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Biochemical characterization of Maize peroxidase under fungicide treatment

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ABSTRACT
This work was carried out to investigate the effect of the fungicide, metalaxyl as a seed treatment on the defense enzyme peroxidase in maize during early germination. Maize seeds were soaked in different concentrations of metalaxyl and a control was maintained. Increased peroxidase activity observed in control and metalaxyl treated seedlings with growth is a common response to oxidative and abiotic stresses which suggest the role of peroxidase in detoxification of H_20_2 produced during plant growth period and in fungicide stress caused due to higher concentration.

Keywords: *Metalaxyl, Fungicide, Peroxidase, Abiotic stress, EGTA*

1. INTRODUCTION

Peroxidases (EC.1.11.1.x) are hydrogen peroxide decomposing enzymes associated with the oxidation of phenolic as well as non- phenolic compounds. The plant peroxidases are grouped into class III peroxidase. It is considered as stress indicator of plants since the concentration of peroxidase increases considerably after any kind of stress stimulation (Cleverson D.T. *et al* 2024, Simin Li *et al* 2024). Therefore, the present study is conducted to understand the activity of induced Peroxidase on treating with a systemic fungicide, Metalaxyl in Maize seedlings during early germination.

2. MATERIAL AND METHODS

2.1 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 25^0C.

2.2 Preparation of crude extract

1 g of seedlings were taken (0-7days) homogenized in 10ml of 0.1 M phosphate buffer pH 7.0, using chilled pestle and mortar. The solution was centrifuged for 20 minutes at 0^0C for 12,000 rpm and supernatant were used as crude enzyme extract for the determination of the activity of peroxidase.

2.3 Estimation of peroxidase (POX)

Peroxidase activity was determined spectrophotometrically using guaiacol as the substrate by the modified method of Chance and Maehly (1956). The specific activity of peroxidase was expressed as µmoles/mg of protein/minute.

2.4 Influence of Ca^{2+} and EGTA on peroxidase activity:

The assay solution containing 50 µl of enzyme extract and 1000 µl of $CaCl_2$ solution (1µM - 1mM) was incubated at 37^0 C for 15 minutes. Then the reaction was started by adding 600 mM hydrogen peroxide (H_2O_2). Initial rate of increase in absorbance was measured over one minute at 470nm. This gives the total activity of peroxidase which is expressed in µmoles/minute/g.

2.5. Statistical Analysis

All the experiments were repeated four to five times at different time intervals and the data collected were subjected to analysis of variance and the means were compared according to Tukeys's (1949) significance test at 5% level using computerized SPSS package version 14.0. There are significant differences between the means of four doses when the probability value (p) is less than or equal to the risk $\alpha = 0.05$ ($p \leq \alpha = 0.05$); highly significant differences when $p \leq \alpha = 0.01$.

3. RESULTS

3.1. Effect of metalaxyl on specific activity of peroxidase:

The result (Table 1) shows highest activity on 7th day in control (4.98µmoles/mg/minute) as well as in 7mg metalaxyl treated seedlings (53.8µmoles/mg/minute). Among the metalaxyl treated seedlings, 6mg concentration showed lowest peroxidase activity on all the days of observation with an average value 10.77µmoles/mg/minute. Similarly, 7mg treated seedlings showed highest average activity on all the days of observation (15.97µmoles/mg/minute).

Table 1: Effect of metalaxyl on specific activity (µmoles/mg of protein/minute) of peroxidase in germinating maize seedlings

