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Suppression of Nitrate Accumulation in Lettuce by Application of Mg and Micronutrients

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Abstract : High rates of nitrogen fertilization dangerously increase the nitrate content of vegetable crops, and the accumulation of nitrate in edible crops is undesirable because of potential risks to human health. Micronutrient solution containing Cu, Mn, Mo, Zn was tested for the suppression of nitrate accumulation in lettuce grown in pots treated with Mg fertilizer under a greenhouse condition. The micronutrient solution was sprayed on leaves at 3 and 4 weeks after transplanting of 20-day old seedlings. Plants were harvested after 5-week growth, and yield, contents of chlorophyll, sugar, micronutrient and nitrate, and also nitrate reductase activity were measured. Fresh weight of lettuce was significantly increased by the application of Mg and micronutrients, and the effect was the most significant in the Mg+micronutrient treatment. Also contents of chlorophyll and micronutrients were higher in the plants of micronutrient treatments. Contents of nitrate were reduced by about 14-18% in lettuce with Mg and/or micronutrient applications. Compared to the plants of control treatment, nitrate reductase activity was also higher in those plants treated with micronutrients, and in the treatment of Mg+micronutrients the enzyme activity was six times as high as that of control treatment. Although the effect of mineral nutrients on the suppression of nitrate accumulation in lettuce was relatively small in this study, an appropriate supply of mineral nutrients could be one of the solutions for the nitrate accumulation in vegetables.

Key words : lettuce, magnesium, micronutrients, nitrate, nitrate reductase

INTRODUCTION

Excessive utilization of chemical fertilizers causes soil and water pollutions and accumulation of certain chemicals in water and agricultural products. High concentrations of nitrate in water and foodstuffs are believed to carry certain direct health risks to humans[1-3]. The most understood, though fortunately very rare, effect is to cause a disease known as methemoglobinemia in infants. Nitrate is converted to nitrite which oxidizes the ferrous iron of blood to ferric, forming methemoglobin instead of hemoglobin, which is inefficient in binding oxygen. The other potential health risk associated with nitrate is their conversion in the digestive process to nitrosoamine. These have been shown to be powerful carcinogens in animals, though with no direct evidence that human cancers are accelerated as a result of nitrate consumption. Based on the premise that dietary nitrate is detrimental to human health, increasingly stringent regulations are being instituted to lower nitrate levels in food and water. This poses a financial challenge to water managements and a threat to vegetable production.

One of the reasons for high levels of nitrate in vegetables is the application of excess amount of chemical fertilizers and compost with intensification of agriculture and food production[4-7]. Therefore, many researches were carried out to improve cultivation techniques, including fertilization, for production of low-nitrate vegetable crops[5-8], but still vegetables are considered to contribute most to daily nitrate intake in developed countries.

Nitrate, the primary nitrogen source for most land plants, is absorbed by a specific nitrate uptake permease in plant roots, reduced by nitrate reductase in the cytoplasm, and then reduced to ammonium by nitrite reductase in the plastid. Therefore, nitrate uptake permease, nitrate reductase, and nitrite reductase constitute the first three enzymes of the nitrate assimilatory pathway and are subject to regulation by several endogenous and environmental stimuli, including nitrate and

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light[9]. Nitrate reductase is a complex enzyme which contains several prosthetic groups, including FAD, cytochrome, and molybdenum. Therefore, Fe and Mo are essential micronutrients in the nitrate reduction system[10]. As would expected, nitrate reductase activity is very low in Mo-deficient plants. Incubation of deficient leaf segments in solutions containing Mo markedly increases enzyme activity[11]. In green leaves a close correlation exists between light intensity and nitrate reduction[12,13]. According to the mechanism of nitrate reduction, this light effect reflects fluctuations in the carbohydrate level and in the corresponding supply of reducing equivalents (ferredoxin and NADPH). Magnesium and other micronutrients including Fe, Mn, and Cu can also indirectly influence nitrate assimilation. They are essential in photosynthetic system and hence in the flow of e^- from water via ferrodoxin to nitrate reduction. Therefore the maintaining of appropriate levels of those micronutrients in plants is believed to increase activities of photosynthesis and nitrogen assimilation. Magnesium fertilization is highly desirable for plants to increase photosynthesis, and then not only assists in producing a higher crop yield but at the same time it also decreases the plant nitrate content[14].

In this study, we examined the suppression of nitrate accumulation in lettuce by Mg and micronutrient applications.

MATERIALS AND METHODS

Preparation of Micronutrient Solution

Micronutrient solution containing Ca, Cu, Mn, Mo, and Zn was prepared to test the effect on the suppression of nitrate accumulation in leaves of lettuce. Except the micronutrients, the solution contained chitosan which is known to stimulate plant growth. Composition of the micronutrient solution is presented in Table 1.

