



Efficiency of vitamin B6 (Pyridoxine HCl) on production of Endogenous IAA for growth promotion in *Zea mays* varieties (Azam & Jalal)

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Abstract

Vitamin B6 act as a versatile cofactor for many enzymes catalyzing important biochemical reactions, it has also been shown to function as a potent antioxidant molecule. B6 Vitamers is essential for plant development and their metabolism by modulating the endogenous phytohormone production, such as auxin (IAA). The recent explanation of the vitamin B6 biosynthesis pathways in plants provides opportunities for characterizing their importance during developmental processes and contact to stress. In plants, vitamin B6 are biosynthesized through salvage pathway is very similar to the bacterial variant. Pyridoxine, pyridoxal and pyridoxamine are inactive forms of vitamin B6 by the process of phosphorylation converted to active coenzymes forms pyridoxine 5' phosphate (PNP) Pyridoxal 5' phosphate (PLP) and pyridoxamine 5' phosphate (PMP) respectively. This research was performed on different varieties (Azam & Jalal) of *Zea mays* to check the natural variation response of various *Zea mays* varieties (Azam & Jalal) for efficacy of exogenously supplied pyridoxine-HCl to induce endogenous (IAA) production for growth promotion. Moreover, it is revealed that increased level of pyridoxine-HCl in *Zea mays* varieties (Azam & Jalal), differentially promoted the (IAA) level in plants in comparison to control. This research demonstrates an important link between vitamin B6 homeostasis and (IAA) biosynthesis in *Zea mays*.

Keywords: pyridoxine HCl, IAA, azam and jalal *Zea mays* varieties, chlorophyll a and b, anthocyanin, carotenoids

Introduction

Vitamins are compounds that cannot be synthesized by humans and thus need to be taken up in the diet. They have complex biochemistry and play an essential role in human nutrition and health. Vitamin deficiencies cause diseases that can be severe and even lethal in some cases. For instance, vitamin A deficiency is a major health problem in low-resource countries, putting an estimated 125– 130 million children at increased risk of morbidity and mortality from infectious diseases Kramer, K. *et al.* (2008) [7]. Well-known human vitamin-related disorders include, among others, blindness (vitamin A), beriberi (vitamin B1), pellagra (vitamin B3), anemia (vitamin B6), scurvy (vitamin C) and rickets (vitamin D). In much of the developed world, such deficiencies are, however, rare due to an adequate supply of food and/or the addition of vitamins to common foods, often called fortification. For instance, the deficiency of folic acid (vitamin B9) that generally occurs during pregnancy is avoided through supplementation, therefore preventing neural tube defects in the fetus (Wright, A.J.A. *et al.* 2007) [9]. Alternatively, vitamins in plant-derived foods can also be increased either through optimization of growth conditions, conventional plant breeding or through the use of transgenic techniques, a process known as biofortification (Bouis, H.E. and Welch, R.M. 2010) [1]. Plants contain a wide range of vitamins that are essential not only for human metabolism but also for plants, because of their redox chemistry and role as cofactors, and some of them also have

strong antioxidant potential. The antioxidant vitamins that have been the focus of most attention in plants are carotenoids (pro-vitamin A), ascorbate (vitamin C) and tocopherols (vitamin E, including both tocopherols and tocotrienols) (reviewed in (Demmig-Adams, *et al.*, 2002) [4] (Della Penna *et al.*, 2006) [5]. However, recent evidence indicates that vitamin B compounds could also play a significant role as antioxidants in plants. Thiamine (vitamin B1) has been shown to alleviate the effects of several environmental stresses on *Arabidopsis* (*Arabidopsis thaliana*), presumably by protecting the plant from oxidative damage (Tunc-Ozdemir, M. *et al.* 2009) [8]. The antioxidant role of thiamine can be indirect, by providing NADH and NADPH to the antioxidant network, or direct, by acting as an antioxidant. Another important finding is increased sensitivity to photooxidative stress in vitamin B6-deficient *Arabidopsis* plants (Havaux, M. *et al.* 2009) [6]. Pyridoxine, pyridoxal, pyridoxamine, and phosphorylated derivatives are collectively known as vitamin B6. Singlet oxygen levels increase in the *pdx1* mutant, which is deficient in *de novo* vitamin B6 biosynthesis. Furthermore, the *pdx1* mutation enhances the photosensitivity of the *vt1 npq1* *Arabidopsis* double mutant, which indicates interplay between vitamin B6, tocopherols, and carotenoids in chloroplasts (Havaux, M. *et al.* 2009) [6]. Other compounds with potential antioxidant activity within the plant cell include folates (vitamin B9) and phyloquinone (vitamin K1). This review focuses on the occurrence, biosynthesis, and antioxidant

function of vitamins in plants, including classical groups (vitamins A, C, and E) and other compounds (vitamins B and K). We emphasize the common points of their biosynthetic pathways and discuss which vitamins can be considered antioxidants in plants in vivo.

