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# Effect of Zinc and Phosphorus Supply on the Activity of Carbonic Anhydrase and the Ultrastructure of Chloroplast in Sweet Corn (*Zea mays* var. *saccharata*)

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**Abstract:** Phosphorus (P) and zinc (Zn) interact with each other and this interaction can affect the yield of corn plants. This study was conducted to examine the effect of different levels of P and Zn on the ultrastructure of chloroplast and physiological characteristics of corn plants. Sweet corn was grown in nutrient culture containing all combinations of P at levels of 0.0 and 80.0 mg $L^{-1}$ as $KH_2PO_4$ and Zn at levels of 0.0 and 20.0 mg $L^{-1}$ as $ZnSO_4.7H_2O$ and harvested at 14 and 28 days after transplanting. Phosphorus (P) and zinc (Zn) concentrations in leaves increased with increasing P and Zn concentration in nutrient solution. Zinc supply did not affect P concentration but Zn concentration reduced with increasing P supply in nutrient solution at both harvests. The lowest amount of chlorophyll content was recorded in $Zn_{20}P_0$ treatment due to the interaction of Zn with iron in the growth medium. Carbonic anhydrase activity in leaves was enhanced with increasing Zn levels and decreased with increasing P levels at both harvest times. Carbonic anhydrase activity is a better indicator of Zn nutritional status than Zn concentration alone. The ultrastructure of chloroplast was affected with P and Zn supply.

**Key words:** Carbonic anhydrase, chloroplast ultrastructure, phosphorus, transmission electron microscopy, zinc

## INTRODUCTION

Zinc (Zn) is absorbed by plants as a cation ($Zn^{2+}$) and phosphorus (P) is taken up by plants as phosphate ions ($H_2PO_4^-$ or $HPO_4^{-2}$). These oppositely charged ions attract each other which facilitates the formation of chemical bonds in soil and plant tissues. If excess soil or plant P binds a large quantity of Zn normally available to the plant, the result can be a P-induced Zn deficiency by changing the physical, chemical and biological properties in soil-plant systems (Brown and Tiffin, 1962; Cakmak and Marschner, 1987; Christensen, 1972; Lindsey, 1973; Singh *et al*., 1986, 1988; Marschner, 2002). Phosphorus (P) and zinc (Zn) deficiencies are global nutritional problems for crop production in many areas of the world and P-Zn interactions have been widely studied (Edwards and Kamprath, 1974; Schwartz *et al*., 1987; Singh *et al*., 1988; Loneragan and Webb, 1993; Hopkins *et al*., 1998; Rupa *et al*., 2003).

When a significant amount of a nutrient accumulates in leaves in a physiologically inactive form, determination of total nutrient concentration in leaves is not precise and overestimates the true nutritional status (Gibson and Leece, 1981). Within plants there are several Zn-dependent processes which may be correlated with levels of active Zn in tissue. Carbonic Anhydrase (CA) activity is one of these Zn-dependent processes which was employed previously by Wood and Sibly (1952) in tomato, Bar-Akiva and Lavon (1969) in citrus, Randall and Bouma (1973) in spinach, Ohki (1978) in soybean, Gangwar *et al*. (1989) in rice, Pandey and Sharma (1989) in sunflower and Rengel (1995) in wheat to determine the Zn status in plants.

Carbonic anhydrase is a ubiquitous enzyme (found in animals, terrestrial plants, eukaryotic algae, cyanobacteria) which catalyzes the rapid inter-conversion of carbon dioxide and water into carbonic acid, proton and bicarbonate ions. The conversion of bicarbonate to $CO_2$ facilitates its transport into the cell, while the conversion of $CO_2$ to bicarbonate helps trap $CO_2$ in the cell. This reaction involves a two-step mechanism. The first step is the nucleophilic attack of a Zn-bound hydroxide ion on $CO_2$ (Rengel, 1995). The second step is the regeneration of the active site by ionization of the Zn-bound water molecule and removal of a proton from the active site. Carbonic anhydrase from dicotyledons consists of six subunits, has a molecular weight of 180 kDa and six Zn atoms per molecule (Sandmann and Boger, 1983). The enzyme is located in the cytosol, root plastids and chloroplasts. As CA is available in leaves of higher plants in plentiful quantities (1-2% of total leaf protein) (Okabe *et al*., 1984), it can be a source of Zn in leaf cells and a biological marker to estimate physiological Zn availability.

