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The Effect of Mixed Amino Acids on Nitrate Uptake and Nitrate Assimilation in Leafy Radish

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ABSTRACT: The objective of the present work was to determine the corresponding uptake and assimilation of NO_3^- in roots and shoots of leafy radish by applying of mixed amino acids (MAA). The amino acids used in this experiment were alanine (Ala), β-alanine (β-Ala), aspartic acid (Asp), asparagines (Asn), glutamic acid (Glu), glutamine (Gln), and glycine (Gly). Leafy radish was grown by conventional fertilization with macro- and micronutrients under controlled conditions. The 15-day-old seedlings were treated 0, 0.3 and 3.0 mM of MAA containing 5 mM NO_3^- in growth medium. Nitrate uptake was determined by following NO_3^- depletion from the uptake solution. The activity of the enzymes related to the process of NO_3^- reduction (NR: nitrate reductase; NiR: nitrite reductase; GS: glutamine synthetase) and the content of NO_2^- and NO_3^- were analyzed in shoots and roots. The results of this study showed that NO_3^- uptake was inhibited 38% with treatment of 0.3 mM of MAA. However, there was more than three times increase of NO_3^- uptake in 3.0 mM MAA. In addition, the enzymatic activities were positively affected by the high MAA rate. Finally, the NO_3^- content was increased slightly both in shoots and roots of leafy radish by MAA treatments.

Key Words: leafy radish, nitrate uptake, amino acid, nitrate assimilation

INTRODUCTION

Nitrate, the most abundant N source for the higher plants, is absorbed by the plasma membrane-bound transporter, reduced to nitrite by nitrate reductase (NR) in the cytoplasm, and then reduced to ammonium by nitrite reductase (NiR), this ammonium is then incorporated into amino acids by the glutamine synthetase-2-oxoglutarate amidotransferase (GS - GOGAT) enzyme system, giving rise to glutamine (Gln) and ultimately other amino acids and their metabolites[1,2]. Therefore, NR, NiR and GS consist of the most important three enzymes of the NO_3^- assimilatory pathway. The NR activity is the limiting step of N-NO_3^- conversion to amino acid synthesis[3]. In most plant species only a proportion of the absorbed NO_3^- is assimilated in the root, the remainder being transported upwards through the xylem for assimilation in the shoot where it is reduced and incorporated into amino acids[4].

During the last decade, a number of studies have confirmed the ability of plants to take up and use amino acids as a source of nitrogen[5-8]. The effect of amino acids on nitrate uptake and reduction was investigated[1,9-15]. The amino acids, whether accumulated in the plant internally or supplied externally, usually were showed to regulate the induction of NO_3^- uptake and reduction systems.

Regulation of induction of the NO_3^- uptake and reduction systems by nitrogen metabolites has been attributed to feed-back inhibition[16]. It was found that nitrate uptake rate follows a biphasic relationship with external nitrate concentration, suggesting the existence of at least two different uptake systems[17]. At high external nitrate concentration (> 0.5 mM), a low affinity transport system (LATS), which shows

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linear kinetics, contributes significantly to the uptake rate and appears to be constitutively expressed and essentially unregulated. At low external concentrations (< 0.5 mM), two high affinity transport systems (HATS) operate, one of these being constitutive whereas the other is induced by nitrate. The HATS for nitrate uptake is sensitive to metabolic inhibitors and appears to be an active transport system[18].

Effect of amino acids on the regulation of NR gene expression has been studied at the molecular level. Deng *et al.*[19] reported that the addition of 5 mM glutamine to the nutrient solution of tobacco plants grown in 1 mM NO_3^- resulted in a pronounced inhibition of NR mRNA accumulation in the roots. Vincentz *et al.*[20] showed, under low light conditions (limiting photo synthetic conditions), the supply of glutamine or glutamate led to a drop in the level of NR mRNA. However glutamine and glutamate were less efficient at decreasing NiR mRNA than NR mRNA levels. Li *et al.*[11] also demonstrated 5 mM glutamine added together with NO_3^- resulted in reduced levels of NR mRNA in both root and shoot of maize.

Although the regulatory effect of amino acids on NO_3^- uptake and NR has been examined extensively, its effect on GS has not been examined in detail. Otherwise, many types of amino acids were investigated about their regulation on NO_3^- uptake and assimilation, but very little information has been reported about effect of mixed amino acids (MAA).

