Egyptian Journal of Biology, 2004, Vol. 6, pp 62-71
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# Protective systems against active oxygen species in Spinach: response to high light stress and Mg-deficiency.

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**ABSTRACT**

The influence of different light intensities (250, 500 &1000 W/m$^2$) and sufficient and deficient magnesium supplies on the antioxidant defense system were studied in *Spinacia oleracea* plants. It was found that high light intensity in combination with Mg-deficiency induced significant decrease in dry matter and significant increases in total carbohydrates and anthocyanin contents. However, significant decreases in aminolevulinic acid (ALA), Chl.a&b and carotenoids were recorded. Lipid peroxidation and electrolyte leakage were significantly increased as a response to high light stress and Mg deficiency. On the other hand, ASPX & CuZn-SOD activities and glutathione content were significantly increased while catalase was slightly decreased under high light stress with both sufficient and deficient magnesium. It could be concluded that high light intensity and Mg-deficiency trigger the antioxidant defense system of *Spinacia oleracea* plant to oppose the harmful effect of free radical damage.

**Keywords**: Antioxidants- high light stress- Mg-deficiency- *Spinacia oleracea*

## INTRODUCTION

Light is a critical environmental signal that affects nearly every aspect of plant development, including germination, seedling morphogenesis, and floral initiation (Pepper *et al.* 2001). Under conditions of mineral deficiency, the amount of excess light that a plant absorbs can increase due to stress-induced decreases in the capacity of photosynthesis (Verhoeven *et al*. 1997). Excess absorbed light energy can lead to the formation of dangerous oxygen radicals (Müller *et al*. 2001). The production of superoxide radical ($O_2^{\cdot}$) can induce lipid peroxidation and oxidation of proteins and nucleic acids.

As protection against toxic $O_2$ species, chloroplasts are equipped with several antioxidants and defense enzymes such as superoxide dismutase (SOD), ascorbic acid peroxidase and glutathione (Jackson *et al*. 1978).

Many metabolic processes are directly affected by Mg, including enzymatic reactions that depend on the presence of Mg (Marschner 1995). Photosynthesis, net assimilation and transpiration rates are decreased under Mg-deficiency. (Cao and Tabbits 1992). Magnesium deficiency in plants often results in ultrastructure changes (Pucch & Mehn-Jakobs 1997), especially in the chloroplast, well before visible foliage symptoms are obvious. This is accompanied by impairment of photosynthesis (Sun & Payn 1999).

In response to light, etiolated seedlings of some sorghum (*Sorghum bicolor*) cultivars accumulate anthocyanin pigments in epidermal tissue of the mesocotyl (Orczyk *et al*. 1996; Weiergang *et al*. 1996). Because they are photoinduced, researchers surmise that anthocyanins would have a photoprotective function, either against light-induced photooxidation or against UV-B damage (Chalker-Scott 1999). Anthocyanins protect against damaging levels of light, especially high-energy blue wavelengths that can damage protochlorophyll in developing leaves (Drumm-Herrel 1984). The levels of antioxidant

enzymes such as superoxide dismutase showed either slight increases or actually decreased in conjunction with photoinduced anthocyanin production in *Mahonia repens* (Grace *et al.* 1995).

It was found that Mg depletion induced decreases in chlorophyll and leaf protein content (Anza & Riga 2002). They also mentioned that glutathione reductase (GR), (SOD) and ascorbic acid peroxidase (ASPX) activities were increased in magnesium deficient than in magnesium sufficient leaves. The photosynthetic apparatus and pigments should be most susceptible to excessive irradiance and Mg deficiency. The present study hypothesized that growing under conditions of high light intensity and Mg-deficiency may induce specific mechanisms to protect the photosynthetic apparatus. Therefore, as an initial approach to this problem, the study examined some compounds of the water-water cycle (ASPX, CuZn-SOD, glutathione) and carotenoids and anthocyanin pigments as photoprotective pigments.