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This study was undertaken with three aims: To assess the suitability of CA activity as indicators of functional Zn availability in different ranges of Zn tissue concentrations, to investigate the effect of P-Zn interaction on the chloroplast ultrastructure and chlorophyll content of sweet corn plants and to determine the effects of P-Zn interaction on root growth of sweet corn plants.

## MATERIALS AND METHODS

**Experimental design:** The nutrient culture method was used in this experiment. The experiment was performed at the Department of Land Management, Faculty of Agriculture, University Putra Malaysia (UPM). Sweet corn seeds hybrid 926 from Green World Genetics in Malaysia were used as the indicator plant. The seeds were soaked in water for 24 h and then geminated in rolled paper towels saturated with deionized water in the laboratory at 24°C. The paper towels were kept saturated for 5 days and the sweet corn seedlings were transplanted into 2 L capacity plastic pots containing nutrient solution at the rate of four seedlings per pot.

The basic nutrient solution was according to Trostle *et al.* (2001) which contained 0.75 mM $K_2SO_4$, 0.65 mM $MgSO_4$, 0.1 mM KCl, 2 mM $Ca(NO_3)_2$, 0.0001 mM $CuSO_4$, 0.1 mM EDTAFe, 0.001 mM $MnSO_4$, 0.01 mM $H_3BO_3$ and $5\times10^{-6}$ mM $(NH_4)_6Mo_7O_{24}$. The pH was adjusted to 6.8 by using 0.1 M KOH or HCl solution. All combinations of Zn treatments (in the form of $ZnSO_4.7H_2O$) at levels of 0.0 and 20.0 mg $L^{-1}$ and of P treatments (in the form of $KH_2PO_4$) at levels of 0.0 and 80.0 mg $L^{-1}$ were included. The nutrient solution was changed every three days. The experimental design was a randomized complete block consisting of 5 blocks (replications). The plants were grown at ambient sunlight. The temperature and humidity were 24-33°C and 70-88%, respectively.

**Measurements:** Plants were harvested at 14 (V5- plants had 5 leaves) and 28 (V8- plants had 8 leaves) days after transplanting. The roots and shoots were then separated. The plant samples were rinsed with distilled water, dried at 70±2°C for 48 h, weighed, and ashed at 300°C for 3 h followed by 500°C for 2 h in a muffle furnace. The ash was dissolved in concentrated HCl and 20% $HNO_3$. Phosphorus (P) was measured using an autoanalyzer (8000 series, Lachat QuickChem FIA+, USA) and Zn was determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) (Optima 8300, PerkinElmer, USA). For chlorophyll content analysis, the fresh leaves (0.2 g) were placed into the 20 mL glass vials. 5 mL concentrated dimethyl sulfoxide (DMSO) was added and the glass vials placed in an oven at 70°C for 1 h. Under the wavelength of 645 and 663 nm, the contents of chlorophyll b and a were measured, respectively, using spectrophotometer (1000 series, Cecil CE 1011, Auckland, New Zealand). Arnon's equation (Arnon, 1949) was used to convert absorbance measurements to mg chlorophyll per g in leaf tissue.

For CA activity determination, all manipulations during the extraction procedure were done on ice; centrifugation was done at 0°C. 400 mg of leaf material was ground in a chilled glass mortar with 10 mL of ice-cold 10 mM tris-HCl buffer, pH 8.2, containing 5 mM 2-mercapto-ethanol, and centrifuged at 12,000×g for 10 min. The pale green supernatant was saved for the enzyme analysis. Tests showed no loss of enzyme activity for up to 2 h after grinding. Carbonic anhydrase activity was generally assayed during fractionation by colorimetric method of Wilbur and Anderson (1948). Activity of CA was estimated in a solution consisting of 2 mL of 25 mM vernol buffer (barbitone, 5-5-diethyl barbituric acid), pH 8.2, 0.1 mL of 1% (w/v) of bromthymol blue and 1 mL of a sample (or extraction buffer for a blank), at 2°C. Two milliliter of a cold, saturated $CO_2$ solution were injected by means of a syringe into the vernol buffer. The experimenter recorded the time from the moment of injection to the color change of the indicator from blue to greenish yellow.

