Pak.J.Bot., 31(2): 347-359, 1999.

ISOZYME MARKERS IN COTTON BREEDING-II: INTER AND INTRAVARIETAL VARIATION IN THE ACTIVITY OF ISOZYMES OF THE ENZYME PEROXIDASE AS AFFECTED BY AREA OF COTTON CULTIVATION

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Abstract

Isozyme peroxidase was studied in different varieties of cotton (*Gossypium hirsutum*) growing at three different locations to detect possible variation in the activity of peroxidase within a variety growing at three different locations, between different cotton varieties growing at a particular location and to identify possible reason(s) of the observed (if any) variation in Pox. activity as measured by the intensity of isozyme bands observed in the extract of the material collected from different areas. Significant differences were observed in peroxidase activity within the plants of a variety growing at a particular location, between different varieties growing at one location, within a variety growing at three locations and between different loci of a variety growing at all the three locations. Isozymic phenotypes of varieties growing at locations III were significantly different from those growing at other two locations. Locus 4b expressed fully at location I and II while it did not show any activity at location III. Also, locus 3b showed maximum activity in varieties growing at location III while there was no activity at this locus in varieties growing at locations I and II. Prevalence of cotton leaf curl virus (CLCuV) disease at location III could be one of the reasons of observed isozymic variation. It was inferred that instead of different varieties, if similar varieties growing simultaneously at all the three locations were studied, it would have been possible to find a locus and/or loci which may show effect of CLCuV. Possibilities of success in such studies and their utilization in breeding cotton varieties having resistance agaist cotton leaf curl virus disease are discussed.

Introduction

Peroxidases are a class of enzymes in which every isozyme has a unique physiological and developmental role (Gasper *et al.*, 1982). Wall associated defense mechanism is one of such roles and is known to be implicated in processes like suberization (Robb *et al.*, 1991), lignification (Lagrimini & Rothstein, 1987) extension of cross linking (Showalter & Varner, 1989) and clearance of H_2O_2 (Bowels, 1990). Peroxidases are also reported to have played significant role in wound healing (Birecka & Miller, 1974., Parent *et al.*, 1985) and disease resistance (Nessel & Mader, 1977; Weststeijn, 1976). In our efforts to use isozymes in breeding cotton varieties for resistance against cotton leaf curl virus disease, we studied different isozyme systems (Farooq, 1999) including peroxidase which exhibited complex profile as it was coded by 7 loci. The enzyme activity was significantly different at different loci and in different varieties. In this study allelic polymorphism was not detected in any of the cotton varieties and only qualitative and quantitative differences were observed. It was

therefore anticipated that either the physiological state of the tissue or a particular environment in which the plant is growing may have influenced a particular isozyme phenotype. In order to know the extent of such variation, we studied profiles of isozyme peroxidase in different varieties of cotton (*G. hirsutum*) growing at three locations in the south of Punjab which is considered "cotton belt" of the country. The objectives were to detect possible variation i) in the activity of peroxidase isozyme within and between different cotton varieties growing at a particular location and ii) between different varieties growing under different areas of cotton cultivation.

Materials and Methods

a) **Description of material:** Material used in this study was collected mainly from two different cotton experimental farms of Central Cotton Research Insitutue (CCRI), Multan, and Punjab Seed Corporation, Khanewal. Cotton planted at one of the two farms at CCRI, Multan was named as Varietal Trial (hereafter known as "Location-1") and comprised CIM-435, CIM-443, CIM-448, CIM-1100, CIM-240 and S-14. Cotton planted at the other farm was named as Standard Commercial Varieties (hereafter known as "Location-II") and comprised CIM-109, CIM-240, CIM-1100, NIAB-78, BH-36, FH-682, Cris-9 and S-14. The recommended agronomic practices were applied in the experiments (Anon., 1998). Cotton was also planted at the farm of Punjab Seed Corporation, Khanewal, a hot spot for cotton leaf curl virus disease (Anon., 1995a) and would hereafter be known as "Location-III". This location comprised cotton varieties CIM-1100, CIM-435, CIM-443, CIM-448, CIM-240, CIM-109, FH-682, NIAB-78, BH-36 and S-14 which were mainly planted for screening different cotton varieties against cotton leaf curl virus disease.