## MATERIALS AND METHODS

Spinach seeds (*Spinacia oleracea*) were germinated in plastic pots half-filled with acid washed sandy soils and irrigated with 0.25 strength Hoagland solution. After 2 weeks, plants were transferred for growing under controlled conditions. The growth conditions were 12/12h (light/dark), 27/22 ˚C day/night. They received different light intensities: 250, 500 and 1000 W/m$^2$. This was referred to as low, medium and high light, respectively. Under each light intensity, pots were divided to 2 sub-groups. One of which irrigated with half-strength Hoagland nutrient solution (Mg-sufficient), and the other was irrigated with the same solution without magnesium (Mg-deficient).

Plants were harvested after 7 and 14 d growth under the controlled conditions. At harvest, plants were separated for the different analyses (dried at 70ºC for determination of dry weight and carbohydrates), or freezed for the analyses of enzymes, pigments and protein. Weekly productivity was calculated by subtracting the 1$^{st}$ week data from those of the 2$^{nd}$ week's.

**Total carbohydrate and Total protein contents:** Total carbohydrates were extracted with 2.5 N HCl. The samples were analyzed by the method of Hedge & Hofreiter (1962) using glucose as a standard. Total protein was extracted and analyzed by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

**Estimation of 5-aminolevulinic acid (ALA):** Samples of leaves material were extracted in 1 ml 4% (w/v) Trichloroacetic acid at 100°C for 15 min, according to the method of Stobart & Ameen-Bukhari (1984). The concentration of ALA was determined using a calculated molar extinction value of 7.24x10$^4$.

**Estimation of pigments**: The pigments were extracted in 80% chilled acetone. The amounts of total chlorophylls a&b and carotenoids were estimated spectrophotometrically according to Lichtenthaler (1987).

**Anthocyanin**: Seedling leaves were homogenized in a mixture composed of 350 µL of 18% 1-propanol, 1% HCl, and 81% water. The amount of anthocyanins in the resulting extract was quantified spectrophotometrically. The values are reported as $A_{535} - 0.25\ (A_{650})$ /g fresh weight (Lange *et al*. 1970).

**Lipid peroxidation as accumulation of malondialdehyde (MDA) and electrolyte leakage**: Estimation of lipid peroxidation was assessed spectrophotometrically using TBA-MDA assay (Hodges *et al*. 1999). The amount of MDA was estimated by using extinction coefficient of 155 mM/cm. Measurement of electrolytes leakage from spinach leaves was performed as described by Panavas *et al*. (1998). One g leaves was incubated in 50 ml double distilled water for 1 h under gentle shaking. This solution was tested for

sample conductivity. Then, the leaves were boiled in 50 ml bi-distilled water for 5 min, and this solution was measured to obtain subtotal conductivity. Membrane leakage is represented by the relative conductivity, which was calculated as sample conductivity divided by total conductivity (the sum of sample conductivity and subtotal conductivity).

**Antioxidant enzymes and glutathione content**: Enzyme extracts were prepared by homogenizing spinach leaves in a prechilled mortar in 20 ml chilled extraction buffer (pH 7.5). Extracts were then centrifuged at 6000 rpm for 20 min at 5 °C. Enzyme assays were conducted immediately following extraction. ASPX activity was determined using the method of Nakano & Asada (1987). Activity was determined by following the $H_2O_2$-dependent decomposition of ascorbate at 290 nm. Catalase activity was determined by following the decomposition of $H_2O_2$ at 240 nm (Aebi 1983). SOD was measured following the photochemical method, as described by Giannopolitis & Ries (1977). Assays were carried out under illumination. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of ρ–nitro blue tetrazolium chloride reduction at 560. Total glutathione content was determined spectrophotometrically following the method described by Griffith (1980).

**Statistical analysis**: Analysis of variance (ANOVA) and Pearson correlation test were performed with all data using SPSS program (version 8.0).

## RESULTS

**Dry matter**: The data in Fig. (1,a) show that increasing light intensity significantly decreased the weekly production of dry matter in the Mg-sufficient {$F(5,12) = 892.94$, $p< 0.01$} and the Mg-deficient plants {$F(5,12) = 819.23$, $p<0.01$}.

Pearson correlation test revealed a highly significant negative correlation between dry matter accumulation and light intensity {$r(6) = -0.975$, $p<0.01$} and between dry matter and Mg deficiency {$r(6)= -0.999$, $p<0.01$}.