CA activity was expressed in enzyme units (EU) on a leaf fresh weight basis and was calculated from the following equation (Gibson and Leece, 1981):

$$EU/g = \frac{10\left(\frac{Tb}{Te}-1\right)}{g}$$

where, $T_b$ is the time for the uncatalyzed reaction and $T_e$ is the time for the reaction with active enzyme added. In this method, the time required for the pH to drop from approximately pH 8 to 6.3 is measured. Three determinations were done per replicate. As the experiment contained 5 replications, results presented here are averages of 15 determinations.

Root parameters such as average root diameter, root length, root surface area and root volume were measured immediately after harvest by WinRHIZO 2012b software (Regent Instruments Inc., Quebec, Canada).

For TEM analysis, one extra treatment was considered which had plants grown under full-nutrient solution. All tissues were obtained from the middle region of the leaflet blades adjacent to the main veins. Sections of the leaf samples were cut (1×1 mm) and then placed into separate vials containing the fixation solution which was composed of 4% glutaraldehyde at pH 8 for 2 days at 4°C.

Then the samples were washed with 0.1 M sodium cacodylate buffer (pH 7.2) for 3 changes of 30 min each. Samples were post-fixed in 1% osmium tetroxide, dehydrated by acetone, infiltrated with acetone and resin mixture, and embedded with epoxy resin (Epon-812) for 48 h. Sections were cut on a Leica Ultracut UCT ultramicrotome, post-stained with 4% (w/v) aqueous uranyl acetate and Reynild's lead citrate (Reynolds, 1963). To check the configuration of cells, the sections were examined with Transmission Electron Microscopy (TEM) LEO 912 AB EFTEM under the working voltage of 120 kV.

**Statistical analysis:** Data was analyzed statistically by using SAS 9.2 software (SAS, 2010).

## RESULTS AND DISCUSSION

Leaf P concentration increased with increasing P concentration in solution at all harvests (Table 1). Zinc supply did not show any significant effect on P concentration in leaves at both harvesting times ($p>0.05$). Similar results were obtained by Li *et al.* (2003) in barely. Contrary results were reported by Loneragan *et al.* (1982). They demonstrated that P concentration in leaves enhanced with reduction of Zn concentration in nutrient solution and stated that this phenomenon can be explained by increased P absorption by roots and transportation to upper plant parts and Huang *et al.* (2000) showed that Zn deficiency causes a rise in the expression of P transporter genes in barely roots and may result in P toxicity in plants. In this experiment, Zn deficiency was not obvious even in $Zn_0$ treatments, so P concentration in shoots was not affected by Zn supply. Phosphorus (P) concentrations in all the treatments were sufficient at 14 and 28 DAT (0.295% according to Tyner, 1946). The interaction of P with Zn did not have a significant effect on Phosphorus (P) concentration in leaves of maize at 14 and 28 DAT ($p>0.05$).

As expected, Zn concentration in shoots enhanced as Zn concentration in nutrient solution increased (Table 1). Zinc (Zn) concentration in shoots was several folds higher in Zn-supplied plants as compared to non-applied ones. The Zn concentration decreased with rise in P level in growth medium at both harvests. Phosphorus (P) can reduce Zn solubility or hamper Zn translocation to its functional location in the plant. Terman *et al.* (1972) demonstrated that P decreased Zn translocation from the roots to the upper plant parts in corn plants. Increased Zn concentration in the root cell walls as a function of added P could explain this phenomenon. Leece (1978) reported that Zn may be bound to cell walls or chelated by organic ligands as a function of increased P. Similar results were observed by Loneragan (1951) in flax and Stukenholtz *et al.* (1966) in corn. In this experiment, Zn concentrations in leaves were sufficient (>20 μg $g^{-1}$ according to Kuldeep (2009)) at both harvesting times and hence, a P-induced Zn deficiency was not observed as P levels increased. With regard to P/Zn ratio, increased P plays a moderating or balancing role in shoot Zn concentration. Perhaps more impact would have been observed if plants had been allowed to grow for a longer period of time. The P-Zn interaction was significant for Zn concentration in shoots only at 14 DAT ($p\leq0.05$).

