Aluminium modulation of the photosynthetic carbon reduction cycle in *Zea mays*

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Abstract

Two-weeks-old maize (*Zea mays* L. cv. XL-72.3) plants were submitted to Al concentrations of 0-81 g m^{-3} for 20 d, after which the Al concentration-dependent effects on CO_2 uptake by the mesophyll tissue and subsequent CO_2 assimilation in the photosynthetic carbon reduction cycle of bundle sheath cells were investigated. The net photosynthetic rate (P_N) and stomatal conductance (g_s) increased continuously up to 27 g m^{-3} Al, whereas the intercellular CO_2 concentration showed minimum values with the 27 g m^{-3} Al treatment. Moreover, the starch and saccharide concentrations, and fructose-1,6-bisphosphatase did not change significantly with increasing Al concentrations. The photosynthetic electron transport rates along with photosystems 2 and 1 started falling from 9 g m^{-3} Al onwards, while thylakoid acyl lipid composition did not show a clear pattern. With the Al concentration at 81 g m^{-3}, NADP-malate dehydrogenase activity decreased to minimum values, whereas the opposite occurred with those of pyruvate dikinase, NADP-malic enzyme, and phosphoenolpyruvate carboxylase. Thus *in vivo* Al concentrations modulate the

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Abbreviations: BSA = bovine serum albumin; C_i = intercellular CO_2 concentration; DGDG = digalactosyldiacylglycerol; DTT = dithiothreitol; FBP = fructose 1,6 bisphosphate; FBPase = fructose-1,6-bisphosphatase; GL = total galactolipids; g_s = stomatal conductance; LDH = lactate dehydrogenase; MGDG = monogalactosyldiacylglycerol; MV = methyl viologen; NADP-MDH = NADP-malate dehydrogenase; NADP-ME = NADP-malic enzyme; OAA = oxaloacetate; PC = phosphatidylcholine; PCR = photosynthetic carbon reduction; PEP = phosphoenolpyruvate; PEPC = phosphoenolpyruvate carboxylase; PG = phosphatidylglycerol; PI = phosphatidylinositol; PL = total phospholipids; P_N = net photosynthetic rate; PPDK = pyruvate P_i dikinase; PS = photosystem.

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photosynthetic reduction cycle, possibly by interacting with the carbon flow rate exported to the cytosol. Although the inhibition of NADP-malate dehydrogenase activity might limit pyruvate dikinase, NADP-malic enzyme, and phosphoenol-pyruvate carboxylase activities, *in vivo* the balance between phosphoenolpyruvate production and its carboxylation remains unaffected.

Additional key words: Al; fructose-1,6-bisphosphatase; intercellular CO_2 concentration; maize; NADP-malate dehydrogenase; net photosynthetic rate; photosystems 1 and 2; saccharides; stomatal conductance.

Introduction

The CO_2 diffusion in C_4 leaves between bundle sheath and mesophyll cells is highly restricted (Hatch and Osmond 1976, Hatch 1987, Jenkins *et al.* 1989). The restriction may be associated with the existence of suberized lamellae (Hattersley and Perry 1984) or related to specific structures of bundle sheath cell walls (Weiner *et al.* 1988). Such barriers would be necessary for the development of relatively high CO_2 concentrations in the bundle sheath cells (possibly in excess of 20 times atmospheric concentrations, and largely in the form of bicarbonate at a physiological pH, when the thermodynamic equilibrium is reached) during photosynthesis (Hatch and Osmond 1976, Furbank and Hatch 1987, Furbank *et al.* 1989, Jenkins *et al.* 1989). The resulting suppresssion of ribulose-1,5-bisphosphate oxygenase activity and corresponding reduction in photorespiration account for many of the physiological features of C_4 species (Edwards and Walker 1983, Hatch 1987). Depending on growth conditions, Al mediates inhibition or stimulation of photosynthesis (Hampp and Schnabl 1975, McLean 1979, Cambraia and Calbo 1980, Foy 1984, Haug 1984, Ohki 1986, Simon *et al.* 1994) and the content of adenylates (Lorenc-Plucińska and Ziegler 1996). Moreover, an excessive Al concentration affects the structure of photosynthetic membranes (Moustakas and Ouzounidou 1994). Accordingly, the purpose of this study was to investigate the photosynthesis related parameters modulation by a broad range of Al concentrations on the CO_2 uptake, and by the mesophyll tissue with its concomitant assimilation in the PCR cycle of bundle sheath cells.

Materials and methods

Maize (*Zea mays* L. cv. XL-72.3) seeds were washed in distilled water and sterilized by immersion in a 0.1 % mercury dichloride solution for 2 min. Then they were washed 5 times in deionized water and placed in an oven at 28 °C for 24 h. Further they were germinated on moist filter paper at 28 °C for 3 d. The 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, and then irrigated for 20 d with Al solutions ranging from 0 to 81 g m^{-3} (pH 4). Al was applied in the form of $Al_2(SO_4)_3$. The experiments were done on the second youngest leaf of each plant collected after 20 d of germination.

