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Changes in the activity of Metabolic and Antioxidant Enzymes in Fungicide treated Maize seedlings

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ABSTRACT
The present investigation was aimed at finding out the enzymatic changes induced by the fungicide seed treatments in maize seedlings. Induction of various antioxidant enzymes and other defensive compounds is a common phenomenon in plants in response to different biotic and abiotic stresses. Hydrolytic enzymes play a set of roles in the biochemical mechanism of germination. The present study gives an insight into a protective effect of the system with an increased production of proline and decreased protease activity, and hydrolytic enzymes. At the same time, increased defense enzymes were observed during the later stages of germination for a particular concentration of metalaxyl. This may be due to the synthesis of novel proteins involved in the defense mechanism and regulation process. This indicates the dual role of metalaxyl. Further work is in progress in understanding the structural and functional aspects of these enzymes.

Keywords: Metalaxyl, germination, Peroxidase (POX), Polyphenol Oxidase (PPO), Catalase (CAT), Phenyl alanine ammonia lyase (PAL) α-amylase, β-amylase, Protease.

Introduction

Germination requires the metabolic energy for the synthesis of new protoplasm, structural components and the embryo development which is provided by the breakdown of the reserve food mainly carbohydrates, lipids and proteins. Germination increases the activity of number of enzymes (Fabiola Araceli Guzmán-Ortiz 2018). During pre-germination, starch is broken down by a cascade of enzymatic reactions to release required components for the germination process. Various new enzymes and the structural proteins are synthesized from the amino acids which are the products of the hydrolysis of the proteins by hydrolytic enzymes (Gepstin and Han,1980), while lipids play a role as a source of reduced carbon for the metabolism (Evelyn P, 1973). In cereals, the endosperm stores the starch and proteins required for germination (Olsen 2001).
Hydrolytic enzymes like amylases and proteases play an essential role for the breakdown of reserve starch and proteins which is not only essential for the emergence of radical but also reduces the dietary bulk and improves the digestibility of starch and protein (A.Negi.P 1996, K.Muntz, 1996, A.Kataria 1992). The level of monosaccharide is increased on germination by the activity of amylases (A. Akinlosottu and I.O.Akinyele, 1991), which is the source of energy and accompanied with an increase in oxygen consumption and also with the more palatability. Proteolytic enzymes play a central role in the biochemical mechanism of germination (Muntz K et al 2001).
Plants have evolved a multifaceted system of defense against pathogens, some of which are highly specific to a particular microbial species (Doehlemann G 2008). The host response typically involves a concerted series of events ranging from the rapid generation of reactive oxygen and nitrogen species, through changes in ion flux across the plasma membrane, proteolysis, and the reprogramming of hundreds of genes (Dangl J, Jones J 2001). High levels of antioxidant enzymes are found in number of plants to several abiotic stress conditions (Hussain, A *et al.,* 2020, Kusvuran, S., Kiran, S., & Ellialtioglu, S. S. 2016).
Prominent among the enzymes involved in reactive oxygen species scavenging are phenylalanine ammonia lyase (PAL), various peroxidases (POD) and various polyphenol oxidases (PPO) and catalase (Averyanov A, 2009, Kumar N, 2022). Increased levels of POD activity have been observed in conjunction with both local and systemic disease resistance. PPO catalyzes the oxygen dependent oxidation of phenolics to quinines (Hao Z *et al.,* 2012).
Metalaxyl is a systemic fungicide acts by suppressing sporangial formation, mycelia growth and establishment of new infection (Fisher DJ 1982). Much of our knowledge of reserve mobilization and its control processes during germination is very well understood. In contrast, available information on metalaxyl is limited to its effect on the pathogen and not much data is available on the effect of metalaxyl in metabolism and in defense processes of maize seeds. Hence, the present work is an attempt for furthering our knowledge towards better understanding of the effect of metalaxyl in germinating maize seeds focusing on the metabolic and defense related changes during germination.