**Total Carbohydrate content**: Increasing light intensity induced significant enhancement in the weekly production of carbohydrate contents in the Mg-sufficient treatment. However, the combination between Mg-deficiency and increased light intensity showed a non-significant decrease in the weekly production of carbohydrates (Fig. 1,b). There was a significant negative correlation between carbohydrate contents and Mg-deficiency {$r(6)= -0.990$, $p<0.01$}. On the other hand, a non-significant correlation was observed between the carbohydrate content and elevation of light intensity.

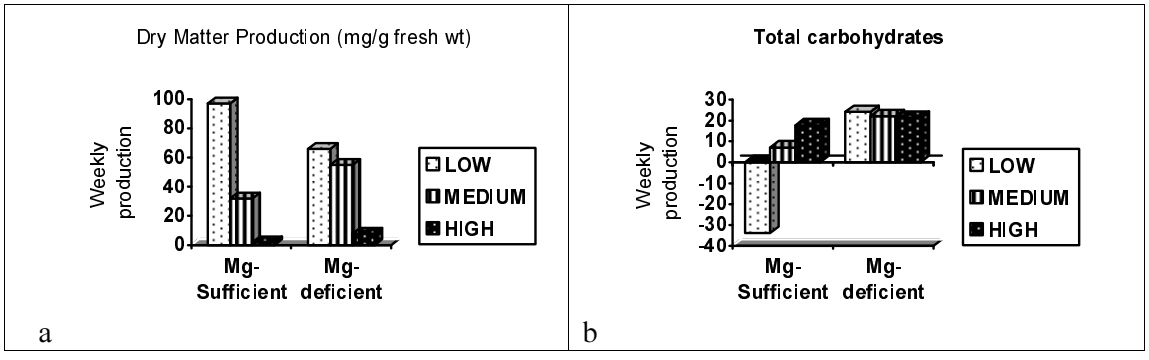


Figure 1: Effect of different light intensities (250, 500 and 1000 W/m$^2$) with sufficient and deficient magnesium supplies on the weekly production of dry matter and total carbohydrates content of spinach plants.

**Protein (Fig. 2,a)**: The data obtained showed that, weekly production of protein was significantly decreased in Mg-deficient leaves with increase of light intensity {F(5,12)= 24.98, $p$<0.01}.

**Aminolevulinic acid (ALA) content:** Fig. (2,b) shows that ALA content was significantly increased at high light intensity in Mg-sufficient {F(5,12)= 9670.9, p<0.01}. In the Mg-deficient plants, increasing light intensity caused significant decrease of the weekly production of ALA {F(5,12)= 9670.9, $p$<0.01}. The results also revealed a significant negative correlation between ALA content and increasing light intensity {r(6)= -0.998, $p$<0.01}, and between ALA and Mg-deficiency {r(6)= -0.995, $p$<0.01}.

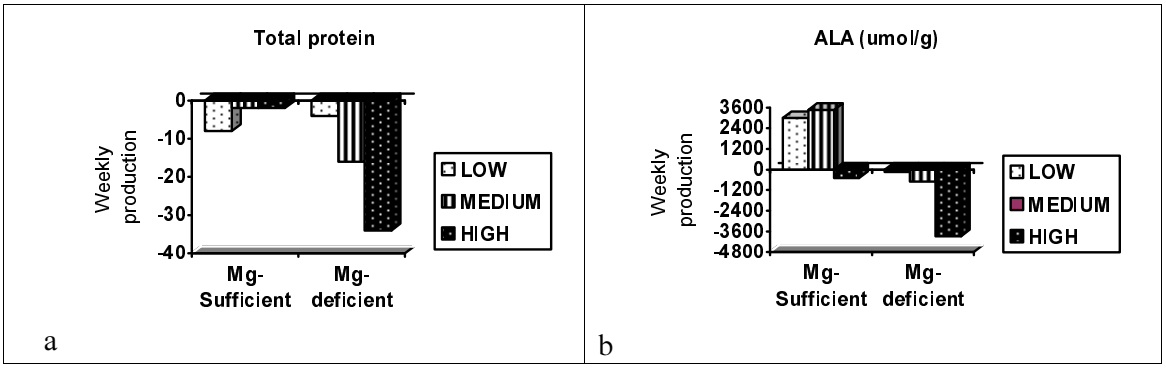


**Figure** 2: Effect of different light intensities (250, 500 and 1000 W/m$^2$) with sufficient and deficient magnesium supplies on the weekly production of total protein, aminolevulinic acid contents of spinach plants.

