Aluminium toxicity modulates nitrate to ammonia reduction

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Abstract

Two weeks-old maize (*Zea mays* cv. XL-72.3) plants were exposed to Al concentrations 0 (Al_0), 9 (Al_9), 27 (Al_{27}) or 81 (Al_{81}) g m^{-3} for 20 d in a growth medium with low ionic strength. Thereafter, the Al concentration-dependent interactions on root nitrate uptake, and its subsequent reduction to ammonia in the leaves were investigated. Al concentrations in the roots sharply increased with increasing Al concentrations while root elongation correspondingly decreased. Root fresh and dry masses, acidification capacity, and nitrate and nitrogen contents decreased from Al_{27} onwards, whereas leaf nitrogen, nitrate, nitrite, and ammonia concentrations decreased starting with Al_9. Electrolytic conductance increased by 60 % in root tissues from Al_0 to Al_{81} but it did not increase significantly in the leaves. In Al_9, Al_{27}, and Al_{81} plants a decrease in shoot fresh and dry masses was observed. Al concentrations between 0 and 27 g m^{-3} increased net photosynthetic rate, stomatal conductance, and the quantum yield of photosynthetic electron transport, whereas the intercellular CO_2 concentration was minimum in Al_{27} plants. In the leaves, nitrate reductase (E.C. 1.6.6.1) activity increased until Al_{27}, and nitrite reductase (E.C. 1.6.6.4) activity until Al_{81}. Hence there may be an Al mediated extracellular and intracellular regulation of root net nitrate uptake. Nitrate accumulation in the roots affects the translocation rates and, therefore, the nitrate concentration in the leaves. The *in vivo* reducing power generated by the photosynthetic electron flow does not limit nitrate to ammonia reduction, and the increase of maximum nitrate and nitrite reductase activities parallels the decreasing nitrate, nitrite, and ammonia concentrations.

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Abbreviations: Chl, chlorophyll; C_i, intercellular CO_2 concentration; g_s, stomatal conductance; P_N, net photosynthetic rate; Φ_e, quantum yield of photosynthetic non-cyclic electron transport.
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Additional key words: Al toxicity; ammonia; fresh and dry mass; maize; nitrate; nitrate reductase; nitrite; nitrite reductase; photosynthesis; *Zea mays*.

Introduction

Nitrate uptake is coupled to either an influx of protons or an efflux of anions. Thereafter, most of the nitrate taken up by the roots is converted into ammonia by the successive action of nitrate and nitrite reductases in the leaves. This reduction depends on irradiance, because it utilizes reducing power generated by the photosynthetic electron transport. Moreover, depending on the growth conditions (Al concentration applied in the nutrient solution being the decisive factor), Al mediates inhibition or stimulation of nitrate uptake (Keltjens and van Ulden 1987, Keltjens 1988, Klotz and Horst 1988, Taylor 1991, Nichol *et al.* 1993, Lazof *et al.* 1994). This may be connected with the decline in adenylate contents induced by Al (Lorenc-Plucińska and Ziegler 1996). Accordingly, we hypothesise that Al concentrations in the roots interact with nitrate uptake, thus affecting the translocation rates, which in turn modulates the reduction of nitrate to ammonia in the leaves. The aim of this work was to improve the understanding of the Al mediated regulation of nitrate metabolism. Following Durieux *et al.* (1993), the entire root and the second youngest leaf of maize were used as the test system. The entire roots allowed the *in vivo* determination of Al, N, and nitrate concentrations and acidification capacity as well as the extent of membrane permeability.

Materials and methods

Maize (*Zea mays* L. cv XL-72.3) seeds were washed with distilled water and sterilized by immersion in a 0.1 % mercury bichloride solution for 2 min. Seeds were then washed 5 times with deionized water and placed in an oven at 28 °C for 24 h. The seeds were germinated on moist filter paper at 28 °C for 3 d. The obtained seedlings were grown in a glasshouse (30-37/15-19 °C day/night temperature) in pots filled with a mixture of vermiculite and *Trio-hum* tray substrate (2 : 3) for two weeks. Then they were irrigated for 20 d with Al solutions between 0 and 81 g m^{-3} (pH 4). Al was applied in the form of $Al(SO_4)_3$, and the experiments were done on entire roots and the second youngest mature leaf (except the measurements of N contents, and fresh and dry masses). Root Al content was measured by the method of Chapman and Pratt (1961), and N concentrations were determined in both root and shoot tissues according to NP-1996 (1982).

