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Diagnosis of Copper Deficiency Through Growth, Nutrient Uptake and Some Biochemical Reactions in *Pisum sativum* L.

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Abstract: A water culture experiment was carried out on *Pisum sativum* L. (cv. Master lee) cultivar to test and compare the possible use of changes in growth (as dry weight), nutrient uptake, nutrient concentration shoot /root ratios, (super oxide dismutase SOD (EC. 1. 15. 1.1) and ascorbate oxidase AO (EC.1. 10. 3.3) activities, chlorophyll content, proton release mechanism and protein profiles as parameters for diagnosis of Cu deficiency. Copper was supplied in two levels (Zero- 1μmol) as $CuCl_2$. The results indicate that sufficient level of Cu caused increases in shoot and root dry weights as well as nutrient uptake. Mg, P, Fe, Mn and Cu concentration shoot/root ratios showed pronounced increases. In contrary the Zn shoot/root ratio was decreased. Under Cu deficient conditions, the activities of SOD and AO were reduced by 28 and 68% as well as proton release mechanism (71%). Moreover total chlorophyll was reduced by (49%) as a result of absence of Cu in the growth root medium. On the other hand protein profiles showed over expression of two high molecular weight bands at 94kD and 67kD with high intensity and one band at 43 kD were recorded. Therefore, study appreciated the possible use of proton release, AO level, chlorophyll content, Cu-specific changes in SDS-PAGE proteins and SOD level as rapid and sensitive biochemical assays to assess the Cu nutrition status in pea plants.

Key words: Cu deficiency, growth, nutrient uptake, SOD, AO activities, proton release and SDS-PAGE protein profiles in *Pisum sativum* L.

Introduction

Copper is an essential element in plant metabolism, in low micro molar concentrations (Loneragan, 1981). Copper deficiency is now known to be a significant cause of low yields for many crops (Owuoche *et al.*, 1994). Mostly copper deficiency in soils can be caused by abounding Cu in a form unavailable to plants (Clark, 1983). It was found that copper interferes with several physiological functions, as it is a component of several enzymes, mainly of those participating in redox system mechanisms. Proton release by intact roots has been found to be a good tool for diagnosing the Cu status in plants (Rayle and Cleland, 1977). Furthermore, (Del Rio *et al.*, 1978; Cakmak and Marschner, 1992; Cakmak *et al.*, 1995) recommended that SOD and AO levels can be used as biochemical assay to diagnose the Cu-deficiency and pigment changes were also found as good tool to diagnose Cu-deficiency in plants (Walker and Webb, 1981). The changes in protein synthesis are considered as a rapid and efficient assay to detect Cu deficiency (Delhaize *et al.*, 1986).

Therefore, the present study aimed to test and compare together some biochemical assays used for recognizing the Cu nutritional status in pea plants.

Materials and Methods

Plant growth conditions: A water culture experiment was carried out with pea under control conditions in the laboratory of the Program "Micronutrients and Other Plant Nutrition Problems in Egypt" National Research Center, Dokki, Cairo, Egypt. Seeds of pea (*Pisum sativum* L. cv. Master lee) were germinated in February 2001 in moistened sand for 7 days and then seedlings were transferred in plastic vessels containing full nutrient solution (FNS) of Hoagland and Arnon (1950) for 4 days. On 12th day seedlings were provided with two levels of Cu (Zero - 1μmol) as copper chloride. Seedlings grew at day/night temperature 25/20 °C, relative humidity about 60% with 16h light and 8h dark periods at light intensity 300-350 μM m^{-2}-sec^{-1}. Plants were harvested and prepared for nutrient analyses and enzyme activity determinations on 21st day.

Enzymatic extraction and determination: The activities of the Cu-containing enzymes super oxide dismutase (SOD) and ascorbate oxidase in control and Cu-deficient plants were determined in the extract of the 3rd leaf at fully mature stage. Leaf samples were ground with saccrose tris buffer (pH 7.8) in a cold mortar with ice. The homogenate was filtered through 4 layers of nylon cloth and centrifuged at 15000 rpm for 15 min. The resulting crude supernatant was used for enzyme determination.

Superoxide dismiutase (SOD) was assayed according to the photochemical method of Beauchamp and Fridovich (1971) as modified by Giannopolitis and Ries (1977). The activity (unit/mg protein/min) was measured by determining the amount of enzyme required producing 50% inhibition of the rate of NBT reduction. Protein concentration was measured by the method of Bradford (1976).

