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Research Journal of Agricultural Sciences
An International Journal

P- ISSN: 0976-1675
E- ISSN: 2249-4538

Volume: 13
Issue: 05

Res. Jr. of Agril. Sci. (2022) 13: 1576–1579

CARAS

Res. Jr. of Agril. Sci. (Sep-Oct 2022)
13(5): 1576–1579

ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

www.rjas.org

Full Length Research Article

Electrophoretic Study of Isoenzymic forms of Peroxidase Enzyme in Seeds and Seedlings of Sorghum Carrying Infection of *Macrophomina phaseolina*

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Received: 25 Jul 2022 | Revised accepted: 18 Sep 2022 | Published online: 15 Oct 2022

A B S T R A C T

Comparison of peroxidase isozymes among infected seeds and seedlings of sorghum (naturally infected and artificially inoculated to *Macrophomina phaseolina*) were investigated. During electrophoretic study no significant qualitative difference in isoenzymic patterns were seen in different categories of seeds viz. healthy, naturally infected and artificially inoculated whereas results of seedlings indicated difference in banding pattern and band intensity in healthy, naturally infected and artificially inoculated seedlings. Band intensity was in increasing order from 10th to 30th day i.e., it was highest in samples of 30th day of sowing. Peroxidase isozymes analysis gave only a possibility of defence mechanism against *Macrophomina phaseolina* by introducing new isozymes of peroxidase.

***Key words*:** *Macrophomina phaseolina,* Electrophoretic study, Isoenzymes, Peroxidase

In the natural environment, plants are subject to various abiotic and biotic stresses, leading to excess production of reactive oxygen species (ROS) [1]. To decrease the damage caused by ROS, plants activate the enzymatic system, which mainly comprises large families of peroxidases. These enzymes also play an important role in abiotic stress tolerance and pathogen resistance. Peroxidase is an enzyme belonging to the class of oxidoreductase, which is actively involved in the oxidation of phenols, suberinization and lignification of plant cell walls in response to the action of phytopathogenic microorganisms [2]. This resistance mechanism is related to the induction of peroxidase enzyme activity [3].

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. PAGE is an electrophoretic technique established in the early 1980s, which is a kind of electrophoresis that maintains the enzyme biological activity after electrophoresis [4]. Peroxidases can largely reflect the characteristics of plant growth and development, bio metabolic status, ability to adapt to the external environment and genetic differences among varieties. The electrophoretic profiles of peroxidases are relatively stable under certain conditions and are as species-specific as morphological trait indicators [5-7]. It has been widely used as a genetic marker in plant variety identification, genetic diversity analysis, plant disease resistance analysis, plant growth and development analysis [8]. The POX characterized from Fagopyrum esculentum by native gel electrophoresis revealed two isozymes, i.e., POX I and POX II [9].

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MATERIALS AND METHODS

Seeds of healthy, naturally infected (three categories weakly, moderately and heavily) and artificially inoculated with *Macrophomina phaseolina* of sample acc No. 49 and their seedlings after 10 days, 20 days and 30 days of sowing, were taken for conducting studies.

Raising of crop: the crop was raised in earthen pots (height 35 cm, diameter 25 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 [10] and were detected by the method of [11].

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:

Chemical 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'-Tetramethylethylenediamine), 0.23ml and water to make volume 100ml.

Solution B: Acrylamide 28 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 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 6g, glycine 27.3g 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 hour 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 cold water in order to facilitate the removal of the gels.

Staining of isoenzymes: the method of [12] was followed for staining of peroxidase isoenzymes. The staining mixture consisted of the solution (a and b) in the ratio of 1:1 (v/v). These solutions were:

- 1.0% Benzidine dissolved in 25% acetic acid and
- 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% 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.

RESULTS AND DISCUSSION

Seeds

In the present study, peroxidase isozymes on *Macrophomina phaseolina* infected seeds revealed that intensity of bands were almost similar but the number of bands were different in all seed categories i.e., healthy, weakly infected, moderately infected, heavily infected and artificially inoculated. Bands observed in healthy, weakly and moderately infected seeds were 4 and in heavily infected and artificially inoculated seeds were 3. Rf values of isozyme bands found in healthy to moderately infected seeds were 0.8, 0.4, 0.1, 0.09 whereas Rf values of isozyme bands found in heavily and artificially inoculated seeds were 0.8, 0.4, 0.1. Last isozyme band with value 0.09 was not found in heavily infected and artificially inoculated seeds.