This study was carried out to investigate the regulation of the induction of NO_3^- uptake, NRA, NiRA and GSA in leafy radish by applying mixed amino acids (MAA) under the conventional fertilization. The amino acids used in this experiment were alanine (Ala), β -alanine (β-Ala), aspartic acid (Asp), asparagines (Asn), glutamic acid (Glu), glutamine (Gln) and glycine (Gly).

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of leafy radish soaked for 6 h allowed to germinate on paper towels soaked in water in the dark. After 5 days the seedling were transferred to 50 ml tubes containing 10 ml inorganic nutrient solution. The nutrient solution was renewed every day. The composition of the inorganic nutrient solution is given in Table 1. Boron (H_3BO_3), Manganese ($MnCl_2 \cdot 4H_2O$), Zinc ($ZnSO_4 \cdot 7H_2O$), Copper ($CuSO_4 \cdot 5H_2O$) and Molybdenum ($H_2MoO_4 \cdot H_2O$) were supplied to all treatments at rates of 460, 90, 7.7, 3.2 and 0.1 μM, respectively[21]. Seedlings were grown in a growth chamber maintained 25℃ temperature, 70-80% relative humidity, with a light 14-h light/10-h dark cycle and a light intensity of 300 μmol $m^{-2}S^{-1}$.

Table 1. Composition of the nutrient solution (mM)

	K^+	NO_3^-	Ca_2^+	$H_2PO_4^-$	Mg_2^+	SO_4^{2-}
KNO_3	1.25	1.25				
$Ca(NO_3)_2$		2.50	1.25			
KH_2PO_4	0.25			0.25		
$MgSO_4$					0.50	0.50
Total	1.50	3.75	1.25	0.25	0.50	0.50

Mixed amino acids treatments

The mixed amino acids (MAA) solution contained 7 equal concentration of amino acids were as follows: alanine (Ala), β-alanine (β-Ala), aspartic acid (Asp), asparagines (Asn), glutamic acid (Glu), glutamine (Gln) and glycine (Gly). At 10 days after transferring, radish seedlings were placed in 10 ml inorganic nutrient solution containing 5.0 mM NO_3^- and 0, 0.3 or 3.0 mM MAA, as indicated in Table 2. The pH of the nutrient solutions were maintained between 6.0-6.1 by adding KOH as appropriate. The nutrient solutions were renewed at 4, 8 and 16 h. Plants were harvested 24 h after treatment and separated into roots and shoots.

Measure of NO_3^- uptake

Net NO_3^- uptake rates were determined by amount of NO_3^- disappeared from the initially treated solution.

Table 2. Different composition of the treatment solutions (mM)

Treatment	K^+	NO_3^-	Ala	β-Ala	Asp	Asn	Glu	Gln	Gly
A0	5.25	5.0	—	—	—	—	—	—	—
A1	6.78	5.0	0.3	0.3	0.3	0.3	0.3	0.3	0.3
A2	13.10	5.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0

The solution samples were filtered through 0.45-μm filters and diluted 10 or 20 times by ultrapure water to nitrate determination. Nitrate was analyzed on an Ion Chromatograph system (model DX-500, Dionex) using an IonPac AG-4A-SC column. Nitrate uptake was expressed as mmol NO_3^- g^{-1} fresh weight (FW).

Preparation of extracts

Plant shoots 2 g and roots 1 g were ground, with a ratio of 1:5 (w/v), in a mortar at 0℃ in 50 mM KH_2PO_4 buffer pH 7.5, containing 2 mM EDTA, 1.5% (w/v) soluble casein, 2 mM dithiothreitol (DTT) and 1% (w/v) insoluble polyvinylpolypyrrolidone (PVP). The homogenate was filtered through two layers of cheese cloth and centrifuged at 3000 g for 5 min and then the supernatant was centrifuged at 20000 g for 20 min. The resulting extract was used to measure NO_3^-, NO_2^- and enzyme activities (NR, NiR and GS). The extraction medium was optimized for the enzymatic activities so that these could be extracted jointly by the same method[22].

