Research Paper :

Role of oxidyzing enzymes in host plant resistance to cotton mealybugs (*Phenococcus solenopsis*)

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SUMMARY

A laboratory investigation was conducted to find out the role of oxidizing enzymes in defense mechanism of cotton cultivars against mealybugs in Insect Biotechnology Laboratory of Department of Agricultural Entomology,Dr. PDKV, Akola during 2008-09. Quantitative and qualitative studies were undertaken for estimation of oxidizing enzymes like superoxide dismutase, polyphenol oxidase, polyphenol peroxidase and catalase from different cotton cultivars. CAHH-231 (pigmented hybrid) recorded higher activity of polyphenol oxidase (0.95 unit mg protein^{-1} min^{-1}) and polyphenol peroxidase (0.87 unit mg protein^{-1} min^{-1}). Catalase activity was found higher in susceptible cultivars. PKV Rajat showed highest SOD activity 2.84 unit mg^{-1} followed by Bunny-Bt. Polyphenol oxidase, polyphenol peroxidase and catalase activity was found higher in Bunny-Bt and CAHH-231 (Pigmented hybrid) which are susceptible to the sucking pests. The study will be helpful in understanding the biochemical basis of mealy bug resistance in cotton. The outcome of the present investigation will act as stepping stone to develop mealy bug resistant cotton variety.

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Cotton is known as "white gold" or "king of fibre" plays a prominent role in Indian economy. In cotton, insect pest is the major limiting factor for increasing the productivity of crop. Bollworms are suppressed by inserting Bt gene in to cotton plants, but for the sucking pest there is no such control measure developed till date. Recently in India, the cotton crop in Punjab, Rajasthan, Maharashtra and Gujarat is being seriously infested with mealy bug. During 2005, the sudden appearance of the pest in cotton in Multan, Sanghar, Mirpurkhas and Tando Allahyar of Pakistan destroyed the entire crop within a few days (Tanwar *et al.*, 2007).

In current decade, the trend of increased build up of various mealy bug species in crop plants is observed, which might be due to certain abiotic changes in climate and environment. This pest showed very detrimental effects on yield of the crop. Chemicals recommended for its management have also shown some adverse effect. Hence, it is necessary to search for the alternative like host plant resistance. During the last few years mealy bugs, which were considered to be the minor pests in many crops have acquired the status of the major pest causing damage to important agronomic crops.

Today's trend in insect control is to decrease the use of conventional insecticides not only because of the cost but also to minimize environmental pollution and to the further development of pesticide resistance. The ability of plant to withstand the attack of insect pests is due to certain biochemical characteristics which exert unfavourable effects on the insects.

Plants challenged by insects respond through changes in the composition and physical properties of the cell wall as well as the biosynthesis of secondary metabolites (Hopkins and Huner, 2004). Herbivory by the phloem feeding three corned alfalfa hopper caused increase in the activities of several oxidative enzymes including lipoxygenases, peroxidases, ascorbate oxidase and polyphenol oxidase studied by Felton *et al.* (1994). Also,

Khattab (2007) focused on the defense mechanism of cabbage plant (*Brassica olereaceae* var. capitata) against phloem sucking aphid (*Brevicoryne brassicae* L.). Argandona *et al.* (2001) studied on the peroxidase activity in barley after infestation of aphid (*S. graminum*). *S. graminum* infestation increased hydrogen peroxide content and total soluble peroxidase activity in cv. Frontera. The oxidative enzyme activities (superoxide dismutase, ascorbate peroxidase, ascrobate oxidase) were significantly reduced whereas polyphenol oxidase and peroxidase activities were enhanced by insect infestation. Phenol peroxidases, oxidases play a role in the defense mechanism of aphid infested cabbage leaves, thereby delay their death.

The aim of present investigation was, therefore, to study the defense mechanism of cotton cultivars against mealy bugs, the oxidative responses since they thought to have an important role as antiherbivores.