The leaf samples from the plants growing at all the three locations were collected for three consecutive days in the month of July, 1996. Since in the isozyme studies, age and stage of the plant growth is very important to get reproducible profiles (Endo, 1981., Tyson *et al.*, 1985) therefore, specific efforts were made to collect the samples from the healthy plants of similar age and date of sowing. For each variety growing in a particular field and location, 10 plants were selected at random and from each plant, leaf samples from identical position were collected. All the samples were collected in the polythene bags, sealed and stored immediately in the box containing dry ice before they were transported to NIAB, Faisalabad for ultimate storage at -70°C in a deep freezer.

For extraction, electrophoreses and staining of enzyme peroxidase methods of Davis (1964) and Vallejose (1983) with slight modifications (Farooq, 1999) were used. Preparation of zymogram and photography of the gels was according to the procedure described by Farooq, *et al.*, (1997). In experiment-1, individual plant of each variety were analyzed to detect within the variety variation in the activity of peroxidase at any of the loci. In experiment-2, plants from one variety having nearly identical profiles were pooled and variation exhibited by the pooled samples at different loci were compared with other varieties growing at three different locations. In the experiment-3, variation in the peroxidase activity was measured in three different varieties growing simultaneously at all the three locations.

Data were statistically analyzed by giving a numerical number to an isozyme band according to its intensity (1 = no band, 2 = very faint band, 3 = faint band, 4 = medium intensity, and 5 = intense band). Such type of data for 7 isozyme loci of each plant and variety were collected from all the locations and stored as data files in a software programme MSTATC as has already been described by Farooq *et al.*, (1997). The variation in mean Pox. activity (as measured by the intensity of isozyme bands) among different varieties growing at different locations were measured by the analysis of variance and significance of these variations was measured by Duncan's Multiple Range Test (DMRT). Using this analyses, variation in the intensity of isozyme peroxidase at, 1) different loci in a particular variety growing at one location, 2) different cotton varieties growing at three different locations, 3) three cotton varieties growing simultaneously at all the three locations and 4) variation in peroxidase activity observed due to difference in different loci and in varieties was determined.

Results and Discussion

Exp-1. Variation within a variety: All the cotton varieties exhibited seven loci for peroxidase however, within the line variation in the intensity of certain bands were detected in all the commercial cotton varieties. The variations in intensity were mostly observed at loci 2, 3a, 4a, 5 and 6 and were both qualitative and quantitative. For example in cotton variety CIM-240 (Fig.1), the activity at locus 6 was very low as