Determination of root acidification capacity followed the method of Zocchi and Cocucci (1990). Roots were washed in distilled water and transferred to 50 cm^3 Erlenmeyer flasks, which were submerged in a temperature regulated shaking-bath at 25 °C, with 100 oscillations per min. After pre-incubation in 10 cm^3 of 5 mM $CaSO_4$ (pH 6.2) for 60 min, the solution was replaced with 10 cm^3 of an identical, but fresh solution containing 0.1 mM $CuSO_4$. During the incubation time (120 min), no precipitate was observed. The pH of the medium was recorded using a *Radiometer 64*

pH-meter.

Electrolytic conductance was determined with a *Crison 522* conductimeter using the modified method of Ketchie (1969). Roots were incubated for 5 h with 500 cm^3 of deionized water, while leaf discs with an area of 1.33 cm^2 were incubated for 4 h with 10 cm^3 of deionized water.

Nitrate and nitrite contents were measured colorimetrically according to Singh (1988). Plant material (3 g f.m.) was crushed thoroughly, 50 cm^3 of 2 % acetic acid were added, and the mixture was filtered through filter paper. The aqueous extract was diluted to contain 5 to 10 g m^{-3} nitrate in a final volume of 10 cm^3 in a glass stoppered test tube. To each sample, 0.4 g of a powder mixture containing 37 g citric acid, 5 g manganese sulfate monohydrate, 2 g sulphanilamide, 1 g N-(1-naphthyl)-ethylenediamine dihydrochloride, and 1 g finely powdered zinc were added. The test tube was shaken immediately and then centrifuged at 1 500×*g* for 5 min. Absorbance of the supernatant was measured in a colorimeter at 540 nm. Determination of nitrite was made using the same powder mixture, except that zinc and manganese sulphate were omitted. The nitrate and nitrite contents of the samples were estimated from a standard curve prepared for 0 to 10 g m^{-3} nitrate or nitrite solutions.

P_N, g_s, and C_i was determined on 8.25 cm^2 of an attached leaf blade, using a Portable Photosynthesis System (*LI-6200, Li-Cor*, Lincoln, USA). Plants were kept in light for 2-3 h prior to measurements, which were made under natural daylight with temperature around 30 °C, and irradiance of about 1100-1200 μmol m^{-2} s^{-1} (which was observed to be saturating).

The quantum yield of photosynthetic electron transport ($\Phi_e = q_p \times F_v'/F_m'$) was calculated according to Genty *et al.* (1989), using the *PAM 2000* Chl fluorescence measuring system (*Walz*, Effeltrich, Germany). For fluorescence measurements, leaf discs were placed in an *LD2/2* oxygen electrode chamber (*Hansatech*, Kings Lynn, UK) at 25 °C with a CO_2 saturated atmosphere (5-7 %). Photochemical quenching was calculated according to van Kooten and Snel (1990).

Ammonia concentration was measured colorimetrically based on Solorzano (1969). Plant material (500 g f.m.) was homogenized, suspended in H_2O (4 cm^3), and incubated at 45 °C for 60 min. The homogenate was then filtered, centrifuged at 2000 ×g (3 min), and 0.5 cm^3 of the supernatant was added to 8.9 cm^3 of H_2O. Thereafter, 0.3 cm^3 of solution A (175 mg phenol and 5 mg sodium nitroprussiate mixed with 5 cm^3 deionized water) and 0.3 cm^3 of solution B (1.4 g sodium citrate and 0.11 g sodium hydroxide mixed with 4.7 cm^3 deionized water and 0.22 cm^3 sodium hypochloride) were added. The samples were kept in the dark for 6 h at 20 °C. The absorbance of this solution was determined at 610 nm.

Nitrate reductase activity was assayed by the method of Wakasa *et al.* (1984), after enzyme extraction by the procedure of Kamada and Harada (1984) with minor modifications. Leaf tissue (2 g) was homogenized for 3 min at 4 °C with 10 cm^3 of 250 mM potassium phosphate buffer (pH 7.4), containing 2-mercaptoethanol (1 mM) and EDTA (1 mM). The homogenate was centrifuged at 5000×*g* for 20 min at 4 °C, and the supernatant was then re-centrifuged at 10 000×*g* for 60 min at 4 °C. Enzyme activities in this supernatant were assayed. The reaction mixture was composed of 50

mM potassium phosphate buffer (pH 7.5; 1 cm^3), 2 mM potassium nitrate (0.4 cm^3), the enzyme solution (0.6 cm^3), and 2 mM NADH dissolved in 50 mM $NaHCO_3$ (0.1 cm^3). Enzyme reaction was performed at 30-32 °C for 10 min. After that, 2 cm^3 of 1 % (m/v) sulfanilamide solution in 1.5 M HCl and 2 cm^3 of 1 % (m/v) N-(1-naphthyl)ethylenediamine dihydrochloride were added. After 10 min, 1 cm^3 of cold 25 % (m/v) trichloroacetic acid was added, and after 20 min the solution was centrifuged at 3000×*g* for 3 min. The activity of nitrate reductase was measured by recording the absorbance of the supernatant at 540 nm.