Material and Methodology

Conditions for plant material and growth

At the beginning of the experiment, the *Zea mays* mature seeds were sterilized. The experiment was carried out in the disposable glasses (scientific equipment) to characterize *Zea mays* response to vitamin B6 (pyridoxal-HCL). All vitamin B6 (pyridoxal-HCL) treatments were taken in different concentrations (Control, 10 ppm, 50 ppm, 100 ppm). 11 days old *Zea mays* plants were treated in different concentrations (Control, 10 ppm, 50 ppm, 100 ppm). The plants were returned to normal growth at room temperature for 9 days after initiation and subsequently exposed to treatments of vitamin B6 (pyridoxal-HCL). The following metabolisms of *Zea mays* treated with vitamin B6 (pyridoxal-HCL) plant groups have been achieved using a spectrophotometer.

Determination of IAA

- 0.3g (300mg) of plant tissue was collected and grinded with 3ml pre-cooled distill water.
- The mixture was Centrifuged at 10,000 rpm for 10 min at 4°C.
- Supernatant was collected and mixed with pre-cooled ethyl acetate (1:2). After vigorous shaking the mixture was allowed to stand for 10 - 30 min.
- IAA was extracted by separating the solvent layer (organic phase) into separate vial. The procedure might be repeated 3 to 4 times.
- The organic phase was diluted with 2 ml 100 % ethanol (pre-cooled).
- Equal volume of extract (1 ml each) was taken into three vials for three replications.
- 1ml supernatant was taken into test tube and 2ml of Salkowski's reagent was added (shake well).
- The mixture was then Incubated at room temperature in the dark for 30 min.
- After 30 min, IAA production in the plant tissue extract was observed by a characteristic indication of reddish to pinkish color in the solution.
- After 30 min the absorbance by color changed of each replicates was monitored at 540 nm using UV-VIS Spectrophotometer (Bio Tek Instruments, Inc, ELX800, US).
- In herbal tissue samples (Benizri *et al.*, 1998) ^[3], the IAA content was based on OD of samples using the Salkowski reagent. The standard IAA curve was used to generate a Linear Regression Equation ($Y = A + BX$) as defined by Beets and Dubery (2011) ^[2] with the aim of quantifying the IAA content in ug/ml.

Quantification of Chlorophyll a, b, Total Chlorophyll and Carotenoid Content

- 0.3g (300mg) of plant tissue was collected and grinded with 3ml pre-cooled 80 % acetone and mix thoroughly.

- The mixture was centrifuged at 12,000rpm for 5 min at room temperature.
- The supernatant was transferred to new tubes in triplicates and Centrifuged at 12,000rpm for 5 min at room temperature.
- The supernatant was Transfer to new tube and the absorbance was measured at 530 and 637nm.
- Absorbance was measured at 645 nm and 663nm for chlorophyll a and b, while 470nm for carotenoid by using UV-VIS Spectrophotometer (Bio Tek Instruments, Inc, ELX800, US).

Quantification of Total Chlorophyll

In the Maclachlan and Zalik Protocol (1963) the concentration of chlorophyll has been estimated. This protocol has been followed in order to determine the total chlorophyll content by using following formulas:

Quantifications were done using the formula below.

$$\text{Chlorophyll a } (\mu\text{g/ml}) = 12.7 (A_{663}) - 2.69 (A_{645})$$

$$\text{Chlorophyll b } (\mu\text{g/ml}) = 22.9 (A_{645}) - 4.68 (A_{663})$$

$$\text{Total chlorophyll } (\mu\text{g/ml}) = 20.2 (A_{645}) + 8.02 (A_{663})$$

$$\text{Formula for Carotenoids (mg.ml}^{-1}\text{)} = \{ (1000 * \text{Abs}_{470}) - (1.82 * \text{chl.a}) - (85.02 * \text{chl.b}) \} / 198$$

Quantification of Anthocyanin Content

Laboratory Procedure

- 0.3g (300mg) of plant tissue was collected and grinded with 3ml pre-cooled extraction buffer and mix thoroughly.
- The mixture was centrifuged at 12,000rpm for 5min at room temperature.
- The supernatant was transfer to new tube in three replications and centrifuge at 12,000rpm for 5 min at room temperature.
- The supernatant was transfer to new tube and the absorbance was measured at 530 and 637 nm using UV-VIS Spectrophotometer (Bio Tek Instruments, Inc, ELX800, US).

