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Effect of inland salt-alkaline stress on C_4 enzymes, pigments, antioxidant enzymes, and photosynthesis in leaf, bark, and branch chlorenchyma of poplars

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Abstract

The effects of soil salt-alkaline (SA) stress on leaf physiological processes are well studied in the laboratory, but less is known about their effect on leaf, bark and branch chlorenchyma and no reports exist on their effect on C_4 enzymes in field conditions. Our results demonstrated that activities of C_4 enzymes, such as phospho*enol*pyruvate carboxylase (PEPC), NADP-malic enzyme (NADP-ME), pyruvate orthophosphate dikinase (PPDK), and NADP-dependent malate dehydrogenase (NADP-MDH), could also be regulated by soil salinity/alkalinity in poplar (*Populus alba* × *P. berolinensis*) trees, similarly as the already documented changes in activities of antioxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR), pigment composition, photosynthesis, and respiration. However, compared with 50–90% changes in a leaf and young branch chlorenchyma, much smaller changes in malondialdehyde (MDA), antioxidative enzymes, and C_4 enzymatic activities were observed in bark chlorenchyma, showing that the effect of soil salinity/alkalinity on enzymatic activities was organ-dependent. This suggests that C_4 enzymatic ratios between nonleaf chlorenchyma and leaf (the commonly used parameter to discern the operation of the C_4 photosynthetic pathway in nonleaf chlorenchyma), were dependent on SA stress. Moreover, much smaller enhancement of these ratios was seen in an improved soil contrary to SA soil, when the fresh mass (FM) was used as the unit compared with a calculation on a chlorophyll (Chl) unit. An identification of the C_4 photosynthesis pathway *via* C_4 enzyme difference between chlorenchyma and leaf should take this environmental regulation and unit-based difference into account.

Additional key words: NADP-dependent malate dehydrogenase; NADP-dependent malic enzyme; phospho*enol*pyruvate carboxylase; photosynthetic pathway discrimination; pyruvate orthophosphate dikinase; woody chlorenchyma.

Introduction

About 15% of the world total land area has been degraded by soil erosion or by physical and chemical degradation, including soil salinization (Wild 2003). Salinity problems get worse, when the affected soils are also alkaline. Areas in China, such as the Songnen Plain, are characterized by poor soil physical properties, infertility, excessive Na_2CO_3 and $NaHCO_3$, and high pH. These conditions are even more toxic to plants than a saline soil alone (Yang *et al.* 2008). Many C_4 photosynthetic dicots originated in arid regions or SA soils, showing that the combined effects of heat, drought, and/or salinity are important conditions for promoting the evolution of C_4 photosynthesis (Pyankov *et al.* 2000, Tang and Liu 2001, Voznesenskaya *et al.* 2001, Han *et al.* 2006, Urban *et al.* 2010). The C_4 photosynthetic pathway is more efficient than the C_3 pathway owing to the special enzymatic system together with the specialized anatomy of bundle sheath used to pump up CO_2. C_4 plants can survive better in the harsh environments such as those with SA soils compared to C_3 plants (Osmond *et al.* 1982, Pyankov *et al.* 2000, Voznesenskaya *et al.* 2001, Sage 2004). It is well reported that SA stress induces significant changes in photosynthesis and respiration, antioxidative activities, and membrane leakage in

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Abbreviations: Car – carotenoids; CAT – catalase; Chl – chlorophyll; DTT – dithiothreitol; EC – electrical conductivity; FM – fresh mass; GR – glutathione reductase; MDA – malondialdehyde; NADP-MDH – NADP-dependent malate dehydrogenase; NADP-ME – NADP-dependent malic enzyme; P_N – net photosynthetic rate; PEPC – phospho*enol*pyruvate carboxylase; PPDK – pyruvate orthophosphate dikinase; PSII – photosystem II; SA – salt-alkaline; SOD – superoxide dismutase; R_D – dark respiration rate.
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leaves (Dickson and Isebrands 1991, Yan *et al*. 2009, Yan *et al*. 2010, Yang *et al*. 2008). Therefore it seemed reasonable to postulate,as one of the main characteristics for the evolution of C_4 (Sage 2004, Pan 2004) that C_4 photosynthetic enzymes might be also involved in the physiological acclimatization of plants to SA stress (Doubnerová and Ryšlavá 2011).

Hibberd and Quick (2002) reported that the stems of typical C_3 plants had more than 10 times greater activity of NADP-ME, NADP-ME, and PEPC than in leaf blades. Similarly, Berveiller and Damesin (2008) found that the PEPC and NADP-ME activity were 13 and 30 times greater, respectively, in a woody branch chlorenchyma than in leaf blades. These data on C_4 enzyme activities are within the range of ratios seen in typical C_4 leaves compared to C_3 plants (Hibberd and Quick 2002). Therefore it was concluded that these plants have a C_4 photosynthetic pathway in a nonleaf chlorenchyma. This shows the importance of examining C_4 enzymatic ratios, between woody chlorenchyma and leaf blades, in photosynthetic pathway discrimination. However, less attention has been given to an environmental influence (such as soil salinity/alkalinity) on C_4 enzymatic activities of leaves and no reports are available on a nonleaf chlorenchyma of branches and bark. Given the same effect on a leaf, branch and bark chlorenchyma, C_4 enzymatic ratios between a nonleaf chlorenchyma and leaf should be conservative with soil salinity/alkalinity; or else, these ratios would be variable and be unsuitable for photosynthetic pathway discrimination. This was tested in this paper.

