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Zymographic Analysis of Maize Peroxidase Isoenzymes in Response to Fungicide Stress

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ABSTRACT: The peroxidase isoenzymes have essential role in the oxidative protection of plants under abiotic stress. The isoenzymes are a set of proteins catalysing the same reaction but they differ in number of properties. Analysis of these isoenzymes helps in finding the mutagenic potential of plants in response to environmental conditions. The native PAGE analysis was conducted which separates the isoenzymes based on their electrophoretic mobility and then visualized using staining solution containing substrate. The experiment showed Up-regulations of few isoenzymes and also down-regulations of some of the isoenzymes is observed in different concentrations of the fungicide *i.e.*, metalaxyl treatment. The study shows the induced peroxidase is Ca^{2+} dependent. However, inclusion of EGTA could not effectively inhibit the peroxidase activity which clearly shows the involvement of multiple signal pathways.

Keywords: Peroxidase, isoenzymes, native PAGE, EGTA.

INTRODUCTION

The enzyme Peroxidases are efficient components of the antioxidative system induced in response to environmental stress, such as pathogen attack, metal excess, salinity, drought and high light intensity (Veljović *et al.*, 2018; Aftab *et al.*, 2022). The isoforms of Class III Peroxidases in plants are shown to be expressed in many kinds of biotic and abiotic stress conditions (Aleem *et al.*, 2022) and have low substrate specificities (Aleem *et al.*, 2022). The differences in their relative activity which can be induced by any external stimuli is exploited to identify their role as biomarker in many developmental stages and stress conditions (Stival Sena *et al.*, 2018). In view of this the present study aimed at analyzing the activity of the enzyme peroxidase on treating the maize seeds with metalaxyl, a systemic fungicide by using electrophoretic technique. The activity of peroxidase can be analyzed directly on the gel by addition of a suitable substrate, like guaiacol (Achar *et al.*, 2014) known as Zymographic analysis.

MATERIAL AND METHODS

Collection of seeds and Treatments. Maize seeds procured from VC farm, University of Agriculture Science, Mandya, Karnataka. Seeds of uniform size were selected and soaked for 24 hours in distilled water (control) and with different concentrations (mg/g) of metalaxyl for 24 hours. Five seeds in triplicate were placed on Petri dish with 8-10 layer of soaked filter paper and incubated at 25°C.

Preparation of enzyme extract and electrophoresis. The extraction of peroxidase enzyme was performed by using phosphate buffer PH 7.0. The isoenzyme pattern was analysed for 0, 5th and 7th day enzyme extracts. NATIVE-PAGE was performed to separate the isoenzymes in a BIORAD Mini Gel electrophoretic system according to the method of Laemmli (1970).

Effect of Ca^{2+} and EGTA on peroxidise. Different concentrations of $CaCl_2$ (500µM) and EGTA (50µM) were added to the enzyme samples from control and 7mg treated seedlings. And then the isoenzyme patterns were tested for 0-day, 5th day and 7th day of germination.

Visualization of enzyme activity and analysis: Staining of gels for peroxidase activity was performed by the method described by Sadasivam and Manickam (1991). The gels were washed thrice with distilled water and were stained with freshly prepared Benzidine solution. Benzidine solution was prepared by dissolving 0.05 g of benzidine in 0.5 ml of absolute alcohol and the volume was made up to 20 ml with distilled water. Enzyme activity was assayed by using histochemical stains that produce an insoluble dye where enzyme activity was present. The gels were photographed and scored for isozyme bands using Vilbert Lourmat Gel documentation system. Data generated for each enzyme were recorded in a matrix identifying the presence or absence of a particular band and Rf (resolving front) values were calculated. Rf = distance travelled by the enzyme band in the gel/ distance travelled by the indicator (Palem and Padmaja 2014).

