Influence of Magnesium Deficiency on Induction of Activity of Antioxidative Enzymes and Increased Tolerance to Active Oxygen-Generating Herbicides in Cucumber and Kidney Bean Plants*

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Abstract: The influence of a deficient ($7\mu\text{M}$) or sufficient ($1000\mu\text{M}$) supply of magnesium (Mg^{2+}) on the content of Mg^{2+} , chlorophyll and protein, and the activity of superoxide dismutase (SOD), ascorbate peroxidase (AP) and glutathione reductase (GR) were studied in cucumber (*Cucumis sativus* L.) and kidney bean (*Phaseolus vulgaris* L.) plants over an 11-day period. Tolerance of kidney bean to paraquat and oxyfluorfen dependent injury were also determined with intact primary leaves. Mg^{2+} content in the primary leaves of cucumber and kidney bean plants grown in nutrient solution with sufficient Mg^{2+} supply was increased, while that in the leaves of plants grown with insufficient Mg^{2+} was very low. Mg^{2+} deficiency caused more severe chlorosis and protein reduction in cucumber than in kidney bean. The activities of AP, GR and SOD of Mg^{2+} -deficient cucumber leaves were greatly enhanced compared to those of Mg^{2+} -sufficient leaves. In Mg^{2+} -deficient kidney bean leaves, AP and SOD activities were enhanced,

but GR activity was decreased. The results suggested that Mg^{2+} deficiency enhances the antioxidative enzymes in plants. Foliar application of paraquat and oxyfluorfen, active oxygen-generating herbicides, to kidney bean plants caused herbicidal damage. However, the chlorosis and water loss of kidney bean by the herbicides were much lower in Mg^{2+} -deficient leaves than in Mg^{2+} -sufficient ones. The data demonstrated that Mg^{2+} -deficient kidney bean plants became highly tolerant to paraquat and oxyfluorfen injury.

Key words: Mg^{2+} deficiency, antioxidative enzymes, paraquat, oxyfluorfen

Introduction

Oxygen molecules in the atmosphere are relatively stable, but when they come into contact with the metabolic pathway they can be transformed into active forms such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxy radical ($\cdot\text{OH}$) and singlet oxygen ($^1\text{O}_2$)²⁶⁾. These oxygen species are often called active oxygen. They interact with a wide range of biological molecules and cause oxidative damage. The plant response under extreme environmental conditions is known to be associated with free radicals or oxidative damage. These conditions are caused by herbicides^{2,23)}, air pollutants²²⁾, toxic metals⁶⁾, temperature extremes¹⁶⁾, salt stress²⁶⁾ and so

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forth. Plant species have acquired a kind of defensive ability to overcome oxidative stress in the course of evolution. Their defense mechanisms contain antioxidants such as Vitamin C, E, carotenoids and glutathione, and antioxidative enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (AP), glutathione reductase (GR) and catalase^{9,26)}

Magnesium is a main unit of chlorophyll. It also is a cofactor of chloroplast ribosome (a 70S ribosome) that is responsible for biosynthesis of large subunits of ribulose 1, 5-bisphosphate (RUBP) carboxylase¹³⁾. In Mg^{2+} -deficient chloroplasts, CO_2 fixation can be impaired by the inhibition of biosynthesis of RUBP carboxylase. This may lead to the generation of harmful active oxygen species by photosystem I and II.

It is known that paraquat, a bipyridylum herbicide, and oxyfluorfen, a photobleaching diphenyl ether herbicide, injure plants by photogeneration of toxic oxygen species^{1,8)}. The active oxygen species formed by paraquat⁷⁾ and oxyfluorfen⁸⁾ are potentially capable of peroxidizing cellular components such as chlorophyll and membrane lipids; however, plant species vary in their tolerance to paraquat^{5,25)} and oxyfluorfen¹⁹⁾. As plants have an antioxidative system composed of antioxidants and enzymes responsible for quenching the radicals and maintaining a protective redox state, different levels of antioxidants^{10,19)} or protective enzyme activities^{14,21)} among the species could be possible be factors determining their tolerance to the herbicides.