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Material And Methods

Collection of seeds and seed treatment

Maize seeds were procured from VC farm, University of Agriculture Science, Mandya, Karnataka. All the chemicals were purchased from SLR and MERCK and the chemicals were of analytical grade. Seeds were surface sterilized with 0.1% mercuric chloride for 10 minutes and repeatedly washed with distilled water for 4-5 times. Seeds of uniform size were selected and soaked for 24 hours in distilled water (control) and with different concentrations (mg/g) of metalaxyl, 1.5, 3, 4.5, 6 and 7mg/gm of the seeds (1:5weight/volume) 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 both in light and dark condition. Uniform seedlings were selected and processed for further studies. Everyday filter paper was wetted with 10ml of distilled water (ISTA 2003).

Preparation of crude extract

Around 1-2 gram of seedling treated with different concentrations of the metalaxyl and the untreated seeds were taken each day, (up to 7 days) homogenized in ice cold saline (5ml) using pestle and mortar. The solution was centrifuged for 10 minutes at 10,000g and supernatant were used for further analysis.

Biochemical studies

Protein was estimated as described by Lowry *et al*. (1951) using BSA as standard. The activity of protease was determined following the procedure of Kunitz (1947). The activities of α-amylase (Bernfield, P.1951), β-amylase (Baun, L.C 1970) was determined in the extracts. Catalase activity and Phenyl alanine ammonia lyase (PAL) was determined spectrophotometrically (Aebi, H.,1984). Peroxidase activity was determined spectrophotometrically with guaiacol as the substrate by the modified method of Chance and Maehly (1955). Polyphenol oxidase activity was determined spectrophotometrically by using the method of Mayer *et al*. (1979) Total phenols was determined by Abbaspour H. 2012.

Statistical Analysis

The data are expressed as the mean ± SEM analyzed by one-way analysis of variance (ANOVA) and Dunnett's *t*-test was used as the test of significance. P value < 0.05 was considered as the minimum level of significance. All statistical tests were carried out using SPSS statistical software.

Results and Discussion

Activity of Protease

The specific activities of protease during germination of maize seeds treated with Metalaxyl are shown in Table 1. Both control and treated seeds showed increasing specific activity up to 3rd day of germination and decreased then onwards.
The specific activity was decreased by 46%, 81%, 88%, 97%, and 100% with 1.5mg, 3mg, 4.5mg, 6mg and 7mg concentration of metalaxyl on 3rd day of germination.
When compared to the control. Increasing in proteolytic activity with concomitant reserve protein depletion agrees with the findings of earlier studies on other seeds; *Phaseolus vulgaris* (Abbaspour H. 2012), *Lupinus albus* (Ferreira RB 1995), *Vicia sativa* (Schlereth A 2001) and *Macrotyloma uniflorum* (Rajeswari J 2003). The result from our study is not parallel with the above data as there is a dose dependent inhibition of protease activity in metalaxyl treated maize seeds on 3rd day of germination. Impairment of proteasome functionality and decreased protease activities seems to be a common feature involved in metal toxicity in plants (Wang C 2010, Martinez M *et al.,* 2019).

Table 1: Effect of metalaxyl on the specific activity of protease (10^{-4}mM of tyrosine liberated /mg of protein/min.) in the germinating maize seedlings

Germination in days	Control	Concentration of metalaxyl(mg/g)					Mean
		1.5	3	4.5	6	7	
0	0.3ab	0.3ab	0.2ab	0.2a	0.1a	0.4b	0.242a
1	0.76a	0.6a	0.7a	0.65a	0.64a	0.79a	0.673b
2	2.5c	1.5b	0.51a	0.62a	0.364a	0.4a	0.982c
3	5.0c	2.7b	0.91a	0.6a	0.164a	0.03a	1.527d
4	3.0c	1.8b	0.63a	0.43a	0.1a	0.7a	1.144c
5	0.2ab	0.73c	0.25ab	0.28b	0.13a	0.11ab	0.283a
6	0.16a	0.4b	0.23b	0.13a	0.16a	0.15a	0.186a
7	0.12ab	0.1a	0.2ab	0.15b	0.18ab	0.19ab	0.159a
Mean	1.548d	0.963c	0.453b	0.388ab	0.214a	0.329ab	0.649
F value	Concentrations F (5,95) = 121.598**, Days F (7, 96) = 93.994** Concentration * Days = F (35, 96) = 32.773**						

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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

Activity Amylase (α and β):

α Amylase activity and β-Amylase activity (Figure 1a and 1b) showed increased activity during germination in control seedlings by 18 & 10 folds. The amylolytic breakdown of starch is one of the central biochemical events in the germination and principal enzyme is amylase. Several fold increased activities of amylase during the seed germination have been reported (Mohamed *et al.,* 2009).