Furthermore, the unit basis for expressing C_4 enzymatic activity may modify these ratios between a nonleaf chlorenchyma and leaf to a greater or lesser extent. Given that there are similar enzymatic activities on FM basis, the C_4 enzymatic activities per one unit of Chl in a woody chlorenchyma should be several times higher than those in a leaf because the leaf Chl is several-fold higher than in a woody chlorenchyma (Wang *et al*. 2007). At present, most studies have used the Chl-based unit, and it is necessary to check the influence of a different unit-basis in calculating enzymatic ratios between a nonleaf chlorenchyma and a leaf.

In order to provide an accurate understanding of physiological processes in nature, the present paper studied the effects of SA stress on poplar trees in the field conditions. C_4 enzymes, pigments, antioxidant enzymes, and photosynthetic capacity of leaves, bark, and branch chlorenchyma were determined in poplar trees grown in both improved soil and the control, SA soil conditions. The following questions were investigated: (*1*) Are C_4 enzyme activities similar to pigments, antioxidant enzymes and gas exchange parameters for indicating the biochemical regulation of a plant to soil SA stress? What are the differences between a leaf and woody chlorenchyma of branches and bark in variable C4 enzymatic activities? (*2*) How could soil SA stress and unit expression (FM basis and Chl basis) affect the C_4 enzymatic ratios between a nonleaf chlorenchyma and leaf, typical parameters indicative of the C_4 photosynthetic pathway?

Materials and methods

Study site: The Songnen Plain is located in the central part of northeastern China. This plain is undergoing rapidly increasing alkalization and desertification (Lin and Tang 2003, Wang *et al.* 2009). The study site is located in the economic development zone of Zhaodong City (125°58′E, 46°04′N), Heilongjiang Province, China. This region is in a cold-temperate zone. The weather in the spring is windy with little rain. The summer is hot and wet, the autumn is cool but prone to early frost, and the winter is very cold and dry. The period without frost is on average 140 d, the annual precipitation ranges from 293 to 656 mm with an average of 448 mm, and the mean air temperature is 3.2°C.

Treatments: Laboratory pot simulations can control soil salinity and alkalinity (Yan *et al.* 2009, 2010), but it is difficult to simulate the physical soil properties occurring in the field (*e.g.* poor soil quality with high bulk density and so poor infiltration capacity). SA stress in the field is a compound stress of high salinity, alkalinity, poor soil physical properties, and nutrient deficiency (HLJTR 1993, Wang *et al.* 2011). Thus, field treatments instead of laboratory pot simulations were used in this paper, *i.e.* 3-year-old poplar trees (*Populus alba* × *P. berolinensis*) (with an average trunk diameter at breast height of 4.0 cm) were planted in the field with an untreated and improved SA soil at a density of 2,500 trees ha^{-1}. In the SA soil control, the trees were planted in holes 40 cm in diameter and 40 cm in depth without any modification of the soil. In the improved soil (an area of approximately 3,000 m^2 adjacent to the control), composted cornstalks and dairy manure (1 part), and dark brown forest soil (high in soil organic matter) (1 part) were mixed fully with the SA soil from the hole dug out for tree planting (50 cm in diameter and 50 cm in depth) (2 parts). Moreover, about 10 cm of cornstalk and dairy manure were layered at the bottom and sides of the holes, in order to prevent SA invasion; about 0.5 L of hydrolyzed polymaleic anhydride (HPMA) soil conditioner was put into the each hole for the improved soil conditions (Wang *et al.* 2011).

Measurements and sampling: The duration of SA stress treatment used in this paper was 1 year, long enough to strongly affect the growth of poplar trees (HLJTR, 1993). The following measurements were made on 5 trees from

each treatment. Photosynthetic rate, respiration, and Chl fluorescence were measured in the field. Samples were also collected for laboratory measurements. In August, the branch chlorenchyma was collected from 1-year-old branches, and a knife blade was used to exclude the inner woody xylem. The bark chlorenchyma was collected from the main trunk and cut as strips of 2 cm in a width, 10 cm in a length, and a depth down to the main woody xylem using a knife blade. Leaf samples were collected and the main leaf veins were cut out using a knife blade. All samples of leaves (minus main vein), branches, and bark chlorenchyma were rinsed with distilled water, then stored in a liquid nitrogen for transporting to the laboratory, where they were finally stored at –70°C prior to determinations of pigments, C_4 and antioxidant enzyme activities.