Seedlings

In seedlings no significant difference found in number of isozymic bands but found difference in band intensity and pattern from 10^{th} to 30^{th} day of sowing. Highest band intensity found in samples of 30^{th} day of sowing.

10^{th} day of sowing

3 isozymic bands of peroxidase with Rf values 0.8, 0.5, 0.2 found on polyacrylamide gel during electrophoretic study in all the categories of seedling. No significant difference was observed in Rf values of bands in seedling categories.

20^{th} day of sowing

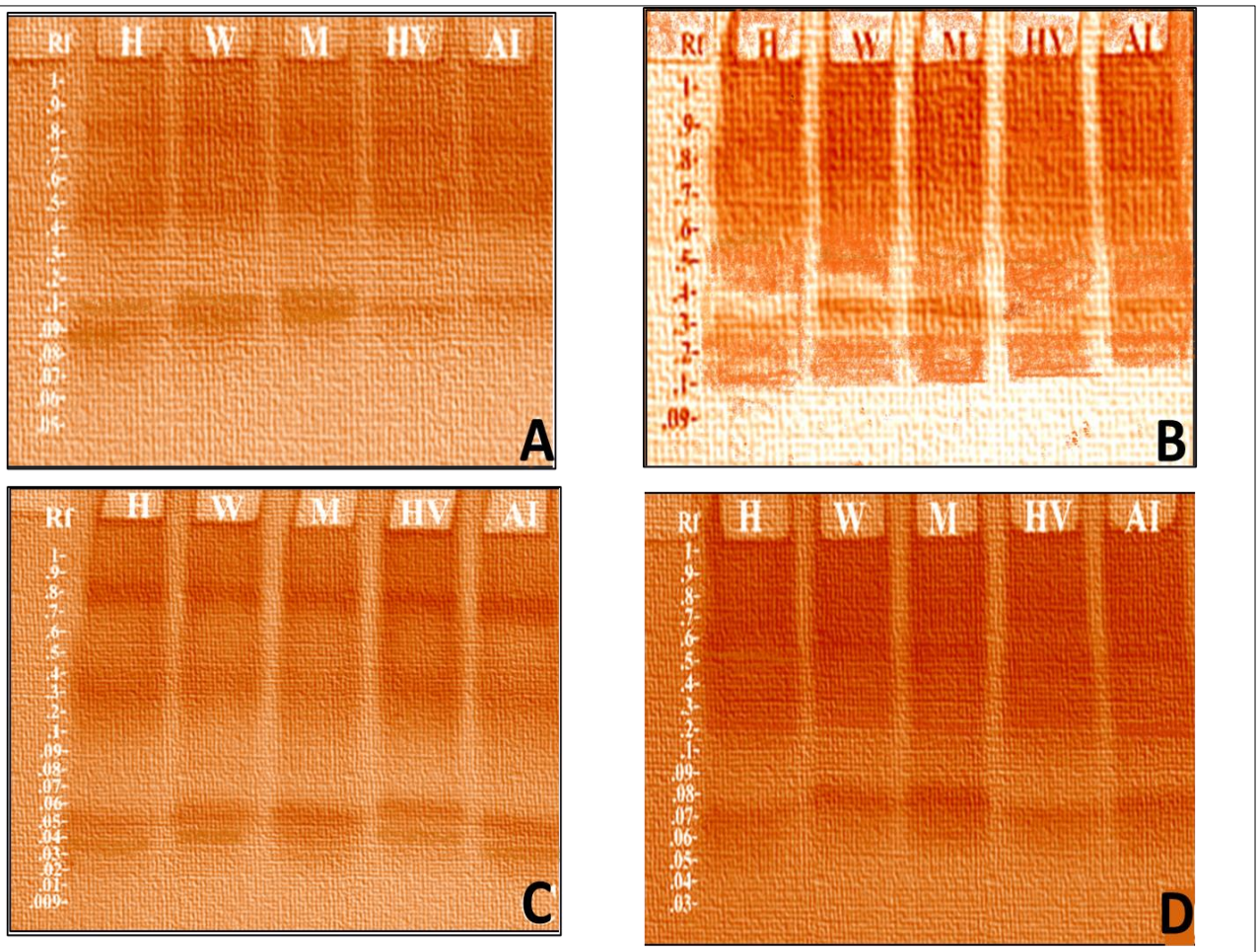
4 isozymic bands of peroxidase with Rf values of 0.8, 0.3, 0.05, 0.04 found on polyacrylamide gel during electrophoretic study in all the categories of seedling. No significant difference was observed in Rf values of bands in seedling categories.