Production of chlorophyll a & b significantly decreased with increasing light intensity in both the Mg treatments (Fig 3).

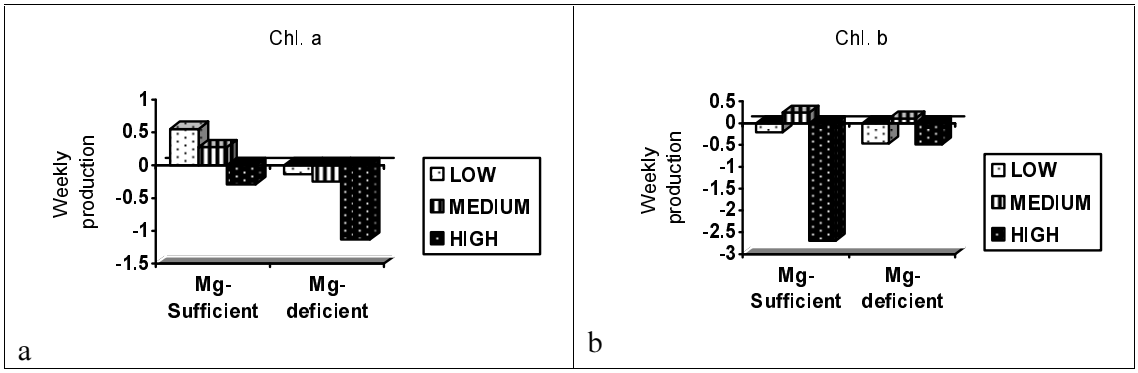


Figure 3: Effect of different light intensities (250, 500 and 1000 W/m$^2$) with sufficient and deficient magnesium supplies on the weekly production of chlorophyll a and b concentration of spinach plants.

Magnesium deficiency induced a highly significant increase in anthocyanin concentration (Fig. 4a), highest at high-light treatment. High light intensity appeared to induce a strong decrease in carotenoid concentration (Fig. 4b).

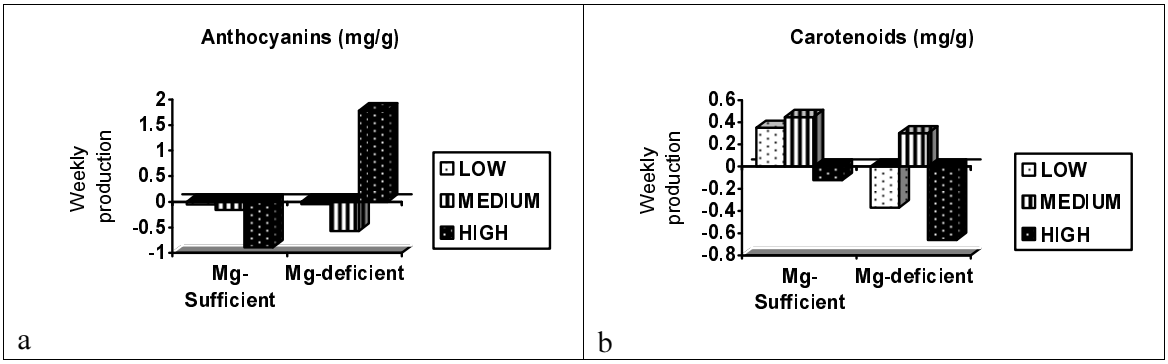


Figure 4: Effect of different light intensities (250, 500 and 1000 W/m$^2$) with sufficient and deficient magnesium supplies on the weekly production of anthocyanin and carotenoids concentration of spinach plants.

## Free radical damage

Production of lipid peroxidation (Fig. 5 a&b) reaches its highest value in the Mg-deficient and high light intensity treated plants.