RESULTS

Isoforms of maize peroxidase on 0-day, 5th day and 7th day of germination: On 0 day of germination, native gel stained for peroxidase activity reveals the presence of isoforms in control. Similar patterns of

isoforms were detected in metalaxyl treated maize seedlings in concentrations 4.5, 6 and 7 mg/g of metalaxyl treatment (Fig. 1a and 1b).

The zymogram study also showed that there was changes in the intensity and number of isoenzyme bands under normal and metalaxyl treated condition. Analysis of peroxidase zymogram pattern showed varied expression of isoforms in control. These isoforms could not be detected in concentration 1.5 and 3.0mg of metalaxyl treated seedlings and the intensity was low compared to the control seeds.

On the otherhand peroxidase expressed isoforms 1 and 2 when exposed to 4.5, 6.0 and 7mg metalaxyl treated seedlings. But variation in the intensity of the bands was observed among different concentrations. In 4.5mg metalaxyl treatment the intensity of the bands formed were 0.8% and 0.5% whereas in 6.0mg treated seedlings the intensity was 2.2%, 1.2% and in 7mg it was 1.3% and 0.8%. However no isoforms could be detected in the concentrations 1.5 and 3.0mg metalaxyl treatments.

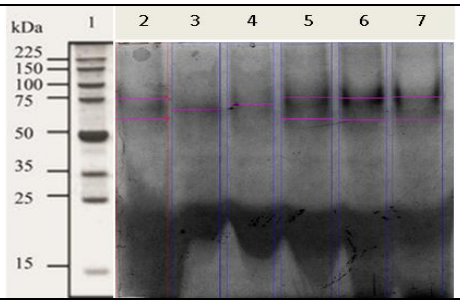
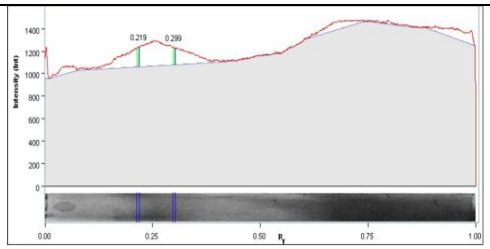


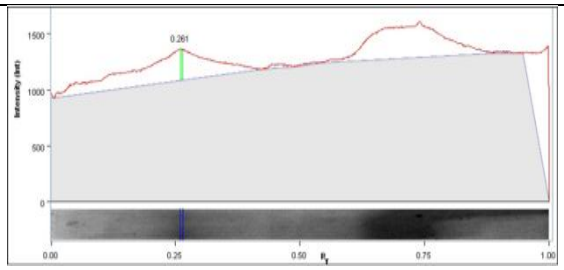
Fig. 1a. Peroxidase isozyme banding profiles in maize seedlings on 0-day.
Lane 1: Marker; Lane 2: Control; Lane 3-7: Metalaxyl treatment viz, 1.5, 3.0, 4.5, 6.0 and 7mg/g concentrations.

Lane 2:



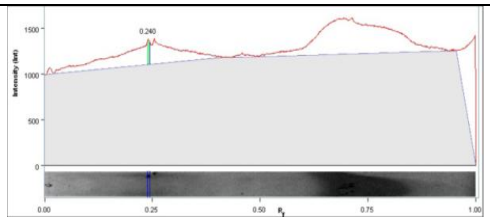
Band No.	Relative front	Lane %
1.	0.219	2.4
2.	0.299	2.1

Lane 3:



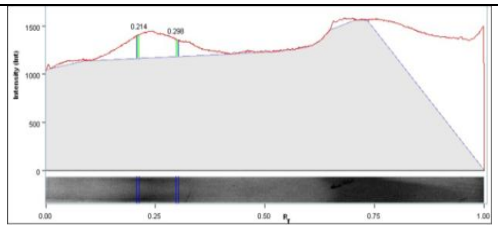
Band No.	Relative front	Lane %
1.	0.261	1.4

Lane 4:



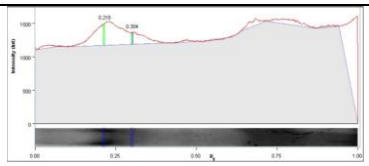
Band No.	Relative front	Lane %
1.	0.240	1.2

Lane 5:



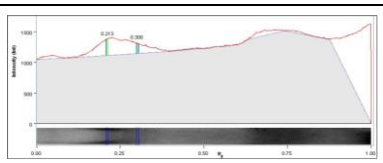
Band No.	Relative front	Lane %
1.	0.214	0.8
2.	0.298	0.5

Lane 6:



Band No.	Relative front	Lane %
1.	0.218	2.2
2.	0.301	1.2

Lane 7:



Band No.	Relative front	Lane %
1.	0.213	1.3
2.	0.300	0.8

Fig. 1b. Densitometric scan, Rf value and intensity of peroxidase isoenzymes in control and fungicide treatment on 0-day of germination.

The zymogram study on 5th day germinated seedlings also showed similar pattern of isoforms (Fig. 2a and 2b). However the intensity of the band has been increased which correlates with the increased peroxidase activity on the 5th day of germination. Peroxidase isoforms was not observed in 1.5, 3.0, 4.5 and 6.0mg of metalaxyl treated seedlings. Two isoforms with the same intensity as control was observed in the 7mg treated seedlings. In all the other concentrations only one band with almost the same intensity was observed.

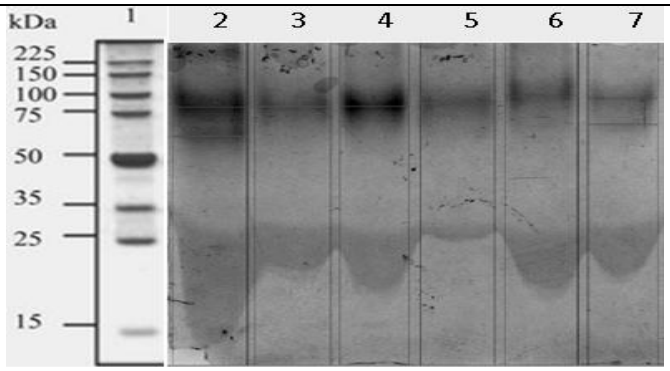
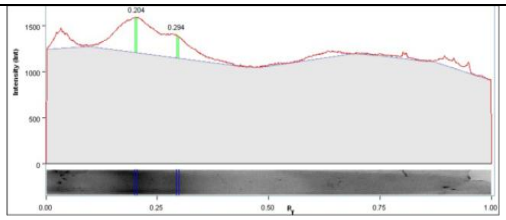


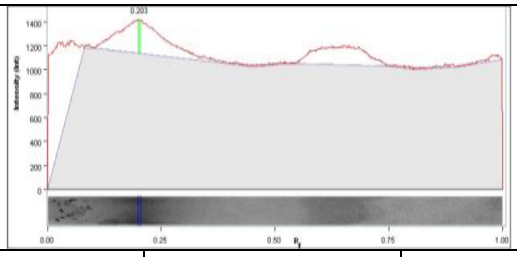
Fig. 2a. Peroxidase isozyme banding profiles in maize seedlings on 5th day.
Lane 1: Marker; Lane 2: Control; Lane 3-7: Metalaxyl treatment *viz.*, 1.5, 3.0, 4.5, 6.0 and 7mg/g concentrations.

