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Electrophoretic study of isoenzymic forms of peroxidase enzyme in seeds and seedlings of Barley carrying Infection of *Drechslera graminea*

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ABSTRACT

Comparisons of peroxidase isozymes among seeds and seedlings of barley, naturally infected and artificially inoculated to *Drechslera gramnea* were investigated. Electrophoretic comparisons revealed no significant qualitative differences in peroxidase isoenzymic patterns in seeds of different categories *viz*. healthy, naturally infected and artificially inoculated. Results of electrophoretic study of peroxidase isozymes on *Drechslera graminea* infected seedlings indicated difference in banding pattern and band intensity in healthy, naturally infected and artificially inoculated seedlings during examination periods. Band intensity was in increasing order from 10th to 30th day, was highest in samples of 30th day of sowing. peroxidase isozymes analysis gave only a possibility of defence mechanism against *Drechslera graminea* by introducing new isozymes of peroxidase.

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Introduction

Peroxidases (EC 1.11.1.7) have many physiological roles in several primary and secondary metabolic processes, such as scavenging of peroxide, participation in lignification, regulation of cell growth and differentiation, hormonal signaling, plant defense, IAA catabolism, oxidation of toxic compounds, and ethylene biosynthesis (Campa, 1991). Multiple isoenzymes of Peroxidases exist in plants normally. The existence of multiple forms of peroxidase in plants has been known for a number of years, but the relationship of individual isozymes to specific biological functions is not clear. A positive relationship found between peroxidase isozymes and resistance development in plants. Plant peroxidases have been proposed to catalyze the last enzymatic steps in the biosynthesis of both lignin and hydrogen peroxide.

Gel electrophoresis of enzymes is a very useful and powerful analytical method, which is at present widely used in many distinct fields of both biological and medical sciences and successfully applied in many different fields of human activity.

Six extracellular isoenzymes of peroxidase are detectable in healthy suspension cultures of castor bean by native gel electrophoresis. The differential regulation of expression of peroxidase isoenzymes following elicitor treatment suggests that individual isoenzymes of peroxidase may have specific functional roles in the biosynthesis of disease-lignin (Bruce and West, 1989). Tyagi *et al*. (1998, 2000) observed higher activities of isoenzymes of peroxidase in wheat infected with *Alternaria triticina*. They observed an extra band in plants exposed to the pathogen.

Gogoi *et al.* (2001) investigated the effects of the highly aggressive isolate KB-2 of the Karnal bunt pathogen (*Neovossia indica*) on peroxidase (POX) and its isoenzymes in wheat. Result revealed that the activity of POX was highest at 2 d.a.i. in the two resistant wheats, but increased more slowly to a peak at 6 d.a.i. in the susceptible wheat. The number of isoenzymes of POX detected by polyacrylamide gel electrophoresis (PAGE) changed after inoculation with KB-2. The maximum number of isoenzymes occurred at 2 day after inoculation in the two resistant wheats and at 6 d.a.i. in the susceptible wheat. Although the isoenzymes detected in seedlings were not identical to those detected in seeds, the PAGE banding patterns of seeds and seedlings were the same for the two resistant wheats.

Material and Method

Material

Seeds of healthy, naturally infected (three categories weakly, moderately and heavily) and artificially inoculated with *Drechslera graminea* of sample acc. No. Br32 and their seedlings after 10 days, 20 days and 30 days of sowing, were taken for conducting studies. Artificial inoculation of *Drechslera graminea* was made in healthy seeds of acc. No. Br32.

Raising of crop: The crop was raised in earthen pots (height 30 cm, diameter 20 cm) filled with sterile coarse sand (pH 8.3). Seedlings were harvested at different time intervals for conducting the electrophoretic studies of peroxidase isoenzymes.

Method

Alterations in different isoenzymic forms of peroxidase were examined by PAGE (poly acrylamide gel electrophoresis) according to the method of Davis (1964) and were detected by the method of Seigel and Galston (1967).