30^{th} day of sowing

Zymogram of healthy seedlings had 4 isozyme bands with Rf values of 0.6, 0.4, 0.2, 0.07 while all the categories of naturally infected and artificially inoculated seedlings had 3 isozymes of Rf values 0.6, 0.4, 0.07.

Data obtained through this study showed a positive relation between severity of disease and intensity of bands of peroxidase enzymes. At 30^{th} day of sowing darker bands of peroxidase enzymes showed high severity of charcoal disease. But there is no relation was found between number of bands and disease severity, as number 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, as peroxidase is the final enzyme in the lignin biosynthetic pathway. So,

lignification and peroxidase activity occurred in all categories so might be it is a reason for same number of bands. And also, the infected plant proteins show not much difference from the healthy one, however in the separation of SDS PAGE the significant band pattern of infected and healthy plants did not show much difference. Due to which the slight difference the bands merge together. [13] reported that isozyme patterns of acid phosphatase, polyphenol oxidase and superoxide dismutase from healthy and diseased Mesta plants infected by Yellow Vein Mosaic Virus produced similar types of band patterns. [14] mentioned that the interaction between the pathogen and the host plant induces some changes in cell metabolism, primarily activity of enzymes, particularly peroxidase (POX) and polyphenoloxidase (PPO) such plant enzymes were shown to be involved in defense reactions against plant pathogens. So, POX activity was more in tolerant sesame cultivar than susceptible sesame cultivar to the *Macrophomina phaseolina*.



Gel photograph of native PAGE showing electrophoretic separation of peroxidase isoenzymes (A) in sorghum seeds of healthy (control), naturally infected (weakly, moderately, heavily) and artificially inoculated with *Macrophomina phaseolina* 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

Increased peroxidase activity has been reported in many plants exposed to external biotic stresses. The peroxidase pattern in nematode infected 11 resistant and 11 susceptible sugarcane clones a total of eight bands were appeared in leaves. In roots totally 11 bands were recorded. Together roots and leaves 0.36 band (Rf value) was present in all resistant clones but the same was not found in susceptible clones. Hence, this peroxidase isoform shall be used as a selection marker in breeding for nematode resistance [15]. [16] found that the isozyme profile of polyphenol oxidase in general revealed ten isoforms in cowpea. After SA spray and infection, a quantitative change in enzyme activity corresponding to the mainly anodic isoforms was observed. A clear and distinct isoform (PPO-10) over-expressed in SA-applied-Rizoctonia solani inoculated UPC-4200 genotype.

Soyabean plant responses to *Heterodera glycines* were compared between resistance and susceptible cultivars at 10, 20 days after inoculation and the POD isozyme patterns were characterized by different electrophoretic mobility and Rf values. Difference found between the isozyme band width between inoculated and control cultivars and wider isozyme bands found in inoculated cultivars [17]. [18] found that a high variability of the peroxidase spectrum is noted in the Scots pine needles under the influence of technogenic pollution. [19] found that isozyme bands of rice suffering from Tungro disease caused by double infection of Rice Tungro Baciliform Virus (RTBV) and Rice Tungro Sphiracle virus (RTSV) appeared thicker compared to the bands of healthy plants.

The basis of host defense response was analyzed by [20], 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 enzymes was detected in most resistant 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.

CONCLUSION

Charcoal rot is a major disease of sorghum (*Sorghum bicolor*) caused by *Macrophomina phaseolina* and results in significant loss in yield and seed quality. The present study demonstrates that specific activities of Peroxidase and IAA oxidase enzymes increased in diseased tissues as the disease response because these enzymes play important roles in defence mechanism.

Acknowledgement

The authors are grateful to the CSIR, for providing funds and to the head, Department of Botany, Govt. M. S. J. P.G. College, Bharatpur, India, for providing all the facilities to carry out the work.

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