Calculation of Anthocyanin Content

Anthocyanin content was calculated using the formula: $(\text{Abs}_{530} / \text{g F.W.})$ by $[\text{Abs}_{530} - (0.25 \times \text{Abs}_{657})] \times 3$.

Results and Discussion

Phenotypic Analysis

11 days old *Zea mays* plants were treated for 3 times in different concentration (10ppm, 50ppm, 100ppm), at room temperature. The plants were returned to normal growth at room temperature (July month) for 11 days after initiation treatment of vitamin B6 in different concentration (10ppm, 50ppm, 100ppm). Plants were then moved to normal growth. The result below shown two different varieties of *Zea mays* plants that is, *Azam* and *Jalal* shown in the (Figure: 1).

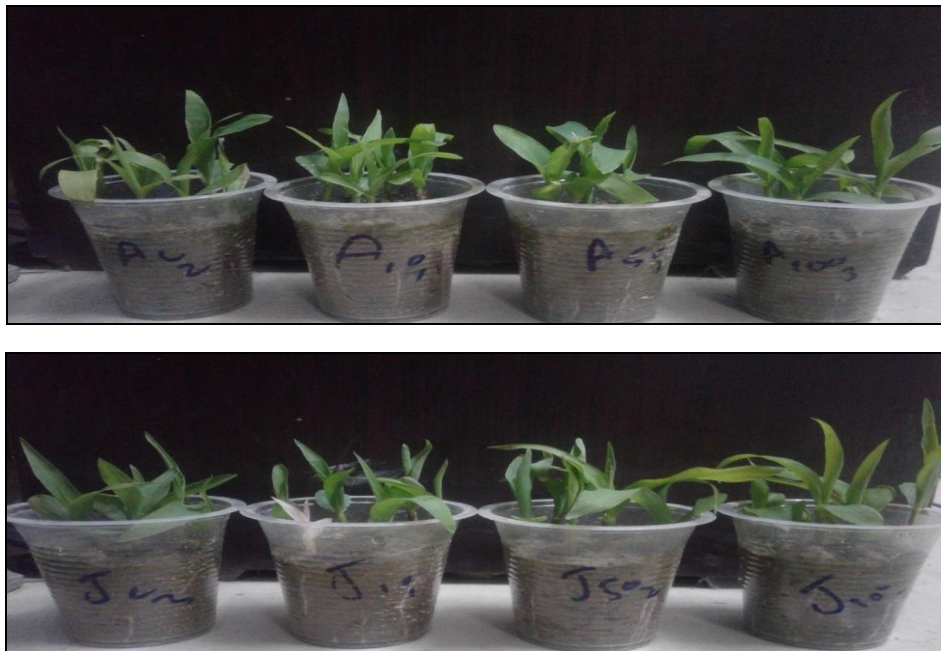


Fig 1: Phenotypically representation of two different varieties of *Zea mays* plants upon vitamin B6 different treatments

- (A) Indicates *Azam* treated plants at room temperature.
- (J) Indicates *Jalal* treated plants with vitamin B6 in different concentration (10ppm, 50ppm, 100ppm).

Chlorophyll A Content Determination in *Zea mays*

For the Analysis of Chlorophyll A content from fresh leaves of two *Zea mays* varieties, we have four Treatments for each variety which are: control, 10 ppm, 50 ppm and 100 ppm of pyridoxine Hcl solution. In *Azam* variety, the *Chlorophyll A* content is 0.30 µg/ml while it has been increased greatly in the treatments of 10 ppm and remained same in 50 ppm and 100 ppm. For 10 ppm treatment, the *Chlorophyll A* content is 0.81 µg/ml which is 37% increased as compared to control. This means that pyridoxine Hcl greatly effects the *Chlorophyll A* content and increases it. In the other treatment that included 50 ppm pyridoxine Hcl, the

Chlorophyll A content is 0.37 µg/ml which is 23% increased as compare to control and only 0.14 % decreased as compared to 10 ppm treatment of pyridoxine Hcl. For 100 ppm treatment, the *Chlorophyll A* content is decreased to 0.31 µg/ml which is 03% increased as compared to control. In *Jalal* variety, 0.31 µg/ml *Chlorophyll A* content was found in control which increased to 0.40 µg/ml (0.09%) in 10 ppm treatment of pyridoxine Hcl. while in 50 ppm treatment of pyridoxine Hcl, it decreased to 0.19 µg/ml (21%), and in 100 ppm the *Chlorophyll A* content is greatly increased 1.1 µg/ml which is 46% increased as compared to control which shows that pyridoxine Hcl increases *Chlorophyll A* content when applied in higher concentration while in lower concentration, the effect is low, as shown in (Figure 2.).