Plant Material and Growth Conditions

Lettuce, one of the most common fresh vegetable consumed in great quantities, was selected for the experiment. Seeds of *Lactuc sativa* L. cv 'Chungchima' were sown in polystyrene seed-boxes filled with a mixture of perlite and vermiculite (1:1, v/v) and grown in a greenhouse. Seed-boxes were watered twice each day with tap water.

Table 1. Composition of the micronutrient solution

Chitosan	Lactic acid	Mo	Ca	Cu	Zn	Mn
----------	----------	----------	%	----------	----------	----------
1	0.66	1	0.5	0.02	0.02	0.02

After three weeks, plants were transplanted to pots (WxL xD, 18x58x13 cm) filled with a sandy loam soil (10 plants in a pot). Some physicochemical properties of the soil are presented in Table 2. Four treatments - control, Mg treatment, micronutrient treatment, and Mg and micronutrient treatment - were included in this experiment. For each treatment 2 pots were prepared and the pots were arranged in a randomized complete block design. To each pot, 7 g of a complex fertilizer (21-17-17) was added and for the pots of Mg treatment 5 g of $Mg(OH)_2$ was additionally added and thoroughly mixed with soil. At three and four weeks after transplanting, 30 mL of micronutrient solution (2000x dilution) was sprayed on leaves. Five weeks after transplanting, the aerial parts of the lettuce were harvested and fresh weight was determined. Then the samples were used for analysis of nitrate, nitrate reductase activity, chlorophyll, Brix value, and mineral elements.

Analytical Methods

Nitrate reductase activity was determined for 5 plants in each treatment using the method of Scheible et al.[15]. Leaf sample stored in liquid nitrogen was ground to a fine powder in a mortar that had been precooled with liquid nitrogen. The powdered material was throughly mixed and extracted with four volumes of ice-cooled buffer (100 mM HEPES-KOH (pH 7.5), 5 mM $Mg(OAc)_2$, 1 mM EDTA, 10% glycerol, 5 mM DTT, 1% BSA, 0.1% Triton X-100, 0.5 mM PMSF, 20 μM FAD, 25 μM leupeptin, 5 μM Na_2MoO_4, 1% PVPP). Nitrate reductase activity was measured immediately by mixing one volume of extract with five volumes of prewarmed (25°C) assay buffer (100 mM HEPES-KOH, pH 7.5), 5 mM KNO_3, 5 mM EDTA, 0.25 mM NADH). After 1 hour at 25°C, 300 μL aliquot was removed from the assay mixture and the reaction was stopped by adding 75 μL의 150 μM phenazine methosulphate and 25 μL 0.6 M zinc acetate, mixed, and held for 15 min before adding 300 μL의 1% (w/v) sulphanilamide in 3 N HCl plus 와 300 μL 0.02% (w/v) N-(1-naphthyl)-ethylenediamine in bidistilled water. Tubes were allowed to stand for 20 min at room temperature, centrifuged at 14000 *g* and the absorbance of the produced azo-dye in the supernatant wasmeasured at 540 nm. Time zero controls in the presence or

Table 2. Selected properties of soil used in the experiment

pH (1:5 H_2O)	OM	NO_3-N	Total N	Exch. Cations				DTPA Extractable Cations					Texture
				Ca	Mg	K	Na	Cu	Mg	Mn	Mo	Zn	
	%	mg/kg	%	cmol/kg				mg/kg					
6.8	2.87	56	0.09	11.8	8.2	6.8	1.2	1.9	192	6.5	0.1	12.9	Sandy loam

Table 3. Concentration of Mg and micronutrients in lettuce as affected by the application of Mg and micronutrients

Treatments	Mg	Cu	Mn	Mo	Zn
	%	mg/kg			
Control	0.34	17.0	54.5	5.2	52.5
Mg	0.45	17.9	54.2	5.8	52.1
Micronutrients	0.39	21.5	58.5	85.3	66.2
Mg + Micronutrients	0.43	19.6	59.4	79.4	56.7

absence of 10 nmol nitrate were performed under identical conditions for all extracts.

Sugar content in lettuce leaf was estimated by measuring the Brix value. Fresh leaf sample was ground to a fine powder in a mortar and centrifuged at 5000 *g*. Brix value of the supernatant was measured using a refractometer (ATAGO ATC-1, Japan).