Chl content was determined using dimethyl sulfoxide (DMSO) as the extraction solvent (Wellburn *et al.* 1994). Plant materials were extracted at 60°C for a period of 2 h, the temperature was maintained by a water bath with a thermostat, and light was kept out by wrapping the sample with an aluminum foil. However, the samples were stored in the dark until all greenish remnants had been extracted by the DMSO solution, if any Chl remained in the plant material The supernatant was decanted and the absorbance was read at a wavelength of 480 nm, 665 nm, and 649 nm using a *UV-Vis* spectrophotometer *UV-2550* (*Shimadzu*, Japan). Chl *a*, Chl *b*, and total carotenoids (Car, carotenes plus xanthophylls, C_{x+c}) were quantified according to the methods described by Wellburn *et al.* (1994).

C_4 enzymes were extracted according to the method of Sayre *et al.* (1979), with some modifications. Frozen samples of leaf blades, bark, and branch chlorenchyma (*ca*. 0.5 g) were ground rapidly using a precooled pestle and mortar on an ice bath, together with 8 ml of the extraction buffer containing 50 mM Tris-HCl (pH7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), and 2% (w/v) insoluble polyvinylpolypyrrolidone (PVPP). PVPP was included to prevent the oxidation and polymerization of phenolic compounds in the crude enzyme preparation (Schaedle *et al.* 1986). The homogenates were filtered through 4 layers of gauze and the supernatant was centrifuged, first at 12,000 × *g* and 4°C for 8 min, then at 15,000 × *g* at 4°C for 12 min. Thereafter, the supernatant was desalted on a *Sephadex G-25* column with a desalting buffer containing only Tris/HCl and DTT. This desalting supernatant was immediately used to assay activities of PEPC, PPDK, NADP-ME, and NADP-MDH.

Following the method of Gonzalez *et al.* (1984), PEPC (EC 4.1.1.31) activity was assayed in a 3 ml mixture containing 50 mM Hepes-KOH (pH 8.0), 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.2 mM NADH, 2 mM phospho*enol*-pyruvate (PEP), and 1.5 unit NAD-MDH. The reaction was initiated by adding the crude enzyme extract (100 µl).

PPDK (EC 2.7.9.1) activity was assayed as described by Hatch and Slack (1975). The total volume of the reaction mixture 3 ml comprised of 25 mM Hepes-KOH (pH 8.0), 8 mM $MgSO_4$, 10 mM DTT, 10 mM $NaHCO_3$, 2 mM pyruvic acid (sodium salt), 5 mM $(NH_4)_2SO_4$, 2.5 mM KH_2PO_4, 0.5 units PEP, 2 units NAD-MDH, and 1 mmol L^{-1} ATP. The reaction was initiated by adding the crude enzyme extract (100 µl).

NADP-ME (EC 1.11.1.40) activity was assayed as described by Johnson and Hatch (1970), following NADPH production. The standard assay medium (3 ml) contained 50 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM EDTA, 0.33 mM NADP, and 5 mM L-malate. The reaction was initiated by adding the crude enzyme extract (100 µl).

NADP-MDH (EC 1.1.1.37) activity was determined as described by Johnson and Hatch (1970). To determine the maximal potential activity of NADP-MDH, the crude enzyme preparation was supplemented with 0.1 mM DTT and 0.2 mM $MgCl_2$, and was incubated at 30°C for 1.5 h prior to assay. The total volume of reaction medium comprised 100 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.18 mM NADPH. The reaction was started by adding of 0.5 mM oxaloacetic acid to a final volume of 3 ml. The changing rate in the absorbance at 340 nm was monitored spectrophotometrically and millimolar extinction coefficient 6.22 mM^{-1} cm^{-1} was used for the computation of the above enzyme activities. The same *UV-2550* spectrophotometer was used throughout this study, and enzyme content was expressed, in this work, using two types of unit, one Chl-based and the other per unit of FM.

Net photosynthetic rate (P_N) and dark respiration rate (R_D) were measured using a *Li-6400* photosynthesis system (*Licor,* Nebraska, USA) with a standard chamber (2 × 3 cm broadleaf chamber fitted with a *6400-02B* red/blue LED light source). Leaf gas exchange was measured using intact leaf samples, whereas measurements made on branches and bark chlorenchyma were done on detached samples. Upon an excision, the detached branches were immediately placed in water and carefully defoliated; vaseline was used to seal off the wounds. Then, 3–5 branches (< 4 mm) were selected for P_N and R_D measurements. R_D was recorded after 5 min, then the *6400-02B* red/blue LED light source was used to adjust photosynthetic active radiation to saturated light level (1,000 µmol m^{-2} s^{-1}) for measuring P_N (*ca*. 5 min). Similarly, one slice of the bark chlorenchyma was placed in the chamber, which was sealed off by vaseline, for bark P_N and R_D measurement. The system was confirmed as airtight by blowing near the chamber prior to measuring each sample. The chamber temperature was maintained at 23°C (ambient air temperature) and a CO_2 injector (*Li-6400-01* CO_2 injector system (*Licor,* Nebraska, USA) was used to maintain the CO_2 concentration at 380 ppm. The humidity in the measuring chamber was maintained at 35 ± 10%.