In this paper, as part of our attempt to understand the process of acclimation of plants to environmental stress, we studied the responses of O_2^- and H_2O_2 scavenging enzymes in cucumber and kidney bean plants grown with an insufficient Mg^{2+} supply.

Tolerance against paraquat and oxyfluorfen toxicity was also studied in kidney bean.

Materials and Methods

Plant growth

Cucumber (*Cucumis sativus* L.) and kidney bean (*Phaseolus vulgaris* L.) were grown in a controlled environmental chamber (light/dark regimes 14/10h at 27/22°C, relative humidity 65–75%) with light intensity of $250 \mu \text{Em}^{-2}\text{s}^{-1}$ provided by fluorescent and incandescent lamps. Seeds were germinated in vermiculite saturated with water. After 7 days, the seedlings were transferred to a nutrient solution. The nutrient solution was made by a slightly modified composition of that described by Cakmak and Marschner⁴⁾. Composition of the nutrient solution used was as follows: $0.88 \times 10^{-3}\text{M}$ K_2SO_4 ; $2 \times 10^{-3}\text{M}$ KCl ; $1 \times 10^{-5}\text{M}$ H_3BO_3 ; $4 \times 10^{-5}\text{M}$ $FeEDTA$; $1 \times 10^{-6}\text{M}$ $MnSO_4$; $1 \times 10^{-6}\text{M}$ $ZnSO_4$; $1 \times 10^{-7}\text{M}$ $CuSO_4$; $1 \times 10^{-8}\text{M}$ $(NH_4)_6MoO_{24}$; $1 \times 10^{-3}\text{M}$ Mg^{2+} (sufficient condition) or $7 \times 10^{-6}\text{M}$ Mg^{2+} (deficient condition) as $MgSO_4$. Plants were harvested 5 to 11 days after transfer to the nutrient solution. At harvest, primary leaves were separated and dried at 105°C for 24 hr to determine Mg^{2+} concentration. For analysis of enzyme activities and chlorophyll contents, the primary leaves were harvested and stored at -70°C . For paraquat and oxyfluorfen treatment, kidney bean plants grown for 11 days in nutrient solution with deficient and sufficient Mg^{2+} supply were used.

Enzyme determinations

About 0.5g of leaf material without the main midrib was ground with liquid nitrogen and homogenized with 5ml of 100mM potassium phosphate buffer (pH 7.0) containing 5mM ethylenediaminetetraacetic acid (EDTA), 1mM ascorbic acid (AsA) and 20% polyvinylpyrrolidone (PVP : w/v) with a

mortar and pestle¹⁹). The homogenate was centrifuged at 12,000g for 20min. The supernatant was used for determination of enzyme activities. All enzyme activities were measured at 25°C in a final volume of 1 ml using aliquots of the supernatants (25–50 μ l for ascorbate peroxidase (AP) ; 100 μ l for glutathione reductase (GR) ; and 25–100 μ l for superoxide dismutase (SOD)).

Activity of AP was measured by monitoring the rate of ascorbate oxidation at 290nm ($E = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$). The reaction mixture consisted of 50mM potassium phosphate buffer (pH 7.0), 0.1mM H_2O_2 , 0.5mM ascorbate, and the enzyme aliquot. Glutathione reductase was assayed according to the method of Foyer and Halliwell¹¹) by following the decrease in absorbance at 340nm due to NADPH oxidation ($E = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$). The reaction mixture contained 100mM potassium phosphate buffer (pH 7.8), 3mM MgCl_2 , 0.5mM GSSG, 0.2mM NADPH, and the enzyme aliquot. SOD was measured by the method described by McCord and Fridovich²⁰). Prior to assay, crude enzyme was dialyzed for 24 hr at 2°C. The assay mixture was comprised of 50mM potassium phosphate buffer (pH 7.8), 0.1mM xanthine monosodium salt, 10mM cytochrome C, and an aliquot of xanthine oxidase (XOD) which caused a 0.04 increment of the absorbance at 550nm during 120 sec, and the crude enzyme aliquot. One unit of SOD activity was defined the amount of enzyme to required to reduce 50% of the absorbance which was increased by XOD addition.