Quantification of NO_3^- and NO_2^-

Nitrate concentration in plant shoots and roots was measured by the salicylic acid method[23]. The enzyme extracts 0.2 ml were pipetted into 50 ml tubes and mixed thoroughly with 0.8 ml of 5% (w/v) salicylic acid in concentrated H_2SO_4. After 20 minutes at room temperature, 19 ml of 2 M NaOH were added slowly to raise the pH above 12. Samples were cooled to room temperature and absorbance at 410 nm was determined by spectrophotometry. A separate blank was required for each sample because of pigmentation in the extracts. The blank consisted of the extract, 0.8 ml of concentrated H_2SO_4 (without salicylic acid) and 19 ml of 2 M NaOH. The results were expressed as μmol g^{-1} fresh weight (FW). NO_2^- was determined by measuring absorbance at 540 nm after color development for 15 min with a 1:1 mixture of 1% (w/v) sulfanilamide in 1.5 M HCl and 0.02% (w/v) n-1-napthyl- ethylenediamine dihydrochloride. The NO_2^- was expressed as nmol g^{-1} fresh weight (FW).

NR activity assay

Each assay mixture tube contained 0.1 ml potassium phosphate buffer, pH 7.5; 0.1 ml NADH (1mg ml^{-1}), 0.2 ml of 0.1 M KNO_3 and 0.2 ml extract made up to a final volume of 2 ml with distilled water. After 15 min incubation at 28℃, the reaction was stopped by the addition of 1 ml of 1% (w/v) sulphanilamide in 1.5 M HCl and 1 ml of 0.02% (w/v) n-1-napthyl-ethylenediamine dihydrochloride solution. All samples were centrifuged at 3000 g for 10 min to remove suspended matter. NO_2^- was determined by measuring absorbance at 540 nm[24]. Triplicate aliquots of crude extract were assayed in each experiment. The NR activity was expressed as μmol NO_2^- formed g^{-1} fresh weight (FW) h^{-1}.

NiR activity assay

The NiR activity was determined on its basis of the drop in NO_2^- concentration in the reaction medium. After incubation at 30℃ for 30 min, the NO_2^- content was determined by measuring absorbance at 540 nm after color development and the activity was expressed as μmol NO_2^- reduced g^{-1} fresh weight (FW) h^{-1}. Triplicate aliquots of crude extract were assayed in each experiment.

GS activity assay

Each assay tube contained in a final volume of 2 ml reaction mixture: 184 μmol L-glutamic acid, 90 μmol $MgSO_4$, 12 μmol hydroxylamine, 100 μmol imidazole, 36 μmol ATP and 0.2 ml crude extract. The reaction tubes were incubated for 15 min at 28℃ and the reaction was stopped by the addition of 1 ml ferric chloride reagent (4 g trichloroacetic acid, 3.2 g anhydrous ferric chloride in 100 ml of 0.5 M HCl). Each tube was centrifuge at 2500 g for 10 min and glutamyl hydroxamate was determined by measuring absorbance at 500 nm[24]. Each determination of GSA was carried out in triplicate. The GS activity was expressed as mmol glutamyl hydroxamate g^{-1} fresh weight (FW) h^{-1}.

The experiments were repeated 2 to 3 times and the results of representative experiments are reported. The data are means ± SE of five replicates. All results are reported on the basis of fresh weight.

RESULTS AND DISCUSSION

Effect on NO_3^- uptake

The MAA treatments showed different effect on

nitrate uptake depending on the concentrations (Fig. 1). The NO_3^- uptake in A1 was similar with A0 after 8 h exposure to NO_3^-. However, after longer time (16 or 24 h) exposure to 0.3 mM MAA the NO_3^- uptake was inhibited 38% than A0. In contrast, the highest NO_3^- uptake was found in treatment A2 that showed 305% than A0.

Several authors reported that free amino acids could lower NO_3^- uptake. It was found that exogenously supplied amino acids and amides could decrease the uptake of NO_3^- by soybean[9]; wheat[10]; maize[1,12,13]; barley[15]. In our experiment, the effectiveness of the MAA treatments on NO_3^- uptake was similar to above references at low MAA treatment rate (0.3 mM MAA, Fig. 1). However, contrary result was found at high MAA treatment rate (3.0 mM MAA, Fig. 1), which NO_3^- uptake was 4-fold higher than the control. This result was similar to rice, pea, cucumber and red pepper, which were treated with 5.0 mM MAA[21].

