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Chemical stress induced changes in carbohydrate content and the enzymes of carbohydrate metabolism in germinating maize seedlings

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Abstract
In the present work, the effect of different concentrations of Metalaxyl, a systemic fungicide, on the carbohydrate content and the enzymes of carbohydrate metabolism during early germination (0-7 days) of maize seedlings is studied. In our study α–amylase activity increased progressively in control seeds till 7th day of germination. Maximum activity of α–Amylase with nearly 100 fold increase was observed with 4.5mg/g treated seeds on 3rd day of germination with a gradual decline. Similar pattern of activity was observed with β-amylase. A gradual decline in the invertase activity was seen in control seeds till 3rd day and further increase on subsequent days. The seeds treated 4.5mg/g of fungicide showed an enhanced activity of invertase on 3rd day. Maximum activity of invertase was observed in both control and fungicide treated seeds. Similar pattern could also be observed with the total carbohydrates, reducing and non-reducing sugars on different days of germination in control and treated seeds. Further work is in progress to understand the mechanism of the interaction of metalaxyl in carbohydrate metabolism, during germination.

Keywords: Metalaxyl, germination, αand β-Amylases, invertase, total carbohydrates.

Introduction
Maize is extensively cultivated crop in India for its nutritive value and use as a fodder. It is subject to a wide range of pathogens of which fungi is the major cause of diseases in maize (Shurtleff 1980) [49] which in turn affects the yield and economy of the country. Therefore efforts are needed to enhance the productivity of maize.
Pre-sowing seed treatment with pesticides is an efficient, economic means of plant protection (Rotrekl, J., Cejtcham, 2008) [41]. The plant absorbs a certain amount of pesticides that changes the plant's metabolism (Koehle, H., *et al* 2002) [27] but the rate of change varies from crop to crop. The external morphology of development is often marked by biochemical changes of seed reserves and enzymes of the internal tissues and is considered as markers of growth and development. During seed germination and seedling growth, the seed reserve gets hydrolyzed and a change in the cellular and organelle constituents such as proteins, lipids and carbohydrates takes place (Rangwala Tasneem *et al* 2013) [39-40].
Hence the present study was aimed at evaluation of the effect of the systemic fungicide Metalaxyl with different concentration on seed reserves carbohydrates contents *viz*. reducing sugar, total soluble sugar, starch, total carbohydrates, and the activity of the enzymes; α amylase, β amylase and invertase during different days of germination (0-7 days).

Materials and Methods
Collection of seeds and treatments
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(1:5 weight/volume) for 24hours. Five seeds in triplicate were placed on petridish with 8-10 layer of soaked filter paper and incubated at 25^{0}c 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.

Extraction and estimation of starch and total carbohydrate:
Extraction of starch and total carbohydrate was carried out as described by Sadasivam and Manickam (2008) [42] and it was estimated by Anthrone method (Clegg KM.1956) [14]

Extraction and estimation of reducing sugars:
Sugars were extracted twice with 80% ethanol at 90^{o}C followed by 4 times extraction with 70% ethanol as described in Gill *et al* (Gill PK *et al* 2003) [19] from this extract the reducing sugars were quantitatively estimated by DNS method (Miller, G.L. 1959) [33]

Extraction of α amylase and determination of activity
The extraction of α -amylase was carried out by following Gupta *et al* (1993) [22, 26] using 50 mM calcium acetate (pH 6.0) and 50 mM sodium acetate (3.6) extracting buffers respectively. Amylases were extracted by crushing 0.1 g of tissue in a pestle and mortar with 50 mM calcium acetate (pH 6.0). The mixture was kept for 1 hour at room temperature and centrifuged at 10.000 rpm for 10 min. The supernatant was heated at 70^{o}C for 20 min to inactivate the β-amylase and then cool to 0^{o}C. The precipitates were removed by centrifugation at 10,000rpm for 10 min and supernatant was used for measuring the α-amylase activity.