According to Prasad *et al.* (1971), P/Zn ratio of 25-154 can be stated as normal nutrient ratios in corn plants at these growth stages. In $Zn_0P_{80}$ treatment at 14 and 28 DAT, this ratio was higher than the critical range (Table 1) which could be indication of latent Zn deficiency, although the Zn concentration in these treatments were 49.8 and 51.8 μg $g^{-1}$, respectively. This high P/Zn ratio caused by high P concentration in tissues could be a better indicator for Zn nutritional status and plant metabolism than Zn concentration alone. P/Zn ratio in $Zn_{20}P_0$ treatment dropped to 16.2 and 13.1 at 14 and 28 DAT, respectively which could show Zn excess in these treatments. The Zn concentrations in these treatments were higher than the threshold level and resulted in Zn toxicity. In $Zn_{20}P_{80}$ treatment, Zn concentration was also toxic but P application increased P/Zn ratio to reduce the toxicity of Zn. The P×Zn interaction had a significant effect on P/Zn ratio at both harvests ($p\leq0.01$). This ratio can affect both Zn status and total Zn content. It shows that Zn or P application alone can cause the reduction of the non-applied one in the leaves. Millikan (1951, 1963), Boawn and Leggett (1964), Watanabe *et al.* (1965), Boawn

Table 1: Phosphorus (P) (%) and Zinc (Zn) (μg $g^{-1}$) concentration and P/Zn ratio in leaves of sweet corn plants in nutrient solution with different Zn and P levels

| Treatments (mg $L^{-1}$) | | 14 days | | | 28 days | | |
|---|---|---|---|---|---|---|---|
| Zn | P | P (%) | Zn (μg $g^{-1}$) | P/Zn | P (%) | Zn (μg $g^{-1}$) | P/Zn |
| 0 | 0 | 0.451 | 79.4 | 56.8 | 0.589 | 64.8 | 93.5 |
| 0 | 80 | 0.878 | 49.8 | 176.6 | 1.184 | 51.8 | 234.4 |
| 20 | 0 | 0.608 | 374.2 | 16.2 | 0.542 | 417.6 | 13.1 |
| 20 | 80 | 1.076 | 277.4 | 39.5 | 1.084 | 337.8 | 33.6 |
| LSD (5%) | | 0.103 | 36.1 | 8.7 | 0.195 | 52.5 | 48.1 |
| P×Zn interaction | | ns | * | ** | ns | ns | ** |

ns: Non significant, *,**Significant at the 5 and 1% level, respectively

Table 2: Carbonic anhydrase activity (EU $g^{-1}$ fresh tissue) and total chlorophyll content (mg $g^{-1}$ fresh tissue) in leaves and average root diameter (mm) of sweet corn plants in nutrient solution with different Zn and P levels

| Treatments (mg $L^{-1}$) | | 14 days | | | 28 days | | |
|---|---|---|---|---|---|---|---|
| Zn | P | Carbonic anhydrase (EU $g^{-1}$ fresh tissue) | Total chlorophyll content (mg $g^{-1}$ fresh tissue) | Average root diameter (mm) | Carbonic anhydrase (EU $g^{-1}$ fresh tissue) | Total chlorophyll content (EU $g^{-1}$ fresh tissue) | Average root (mm) |
| 0 | 0 | 513.0 | 0.798 | 0.478 | 268.0 | 1.466 | 0.352 |
| 0 | 80 | 353.0 | 0.442 | 0.495 | 117.0 | 1.091 | 0.337 |
| 20 | 0 | 1260.0 | 0.398 | 0.544 | 837.0 | 0.854 | 0.384 |
| 20 | 80 | 1098.0 | 0.671 | 0.494 | 754.0 | 0.930 | 0.284 |
| LSD (5%) | | 49.0 | 0.207 | 0.134 | 35.0 | 0.510 | 0.085 |
| P×Zn interaction | | ns | * | ns | * | ns | ns |

ns: Non significant, *Significant at the 5 level

and Brown (1968) and Millikan *et al.* (1968) demonstrated that symptoms of Zn deficiency in leaves was intensified with P application without any change in leaf Zn concentration. This intensification was correlated with P/Zn ratio, leading to the suggestion that enhancement of P concentrations in leaves caused a higher physiological Zn demand by inactivating Zn in some way (Olsen, 1972; Leece, 1978).

