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Effects of nitrate supply on plant growth, nitrate accumulation, metabolic nitrate concentration and nitrate reductase activity in three leafy vegetables

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Abstract

Three leafy vegetables, rape (*Brassica campestris* L.), Chinese cabbage (*Brassica chinensis* var. *Oleifera Makino et Nenoto*) and spinach (*Spinacia oleracea* L.), were grown in plastic pots with 5 kg soil per pot at five nitrate supply rates, 0.00 (N_1), 0.15 (N_2), 0.30 (N_3), 0.45 (N_4), and 0.60 (N_5) g N kg^{-1} soil to investigate the effects of nitrate supply on plant growth, nitrate accumulation and nitrate reductase activity (NRA) 9 weeks after sowing. The optimum yield appeared at N_3, while above N_4, a strong decrease in plant growth occurred. The nitrate concentration increased with nitrate supply in the whole plant and the different organs except in roots where nitrate concentration at N_5 decreased compared with N_4. The nitrate concentration in both the metabolic pool (MP) and the storage pool (SP) of the leaf blades increased with nitrate supply. From N_1 to N_2, NRA increased most rapidly. The highest NRA occurred at N_4. However, nitrate reductase (NR) activities were not significantly different between N_3, N_4 and N_5, which imply that there is a threshold of nitrate concentration in MP (NMP) to induce NRA. The parameters of NR for nitrate were measured by the *in vivo* method. The K_m values we obtained were similar to the reported values by the *in vitro* method, which confirms the feasibility of the anaerobic method for determining NRA and NMP. Finally, the effects of the posttranslational regulation of NR were discussed.

Keywords: Compartment; Metabolic nitrate; Nitrate; Nitrate reductase; Vegetable

1. Introduction

More and more nitrogen fertilizers are applied in fields, since N fertilizer plays a significant role both in crop yield and quality [1–3]. Nitrate is often the major source of N available to higher plants [4], especially to vegetables. Nitrate uptake and distribution in crops is of major importance with respect to both environmental concerns and the quality of crop products. Nitrate, not taken up by a crop, may potentially contribute to ground and surface water pollution through nitrate leaching and soil erosion [2,3]. On the other hand, nitrate taken up by plants causes high nitrate accumulation in plants, especially in most vegetables. Because edible parts contain very high concentrations of nitrate that has been implicated in the occurrence of methaemoglobinemia and possibly in gastric cancer as well as other diseases [5–7], nitrate accumulation in plants is a major concern, and is known to be a problem in most crops [8–10].

Although most higher plants are capable of reducing NO_3^- in both roots and shoots [4], nitrate is reduced more efficiently in leaves than in roots because of the readily available reductants, energy and carbon skeletons produced by photosynthesis, which is dependent on plant species [11,12]. This is also true for most leafy vegetables. Nitrate taken up by a plant is either reduced or stored in the vacuoles or transported in the xylem transpiration stream to the leaf for reduction, and most is stored in the vacuole until

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released for reduction in the cytosol [10]. In addition, NR exists in the cytosol, therefore, nitrate in cytosol is called the nitrate metabolic pool (MP), and nitrate in the vacuole is called the nitrate storage pool (SP) [13–15]. Since NR is assumed to be the rate-limiting step for nitrate assimilation [16,17], and the NR is an inducible enzyme, there is a close relationship between NR activity (NRA) and nitrate concentration in plants [18]. Furthermore, nitrate induces the expression of both the uptake and reduction systems [19]. Thus it can be seen that nitrate accumulation might be regulated by many factors, such as plant growth, endogenous nitrate, and nitrate uptake and reduction.

Most of the above studies were carried out either under two to three nitrate levels, or with seedlings, or in hydroponics, or with short-term nitrate induction, while the present work was conducted at a gradient of five nitrate supply rates (from nitrate deficiency to surplus), with flourishing plants at harvest time (vegetative stage), and with soil culture, such conditions being closer to natural environment and agricultural production. Three leafy vegetables, rape (*Brassica campestris* L.), Chinese cabbage (*Brassica chinensis* var. *Oleifera Makino et Nenoto*) and spinach (*Spinacia oleracea* L.), were planted in plastic pots with tilth layer soil. The purpose was to investigate plant growth, nitrate accumulation, the allocation of nitrate throughout the plant, the compartmental distribution in leaves, and the relationship between leaf NRA and nitrate accumulation at different nitrate supply rates under natural environment. We also analyzed the relationship between NRA and nitrate distribution in MP and SP in leaf blades at the five different nitrate supply rates for the three leafy vegetables. All the analyses were performed at the level of tissue and whole plants.

2. Materials and methods

2.1. Plant material and growth conditions

The experiment was conducted in No. 1 Crop Experimental Base of Northwestern Science-Technology University of Agriculture and Forestry (NWSUAF), Yangling, Shaanxi Province, China. Three leafy vegetables—rape (*Brassica campestris* L.), Chinese cabbage (*Brassica chinensis* var. *Oleifera Makino et Nenoto*) and spinach (*Spinacia oleracea* L.)—were sown in 25 cm × 30 cm (diameter × depth) plastic pots filled with 5 kg field soil. Soils were taken from a fallow field of the college of agronomy of NWSUAF, then passed 4 mm sieve for removing plant litter, and were mixed uniformly. Soil organic matter, total N, NO_3^--N, NH_4^+-N, Olsen-phosphorus, available potassium and pH were 11.19 g kg^{-1}, 0.91 g kg^{-1}, 37.45 mg kg^{-1}, 13.15 mg kg^{-1}, 23.93 mg kg^{-1}, 97.65 mg kg^{-1} and 7.78 mg kg^{-1}, respectively. Before sowing, soil was irrigated to 20% water content. Half of the fertilizer was applied with irrigation water prior to sowing. Twelve plants per pot were sown at six points with the same distance apart on 4th September 2001. One week after sowing, six uniform plants were left per pot. The pots were placed in the glasshouse, weeded and scarified weekly. Soil water content was controlled to 20% by the commonly used weight method. Each treatment was planted in four replicated pots.

2.2. Treatments

The application of five nitrogen treatments (0.00, 0.15, 0.30, 0.45, 0.60 g N) in the form of KNO_3 was split into two. One half was fertigated before sowing, and the other half was fertigated 4 weeks after sowing. Besides, 0.20 g P_2O_5 kg^{-1} soil was applied as the basal fertilizer.