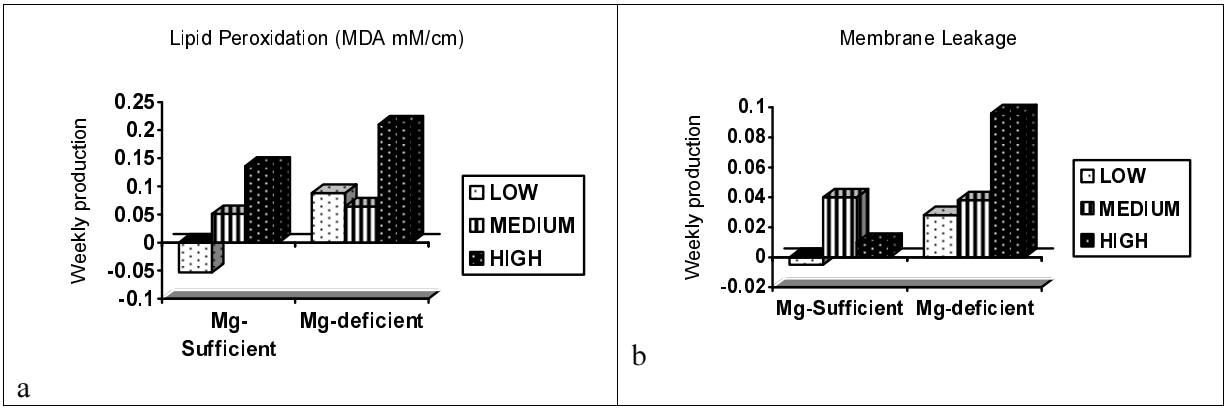


Figure 5: Effect of different light intensities (250, 500 and 1000 W/m$^2$) with sufficient and deficient magnesium supplies on the weekly production of lipid peroxidation and membrane leakage of spinach plants.

As light intensity increased, a significant increase in electrolyte leakage was observed in the Mg-deficient plants.

**Antioxidant enzymes and glutathione content (Fig. 6, a&b and 7, a&b)**:

The combination between increasing light intensity and magnesium deficiency treatments induced a highly significant increase in ASPX activity {$F(1,4)= 4146.19$, $p<0.01$} and in SOD activity {$F(1,4)= 3741.30$, $p<0.01$) while they induced a significant decrease in catalase activity. There was a non significant change in the weekly production of catalase activity in all treatments.

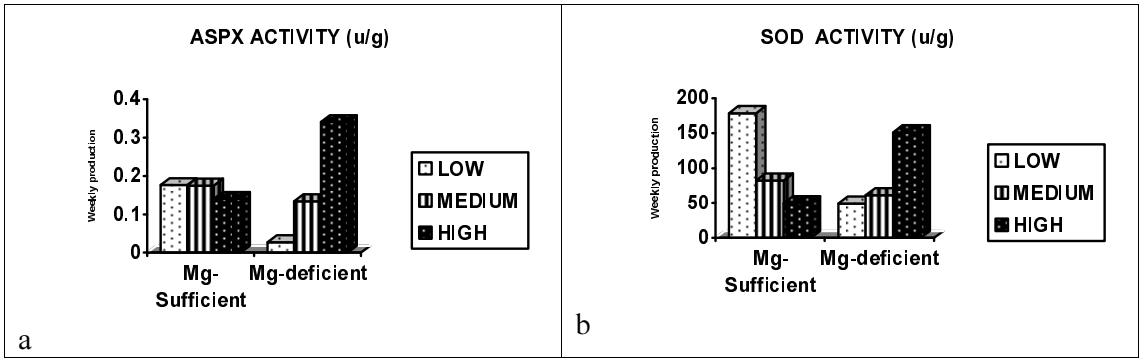


Figure 6: Effect of different light intensities (250, 500 and 1000 W/m$^2$) with sufficient and deficient magnesium supplies on the weekly production of ASPX and SOD activities of spinach plants.

The weekly production of glutathione content was significantly increased as light intensity increased in both the Mg-sufficient and the Mg-deficient plants. Meanwhile, there was a highly significant positive correlation between glutathione content and increasing light intensity {r(6)= 1.000, $p$<0.01}.