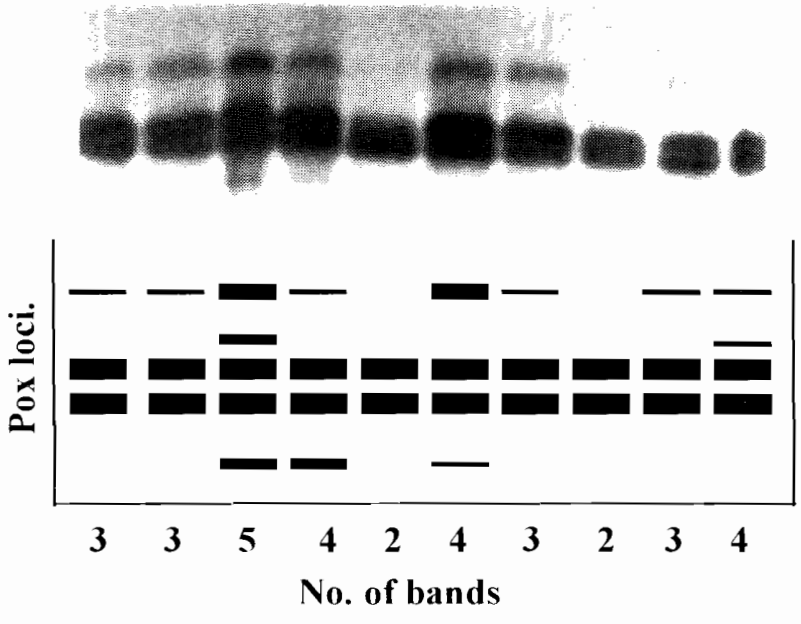


Fig.1. Profile and zymogram of isozymes of the enzyme Peroxidase in cotton variety CIM-240 growing at location II. Figure clearly exhibited variation in the intensity of the isozyme peroxidase at different loci in 10 plants selected randomly from one variety.

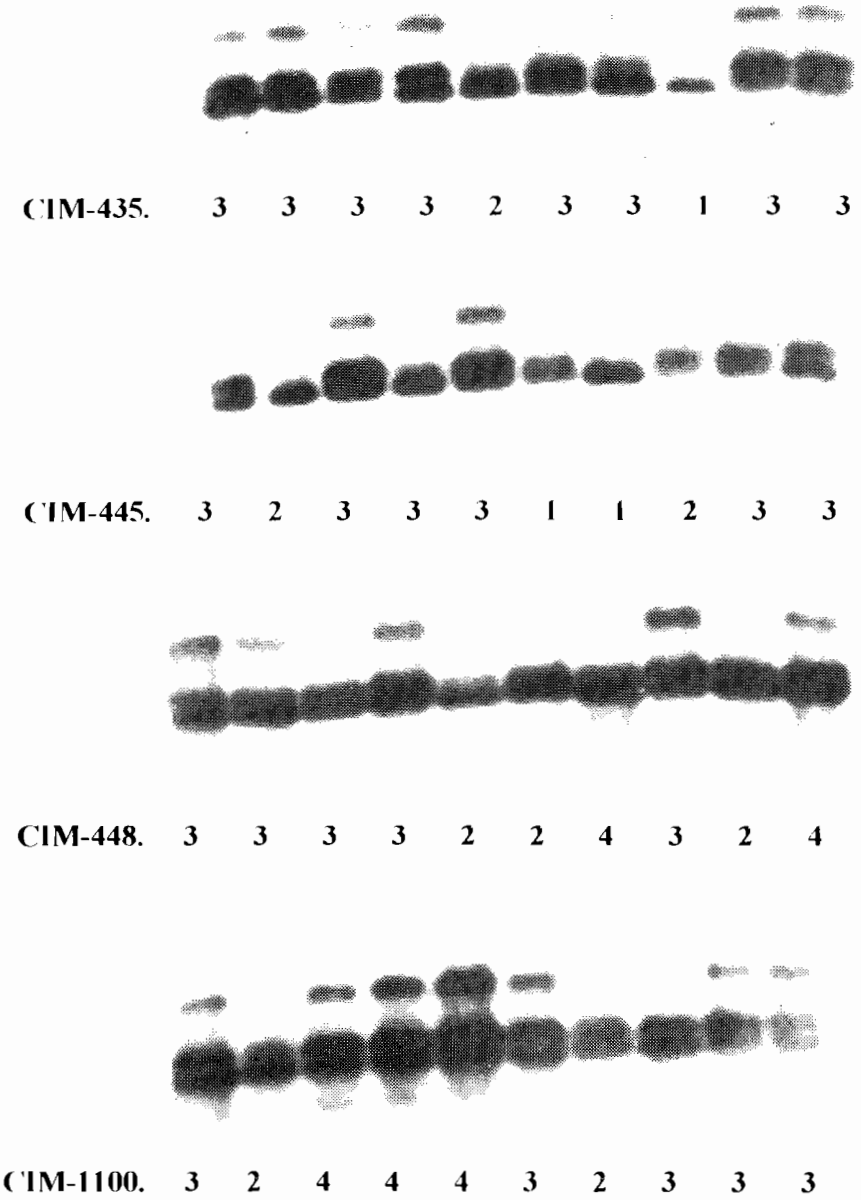


Fig.2. Profiles of isozymes of the enzyme peroxidase exhibiting within the line variations in the intensity of the isozyme detected in 10 plants selected randomly from cotton varieties CIM-435, CIM-445, CIM-448 and CIM-1100 growing at location II.