Ascorbate oxidase activity (AO) as (unit/mg protein/min) was measured spectrophotometrically as described by (Tono and Fujita, 1982). The decrease in absorbance at 265 nm (extinction coefficient of ascorbate: 7,000 cm^{-1} M^{-1}) was then monitored.

Chlorophyll was extracted by acetone 80%, according to Maclachlane and Zalik (1963).

Plant analyses: Samples were taken for growth measurements in terms of shoots and roots dry weight and the determination of nutrient concentrations was done according to Chapman and Pratt (1978).

Proton release: The pH change during experimental period was measured and the release of protons (net H^+ flux) was expressed in μ mol H^+g^{-1} Fw h^{-1} after subtraction from pH of the root free control reaction medium for the same period of time, using a pH meter, Jenway pH 3020 model.

Electrophoresis SDS -PAGE -protein profiles: Fresh leaf (0.5g) material was homogenized in 1 ml of sodium phosphate buffer (pH 6.8), and centrifuged for 10 min at 10000 rpm. The supernatant was used for protein electropherogram protein profiles according to Laemmli (1970) using 10% acrylamide in the separating gel and 3% in stacking gel. The separation was carried out using EC minigel unit at 60 V/4h. Gels were

stained with Coomassie Brilliant Blue R-250 and destained with 40% methanol in 10% acetic acid. Low molecular weight standard protein of Pharmacia 2 was electrophoresed along with the samples was used for the determination of molecular weights of the polypeptide.

Results and Discussion

Growth: Copper treatment (1μmol) led to both leaves and roots dry weight increases by 50 and 28% respectively (Fig. 1). Dry weight increase may attribute to the stimulation took place in the nutrient uptake and balance. As Cu plays a major role in photosynthesis as well as carbohydrates and nitrogen metabolism, its deficiency can induce lower contents of soluble carbohydrates (Brown and Clark, 1977), which lead to low dry biomass accumulation.

Nutrient uptake: Copper treatment (1μmol) found to enhance the uptake of P, Mg, Fe, Zn, Mn and Cu in pea plants (Fig. 2). In the presence of adequate copper as the plants were Cu-treated, most of P, Mg and Fe were migrated to the leaves. So, Mg, P, Fe and Cu shoot/root ratios were increased (Table 1). This may be explained by the ability of Cu to displace other nutrients from the roots, as it strongly bound in the root free space (Mengel and Kirkby, 1987). It was observed also that relatively high portion of both P and Mn was accumulated in roots in the absence of copper, while in Cu-presence, most of P and relatively higher portion of Mn is translocated to leaves. This may give clear evidence that copper plays a role in energizing the translocation and assimilation of these elements. On the other hand, a high portion of Zn was found to accumulate in the roots with 1μmol Cu in the growth root medium which may take place because of the known antagonism between the two elements (Bowen, 1969).

Enzymes activity: When the copper containing enzymes (super oxide dismutase and ascorbate oxidase) were tested, the Cu deficient leaves exhibited a decrease in AO activity

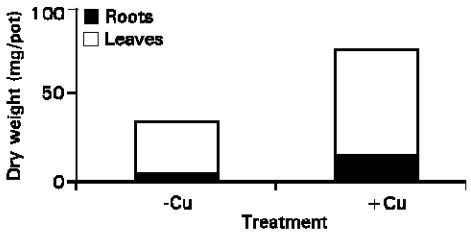

Fig. 1: *Pea* Root and leaf dry weight as affected by copper treatment

Table 1: Nutrient concentration shoot/root ratios in plants as affected by Cu deficiency

Nutrient s/r ratio	Mg	P	Fe	Mn	Zn	Cu
Zero Cu	0.37	0.32	0.80	0.04	0.61	0.11
1 μmol Cu	0.46	9.77	1.60	0.21	0.10	0.77

which corresponded well with Cu uptake (Fig. 3).
This demonstrated that Cu deficiency inhibits the biosynthesis of this enzyme and apo proteins of this metalloenzyme are either absent or cannot be reactivated even if Cu becomes available (Delhaize *et al.*, 1986). However under condition of Cu-deficiency SOD level exhibited less decrease (28%) than AO level (68%). This may be due to that SOD (Cu - Zn - SOD) plays an important role in detoxification of superoxide radicals (O_2^-) and the protection of membrane lipids against photosynthesis and respiration (Foyer *et al.*, 1994).