A second *Li-6400* system with a *6400-40* chamber (*Licor,* Nebraska, USA) was used to measure a light-adapted photosystem II (PSII) efficiency ($\Delta F/F_m'$) (simultaneously with the P_N and R_D measurement to minimize the time period of detached measurements). The intact leaf adapted to a natural light intensity, the detached branch, and the bark chlorenchyma were placed into the chamber for measurement. Data were taken, when fluorescence came to a steady state (dF/dt, the rate of fluorescence signal change per minute over the past 20 readings; the measuring period was less than 5 min, the waiting period was generally less than 10 min). The actinic light intensity was 1,000 ± 185 μmol m^{-2} s^{-1} with the build-in LED light source (blue 10%, centered wavelength of 470 nm and red 90%, centered wavelength of 630 nm). The saturating light intensity was 5,500–5,700 μmol m^{-2} s^{-1} centered at the wavelength of 630 nm. Although detached samples may bias the gas-exchange rate and fluorescence intensity, the same procedures were used for both treatments making it possible to compare them. A similar procedure has been used previously (Ivanov *et al.* 2006, Berveiller *et al.* 2007).

MDA content was measured according to the method of Sofo *et al.* (2004), with minor changes. In summary, 1 g of tree bark, branches, or leaves was homogenized in 10.0 ml of 10% (w/v) trichloroacetic acid and was centrifuged at 10,000 × *g* for 5 min. A 2 ml aliquot of supernatant was added to 2.0 ml of 0.6% (w/v) thio-barbituric acid in 10% (w/v) trichloroacetic acid. The mixture was heated at 100°C for 15 min and then cooled rapidly in an ice bath. After centrifugation at 10,000 × *g* for 10 min, the absorbance values at 532, 600, and 450 nm (A_{532}, A_{600}, and A_{450}) of the supernatant were recorded. The value for nonspecific absorption at 600 nm was subtracted, and a standard curve for sucrose (from 2.5 to 10 μmol ml^{-1}) was used to rectify the results from the interference of soluble sugars in the samples (at A_{532} and A_{450}). The MDA content was calculated from the absorption coefficient of 157 $mmol^{-1}$ cm^{-1} and was expressed as μmol MDA g^{-1}(FM).

Antioxidant enzymes: The extractions and assays followed the methods of Zhang *et al.* (2007) with minor revisions. Frozen tree bark, branch, or leaf segments (0.5 g) were homogenized in 10 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% PVPP. The homogenates were centrifuged at 10,000 × *g* for 30 min at 4°C and the supernatant was desalted by a *Sephadex G-25* column with a desalting buffer, which was the same as the extraction buffer.

Total SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium. One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of the reduction of nitroblue tetrazolium, as monitored at 560 nm.

Total CAT (EC 1.11.1.6) activity was assayed by measuring the rate of decomposition of H_2O_2 at 240 nm (extinction coefficient 39.4 mM^{-1} cm^{-1}). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2, and 200 μl of enzyme extract in 3 ml.

Total GR (EC 1.8.1.7) activity was measured by following the change in absorbance at 340 nm in the oxidized glutathione-dependent oxidation of NADPH (extinction coefficient 6.22 mM^{-1} cm^{-1}) for 3 min in 1 ml of an assay mixture containing 50 mM potassium phosphate buffer (pH 7.8), 2 mM Na_2EDTA, 0.15 mM NADPH, 0.5 mM GSSG (oxidized glutathione), and 200 μl of enzyme extracts. The reaction was initiated by adding NADPH. Corrections were made for the back-ground absorbance at 340 nm, without NADPH.

Soil properties: Samples from the SA and adjacent, improved soil were sampled and their physico-chemical properties were measured. The soil pH and soil electrical conductivity (EC) in a solution of 1 part soil to 5 parts water (by FM) were measured with a pH meter (*PB10*, *Sartorius*, Shanghai, China) and an EC meter (*DDS307*, *Leici*, Shanghai, China). The total porosity was measured by the method described by Lao (1988) and air-dried bulk density was measured by a 100 cm^{-3} soil cutting ring. A field *Scout SC-900* soil compaction meter (*Spectrum,* USA) was used to measure the soil compaction status at two sites. Soil total N was measured by the Kjeldahl method, total P was measured by the NaOH fusion-anti-molybdenum antimony colorimetry method (Lao 1988) and total K was measured by the NaOH fusion-flame photometric method (Lao 1988).

Tree growth was quantified as the difference between two measurements of diameters (at breast height) and tree heights at the start and the end of the growing season (the beginning of May–the end of October).

Statistical analyses were carried out using the *SPSS13.0* software package (*SPSS*, USA). Analysis of variance (*ANOVA*) was used to determine the statistical significance of differences in parameters (soil properties, tree growth, C_4 enzymatic activities, antioxidative enzymatic activities, *etc*.) between SA soil and the improved soil. Regression analysis was used to best fit the relations between C_4 enzymes, Chl, and the photosynthetic- or respiration rate of different organs.

Results

Soil properties and growth: There were statistically significant differences in the physico-chemical properties of the two soils (Table 1). The pH values of the improved soil were 1 unit lower than for the SA soil control (10.15).