Separation of different isoenzymes of peroxidase was carried out using 7 % acrylamide gel electrophoresis. For the preparation of gels, only running gel system was used for isoenzymes separation, the procedure followed was as follows:

Chemicals and Reagents used

Stock solutions:

Solution A: 1 N HCl, 48 ml Tris (Tris hydroxyl methyl amino methane), 36.6 g TEMED (N,N, N', N' tetramethyl ethylene diamine), 0.23 ml and water to make volume 100 ml.

Solution B: Acrylamide 28.0 g, Bisacrylamide 0.735 g and water to make volume 100 ml.

Solution C: 0.14% Ammonium persulphate solution in water.

Preparation of working solution: The stock solutions were allowed to attain the room temperature on removal from the

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refrigerator. For gel polymerization, mixed one part of solution A, two part of solution B, one part of water and then four parts of solution C.

The above gel polymerization solution was immediately dispersed with a disposable pipette into the gel chamber avoiding trapping of air bubbles, after one minute of filling the gel, one drop of water was layered in order to attain a flat smooth top of the gel.

Preparation of electrolyte buffer: Tris glycine buffer (PH 8.3) was employed for filling the chambers and was prepared by taking, Tris 6 g, glycine 27.3 g and water to make volume to 1000 ml. This served as the stock buffer solution which was diluted ten times with water at the time of use.

Method

Upon completion of polymerization of the gels the water from the gels chamber was removed carefully with small filter paper stripes. The gel chamber was inserted into the grammutes of the upper buffer reservoir and the enzyme extract was applied on the wells of gel with the help of pipette in the form small aliquots containing 150-200 µg of protein. Then the upper buffer reservoir was filled with about 200 ml of the buffer solution. Now one part of 0.001% aqueous bromophenol blue was stirred into the upper buffer reservoir. Any air space if present in the gel chamber above the sample was displaced with buffer by means of pipette. A hanging drop of buffer was placed on the bottom of gel chamber, to prevent trapping of bubbles and then upper reservoir was lowered so that the bottom ends of the gel chamber was immersed to 0.5 cm in the buffer of the lower reservoir. The power supply was connected with the lower reservoir to the anode and upper reservoir to the cathode.

The current was initially adjusted to 1.5 m A per gel well about 10 mm and subsequently to 3 m A per gel well. Electrophoresis was conducted at 4˚ C for 2- 2.5 h till the maker dye travelled up to about 1 cm from bottom of the gel chamber. When the electrophoretic run was complete, the power supply was turned off and buffer solutions were decanted. The gel chamber was removed from the upper reservoir. This was immediately placed in ice coldwater in order to facilitate the removal of the gels.

Staining of isoenzymes: The method of Mitra *et al.* (1970) was followed for staining of peroxidase isoenzymes. The staining mixture consisted of the solutions (a and b) in the ratio of 1:1 (v/v). These solutions were

(a) 1.0% Benzidine dissolved in 25% acetic acid and

(b) 1.0% H_2O_2

The solution was poured on to the gels and after the staining was over (approx. 5 min.) the gels were kept in 7.0 % acetic acid at 4˚ C.

Scanning of the gels: The gels were scanned manually in a proper light arrangement for identification of individual isoenzyme bands. Each band was characterized with a contain Rf value which was calculated as below:

$$Rf = \frac{\text{Distance travelled by the band}}{\text{Distance travelled by marker}}$$

After staining they were photographed. For the expression purpose proper diagrammatic representation was drawn out and also a tabular expression was made for the description of the isoenzymic bands spectrum based on colour intensity and relative mobility of the bands.

Result and Discussion

Seeds

Electrophoretic study of peroxidase isozymes on *Drechslera graminea* infected seeds revealed that band intensity was almost similar but number of bands was different in all seed categories healthy, naturally infected and artificially inoculated. Numbers of bands recorded in healthy, weakly infected and moderately infected seeds were 4 and in heavily infected and artificially inoculated seeds were 3. Prominent bands were 3 in all the seed categories. Rf values of isoenzyme bands were 0.8 and 0.5, 0.09 and 0.08. Last band 0.08 Rf value was not detected in heavily infected and artificially inoculated seeds. The lesser bands of peroxidase isoenzymes in heavily infected and artificially inoculated seeds might be a result of defence reaction of the host. Arora and Bajaj (1985) recorded the variability in peroxidase enzyme banding pattern in hypocotyls of mungbean after infection with *Rhizoctonia solani.*

Seedlings

Results of electrophoretic study of peroxidase isozymes on *Drechslera graminea* infected seedlings indicated difference in banding pattern and band intensity in healthy, naturally infected and artificially inoculated seedlings during examination periods. Band intensity was in increasing order from 10th to 30th day, was highest in samples of 30th day of sowing.