2.3. Sampling

Fully expanded leaves from the same position in three plants per pot, and four pots of each treatment were sampled 9 weeks after planting. The leaves without midrib were left to determine NRA, nitrate concentration in MP (NMP) and total nitrate concentration. The rest three plants per pot were flushed and washed with flowing water to obtain the whole plants. Then they were divided into root, petiole, leaf and stem to determine nitrate concentration in different organs.

2.4. Determination of in vivo NRA

Fresh leaves without midrib were cut into 5 mm × 5 mm pieces. The samples were infiltrated and the intracellular spaces of the tissues were flushed with buffer using a vacuum. The NR activity was measured based on the method described by Brunetti and Hageman [20] and Aslam *et al.* [21]. The samples (0.50 g) were placed in 10 ml of the incubation medium which is 0.1 M potassium phosphate buffer (pH 7.5) containing 0.1 M KNO_3 with 1% (v/v) propanol. Prior to assay the buffer solution was purged with N_2 gas for 30 min to remove dissolved O_2. Then the samples were vacuum-infiltrated (two times), and the reaction flasks were purged with N_2 gas for 5 min, were sealed with rubber stoppers and incubated in a water bath at 30 °C for 30 min. After incubation the samples were placed in a boiling water bath to stop the NR activity. The nitrite released to the medium was measured by the method of Snell and Snell [22], and NRA was expressed as μmol NO_2^- g^{-1} FW h^{-1}.

2.5. Determination of nitrate concentration in MP (NMP) and nitrate concentration in SP (NSP)

The NMP was assayed using the anaerobic incubation method described by Ferrari *et al.* [13], Steingröver *et al.* [14] and Aslam *et al.* [21]. The samples were washed with incubation medium to rinse off nitrate and sugars leaking from cuts. Prior to assay the buffer solution was purged with N_2 gas for 30 min to remove dissolved O_2. Incubation occurred at 30 °C in darkness for 4–6 h in a shaking water bath after flushing the samples with N_2 gas for 3 min,

Table 1
Fresh weight (FW) of three 9-week-old vegetables grown under five nitrate supply rates

Nitrate supply (g N kg^{-1} soil)	Shoot (g FW plant^{-1})			Whole plant (g FW plant^{-1})		
	Rape	Cabbage	Spinach	Rape	Cabbage	Spinach
0.00 (N_1)	8.2 ± 1.44d	12.3 ± 1.57d	6.2 ± 0.41d	12.2 ± 1.99c	15.2 ± 1.41c	8.5 ± 0.21c
0.15 (N_2)	19.9 ± 3.91b	39.5 ± 4.81b	17.1 ± 1.82b	23.0 ± 4.20b	42.0 ± 4.86b	19.0 ± 2.10b
0.30 (N_3)	30.0 ± 2.48a	50.2 ± 6.32a	20.9 ± 2.36a	33.3 ± 2.59a	54.3 ± 6.57a	24.3 ± 2.46a
0.45 (N_4)	30.1 ± 2.72a	39.6 ± 8.9b	15.3 ± 1.74b	32.8 ± 2.60a	43.4 ± 8.74b	17.8 ± 1.43b
0.60 (N_5)	14.6 ± 3.61c	22.1 ± 3.21c	8.7 ± 0.84c	16.6 ± 3.72c	23.6 ± 3.25c	9.9 ± 0.78c

Data are arithmetic means ± S.D. Each value is the mean of four replicates. Differences in plant biomass were tested according to one-way ANOVA followed by Duncan's test. In a column, means followed by different letter are significantly different at $P < 0.05$.

with the incubation medium being 10 ml phosphate buffer (100 mM K-phosphate buffer, pH 7.5). The nitrite production was detected every hour during the 4–6 h incubation, during which process each flask was sampled only once. The amount of nitrite produced under anaerobiosis was equivalent to the NMP.

The NSP is the total nitrate concentration in leaf blades minus NMP.

2.6. Determination of nitrate concentration in plant

Nitrate concentration was measured according to the method described by Wang and Li [23]. Nitrate was extracted with distilled water. After 2 g fresh samples adding a little (0.50 g) arenaceous quartz were ground with a pestle and mortar, they were completely transmitted to centrifugal tube with a definite quantity of distilled water. After centrifugation, the supernatant was used for nitrate concentration assay with flow-injection-analysis after Cd-catalysed reduction to NO_2^- (FIASTAR 5000).

2.7. Statistical analysis

Standard deviation was calculated for each treatment. Effects of nitrate supply on the plant fresh weight, nitrate concentration and NRA were tested with one-way ANOVA followed by Duncan's test at $P < 0.05$ (SPSS 10.0).

3. Results

3.1. Effect of nitrate supply on plant growth

Nitrate supply exerted significant effects on plant growth (Table 1). Within lower supply (N_1–N_3), plant biomass

Table 2
The NO_3^- concentrations in different organs of three 9-week-old vegetables

Vegetables	Nitrate supply (g N kg^{-1} soil)	Nitrate concentration (μmol NO_3^- g^{-1} FW)			Nitrate concentration (μmol NO_3^- plant^{-1})
		Root	Petiole + stem[a]	Leaf	Whole plant[b]
Rape	0.00 (N_1)	3.94 ± 0.45d	4.27 ± 0.49c	0.96 ± 0.09d	3.61 ± 0.38c
	0.15 (N_2)	6.73 ± 0.84d	11.07 ± 3.86c	4.54 ± 0.79c	8.43 ± 2.11c
	0.30 (N_3)	27.14 ± 2.84c	72.18 ± 8.39b	31.76 ± 3.40b	54.86 ± 6.07b
	0.45 (N_4)	47.35 ± 2.17a	113.69 ± 6.44a	55.62 ± 1.09a	87.43 ± 3.05a
	0.60 (N_5)	31.24 ± 4.32b	119.14 ± 5.55a	57.78 ± 2.26a	86.54 ± 4.46a
Cabbage	0.00 (N_1)	4.05 ± 2.11e	3.39 ± 0.18e	0.75 ± 0.05e	2.72 ± 0.57e
	0.15 (N_2)	12.63 ± 1.46d	24.28 ± 3.42d	5.40 ± 0.70d	17.39 ± 2.33d
	0.30 (N_3)	32.16 ± 3.80c	82.24 ± 7.12c	30.19 ± 3.03c	61.98 ± 2.74c
	0.45 (N_4)	47.07 ± 2.82a	116.02 ± 8.73b	49.70 ± 2.93b	87.08 ± 2.86b
	0.60 (N_5)	38.32 ± 3.14b	130.03 ± 10.69a	59.16 ± 4.14a	96.64 ± 3.70a
Spinach	0.00 (N_1)	3.80 ± 1.51d	7.77 ± 3.75d	1.36 ± 0.07e	3.83 ± 1.26e
	0.15 (N_2)	8.05 ± 1.77c	30.59 ± 4.88c	9.22 ± 1.62d	17.48 ± 2.52d
	0.30 (N_3)	17.82 ± 1.91b	75.20 ± 3.28b	26.27 ± 3.42c	44.52 ± 1.85c
	0.45 (N_4)	29.63 ± 0.90a	81.37 ± 3.14b	41.46 ± 2.13b	56.22 ± 1.34b
	0.60 (N_5)	27.33 ± 2.81a	97.58 ± 6.55a	46.17 ± 3.28a	64.05 ± 2.91a