Lane 2:



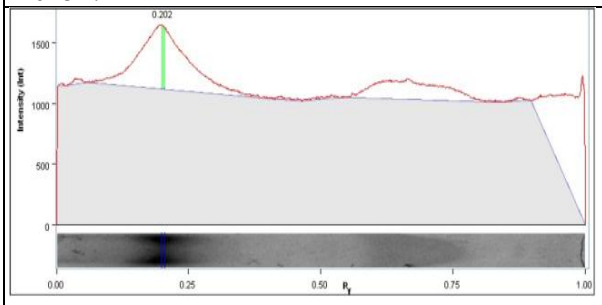
Band No.	Relative front	Lane %
1.	0.204	3.7
2.	0.294	2.3

Lane 3:



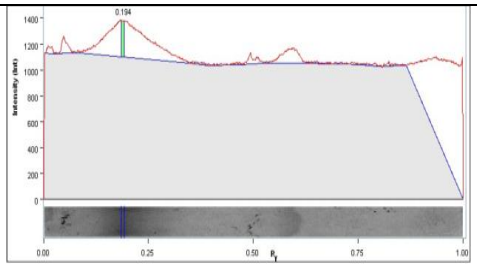
Band No.	Relative front	Lane %
1.	0.203	2.0

Lane 4:



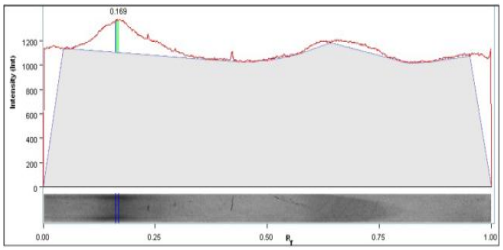
Band No.	Relative front	Lane %
1.	0.202	2.8

Lane 5:



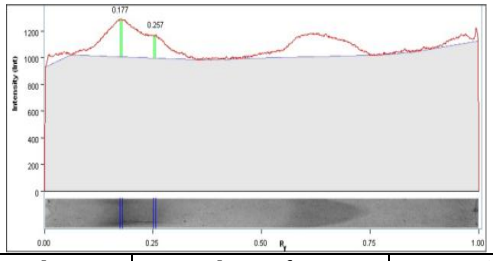
Band No.	Relative front	Lane %
1.	0.194	1.8

Lane 6:



Band No.	Relative front	Lane %
1.	0.169	2.2

Lane 7:



Band No.	Relative front	Lane %
1.	0.177	3.0
2.	0.257	1.8

Fig. 2b. Densitometric scan, Rf value and intensity of peroxidase isoenzymes in control and fungicide treatment on 5th day of germination.

On the 7th day of germination (Fig. 3a and 3b), the activity of peroxidase in the control maize seedlings as well as in the highest metalaxyl treated maize seedlings of concentration- 7 mg/g of seed weight was more than that on the 5th day of germination in the same concentrations. However, on the 7th day of germination, the activity of peroxidase in the concentrations- 1.5, 3, 4.5 and 6 was decreased when compared to that on the 5th day of germination in the same concentrations.

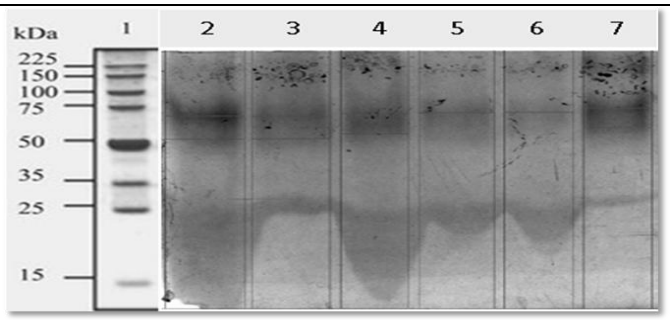
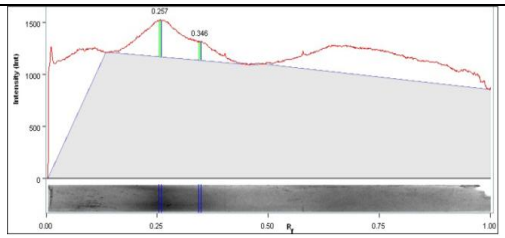


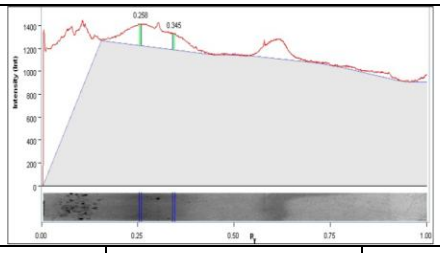
Fig. 3a. Peroxidase isozyme banding profiles in maize seedlings on 7th day.
Lane 1: Marker; Lane 2: Control; Lane 3-7: Metalaxyl treatment *viz.*, 1.5, 3.0, 4.5, 6.0 and 7mg/g concentrations.