indicated by faint bands in plant nos.,1, 2 and 10 while there was no activity at this locus in plant nos., 5 and 8 as no band was visible. Similar variations were also observed in other cotton varieties viz., CIM-435, CIM-445, CIM-448 and CIM-1100 (Fig.2) as well as in N-78, FH-682, Cris-9 and S-14 (Fig. 3) growing at location II and location III, respectively. Variations in Pox. intensity were also observed in a variety (CIM-240) growing at two different locations (Fig. 4). Fedak & Rajhathy (1972) and Almgard & Lendegren (1974) made two independent studies to find isozymic uniformity in 71 and 32 commercial barley cultivars of which 37 and 12 cultivars, respectively, exhibited intravarietal variation. Similarly, Almagard & Clapham (1975) studied 18 and Singh *et al.*, (1973) 10 oat cultivars and found 7 and 3 cultivars respectively with intravarietal variation in isozymes with many possessing 3-4 different isozymic phenotypes.

Intravarietal variation in isozyme phenotypes have been studied with great interest because of their significance in plant breeding rights. Such variation arises when several morphologically similar lines take part in the development of a cultivar. Since these lines are mostly selected for physiological and morphological uniformity and not for isozymic uniformity, therefore, any cultivar which has not been independently evaluated or selected for a particular isozyme, may and often will exhibit significant intravarietal variation in their isozymic profile (Bailey, 1983). Such variation can be eliminated by selecting and bulking of plants showing nearly identical profiles of a particular isozyme as suggested by Bailey (1983). In the present study also, plants of a variety exhibiting nearly identical isozymic phenotypes (Fig 5) were pooled and used in the experiments conducted to study variation in peroxidase activity due to differences in varieties and of different locations.

Exp-2. a) Variation in peroxidase activity due to differences in loci and varieties: Among different loci of 6 varieties growing at location-1, maximum peroxidase activity was observed by locus 4b and a minimum by loci 3a, 3b, and 4a as evidenced by very intense and faint band respectively while among different varieties, maximum mean Pox. activity was observed in CIM-443 and minimum in CIM-240. Within a variety, differences in Pox. activity due to different loci were significant while among different varieties, differences in the activity of any specific locus were mostly non-significant. Among different varieties, differences in mean pox. activity of all the loci were not significant however, among different loci, the differences in mean Pox. activity of the 6 varieties were significant (Table 1).

At location-11, 8 varieties were studied of which three (S-14, CIM-1100 and CIM-240) were included in studies conducted at location-I, while the remaining 5 were new entries. Unlike location 1, at location-II, three of the seven Pox. loci (3a, 3b and 4a) did not exhibit any activity at all in any of the varieties whereas like location-I, locus 4b exhibited maximum activity. Differences due to varieties in mean peroxidase activity of different loci were not significant. Maximum mean varietal activity was exhibited by Cris-9 and the minimum by CIM-1100. Differences in activity due to loci were more profound than those due to differences in varieties (Table 2).

At location-III, 10 varieties were studied of which 6 (CIM-1100, CIM-240, S-14, CIM-443, CIM-448 and CIM-435) were common with location-I and the remaining 4 (CIM-109, NIAB-78, BH-36 and FH-682) with location-II. Like location I and II