These plants were separated into root, leaf blade, petiole and stem. Then the nitrate concentrations in different organs of the three vegetables were measured respectively. Data are arithmetic means ± S.D. Each value is the mean of four replicates. Differences in NO_3^- concentrations were tested according to one-way ANOVA followed by Duncan's test. Within the same parts of one vegetable but different N treatments, means followed by different letter are significantly different at $P < 0.05$.

[a] Because stem was very small at vegetative stage in spinach, its weight no less than 1 g, the stem and petiole of a same sample were mixed for nitrate determination. While to cabbage and rape, the nitrate concentration of petiole and stem were detected separately, and the nitrate of petiole + stem was weighted mean of petiole and stem.

[b] The nitrate concentration of whole plant was weighted mean of root, petiole + stem and leaf.

increased with the increase of nitrate supply, while plant biomass decreased above N_3, and a large reduction occurred at N_5. The fresh weight of the three leafy vegetables showed a similar trend. In general, the maximum fresh yield occurred at N_3, the minimum occurred at N_1. However, there was a discrepancy of biomass at different N treatments among the three vegetables. The concrete results are shown in Table 1.

3.2. Effect of nitrate supply on nitrate distribution in plants

Nitrate supply had a significant effect on nitrate accumulation in the plants (Table 2). Nitrate concentration increased with nitrate supply in different parts except in the roots. Nitrate concentration in the roots at N_5 decreased compared with that at N_4 with the largest decrease in rape, the smallest in spinach. The distribution of nitrate concentration of the three vegetables showed a similar trend. Generally, the highest nitrate concentration existed in the petiole-stems, the lowest in the roots. The discrepancy of nitrate concentration at different N treatments among the three vegetables demonstrated little difference. The final results are shown in Table 2.

3.3. Effect of nitrate supply on nitrate distribution in two pools

The results indicate that nitrate supply had significant effect on nitrate distribution both in MP and SP of leaf blades, but the discrepancy between the treatments was not significantly different (Fig. 1). The NMP and NSP increased with the increase of nitrate supply, while at the higher nitrate supplies, no significant increase of NMP occurred except in spinach, and there was no significant increase of NSP except in cabbage. Although NMP, NSP and NMP/NSP ratio of the three vegetables showed a similar trend with nitrate supply, among the three vegetables, the three above indexes at the same nitrate supply showed different changes. NMP of spinach was the highest, while NSP was the lowest, which resulted in the highest NMP/NSP ratio in spinach.

In addition, the comparison between the NSP (Fig. 1B) with the nitrate concentration in whole leaf (NMP + NSP, Fig. 1A and B) suggested that nitrate was mainly (around 90%) stored in SP, which is consistent with the results (88.8% in the vacuole of barley root and 89.3% in maize root) measured by double-barrelled nitrate-selective microelectrodes [15]. This, to some extent, tested the reliability of the anaerobic method for determining NMP and NSP.

3.4. Effect of nitrate supply on NRA

Nitrate supply had a significant effect on NRA in leaf blades (Table 3). NRA increased most rapidly from N_1 to N_2. However, the increase of NRA was not significant among the other three nitrate supplies. In general, among the three vegetables, the NRA of rape was the highest with that of

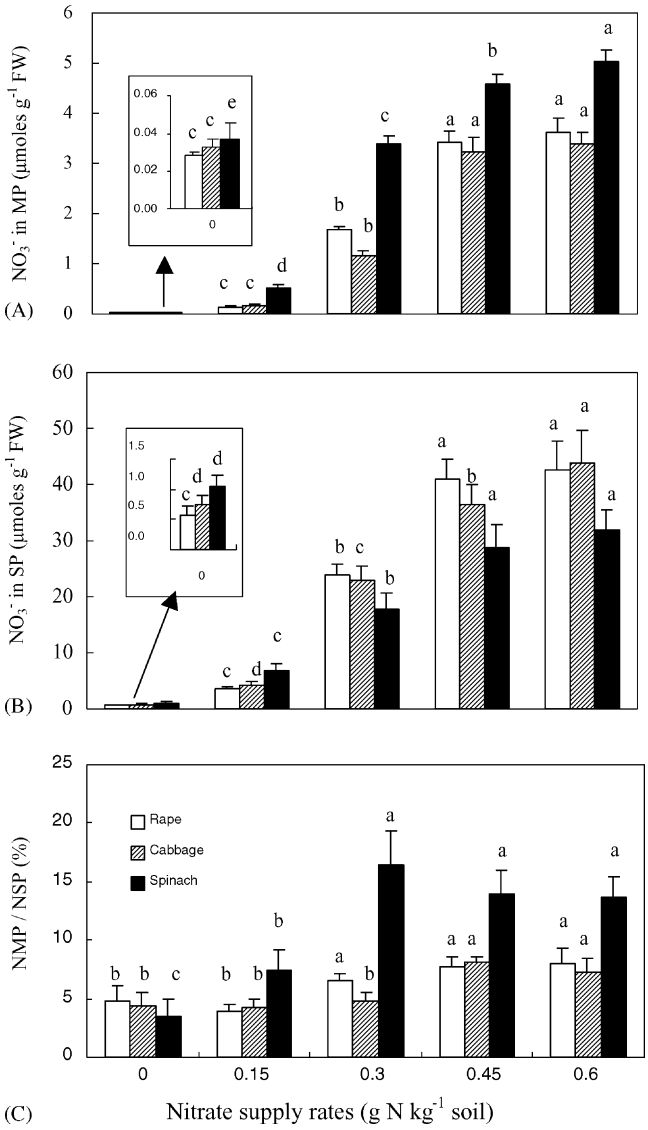

Fig. 1. Nitrate concentration in MP (NMP) and SP (NSP) in fully expanded leaf blades of the three 9-week-old vegetables grown under five N applications in pots. (A) Nitrate in MP; (B) nitrate in SP; (C) NMP/NSP ratio. Each value represents the mean of four replicates. Error bars indicate S.D. values. Means followed by different letters in same vegetable are significantly different at $P < 0.05$ (one-way ANOVA's Duncan test).

cabbage being the lowest, which indicate that the response of NRA to nitrate supply in rape is the most sensitive, while that in spinach is the least sensitive.