Table 1. Differences in the soil physico-chemical properties and the growth of trees in the improved and untreated SA soil. The data in the table were the means ± SD of at least 3 replicates. *Different letters* in the same row indicate significant differences between two soils (p<0.05), while *the same letters* indicate no significant difference (p>0.05). EC – electrical conductivity.

	Salt-alkaline soil control	Improved soil
pH	10.15 ± 0.06[a]	9.17 ± 0.13[b]
EC [μS cm^{-1}]	1220.0 ± 130[a]	372.8 ± 46.5[b]
Bulk density [g cm^{-3}]	1.33 ± 0.12[a]	1.09 ± 0.06[b]
Soil compaction degree [kPa]	2,199.2 ± 443.4[a]	1,170.0 ± 271.1[b]
Total soil porosity [%]	53.0 ± 7.6[a]	67.9 ± 8.6[b]
Soil N [g kg^{-1}]	0.72 ± 0.10[a]	1.53 ± 0.12[b]
Soil P [g kg^{-1}]	0.10 ± 0.02[a]	0.30±0.23[a]
Soil K [g kg^{-1}]	12.8 ± 1.2[a]	12.2±0.4[a]
Height increment [m season^{-1}]	0.58 ± 0.12 [a]	1.636 ± 0.512[b]
Basal diameter increment [mm season^{-1}]	0.861 ± 0.43 [a]	2.717 ± 0.56[b]

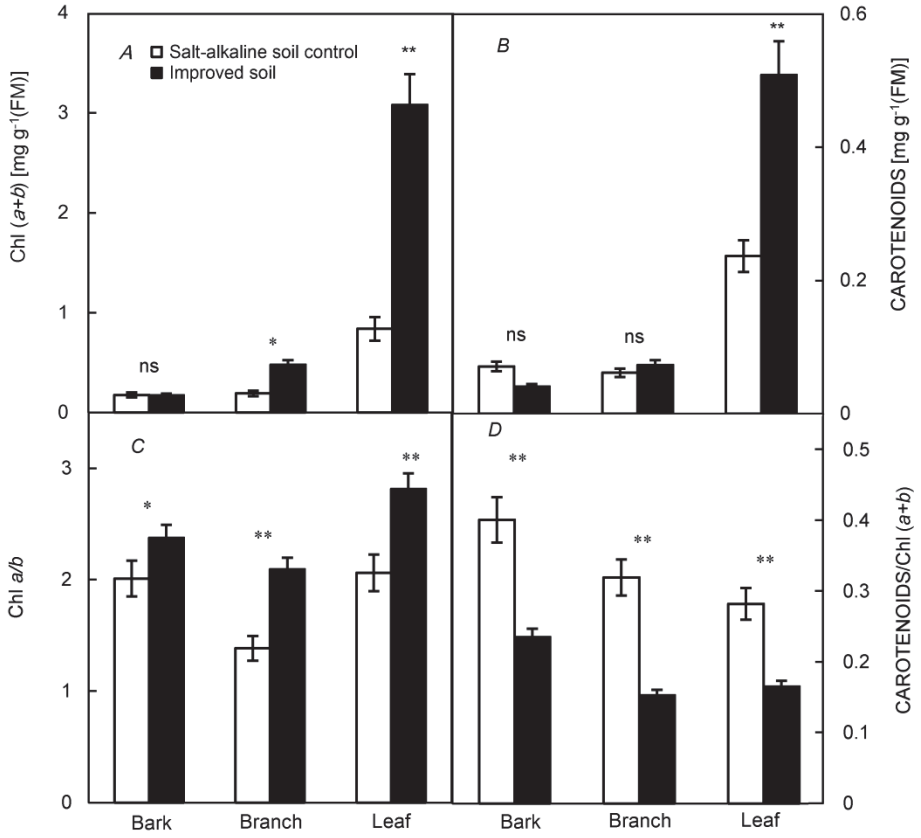

Fig. 1. Effect of salt-alkaline soil amelioration on the pigment content (*A,B*) and composition (*C,D*). *A*: Chl (*a*+*b*); *B*: Carotenoids; *C*: Chl *a*/*b*; *D*: Carotenoids/Chl (*a*+*b*). Means ± SD; n = 5. * – significant difference between the control and the improved soil (p<0.05);** – the significance level at p<0.01; ns – no significant difference (p>0.05). Chl – chlorophyll; FM – fresh mass.

The EC of the SA soil control was 3.5 times higher than that of the improved soil. The bulk density of the improved soil decreased from 1.33 to 1.09 g cm^{-3}, the soil compaction was approximately half, and the soil porosity increased from 53 to nearly 70%. A twofold difference in soil N and P was observed in the improved soil compared with that of SA soil, while no statistically significant difference was found in the soil K between these two soils. The growth of poplar trees differed significantly between the two sites, SA soil amelioration led to at least a 3-fold increase in both the tree diameter and tree height (Table 1), *i.e.*, the height increment in the control was 0.58 m season^{-1}, while it was 1.636 m season^{-1} in the improved soil; the basal diameter increment in the untreated control was 0.861 mm season^{-1}, while it was 2.717 mm season^{-1} in the improved soil.