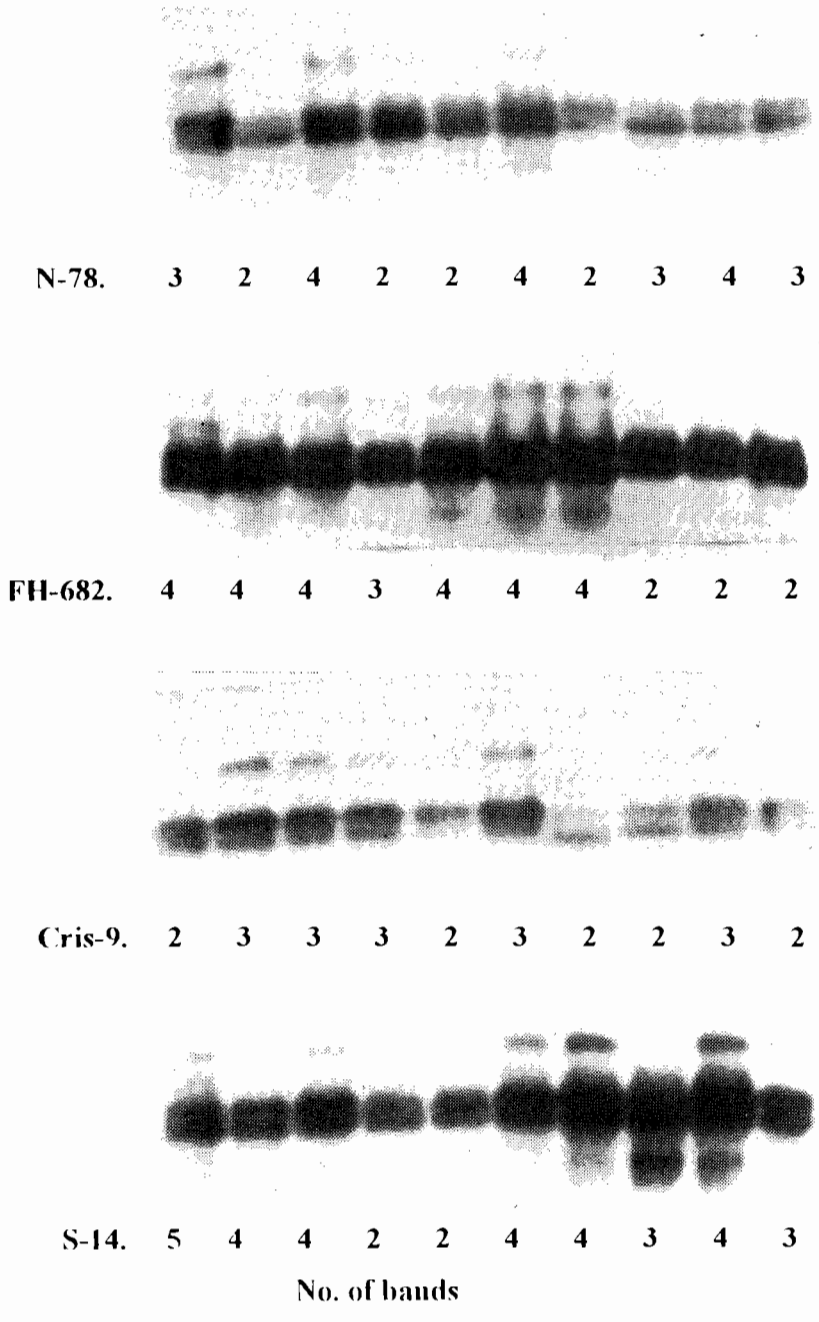
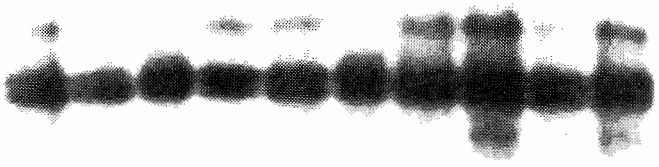


Fig.3. Profiles of isozymes of the enzyme peroxidase exhibiting within the line variations in the intensity of the isozymes detected in 10 plants selected randomly from cotton varieties NIAB-78, FH-682, Cris-9 and S-14 growing at location III.