Extraction of β-amylase and determination of activity

The extraction of β-amylase was carried out by following Gupta *et al* (1993) [22, 26]. The tissues were homogenized in 50 mM sodium acetate (pH 3.6) containing 0.1 mM EDTA. The suspension was left for 1 h at 0ºC, centrifuged and the clear supernatant assayed for β-amylase. The amylase activities were assayed by measuring the reducing sugars released as described in Sawheny and Singh (2000) One unit (U) of amylase (α and β) is equivalent to the amount of enzyme liberating µmole of product per min under assay conditions.

Extraction of Invertase and determination of activity

The invertase was extracted from the seedling following the method of Sawhney and Singh (2000) by grinding the tissue in mortal and pestle at 0-4^{0}C using sodium acetate buffer pH 4.5. The homogenate was centrifuged at 12000rpm for 15 min. and the supernatant was collected. Invertase activity was assayed by measuring the reducing sugar released. One unit (U) of invertase is equivalent to the amount of enzyme liberating I µmole of product per min.under assay condition.

Statistical Analysis

The data obtained were subjected to analysis of variance using SPSS package version 20.0. The data are expressed as the mean analyzed by two way analysis of variance (ANOVA) and Scheffee was used as the test of significance.

Results and Discussions

Use of systemic fungicides caused a significant decrease in protein and carbohydrate content as compared to the control (Siddiqui Z.S. and Ahmed 2002) [50]. In earlier research, Gill and Singh (1985) [20] has reported that the germination, growth, respiration and other related processes could be affected in seeds that are subjected to environmental stresses. Changes in any one of these processes can affect other metabolic activities, particularly the carbohydrate metabolism that plays an important role in germination and seed development.

However there are few studies on the modulator effect of fungicide on the carbohydrate metabolism and the related changes in the germinating maize seeds by physical or chemical stress as reported in our previous study on protein metabolism (Shobha. N *et al* 2014) [48]. In this study we present a detail of the changes in carbohydrate content in correlation with amylase and invertase activity during early seed germination on treating with different concentration of metalaxyl.

Total soluble sugars

Sugars are the primary photosynthetic products which form the building blocks for all other chemical constituents of the plants. The growth and development depend upon the availability of carbohydrates in the conductive system of plant. Sugars are the source of food for plant cells broken down chemically by respiration to supply energy for all plant functions. Nutrient availability, water supply and carbon dioxide, temperature, sunlight, and the presence of toxic substances influence the rate of photosynthesis (Sances *et al* 1982) [44]

It has been found that metalaxyl increases the total sugars in germinating seed and it was maximum on 3rd day with all the concentration and gradually declined on the 7th day of germination. Total sugar content of maize seedling in control was 0.552mg/g on 1st day of germination which gradually declined by 7th day and it was 0.216mg/g. when the seeds were treated with different concentration of metalaxyl it was found to be 0.277mg, 0.438mg, 0.1mg, 0.402mg and 0.298mg with 1.5, 3, 4.5 6 and 7 mg/g of metalaxyl respectively on first day of germination as shown in Figure-1. The percentage of decrease in total soluble sugar was 50%, 20%, 55%, 20%and 30% as compared to control. The highest percentage of decrease was with 1.5mg of metalaxyl on 1st day of germination. With the progress of germination similar decline was observed with different concentration of metalaxyl while maximum decline was observed with 1.5mg, 3mg and 4.5mg. Not much variation was observed on 7th day of germination with the highest concentration of metalaxyl.

Similar to these results, Kamble and Sabale (2002) [25] found total sugar content in *Trigonella* seeds increased with increasing concentrations of Carbandazim while Monocrotophos decreased total sugar with increasing concentrations. However, Santhaguru *et al* (1990) reported reduction in soluble sugars in *Cyamopsis tetragonoloba* L (taub.) with increasing concentration of rogor. Taylorson (1966) [53] found that high rates of diphenamid decreased the total sugars content in freshly seeded tomatoes. In present investigation, the metalaxyl decreases the total sugar content with its increasing concentrations suggesting that metalaxyl adversely affect the metabolic enzymes and inhibits the functioning of the photosynthetic enzymes. With this result the sugar contents in germinating seeds is directly related to stress factor.