3.5. Relationship between NMP and NRA in leaf blades

Fig. 2A was plotted combining NRA with NMP at the five nitrate supplies. At the lower nitrate supply, NRA increased rapidly with the increase of NMP, conversely in higher NMP, nitrate had no significant effect on NRA, and NRA almost reached a plateau value or even decreased. The NRA of the three vegetables had similar trend with NMP under five nitrogen regimes (Fig. 2A). The curves were not linear but

Table 3
NRA (μmol NO_2^- g^{-1} FW h^{-1}) in leaf blades of the three vegetables

Nitrate supply (g N kg^{-1} soil)	Rape	Cabbage	Spinach
0.00 (N_1)	0.87 ± 0.05c	0.35 ± 0.09c	1.10 ± 0.23b
0.15 (N_2)	1.68 ± 0.04b	0.86 ± 0.33b	1.98 ± 0.22a
0.30 (N_3)	2.23 ± 0.14a	1.59 ± 0.24a	1.94 ± 0.12a
0.45 (N_4)	2.46 ± 0.39a	1.71 ± 0.30a	2.02 ± 0.30a
0.60 (N_5)	2.14 ± 0.33a	1.36 ± 0.31a	2.02 ± 0.26a

Data are arithmetic means ± S.D. Each value is the mean of four replicates. Differences in NRA were tested according to one-way ANOVA followed by Duncan's test. Within the same vegetable but different nitrate treatments, means followed by different letter are significantly different at $P < 0.05$.

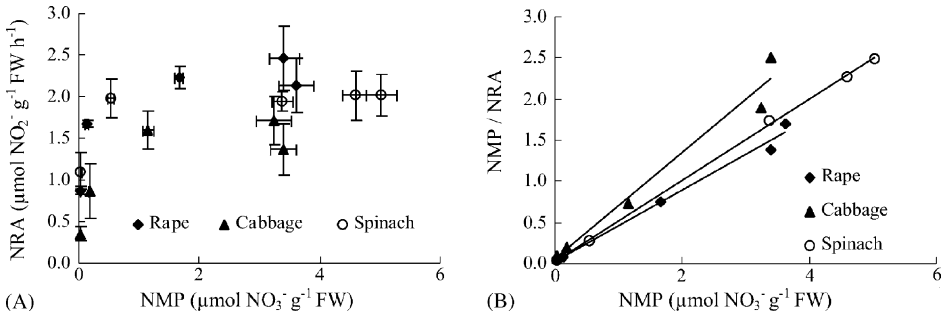

Fig. 2. The relationship between NMP and NRA in fully expanded leaf blades of the three 9-week-old vegetables and the parameters of NR for NMP. (A) The relationship between NMP and NRA. (B) The kinetic parameters for NR, presented as a Hanes plot (NMP against NMP/NRA). For each vegetable, the five data points are values under the five nitrogen regimes. All values are means ± S.D. of four replicates.

Table 4
The parameters of NR for NMP (μmol NO_2^- g^{-1} FW h^{-1}) were estimated according to the Hanes plot shown in Fig 2B

Vegetables	The linear equation of Hanes plot	K_m (mM)	V_{max} (μmol g^{-1} FW h^{-1})
Rape	$y = 0.4341x + 0.0193$ ($R^2 = 0.9896$*)	0.0445	2.304
Cabbage	$y = 0.6445x + 0.0505$ ($R^2 = 0.9706$*)	0.0784	1.552
Spinach	$y = 0.4954x + 0.0188$ ($R^2 = 0.9993$*)	0.0379	2.019

The slopes of regression lines provide $1/V_{max}$, and the y-intercepts generate estimates of K_m/V_{max}, on the basis of which, the K_m and V_{max} for each vegetable were estimated.
* $P < 0.01$.

fitted the Michaelis-Menten equation and the Hanes plot (NMP against NMP/NRA) were plotted based on the data in Fig. 2A, which was presented in Fig. 2B.

3.6. Parameters of NR for nitrate based on NMP and in vivo NRA in leaf blades

The parameters of NR for nitrate were determined based on the *in vivo* NRA and NMP at the five different nitrate supply. It should be noted that the unit of NMP (μmol g^{-1} FW) was different from the reported K_m values unit (mM) due to the different measurements. But it can be assumed that 1 μmol g^{-1} FW nearly equaled to 1 mM because 1 g fresh leaves approximately equaled to 1 ml in that the water content of fresh leaves was about 90%. The values were estimated according to the Hanes plot shown in Fig. 2B, and the results were shown in Table 4. The K_m values showed an identical ranking of the three species with the cabbage having the highest and the spinach the lowest. The comparison of K_m values between the present and other reported was discussed in Section 4.4. The highest V_{max} was represented in rape and the lowest in cabbage. The V_{max} values are two- to three-fold higher than the reported V_{max} (0.69 μmol g^{-1} FW h^{-1}) for barley root which was also obtained by the *in vivo* NRA [24].

4. Discussion

In this study, we investigated the effect of nitrate supply on plant growth and nitrate distribution and NRA in the three leafy vegetables. To make explicit the significant differences of available nitrate content in soil among the five nitrate supply rates, the nitrate concentrations in soil were determined twice, 4 weeks after sowing and before sampling. The results showed that there were significant differences of nitrate content in soil among the five nitrate supplies, and the higher nitrate residues being tested in soil at the higher nitrate supply (data not shown).