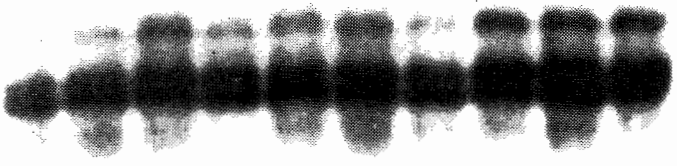
CIM-240. Loc.II 3 3 4 4 2 3 3 2 3 3



CIM-240. Loc.III 3 3 3 3 3 3 3 4 3 4

No. of bands

Fig.4. Profiles of isozymes of the enzyme peroxidase showing variation in the intensity of the bands exhibited by 10 plants selected randomly from a cotton variety CIM-240 growing at location II (top) and location III (bottom).



S-14. 5 5 5 5 5 5 5 5 5 5

No. of bands

Fig.5. Nearly identical profile of isozymes of the enzyme peroxidase exhibited by 10 plants selected randomly from cotton variety growing at location III.

Table 1. Variation in the intensity of isozyme peroxidase exhibited by bands appeared at different loci in different varieties of cotton growing at location-I.

Isozyme loci:	Intensity of bands at different loci in cotton variety CIM-435	CIM-443	CIM-448	CIM-1100	CIM-240	S-12	Mean
Pox-1	1.0	2.4	1.0	1.0	1.0	2.4	1.47c
Pox-2	3.0	3.0	2.6	2.6	2.6	3.0	2.80b
Pox-3a	1.0	1.0	1.0	1.0	1.0	1.0	1.0c
Pox-3b	1.0	1.0	1.0	1.0	1.0	1.0	1.0c
Pox-4a	1.0	1.0	1.0	1.0	1.0	1.0	1.0c
Pox-4b	5.0	5.0	5.0	5.0	4.8	5.0	4.97a
Pox-5	1.0	1.0	1.4	1.0	1.0	1.0	1.07c
Pox-6	1.0	1.0	1.6	1.6	1.0	1.0	1.20c
Pox-7	5.0	4.6	3.6	4.4	3.8	4.0	4.23a
Mean	2.11a	2.22a	2.02a	2.06a	1.91a	2.15a	---

Figures followed by the same letters in one row and a column are not significantly different from each other at 5% level of significance according to Duncan's Multiple Range Test.

Table 2. Variation in the intensity of isozyme peroxidase exhibited by bands appeared at different loci in different varieties of cotton growing at location-II.

Isozyme loci	Intensity of bands at different loci in cotton variety CIM-109	CIM-240	N-78	BH-36	FH-682	CRIS-9	S-14	CIM-1100	Mean
Pox-1	1.0	1.0	3.4	2.0	3.0	2.2	2.6	1.0	2.03^{d}
Pox-2	3.4	3.8	3.6	3.4	2.2	3.6	3.4	2.6	3.25^{c}
Pox-3a	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pox-3b	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pox-4a	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pox-4b	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0^{a}
Pox-5	1.0	1.0	1.0	1.0	1.4	1.0	1.0	1.0	1.05^{e}
Pox-6	2.4	1.0	2.0	2.4	1.6	2.4	2.2	1.0	1.87^{d}
Pox-7	3.2	3.8	3.6	4.8	4.0	4.6	4.2	4.2	4.05^{b}
Mean	1.78^{a}	1.73^{a}	2.07^{a}	2.07^{a}	1.91^{a}	2.09^{a}	2.04^{a}	1.64^{a}	--

Figures followed by the same letter in a row and a column are not significantly different from each other at 5% level of significance according to Duncan's Multiple Range Test.

differences due to varieties in mean peroxidase activity exhibited by different loci were not significant. Also, no activity was observed at locus 4b (Table 3). The maximum activity was exhibited by locus 3b and the minimum by locus 1 and 2. NIAB-78 exhibited maximum mean peroxidase activity and CIM-1100 the minimum. Again, differences in mean Pox. activity due to difference in loci were more profound than due to differences in the varieties (Table 3).

b. Variation in Pox. activity due to difference in loci and locations: When the mean peroxidase activity of different loci in varieties growing at one location or at all the three locations was compared, differences in mean varietal activity appeared non significant at all the 3 locations. However, since at all the three locations different varieties were growing therefore, real differences due to locations in mean varietal Pox. activity may not be detected unless, same varieties growing at all the places were studied.

