath. To this, 10 ml of distilled water added and sugars were allowed to dissolve completely. From this, 1.5 ml of alcohol free extract was taken and the final volume made up to 3 ml with distilled water. To this, 3 ml of dinitrosalicylic acid reagent was added and mixed gently. The reaction mixture was kept in boiling water bath for 5 minutes and allowed to develop colour. In this mixture 1 ml of 40% Rochelle salts solution was added, the contents were mixed, cooled and the absorbance were measured at 510 nm. The total reducing sugars of the unknown samples were calculated through the standard graph of glucose developed by the known concentrations of glucose (100– 500 µg) by following the above same equation as used in the determination of total protein content.

Total soluble sugars: Total soluble sugars were determined by the method of Hodge and Hofreiter (1962) with little alterations. Fresh shoot of mealybug infested and healthy cotton plants (250 mg each) hydrolyzed in 5 ml of 2.5 N HCl for 3 hours in water bath at 70–75 ^{0}C. Contents were cooled to room temperature and neutralized with solid Na_2CO_3 till effervescence ceases and the supernatant was collected. Supernatant (200 µl) was taken as sample to which 4 ml anthrone was added; heated in boiling water bath for 10 minutes; cooled to room temperature and the absorbance was measured at 630 nm. A standard graph was prepared by using known concentration of glucose to calculate the concentration of the sample.

Total phenols: Total phenols (µg/g) were determined by following the procedure of Malick and Singh (1980). Shoots of mealybug infested and healthy cotton plant (500 mg each) were boiled at 70^{0}C under water bath in 5ml ethanol (80%) for 10 minutes. Cooled and macerated with 80% ethanol and centrifuged at 10,000 rpm for 5 minutes. Supernatant was allowed to dry under water bath. The residue was dissolved in 1ml of distilled water. To this 1 ml of sample folin's ciocalteau reagent with 2 ml of 20% Na_2CO_3 solution was added and the total volume of reaction mixture was made up to 10 ml with distilled water and the absorbance was measured at 650 nm. A standard curve was prepared using 10-100 µg of catechol. From the standard curve, the concentrations of phenols in the unknown samples were calculated.

Photosynthetic pigments: Fresh leaves (250 mg each) were macerated in 80% acetone with mortar and pestle. The homogenate were filtered through the Whatman filter paper and the filtrate was collected. The absorbance of acetone extracts were measured at 645, 652, 663, 480 and 510 nm using UV- visible spectrophotometer. The chlorophyll a, b, total chlorophyll and total carotenoids were calculated following the equations (2, 3 &4) of Arnon (1949) and equation (5) by Parsons et al. (1984). The photosynthetic pigments chlorophyll a, b, total chlorophyll and total carotenoids were estimated by macerating the fresh leaves (250 mg each) in 80 % acetone under mortar and pestle. The homogenate were then filtered through Whatman filter paper and the filtrate was collected; the absorbance of acetone extracts were determined over 645, 652,663,480 and 510 nm using UV- visible spectrophotometer.

Chlorophyll a: (22.9) x (O.D. 663) – (2.69 x O.D. 645) ……..(2)

Chlorophyll b: (22.9) x (O.D. 645) – (4.68 x O.D. 663) ……..(3)

Total Chlorophyll: (20.2) x (O.D. 645) + (8.02) x (O.D. 663) ……..(4)

Total Carotenoids content: (7.6) x (O.D. 480) – (1.49) x (O.D. 510) ……..(5)

Statistical Analysis: Statistical analysis of the data obtained from the different experiments was carried out according to the "Students T test" using online WASP (ICAR, Goa, India). Significant differences were established at $P<0.05$. The data were also subjected to percentage changes (decrease or increase) in the infested and healthy leaves and calculated equation (6):

$$\% \text{ increase or decrease} = \frac{(\text{Value of healthy plant} - \text{Value of infected plant})}{(\text{Value of healthy plant})} \times 100 \quad \ldots.(6)$$

RESULTS AND DISCUSSION

The results of the study clearly demonstrated that *P. solenopsis* infestation on cotton plant significantly increases ($P<0.05$) total soluble protein and total phenol contents while no significant change in the total reducing sugar and total soluble sugars. Insignificant reduction was observed in photosynthetic pigments viz., Chlorophyll a, Chlorophyll b, total chlorophyll and carotenoids observed.