Lane 2:



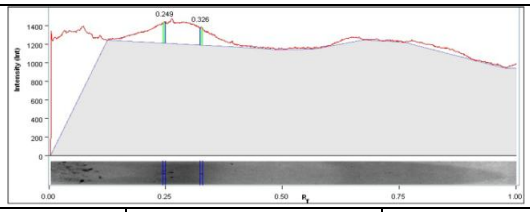
Band No.	Relative front	Lane %
1.	0.204	3.7
2.	0.294	2.3

Lane 3:



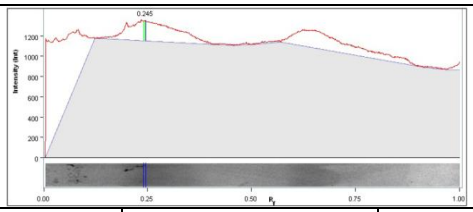
Band No.	Relative front	Lane %
1.	0.227	2.8
2.	0.317	2.1

Lane 4:



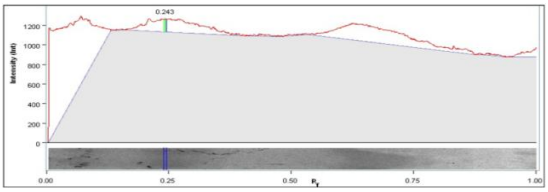
Band No.	Relative front	Lane %
1.	0.227	2.4
2.	0.317	0.4

Lane 5:



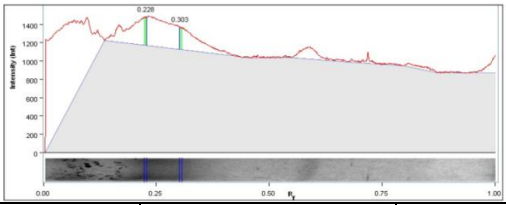
Band No.	Relative front	Lane %
1.	0.228	3.4
2.	0.310	2.7

Lane 6:



Band No.	Relative front	Lane %
1.	0.230	3.1
2.	0.309	2.2

Lane 7:



Band No.	Relative front	Lane %
1.	0.225	1.1
2.	0.310	0.7

Fig. 3b. Densitometric scan, Rf value and intensity of peroxidase isoenzymes in control and fungicide stress on 7th day.

Effect of Ca^{2+} and EGTA on peroxidase: Our results have clearly demonstrated an enhanced activity of peroxidase (Fig. 4a and 4b; 5a and 5b; 6a and 6b) with the addition of exogenous Ca^{2+} (50 µM). This activity could be further inhibited by the addition of Ca^{2+} chelater EGTA, the inhibited activity was restored with the addition of exogenous calcium.

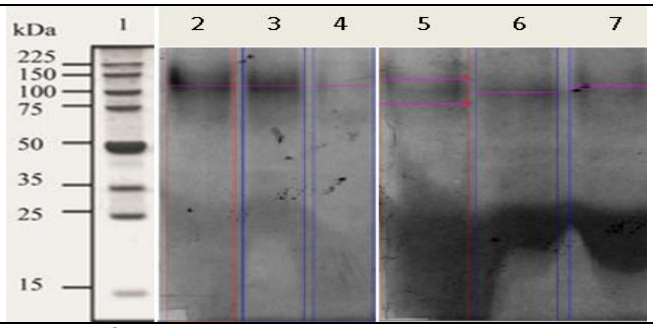
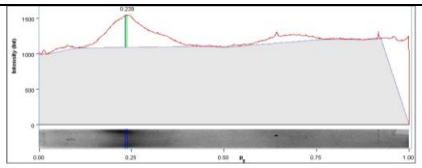