Reducing sugar

The α- amylases which are found virtually in all living cells cleave the - α D-(1-4) linkages at random and bring about conversion of the starch molecule into the reducing sugars (Chow, KW, and Halver, JE 1980) [13].

As compared with the control a significant decrease in the reducing sugar was observed till 5th day of germination. However an increase in reducing sugar was observed on 6th and 7th day of germination with the metalaxyl concentration of 3mg and 7mg/g. As shown in Figure-2 reducing sugar content of maize seedling in control was 0.0047mg and with 7mg treated metalaxyl it was 0.2045mg however with other treated seedlings there was a marked decrease in the reducing sugar to nearly by 80 – 90% on 1st day of germination. High concentration of reducing sugar was observed with the correlation of low starch content with 7mg treated seedlings on the 7th day of germination.

There are few reports available which described effect of pesticides on reducing sugar content in germinating seeds. These include positive as well as negative influence of pesticides. Prasad and Mathur (1983) [38] noticed considerable decrease in reducing sugar in *Vigna mungo* L. with treatment of metasystox and Dalvi *et al.* (1972) also reported a decrease in the amount of reducing sugar in wheat and mung bean seeds treated with menazon. Chopra and Nandra (1969) reported a decrease in formation of reducing sugar in germinating mustard seeds while treated with thiomenton. But carbandazium seed treatment in some plant did not effect on reducing sugar content (Chandra G *et al* 1983) [10]

The decrease in the reducing sugar content on 5th day of metalaxyl treated geminating seeds might be due to some enzymatic changes which are responsible for the conversion of starch to some reducing sugars (Chauhan *et al* 2013). An increased level of reducing sugar in seed may be due to its nonconversion to non-reducing sugar (Elloumi *et al* 2005). This might be mechanism adopted by germinating seed to reduce the effect of the fungicide stress (Gupta *et al* 2009) [22, 26].

Total carbohydrates

Carbohydrates represent a broad group of substances which include the sugars, starches, gums and celluloses (Cho and Halver 1980) Carbohydrate contents were significantly influenced by the treatment of fungicide (Avinash and Hoshamani 2012)

High concentration of total carbohydrate on 1st and 3rd day of germination (1.229 ± 0.03) was observed in control which gradually declined on 7th day of germination. Same correlation was observed in total soluble sugars, starch and reducing sugar was observed in treated seedlings on different days of germination. Least amount of the total carbohydrate was found on 7th day with 1.5mg, 3mg and 4.5mg treated seedlings on 7th day while not much decrease was observed with the highest concentration.

Similar to our results, Avinash *et al*. (2012) and positive increase in 0.1% and 0.3% carbendazim, whereas in the 0.2% concentration the carbohydrate content decreased considerably in jowar. There are few other reports describes adverse effect of pesticides on total carbohydrates content in germinating seeds. A study on *Hibiscus esculantus* and *Capsicum annum* by Ahmed and Siddiqui (1995) reported an increase in carbohydrate contents in the seeds treated with benlate fungicide. Siddiqui and Ahmed (2001) studied significant decrease in total carbohydrate content after the application of systemic fungicide in two varieties of wheat. Bhattacharya *et al*., (2001*)* investigated the effect of Carbofuran, Butachlor and Carbendazim treatments on carbohydrate contents of two summer rice cultivars and found that fungicide Carbendazim application at panicle emergence of rice produced a decreasing trend in carbohydrate content. A similar result was reported by Pablo *et al* (2002) in Carbendazim treated tobacco plants.