$Zn_0P_0$ treatment showed the highest and $Zn_{20}P_0$ treatment showed the lowest chlorophyll content at 14 and 28 DAT (Table 2). The lowest amount of chlorophyll content in $Zn_{20}P_0$ treatment was possibly caused by the interaction of Zn with Fe in growth medium. Iron (Fe) is required for the activity of ALA synthase which catalysis the first identified step of the tetrapyrrole biosynthetic pathway leading to chlorophyll formation. In $Zn_{20}P_{80}$ treatment, Zn and P form the insoluble complexes in the nutrient solution. P also can circumvent Zn in roots by the formation of Zn-phytate or may cause the binding of Zn within the root cells and therefore, reduce the interaction of Zn with Fe (Young, 1969). The results were in agreement with those of Alam and Shereen (2002) in wheat and Soltangheisi *et al.* (2013) in sweet corn. The interaction effect of P and Zn on chlorophyll content were also significant at 14 DAT ($p<0.05$). The chlorophyll content in $Zn_{20}P_{80}$ treatment was higher than $Zn_0P_{80}$ treatment at 14 DAT but there were not significantly different at 28 DAT.

Different levels of Zn and P did not have a significant effect on root parameters such as root length, surface area and root volume (data not shown). Average root diameter at 28 DAT was the only affected parameter by Zn and P application (Table 2). The $Zn_{20}P_0$ treatment had the highest and $Zn_{20}P_{80}$ treatment had the lowest average root diameter at 28 DAT. The differences between these two treatments with $Zn_0P_{80}$ and $Zn_0P_0$ treatments were not significant. Adriano *et al.* (1971) showed that high levels of P depressed corn seedling root growth when other nutrients were at standard levels. The reason why $Zn_{20}P_0$ treatment had the highest average root diameter is unknown and needs more investigation. P×Zn interaction did not have a significant effect on average root diameter at both harvesting times.

Carbonic anhydrase activity in leaves increased with Zn supply but reduced with P supply at both harvesting times (Table 2). Dell and Wilson (1989) observed a positive linear correlation between CA activity and the level of Zn supply and Zn concentrations in leaves of *E. maculate* and subterranean clover but not in the conditions of extreme Zn deficiency. Lopez-Millan *et al.* (2005) showed that with increasing total Zn tissue concentrations, carbonic anhydrase activity increased linearly and then reached a plateau in both leaves and roots of *Medicago truncatula*. In rice plants, Zn deficiency resulted in a decrease in the expression of mRNAs for CA, indicating that the decrease in CA activity by Zn deficiency is due to a reduced amount of the enzyme in C3 plants (Sasaki *et al.*, 1998). The CA activity reduced in a number of Zn deficient plant species (Wood and Sibly, 1952; Bar-Akiva and Lavon, 1969; Randall and Bouma, 1973; Ohki, 1978; Gibson and Leece, 1981; Snir, 1983; Gangwar *et al.*, 1989; Pandey and Sharma, 1989). Zinc can be removed from CA molecules in an irreversible reaction and therefore CA is identified as a source of Zn in this condition. Under conditions of acute Zn deficiency, CA activity is completely inhibited (Rengel, 1995).

Gibson and Leece (1981) reported that 10 day-old maize plants have a CA activity of greater than 1000 EU $g^{-1}$ f.w and less than 500 EU $g^{-1}$ f.w under conditions of sufficiency and deficiency, respectively. They reported that corresponding values for 30 day-old plants would be greater than 700-800 EU $g^{-1}$ f.w and less than 250 EU $g^{-1}$ f.w for healthy and deficient plants, respectively. In this experiment, CA activity of 353 and 117 EU $g^{-1}$ f.w was recorded in $Zn_0P_{80}$ treatment at 14 and 28 DAT, respectively. These values are at the deficiency level according to Gibson and Leece (1981) and are in agreement with P/Zn ratio results which showed that this treatment suffered from Zn deficiency, although Zn concentrations in leaves were within the sufficiency range. The P-Zn imbalance causes the yield reduction in