Herbicide treatments

The primary leaves of kidney bean plants were sprayed with 5ml of 300, 600 and 900 μ M paraquat containing 0.1% tween 20 or 40, 80 and 120 μ M oxyfluorfen dissolved in 30% acetone containing 0.2% tween 20. After the

treatments, surface of the leaves was dried in the dark, and they were then transferred to the growth chamber with light irradiation at 250 $\mu\text{Em}^{-2}\text{s}^{-1}$. Plant injury was determined after 24 hours for paraquat and 48 hours for oxyfluorfen treatment.

Analytical methods

The Mg^{2+} concentrations were determined by an inductively coupled plasma (ICP) spectrometer after grinding dried leaves with a mortar and pestle and extracting with 1N HCl for 12 hr. Concentrations of chlorophyll (a + b) were measured after extraction with dimethyl sulfoxide at 60°C for 12 hr as described by Hiscox and Israelstam¹⁵). Protein concentration was determined by the method of Bradford⁹).

Results and Discussion

Sufficient Mg^{2+} supply significantly increased dry matter production of root and shoots of cucumber and kidney bean plants, however, at the insufficient supply, their dry weight increments were slight (data not shown). Mg^{2+} concentration in the primary leaves of Mg^{2+} -sufficient plants was increased gradually for a given period (Fig. 1). In the leaves of those Mg^{2+} -deficient, however, the concentration was significantly lower than that of the Mg^{2+} -sufficient condition (only about 1/26 for cucumber and 1/23 for kidney bean plants at 11 days).

Chlorophyll diminution in the primary leaves by Mg^{2+} deficiency differed between plant species (Fig. 2). Visual injury (data not shown) and reduction of chlorophyll arose in cucumber plants after 5 days of growth in the nutrient solution with inadequate (7 μ M) Mg^{2+} supply. The symptoms appeared more rapidly than in kidney bean. In kidney bean plants, chlorophyll content was little affected by Mg^{2+} deficiency (Fig. 2(B)), and inter-

Fig. 1. Concentration of Mg in the primary leaves of cucumber and kidney bean plants grown in nutrient solutions with sufficient ($1000\mu\text{M}$) and deficient ($7\mu\text{M}$) Mg supply. Each data point represents the mean of three replicates. Bars indicate SE.

Fig. 2. Concentration of chlorophyll in the primary leaves of cucumber and kidney bean plants grown in nutrient solutions with sufficient ($1000\mu\text{M}$) and deficient ($7\mu\text{M}$) Mg supply. Each data point represents the mean of three replicates. Bars indicate SE.

veinal chlorosis and rugged or rugous surface of the primary leaf appeared with time (data not shown). Regardless of Mg^{2+} status, protein concentration of the tested plant species decreased with time (Fig. 3). The protein content was remarkably lower in Mg^{2+} -deficient cucumber leaves than in Mg^{2+} -sufficient ones (Fig. 3(A)): the concentration was 77% after 5 days and 57% after 11 days compared to leaves grown in nutrient solution with Mg^{2+} sufficient supply. The protein content of kidney bean plants, however, was not reduced by Mg^{2+} deficiency (Fig. 3(B)).

Effects of Mg^{2+} supply on antioxidative enzyme activities of cucumber and kidney bean plants are shown in Fig. 4. The activities of antioxidative enzymes in the leaves of both plant species except GR in kidney bean plants increased greatly with time in Mg^{2+} -deficient condition. After 11 days, AP, GR and SOD activities in cucumber were 3.6, 2.8 and 3.9 times higher in leaves of Mg^{2+} -defi-

cient than Mg^{2+} -sufficient plants, respectively. In kidney bean, AP and SOD activities were 3.7 and 2.1 times higher in Mg^{2+} -deficient plants than Mg^{2+} -sufficient plants, respectively. However GR activity of Mg^{2+} -deficient plants was decreased to 62% compared to that of Mg^{2+} -sufficient leaves.