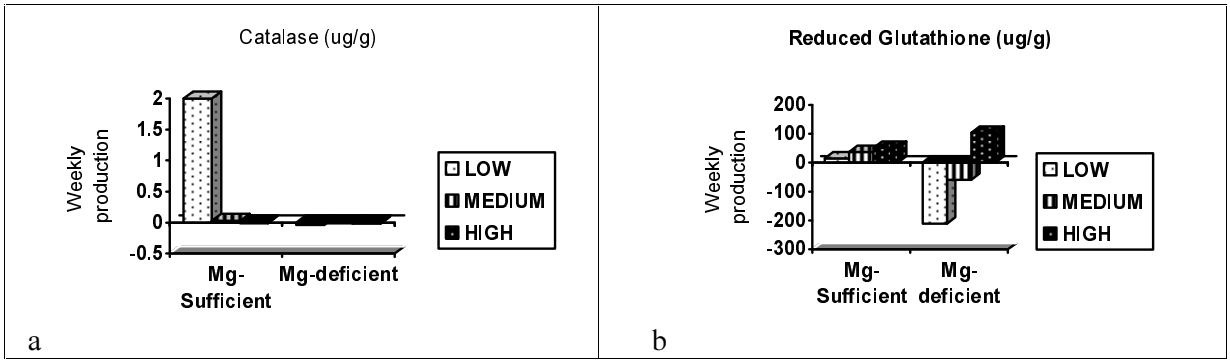


Figure 7: Effect of different light intensities (250, 500 and 1000 W/m$^2$) with sufficient and deficient magnesium supplies on the weekly production of catatlase activity and reduced glutathione content of spinach plants.

## DISSCUSION

The accumulation of carbohydrates in the Mg-sufficient and high light intensity treated spinach plants (Fig. 1) could arise as a result of either a malfunction of phloem loading, or a reduced demand for carbohydrates in growing tissues that depend on assimilates such as roots, trunks or developing shoots (Rufty *et al*. 1988). Nevertheless, growth reductions observed in Mg-deficient plants, indicating that carbohydrate accumulation in assimilating leaves is closely associated with reduced growth rates (Jakobs 1995). A deficiency in Mg nutrition can affect carbon metabolism in many ways causing reductions of specific carbohydrates. Because Mg is a constituent of the chlorophyll molecule and is also a cofactor of a series of enzymes involved in carbon fixation, it is essential for photosynthesis (Laing & Christeller 1976). Magnesium is also required for ATP metabolism and is, therefore, essential for many metabolic processes, including carbohydrate metabolism and the synthesis of proteins, fats and nucleic acids. It is also a key element in membrane transport (Marschner 1986).

The data of the present study showed a significant decrease in chlorophylls a&b and carotenoid concentrations (Figures 3&4). These results are in agreement with those of Abadia (1992) who showed that chlorophyll and carotenoids were simultaneaously decreased as a result of Fe- and Mg- deficiencies. Moreover, light mig ht regulate ALA synthesis, and hence chlorophyll formation (Ilag *et al*. 1994). So the decrease in chlorophyll content as a result of high light intensity and MG-deficiency might be due to the decrease in ALA content (Fig. 2). Carotenoids play a key role in the protection of photosynthetic organisms against the toxic effects of light. They are able to quench triplet excited chlorophyll molecule $^3$3Chl and singlet oxygen $^1O_2$ (Frank & Cogdell 1996). Carotenoids protect chlorophylls from photooxidative degradation (Ghoudhury *et al*. 1994). During the protective action, they themselves get degraded. The primary target of high irradiance induced damage is PSII reaction center, where electron transfer between the primary electron donor and the secondary plastoquinone acceptor occurs (Barber *et al*. 1997). Because carotenoids protect Chlorophyll from photooxidative degradation, the decrease in carotenoids may cause a decrease in chlorophyll content.

Anthocyanin pigments accumulated in response to light and magnesium deficiency in the seedlings of plants under investigation (Fig. 4). This accumulation might be preceded by increased transcription of genes encoding enzymes in the anthocyanin-biosynthetic pathway. A view that has emerged from various photobiological, biochemical and genetic studies is that transcriptional control of the biosynthetic enzymes accounts for the effects of light on anthocyanin accumulation (Mol *et al*. 1996).