The effect on NO_3^- uptake seems to respond to kinds and concentration of amino acids. Muller and Touraine[9] examined the effect of 14 different amino acids on NO_3^- uptake in soybean seedlings supplied with 0.5 mM NO_3^-. After 10 mM single amino acid pretreatment, about half of the tested amino acids had a substantial inhibitory effect on NO_3^- uptake. These were mainly Ala, Glu (almost 100% inhibition), Asn, Arg (about 80%), Asp, β-Ala, Scr, and Gln (from 70% to 48%). However, when supplied 100 mM amino acid to the tip-cut cotyledons, only eight of fourteen amino acids had inhibitory effect and four amino acids had enhanced NO_3^- uptake.

Effect on NO_3^- and NO_2^- accumulation

The application of MAA increased the NO_3^- concentrations both in shoots and in roots regardless of application rates (Table 3), resulting the highest concentration in A2 and the lowest concentration in A0. The high concentration of NO_3^- in A2 was attributed to the high NR activity (Fig. 2). Although A1 treatment showed the lowest uptake of NO_3^- (Fig. 1), the high concentration of NO_3^- in A1 was found due to that low NR activity in A1 (Fig. 2) led to a blocking of the reduction of NO_3^- to NO_2^-. With respect to the NO_2^- values (Table 3), in our experiments, the highest NO_2^- concentrations in both shoots and roots were

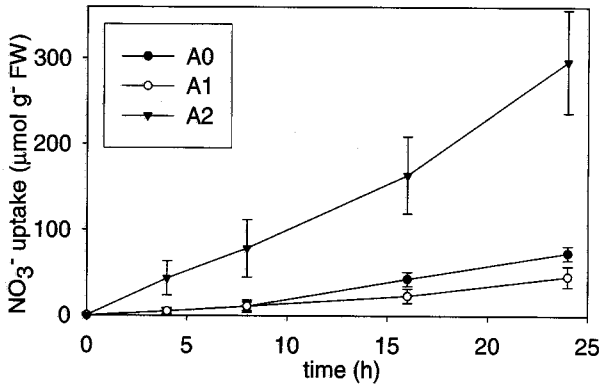

Fig. 1. Effect of MAA treatments (A0, absence of MAA; A1, 0.3 mM MAA; A2, 3.0 mM MAA) on the nitrate uptake in leafy radish supplied with 5.0 mM NO_3^-. Values are means ± SE (n=5).

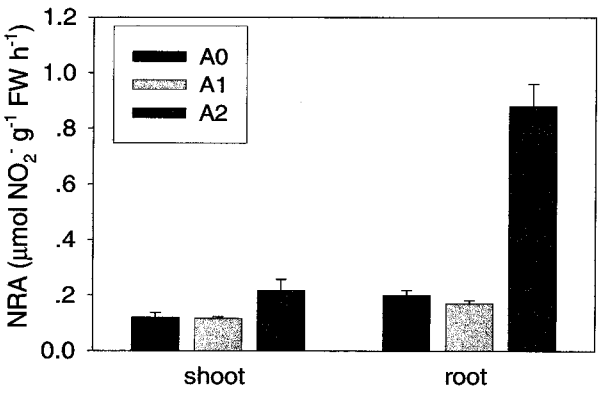

Fig. 2. Effect of MAA treatments (A0, absence of MAA; A1, 0.3 mM MAA; A2, 3.0 mM MAA) on NRA in leafy radish at 24 h after treatment. Values are means ± SE (n=5).

Table 3. Effect of MAA treatments (A0, absence of MAA; A1, 0.3 mM MAA; A2, 3.0 mM MAA) on NO_3^- and NO_2^- concentration in leafy radish at 24 h after treatment

treatment	NO_3^- mmol g^{-1} (FW)		NO_2^- nmol g^{-1} (FW)	
	shoot	root	shoot	root
A0	62.47±4.06	16.3±1.88	6.76±0.62	11.43±1.67
A1	67.73±7.49	22.99±2.23	3.77±0.34	17.14±2.10
A2	63.37±3.58	17.41±1.92	29.70±2.78	30.39±4.13

Data are means ± SE (n=5).

found in the A2. In shoots, the lowest NO_2^- concentration was found in A1 and the lowest in A0 in roots.