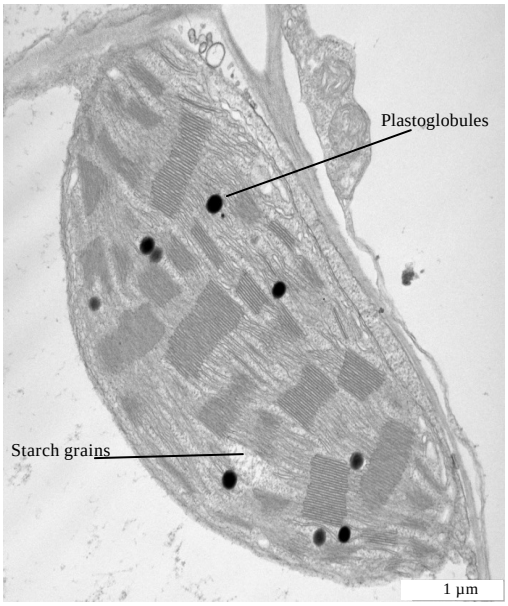


Fig. 1: Chloroplast from full-nutrient plant. Magnification: X20000

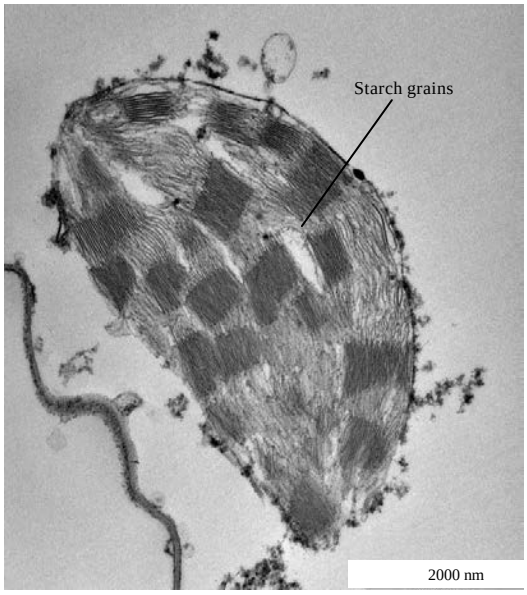


Fig. 2: Chloroplast from $Zn_0P_{80}$ treatment. Magnification: X17000

sweet corn plants although leaf Zn concentration remains at adequate levels for healthy growth (Soltangheisi *et al*., 2013). Cakmak and Marschner (1987) demonstrated that the total and physiologically active Zn are quantitatively different in leaves of cotton plants. Zn may be bound to cell walls or chelated by organic ligands and therefore, become physiologically inactivated in tissues (Lopez-Millan *et al*., 2005). In this experiment, most of the Zn in leaf tissue was physiologically inactive and this inactivation was intensified with P supply and, therefore, Zn leaf concentration overestimates the true nutritional status and is not well correlated with Zn nutritional status of corn plants. In such situations, determination of CA activity can be used as a biochemical assay to measure the physiologically-active component of total Zn. It can be stated that CA activity is more sensitive to the deficient levels of active Zn as compared to leaf Zn concentration and plant growth at these growth stages and exhibits the Zn deficiency before growth is affected. It has been suggested that CA activity is a better indicator for diagnosing Zn deficiency in sweet corn plants than leaf Zn concentration. The assay is easy to do and fast and needs the fresh leaf tissue. The interaction effect between P and Zn did not show any significant ffect on CA activity of corn plants at 14 DAT but this interaction effect was significant at 28 DAT ($p \leq 0.05$). It shows that the combined effect of P and Zn on CA activity was intensified with plant age.

Ultrastructural changes of all the treatments were examined at 14 and 28 DAT. At 14 DAT, the chloroplast fine structures were not different form full-nutrient chloroplasts (images not shown). However, after 28 DAT chloroplasts from each of the treatments showed distinct changes in fine structure and investigated here. The normal chloroplast of leaf cell which was treated with the basic nutrient solution was intact, in elliptical shape and with normal membrane which protect the chloroplast from its surroundings. Plastoglobules and starch grains were present in the chloroplasts (Fig. 1).

When the plants grown in the solution with only added P at a concentration of 80 mg $L^{-1}$ without adding external Zn, the chloroplast of the leaf cell appeared as is shown in Fig. 2. Chloroplasts are of regular ellipsoidal shape but starch deposits were larger and more extensive than the normal chloroplast and they did not contain plastoglobules. Kim and Wetzstein (2003) observed extensive starch deposits in Zn-deficient pecan leaves.