Nitrite reductase activity was assayed on thylakoid fractions according to Brunswick and Cresswell (1988) with minor modifications. After isolation the chloroplasts, osmotically burst by re-suspension in a 175 mM sorbitol solution for 3 min at 4 °C, were incubated in 1.2 cm^3 of mixture containing 380 mM sorbitol, 80 mM Hepes-KOH at pH 7.7, 27 mM sodium bicarbonate, 17.4 mM sodium dithionite, and 0.6 mM methyl viologen in 330 mM sorbitol, 70 mM Hepes-KOH at pH 7.7; 1.5 mg Chl was in the incubation mixture.

Fresh and dry masses were determined 34 d after germination using the entire root and shoot system. The ratio between fresh and dry masses of the shoot were also compared with those of the second youngest leaf: they did not vary significantly. Dry mass was measured after drying the samples at 100 °C for 10 d.

Chl determination was based on subsamples of leaf tissues and followed the method of Arnon (1949). Protein was determined according to Lowry *et al.* (1951), using a bovine serum albumin standard curve.

Results

Under the defined growth conditions, Al concentrations in root tissues increased continuously with increasing concentration of Al in the nutrient solution (Table 1).

Table 1. Root Al concentrations [mg kg^{-1}(d.m.)], and fresh and dry masses [g], N contents [g kg^{-1}(d.m.)], and electrolytic conductances of maize plants [mS m^{-1}] determined 34 d after germination when treated during the last 20 d with increasing Al concentrations. Each value is the mean ± S.E. based on three replicates of three independent series. The S.E. of root Al concentrations and N contents was equal to or less than 10 %.

Al [g m^{-3}]	Root Al	Fresh mass		Dry mass		N content		Conductance	
		roots	shoots	roots	shoots	roots	shoots	roots	shoots
0	53.7	8.3±1.0	31.6±1.0	0.9±0.1	3.3±0.1	8.8	18.6	2.0±0.4	0.35±0.01
9	73.8	13.8±1.0	47.6±2.0	1.1±0.1	4.6±0.1	12.5	17.5	2.9±0.2	0.37±0.02
27	114.2	8.2±0.2	32.0±1.0	0.9±0.0	3.3±0.1	9.8	14.6	3.0±0.3	0.38±0.06
81	163.2	7.0±0.1	19.6±1.0	0.8±0.0	2.1±0.0	9.8	11.8	3.2±0.3	0.39±0.02

The Al concentration-dependent effects on roots were a decreased elongation in all Al treatments (Fig. 1*A*) and decreased fresh and dry masses and root acidification capacity in concentrations greater than 9 g m^{-3} Al (Table 1, Fig. 1*B*). The electrolytic

conductance showed a 60 % increase until Al_{81} (Table 1). Nitrate concentration of the roots increased by 26 % in Al_9 but thereafter decreased upto 58 % of the control