Total soluble protein: The soluble total protein contents estimated from the shoots of the healthy and infested cotton plants showed significant difference ($P<0.05$). The soluble total protein contents increased as high as 50.5 % in the mealybug infested plants (4.29 mg/g) over the healthy cotton plants (2.85mg/g) (Table 1). Significant increase in soluble protein contents was observed by the infestation of *H. armigera* and *A. craccivora* in groundnut as compared to the control plants (War *et al.,* 2013). Aphids *Rhopalosiphum padi* and *Diuraphis noxia* feeding caused significant increase of total protein contents in comparison with the control of leaves of four cereals *viz.*, Russian wheat aphid, *Diuraphis noxia*-susceptible 'Arapahoe' and -resistant 'Halt' wheat, *D. noxia*-susceptible 'Morex' barley, and *D. noxia*-resistant 'Border' oat (Ni *et al*., 2001). The total soluble proteins increased by 17.8% with infestation of pink hibiscus mealybug *(Maconellicoccus hirsutus)* on host plant (*Terminalia arjuna*) of tussar silkworm (Velide *et al.,* 2013). These studies support present findings. The results are comparable with Chen et al. (2009) who stated that increase in protein content is a general phenomenon in plants in response to insect damage as defence mechanism.

When plants are under stress, protein based defencive compounds get accumulated in the plants (Chen *et al*., 2009) and this is an important and widely studied defence in plants against insect feeding and other stresses. Increase in defence related protein after mealybug infestation mediate a range of responses in plants that include inhibition of feeding, oviposition and survival of mealybugs. Similarly increased in defencive protein contents after insect infestation was also reported earlier in other crops (Ni *et al*., 2001; Sinha *et al.* 2005; Chen *et al*., 2009). In one isolated study, decrease in protein content was observed with Leaf folder (*Cnaphalocrocis medinalis)* infested rice genotypes (Punithavalli *et al*., 2013).

Total reducing sugars: Under present study, no significant change in total reducing sugars was observed in both the plants. (Table 1). The present results are not in conformity with the results that stated significant reduction in total reducing sugar with the infestation of leafroller (*Diaphania pulverulentalis*) (Narayanaswamy, 2003; Mahadeva and Nagaveni 2011), mealybug (*M. hirsutus)* (Bose *et al.*, 1992) and jassid (Shree and Mahadeva 2005) in mulberry. Total reducing sugar was decreased by 64.9% by *M. hirsutus* in *T. arjuna* (Velide *et al.*, 2013). Host variation and species infested might be the reason behind variation of reduction in total reducing sugars (Prasad *et al.*, 2002; Mahadeva and Nagaveni, 2011, Mahadeva, 2016).

Total soluble sugars: The level of total soluble sugars was increased marginally by 11.1% in the mealybug infested plants (1.00 µg/g) but was not statistically significant with healthy cotton plants (0.90 µg/g) (Table 1). Similar increased in total soluble sugars by 31.3% was seen with the infestation of mealybug (*M. hirsutus***)** in *T. arjuna* (Velide *et al.,* 2013). Contrarily, Mahadeva and Nagaveni (2011) indicated decreased in total reducing sugars in majority of the mulberry (*Morus alba* L) varieties due to the infestation by leafroller (*Diaphania pulverulentalis* Hampson). Lokeshwari and co-workers (2014) reported significant reduction in the amount of total soluble sugars in the shoots of mango plants upon varying levels of aphid (*Aphis odinae)* feeding. Total soluble sugars is one of the major components largely contributes to the total carbohydrate of plant and has proactive role in the synthesis of phenolic compounds, lectins, etc. as defence mechanism.

Total phenols: The infestation by *P. solenopsis* resulted in insignificant increase (0.13 µg/g) in the total phenol contents in the mealybug infested plants (0.20 µg/g) than the healthy cotton plants (0.07 µg/g). The resultant increase of the phenol contents due to mealybug infestation was registered as high as 185.7% (Table 1). Increase in phenol contents is common response of plant to counter the insect attack and inhibit the oviposition, population build up and continued existence of the attacking insect. It was observed that mealybug feeding has changed the phenol contents in the infested plants than the healthy cotton plants. It can be allied with the earlier finding (Hori *et al.,* 1973) who observed increased phenol compound in the sugarbeets infested with lygus bug which inhibited the