4.1. Relationship of plant growth and nitrate accumulation

It is well established that plants supplied with nitrate in excess of current demand have the ability to accumulate nitrate [25], and nitrate uptake and accumulation are highly related to exogenous nitrate [26]. Therefore, nitrate supply plays a very important role both in nitrate accumulation and plant growth. Our results show that the growth response was highly dependent on nitrate supply with optimum at N_3, while above N_4 a strong decrease in plant growth occurred (Table 1). Here, nitrate concentration was generally on the rise with nitrate supply in spite of a relatively slow increase trend above N_4 (Table 2). It showed that plant growth with high nitrate supply exhibited toxicity symptoms and depressed growth, but had higher nitrate concentrations than those of plants with maximum yield, which might be due to the "concentrated effect" that resulted from the decrease of plant biomass. Moreover, recent investigations indicate that high nitrate accumulation results in nitrite production which was converted into nitric oxide (NO) in plants, while, in turn, NO and O_2^- could be rapidly catalyzed by NR into peroxynitrite ($ONOO^-$), which is highly toxic to plant (see recent reviews) [27,28]. Therefore, the high nitrate accumulation in plants resulted from high nitrate supply was not only harmful to human health [5–7] but also detrimental to plant growth [29].

In return, plant growth plays an important role in nitrate uptake. The efficiency of net nitrate uptake rate (NNUR) substantially increased with the increase of plant relative growth rate increases [30]. Thus, it can be seen that above N_4, the relative slow increase of nitrate concentration might be attributable to the decrease in the nitrate uptake owing to the decrease of the plant growth. Besides, nitrate uptake was regulated by nitrate concentration in plant, and NNUR is under negative feedback control by endogenous nitrate [31]. Therefore, when nitrate supply was higher than N_4, the slow increase of nitrate accumulation might be due to the decrease of nitrate uptake as a result of the negative feedback regulation of the higher nitrate concentration in plants per se.

Although nitrate concentration increased with nitrate supply, a great decrease of the absolute nitrate content (nitrate concentration × plant fresh weight) occurred above N_4, which was chiefly due to the decrease of plant growth. This implies that an excess of nitrate supply in soil led to a decrease of both plant yield and absolute nitrate accumulation, which resulted in more nitrate residues in soil, more nitrate leaching and pollution.

4.2. Relationship between nitrate accumulation and NRA in leaf blades

NR is considered as a limiting factor for nitrate assimilation in higher plants, and is induced by nitrate. Thus, NR has great influence on nitrate accumulation in plants. However, the relationship between NRA and nitrate concentration is still uncertain. Some studies indicate that the higher NRA was, the more nitrate might be reduced, so there was negative relationship between NRA and nitrate concentration [32]. Most studies show that, with NR being a substrate-induced enzyme, the higher substrate-nitrate concentration was in plant, the higher NRA might be, so there was positive correlation between them [18,29,33]. Some investigations show that a very small amount of nitrate is sufficient for induction [34–36], namely, NRA is not induced by nitrate when nitrate concentration was higher than a certain level [37]. Besides, NR expression is controlled by either the N-flux or plant N-status [34]. In our experiments, only at the lower nitrate supplies, the nitrate has positive effect on NRA, while at the higher nitrate supplies, the NRA reached a plateau value or even decreased (Table 3). The present results suggest that the relationship between NRA and nitrate accumulation is dependent on the exogenous nitrate, which might be mainly due to the cellular compartmentation of nitrate [13,38], and it is insufficient to evaluate the nitrate accumulation only by NRA.

4.3. Relationship between NRA and NMP

Nitrate, stored mainly in vacuole where it functions as an osmotic ion [39], might compensate for low levels of organic solutes [40], might be available for assimilation, and might serve as a reservoir to sustain growth processes during subsequent periods when the external nitrogen supply becomes limiting [14,38]. The nitrate in MP can be reduced directly by NR and the stored nitrate can pass through tonoplast and get into MP for reduction under the deficiency of nitrate in MP [13,38,41]. Our study indicates that nitrate supply had an obvious effect on both NRA and NMP (Fig. 1). The NRA increased with nitrate supply at the lower nitrate supplies, while above N_3 the NRA appear constant or even decreased. The nitrate concentration in MP increased with nitrate supply, while no significant increase occurred above N_3 (Fig. 1A). The NRA increased rapidly with the increase of NMP within the lower NMP, and kept constant or even decreased when NMP was higher than that at N_3 (Fig. 2). The results suggest that the substrate nitrate has a positive effect on NRA when nitrate supply was below N_3. However, a role for nitrate seems unlikely at first sight, because the leaf nitrate concentration is always above 40 mM (Table 2), and far smaller levels of nitrate suffice to induce NRA [35,36]. This is probably the reason why NRA keeps constant when NMP increases at the higher nitrate supplies, which implies that there is a threshold of NMP to induce NRA, and it might be dependent on plant species and environmental factors such as soil available nitrogen, light intensity and water content both in soil and in plant and so on.

To some extent, the NRA/NMP ratio can reflect the relative reducing ability of NR or relative rate of nitrate reduction. The higher the ratio is, the more nitrate is reduced by NR in the case of its own NMP. Based on the data from Fig. 2A, it can be seen that the NRA/NMP ratio decreased rapidly with the increase of nitrate supplies. The results in-

dicate that at the lower nitrate supplies, especially at N deficiency, the relative rate of nitrate reduction was higher than that at the higher nitrate supplies. This is consistent with the result that the NR activation state under lower nitrate supplies was higher than under higher nitrate supplies [42].

4.4. K_m values of NR for nitrate

In general, the parameters of NR for nitrate can be measured based on the *in vitro* NRA and the purified or partially purified NR in different nitrate concentration mediums. Many reported K_m values of NR for nitrate in spinach leaves were from 0.013 to 0.11 mM measured by the above method [43–46]. Our results obtained by the *in vivo* method show that the K_m value of spinach, 0.038 mM (Table 4), is within the range of the reported K_m values by the *in vitro* method and is close to the reported K_m of spinach, 0.04 mM [46], which indicate that the anaerobic NR and NMP assay is an effective method of evaluating the K_m values of NR for nitrate. The slight discrepancy of the K_m values is probably due to the different growing conditions and different NR assays.

The K_m values of corn and squash leaves are 0.15 and 0.04 mM, respectively [47]. In the review of Guerrero, the K_m values of six different plant species varied from 0.084 to 0.69 mM [48]. Usually the K_m of NADH:NR for nitrate is higher than that of NAD(P)H:NR, the K_m values of NADH:NR and NAD(P)H:NR for nitrate of soybean leaves were 0.11 and 4.5 mM [49], and those of mustard were 0.05 and 2.5 mM [50], respectively. It seems reasonable that the differences of K_m might occur among plant species and cultivars of the same species (Table 4) [24], which might be due to different enzyme forms or different electron donor [24,48].