Since kidney bean showed less chlorosis than cucumber by Mg^{2+} deficiency, it was used as test plant species to evaluate of herbicidal damage. The extent of chlorosis in the primary leaves clearly indicated that the tolerance of kidney bean plants to paraquat and oxyfluorfen was increased greatly by Mg^{2+} deficiency (Figs. 5 and 6). After being sprayed with paraquat or oxyfluorfen, Mg^{2+} -sufficient leaves showed intensive chlorosis with increasing herbicide concentration, whereas in Mg^{2+} -deficient leaves, the chlorosis was remarkably diminished (Fig. 5). Toxic oxygen species produced by paraquat¹⁾ and oxyfluorfen⁸⁾ attack unsaturated fatty

Fig. 3. Changes of protein contents in the primary leaves of cucumber and kidney bean plants grown in nutrient solutions with sufficient ($1000\mu M$) and deficient ($7\mu M$) Mg supply. Each data point represents the mean of three replicates. Bars indicate SE.

Fig. 4. Activities of ascorbate peroxidase, glutathione reductase and superoxide dismutase in the primary leaves of cucumber and kidney bean plants grown in nutrient solutions with sufficient (1000μM) and deficient (7μM) Mg supply. Each data point represents the mean of three replicates. Bars indicate SE.

acids in the plant cell membrane, and initiate a chain reaction of lipid peroxidation⁵. Disintegration of plasmalemma by attacks of the herbicide-generated active oxygen species is

accompanied by loss of water¹⁸ and electrolyte¹⁷ from cells. The phytotoxic effect of paraquat and oxyfluorfen was evaluated by water loss (Fig. 6). Prior to herbicide treat-

Fig. 5. Leaf injury by paraquat (A) and oxyfluorfen (B) in kidney bean plants grown in Mg-sufficient and deficient nutrient solutions for 11 days. Paraquat or oxyfluorfen solution was sprayed on primary 11 day old leaves, and the plants were kept under light for 24 or 48 hours, respectively.

Fig. 6. Water loss of primary leaves by paraquat and oxyfluorfen spray to intact kidney bean plants grown in Mg-sufficient ($1000\mu\text{M}$) and deficient ($7\mu\text{M}$) nutrient solution for 11 days. The indices are calculated by the formula, $100 - (\text{water content (\%)} \text{ of treated leaves} / \text{water content (\%)} \text{ of untreated leaves})$. Each data point represents the mean of three replicates. Bars indicate SE.

ment, there was no difference in water content between Mg^{2+} -sufficient and Mg^{2+} -deficient plants (data not shown). Water loss increased with herbicide concentration, which became more severe in Mg^{2+} -sufficient than in Mg^{2+} -deficient leaves. In treatment with 300 to $900\mu\text{M}$ paraquat, the water loss index increased from 15.3 to 34.2 in Mg^{2+} -sufficient leaves, although only from 0.4 to 18.9 in Mg^{2+} -deficient leaves. With oxyfluorfen treatments, water loss index was 66 in Mg^{2+} -sufficient leaves, but only 11 in Mg^{2+} -deficient leaves at $120\mu\text{M}$ concentration. Thus herbicidal injury was much greater in Mg^{2+} -sufficient leaves than Mg^{2+} -deficient leaves.

In cucumber, there was a rapid increase in the activities of all tested antioxidative enzymes with Mg^{2+} deficiency (Fig. 4), and in kidney bean, the activities of AP and SOD increased with Mg^{2+} deficiency (Fig. 4). However, the activity of GR in Mg^{2+} -deficient kidney bean plants decreased with time. Although Cakmak and Marscher⁴⁾ reported

that GR activity of kidney bean plants was enhanced by Mg^{2+} deficiency, our data indicated that the enzyme activity of those plants rapidly decreased. Since GR was made invalid by dialysis and was reactivated by Mg^{2+} addition *in vitro* (data not shown), we assumed that Mg^{2+} deficiency caused a decrease of this activity in kidney bean plants even *in vivo*. The opposite response of GR activity between cucumber and kidney bean is probably due to their dissimilar physiological response against Mg^{2+} deficiency.