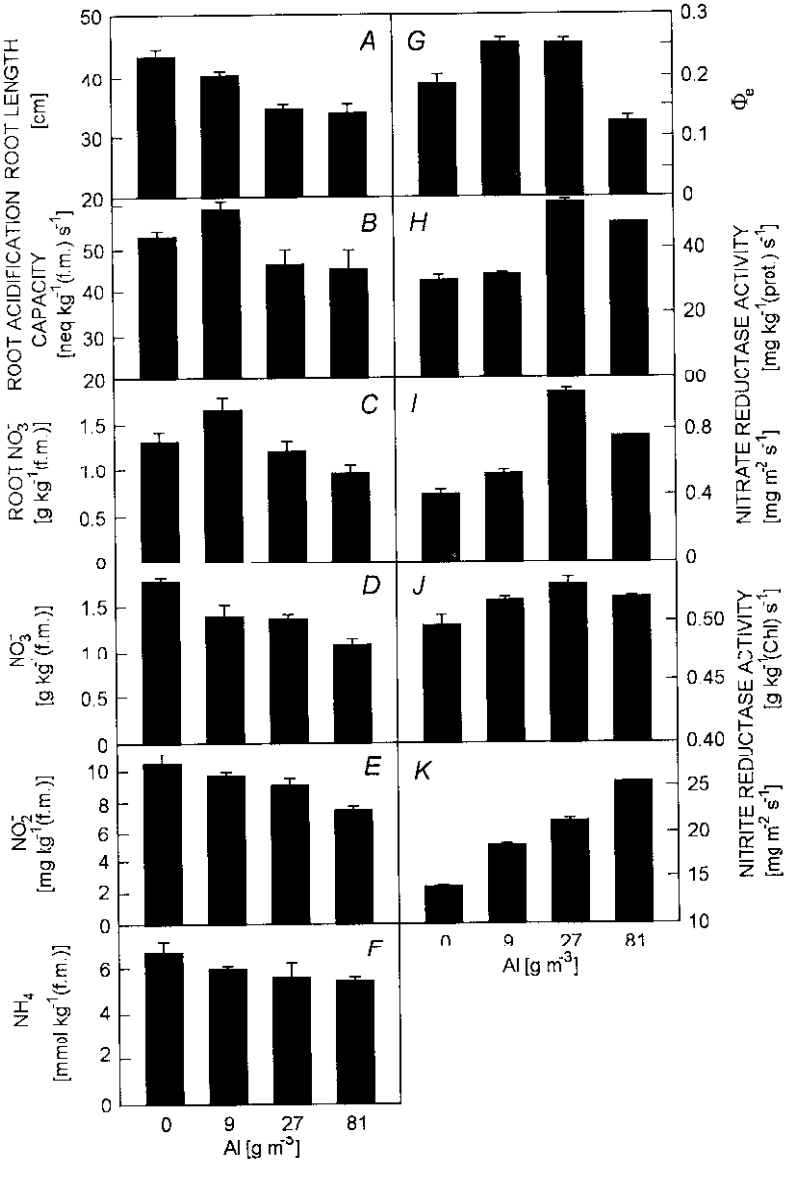

Fig. 1. Root elongation (*A*) and acidification capacity (*B*), root nitrate concentration (*C*), the second youngest leaf nitrate (*D*), nitrite (*E*) and ammonia (*F*) concentrations and nitrate reductase (*H, I*) and nitrite reductase (*J, K*) activities, as well as quantum yield of non-cyclic electron transport, Φ_e (*G*) of maize plants grown in a mixture of vermiculite and *Trio-hum tray* substrate (2 : 3) for two weeks and then irrigated for 20 d with various Al concentrations. Enzyme activities were calculated per different units (protein, chlorophyll, leaf area). Each value is the mean ± S.E. based on six (*A, B*) or three (*C* to *K*) replicates.

in Al_{81} (Fig. 1*C*).

Shoot fresh and dry masses sharply decreased from the Al_9 onwards (Table 1). Nitrate, nitrite, and ammonia concentrations in the leaves decreased in all treatments, and in the Al_{81} plants they were 62, 71, and 82 % of the Al_0 values (Fig. 1*D,E,F*). Leaf electrolytic conductance did not increase significantly in any treatment (Table 1).

Between Al_0 and Al_{27} the P_N increased by 62 % and g_s by 51 %, while C_i was minimum in the Al_{27} treatment (Table 2). In Al_{81} plants, P_N and g_s also showed higher values then in Al_0. The Φ_e also significantly increased (by about 40 %) between Al_0 and Al_{27} (Fig. 1*G*).

Table 2. Net photosynthetic rate, P_N [μmol(CO_2) m^{-2} s^{-1}], stomatal conductance, g_s [mmol(H_2O) m^{-2} s^{-1}], and intercellular CO_2 concentration, C_i [cm^3 m^{-3}] determined 34 d after maize plant germination when treated during the last 20 d with increasing Al concentrations. Each value is the mean ± S.E. based on three replicates.

	Al [g m^{-3}]			
	0	9	27	81
P_N	8.64 ± 0.80	11.49 ± 0.40	13.98 ± 0.43	9.91 ± 0.89
g_s	116 ± 13	164 ± 11	176 ± 11	133 ± 11
C_i	172 ± 17	179 ± 9	165 ± 4	180 ± 12

Nitrate reductase activity increased until the Al_{27} treatment (by 78 and 151 % on a protein and area bases, respectively) (Fig. 1*H,I*). Nitrite reductase activity per leaf area increased between Al_0 and Al_{81} by 81 % (not varying significantly on a Chl basis) (Fig. 1*J,K*).