Baer and Collet [51] confirmed the feasibility of estimating the K_m values by the *in vivo* method and calculated the apparent K_m of NR for nitrate of six winter wheat genotypes based on the double reciprocal plots of NRA against external nitrate concentration. However, the K_m values (from 1.57 to 22.40 mM) reported by Baer and Collet [51] are much higher than those reported values in spinach by the *in vitro* method [43–46]. It should be mentioned that they used different plants, spinach and wheat, and different methods to estimate the K_m of NR for nitrate. Compared with our results (0.04–0.08 mM), the K_m values reported by Baer and Collet [51] are also much higher, which may be due to either the different plant species or the nitrate concentrations used as the "X variable" to calculate K_m or both. The higher external nitrate concentration (~100 mM) were used by Baer and Collet [51], while the lower nitrate concentration in MP (0–6 mM) were used in our study.

4.5. In vivo NR assay and posttranslational regulation of NR

The *in vivo* NRA and NMP assays are all based on the measurement of NO_2^- production. The incubation is performed under darkness and anaerobic conditions which favor nitrite accumulation to inhibit NO_2^- reduction [13,52]. Several concerns might arise regarding the accuracy of the *in vivo* NR assay. Firstly, NO_2^- production may be controlled by the activity of NR and the supply of reductant [53]. However, the fact that the NO_2^- production continued for several hours indicates that the reductant supply was not the limiting factor for NRA during the experiments [21,54]. Secondly, it is difficult to exclude or control the leakage of the vacuolar nitrate into the cytosol during incubation [24,55], while the vacuolar nitrate might not leak into the cytosol freely, and the vacuolar leakage of nitrate is carefully regulated and controlled by the relative activities of the vacuolar uptake system and an efflux channel [56]. Thirdly, anaerobiosis might not cause complete inhibition of nitrite reductase activity, resulting in further metabolism of nitrite, accordingly resulting in an underestimation of NRA [53].

However, it should be mentioned that the darkness and anaerobic conditions might adversely affect the NR activation state, which is linked with the phosphorylation posttranslational regulation of NR. Last decade it became clear that NR can be phosphorylated on a serine residue (serine 543 in spinach) in the hinge 1 region, creating a binding site for 14-3-3 proteins, and binding of 14-3-3 to P-NR inactivates P-NR completely in the presence of divalent cations (Mg^{2+} or Ca^{2+}) [57–59]. Therefore, the NR activity in the presence of Mg^{2+} (NR_{act}) usually reflects the activity of dephospho-NR. In contrast, all NR-forms remain fully active in the absence of divalent cations (+EDTA), which reflects the total amount of NR (NR_{max}). Thus, the ratio of NR_{max} and NR_{act} is defined as the activation state of NR [59].

The *in vivo* NRA and NMP assays were carried out in darkness and under anaerobic conditions, and these artificial treatments have been found to lead to NR activation in the following manner: the cytosolic concentrations of ATP will decrease, which may be sufficient to restrict NRK [60]; the cytosolic concentration of 5′-AMP will increase, which activates dephosphorylation [46,61]; and the cytosolic pH probably falls as either H^+-pumps become restricted or nitrite accumulated (formed a weak acid HNO_2), which is sufficient to activate NR [62]. At the same time, the data suggest that a drastic increase of nitrite levels occurred in the dark when leaves were kept under anaerobic condition compared with aerobic condition [60]. In addition, the *in vivo* NR assay may improve NRA as a result of the incubation medium used in the *in vivo* NR assay containing phosphate which can stimulate the activation of phospho-NR [57,63]. Based on the above discussion, the *in vivo* NR method activates NRA or nitrite production to some extent.

However, NRA might be inactivated because the cytoplasm contains free Mg^{2+} or Ca^{2+} during the *in vivo* NR assay, and this may be due to the control of NRK activity *in vivo* [57]. Moreover, recent results suggest that the concentration of free Mg^{2+} in the cytosol of leaf cells may be very low in the light but increased to about 0.4 mM in the dark [64], which might also decrease the NR activation

state. However, it is reported that there was no significant difference of the Mg^{2+} content in the wheat shoots under the different nitrate supplies [65], and until now there has been no proof that the cytosolic free Mg^{2+} really fluctuates under natural conditions [62].

Because so many cellular parameters change, no single factor can be identified as being solely responsible *in situ*. In addition, the different mechanisms of NR modulation between *in vitro* and *in vivo* make the NR regulation more complicated [24,66]. Therefore, it is worth mentioning that the effects of the different NR assays on the activation state of NR must be considered. Further investigations of post-translational regulation of NR *in vivo* are needed.