Amylase activity increased under lower doses and decreased under higher doses compared to the control. The maximum activity was observed at 4.5mg/g on 3rd of germination.

With the increase in days of germination, the enzyme activities were also increased up to 3-5-days further increase in the germination period decreases in the enzyme activity was noticed. As per the observation made by the Prasad and Mathur (1983), the amylase activity increased during germination in both control, and Cuman-1 treated seeds. The treatment of pesticides may impose an osmotic stress causing damage to membrane structure (Sari, Afrima 2021, Nijabat 2023).

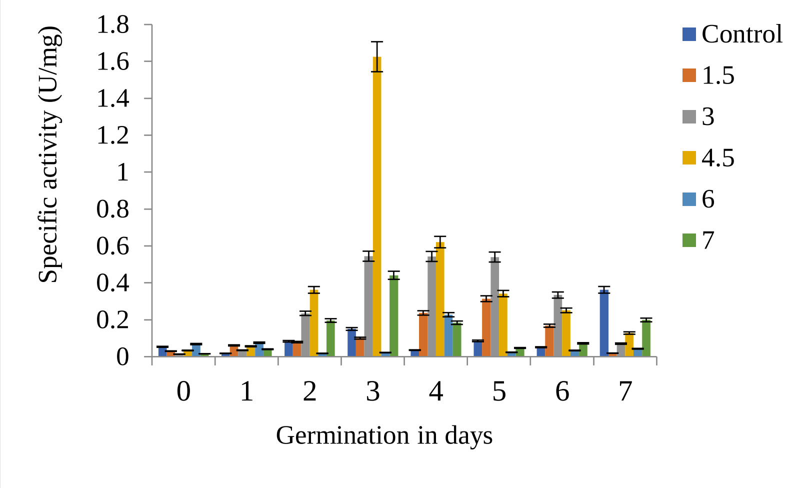

Figure 1a – Effect of metalaxyl on the specific activity of α - Amylase in germinating maize seedlings

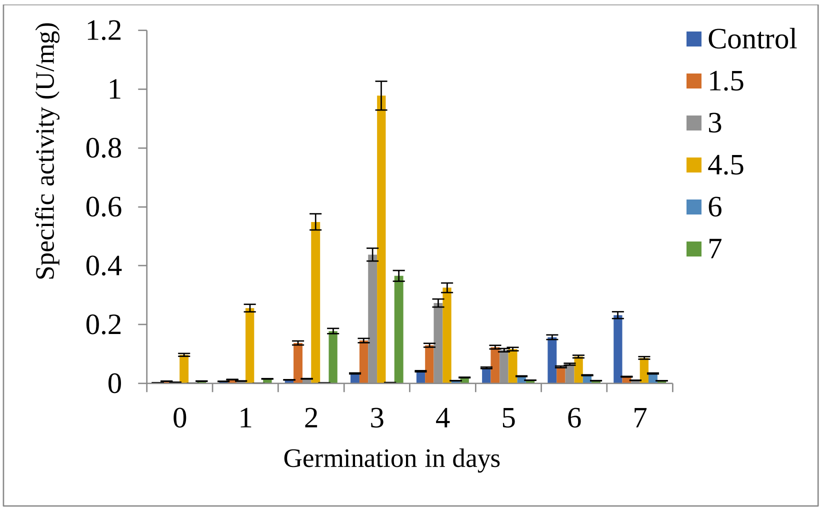

Figure 1b: Effect of metalaxyl on the specific activity of β - Amylase in germinating maize seedlings

Activity of Invertase

A gradual decline in the invertase activity was observed in the control seeds till 3rd day of germination and further increase in the activity was seen on subsequent days (Figure 2) The seeds treated with 7mg/g of fungicide showed an enhanced activity (0.1193U/mg) of invertase on 5th day. Maximum activity of invertase was observed in both control and fungicide treated seeds on 5th day of germination.