To further clear the role of different locations, mean peroxidase activity in the form of banding intensity of 7 loci expressed in three varieties (CIM-1100, CIM-240 and S-14) growing simultaneously at all the three locations was compared. It was interesting to note that among all the places difference in mean varietal peroxidase activity were generally non significant (Table 4). When the mean activity of individual locus in the three varieties growing at three locations was compared, significant differences were observed in the activities of loci 2, 3a, 3b, 4a, and 4b as was expected. As observed earlier in experiment 2a, maximum activity was exhibited by locus 4b in varieties growing at location-I and II while at location-III, no activity was observed at this locus

Table 3. Variation in the intensity of isozyme peroxidase exhibited bands appeared at different loci in different varieties of cotton growing at location-III.

Isozyme Loci	Intensity of bands at differnet loci in cotton variety										
	CIM-1100	CIM-435	CIM-443	CIM-448	FH-682	NIAB-78	BH-36	S-14	CIM-109	CIM-240	Mean
Pox-1	1.0	1.0	1.0	1.0	1.4	1.8	1.0	1.0	1.0	1.0	1.12[d]
Pox-2	1.0	1.0	1.0	1.0	1.4	1.4	1.0	1.0	1.0	1.0	1.08[d]
Pox-3a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.00[d]
Pox-3b	4.2	4.8	5.0	5.0	5.0	4.8	5.0	5.0	5.0	5.0	4.88a
Pox-4a	1.0	5.0	2.8	4.0	3.8	3.4	4.2	3.6	1.0	1.2	3.20[b]
Pox-4b	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0
Pox-5	1.2	1.0	1.0	1.0	1.8	1.0	1.0	1.0	3.4	1.0	1.34[d]
Pox-6	1.8	2.0	1.8	1.8	3.0	3.4	3.4	2.6	1.8	1.6	2.32[c]
Pox-7	2.4	3.0	3.6	4.6	3.8	5.0	5.4	3.4	3.4	3.8	3.78[b]
Mean	1.51[a]	2.08[a]	1.9[a]	2.15[a]	2.35[a]	2.42[a]	2.40[a]	2.06[a]	1.95[a]	1.73[a]	---

Figures followed by the same letters in a row and a column are not significantly different from each other at 5% level of significance according to Duncan's Multiple Range Test.

Table 4. Variation in the intensity of isozyme peroxidase exhibited by bands appeared at different loci in three cotton varieties growing simultaneously at three different locations.

Pox. loci	Isozyme intensity of bands at different loci in three varieties of cotton growing at Location-I CIM-1100	CIM-240	S-14	Mean	Location-II CIM-1100	CIM-240	S-14	Mean	Location-III CIM-1100	CIM-240	S-14	Mean
1	1.0	1.0	2.2	1.4^{d}	1.0	1.0	2.6	1.53^{c}	1.0	1.0	1.0	1.0^{d}
2	2.6	2.6	3.0	2.73^{c}	2.6	3.8	3.4	3.26^{b}	1.0	1.0	1.0	1.0^{d}
3a	1.0	1.0	1.0	1.0^{d}	0.0	0.0	0.0	0.0	1.0	5.0	1.0	2.3^{c}
3b	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.2	5.0	5.0	4.7^{a}
4a	1.0	1.0	1.0	1.0^{d}	0.0	0.0	0.0	0.0	1.0	1.20	4.0	2.06^{c}
4b	5.0	5.0	5.0	5.0^{a}	5.0	5.0	5.0	5.0^{a}	0.0	0.0	0.0	0.0
5	1.0	1.0	1.0	1.0^{d}	1.0	1.0	1.0^{d}	1.0	1.2	1.0	1.06	1.06^{d}
6	1.6	1.0	1.0	1.2^{d}	1.0	1.0	2.2	1.4^{cd}	1 8	1.6	2.6	2.0^{c}
7	4.4	3.8	4.0	4.06^{b}	3.8	4.2	4.6	4.2^{ab}	2.4	3.8	5 0	3.86^{b}
Mean	1.9^{a}	1.8^{a}	2.0^{a}		1.6^{a}	1.78^{a}	2.07^{a}		1.62^{a}	2.17^{a}	2.3^{a}	