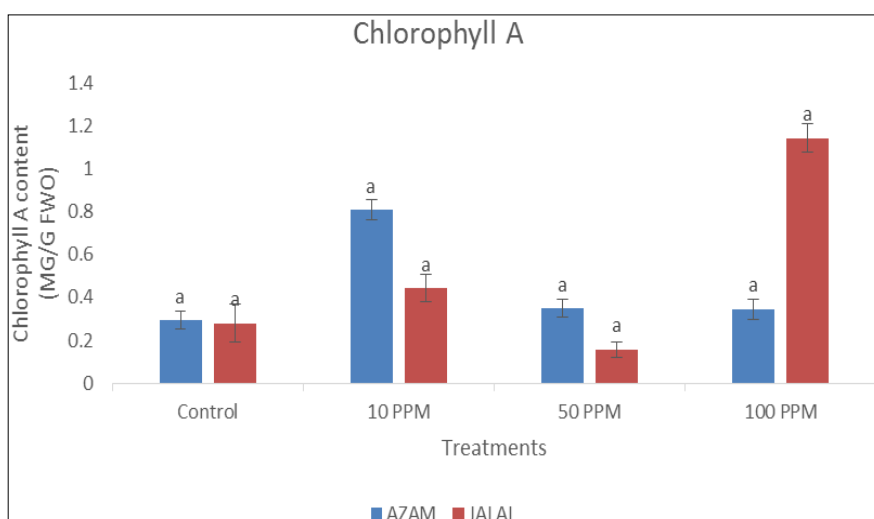


Fig 2: Determination of Chlorophyll A content from *Zea mays* with different pyridoxine HCL treatment

The experiments shows that pyridoxine Hcl affects *Chlorophyll A* content positively. The small letters (a, b) indicate suggestively different means (p 0.05) inside the same variety compared to the matching each other according to one-way ANOVA and Duncun multiple

comparison of means individually which shown in the (Figure 2.). In *Jalal* variety, between the (control, 10 ppm and 50 ppm) treatment are of no significant difference and for 100 ppm treatment there is significance different as compare to control, 10 ppm and 50 ppm pyridoxine Hcl.

However, In *Azam* variety, there is no significant difference between the treatments (control, 50 ppm, 10 ppm & 100 ppm).

Chlorophyll B content Determination in *Zea mays*

For the Analysis of *Chlorophyll B* Content from fresh leaves of two *Zea mays* varieties, we have four treatments for each variety which are: control, 10 ppm, 50 ppm and 100 ppm pyridoxine Hcl solution. In *Azam* variety, the *Chlorophyll B* content is 1.0 µg/ml while it has been increased greatly in the treatments of 10 ppm and remained same in 50 ppm and 100 ppm. For 10 ppm treatment, the *Chlorophyll B* content is 1 µg/ml which is 40% increased as compared to control. This means that pyridoxine Hcl greatly affects the *Chlorophyll B* content and increases it. In the other treatment that included 50 ppm pyridoxine Hcl, the *Chlorophyll B* content is 0.50 µg/ml which is 23% increased as compared to control and only 0.14 % decreased as compared to 10 ppm treatment of pyridoxine Hcl. For 100 ppm treatment, the *Chlorophyll B* content is decreased to 0.40 µg/ml which is 03% increased as compared to control. In *Jalal* variety, 0.4 µg/ml *Chlorophyll B* content was found in control which was increased to 0.9 µg/ml (0.09%) in 10 ppm treatment of pyridoxine Hcl. while in 50 ppm treatment the pyridoxine Hcl was decreased to 0.19 µg/ml (21%), and in 100 ppm treatment the *Chlorophyll B* content is greatly increased 2 µg/ml which is 46% increased as compared to control which shows that pyridoxine Hcl increases *Chlorophyll B* content when applied in higher concentration while in lower concentration while in lower concentration, the effect is low, as shown in (Figure 3.)

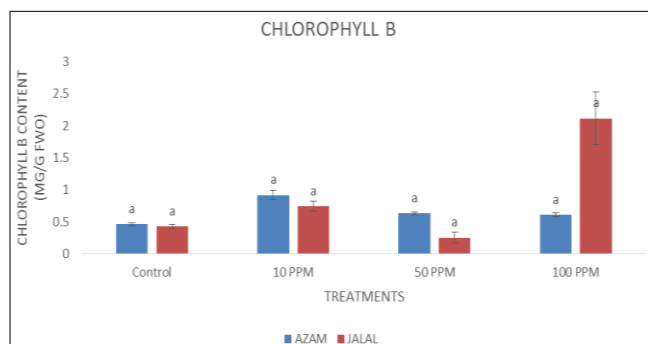


Fig 3: Determination of Chlorophyll B content from *Zea mays* with different pyridoxine HCL treatment

The experiments shows that pyridoxine Hcl affects *Chlorophyll B* content positively. The small letters (a, b) indicate suggestively different means (p 0.05) inside the same variety compared to the matching each other according to one-way ANOVA and Duncan multiple comparison of means individually which shown in the (Figure 3). In *Jalal* variety, between the (control, 10 ppm and 50 ppm) treatment are of no significant difference and 100 ppm treatment have significantly different as compare to control 10 ppm and 50 ppm pyridoxine Hcl. However, In *Azam* variety, there is no significant difference between the treatments (control, 50 ppm, 10 ppm & 100 ppm).