Table 2. Effects of saline-alkalinity on the ratio between the woody chlorenchyma (branch and stem) and the leaf. The data in the table were the means ± SD of at least 3 replicates. *Different letters* in the same row indicate significant differences between two soils (p<0.05), while *the same letters* indicate no significant difference (p>0.05). CAT – catalase; Chl – chlorophyll; FM – fresh mass; GR – glutathione reductase; MDA – malondialdehyde; NADP-MDH – NADP-dependent malate dehydrogenase; NADP-ME – NADP-dependent malic enzyme; PEPC – phosphoenolpyruvate carboxylase; PPDK – pyruvate orthophosphate dikinase; SOD – superoxide dismutase.

Pigments	Ratio	Salt-alkaline land	Improved soil
Chl (*a*+*b*) [mg g^{-1}(FM)]	Bark/leaf	0.21 ± 0.05^a	0.06 ± 0.03^b
	Branch/leaf	0.23 ± 0.05^a	0.16 ± 0.03^b
Carotenoids [mg g^{-1}(FM)]	Bark/leaf	0.30 ± 0.06^a	0.08 ± 0.03^b
	Branch/leaf	0.26 ± 0.06^a	0.14 ± 0.05^b
Chl-based enzymatic activities and MDA			
PEPC [mmol min^{-1} mg^{-1}(Chl)]	Bark/leaf	4.35 ± 0.89^a	8.58 ± 2.45^b
	Branch/leaf	3.10 ± 0.68^a	2.07 ± 0.62^a
NADP-ME [mmol min^{-1} mg^{-1}(Chl)]	Bark/leaf	1.74 ± 0.43^a	17.16 ± 4.37^b
	Branch/leaf	2.05 ± 0.29^a	6.45 ± 1.12^b
PPDK [mmol min^{-1} mg^{-1}(Chl)]	Bark/leaf	5.59 ± 0.48^a	28.61 ± 5.54^b
	Branch/leaf	4.92 ± 0.51^a	15.05 ± 2.01^b
NADP-MDH [mmol min^{-1} mg^{-1}(Chl)]	Bark/leaf	1.74 ± 0.25^a	13.73 ± 2.97^b
	Branch/leaf	1.38 ± 0.23^a	6.45 ± 1.03^b
MDA [µmol g^{-1}(Chl)]	Bark/leaf	2.27 ± 0.54^a	9.68 ± 1.98^b
	Branch/leaf	2.49 ± 0.61^a	2.50 ± 0.65^a
CAT [µmol min^{-1} mg^{-1}(Chl)]	Bark/leaf	3.47 ± 0.98^a	14.98 ± 2.35^b
	Branch/leaf	3.48 ± 0.89^a	3.73 ± 1.21^a
GR [µmol min^{-1} mg^{-1}(Chl)]	Bark/leaf	6.21 ± 0.25^a	13.14 ± 1.45^b
	Branch/leaf	13.9 ± 2.31^a	9.53 ± 1.67^a
SOD [U mg^{-1}(Chl)]	Bark/leaf	6.52 ± 1.67^a	17.66 ± 2.67^b
	Branch/leaf	4.67 ± 0.98^a	5.33 ± 1.31^a
FM-based enzymatic activities and MDA			
PEPC [µmol h^{-1} g^{-1}(FM)]	Bark/leaf	0.92 ± 0.10^a	0.48 ± 0.13^b
	Branch/leaf	0.71 ± 0.11^a	0.40 ± 0.12^b
NADP-ME [µmol h^{-1} g^{-1}(FM)]	Bark/leaf	0.37 ± 0.09^a	0.96 ± 0.15^b
	Branch/leaf	0.47 ± 0.14^a	1.00 ± 0.21^b
PPDK [µmol h^{-1} g^{-1}(FM)]	Bark/leaf	1.18 ± 0.21^a	1.60 ± 0.23^a
	Branch/leaf	1.13 ± 0.15^a	2.33 ± 0.28^b
NADP-MDH [µmol h^{-1} g^{-1}(FM)]	Bark/leaf	0.37 ± 0.15^a	0.77 ± 0.25^b
	Branch/leaf	0.32 ± 0.14^a	1.00 ± 0.25^b
MDA [µmol kg^{-1}(FM)]	Bark/leaf	0.48 ± 0.15^a	0.54 ± 0.24^a
	Branch/leaf	0.57 ± 0.21^a	0.39 ± 0.23^a
CAT [µmol min^{-1} g^{-1}(FM)]	Bark/leaf	0.73 ± 0.25^a	0.84 ± 0.19^a
	Branch/leaf	0.80 ± 0.24^a	0.58 ± 0.24^a
GR [µmol min^{-1} g^{-1}(FM)]	Bark/leaf	1.31 ± 0.22^a	0.74 ± 0.21^b
	Branch/leaf	3.19 ± 0.21^a	1.48 ± 0.23^b
SOD [U g^{-1}(FM)]	Bark/leaf	1.38 ± 0.31^a	0.99 ± 0.31^a
	Branch/leaf	1.07 ± 0.32^a	0.83 ± 0.32^a

Pigment composition: The differences were larger in the leaves compared to either the bark or branches (Fig. 1). The small change in the composition of Chl (*a*+*b*) and Car in the bark was not significant, contrary to the leaves, where it was highly significant. The soil improvement resulted in a twofold increase in the branch Chl (*a*+*b*) and a threefold increase in the leaf Chl (*a*+*b*). Similarly, the soil improvement doubled the leaf Car. Corresponding to the pigment composition, soil SA stress significantly decreased the ratio of Chl *a*/*b* (p<0.05), but significantly increased the Car/Chl (*a*+*b*) ratio in all organs (Fig. 1.).