MATERIALS AND METHODS

The shoot tips of different cotton cultivars *viz.,* PKV-Hy-2,PKV-Rajat,,AKH-081,AKA-8,AKH-3614-10 (Hirsutum pigmented),CAHH-231(pigmented hybrid) and Bunny Bt 30 DAE were obtained from the field, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola (Table 1).

Preparation of sample:

Shoot tips of different cotton cultivars were collected from the field of Entomology. Samples were finally powdered and also crude homogenate was prepared with mortar and pestle and centrifugation method and preserved in refrigerator at 4^0C.

Quantitative estimation of enzymes:

Quantitative estimation of superoxide dismutase :

Superoxide dismutase was estimated by method given by Winterbourn (1975). Different concentrations of samples ranging from 0.1 to 10 micrograms were used in this assay. A tube containing approximately 100 micrograms generally produced maximum inhibition. The tubes were placed in a light box providing uniform light intensity. The tubes were incubated for 5-8 minutes to achieve a standard temperature. At zero time and at timed intervals 0.05 ml riboflavin was added. All tubes were incubated in the light box for 12 minutes and at timed intervals read A_{560}. Per cent inhibition of NBT production was calculated by plotting per cent inhibition versus amount of enzyme from samples test. The amount of enzyme was determined by resulting in one half of maximum inhibition. Calculation was done with following formula:

$$\text{Unit / mg} = \frac{1000}{\mu\text{g enzyme resulting in } \frac{1}{2} \text{ max. inhibition}}$$

Quantitative estimation of polyphenol oxidase:

Polyphenol oxidase was estimated by the method given by Anderson *et al.* (1989). 50 µl of enzyme was added in wells and volume was made upto 150 µl by using 100 µl 0.01 M phosphate buffer (pH 7.0). Followed by 15 minutes incubation, 50 µl of substrate solution (0.004 g Dopamine in 1.0 ml distilled water) was added to the plate and was incubated in dark at room temperature for 10 minutes and was read in microplate reader equipped with a 490 nm filter.

Quantitative estimation of polyphenol peroxidase:

Polyphenol peroxidase was estimated by the method given by Summer and Gjessing (1943). 1 ml of o-dianisidine, 0.5 ml of H_2O_2, 1 ml of phosphate buffer and 2.4 ml of distilled water was pipetted out into a test tube. Blank was prepared by excluding H_2O_2 but added additional volume of water. The reaction mixture was incubated at 30^0C and started the reaction by adding 0.2 ml of enzyme. After 5 min incubation, the reaction was stopped by adding 1 ml of 2 N H_2SO_4. Reading was taken at 430 nm.

Quantitative estimation of catalase:

Catalase was estimated by the method given by Beers and Sizer (1952). Spectrophotometer was adjusted to 240 nm and 25^0C. Each cuvette having reagent grade distilled water (0.95 ml) and 0.059 M hydrogen peroxide (0.5 ml) was incubated in spectrophotometer for 4-5 minutes temperature achieved to equilibration and established blank rate if any. 50 ml diluted enzyme was added and decrease in absorbance at 240 nm for 2-3 minutes was recorded. D A_{240}/ min was calculated from the initial linear portion of the curve.

Calculation:

$$\text{Unit / mg} = \frac{\Delta A_{240}/\text{min} \times 1000}{43.6 \times \text{mg enzyme/ml reaction mixture}}$$

Qualitative analysis enzymes:

Study of oxidizing enzymes by electrophoresis:

Non-denaturing Polyacrylamide Gel Electrophoresis (PAGE) was performed in Hoefer SE

600 slab gel unit (Hoefer, San Fransisco, (A) using Tris-Glycine (pH 8.8) buffer system, 10% running gel and 5% stacking gel were prepared according to manual provided with the Hoefer system. Gel was loaded with the enzyme preparation and electrophoresis was conducted at a constant current of 2 mA per well (Hoefer PS-500 power unit) for 7-8 hrs at 4^0C.