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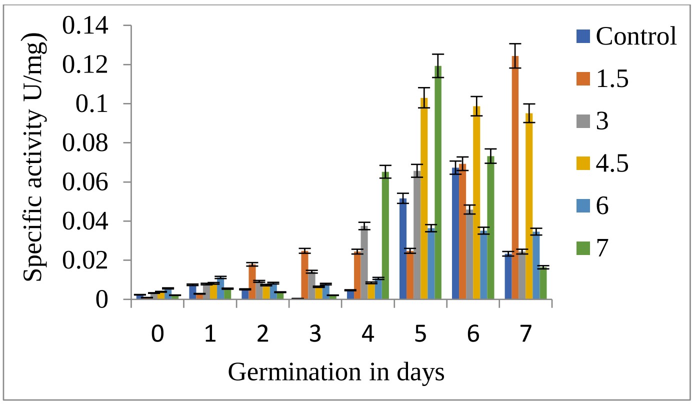

Figure 2: Effect of metalaxyl on the specific activity of Invertase in germinating maize seedlings

Invertase hydrolyzes sucrose into glucose and fructose and plays a major role in plant development and in response to biotic and abiotic stresses (Sturm, 1999, Essmann *et al*., 2008). A review of plant invertases by Fotopoulos (2005) suggests the role of invertases in various abiotic stress factors like low temperature, oxygen deficiency, wounding, drought and salinity.

Our investigation showed a gradual decline in the invertase activity in control seedlings till 3rd day and further increase on subsequent days of germination. Acid invertase increased with the increase in concentration of chromium in the radicle and plumule of the tested pea cultivars (Surekha and Duhan, 2012). On the contrary Dua and Sawhney (1991) report a decrease in the invertase activity with the increasing dose of chromium treatment. Jha and Dubey (2005) also reported an increase in acid invertase activity in endosperm as well as in embryonic axis under arsenic treatment in rice seedlings.

Changes in invertase activity have also been reported to correlate with reducing sugar content in maize (Trouverie *et al.,* 2003) and in chickpea (Kaur *et al.*, 2003) in water and salt stress respectively. The induction of invertase by abiotic stimuli supports the suggestion that invertase is an important component of stress response (Meenu and Sharma, 2005). With the above observations, enhancement in the invertase activity can be attributed to its role in alleviating the stress response in abiotic stress conditions.

Increased invertase activity observed on 6th day can be due to the increased requirement of carbon metabolism with growth of the plant during germination and the seeds treated 7mg/g of fungicide showed an enhanced activity of invertase on 5th day, which can be also due to stress response of the plants under high concentrations of metalaxyl.

Activity of Peroxidase

The maximum Peroxidase activity could be seen on 3rd day of germination in both treated and untreated seeds (Figure 3). This activity decline till the 6th day of germination and a marginal increase could be seen on the 7th day of germination. When compared to control 1-1.5fold of increase in activity was observed with the different concentration of fungicide treatment and nearly 2 folds of activity were observed with 6mM of fungicide treatment. An enormous increased activity of peroxidase was seen in the seeds challenged with highest (10 folds) concentration of fungicide (7mM) on seventh day of germination.

Peroxidase activity or Peroxidase gene expression in higher plants is, induced by fungi, bacteria, viruses, and viroids (Gurupreet Singh and Daljit Kaur 2016). Peroxidase oxidizes phenolic to highly toxic quinones hence has been assigned a role in disease resistance (Hammerschmidt, R 1982). This clearly shows increase in the peroxidase activity is a mean of protection from stress and our results are in parallel.