P_N, g_s, and C_i were determined on 8.25 cm^2 attached leaf blade, using a Portable Photosynthesis System (*LI-6200, Li-cor*, Lincoln, USA). The plants were left in the daylight for 2-3 h prior to measurements which were taken under the temperature of around 30 °C and irradiances of about 1100-1200 μmol m^{-2} s^{-1} (which was observed to be saturating).

Soluble saccharides and starch concentrations were measured according to Ashwell (1957) and McCready *et al.* (1950), respectively. The determination of photosynthetic electron transport rates associated to PS2 and PS1 ($H_2O \rightarrow MV$) as well as preparation of subchloroplast fractions followed the methods described by Droppa *et al.* (1987).

Extraction and analysis of chloroplast lipids were performed with minor modifications, as described by Droppa *et al.* (1987). After chloroplast isolation, phospholipase D was inactivated by boiling the probes in isopropyl alcohol for 2 min. The extract, recovered in chloroform containing 0.05 % butylated hydroxytoluene, was spotted onto *Silicagel 60* plates to separate the polar lipids in acetone/benzene/water (91:30:8, v/v/v) as solvent. The plates were sprayed with 1 % 8-anilinonaphtalene sulphonic acid in methanol, and viewed under an UV radiation. Standards were used for the identification. Samples were therefore scraped, and galactolipid and phospholipid concentrations were estimated by measuring galactose and phosphorus following Ashwell (1957) and Fiske and Subbarow (1925), respectively.

PPDK was extracted according to Bauwe (1984) and assayed spectrophotometrically by measuring pyruvate production in the presence of LDH (Andrews and Hatch 1969). At room temperature, 1 g fresh mass was homogenized in 3 cm^3 0.05 M HEPES-KOH, pH 7.5, 5 mM DTT, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 2 mM pyruvate-Na, and 1 % BSA, for 15 min with an addition of 100 mg *Dowex 1x4*. After centrifugation at 20 000×*g*, the supernatant was used for the enzyme assay. The reaction mixtures contained 50 mM Tris-HCl, pH 8.3, 6 mM $MgSO_4$, 10 mM DTT, 0.15 mM NADH, 1 mM PEP, 1 mM AMP, 1 mM P_i, and 6 units of LDH. The rate of decrease in A_{340} was measured at 30 °C.

NADP-MDH, NADP-ME, and PEPC were extracted according to Bauwe (1984). About 0.5 g of fresh mass was homogenized in a cold mortar in a fivefold amount of 0.1 M Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 2 mM EDTA, 5 mM DTT, 0.002 % pyridoxal phosphate, and 50 mg *Dowex 1x4*. The assays of NADP-MDH and NADP-ME included a preincubation period (equal volumes of extract and 10 mM DTT/25 mM $MgCl_2$ were mixed and incubated for 60 min at 25 °C). Thereafter, 50 mm^3 of the mixture was added to 50 mM Tricine-KOH, pH 8.0, 3 mM OAA, and 0.15 mM NADPH, and the activity of NADP-MDH was measured spectrophotometrically at 340 nm according to Bauwe (1984). NADP-ME activity was measured using 50 mm^3 of the preincubated mixture in 50 mM Tricine-KOH, pH 8.0, 0.2 mM EDTA, 5 mM L-malate, 2.5 mM DTT, and 0.5 mM NADP (Bauwe 1984). After recording a steady-state blank rate at 340 nm due to the action of NADP-MDH, the decarboxylation reaction was started by the addition of 5 mM $MnCl_2$. PEPC was assayed spectrophotometrically at 32 °C, as described by Silva *et al.* (1991). The assay medium contained 50 mM HEPES-KOH (pH 8.0), 10 mM

$MgCl_2$, 0.12 mM EDTA, 5 mM DTT, 10 mM $NaHCO_3$, 6 units of MDH, 0.1 mM NADH, and 10 mM PEP. The reaction was started by the addition of 100 mm^3 of extract. FBPase activity was assayed by measuring the phosphate release from FBP, following Graham *et al.* (1970). The reaction mixture (0.5 cm^3) contained 100 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, 1.6 mM EDTA, and 5 mM FBP. Prior to the measurement of inorganic phosphate, the reaction was stopped by adding 0.5 cm^3 of 10 % perchloric acid.

The protein content was determined according to Lowry *et al.* (1951) using a BSA standard curve.

Table 1. Net photosynthetic rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), acyl lipids' composition of thylakoids, and NADP-MDH, NADP-ME, PEPC, and PPDK activities of two-weeks old maize plants submitted to increasing Al concentrations for 20 d. Each value is the mean ± S.E. based on three replicates.