Figures followed by the same letters in one row and a column are not significantly different from each other at 5% level of significance according to Duncan's Multiple Range Test.

in any of the varieties. Similarly, in varieties growing at location-III, the highest activity was exhibited by locus 3b while in varieties growing at location I and III, there was no activity at all at this locus. Also, loci 3a, 3b and 4a exhibited negligible to no activity in varieties growing at location I and II while in varieties growing at location III, these loci exhibited significant differences in activity which ranged from 1 to 5 with maximum exhibited by locus 3b (Table 4).

In the present study, quantitative differences in the activity of isozymes of peroxidase were observed between different varieties growing at one location, between a variety growing at three different locations and between different loci of one variety. Since quantitative variation exists due to difference in staining intensity of a particular band and thus, does not provide clear and distinct difference between the two varieties (Bailey, 1983). Such variations have also been reported in soybean (Brim *et al.*, 1969) where high and low peroxidase activity in seed coat have been used in varietal identification (Buttery & Buzzel, 1968). The observed difference was genetically controlled (Buzzel & Buttery, 1969) and was so distinct that extraction of isozyme was not necessary as seed treatment with H_2O_2 produced intense color in high activity varieties while seeds with no Pox. activity remained colourless. Classification of blue grass cultivars on the basis of differences in peroxidase staining intensity has also been reported (Wehner *et al.*, 1976). In this study, intensity of some of the bands was so low that statistical methods were applied to distinguish them from each other as we did in the present study.

Quantitative variation in plant tissue may arise due to ontogenetic differences among the tissue samples which may affect their relative physiological activity, (Kuhns & Fretz, 1978; Menancio & Ramirez, 1977). In the present study this should not be the reason of observed quantitative variation as at all the locations, leaf samples of uniform size were collected at one time from varieties of one sowing date in one field. Among the other reason (s), variables of growth environment such as effect of temperature (Alamgard & Clapham, 1975), light (Siegel & Galston, 1967), disease infestation of plant (Stahmann & Demorest, 1973), field versus laboratory conditions (Gates & Boulter, 1979) and year of harvest (Alamgard & Clapham, 1977) may be considered. Since the studies were made on the plants growing under the fields of which two were located at one campus (location- I and II) and there was no possibility of significant differences in growth environment and temperature that ranged between 47/30.5°C (day and night) and 31.1/16.8°C during the active growth period (Anon., 1995b). The location-III on the other hand, was located about 50 kilometers north of location-I and II and is known as one of the hot spots for the cotton leaf curl virus (CLCuV) disease. Therefore, there is every possibility that growth environment of this location may differ significantly from the other two locations. The prevalence of CLCuV at this location could be one of the reason (s) that our results showed significant differences in peroxidase activity due to difference in locations. The present study was made on leaves collected in the month of July, when incidence of disease has been reported to be over 80% (Anon., 1995b) and it is possible that locus 3b specifically and 3a and 4a generally play role in protecting the plants against this disease. However, to fully understand the role of disease prevalence on the observed variation in the Pox. activity, it is necessary that comparative and comprehensive studies on isoyzme peroxidase be made on plant samples collected between the end of August and beginning of September when leaf curl virus disease reached its peak (Anon., 1995a). Such studies may help in identification of a locus and/or loci in a variety which may show effect of cotton leaf curl virus.

Acknowledgement

This work was supported in part by a grant from Asian Development Bank (ADB) and Ministry of Food, Agriculture and Livestock (MINFAL) for the project titled "Identification of genetic structure of cotton leaf curl virus resistant and susceptible varieties of cotton through the use of Isozyme" for which the author is grateful. Technical help of Dr. Farooq-e-Azam PSO and Ms. Anjuman Ara Zaidi, SO, are also gratefully acknowledged. The author is also grateful to the authorities at CCRI, Multan and PSC, Khanewal for providing the material used in this study.

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(Received for publication 2 April 199[illegible])