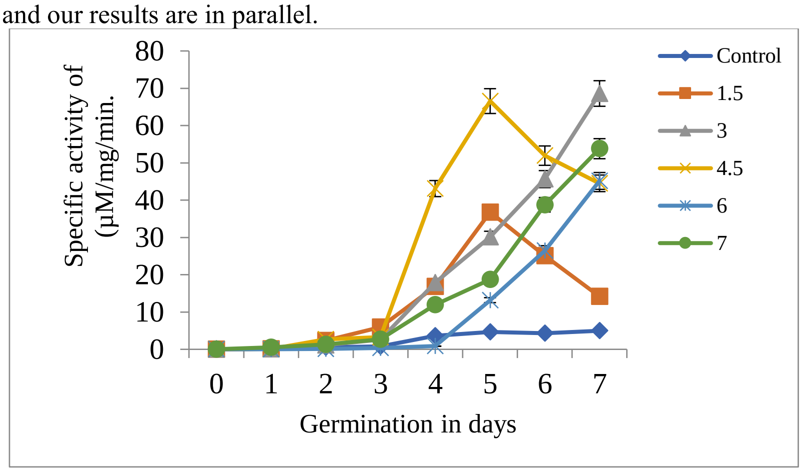

Figure 3: Effect of metalaxyl on the specific activity of Peroxidase in germinating maize seedlings

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Activity of Catalase:
A basal activity of Catalase (Figure 4) was observed on the first day of germination which increased gradually and a maximum activity nearly 6-8 folds on the third day of germination. However, when the seeds were treated with the different concentration of metalaxyl, the higher catalase activity was seen in 4.5, 6, and7 mg/g. In the other concentration a basal activity was seen on the first day peaked to maximum on the third day of germination. When compared, a maximum activity of 1mM/mg of protein/minute was obtained on the third day with 6mM concentration which was almost the double than that of control seeds. CAT activity exhibited a significant increase in the treated seeds comparatively to the untreated seeds.

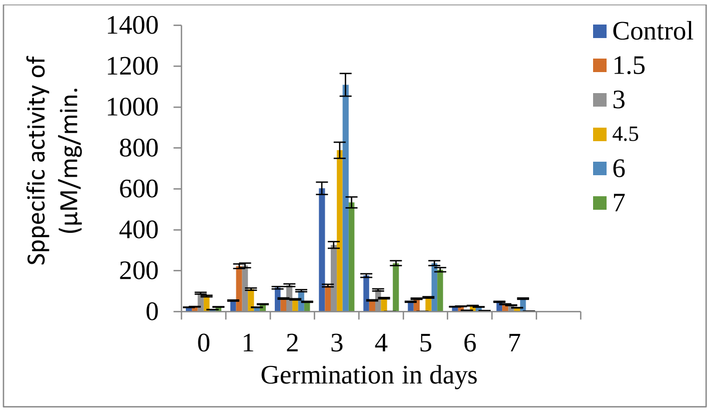

Figure 4: Effect of Metalaxyl on the specific activity of catalase in germinating maize seedlings

A positive relationship occurs as there is a dose dependent increase in the catalase activity. In our studies also we have shown when the seeds were treated with the fungicide which is also a stress to the plant there was drastic increase in the catalase activity on the third and fourth day of germination. Catalase activity differ in different concentration of fungicide treatment and we could get the maximum activity on third day with 6mM concentration and least activity on first day of germination. This clearly suggests the protective mechanism varies and it depends on stress level and corresponds to their tolerance towards the stress (Mhamdi et al 2010, Franklin Aragao Gondim 2012, Leung, DWM 2018, Jablonkai, I. 2022). Hence this result can also be used as a practical biochemical indicator for the selection of fungicide treatment at seedling stage.

Activity of Polyphenol oxidase:
In the case of control, a basal activity is seen on the 7th day of germination (Figure 5) and a maximum activity nearly 0 - 0.2 folds on the 3rd day of germination. However, when the seeds were treated with the fungicide the PPO activity triggered on 3rd day with a gradual decline till the 7th day. The activity was maximum i.e., 220µmoles/mg/minute with 7mg concentration on the 3rd day of germination. In all other concentrations a basal activity which was seen on 1st day peaked to maximum on the 3rd day of germination.