Total Chlorophyll Content Determination in *Zea mays*

For the Analysis of *total Chlorophyll* Content from fresh leaves of two *Zea mays* varieties, we have four treatments for each variety which are: control, 10 ppm, 50 ppm and 100 ppm pyridoxine Hcl solution. In *Azam* variety, the *total*

Chlorophyll content is 0.7 µg/ml while it has been increased greatly in the treatments of 10 ppm and remained same in 50 ppm and 100 ppm. For 10 ppm treatment, the *total Chlorophyll* content is 0.6 µg/ml which is 10% decreased as compared to control. This means that pyridoxine Hcl greatly effects the *total Chlorophyll* content and increases it. In the other treatments which included 50 ppm pyridoxine Hcl, the *total Chlorophyll* content is 0.9 µg/ml which is 23% increased as compare to control and only 0.14 % increased as compared to 10 ppm treatment of pyridoxine Hcl. For 100 ppm treatment, the *total Chlorophyll* content is increased to 0.9 µg/ml which is 0.3% increased as compared to control. In *Jalal* variety, 0.6 µg/ml *total Chlorophyll* content was found in control which increased to 1.1 µg/ml in 10 ppm treatment of pyridoxine Hcl while in 50 ppm treatment of pyridoxine Hcl, it wsa decreased to 0.3 µg/ml (21%), and in 100 ppm the *total Chlorophyll* content is greatly increased 3 µg/ml which is 46% increased as compared to control which shows that pyridoxine Hcl increases *Chlorophyll B* content when applied in higher concentration while in lower concentration, the effect is low, as shown in (Figure 4).

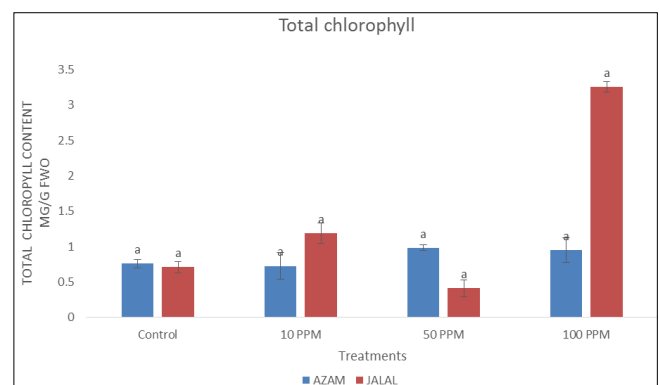


Fig 4: Determination of Total Chlorophyll content from *Zea mays* with different pyridoxine HCL treatments

The experiments suggest that pyridoxine Hcl affects *Total Chlorophyll* content positively. The small letters (a, b) indicate suggestively different means (p 0.05) inside the same variety compared to the matching each other according to one-way ANOVA and Duncan multiple comparison of means individually which shown in the (Figure 4). In *Jalal* variety, between the (control, 10 ppm and 50 ppm) treatment are of no significant difference and 100 ppm treatment have significantly different as compare to control 10 ppm and 50 ppm pyridoxine Hcl. However, In *Azam* variety, there is no significant difference between the treatments (control, 50 ppm, 10 ppm & 100 ppm).

Determination of IAA content in *zea mays*

For the Analysis of *IAA* Content from fresh leaves of two *Zea mays* varieties, we have four treatments for each variety which are: control, 10 ppm, 50 ppm and 100 ppm pyridoxine Hcl solution. In *Azam* variety, the *IAA* content is 0.3 µg/ml while it has been decreased in the treatments of 10 ppm and remained same in 50 ppm and 100 ppm. For 10 ppm treatment, the *IAA* content is 0.31 µg/ml which is 10% increased as compared to control. This means that pyridoxine Hcl greatly affects the *IAA* content and increases it. In the other treatment that included 50 ppm pyridoxine Hcl, the *IAA* content is 0.25 µg/ml which is 23% decreased

as compare to control and only 0.14 % increased as compared to 10 ppm treatment of pyridoxine Hcl. For 100 ppm treatment, the IAA content is decreased 0.27 µg/ml which is 03% increased as compared to control. In *Jalal* variety, 0.28 µg/ml IAA content was found in control which was decreased to 0.3 µg/ml in 10 ppm treatment of pyridoxine Hcl is 0.25µg/ml while in 50 ppm treatment of pyridoxine Hcl, it was decreased to 0.24 µg/ml (21%), and in 100 ppm the IAA content is greatly decreased 0.21 µg/ml which is 46% decreased as compared to control which shows that pyridoxine Hcl increases IAA content when applied in higher concentration while in lower concentration, the effect is low, as shown in (Figure 5).