Chlorophyll content: Pea plants grown for 21 days at two levels of Cu (Zero and 1μmol) showed an decrease in total chlorophyll concentration from 2.43 mg. g^{-1}.FW under sufficient level to 1.24 mg g^{-1} Fw with deficient level (Fig. 4). The percentage decrease was 49% when Cu was absent. These results may suggest that Cu play an important role in regulation of the synthesis of leaf pigments (Horvath *et al.*, 1983).

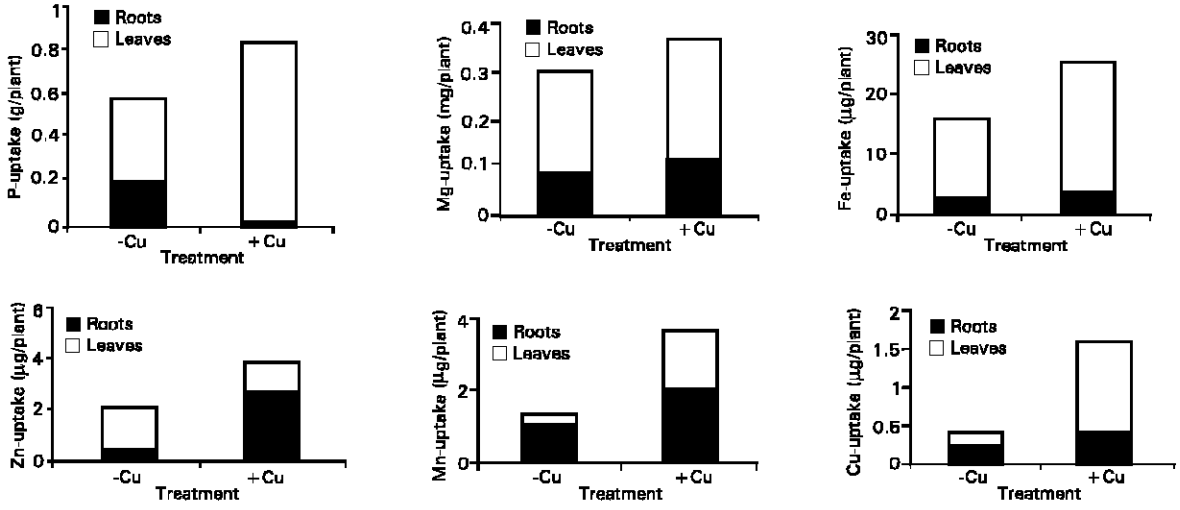

Fig. 2: Uptake of P, Mg, Fe, Zn, Mn and Cu by pea plants as affected by copper treatment.

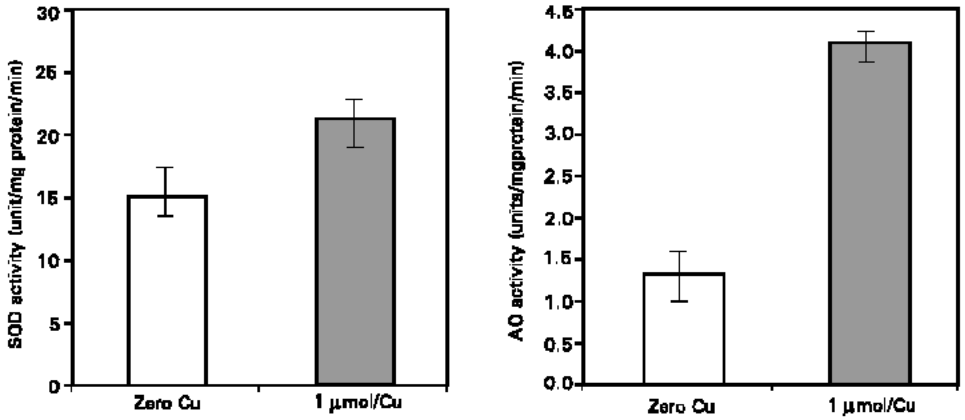

Fig.3: Changes in SOD and AO activities in leaves of pea, 21-days old as affected by Cu deficiency. Bars indicate ±SD with n = 3