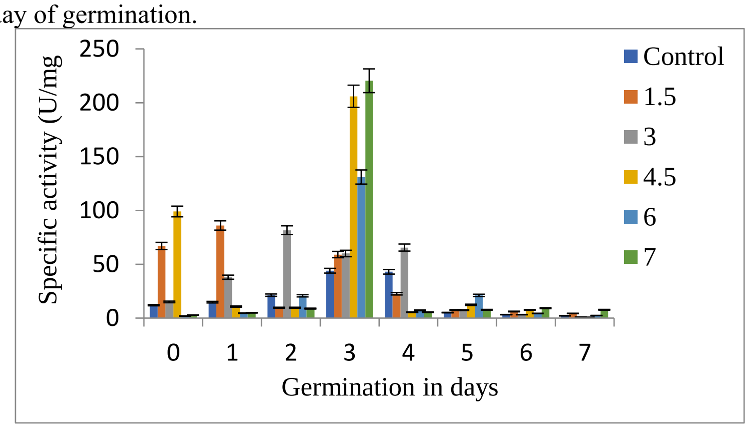

Figure 5: Effect of metalaxyl on the specific activity of Polyphenol oxidase in germinating maize seedlings

In the case of control, a basal activity is seen on the 7th day of germination and a maximum activity nearly 0 - 0.2 folds on the 3rd day of germination. However, when the seeds were treated with the fungicide the PPO activity triggered on 3rd day with a gradual decline till the 7th day. The activity was maximum i.e., 220µmoles/mg/minute with 7mg concentration on the 3rd day of germination. In all other concentrations a basal activity which was seen on 1st day peaked to maximum on the 3rd day of germination.

PPOs are a group of copper containing enzymes that catalyze oxidation of hydroxy phenols to their Quinone derivatives, which have antimicrobial activity. Because of its reaction products and wound inducibility, PPO plays a role in defense against plant pathogens. Plants immediately respond to pathogen so there is immediate rise in PPO indicating immediate synthesis of antimicrobials to ward off pathogen. Increasing in the oxidizing enzyme particularly polyphenol oxidase (PPO) has a tremendous impact on host physiology and predominantly genes responsible for the resistant in cultivars (Zhang J, Sun X 2021)

Activity of Phenylalanine Ammonia Lyase:
PAL is the first enzyme of Phenyl propanoid metabolism in higher plants and has been suggested to play a significant role in regulating the accumulation of phenolics (Massala, A., 1980). Our study has a similar co relation and a maximum activity was seen with 4.5 and 7mM concentration in the treated germinating seedlings on third day and fifth day (Figure 6). The host defense responses are trigger during the pathogens and the activation of induced resistance may be co-related with polyphenol oxidase and Phenyl alanine ammonia lyase activity.
A gradual increase in the phenylalanine ammonia lyase is seen during the different days of germination. A maximum activity of 14.62µmoles/mg/hour was seen on the 5th day of germination with 1.5mg metalaxyl treated seedlings followed by 12.64µmoles/mg/hour on 3rd day of germination in 6mg of metalaxyl treated seedlings. A gradual decrease was seen till the 7th day of germination in both control and metalaxyl treated seedlings on 6th and 7th day of germination. The activity was found to be decreased with the increasing concentration of fungicide. A maximum inhibition was seen with the highest concentration i.e.,7mg treated seedlings, only 34% of the phenylalanine ammonia lyase activity was observed in that concentration (1.26 µmoles/mg/hour) compared to the control (3.69 µmoles/mg/hour).

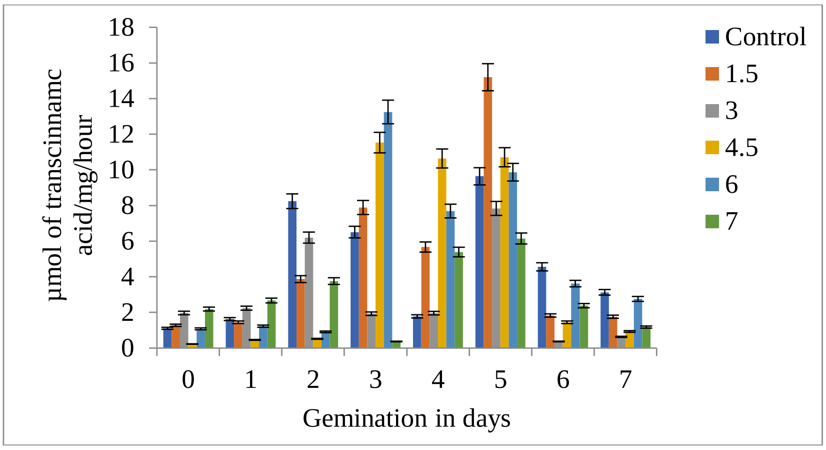

Figure 6: Effect of metalaxyl on the specific activity of Phenylalanine Ammonia Lyase in germinating maize seedlings