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References

[1] V.A. Sisson, T.W. Rufty, R.E. Williamson, Nitrogen-use efficiency among flue-cured tobacco genotypes, Crop Sci. 31 (1991) 1615–1620.
[2] F. Gastal, G. Lemaire, N uptake and distribution in crops: an agronomical and ecophysiological perspective, J. Exp. Bot. 53 (2002) 789–799.
[3] Z.-H. Wang, Z.-Q. Zong, S.-X. Li, B.-M. Chen, Nitrate accumulation in vegetables and its residual in vegetable fields, Environ. Sci. 23 (2002) 79–83.
[4] H. Marschner, Mineral Nutrition of Higher Plants, Academic Press, London, 1995, pp. 229–312.
[5] C.S. Bruning-Fann, J.B. Kaneene, The effects of nitrate, nitrite and N-nitroso compounds on human health: a review, Vet. Hum. Toxicol. 35 (1993) 521–538.
[6] Y. Ikemoto, M. Teraguchi, Y. Kogayashi, Plasma level of nitrate in congenital heart disease: comparison with healthy children, Pediatr. Cardiol. 23 (2002) 132–136.
[7] H. Ishiwata, T. Yamada, N. Yoshiike, M. Nishijima, A. Kawamoto, Y. Uyama, Daily intake of food additives in Japan in five age groups estimated by the market basket method, Eur. Food Res. Technol. 215 (2002) 367–374.
[8] J. Van der Boon, J.W. Steenhuizen, E.G. Steingröver, Growth and nitrate concentration of lettuce as affected by total nitrogen and chloride concentration, NH_4/NO_3 ratio and temperature of the recirculating nutrient solution, J. Hortic. Sci. 65 (1990) 309–321.
[9] I. Quillere, C. Dufossé, Y. Roux, C.H. Foyer, M. Caboche, J.F. Morot-Gaudry, The effects of deregulation of NR genes expression on growth and nitrogen metabolism of nicotiana plumbaginifolia plants, J. Exp. Bot. 45 (1994) 1205–1211.
[10] R. Cárdenas-Navarro, S. Adamowicz, P. Robin, Nitrate accumulation in plants: a role for water, J. Exp. Bot. 50 (1999) 613–624.
[11] L.P. Solomonson, M.J. Barber, Assimilatory nitrate reductase: functional properties and regulation, Annu. Rev. Plant Biol. 41 (1990) 225–253.
[12] A. Oaks, Primary nitrogen assimilation in higher plants and its regulation, Can. J. Bot. 72 (1994) 739–750.
[13] T.E. Ferrari, O.C. Yoder, D. Filner, Anaerobic nitrite production by plant cells and tissues: evidence for two nitrate pools, Plant Physiol. 51 (1973) 423–431.
[14] E. Steingröver, P. Ratering, J. Siesling, Daily changes in uptake, reduction and storage of nitrate in spinach grown at low light intensity, Physiol. Plant 66 (1986) 550–556.
[15] A.J. Miller, S.J. Smith, Nitrate transport and compartmentation in cereal root cells, J. Exp. Bot. 47 (1996) 843–854.
[16] J.M. Caba, C. Lluch, F. Ligero, Distribution of nitrate reductase activity in *Vicia faba*: effect of nitrate and plant genotype, Physiol. Plant 93 (1995) 667–672.
[17] C. Bussi, A. Gojon, L. Passama, *In situ* nitrate reductase activity in leaves of adult peach trees, J. Hortic. Sci. 72 (1997) 347–353.
[18] V. Skrdleta, A. Gaudinova, M. Nemcova, Relationships between nitrate level, nitrate reductase activity and anaerobic nitrite production in *Pisum sativum* leaf tissue, Biol. Plant 21 (1979) 307–310.
[19] S. Sivasankar, S. Rothstein, A. Oaks, Regulation of the accumulation and reduction of nitrate by nitrogen and carbon metabolites in maize seedlings, Plant Physiol. 114 (1997) 583–589.
[20] N. Brunetti, R.H. Hageman, Comparision of *in vivo* and *in vitro* assays of nitrate reductase in wheat (*Triticum aestivum* L.) seedlings, Plant Physiol. 58 (1976) 583–587.
[21] M. Aslam, R.L. Travis, D.W. Rains, Enhancement of nitrate reductase activity and metabolic nitrate concentration by methionine sulfoximine in barley roots, Plant Sci. 161 (2001) 133–142.
[22] F.D. Snell, C.T. Snell, Colorimetric Methods of Analysis, vol. 2, Van Nostrand, New York, 1949, pp. 802–807.
[23] Z.-H. Wang, S.-X. Li, Relationships between nitrate contents and water, total N as well as total P in different organs of vegetable plants, Plant Nutr. Fertil. Sci. 2 (1996) 144–152.
[24] B.J. King, M.Y. Siddiqi, A.D.M. Glass, Studies of the uptake of nitrate in barley. V. Estimation of root cytoplasmic nitrate concentration using nitrate reductase activity—implications for nitrate influx, Plant Physiol. 99 (1992) 1582–1589.
[25] D.T. Clarkson, Regulation of the absorption and release of nitrate by plant cells: a review of current ideas and methodology, in: H. Lambers, J.J. Neeteson, I. Stulen (Eds.), Fundamental Ecological and Agricultural Aspects of Nitrogen Metabolism in Higher Plants, Martinus Nijhoff Publishers, Dordrecht, 1986, pp. 3–27.
[26] F. Devienne-Barret, E. Justes, J.M. Machet, B. Mary, Integrated control of nitrate uptake by crop growth rate and soil nitrate availability under field conditions, Ann. Bot. 86 (2000) 995–1005.
[27] J. Durner, D.F. Klessig, Nitric oxide as a signal in plants, Curr. Opin. Plant Biol. 2 (1999) 369–374.
[28] L. Lamattina, C. García-Mata, M. Graziano, G. Pagnussat, Nitric oxide: the versatility of an extensive signal molecule, Annu. Rev. Plant Biol. 54 (2003) 109–136.
[29] K.S. Reddy, R.C. Menary, Nitrate reductase and nitrate accumulation in relation to nitrate toxicity in *Boronia megastigma*, Physiol. Plant 78 (1990) 430–434.
[30] M.W. Ter Steege, I. Stulen, P.K. Wiersema, F. Posthumus, W. Vaalburg, Efficiency of nitrate uptake in spinach: impact of external nitrate concentration and relative growth rate on nitrate influx and efflux, Plant Soil 208 (1999) 125–134.
[31] R. Cárdenas-Navarro, S. Adamowiczl, P. Robin, Diurnal nitrate uptake in young tomato (*Lycopersicon esculentum* Mill.) plants: test of a feedback-based model, J. Exp. Bot. 49 (1998) 721–730.
[32] C.-X. Hu, B.-E. Deng, T.-C. Liu, Effects of nitrogen fertilizer on nitrate accumulation by the Chinese cabbage (*Brossica chinenses*) and tomato (*Lycopersicum esculentum*), J. Huazhong Agric. Univ. 11 (1992) 239–243.