While the solution with only Zn added at a concentration of 20 mg $L^{-1}$ without applying external P, the chloroplast of the leaf cell was as shown in Fig. 3. In Fig. 3, the membrane of the chloroplast was ruptured and plastoglobules were not observed. The grana were also destroyed. On treatment with external P, the membrane had a same situation but grana were found in normal structure (Fig. 4). Plastoglobules were present but appeared to be smaller in size than in chloroplast from the full-nutrient corn plants.

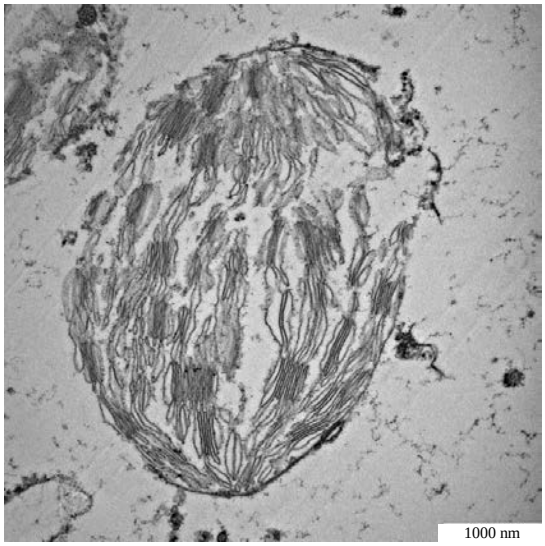


Fig. 3: Chloroplast from $Zn_{20}P_0$ treatment. Magnification: X20000

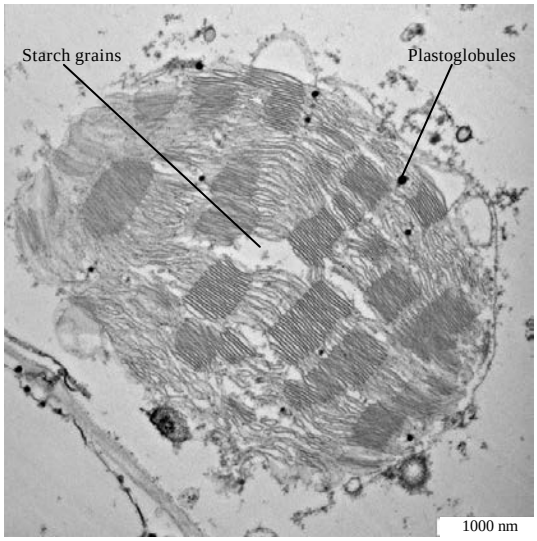


Fig. 4: Chloroplast from $Zn_{20}P_{80}$ treatment. Magnification: X20000

After the sweet corn plants had been treated by nutrient solution without Zn and P for 28 days (Fig. 5), the shape of the chloroplast was distorted from ellipsoidal shape and changed to irregular shape. The membrane of the chloroplast was disrupted in some parts, oversized starch grain was observed within the chloroplast and plastoglobules were not visible. Sharma (1983) in maize and Cakmak *et al.* (1989) in bean observed sucrose and starch accumulation in Zn-deficient leaves.

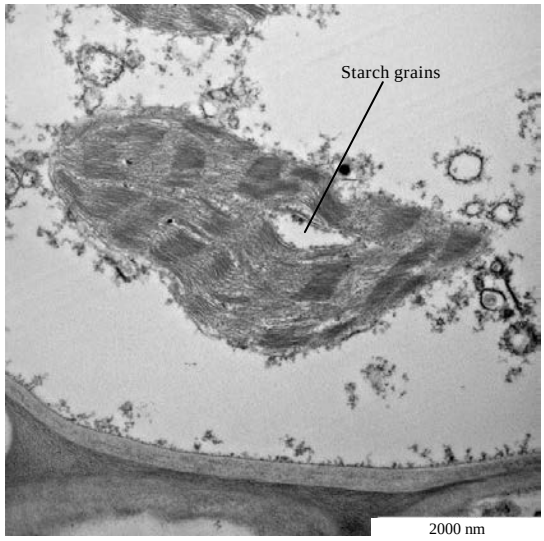


Fig. 5: Chloroplast from $Zn_0P_0$ treatment. Magnification: X17000

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