For chlorophyll determination 500 mg of fresh leaf sample was ground to a fine powder in a mortar with 5 mL of 80% acetone. After adding 10 mL of 80% acetone and throughly mixed and filtered using Whatman No. 2 filter paper. After the residue was washed with 80% acetone, the volume of the filtrate was brought to 100 mL and absorbance was measured at 652 nm.

For nitrate determination the frozen powder of leaf was extracted using boiling water, in the ratio of 250 mg of powder to 10 mL water. The extraction period was 30 min, after which the extracts were filtered through Whatman No. 1 filter paper. After the residue was washed with boiling water, the volume of the filtrate was brought to 10 mL. The sample was again filtered using a 0.45-μm membrane filter prior to nitrate determination. Nitrate was analyzed on the flow injection autoanalyzer FIA-5000 system (FOSS Tecator, Sweden).

Samples of dried ground leaf material were digested using H_2SO_4 and H_2O_2, and digested solutions were filtered using a 0.45-μm membrane filter and analyzed for mineral nutrients by inductively coupled plasma emission spectrometer (Varian Australia Pyt Ltd., Mulgrave, Australia).

RESULTS AND DISCUSSION

Contents of Mg and Micronutrients in Lettuce

The uptake and accumulation of nitrate by vegetables is known to be affected by agronomic management factors. These activities include fertilization with nitrogen and other mineral nutrients and environmental factors including light condition[4,13]. Management of mineral nutrients essential in nitrogen metabolisms directly or indirectly might well be the most useful options to minimize or avoid the accumulation of nitrate in vegetables.

Contents of Mg and micronutrients in lettuce of the four different treatments are shown in Table 3. Magnesium content in lettuce was higher for the two Mg treatments than for the control treatment. And concentrations of the micronutrients was also increased with applications of the micronutrient solution by foliage spray. Among the micronutrients especially Mo concentration was about 16 times higher in lettuce of the micronutrient treatments in comparison to the control treatment.

The significant increase of Mo content in lettuce is of interest. The functions of Mo as a plant nutrient are related to the valency changes it undergoes as a metal component of enzymes. Nitrate reductase contains both heme iron and two

Table 4. Yield response and chlorophyll and sugar contents in lettuce as affected by the application of Mg and micronutrients

Treatments	Fresh Wt.	Chlorophyll	Brix value
	g/plant	mg/g	Brix
Control	14.99 a	16.30 a	5.45
Mg	15.66 ab	21.16 b	4.95
Micronutrients	16.38 ab	21.79 b	5.05
Mg + Micronutrients	17.91 b	21.74 b	5.24

* Values with different letters are significantly different at $P<0.05$.

Table 5. Nitrate concentration and nitrate reductase activity (NRA) in lettuce as affected by the application of Mg and micronutrients

Treatments	NO_3	NRA
	mg/kg fresh wt.	μmol NO_2/g fresh wt./h
Control	3831.6 a	0.28 a
Mg	3201.4 b	0.40 a
Micronutrients	3293.0 b	1.00 b
Mg + Micronutrients	3123.5 b	1.75 c

* Values with different letters are significantly different at $P<0.05$.

atoms of Mo and catalyzes the reduction of nitrate by a reversible valency change. Nitrate reductase activity is low in leaves of Mo-deficient plants but can be readily induced within a few hours by infiltration of the leaf segments with Mo[11]. Also it is known that there is a close connection between Mo supply, the nitrate reductase activity, and the yield of spinach[16]. It is supposed that even low nitrate levels induce the synthesis of nitrate reductase and that this enzyme without the appropriate Mo cofactor may have other activities leading to metabolic disturbances. When nitrate is supplied in the absence of Mo, plants grow poorly and have a low chlorophyll content, but high nitrate levels[16]. Although the increased concentrations of Cu, Mn and Zn would also be expected to improve growth and metabolic processes of lettuce, if we consider the essential roles of Mo in nitrate reduction as described above, the very significant increase of Mo is strongly expected to stimulate the nitrogen assimilation in lettuce.

Growth, Chlorophyll Content, and Brix Value

Results of the measurements of yield response, chlorophyll content and Brix value are given in Table 4. Plant growth was significantly increased by the application of Mg and/or micronutrients. Fresh weight of lettuce was increased by 19% in the treatment of Mg+micronutrients in comparison to the control treatment. Separate application of Mg or micronutrients also increased fresh weight of lettuce. Chlorophyll content was also increased significantly in the treatments of Mg and/or micronutrients. Eom et al. found that foliar applications of micronutrients could improve the growth of lettuce and spinach in a greenhouse condition[17]. In this study the Mg fertilization in addition to the foliar application of micronutrients could improve plant growth further in comparison to the treatment of micronutrients only.