[33] N.V. Ivashikina, O.A. Sokolov, Regulation of nitrate distribution in maize seedlings by nitrate, nitrite, ammonium and glutamate, Plant Sci. 123 (1997) 29–37.
[34] M.E. Samuelson, W.H. Campbell, C.M. Larasson, The influence of cytokinins in nitrate regulation of nitrate reductase activity and expression in barley, Physiol. Plant 93 (1995) 533–539.
[35] W.R. Scheible, M. Lauerer, E.D. Schulze, M. Caboche, M. Stitt, Accumulation of nitrate in the shoot acts as signal to regulate shoot-root allocation in tobacco, Plant J. 11 (1997) 671–691.
[36] P. Matt, M. Geiger, P. Walch-Liu, C. Engels, A. Krapp, M. Stitt, The immediate cause of the diurnal changes of nitrogen metabolism in leaves of nitrate-replete tobacco: a major imbalance between the rate of nitrate reduction and the rates of nitrate uptake and ammonium metabolism during the first part of the light period, Plant Cell Environ. 24 (2001) 177–190.
[37] H.S. Joseph, J.P. Michael, *In vitro* stability of nitrate reductase from wheat leaves, Plant Physiol. 63 (1979) 346–353.
[38] M. Aslam, A. Oaks, R.C. Huffaker, Effect of light and glucose on the induction of nitrate reductase and on the distribution of nitrate in etiolated barley leaves, Plant Physiol. 58 (1976) 588–591.
[39] G.I. McIntyre, The role of nitrate in the osmotic and nutritional control of plant development, Aust. J. Plant Physiol. 24 (1997) 103–118.
[40] M. Blom-Zandstra, J.E.M. Lampe, The role of nitrate in the osmoregulation of lettuce (*Lactuca sativa* L.) grown at different light intensities, J. Exp. Bot. 36 (1985) 1043–1052.
[41] E. Martinoia, U. Heck, A. Wiemken, Vacuoles as storage compartments for nitrate in barley leaves, Nature 289 (1981) 292–293.
[42] H.M. Man, G.K.A. Baki, P. Stegmann, H. Weiner, W.M. Kaiser, The activation state of nitrate reductase is not always correlated with total nitrate reductase activity in leaves, Planta 209 (1999) 462–468.
[43] L.E. Schrader, G.L. Ritenour, G.L. Eilrich, R.H. Hageman, Some characteristics of nitrate reductase from higher plants, Plant Phyisiol. 43 (1968) 930–940.
[44] M.J. Barber, B.A. Notton, Spinach nitrate reductase. Effects of ionic strength and pH on the full and partial enzyme activities, Plant Physiol. 93 (1990) 537–540.
[45] W.M. Kaiser, E. Brendle-Behnisch, Rapid modulation of spinach leaf nitrate reductase activity by photosynthesis. I. Modulation *in vivo* by CO_2 availability, Plant Physiol. 96 (1991) 363–367.
[46] W.M. Kaiser, D. Spill, Rapid modulation of spinach leaf nitrate reductase activity by photosynthesis. II. *In vitro* modulation by ATP and AMP, Plant Physiol. 96 (1991) 368–375.
[47] W.H. Campbell Jr., J. Smarrelli, Purification and kinetics of higher plant NADH:nitrate reductase, Plant Physiol. 61 (1978) 611–616.
[48] M.G. Guerrero, J.M. Vega, M. Losada, The assimilatory nitrate-reducing system and its regulation, Annu. Rev. Plant Physiol. 32 (1981) 169–204.
[49] W.H. Campbell, Separation of soybean leaf nitrate reductase by affinity chromatography, Plant Sci. Lett. 7 (1976) 239–247.
[50] A. Ahmad, M.Z. Abdin, NADH:nitrate reductase and NAD(P)H: nitrate reductase activities in mustard seedlings, Plant Sci. 143 (1999) 1–8.
[51] G.R. Baer, G.F. Collet, *In vivo* determination of parameters of nitrate utilization in wheat (*Triticum aestivum* L.) seedlings grown with low concentration of nitrate in the nutrient solution, Plant Physiol. 68 (1981) 1237–1243.
[52] A.F. Mann, D.P. Hucklesby, E.J. Hewitt, Effect of aerobic and anaerobic conditions on the *in vivo* nitrate reductase assay in spinach leaves, Planta 146 (1979) 83–89.
[53] R.H. Hageman, A.J. Reed, R.A. Femmer, J.H. Sherrard, M.J. Dalling, Some new aspects of the *in vivo* assay for nitrate reductase in Wheat (*Triticum aestivum* L.) leaves. I. Reevaluation of nitrate pool size, Plant Physiol. 65 (1980) 27–32.
[54] B.-M. Chen, Z.-H. Wang, S.-X. Li, Determination of nitrate metabolic pool in spinach leaves, Plant Physiol. Comm. 38 (2002) 124–126.
[55] M.V. Van der Leij, S.J. Smith, A.J. Miller, Remobilization of vacuolar stored nitrate in barley root cells, Planta 205 (1998) 64–72.
[56] A.J. Miller, S.J. Smith, The mechanism of nitrate transport across the tonoplast of barley root cells, Planta 187 (1992) 554–557.
[57] S.C. Huber, M. Bachmann, J.L. Huber, Post-translational regulation of nitrate reductase activity: a role for Ca^{2+} and 14-3-3 proteins, Trends Plant Sci. 1 (1996) 432–438.
[58] S.C. Huber, C. MacKintosh, W.M. Kaiser, Metabolic enzymes as targets for 14-3-3 proteins, Plant Mol. Biol. 50 (2002) 1053–1063.
[59] W.M. Kaiser, H. Weiner, A. Kandlbinder, C.-B. Tsai, P. Rockel, M. Sonoda, E. Planchet, Modulation of nitrate reductase: some new insights, an unusual case and a potentially important side reaction, J. Exp. Bot. 53 (2002) 875–882.
[60] W.M. Kaiser, D. Spill, E. Brendle-Behnisch, Adenine nucleotides are apparently involved in the light-dark modulation of spinach-leaf nitrate reductase, Planta 186 (1992) 236–240.
[61] S.C. Huber, W.M. Kaiser, 5-Aminoimidazole-4-carboxiamide riboside activates nitrate reductase in darkened spinach and pea leaves, Physiol. Plant 98 (1996) 833–837.
[62] W.M. Kaiser, S.C. Huber, Posttranslational regulation of nitrate reductase in higher plants, Plant Physiol. 106 (1994) 817–821.
[63] S.C. Huber, J.L. Huber, W.M. Kaiser, Differential response of nitrate reductase and sucrose-phosphate synthase-activation to inorganic and organic salts, *in vitro* and *in situ*, Physiol. Plant 92 (1994) 302–310.
[64] A.U. Igamberdiev, L.A. Kleczkowski, Implications of adenylate kinase-governed equilibrium of adenylates on contents of free magnesium in plant cells and compartments, Biochem. J. 360 (2001) 225–231.
[65] W.A. Jackson, K.D. Kwik, R.J. Volk, R.G. Butz, Nitrate influx and efflux by intact wheat seedlings: effects of prior nitrate nutrition, Planta 132 (1976) 149–156.
[66] W.M. Kaiser, A. Kandlbinder, M. Stoimenova, J. Glaab, Discrepancy between nitrate reduction rates in intact leaves and nitrate reductase activity in leaf extracts: what limits nitrate reduction *in situ*? Planta 210 (2000) 801–807.