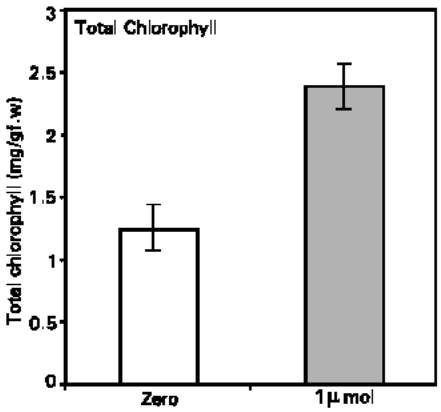

Fig. 4: Changes in total chlorophyll in leaves of pea 21-day old as affected by Cu deficiency. Bars indicate ±SD with n = 3

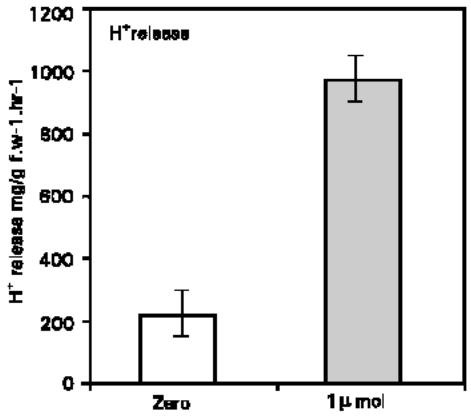

Fig. 5: Changes in proton release by roots of pea 21-days old as affected by Cu deficiency. Bars indicate ± SD with n = 3

As was observed the reduction of total chlorophyll due to Cu deficiency confirmed the results of (Ayala and Sandman, 1988). They showed that in pea leaves with varying Cu

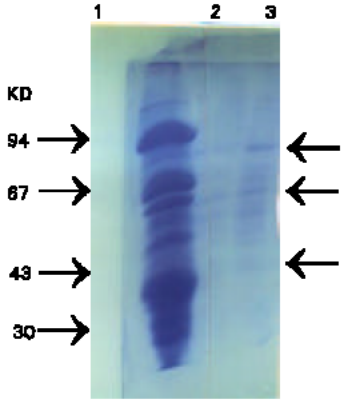

Fig. 6: Effect of Cu nutrition on the profile of proteins. Protein (50µl) were separated by SDS-PAGE on gels then stained with Commassie Blue R-250. Lane-1, mol. Wt standard, lane-2, protein extracted from leaves under deficient level of Cu (zero) and lane-3, protein extracted from leaves under sufficient level of Cu (1µmol). Proteins that stained more intensity in the efficient Cu treatment than in the deficient Cu treatment are marked with arrows.

nutritional status the activity of Cu containing proteins (such as: photosynthesis, respiration and detoxification of free radicals were drastically reduced with Cu-deficiency.

Proton release (H^+ extrusion) mechanism: As was observed in Fig. (5), when copper was absent (zero), the amounts of protons which released by the intact roots reduced by (71%) as a result of decrease in plasma membrane - H^+ ATPase activity (as measured throughout proton extrusion) (Rayle and Cleland, 1977). As previously observed by several authors that H^+ATPase in the plasmalemma of root cells had a key role in H^+ release as a biochemical mechanism and added that the increase in proton pumps from the protoplast induces growth enhancement due to increase in the acidification of growth root medium leading to the loss of cell wall and increased the availability of Cu and other nutrients through cell wall (Schubert, 1995).

SDS page protein patterns of pea plants as affected by copper nutrition: Protein profiles that resulted from SDS - PAGE on gels also showed that there was no major difference in the distribution of proteins. Over expression of two high molecular weight bands at 94kD and 67 kD with high intensity were detected (Fig. 6). One band 43 kD was also recorded. *In vivo* profiles showed that the effects of Cu on protein synthesis affect only few proteins and perhaps some others not resolved by single dimensional gel. In this context, Cherry (1989) reported that when plants exposed to different stress conditions some metabolic and physiological changes occurred during stress, condition. Moreover, the results of present investigation were quiet similar to those of Delhaize *et al.* (1986), who concluded that SDS -PAGE protein can also be used to detect copper deficiency.

Based on the obtained results which support to the previous findings of Babalakova *et al.* (1993) and Schubert (1995) that Cu is responsible for the observed effects on activation of proton pumps and antioxidant activity.

It can be concluded that copper deficiency causes greater biochemical changes in pea seedlings. So, this study suggests that the content of proton release, level of AO, chlorophyll content, and protein profiles as well as SOD level can be used as rapid and sensitive techniques for diagnosing Cu deficiency.

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