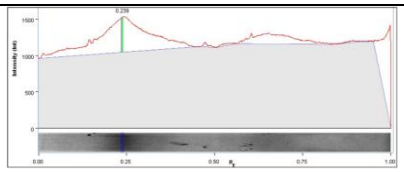
Fig. 4a. Effect of Ca^{2+} and EGTA on peroxidase activity on 0 day of germination.
Lane 1: Marker; Lane 2: Control; Lane 3: Control + $CaCl_2$ (500µM); Lane 4: Control + $CaCl_2$ + EGTA (500µM + 50 µM); Lane 5: Enzyme from 7mg treatment; Lane 6: Enzyme from 7mg treatment + $CaCl_2$ (500µM);
Lane 7: Enzyme from 7mg treatment + $CaCl_2$ + EGTA (500µM + 50 µM).

Lane 2:



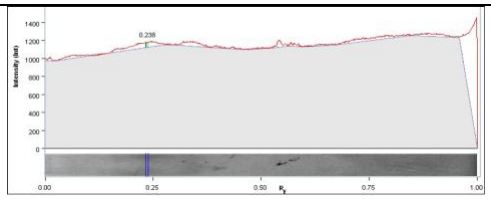
Band No.	Relative front	Lane %
1.	0.239	2.4

Lane 3:



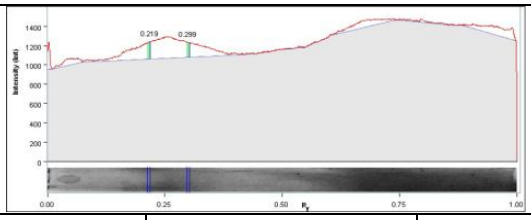
Band No.	Relative front	Lane %
1.	0.239	2.8

Lane 4:



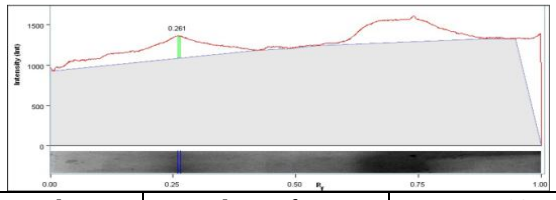
Band No.	Relative front	Lane %
1.	0.238	0.8

Lane 5:



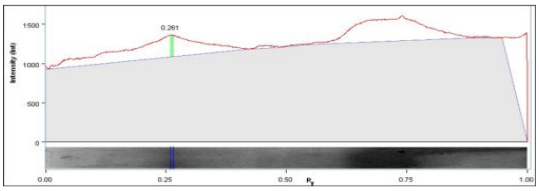
Band No.	Relative front	Lane %
1.	0.219	2.4
2.	0.299	2.1

Lane 6:



Band No.	Relative front	Lane %
1.	0.261	2.8

Lane 7:



Band No.	Relative front	Lane %
1.	0.240	1.2

Fig. 4b. Densitometric scan, Rf value and intensity of peroxidase isoenzymes in presence of $CaCl_2$ and EGTA on 0 day.

DISCUSSION

Expression of different plant peroxidases in rice was induced by different types of wounding (Hiraga *et al.*, 2001). This wide range of peroxidase isozymes and their tight and differential regulation prepares them for various stress conditions (Passardi *et al.*, 2005).

Our result clearly shows that peroxidase exhibited different trends of activity on different days of maize seed germination and a different pattern of expression of peroxidase activity was seen in the different concentrations of metalaxyl. The intensity of isoform bands observed indicates the induction patterns of individual peroxidase isoenzymes since a same amount of protein was used from each preparation for loading on to the gel.