The enhancement of antioxidative enzyme activities by Mg^{2+} -deficiency probably takes place in chloroplasts where $\text{SOD}^{16)}$ and the H_2O_2 detoxifying enzymic cycle²⁴⁾ are predominantly located. The photooxidation of thylakoid constituents may be a major contributing factor in the development of chlorosis. Furthermore, the dissimilarity of plant response between species against Mg^{2+} deficiency may be related to constitutive differences for generation or detoxification of harmful O_2 species in the chloroplasts.

The results presented here show that paraquat and oxyfluorfen toxicity in kidney bean leaves was greatly affected by the nutritional status of Mg^{2+} (Figs. 5, 6). The activities of antioxidative enzymes in kidney bean plants became more distinct with progressing Mg^{2+} deficiency except for GR, and the plant tolerance to paraquat and oxyfluorfen was increased greatly compared to Mg^{2+} sufficiency. The relationship between enhanced antioxidative capacity and tolerance to the herbicides suggests that development of the tolerance is causally related to the elevated detoxification capacity in the leaves. Environmental stress which generates active oxygen species is known to improve the activity of antioxidative enzymes, and to enhance their tolerance to active oxygen generating herbicides, as reported elsewhere^{12,21}. Therefore, kidney bean with Mg^{2+} deficiency may be more tolerant to other O_2 activating stress factors.

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マグネシウム欠乏が抗酸化酵素活性誘導と 活性酸素生成型除草剤に対する 抵抗性増加に及ぼす影響

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摘 要

有害な活性酸素種を生成する環境ストレスに対する植物の適応過程を把握するための一環として、キュウリとツルナシインゲンマメをマグネシウム(Mg^{2+})欠乏の水耕液で栽培した時に発現するクロロフィル含量と Mg^{2+} 濃度の変化、抗酸化酵素の活性変動などを調べた。また、ツルナシインゲンマメの活性酸素生成型除草剤パラコートとオキシフルオルフェンに対する抵抗性に及ぼす Mg^{2+} 欠乏の影響についても検討した。

Mg^{2+} が十分に供給された水耕液で栽培されたキュウリとツルナシインゲンマメでは初生葉の Mg^{2+} 含量が増加したが、 Mg^{2+} 欠乏で栽培された植物では顕著

に減少した(Fig. 1)。 Mg^{2+} 欠乏は、ツルナシインゲンマメよりキュウリにおいて、クロロフィルおよび蛋白質含量を著しく減少させた(Figs. 2, 3)。また、 Mg^{2+} の欠乏によってキュウリではアスコルビン酸ペルオキシダーゼ(AP)、グルタチオンレダクターゼ(GR)ならびにスーパーオキシジスムターゼ(SOD)が、ツルナシインゲンマメではAPおよびSODの活性が著しく高くなることが判明した(Fig. 4)。

ツルナシインゲンマメは活性酸素を生成する作用があるパラコートとオキシフルオルフェンに対し感受性を示したが、 Mg^{2+} 欠乏条件下で育成した植物はクロロシスと水分損失率が少なく、両薬剤により抵抗性になることが明らかになった(Figs. 5, 6)。これらの結果から、 Mg^{2+} の欠乏が供試植物種において、有害な活性酸素種を消去する作用がある抗酸化酵素活性を促進する作用があることが示され、また、ツルナシインゲンマメではAPとSOD活性の増加が、活性酸素種を生成する除草剤に対する植物の抵抗性を高めた一つの原因になっているものと推察される。

キーワード: Mg^{2+} 欠乏, 抗酸化酵素, パラコート, オキシフルオルフェン

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