Germination in days	Control	Concentration of metalaxyl (mg/g)					Mean
		1.5	3.0	4.5	6.0	7.0	
0	0.0027^{b}	0.0081^{c}	0.0024^{ab}	0.0024^{ab}	0.001^{a}	0.034^{d}	0.009^{a}
1	0.134^{d}	0.093^{c}	0.084^{bc}	0.079^{b}	0.05^{a}	0.57^{e}	0.170^{a}
2	0.51^{ab}	2.34^{ab}	1.11^{ab}	2.71^{b}	0.13^{a}	1.32^{ab}	1.353^{b}
3	0.76^{a}	5.92^{c}	2.57^{ab}	3.29^{b}	0.4^{a}	2.67^{ab}	2.599^{c}
4	3.64^{b}	16.87^{d}	17.83^{d}	43.1^{e}	0.84^{a}	11.94^{c}	15.703^{d}
5	4.62^{a}	36.782^{e}	30.15^{d}	66.56^{f}	13.19^{b}	18.72^{c}	28.337^{e}
6	4.35^{a}	25.12^{b}	45.64^{d}	51.93^{e}	26.46^{b}	38.76^{c}	32.043^{f}
7	4.98^{a}	14.19^{b}	68.6^{e}	44.51^{c}	45.15^{c}	53.8^{d}	38.538^{g}
Mean	2.37^{a}	12.66^{b}	20.74^{e}	26.52^{f}	10.77^{b}	15.97^{d}	14.844
F value	Concentrations F (5, 96) = 2497.248 **, Days F (7, 96) = 6922.454** Concentration * Days = F (35, 96) = 663.146**						

Mean ± SD followed by the same superscript are not statistically significant between the concentrations, when subjected to SPSS package ver. 13.0, according to Tukey's mean range test at 5% level. ** Significant at P ≤ 0.01, *significant at ≤ 0.05

3.2. Influence of calcium on peroxidase activity

3.2.1. Peroxidase activity in the presence of calcium on 0th day, 3rd day, 5th day and 7th day of germination:

The results (Table 2) indicate that the activity of peroxidase was gradually increased in the inclusion of $CaCl_2$. In the metalaxyl treated seedlings, peroxidase activity increased with 10 µM of $CaCl_2$ and progressively increased till 500 µM of $CaCl_2$ and declined further.

Whereas on 3rd day (Table 3), the maximum activity was observed with 1.5mg metalaxyl treated seedlings. With 1µM concentration of $CaCl_2$, the activity was lowest (0.72U/minute) and with 1 mM concentration of $CaCl_2$ maximum activity

(7.92U/minute) is recorded. No significant difference found in other treated seedlings but both control and treated seedlings showed an increasing activity with increased concentration of $CaCl_2$.

On 5th day the activity was maximum in control with 10 µM and 1000 µM concentrations of $CaCl_2$. On 7th day of germination, there was a steady increase in the activity with increase in $CaCl_2$. This increase was more prominent in metalaxyl treated seedlings compared to control.

Table 2: Effect of $CaCl_2$ on peroxidase activity (µmoles/min.) in maize seedlings on 0th day of germination

Conc. of $CaCl_2$ µM	Control	Concentration of metalaxyl (mg/g)				
		1.5	3.0	4.5	6	7.0
-	0.539^{b}	1.08^{d}	0.623^{c}	0.401^{a}	0.485^{b}	1.52^{e}
1	0.479^{b}	0.26^{a}	0.284^{a}	2.31^{e}	1.06^{c}	1.22^{d}
10	2.39^{c}	2.85^{d}	2.41^{c}	0.5^{b}	0.18^{a}	4.90^{e}
50	0.73^{b}	1.23^{d}	2.01^{e}	0.41^{a}	0.76^{b}	1.05^{c}
100	1.14^{b}	1.15^{b}	1.94^{d}	1.32^{c}	1.37^{c}	0.86^{a}
500	3.23^{e}	1.70^{c}	1.62^{b}	3.73^{e}	2.33^{d}	1.41^{a}
800	2.39^{b}	5.5^{e}	0.453^{a}	4.87^{d}	2.18^{b}	3.02^{c}
1000	2.00^{a}	3.4^{c}	2.226^{b}	4.25^{e}	2.10^{a}	3.62^{d}
Mean	1.61^{c}	2.146^{d}	1.44^{b}	2.223^{e}	1.308^{a}	2.2^{e}
F value	Metalaxyl F (5,96) = 1.821** $CaCl_2$ (F, 7,96) =196.824* Metalaxyl * CaCl2 = F (35,96) = 54.985**					