In SA soil, the ratio of Chl between the bark and leaf was 0.21, it was 0.30 for Car; in the improved soil, these ratios were as small as 0.06 and 0.08, respectively. Smaller changes, although still significant, were also found in the ratio between the branch and leaf pigments (Table 2).

Antioxidative enzymes and MDA content: When expressed on a total Chl basis, MDA contents decreased by

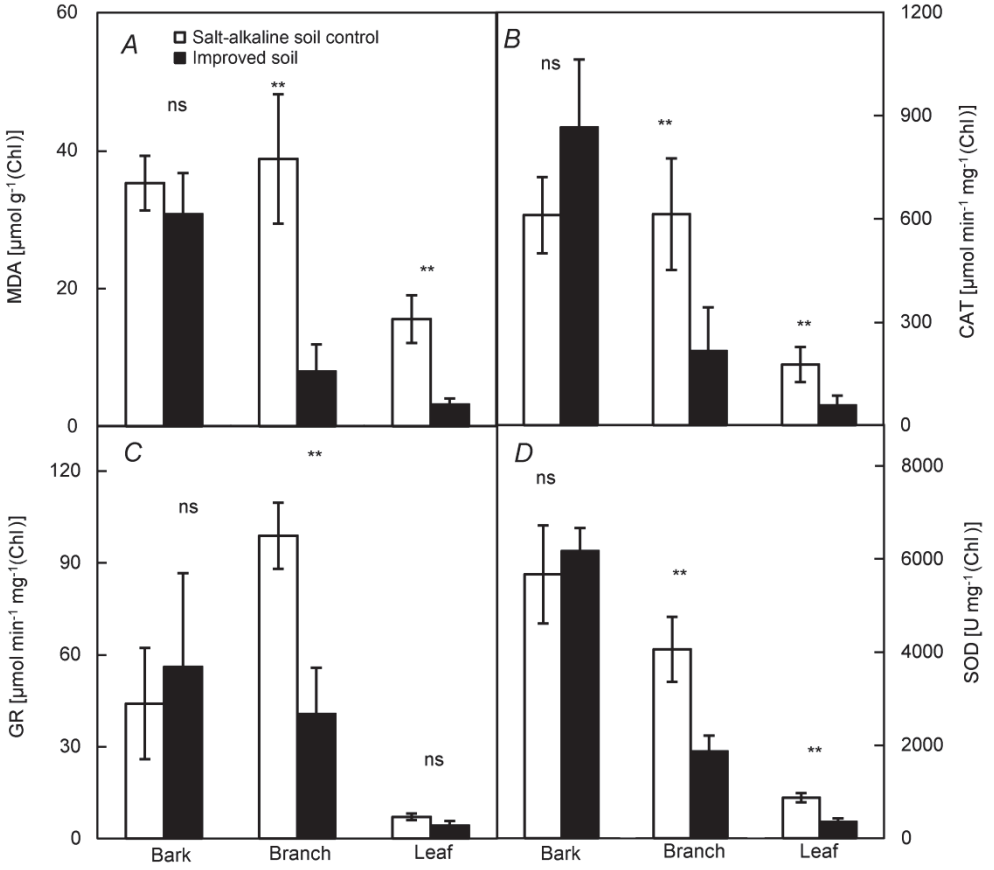

Fig. 2. Effect of salt-alkaline soil improvement on malondialdehyde (MDA) (*A*), catalase (CAT) (*B*), glutathione reductase (GR) (*C*), and superoxide dismutase (SOD) (*D*) activities in bark, branch, and leaf of poplar trees. Means ± SD; $n = 5$. * – significant difference between the control and the improved soil ($p<0.05$);** – the significance level at $p<0.01$; ns – no significant difference ($p>0.05$).

up to 80% in the branch and leaves in the improved soil, while no significant decrease was observed in the bark chlorenchyma (Fig. 2). Similarly, soil salinity/ alkalinity significantly influenced the activities of CAT in the branch and leaf tissue, but not in the bark (Fig. 2). Significantly higher GR activities per unit of Chl were observed in the branch in the SA soil comparing with the improved soil, but no significant changes were found in the leaf and bark chlorenchyma (Fig. 2). Significantly higher SOD activities per unit of Chl were observed in the branch and leaf in the SA soil compared with the improved soil (Fig. 2).