Starch

The principal storage carbohydrate in plants is the polysaccharide, starch. The breakdown of starch to readily utilizable sugar under amylase activity is essential for the growth of seedling (Sengupta 1988). During early seedling growth, starch was mobilized from the endosperm to the embryo, and as a result, the starch content of the endosperm decreased steadily and the starch content of the embryo increased during the first few days of seedling growth (Tonguc *et al* 2012). During these metabolic events ATP and carbon skeleton are provided for anabolic reactions essential in supporting the embryonic plant until it attains an autotrophic condition (Noggle, G. Ray and George Fritz 1992)

In our study the starch content remained almost the same with different concentration of metalaxyl treated seedlings as compared with the control on 1st day of germination. However on the 3rd day of germination nearly 80% of decline in starch was observed with 4.5mg/g treated seedlings and similar results were also found on 7th day of germination. With the highest concentration a gradual decline of starch was observed from the 1st day to the 7th day of germination. On the 7th day of germination and it was least (0.017) with the same concentration. Our results showed the metalaxyl was inhibitory at its higher concentration on the gradual days of germination. The breakdown of starch with metalaxyl treatment correlated with increased amylase activity during seed germination which suggests that amylase activity was influenced by metalaxyl with decrease in starch content. According to Misal and Sabale (1992) the amylase activity is increases or decreases in pesticide stress.

α-Amylase

α- Amylase is important enzyme synthesized during seed germination (Briggs 1972). This enzyme is abundant in the germinating cereals and catalyses a random hydrolysis of α-1, 4 glycosidic linkage in the starch component. The effect of Metalaxyl on specific activity of α-Amylase in germinating seeds of maize was studied in present work and results are depicted in Table-1.

The enzyme α-amylase activity in control was found to be 0.536, 1.788, 15.02, 85.65, 362.36 µmol from 0-7th day of germination. When the seeds were treated with different concentrations of metalaxyl an increase in the α-Amylase was observed but the gradation with the concentration. The order of decreasing activity was with 7mg (15.75µmol), 3mg (7.93 µmol) 6 (6.83 µmol) 4.5(3.38 µmol) and 1.5 (3.01 µmol) on the 0 day of germination. The increase in the specific activity of α-Amylase was dramatic till the 7th day in the control while a maximum activity in treated seedling was found on 3rd day which further declined on subsequent days of germination.

Santhguru and Thamizhchelvan (1990) reported that, the lower concentration of rogor significantly increased the α-amylase activity in germinating seeds of *Cyamopsis tetragonoloba* and similar results were also found by Mathur *et al*. (1982) in *Vigna mungo*. On the other hand Prasad *et al*. (1980) [37] noticed the decreased activity of enzyme amylase in the germinating seeds of *Vigna mungo* by the influence of Metasystox and Cuman-L. The α-amylase activity was enhanced by prolonged darkness (Saeed & Duke, 1990) [43], leaf infection with virus (Heits *et al*., 1991) [24], heat stress (Commuri & Duke, 1997) [17] and water stress (Ashraf *et al*., 1995) [3]. Similar results found in our study suggested that increased activity of amylases may play an important role in stress management.

β-Amylase

The specific activity of β-Amylase is given in Table-2.The increase in the specific activity of β-Amylase was observed in the control while a maximum activity in treated seedling was found on 3rd day which further declined on subsequent days of germination. As observed in α-Amylase activity even the β-Amylase activity was higher on 3rd day with 4.5mg treated seedlings and with all the treated seedlings compared to the control there was an increase in the specific activity which varied from 10-80%. However with the highest concentration minimum activity was observed on 7th day (8.95 µmol) of germination.

Invertase

Carbohydrate is translocated in the maize plant as sucrose (LoowIms, W. E 1945) [29]. The initial reaction in the utilization of sucrose is generally believed to be the invertase-catalyzed hydrolysis of it to glucose and fructose. Invertase (D-fructofuranoside hydrolase, E. C.3.2.1.26) exists in several forms in plant tissues. (T. A. Jaynes 1971) [52].