Table 3: Effect of $CaCl_2$ on peroxidase activity (µmoles/min.) in maize seedlings on 3rd day of germination

Conc. of $CaCl_2$(µM)	Control	Concentration of metalaxyl (mg/g)					Mean
		1.5	3.0	4.5	6	7.0	
-	0.99a	5.1e	2.86c	4.26d	1.90b	2.98c	2.927b
1	0.72a	4.2d	2.0b	3.08c	1.93b	2.11b	2.355a
10	0.82a	5.03e	2.7c	3.34d	1.97b	2.78c	2.784b
50	1.10a	6.4e	3.72c	6.17e	4.34d	3.01b	4.139d
100	1.99a	6.52e	3.89d	3.34c	2.8b	2.78b	3.541c
500	2.2a	7.0e	4.16c	4.95d	3.2b	3.91c	4.242d
800	2.4a	6.91e	4.06c	5.05d	3.8b	4.25c	4.421e
1000	3.2a	7.92d	4.44b	5.3c	4.4b	5.59c	5.147f
Mean	1.653a	6.139e	3.476c	4.413d	3.057b	3.429c	3.695
F value	Metalaxyl F (5,96) = 2.558** $CaCl_2$ (F, 7,96) =780.5088* Metalaxyl * CaCl2 = F (35,96) =37.212**						

Table 4: Effect of $CaCl_2$ on peroxidase activity (µmoles/min.) in maize seedlings on 5th day of germination

Conc. of $CaCl_2$(µM)	Control	Concentration of metalaxyl (mg/g)				
		1.5	3.0	4.5	6	7.0
-	0.647a	3.18d	1.956c	3.70d	1.467b	1.82c
1	0.340a	2.91d	1.354c	2.60d	1.134c	0.64b
10	3.37c	1.02a	4.18d	5.75e	3.703c	2.43b
50	2.93d	1.25c	0.66b	0.137a	0.600b	2.68d
100	1.34b	1.51b	3.34c	1.508b	0.76a	4.325d
500	0.783a	4.734e	1.474b	2.84c	0.934a	3.145d
800	2.72b	5.303d	0.632a	7.81e	4.637c	4.68c
1000	3.95	2.99	1.56	6.37	3.169	5.71
Mean	2.01	2.80117	1.97433	4.06917	2.3005	3.82833
F value	Metalaxyl F (5,96) = 2.558** $CaCl_2$ (F, 7,96) =780.5088* Metalaxyl * CaCl2 = F (35,96) =37.212**					

Table 5: Effect of $CaCl_2$ on peroxidase activity (µmoles/min.) in maize seedlings on 7thday of germination

Conc. of $CaCl_2$(µM)	Control	Concentration of metalaxyl (mg/g)				
		1.5	3.0	4.5	6	7.0
-	6.007e	2.75b	1.780a	4.95d	3.90c	6.917f
1	3.99d	1.82b	1.14a	2.724c	4.29d	7.42e
10	5.55d	3.90b	2.011a	4.045c	5.85d	8.85e
50	6.52d	3.62b	2.84a	7.14e	5.58c	11.43f
100	7.35c	3.81b	3.24a	7.67d	7.53d	12.59e
500	8.619d	5.028b	4.15a	11.64e	7.54c	14.68f
800	10.93e	6.13b	4.31a	9.78d	8.94c	14.64f
1000	8.55c	5.604b	4.23a	10.73d	8.98c	15.07e
Mean	7.189d	4.082b	2.962a	7.334d	6.576c	11.449e
F value	Metalaxyl F (5,96) = 2.558** $CaCl_2$ (F, 7,96) =780.5088* Metalaxyl * CaCl2 = F (35,96) =37.212**					

A gradual decrease in the activity was observed (Table 6) with the increase in the concentration (250 µM to 1000µM) of metabolic inhibitor sodium meta bisulphite ($Na_2S_2O_5$). The inhibition had the same effect on the metalaxyl treated seedlings. Maximum decline in the peroxidase activity was observed with 3.0mg treated seedlings.