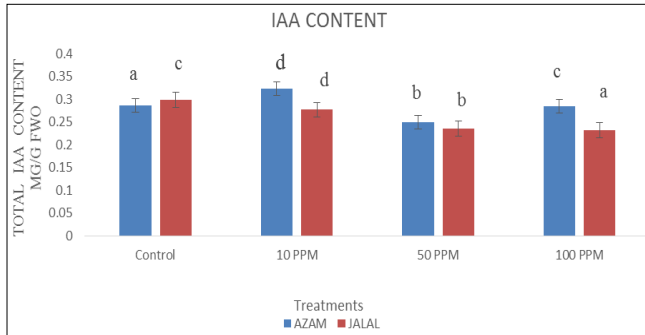


Fig 5: Determination of IAA content from *Zea mays* with different pyridoxine HCL treatments

The experiment shows that pyridoxine Hcl affects IAA content positively. The small letters (a, b) indicate

suggestively different means (p=0.05) inside the same variety compared to the matching each other according to one-way ANOVA and Duncan multiple comparison of means individually which shown in the (Figure 5). In *Azam* variety, between the (control, 50 ppm and 100 ppm) treatment are of no significant difference and 10 ppm treatment have significantly different as compare to control 10 ppm and 50 ppm pyridoxine Hcl. However, In *Jalal* variety, there is no significant difference between the treatments (control, 50 ppm, 10 ppm & 100 ppm).

Determination of Anthocyanin Content in *Zea mays*

For the analysis of *Anthocyanin* Content from fresh leaves of two *Zea mays* varieties, we have four treatments for each variety which are: control, 10 ppm, 50 ppm and 100 ppm pyridoxine Hcl solution. In *Azam* variety, the *anthocyanin* content is 1.0 µg/ml while it has been decreased in the treatments of 10 ppm and 50ppm. For 10 ppm and 50ppm treatments, the *ANTHOCYANIN* content is 0.30µg/ml which remained same as compared to control. In other treatment that included 100ppm pyridoxine Hcl, the *ANTHOCYANIN* content is 1.0µg/ml which is 45% increased as compared to control. In *Jalal* variety 0.23 µg/ml *ANTHOCYANIN* content was found in control which was decreased to 0.2 µg/ml in 10 ppm treatment of pyridoxine Hcl, while in 50 ppm treatment of pyridoxine Hcl, it was increased to 0.24 µg/ml (21%), and in 100 ppm the *ANTHOCYANIN* content is greatly decreased to 0.19µg/ml which is 25% decreased as compared to control (Figure 6).

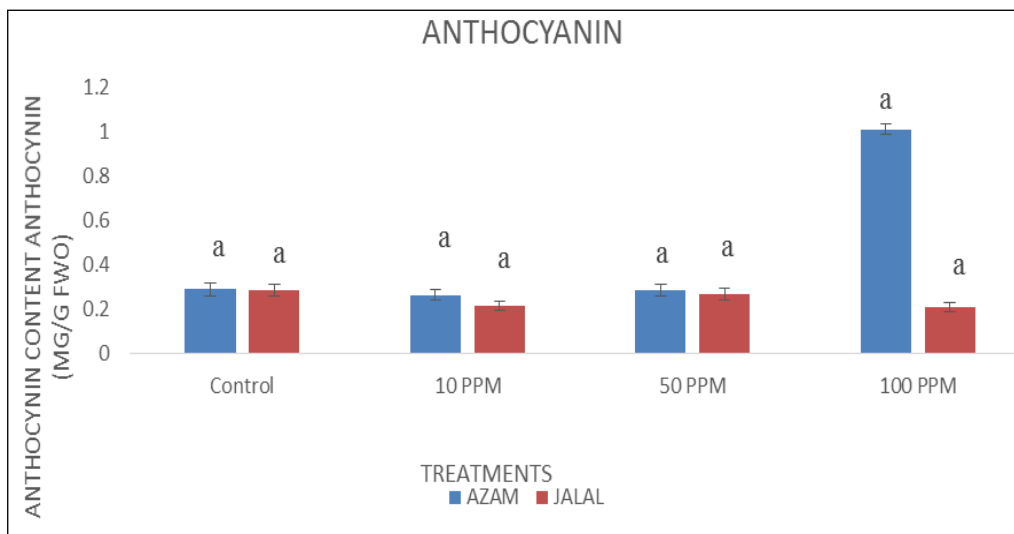


Fig 6: Determination of ANTHOCYANIN content from *Zea mays* with different pyridoxine HCL treatments