Staining for polyphenol oxidase isozyme:

The isozyme of polyphenol oxidase was localized on polyacrylamide gel as per the procedure suggested by Park *et al.* (1980). The gel was incubated in 0.03 M catechol containing 0.05 per cent phenylene diamine in citrate phosphate buffer pH 6.00 (0.1 M solution of citric acid 2.10 g in 100 ml and 0.2 M solution of dibasic sodium phosphate 3.56 g in 100 ml) for one hour.

Staining for polyphenol peroxidase isozyme:

The isozyme bands of peroxidase (Novacky and Hamptom, 1968) were localized first incubating the gel in 0.25% guaicol for 20 minutes followed by incubation in 0.3 per cent hydrogen peroxide for 15 minutes which showed the appearance of reddish brown bands of peroxidase.

RESULTS AND DISCUSSION

The present study was conducted to investigate the biochemical defense of cotton cultivars against mealybugs. Data generated during the period of investigation and discussion pertaining to it is presented here after statistical analysis.

Quantitative study of oxidizing enzymes:

Activity of superoxide dismutase (SOD) from the different cotton cultivars:

SOD activity was found in different cotton cultivars which ranged from 2.55 to 2.84 unit mg^{-1}. There was least difference in the SOD activity amongst all the cultivars. PKV Rajat showed highest SOD activity 2.84 unit mg^{-1} followed by Bunny-Bt, AKH-3614-10 (Hirsutum pigmented), and AKH-081 which possessed 2.83, 2.82 and 2.81 unit mg^{-1}, SOD activity, respectively. Minimum SOD activity was observed in AKA-8 (2.72 unit mg^{-1}) and PKV Hy-2 (2.67 unit mg^{-1}). Whereas, CAHH-231 (pigmented hybrid) showed lowest SOD activity *i.e.* 2.55 unit mg^{-1} (Table 2).

Similar results were found by Stout *et al.* (1999), Ni *et al.* (2001) and Chaman *et al.* (2001) in different plant species.

Table 1 : Cultivars used in the investigation and their reactions against sucking pests

Sr. No.	Cultivars	Insect reaction
1.	PKV Hy-2	Resistant
2.	PKV Rajat	Tolerant
3.	AKH-081	Tolerant
4.	AKA-8	Susceptible
5.	AKH-3614-10 (Hirsutum pigmented)	Highly susceptible
6.	CAHH-231 (Pigmented hybrid)	Highly susceptible
7.	Bunny Bt	Susceptible

Activity of polyphenol oxidase from the different cotton cultivars:

CAHH-231 (pigmented hybrid) recorded higher polyphenol oxidase activity (0.95 unit mg $protein^{-1}$ min^{-1}) followed by Bunny-Bt (0.70 unit mg $protein^{-1}$ min^{-1}) whereas, AKH-081 showed least activity (0.15 unit mg $protein^{-1}$ min^{-1}). The least differences were observed in PKV Hy-2, PKV Rajat, AKA-8 and AKH-3614-10 (Hirsutum pigmented) which recorded minimum polyphenol oxidase activity *i.e.* 0.23, 0.29, 0.30, 0.20 unit mg $protein^{-1}$ min^{-1}, respectively (Table 2).

Polyphenol oxidase activity increased in the susceptible cultivars CAHH-231 (pigmented hybrid) which was followed by Bunny Bt.

Earlier Heng Moss *et al.* (2004) showed that polyphenol oxidase activities were similar between infested and non-infested plants in buffalograsses and the least differences in polyphenol oxidase activity between infested and non-infested plants demonstrated that this enzyme was not likely to be associated with the resistance.