Table 6: Effect of sodium meta bisulphite on peroxidase activity (µmoles/min.) in maize seedlings on 3rd day of germination

Conc. of $Na_2S_2O_5$ (µM)	Control	Concentration of metalaxyl (mg/g)					Mean
		1.5	3.0	4.5	6	7.0	
-	0.99a	5.1c	2.86b	4.26b	1.90ab	2.98b	2.760d
250	0.508a	3.78d	0.596ab	0.80c	0.98c	0.824bc	1.270c
500	0.209a	2.73e	0.38ab	0.536cd	0.9d	0.54bc	0.904b
750	0.156a	1.91c	0.173a	0.114a	0.64b	0.374ab	0.608b
1000	0.119a	1.63b	0.11a	0.11a	0.3a	0.309a	0.519a
Mean	0.331a	3.027c	0.895b	1.077b	0.936b	1.007b	1.212
F value	Metalaxyl F (5,75) = 88.387** $Na_2S_2O_5$(F, 4,75) =111.778** Metalaxyl * $Na_2S_2O_5$ = F (20,75) = 5.233**						

3.6.5. Influence of $CaCl_2$ and EGTA on peroxidase activity

3.6.5.1. Peroxidase activity of maize in the presence of $CaCl_2$ and EGTA on 3rd, 5th and 7th day of germination

In order to determine whether the increased peroxidase activity in metalaxyl treated seedlings also require exogenous $CaCl_2$ was checked. The reaction mixture was preincubated with different concentrations of $CaCl_2$ (50 and 500 µM) with EGTA (25 and 50µM) and the peroxidase activity was determined (Table 7).

Inhibition (20-30%) of the activity was observed with the inclusion of 25 µM and 50 µM of EGTA. The activity could be restored with the further inclusion of $CaCl_2$ to the reaction mixture. Similar results were observed on 5th and 7th day as well (Table 8 and 9). of the presence of EGTA.

Table 7: Peroxidase activity (µmoles/min.) of maize in the presence of $CaCl_2$ and EGTA on 3rd day of germination

Conc. of $CaCl_2$ + EGTA (µM)	Control	Concentration of metalaxyl (mg/g)				
		1.5	3.0	4.5	6	7.0
0 µM $CaCl_2$ +25 µM EGTA	0.79a	4.2d	2.56c	2.5c	2.17b	2.1b
0 µM $CaCl_2$+ 50 µM EGTA	0.68a	3.88d	1.91c	1.4b	1.97c	2.0c
50 µM $CaCl_2$ + 50 µM EGTA	4.81d	5.60e	2.56b	3.5c	1.63a	3.18c
500 µM $CaCl_2$ + 50 µM EGTA	2.0a	6.59d	3.96b	4.20c	3.84b	2.37a
Mean	2.07a	5.06d	2.74c	2.9c	2.40b	2.41b
F value	Metalaxyl F (4,75) = 2.558** CaCl2+EGTA (F, 4,75) = 188.068* Metalaxyl * CaCl2+EGTA = F (20, 75) =16.351**					

Table 8: Peroxidase activity (µmoles/min.) of maize in the presence of $CaCl_2$ and EGTA on 5th day of germination:

Conc. of $CaCl_2$ + EGTA (µM)	Control	Concentration of metalaxyl (mg/g)				
		1.5	3.0	4.5	6	7.0
0 µM $CaCl_2$ +25 µM EGTA	0.238a	1.856d	1.565c	2.5e	0.233a	0.786b
0 µM $CaCl_2$+ 50 µM EGTA	0.179a	1.13c	0.48b	2.07d	0.128a	0.108a
50 µM $CaCl_2$ + 50 µM EGTA	1.23a	2.16c	1.805b	3.87d	1.801b	3.96d

500 µM $CaCl_2$ + 50 µM EGTA	3.032c	2.23b	3.55d	4.52e	0.667a	3.89d
Mean	1.169b	1.844c	1.85c	3.24	0.707a	2.186d
F value	Metalaxyl F (4,75) = 1.8328** CaCl2+EGTA (F, 4,75) =109.9713 * Metalaxyl * CaCl2+EGTA = F (20, 75) =10.997**					