At 10th day of sowing, electrophoretic study revealed 3 isozymes of peroxidase on polyacrylamide gel with Rf values 0.8, 0.6 and 0.3 in all the categories of seedling. No significant difference was observed in Rf values of isoenzyme bands among seedling categories. **At 20th day of sowing**, 5 bands of peroxidase isoenzymes with Rf values 0.8, 0.3, 0.05, 0.03 and 0.02 recorded in all categories of seedlings. No significant difference was observed in Rf values of all five bands in different seedling categories.

At 30th day of sowing, zymogram of healthy seedlings had 4 isozyme bands with Rf values of 0.6, 0.5, 0.3 and 0.07 while, all the categories of naturally infected and artificially inoculated seedlings had 3 isozymes of Rf values 0.6, 0.3 and 0.07.

Regarding the determined peroxidase isozymes in the current investigation, appearance of darker bands of peroxidase isozymes during examination periods in infected plants was closely related to high disease severity of stripe disease. Darker intensity of bands showed higher activity of peroxidase enzymes at 30th day. Generally, data obtained through this investigation showed a positive relation between severity of disease and intensity of bands of peroxidase isozymes. No relation was found between number of bands and disease severity, as numbers of bands were almost similar in all the categories. Result of present investigation showed the multifacial involvement of peroxidase ranging from secondary phenol metabolism to lignin biosynthesis. In this respect such phenomenon was recognized as the primary reflection of stripe disease establishment in young seedlings and later on at the maturity level.

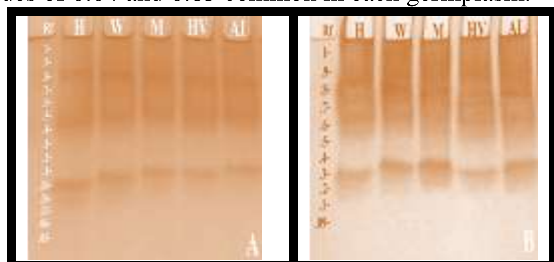
Puchalski *et al.* (1986) observed the induction of three peroxidase isozyme in rye seedlings after inoculation with *Fusarium nivale* and suggested that the isozyme band P x 4 could be used as a maker in identifying resistant genotypes. Chahal *et al.* (1988) investigated the peroxidase isozyme pattern among downy mildew resistant and susceptible pearl millet lines and healthy plants, which indicated the involvement of C5, C6 and C9 isoperoxidases in developing resistance. Aly and Afify (1989) pointed out that the resistance induced in barley plants showed new peroxidase isozymes in infected plants pre-treated with ethephon.

Subashchandra *et al*. (2000) analyzed the banding pattern of peroxidase isozyme in 45 and 60 days old tomato leaf samples against *Ralstonia solanacearum* (bacterial wilt) by using PAGE. They reported that the zymograms of PR x -7 (Rm = 0.361) and

PR x -8 (Rm =0.381) in 45 days old samples and PR x -5 (Rm =0.297) in 60 days old samples could be used as a markers to identify resistant and moderately resistant varieties. Electrophoretic study by Hassan *et al.* (2007) on peroxidase isozymes on pathogens and pretreated faba bean plants with chemical inducers indicated differences in banding pattern, band intensity and also between inoculated and uninoculated plants during examination periods. No new isozymes were detected with the two pathogens *Botrytis fabae* and *B. cinerea*. Result showed peroxidase isozymes analysis which gave only a possibility of defence mechanism induced by spraying faba bean plants with certain chemicals against *Botrytis fabae* and/or *B. cinerea* by introducing new isozymes of peroxidase. Application of chemical inducers resulted in changes in peroxidase isozymes in both healthy and infected plants during examination periods. Some treatments introduced new isozymes and/or increase in concentration of some isozymes especially after infection with the pathogen. The role of oxidative enzymes such as peroxidase could be explained as an oxidation process of phenol compounds to oxidized products (quinones) which may limit the fungal growth.