The results obtained in the present work showed that increasing light intensity and decreasing magnesium supply enhanced lipid peroxidation and electrolyte leakage (Fig. 1 a&b). Oxygen is always available around functional PSII which could be the mediator of free radical reactions and might be involved in the peroxidative degradation of polyunsaturated fatty acyl residues of the thylakoid lipids (Niyogi 1999). Thylakoid lipids are susceptible to fatty acid oxidation due to the predominance of lipids with unsaturated fatty acids (Gounaries *et al*. 1986). Moreover, the quality, quantity and orientation of thylakoid membranes, especially large molecules such as pigment-protein complexes, also differ greatly under Fe- and Mg- deficiency (Lu *et al*. 1995).

The increase of ASPX, CuZn-SOD activities and glutathione content in plants under high light intensity and Mg-deficiency treatment were accompanied with a decrease of catalase activity (Figures 6&7). The increases in antioxidatve defence mechanisms indicate that Mg-deficiency induced enhancement of production of toxic $O_2$ species (Elstner *et al*. 1988). However, because CuZn-SOD indirectly protects chloroplastic membranes from lipid peroxidation (Larson 1988), it was suggested that CuZn-SOD activity is high enough to cope with the predicted increase in superoxide production at high light intensity and Mg-deficiency. A similar response of CuZn-SOD activity to environmental changes has also been reported under other potentially photoinhibitory conditions including cold acclimation (Badiani *et al*. 1993a) and water stress (Moran *et al*. 1994). Dismutation of superoxide generates $H_2O_2$ which is removed by an ASPX with ascorbate as cosubstrate (Asada 1992). Moreover, Badiani *et al*. (1993b) found that monodehydroascorbate reductase (MDHAR) activity was 10 to 20 times higher than the activities of glutathione reductase (GR) and dehydroascorbate reductase (DHAR).

High activities of the enzymes of the antioxidant system, namely CuZn-SOD and ASPX, observed in the present study, suggested that these enzymes catalyze the catabolism of the oxygen reactive species that arise under the stressful conditions of high light and Mg-deficiency. The recorded changes in glutathione contents might also indirectly conclude increased activities of the enzymes involved in the regeneration of the reduced state of glutathione, which provides an additional mechanism for energy dissipation during the

high light stress. This is well documented for such protective mechanisms, induced under stress conditions that stimulate production of toxic $O_2$ species (Elstner *et al*. 1988). The enhancements in activities of antioxidative enzymes by Mg-deficiency most probably take place in chloroplasts where SOD and $H_2O_2$-detoxifying enzymatiic cycle are predominantly located (Gillham & Dodge 1986).

Thus, it might be concluded that high light intensity in combination with magnesium deficiency triggers the antioxidant defense system in *Spinacia oleracea* plants by increasing anthocyanin, CuZn-SOD&ASPX activities and glutathione content. The increase in these antioxidants may represent the earliest cell response under high light stress and Mg deficiency.

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## الملخص العربى

**أنظمة الحماية ضد أنواع الأكسيجين النشط فى السبانخ: الاستجابة لإجهاد الضوء العالى ونقص الماغنسيوم.**

**دينا زين العابدين عبد القادر صالح**
**قسم النبات- كلية العلوم- جامعة قناة السويس.**

تم دراسة التأثير المشترك لاختلاف كل من شدة الاستضاءة (٢٥٠, ٥٠٠, ١٠٠٠ وات/متر٢) ونقص الماغنسيوم على بعض مضادات الاكسدة فى نبات السبانخ. وقد وجد أن نقص الماغنسيوم وشدة الاستضاءة العالية (١٠٠٠) قد تسببا فى نقص معنوى فى محتوى الانثوسيانين و ALA وكلوروفيل أ , ب والكاروتين. بينما تسببا فى زيادة معنوية لفوق اكسدة الدهون وارتشاح الالكتروليتات, وبالنسبة لبعض مضادات الاكسدة المتمثلة فى انزيمات ASPX, CuZn-SOD ومحتوى الجلوتاثيون فقد زادت زيادة معنوية بينما نقص نشاط انزيم الكاتاليز. ويمكن القول بأن تعرض نبات الى نقص فى عنصر الماغنسيوم الى جانب شدة الاستضاءة العالية قد وجه عمليات الايض الى تحفيز نظام مضادات الاكسدة ليقاوم التأثير المدمر للشقوق الحرة الناتجة عن شدة الاسضاءة العالية.