The experiments suggest that pyridoxine Hcl affects *ANTHOCYANIN* content negatively. The small letters (a, b) indicate suggestively different means (p 0.05) inside the same variety compared to the matching each other according to one-way ANOVA and Duncan multiple comparison of means individually which shown in the (Figure 6). In *Azam* variety, between the (control, 10 ppm and 50 ppm) treatment are of no significant difference and 100 ppm treatment have significantly different as compare to control, 10ppm and 50ppm pyridoxine Hcl. However, In *Jalal* variety, there is no significant difference between the treatments (control, 50 ppm, 10 ppm & 100 ppm).

Determination of Carotenoids Content in *Zea mays*

For the Analysis of *CAROTENOIDS* content from fresh leaves of two *Zea mays* varieties, we have four treatments for each variety which are: control, 10 ppm, 50ppm and 100ppm pyridoxine Hcl solution. In *Azam* variety, the *CAROTENOIDS* content is 0.12µg/ml while it has been decreased in the treatments of 50ppm and 100 ppm. For 10ppm treatment, the *CAROTENOIDS* content is 0.11 µg/ml which is greatly increased as compared to control. This means that pyridoxine Hcl greatly effects the *CAROTENOIDS* content. In the other treatment that included 50 ppm pyridoxine Hcl, the *CAROTENOIDS* content is 0.05µg/ml which is 10% increased as compared to

control. For 100 ppm treatment, the *CAROTENOIDS content* is slightly increased as compared to control. In *Jalal* variety, 0.04µg/ml *CAROTENOIDS content* was found in control which increased to 0.14 µg/ml in 100 ppm treatment of pyridoxine HCl, while in 50 ppm treatment of pyridoxine

HCl, the carotenoid content decreased to 0.03µg/ml which is 10% decreased as compared to control and in 100ppm the *CAROTENOIDS content* is greatly increased 0.14 µg/ml which is 45% increased as compared to control in (Figure 7).