In the roots, N concentration increased in the Al_9 treatment, but at higher Al concentrations it declined (Table 1). However, N content of the shoots decreased with increasing Al concentration. Because the ratio between root and shoot biomass changed between the different Al treatments (Table 1), the net N uptake was calculated by adding the mean N contents per root and shoot (Table 3). Maximum net

Table 3. Nitrogen root net uptake and accumulation [mg], and net N translocation to the shoot [%] per maize plant determined 34 d after plant germination and when treated during the last 20 d with increasing Al concentrations. Regression output: constant 0.7355±0.04308, X coefficient 0.00160± 0.00097, 2 degrees of freedom.

Al [g m^{-3}]	Net uptake	Shoot accumulation	Net translocation to shoot
0	69.3	61.4	88.6
9	94.3	80.5	85.4
27	57.0	48.2	84.5
81	32.6	24.8	76.0

N uptake per plant was found in Al_9 plants, showing a pattern similar to that of root

nitrate concentration. By calculating the ratio between the means of shoot content and net uptake of N, the mean translocation rate was determined (Table 3): its pattern was similar to that of nitrate concentration in the second youngest mature leaf. The regression output (computing the Y interception) using as independent and dependent variables the mean N content per shoot and the mean net uptake, respectively, indicated for the different Al treatments a tendency of N net translocation rates that followed an X coeficient of 0.0016 (Table 3).

Discussion

Different plant nutrients show complex interactions which influence their availability for plant uptake. Several ions overcome Al toxicity through competition mechanisms: in soils Al has reduced mobility and, consequently, its bioavailability is low (Coutinho 1989). The high Al concentrations of the irrigating medium extended root Al concentrations (Table 1). However, the sharp increase of root Al concentrations was also closely related to the ionic strength of the nutrient solution as well as to the concentration of Al. With decreasing ionic strength of the nutrient solution, the activity of Al increases changing thus the critical levels of Al toxicity (Pavan and Bingham 1982). Therefore, since an increasing ionic concentration of a nutrient solution increases its ionic strength, the relative activity of Al will concurrently decrease. In our experiments increasing concentrations of Al solutions (without addition of other nutrients) maximized root Al contents thus increasing the toxic effects and minimizing Al interactions with the nutrients of the vermiculite/*Trio-hum tray* substrate mixture. The fairly high Al concentration in root tissues of Al_0 (Table 1) was already related to a direct uptake provided by the vermiculite/*Trio-hum tray* substrate mixture. Al concentrations in the roots were further associated with increasing Al activities of the irrigating medium. The Al concentration-dependent effects on nitrate uptake agree with findings of Rufty *et al.* (1995). The slight increase in root Al concentration between the Al_0 and Al_9 (Table 1) might be associated with increased Al binding to negatively charged sites in the cell walls and at the external surface of membranes (Rufty *et al.* 1995). This would cause increased proton extrusion (Fig. 1*B*) and net nitrate uptake rate (Kinraide 1993), or diminished nitrate efflux (Cakmak and Horst 1991). Moreover, the electrolytic conductance is correlated with membrane permeability (Ketchie 1969) which indicates the integrity of plasma membrane. Accordingly, the Al mediated increase of root membrane permeability indicated that at Al concentrations greater than 9 g m^{-3}, the sharp increase of Al concentration (Table 1) was associated with increasing penetration into the root symplastic areas. Since under these conditions the acidification capacity is inhibited (Durieux *et al.* 1993, Lazof *et al.* 1994), the H^+/NO_3^- co-transport is modulated. In *Zea mays* (Simon *et al.* 1994), excess Al resulting from increased membrane permeability decreases the rate of nitrate uptake, possibly through inhibition of the activity of nitrate transporters. The observed inhibition of biomass production agrees with the symptoms of Al toxicity (Delhaize and Ryan 1995), associated with Al penetration into the symplasm. That may induce

mitosis inhibition (Rengel 1992), possibly through the blocking of DNA synthesis in the root apex (Delhaize and Ryan 1995). In accordance with Kinraide (1993) and Rufty *et al.* (1995), the decrease of acidification capacity at higher than 9 g m^{-3} Al treatment might constitute a prerequisite for the Al associated inhibition of root growth (Fig. 1*A*). The conclusion is that the extent of root Al concentration modulates nitrate accumulation. The initial targets of excess Al seem to implicate only the apoplast, allowing the increase of net nitrate uptake. However, higher root Al concentrations are possibly associated with increased binding of Al to the membrane channel proteins or components of the induction system for net nitrate assimilation. This would increase membrane permeability and decrease both the acidification capacity and the concurrent root nitrate concentrations.