Those micronutrients Cu, Mn, Mo and Zn included in the nutrient solution of foliar application have several essential roles in various metabolic processes and then can assist in producing a higher crop yield. Chitosan compounds included in the nutrient solution are also known to stimulate self-defence system of plants and to promote growth through activation of cell metabolisms[18-21]. Although the detail mech-

anisms are still unknown, foliar application of chitosan is known to increase chlorophyll content of plants[21]. Magnesium applied as a soil amendment is an element essential in chlorophyll structure. And the increased chlorophyll content in lettuce could be due to the Mg amendment in part.

Although higher CO_2 fixation was expected in the treatments of Mg and/or micronutrients, Brix values in the treatments of Mg and/or micronutrients were slightly lower in comparison to the control treatment. Such results would be due to the dilution effect from the higher growth in the treatments of Mg and/or micronutrients. And also the activated photosynthesis and nitrogen metabolism with supplementation of Mg and micronutrients could reduce the content of soluble non-structural carbon compounds in plants.

Nitrate Content and Nitrate Reductase Activity

Effect of Mg and/or micronutrient solution containing chitosan on nitrate content and nitrate reductase activity in lettuce are given in Table 5. In plants treated with Mg and/or micronutrients nitrate content was lowered by 14-18% in comparison to the control treatment. Comparing to the treatments of Mg or micronutrients, average nitrate content was lower in the treatment of Mg+micronutrients. Micronutrient solution used in this experiment was prepared to activate photosynthesis of plants and then energy and electron ($NADH^+$) necessary for nitrate reduction could be supplied easily and also Mo, a cofactor of nitrate reductase enzyme, was included in the solution. Therefore this micronutrient solution was expected to suppress nitrate accumulation in plants by stimulation of the nitrogen assimilation.

As shown in Table 5, nitrate reductase activity was significantly increased in the treatments of Mg and/or micronutrients, and the stimulation effect of Mg was relatively lower than that of micronutrients. In the treatment of Mg+micronutrient, the activity of nitrate reductase was almost six times as high as that of control treatment. Therefore, the above results suggest that application of Mg and micronutrients could increase the content of those elements and chlorophyll and nitrate reductase activity in plants, and such results then can stimulate metabolisms including photosynthesis and nitrogen assimilation, and at the same time nitrate accumulation could be suppressed. Kiss suggested that Mg fertilizers reduce nitrate content of plants; while the nitrogen fertilizers increase the nitrate content of the plants in a measure varying with the species, any form of Mg supplement reduces the plant nitrate content[14]. The energy supplies for nitrate reduction are produced in the course of photoreaction. Magnesium increases CO_2 fixation, and this is achieved in par through an increase in the activity of RUBP-carboxylase.

The decrease in the nitrate level of plants caused by Mg suggests that the Mg also in some way influences nitrate metabolism. Magnesium has no direct effect on the activity of the nitrate reductase enzyme, so it can only influence nitrate reduction in an indirect way. Of various indirect ways, stimulation of photosynthesis and enzyme protein synthesis would be important. Photosynthesis supplies the energy required for nitrate reduction, while the latter ensures the necessary nitrate reductase enzyme concentration. Nitrate reductase, like any other enzyme, is a protein. Continuous nitrate reduction demands a repeated *de novo* synthesis of nitrate reductase, since the half-life of nitrate reductase is as short as 4 hours. Magnesium is known to have a stimulatory effect on this enzyme synthesis[22]. Mg fertilization is highly desirable for plants grown in conditions of limited illumination to increase photosynthesis. Mg supplementation not only assists in producing a higher crop yield but at the same time it also decreases the plant nitrate content.

CONCLUSION

Green vegetables such as lettuce, cabbage and spinach are known as the main sources of nitrate in our diet. The European Communities' Scientific Committee for Food set an ADI for nitrate of 0-3.65 mg/kg body weight[23]. Compared with this ADI, the ingestion of only 100 g of raw vegetables with nitrate concentration of 2190 mg/kg fresh matter corresponds to the whole nitrate ADI for a person of 60 kg. Therefore, the reduction of nitrate content in lettuce from 3831 mg/kg fresh wt. of the control treatment to 3123 mg/kg fresh wt. of the Mg+micronutrients treatment in this study is still not sufficient enough to resolve the harmful effects of nitrate carried to humans by consumption of vegetables. Therefore, in order to maximize the effect of mineral nutrient application on the suppression of nitrate accumulation, nutrient composition in the solution and application time and frequency should be further examined. And, although mineral nutrient supply could be one solution for the nitrate accumulation in vegetables, nitrogen level in soil and other environmental factors including light condition should be considered together.

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