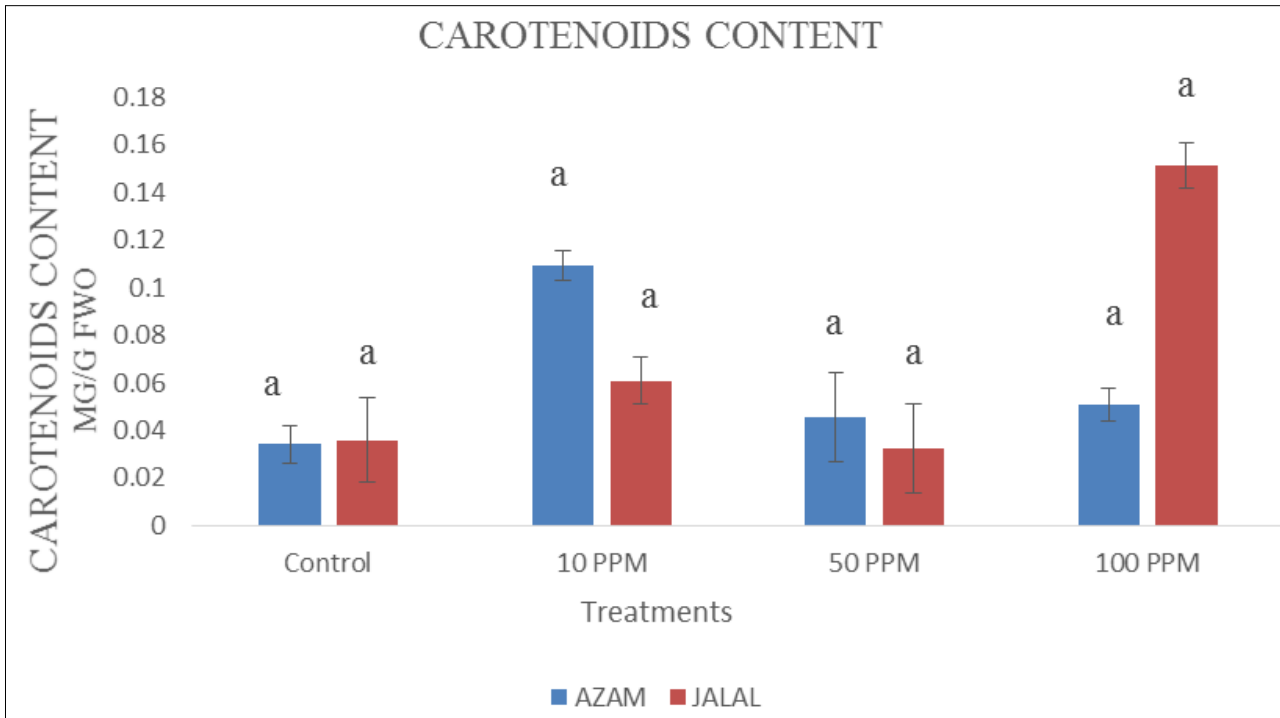


Fig 7: Determination of CAROTENOIDS content in *Zea mays* with different pyridoxine HCL treatments

The experiments Shows that pyridoxine HCl affects *CAROTENOIDS content* positively. The small letters (a, b) indicate suggestively different means (p 0.05) inside the same variety compared to the matching each other according to one-way ANOVA and Duncan multiple comparison of means individually which shown in the (Figure 7). In *Jalal* variety, between the (control, 10 ppm

and 50 ppm) treatment are of no significant difference and 100 ppm treatment have significantly different as compare to control, 10 ppm and 50 ppm pyridoxine HCl. However, In *Azam* variety, there is no significant difference between the treatments (control, 50 ppm & 100 ppm) but 10ppm treatment shows great increased in carotenoid content (0.12µg/ml) which is 40% increased as compared to control.

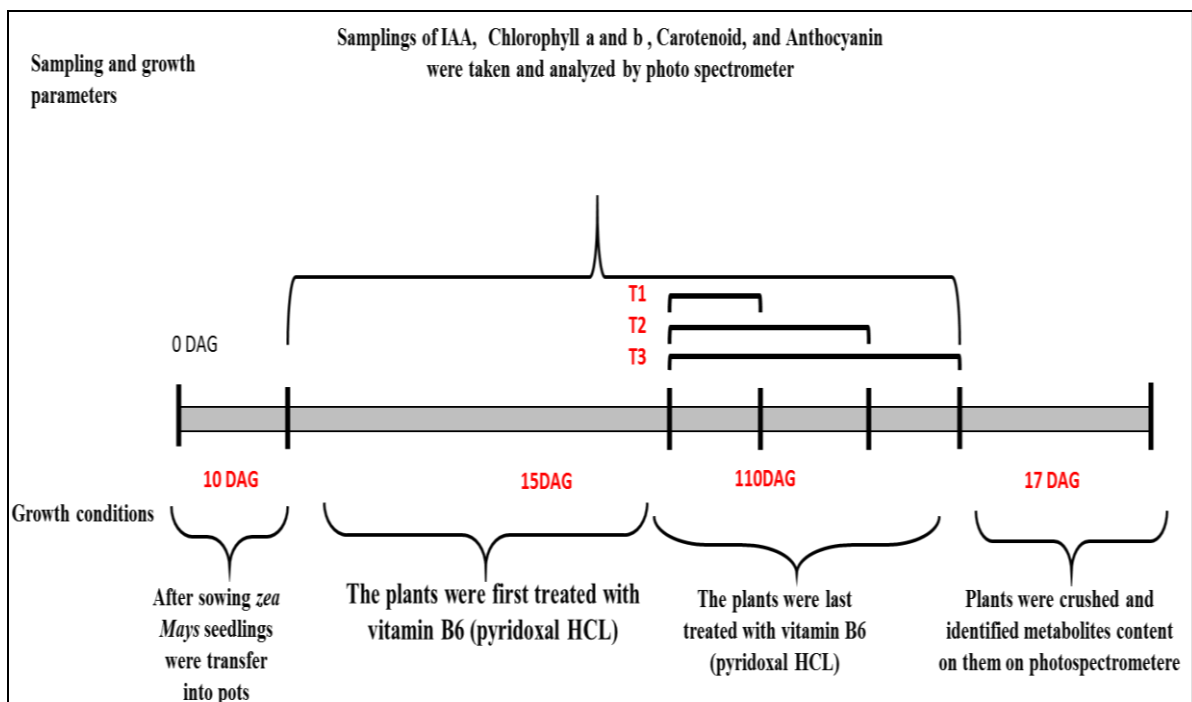


Fig 8: Samples of IAA, Chlorophyll a and b, carotenoid, and Anthocyanin analyzed by spectrophotometer

Conclusion

From the result obtained, it can be concluded that the application of pyridoxine HCl greatly affects the concentration of carotenoid, IAA, Chlorophyll A and B in *JALAL* variety plants. The concentration of anthocyanin content remains normal in *Jalal* variety plants and greatly increased Azam variety.

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