During native PAGE analysis on healthy and *Curvularia penneseti* infected Pearlmillet, Singh (2007) observed that isoenzymes bands showed more colour intensity during the infection and in the treated seedlings with biocontrol agent *Trichoderma viride*. Two strong and two weak bands were more clear, which showed higher activity of peroxidase enzyme. It might be the resultant changes in expression of genes involved in healthy and infected categories.

Jayaraj *et al.* (2010) investigated on oxalic acid-induced resistance to *Rhizoctonia solani* in rice associated with induction of phenolics, peroxidase and pathogenesis-related proteins. Peroxidase isozyme analysis indicated that protein extracts from control plants exhibited two isozymes (PO-1 and PO-2). In the treated plants, two new peroxidases (PO-3 and PO-4) appeared two days after treatment and these peroxidases were present throughout the experimental period of six days. The increased peroxidase isoenzymes in rice leaves treated with Oxalic acid might be involved in lignin biosynthesis which in turn might have contributed to disease resistance. The basis of host defense response was analyzed by Moeen *et al.* (2014), after *Curvularia lunata* (Wakker) Boedijn infection by biochemical and molecular techniques. Five different varieties of *Sorghum bicolor* were challenged with virulent strain of *C. lunata* for development of leaf spot disease. Isozymes of Peroxidase enzyme were visible on polyacrylamide gel with variable Rf values. Maximum number of peroxidase isozymes was detected in most resistant variety 'Indian Gold'. On native gel this variety showed four peroxidase isoforms with Rf values of 0.04, 0.46, 0.813 and 0.850. Moderately resistant variety "Sukhar" had three isozymes with Rf values of 0.04, 0.46, 0.813, and 0.85. While, all the rest of susceptible varieties had two isozymes with Rf values of 0.04 and 0.85 common in each germplasm.



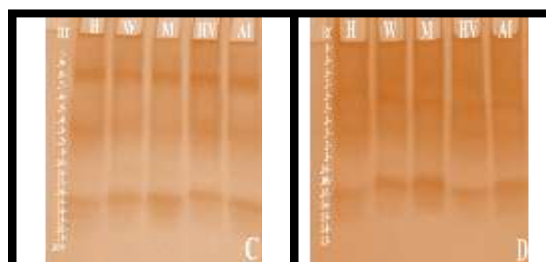


Figure 1: Gel Photograph of native PAGE showing electrophoretic separation of peroxidase isoenzymes (A) in barley seeds of healthy (control), naturally infected (weakly, moderately, heavily) and artificially inoculated with *Drechslera graminea* and (B) their seedlings at 10th day, (C) at 20th day and (D) at 30th day of sowing.

H- Healthy, W- Weakly infected, M- Moderately infected, HV- Heavily infected and AI-Artificially inoculated

A

	H	W	M	HV	A
0.8	–	–	–	–	–
0.5	–	–	–	–	–
0.09	–	–	–	–	–
0.08	–	–	–		

B

	H	W	M	HV	A
0.8	–	–	–	–	–
0.6	–	–	–	–	–
0.3	–	–	–	–	–

C

	H	W	M	HV	A
0.8	–	–	–	–	–
0.3	–	–	–	–	–
0.05	–	–	–	–	–
0.03	–	–	–	–	–
0.02	–	–	–	–	–

D

	H	W	M	HV	A
0.6	–	–	–	–	–
0.5	–				
0.3	–	–	–	–	–
0.07	–	–	–	–	–

Figure 2: Zymogram of native PAGE showing electrophoretic separation of peroxidase isoenzymes (A) in barley seeds of healthy (control), naturally infected (weakly, moderately, heavily) and artificially inoculated with *Drechslera graminea* and (B) their seedlings at 10th day, (C) at 20th day and (D) at 30th day of sowing.

H- Healthy, W- Weakly infected, M- Moderately infected, HV- Heavily infected and A-Artificially inoculated

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