Activity of polyphenol peroxidase from the different cotton cultivars:

The results of polyphenol peroxidase detailed in Table 2 revealed that CAHH-231 (pigmented hybrid) showed maximum activity (0.87 unit mg $protein^{-1}$ min^{-1}) followed by Bunny-Bt (0.70 unit mg $protein^{-1}$ min^{-1}). The least difference was observed in AKH-081, PKV Rajat, PKV Hy.2 and AKH-3614-10 (Hirsutum pigmented) which recorded minimum polyphenol peroxidase activity *i.e.* 0.38, 0.34, 0.25 and 0.25 unit mg $protein^{-1}$ min^{-1}, respectively. Whereas,AKA-8 possessed lowest polyphenol peroxidase activity (0.17 unit mg $protein^{-1}$ min^{-1}).

The peroxidase activity was higher in CAHH-231 (pigmented hybrid) cultivar *i.e.* in susceptible cultivar.

Table 2 : Quantification of oxidative enzymes of shoot tips of different cotton cultivars

Cultivars	Superoxide dismutase	Polyphenol oxidase	Polyphenol peroxidase	Catalase
PKV Hy-2	2.67±0.04	0.23±0.05	0.25±0.07	0.44±0.05
PKV-Rajat	2.84±0.07	0.29±0.07	0.34±0.06	0.53±0.06
AKH-081	2.81±0.03	0.15±0.03	0.38±0.08	0.48±0.07
AKA-8	2.72±0.06	0.30±0.04	0.17±0.05	0.24±0.09
AKH-3614-10(Hirsutum pigmented)	2.82±0.08	0.20±0.06	0.25±0.09	0.35±0.10
CAHH-231 (Pigmented hybrid)	2.55±0.05	0.95±0.08	0.87±0.08	1.26±0.01
Bunny Bt	2.83±0.03	0.70±0.12	0.70±0.11	1.39±0.11

*Mean of three replications ± SE

Similar results were showed by Heng Moss *et al.* (2004) in buffalo grasses. Peroxidase was found to play an important role in herbivore resistance in crop plants (Chittoor *et al.*, 1999 and Constable, 1999).

Activity of catalase from the different cotton cultivars:

The data shown in Table 2 revealed that, Bunny Bt showed highest activities *i.e.* 1.39 unit mg^{-1} followed by CAHH-231 (pigmented hybrid) possess 1.26 unit mg^{-1}. Whereas, AKA-8 possess lowest catalase activity (0.24 unig mg^{-1}).Catalase activity was found higher in susceptible cultivars.Contradictory results were observed earlier Heng Moss *et al.* (2004) .

Qualitative study of oxidizing enzymes on PAGE:

Isozyme pattern of polyphenol oxidase from the different cotton cultivars:

Electrophoretic pattern of polyphenol oxidase showed 2 isozymes (Fig. 1) with molecular weight 69.96 kDa and 81.20 kDa. CAHH-231 (pigmented hybrid) did not show any isozyme. AKA-8 showed single isozyme with molecular weight 81.20 kDa. PKV Hy-2, PKV Rajat, AKH-081, AKH-3614-10 (Hirsutum pigmented) and Bunny-Bt showed two isozymes each (Table 3).

There was absence of isozyme in susceptible cultivar. Whereas, resistant and tolerant cultivar showed two isozyme. Similar results were found by Heng Moss *et al.* (2004) in chinch-bug infested plants.

Isozyme pattern of polyphenol peroxidase from the different cotton cultivars:

All the cultivars possessed single isozyme, except AKH-081 with different molecular weight (Fig. 1). PKV Hy-2 and Bunny-Bt possed dense intensity isozyme with molecular weight 25.05 and 23.12 kDa, respectively (Table 3).