Total Phenolic content:
The result of total phenolic content in control and metalaxyl treated seedlings on different days of germination are shown in the Table 2. When compared to control for the 1st day, a gradual increase in the phenolic content was observed in the treated sample till the 5th day and a gradual decline on the 7th day of germination. The maximum activity was found in 6mg treated seedlings on the 5th day of germination. A dose dependent increase in the phenolic content was observed for all the concentration except with the 7mg of metalaxyl which is the highest concentration in the study. A gradual increase in the phenolic content was observed with the increasing concentration of fungicide and was highest (150mg/g) on 6mg concentration on 5th day of germination. Almost a 10 folds increase in the total phenols was observed after the seeds were challenged with fungicides.
This structural diversity allows phenolic compounds to fulfil a wide range of biological functions within plants (Divekar *et al.,* 2022). Various biotic and abiotic factors influence the synthesis of phenolics (Shomali A 2022). Prominent among the factors that directly influence phenolic metabolism are pesticides and herbicides (Molina *et al.,* 1998). Such large difference in the phenolic content in different species studied can be attributed to differences in abiotic stresses, e.g. type of stress, its intensity, its duration, the stages of plant development and the biological material which are characterized by a great diversity of secondary metabolites (Weidner *et al.,* 2009). The enhanced accumulation of phenolic compounds may be due to increased activity of PAL and PO enzymes which cause an increase in the available phenolic free pool (Jayaraman *et al.,* 2011)
Some phenolic acts as a signaling molecules or antioxidant and thus induce resistance (Burda, S., 1990). This was very much co-related in our results where a gradual increase in the phenolic content was observed with the increasing concentration of fungicide and was maximum on 6mM concentration on fifth day almost a 10th fold increase in the total phenols was observed after the seeds were challenged with fungicides.

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Table 2: Effect of metalaxyl on total phenolic content(mg/g) in germinating maize seedlings

Germination in days	Control	Concentration of metalaxyl (mg/g)					Mean
		1.5	**3**	**4.5**	**6**	**7**	
0	77d	20b	10a	20b	29c	10a	27.61a
1	99e	26c	20b	27c	39d	17a	38.00b
2	60d	28c	10a	19b	29c	10a	26.00a
3	43b	53d	59e	48c	35a	73f	51.83c
4	39c	35b	40c	39c	29a	56d	39.66b
5	130bcd	100a	110ab	140cd	150d	120abc	125.0e
6	100b	60a	100b	70a	100b	60a	81.66d
7	59c	69e	19b	14a	62d	71e	49.00c
Mean	75.87d	48.87ab	46.0a	47.1a	59.1c	52.1b	54.84
F value	Concentrations F (5,96) = 129.759**, Days F (7, 96) = 842.735** Concentration * Days = F (35, 96) = 46.701**						

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 $P \leq 0.01$, *significant at ≤ 0.05

Conclusion

The present study gives an insight in understanding the effect of metalaxyl on metabolic enzymes-protease giving a protective effect to the system. α Amylase and β-Amylase activity were increased suggesting a stress in the treated seeds. In our studies a significant enhancement of peroxidase, catalase, polyphenol oxidase, and phenolic content was observed in a dose dependent manner with different concentration of fungicide treated maize seeds during germination. The data presented here confirmed the induction of defense related enzyme during the fungicide stress. A correlation is seen between the tolerance mechanism and the efficiency of antioxidant system and total phenolic content The assessment of this enzyme in control and fungicide is important for understanding the defense mechanism operating in normal germination and in the stress condition.

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