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

Changes in invertase activity have also been reported to correlate with reducing sugar content in maize (Trouverie *et al.*, 2003) [55] and in chickpea (Kaur *et al.*, 2003) [26].The induction of invertase by a biotic stimuli supports the suggestion that invertase is an important component of stress response (Meenu Thakur and Arun Dev Sharma 2005) [32]

Conclusion

In the present investigation, the use of different concentration of fungicide induced changes in total carbohydrate content and the enzymes of the carbohydrate metabolism, on different days of germination. The noticeable changes might be due to alleviation in carbohydrate metabolic pathway. This may be due to an adaptation to environmental stress which was different on different concentration of fungicide and on different day of germination.

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Figures and Tables

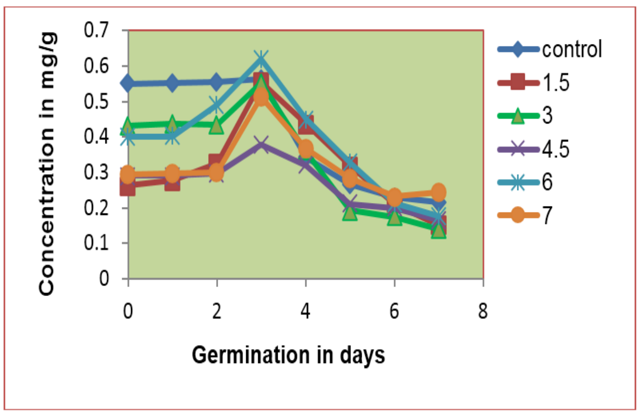

Fig 1: Effect of Metalaxyl on the Total soluble sugar (mg/g)) in the Seedlings of maize

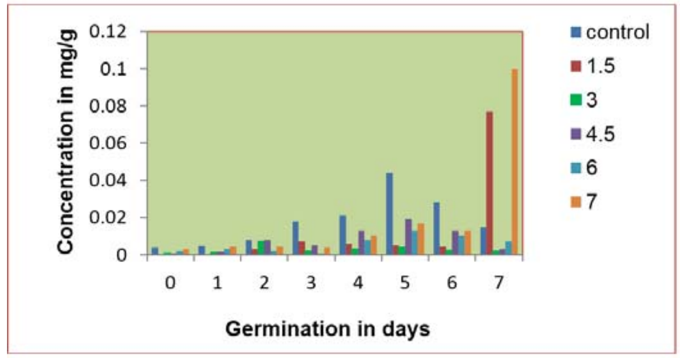

Fig 2: Effect of Metalaxyl on the Reducing sugar (mg/g)) in the Seedlings of maize

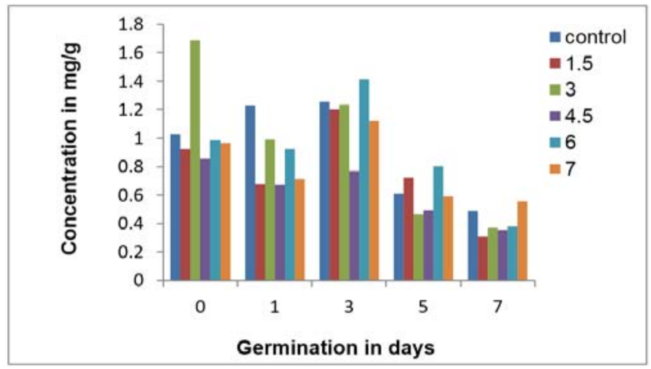

Fig 3: Effect of Metalaxyl on the Total carbohydrate (mg/g)) in the Seedlings of maize