Unchanged electrolytic conductance of leaves (Table 1) indicated that membrane permeability was unaffected by the excess of Al. Hence the decreased accumulation of nitrate in the leaves was not due to plasma membrane degradation which could inhibit nitrate translocation. Determinations of the metabolic products of N do not necessarily indicate the total amount of nitrate absorbed by roots and subsequently translocated to the leaves. However, as nitrate concentrations in both root and leaf tissues were similar, the Al mediated effects on net nitrate uptake by the roots probably modulated the nitrate translocation rates. Moreover, the measurement of N content in both root and leaf tissues allowed to estimate the rates of net N uptake and translocation. As nitrate and ammonium root absorption are the sources of N assimilation, a direct evidence of Al interactions was provided. The conclusion was that, as the integrity of leaf plasma membranes remained the same, independent of root Al concentrations, net N translocation rate was a function of an X coefficient having the value of 0.0016 (Table 3). Al affected N concentrations in the leaves mainly by changing the net uptake rates. Indeed, the absolute amount that was translocated was a function of the absolute N content in the roots. This is a final evidence of the Al modulated effect on N concentrations, subsequently implicating nitrate.

Leaf nitrate reduction is also coupled to a mechanism closely determined by a dynamic balance between substrate accumulation and consumption, implicating the *in vivo* reducing power generated by the photosynthetic electron flow. Fluorescence measurements at room temperature in combination with studies of leaf gas exchange allow to determine the Al interacting effect on the partitioning of excitation energy between photochemical processes responsible for CO_2 reduction and non-photochemical processes. Indeed, the yield of fluorescence emission is inversely related to P_N, and the fluorescence yield of Chl *a* depends on the microenvironment in the thylakoid, therefore indicating "structural" or "organizational" changes of the chloroplast membranes and elucidating the physiological and biochemical bases for changes in the ability of leaves to assimilate CO_2. Similarly as in tomato (Simon *et al.* 1994), excess Al increased P_N in mesophyll cells without stomata limitation until the Al_{27} treatment (Table 2). Furthermore, Φ_e (Fig. 1*G*) also indicated that, independently of structural changes in the photosynthetic apparatus triggered by Al excess, the *in vivo* reducing power generated by the photosynthetic electron flow increased until the same Al treatment. Therefore, the conclusion is that *in vivo* the

photosynthetic electron flow does not inhibit the nitrate to ammonia reduction. According to Dinev and Stancheva (1993), the Al mediated increase of nitrate and nitrite reductases' activities indicates that *in vivo* the nitrate to ammonia reduction in leaves is not limited by these enzymes. However, increasing Al toxicity coupled to the decreasing nitrate accumulation triggered substrate limitations to the functioning of maximum nitrate reductase activity. Under these conditions, the Al mediated modulation of nitrate reductase activity might control metabolic oscillations implicating an increase in nitrate concentrations (Sanchez and Heldt 1990). Indeed, variations in nitrate concentrations implicate concurrent oscillations in nitrate reductase activity, which restore the initial balance between nitrate reduction and accumulation. Since the concentrations of nitrite are somewhat complementary to those of nitrate, Al mediated interactions coupled to nitrate accumulation further control nitrite and ammonia concentrations. As nitrate reductase activity is limited by a decreased accumulation of substrate, the concurrent decrease of nitrite concentrations also becomes a limitation for maximum nitrite reductase activity, as the ammonia concentrations are also affected. Moreover, nitrite reductase activity also parallels the Al mediated modulation of nitrate reductase, limiting the imbalance between nitrite consumption and accumulation.

In agreement with the hypothesis tested, the final remark is that Al modulates the supply of nitrate to the leaves through a concurrent effect on net nitrate uptake by the roots. However, although root nitrate accumulation might affect the extent of the translocation rates and, therefore, the nitrate concentration in leaves, the integrity of plasma membranes is unaffected. The reducing power generated by the photosynthetic electron flow is not a limiting factor for the reduction of nitrate to ammonia. The imbalances of nitrate to ammonia reduction in the leaves are also controlled, since Al modulation of maximum catalytic properties of nitrate and nitrite reductases parallels the antagonistic nitrate, nitrite, and ammonia concentrations.

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