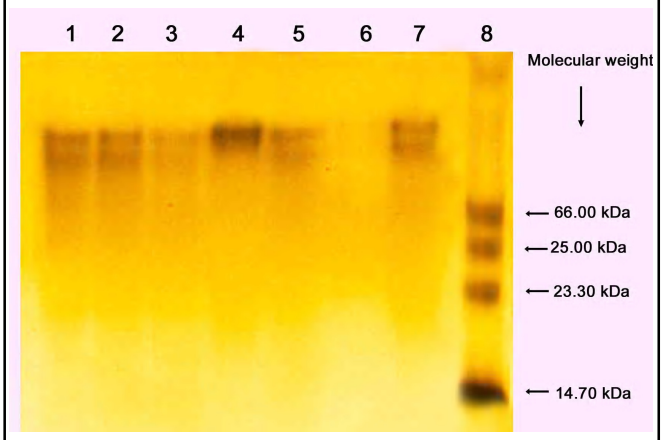
Polyphenol oxidase, polyphenol peroxidase and catalase activity was found higher in Bunny-Bt and CAHH-231 (pigmented hybrid), which are susceptible strain and lower in AKH-081, AKA-8 and PKV Hy-2, which are tolerant and resistant cotton varieties. Plant oxidative enzymes (polyphenol oxidase and polyphenol peroxidase) play an important role in the plants response to biotic and abiotic stresses.

This research offers a new perspective on cotton plant resistance against mealybug and provides a model for studying insect plant interactions. Furthermore, the

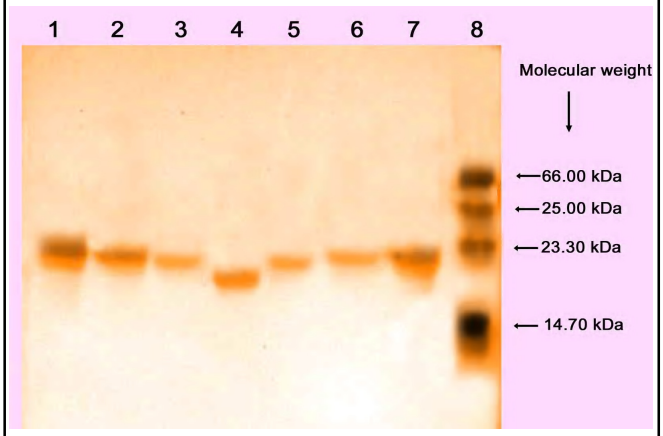
Table 3 : Isozyme pattern of polyphenol oxidase from the different cotton cultivars

Cultivars	Polyphenol peroxidase isozymes with molecular weight					Polyphenol oxidase isozymes with molecular weight		
	Isozyme	PPO1	PPO2	PPO3	PPO4	Total no. of isozymes	PO1	PO2	Total no. of isozymes
	Molecular wt. (kDa)	25.05	23.12	22.81	19.43		81.20	69.96	
	Rf values	0.46	0.47	0.47	0.40		0.20	0.21	
PKV Hy-2		+, D	-	-	-	1	+, M	+, M	2
PKV Rajat		-	+, M	-	-	1	+, M	+, M	2
AKH-081		-		+, L	+, M	2	+, M	+, M	2
AKA-8		-		-	+, M	1	+, D	-	1
AKH-3614-10 (Hirsutum pigmented)		-		+, L	-	1	+, M	+, M	2
CAHH-231 (Pigmented hybrid)		-	+, M	-	-	1	-	-	N.D.
Bunny Bt		-	+, D	-	-	1	+, M	+, M	2

Note: '+' - Presence of band, '-' - Absence of band, L - Light intensity band, M - Medium intensity band, D - Dense intensity band, ND - Not detected, Rf- Relative front.



Polyphenol oxidase isozymes



Polyphenol peroxidase isozymes

Lane 1-PKV Hy-2
Lane 2-PKV Rajat
Lane 3-AKH-081
Lane 4-AKA-8
Lane 5-AKH-3614-10 (Hirsutum pigmented)
Lane 6-CAHH-231 (Pigmented hybrid)
Lane 7-Bunny Bt
Lane 8- Standard Molecular Weight Marker

Fig. 1 : Polyphenol oxidase and polyphenol peroxidase isozymes from different cotton cultivars

identification of biochemical constituents for mealybug resistance provides a novel approach for screening resistant cotton cultivars for further breeding programme or as will act as gene source. Ultimately, biochemical constituents identified from this research will provide a set of tools for screening cotton cultivars for resistance to mealbugs.

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