The expression of isoenzymes was more intense at higher concentration of metalaxyl on 7th day of germination. These results indicates the expression of isoforms of peroxidase depends on the growth stage which may be due to expression of different genes.

Significant changes in the activities of some individual isoenzymes of peroxidase is reported in wheat under drought stress and two isoenzymes showed enhanced activities after the poly ethylene glycol treatment (Csiszar *et al.*, 2008). There were three isoforms of peroxidase was reported in two different ecotypes of *Withania somnifera* in in vivo and in vitro plant

samples but the intensity of isoenzyme bands were different in all the samples with a higher amount in in vitro plants (Kanungo *et al.*, 2013). In order to create crops having higher yields, under adverse abiotic and abiotic stress conditions, plants biomarkers are widely used (Bakhsh and Hussain 2015; Leetanasaksakul *et al.*, 2022).

A total of three isoforms of peroxidase were expressed and its intensity varied between the seeds of eggplant cultivars and even between control and seedlings inoculated with *R. solanacearum* culture (Prakasha and Umesha 2016). The result of our study and the above mentioned reports indicates the expression of peroxidase isoforms depends on the stress level and seedling age which is related to the expression of specific gene. At the same time, the fungicide stress at higher concentration is also responsible for the induction of isoforms. It is very clear from our results since the isoform pox-1 and pox-2 were observed only with the highest concentration (7mg/g) of metalaxyl treated seedlings.

Free Ca^{2+} act as one of the key signal molecules in plants and animals. It is involved in multiple signal transduction pathways, comprising of many intercellular and intracellular interactions. Changes in the cytosolic free Ca^{2+} concentration induce intracellular Ca^{2+} signals. Calcium ions work as a molecular switch for PO activity and exert a protective function, rendering POs heat stable (Plieth and Vollbehr 2012). This change in cellular Ca^{2+} concentration may be due to various environmental stimuli, like salinity, oxygen deficit, cold stress (He *et al*., 2015).

Enhanced activity of antioxidant enzymes and improvement in several physiological and biochemical processes have been reported by several studies. Wang (2010) reported increased peroxidase activity under drought stress in the seedlings treated with calcium. The native gel staining clearly demonstrated the peroxidase activity of both isoforms obtained on 0 day, 5th day and 7th day of germination are Ca^{2+} dependent.

The study shows the induced peroxidase is Ca^{2+} dependent and further studies have to be conducted to understand how Ca^{2+} signaling would protect the plants against the oxidative damage that occurs during and after the stress. A more direct influence of Ca^{2+} has been confirmed from our study and this is in accordance with the many other protective functions that Ca^{2+} in plants.

However, inclusion of EGTA could not effectively inhibit the peroxidase activity which clearly shows the involvement of multiple signal pathways as Xu and Zhang (2015) have shown the involvement of protein kinases in antioxidant defensive signaling. The expression of Ascorbate peroxidase encoding genes is differentially modulated by several abiotic stresses in different plant species (Caverzan *et al.*, 2012). As EGTA can inhibit only the Ca but not the kinases and hence effective inhibition could not be observed. Further work in understanding the signaling pathways, its regulation and cross talk may help us to understand the mechanism involved in abiotic stress response.

CONCLUSIONS

Zymography of peroxidases studied on 0, 5th and 7th day of germination showed the isoenzyme pattern. Some of the isoenzymes were up-regulated whereas others are down-regulated, depending on the stress level. The expression of isoenzymes was more intense at higher concentration of metalaxyl on 7th day of germination. And the zymogram also revealed that induced peroxidase is calcium dependant but the presence of EGTA could not inhibit enzyme activity effectively. This may suggest the involvement of multiple signalling pathways. These results further confirms our findings on the expression of isoforms of peroxidase depends on the growth stage which may be due to expression of different genes.

FUTURE SCOPE

Further study is required to know the specific role of different isoenzymes expressed and their gene analysis.

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Conflict of Interest. None.

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