Table 9: Peroxidase activity (µmoles/min.) of maize in the presence of $CaCl_2$ and EGTA on 7th day of germination

Conc.of $CaCl_2$ + EGTA (µM)	**Control**	**Concentration of metalaxyl (mg/g)**				
		1.5	**3.0**	**4.5**	**6**	**7.0**
0 µM $CaCl_2$ +25 µM EGTA	4.104c	2.46b	0.909a	4.87d	4.53d	7.80e
0 µM $CaCl_2$+ 50 µM EGTA	4.58d	2.17b	0.971a	4.78d	4.375c	2.80b
50 µM $CaCl_2$ + 50 µM EGTA	4.70b	3.27a	3.798b	11.22e	6.40d	3.13a
500 µM $CaCl_2$ + 50 µM EGTA	6.38c	3.41b	1.54a	9.57d	8.78d	12.59e
Mean	4.941c	2.82b	1.80a	7.61e	6.02d	6.58d
F value	Metalaxyl F (4,75) = 4.9640** CaCl2+EGTA (F, 4,75) = 63.56877* Metalaxyl * CaCl2+EGTA = F (20, 75) =29.78425**					

4. DISCUSSION

Influence of calcium on peroxidase activity:

Activity of peroxidase is under the strong influence of calcium ions. Some of the peroxidases also possess calmodulin binding domain. The calcium ions can switch the peroxidase between calcium and calmodulin domains (Christoph, 2012).

The results indicates that the low concentration i.e., 1 µM $CaCl_2$ was almost at par with the control, while towards higher concentration (10 µM -1mM) of $CaCl_2$ the activity was increased by 5-6 folds as compared to control.

Bhattacharjee (2009) reported the *Amaranthus lividus* seedlings grown under heat and chilling stress when treated with with Ca^{2+} expressed higher activities of defence enzymes including peroxidases and total thiol level than the untreated plants. Whereas reduced activity of all the antioxidant enzymes and total thio levels is observed upon EGTA treatment. With this work he suggests the significant role of Ca^{2+} signaling pathway in alleviating the oxidative stress induced by heat and chilling stress.

Liang C *et al* (2021) reported increased activities of SOD, CAT and POD isozymes in rice leaves under SAR stress on treatment with exogenous Ca^{2+}. Edel and Kudla (2016), Marcec *et al* (2019), Wang Ex *et al* (2021), Ghosh et al (2022), Shabbir *et al* (2022), Yejin Kim et al, (2024) have suggested the role of Ca2+ in second messenger and as a modulator in gene expressions.

A gradual decrease in the activity was observed with the increased concentration of metabolic inhibitor sodium meta bisulphite (250 µM to 1000µM). This confirms the induction of peroxidase enzyme in our study. The peroxidase activity in all the treatments could be effectively inhibited with the addition of EGTA. This decline in the activity could be restored by the inclusion of additional $CaCl_2$. These results confirm the induction of peroxidase under Metalaxyl treatment. Olusesan omidiji *et al.,* (2002) has reported that peroxidase activity in germinating S*orghum bicolor* was strongly inhibited by dithiothreitol and sodium meta bisulphite.

5. CONCLUSION

From the overall findings, the present study showed that Peroxidases were found to increase on 7th day of germination in both control and metalaxyl treated maize seedlings. Increased peroxidase activity is reported in germination as well as abiotic stress conditions. Therefore, growth stage and higher concentrations of fungicide are the reasons for increasing oxidative stress and that is correlated with increased defense enzyme on 3rd and 4th day of germination which decreases on further days of germination. This induced peroxidase like any other plant peroxidases found to be calcium dependant enzyme. Calcium

as activator of the peroxidase enzyme was proved by the addition of varying concentrations of $CaCl_2$ and the inhibition could be showed by adding metal chelating agent EGTA.

Since at higher concentration of calcium(1mM) used for the study could increase the activity of peroxidase on all the days of observation, a more direct effect of calcium has now been confirmed by the findings.

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