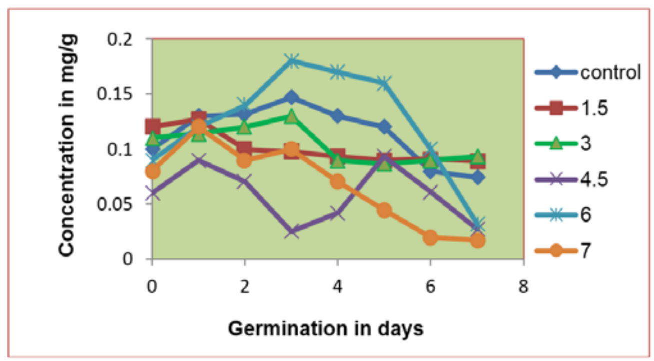

Fig 4: Effect of Metalaxyl on Starch (mg/g)) in the Seedlings of maize

Table 1: Effect of Metalaxyl on the Specific activity (μmol/g/min) of α- Amylase during germination of Maize

Seedling age in days	Concentration of fungicide in mg/g					
	control	1.5	3	4.5	6	7
0	0.536 ± 0.03	3.01 ± 0.07	7.93 ± 0.08	3.38 ± 0.04	6.83 ± 0.08	15.75 ± 1.3
1	1.788 ± 0.07	6.12 ± 0.43	17.49 ± 2.1	7.63 ± 0.08	15.65 ± 1.1	40.42 ± 2.2
3	15.02 ± 1.26	100.5 ± 8.65	544.49 ± 18.5	1625.4 ±53.5	22.35 ±2.1	441 ±12.3
5	85.65 ± 5.3	314.21 ± 26.1	540.02 ±16.3	342 ±12.8	23.78 ±1.9	47.2 ±7.6
7	362.36 ±12.76	19.06 ± 1.21	70.76 ±3.8	128.2 ±6.5	43.04 ±5.8	199 ± 9.7

Table 2: Effect of Metalaxyl on the Specific activity (μmol/g/min) of β- Amylase during germination of Maize

Seedling age in days	Concentration of fungicide in mg/g					
	control	1.5	3	4.5	6	7
0	0.536 ± 0.03	3.01 ± 0.07	7.93 ± 0.08	3.38 ± 0.04	6.83 ± 0.08	15.75 ± 1.3
1	1.788 ± 0.07	6.12 ± 0.43	17.49 ± 2.1	7.63 ± 0.08	15.65 ± 1.1	40.42 ± 2.2
3	15.02 ± 1.26	100.5 ± 8.65	544.49 ± 18.5	1625.4 ±53.5	22.35 ±2.1	441 ±12.3
5	85.65 ± 5.3	314.21 ± 26.1	540.02 ±16.3	342 ±12.8	23.78 ±1.9	47.2 ±7.6
7	362.36 ±12.76	19.06 ± 1.21	70.76 ±3.8	128.2 ±6.5	43.04 ±5.8	199 ± 9.7

Table 3: Effect of Metalaxyl on the Specific activity (µmol/g/min) of Invertase during germination of Maize

Seedling age in days		Concentration of fungicide in mg/g				
	control	1.5	3	4.5	6	7
0	23.69 ± 3.2	0.93 ± 0.03	3.24 ±0.84	3.89 ± 0.07	5.61 ± 0.86	2.13 ± 0.07
1	74.52 ± 8.7	2.86 ± 0.08	7.83 ± 0.96	8.2 ± 1.0	11.2 ± 1.5	5.48 ± 0.8
3	0.42 ±. 03	24.8 ± 1.23	14.13 ± 1.06	6.44 ± 0.86	7.8 ± 0.93	2.11 ± 0.6
5	51.62 ± 2.8	24.88 ± 1.06	65.68 ± 4.6	103 ± 5.8	36.38 ± 3.5	119.3 ± 7.5
7	23.3 ± 2.9	124.4 ± 8.9	24.4 ± 2.3	95.1 ±3.5	34.6 ± 2.8	16.4 ±2.36

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