Although many authors agree with that amino acid can negatively regulate nitrate content in higher plants[25-28], our results do not support this interpretation. Both in shoots and in roots, the MAA used in this study led to little increase of NO_3^- concentrations (Table 3). The contradiction may reside in amino acids treatment method. It was demonstrated in other studies that amino acid pretreatment decreased NO_3^- accumulation slightly, but Gln and Asn led to NO_3^- concentration increase in barley roots when they were used together with nitrate[15].

As interim product of NO_3^- assimilation procedure, the concentration of NO_2^- depended on the reduction rate of nitrate and nitrite. The highest concentration of NO_2^- had been found in A2 (Table 3) due to high NR activity (Fig. 2), and the lowest concentration of NO_2^- in shoots in A1 (Table 3) was due to low NR activity (Fig. 2) too. However, low NiR activity (Fig. 3) led to a blocking of the reduction of NO_2^- to NH_4^+ in roots in A1, so that concentration of NO_2^- was showed higher than A0 (Table 3).

Effect on NRA, NiRA and GSA

For NO_3^- assimilation, NO_3^- is reduced to NO_2^- by catalysis of NR. In our experiment, low concentration and high concentration of MAA treatments led to different effects on NR activity (Fig. 2). Both in the shoots and in the roots NR activities were inhibited slightly in A1. Significant increases of NR activities were found in A2 treatment, with 75 and 340% respectively in relation to A0.

There are contradictory results for the possible regulation of NR activity by amino acids for higher plants. For example, Radin[29,30] has shown that the reduction of NO_3^- to NO_2^- in cotton roots is inhibited by specific amino acids. On the other hand, Oaks[31] has found that amino acids inhibited minor levels of NR in both intact and excised corn roots using an *in vitro* assay. Aslam *et al.*,[15] reported that the amino acids partially inhibited the increase of NR activity in barley roots where most NO_3^- uptake was facilitated via inducible high affinity transport system (IHATS) but had little effect where LATS is operative. It has been suggested that glutamate inhibited NR activity in roots, but no inhibition in shoots[13]. Sivasankar *et al.*,[1] observed that Gln and asparagine (Asn) inhibited the induction of NR activity in corn roots at both 250 μM and 5mM external NO_3^- supply. They concluded that inhibition was not the result of altered NO_3^- uptake, and tissue nitrate accumulation was reduced at 250 μM external nitrate in the presence of 1 mM Asn, but not at 5 mM Asn.

In studies of the possible regulation of NR activity by multiple amino acids in higher plants, the conclusions are also contradictory. The inhibition on NR activity by glycine, asparagines, and glutamine could be partially or wholly prevented by the presence of other amino acids during the induction[30]. However when glutamine and asparagines were included along with the "corn amino acid mixture", the inhibition on the induction of NR in corn roots was more severe[31]. Chen and Gao[27] have applied different mixture of glycine, isoleucine and proline to Chinese cabbage and lettuce in hydroponics. They found the amino acids treatment enhanced NR activity in Chinese cabbage, while decreasing it slightly in lettuce.

In our experiment, at 5.0 mM NO_3^- which is facilitated by LATS, the presence of 0.3 mM MAA partially inhibited NR activity, as observed in other works[9-11], whereas the 3.0 mM MAA increased the NR activity more than 4 times (Fig. 2). In addition, the very high NO_2^- content was found in A2 (Table 3). These results suggest that high concentration MAA can increase NO_3^- uptake by enhancing NR activity in leafy radish, especially in roots.

The next step in NO_3^- assimilation is the conversion of the NO_2^- to NH_4^+ by the action of NiR. Both enzymes, NR and NiR, are induced by the same factors[32], and therefore the response of NiR to the MAA treatments resembled that of NR in roots, but was a little different with that of the NR in shoots (Fig. 3). NiR activities in shoots and roots in A1 were inhibited 17 and 52% respectively in relation to A0. In A2, NiR activity was inhibited 15% in shoots and enhanced 8 times in roots. The decrease of the level of total amino acids in shoot of red pepper was observed when treated with MAA (X.Q. Liu, and K.S. Lee, unpublished results). In the present study, the decrease of NiR activity in shoots in A2 might be attributed to the low concentration of amino acids in shoots too. The increase of NiR activity in roots in A2 was due to same reason with NR.