Parameter	Al [g m^{-3}]			
	0	9	27	81
P_N [μmol(CO_2) m^{-2} s^{-1}]	8.64 ± 0.80	11.49 ± 0.40	13.98 ± 0.43	9.91 ± 0.90
g_s [mmol(H_2O) m^{-2} s^{-1}]	116 ± 13	164 ± 11	176 ± 11	133 ± 11
C_i [cm^3 m^{-3}]	172 ± 17	179 ± 9	165 + 4	180 ± 12
DGDG [kg kg^{-1}(Chl)]	1.08 ± 0.09	1.60 ± 0.02	1.32 ± 0.06	2.93 ± 0.09
[% of total]	0.61 ± 0.08	0.83 ± 0.03	0.53 ± 0.01	1.58 ± 0.08
MGDG [kg kg^{-1}(Chl)]	0.47	0.47	0.51	0.46
[% of total]	0.26	0.24	0.21	0.25
GL [% of total]	0.73	0.71	0.72	0.71
PC [kg kg^{-1}(Chl)]	0.17 ± 0.03	0.27 ± 0.01	0.18 ± 0.01	0.48 ± 0.01
[% of total]	0.07	0.08	0.08	0.08
PI [kg kg^{-1}(Chl)]	0.27 ± 0.11	0.39 ± 0.01	0.29 ± 0.02	0.70 ± 0.01
[% of total]	0.11	0.11	0.11	0.11
PG [kg kg^{-1}(Chl)]	0.21 ± 0.04	0.34 ± 0.07	0.26 ± 0.05	0.61 ± 0.12
[% of total]	0.09	0.10	0.10	0.10
PL [% of total]	0.27	0.29	0.29	0.29
PPDK [mmol kg^{-1}(prot.) s^{-1}]	69.4 ± 12.7	197.2 ± 20.0	183.3 + 20.5	400.0± 12.5
NADP-MDH [mol kg^{-1}(prot.) s^{-1}]	10.4 ± 1.9	9.9 ± 2.2	8.3 ± 0.8	7.0 ± 0.4
NADP-ME [mmol kg^{-1}(prot.) s^{-1}]	800 ± 83	1186 ± 36	1124 ± 50	1578 + 126
PEPC [mol kg^{-1}(prot.) s^{-1}]	317 ± 55	275 ± 53	350 ± 42	403 ± 28

Results and discussion

The Al toxicity is closely related to ionic strength of a nutrient solution along with its Al activity (Pavan and Bingham 1982). With decreasing ionic strength of the nutrient solution, the Al activity increases, therefore changing the critical levels of toxicity (Khashawneh 1971, Pavan and Bingham 1982). Thus, as rising ionic concentration of

the nutrient solution increased the ionic strength and decreased the concurrent relative activity of Al (Foy 1978), the irrigating medium (that contained increasing Al concentrations without other nutrients) used in this experiment maximized the toxic effects. Additionally, although maize was grown in a vermiculite/*Trio-hum tray* substrate mixture, increasing Al concentrations in the irrigating medium also overpassed the reduced mobility of Al as well as the consequent low bioavailability. The Al concentration-dependent effects on P_N and g_s were a continuous 1.51-1.62 fold increase until the 27 g m^{-3} Al treatment, whereas C_i showed minimum values at the 27 g m^{-3} Al treatment (Table 1). These results allow the estimation of the Al interacting effects on the partioning of excitation energy between photochemical processes responsible for CO_2 reduction and non-photochemical processes. The increasing P_N and g_s, associated with the variation of C_i, indicated that the ability to assimilate CO_2 increased in mesophyll cells without stomata limitations.