subsequent bug feeding. Usha Rani and Jyothsna (2009) observed that the extent of tissue damaged corresponds to the increased phenol concentration in response to attacking insect feeding. Infestation with *H. armigera* and *A. craccivora* resulted in a tremendous increase in the amounts of phenolic compounds than the uninfected plants of groundnut (War *et al.*, 2013) and due to Leaf folder (*Cnaphalocrocis medinalis*) in rice (Punithavalli *et al*., 2013). Phenols involved in plant resistance against many biotic and abiotic stresses (Noreen and Ashraf 2009; Sharma *et al.*, 2009; Usha Rani and Jyothsna 2010). The enhancement in the phenol contents in response to insect infestation is considered to be a general phenomenon (Ramiro *et al*., 2006; Sharma *et al*., 2009) as it reduces the growth and development of herbivores (Bhonwong *et al.*, 2009; Sharma *et al*., 2009; Usha Rani and Jyothsna, 2010; War *et al*., 2011a; 2011b).

Photosynthetic pigments: Total photosynthetic pigments were estimated using leaf of the mealybug infested and healthy cotton plants. Decreased in the photosynthetic pigments including chlorophyll a, chlorophyll b, total chlorophyll and carotenoids due to the mealybug infestation were observed. The total chlorophyll content was decreased by 21.2% where as chlorophyll a and chlorophyll b was declined by 19.1% and 23.7 % respectively in the mealybug infested cotton plants than healthy cotton plants. Carotenoids found to be reduced by 20.8%. Though there was decreased in the photosynthetic pigments in infested plants, the difference in pigments between the infested and healthy cotton plant was found to be non-significant (Table 2). The reduction in chlorophyll content with *P. solenopsis* infestation was also reported in tomato (Huang *et al.,* 2013). Reduction in photosynthetic pigments with the infestation of Leaf roller (*D. pulverulentalis*) was also observed in mulberry (Mahadeva and Nagaveni 2011). Total chlorophyll, chlorophyll–a, and chlorophyll–b and carotenoids were reduced significantly by 45.4, 45.9 41.9% and 44.9%, respectively with the infestation of *M. hirsutus* in *T. arjuna*, which corroborate the findings of the present study (Velide *et al*., 2013).

The results clearly demonstrated that infestation of *P. solenopsis* had negative impact on chlorophyll and carotenoid contents of the plants. The present study revealed that the concentration of photosynthetic pigments including total chlorophyll, chlorophyll a, chlorophyll b and carotenoids contents are relatively higher in the non-infested leaves of cotton plant compared to the mealybug infested plants. These results are in line with the earlier findings (Golawska *et al.*, 2010) that revealed higher chlorophyll concentrations in non-infested plant.

The present findings are also relatively similar with the results of Huang and coworkers (2014) who have explained the reduction in chlorophyll content resulted in response to the feeding damage caused by hemipteran insect *Bagrada hilaris*. It is also considered that the transition of the photosynthetic pigment can also be due to the adaptive reactions against the insect attack (Golawaska *et al*., 2010). Infestation by *Thrips tabaci* (Linderman) on *Hypericum sampsoni* Hance (Dai *et al*., 2009) and tomato (Buntin *et al*., 1993) apparently synthesised less chlorophyll pigment in damaged leaves. Total chlorophyll and carotenoid concentrations differed among Betta wheat isolines in response to aphid feeding (Heng-Moss *et al*., 2003). The level of photosynthetic pigment like chlorophyll and carotenoids contents of plant in response to the insect attack is mainly determined by the species of host plant and scale of insect abundance. However it can also get influenced with respect to the environmental factors (Mary *et al*., 2006). The changes in the chlorophyll content in the stress leaves due to less synthesis might be part of adaptive response of plant (Golawska *et al.*, 2010). Insect injury has indirect effects on the growth and performance of host plants through photosynthetic suppression, causes uncertain reduction in photosynthesis, which likely depends on the degree of infestation (Huang *et al*., 2013).

Conclusion

The study demonstrated occurrence of biochemical changes due to *P. solenopsis* infestation in cotton plants. Cotton plants responded to biochemical changes associated with feeding of *P. solenopsis*, through the accumulation of phenol (185.7%) and protein (50.5%) contents as defensive mechanism. Losses of the photosynthetic pigments in response to *P. solenopsis* suggest a feeding-induced stress response in the host plants. Thus, the present study provides a better understanding of defensive mechanism by biochemical changes due to the impacts of *P. solenopsis* infestation in cotton.

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