The principal NH_4^+ pathway is the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle. The behavior of GS activities in shoots was not affected by MAA treatments (Fig. 4). However differences were found in roots between treatments, showing 22% inhibition in A1 and 17% increase in A2 in relation to A0.

The NH_4^+ originating in the plant from NO_3^- reduction is incorporated into an organic form primarily by the enzyme GS. In the present experiment, GS activity was inhibited by 0.3 mM MAA treatment in leafy radish roots, whereas 3.0 mM of MAA treatment was enhanced (Fig. 4). It is also striking that effect of MAA on NO_3^- assimilation in the roots was higher than in the shoots, presumably NO_3^- was more available and the MAA content was higher in the roots.

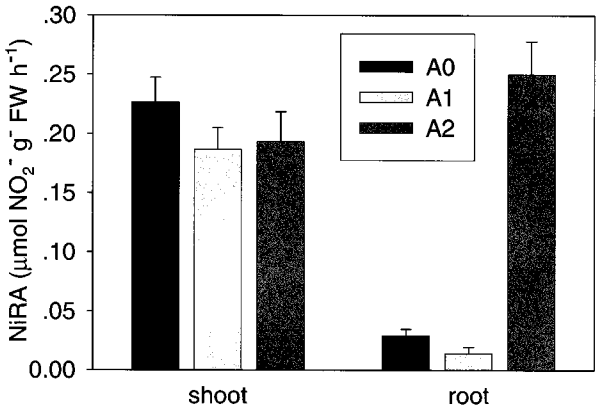

Fig. 3. Effect of MAA treatments (A0, absence of MAA; A1, 0.3 mM MAA; A2, 3.0 mM MAA) on NiRA in leafy radish at 24 h after treatment. Values are means ± SE (n=5).

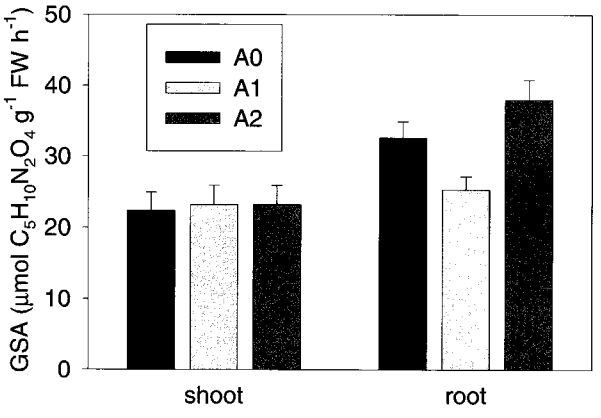

Fig. 4. Effect of MAA treatments (A0, absence of MAA; A1, 0.3 mM MAA; A2, 3.0 mM MAA) on GSA in leafy radish at 24 h after treatment. Values are means ± SE (n=5).

Under normal growth conditions, an adequate availability of free amino acids is necessary in order to meet any changes in protein synthesis and growth rates[33]. In these amino acids, glutamine, as the key intermediate of N assimilation[34], is more important one. Within higher plant glutamine can be converted into other amino acids and incorporated into proteins and nucleic acids[34]. The protein synthesis in higher plant is a complex process which requires many kinds of amino acids. Comparison with single amino acids, multiple amino acids has more significant effect on protein synthesis process. High concentration of MAA supply could lead to an increase in protein synthesis ratio and this increase probably produced a depletion of glutamine, which exerts a negative control on GS[35], so that increase of GS activity and a higher NO_3^- uptake would be induced. On the contrary, low concentration of MAA could not affect protein synthesis significantly and led to glutamine accumulation in roots, where it could contribute to the inhibition of GS activity and NO_3^- uptake. This is probably the main reason of that A1 and A2 showed different effect on NO_3^- uptake in the present study.

The results of this study clearly indicated that NO_3^- uptake and NO_3^- assimilation was regulated by MAA in leafy radish, especially at high concentration of MAA treatment. In conclusion, the application of high MAA rates (principally A2) could be the direct cause of increased activities of the three enzymes (NR, NiR and GS) of the NO_3^- assimilatory pathway and the NO_3^- uptake was enhanced when supplied with LATS range of NO_3^-.

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