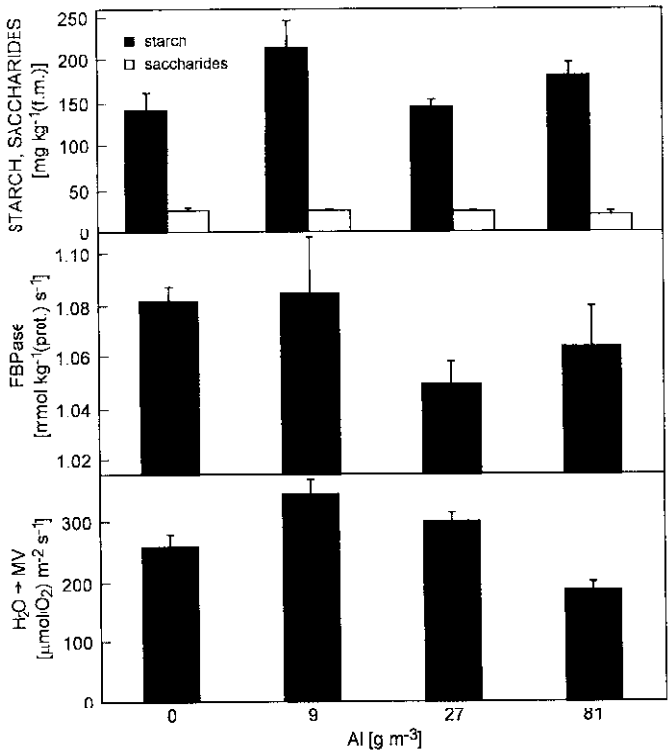

Fig. 1. Contents of starch and soluble saccharides (*top*), activity of fructose-1,6-bisphosphatase, FBPase (*middle*), and rates of $H_2O \rightarrow MV$ reactions (*bottom*) of two weeks old maize plants submitted to increasing Al concentrations for 20 d. Each value is the mean ± S.E. based on three replicates.

Starch and soluble saccharide concentrations as well as FBPase activity did not vary significantly with increasing Al concentrations (Fig. 1). Thus, the increase of CO_2 uptake coupled with the stabilized rate of starch and soluble saccharides accumulation indicated that carbon flux necessary for the regeneration of ribulose bisphosphate was preserved. FBPase activity is a limiting step in the PCR cycle,

further controlling saccharide synthesis (Lawlor 1987). Under the defined conditions the FBPase activity remained almost constant, and hence the rate of saccharide synthesis was unaffected. Moreover, until the 27 g m^{-3} Al treatment, the additional assimilated carbon might be exported to the cytosol *via* a phosphate translocator in the form of triose-P produced by the assimilatory segment of the PCR cycle (Geiger and Servaites 1994).

The rates of $H_2O \rightarrow MV$ reactions showed, on the leaf area basis, an 1.8-fold decrease from 9 to 81 g m^{-3} Al (Fig. 1), while the acyl lipid composition of thylakoids did not show a clear pattern (Table 1). As the measurements of $H_2O \rightarrow MV$ reactions were done without substrate limitations, the rates of thylakoidal electron transport associated with PS2 and PS1 indicated a decreasing rate of ATP synthesis and NADP reduction. Yet, although an Al excess may mediate the degradation of photosynthetic membranes (Moustakas and Ouzounidou 1994), the concentrations of acyl lipids did not change significantly in our experiments and, additionally, the ratio between total galactolipids or phospholipids and total acyl lipids remained constant. These results indicate that Al-mediated effects on the thylakoid structure are probably not directly responsible for the inhibition of photosynthetic electron flow. Although the Al toxicity might potentiate the inhibition of maximum rates of Hill and Mehler reactions, *in vivo* and under all the studied Al treatments, the photosynthetic electron flow rates were (as a result of multiple limiting steps associated to the photosynthetic metabolism) much smaller than the values obtained *in vitro* (therefore allowing the increase of P_N).

In the NADP-ME type enzymatic system coupled to mesophyll and bundle sheath cells, NADP-MDH activity decreased by 67 % between the 0 and 81 g m^{-3} Al treatments, whereas those of PPDK, NADP-ME and PEPC showed, on the protein basis, 5.75, 1.97, and 1.27 fold increases (Table 1). These results agree with reports of Hatch (1977) and Edwards *et al.* (1985) suggesting thus that the inhibition of photosynthetic electron flow rate of *Zea mays* (Fig. 1) is coupled to the inhibition of NADP-MDH activity. Furthermore, under saturating CO_2 concentrations, the inhibition of this enzyme activity might further limit maximum activities of NADP-ME, PPDK, and PEPC. As PPDK is activated by high ratios of ATP/ADP and pyruvate/PEP in the mesophyll cells (Edwards *et al.* 1985), the important regulatory role advanced for PPDK responsible for the regeneration of PEP (Andrews and Hatch 1969) is limited under these conditions. The *in vitro* inhibition of photosynthetic electron transport rates (Fig. 1) also limits O_2 evolution by changing the pseudocyclic phosphorylation rates and/or by changing the electron transport system required for cyclic or pseudocyclic photophosphorylation (Ziem-Hanck and Heber 1980, Edwards *et al.* 1985). Thus, as in maize O_2 is apparently required for PPDK activation (Edwards *et al.* 1985), this might be an additional inhibitory factor for the activity of this enzyme. Nevertheless, the increased CO_2 uptake in the mesophyll cells allows to conclude that, due to the multiple limiting steps associated with this metabolism, the *in vivo* activity of NADP-MDH will also not be achieved and, therefore, OAA and malate will not accumulate the inhibiting PEPC (O'Leary 1982). Furthermore, the reaction proceeding towards PEP synthesis will not be

inhibited, and this will contribute to maintaining a balance between PEP production and its utilization in carboxylation reactions.

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