The soil improvement resulted in a variety of changes in the antioxidant enzyme activity ratios between the woody and leaf chlorenchyma (Table 2). When expressed on a total Chl basis, the MDA ratio between the woody and leaf chlorenchyma ranged from 2.27 to 2.49 in the SA soil, and this ratio increased from 2.50 to 9.68 in the improved soil. Similarly, the range for the CAT ratio was 3.47–3.48 in the SA soil control, while it was 3.73–14.98 in the improved soil. The SOD ratio was 4.67–6.52 in the SA soil, while it was 5.33–17.66 in the improved soil (Table 2). When expressed on FM basis, the ratios of MDA (0.48–0.57), CAT (0.73–0.80), GR (1.31–3.19), and SOD (1.07–1.38) in the SA soil were quite similar to those found in the improved soil (0.39–0.54 for MDA, 0.58–0.84 for CAT,0.74–1.48 for GR and 0.83–0.99 for SOD), showing that the difference in the unit used for the calculation could modify the ratio between the woody and leaf chlorenchyma, and the difference in Chl caused by soil stress was a reason for the differences observed in enzymatic activities, when expressed on a total Chl basis (Table 2).

Photosynthesis, respiration, and PSII efficiency: The photosynthetic and respiratory capacity in the bark and branches responded slightly to the soil SA changes, whereas the response was stronger in the leaves (Fig. 3). No significant differences were observed in P_N of the bark between the SA soil and the improved soil; the same was true for P_N of the branches. Leaf P_N in the SA soil was 1.6 and 3.0 μmol m^{-2} s^{-1} in the improved soil (Fig. 3). Leaf R_D was generally greater than R_D of the bark and branches. The difference in response to the SA soil resulted in smaller respiratory differences between the leaf and nonleaf chlorenchyma, *i.e.*, the SA soil caused 20% rise in the leaf R_D compared with the branch and bark. It was 80% in the improved soil (Fig. 3). The

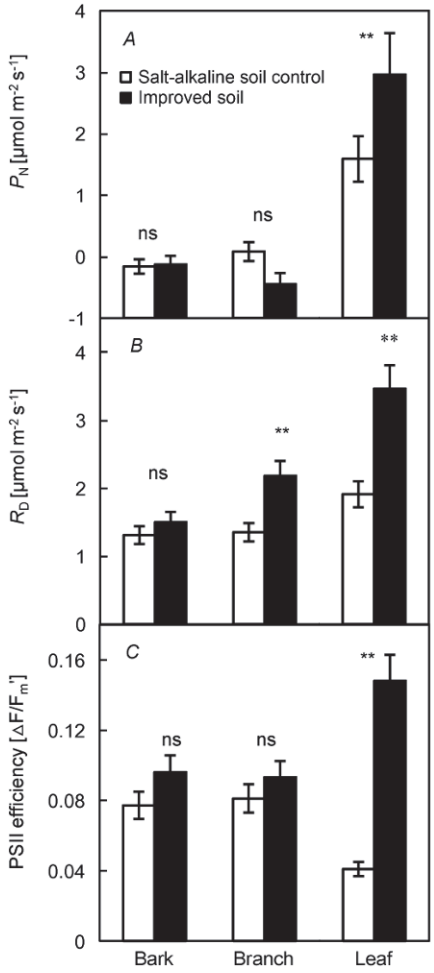

Fig. 3. Effect of salt-alkaline soil improvement on net photosynthetic rate (P_N) (*A*), dark respiration rate (R_D) (*B*), and PSII efficiency ($\Delta F/F_m'$) (*C*). Means ± SD; n = 5. ** – the significance level at $p<0.01$; ns – no significant difference ($p>0.05$).

improved soil enhanced the bark and branch PSII efficiency, but it was not statistically significant, while the leaf PSII efficiency was about 3.5 times higher in the improved soil than that in the SA soil (Fig. 3).

C_4 enzymes: The activity of PEPC was the lowest in the leaves, followed by the branch. The bark had the highest PEPC activity per unit of Chl. The soil improvement resulted in declines of 58% and 50% in PEPC activities on Chl basis in the branch and the leaf, but the activity in the bark remained almost constant (Fig. 4). This organ-dependent response resulted in 8.6- and 2.1-times PEPC activity in bark and branch relative to leaf in the improved soil, and corresponding values were 4.4- and 3.1-times in the SA soil (Table 2). However, PEPC activities calculated per one unit of FM were 92 and 71% in the bark and the branches, respectively, compared with those in the leaf in the SA soil, while the corresponding values were 48 and 40% in the improved soil (Table 2).

Decreasing tendencies of NADP-ME activity per unit of Chl were observed in the order of the bark, the branch and the leaf, both in the SA soil control and the improved soil. The soil improvement significantly reduced NADP-ME activity in the leaf and branch, but it increased significantly in the bark compared with those in the SA soil control (Fig. 4). In the SA soil control, NADP-ME activities were 1.7 and 2.1 times higher in the bark and branch, respectively, than in the leaves, and these differences increased to factors of 17.2 and 6.5 in the improved soil, when calculated on Chl-based unit (Table 2). When the unit was based on FM, the bark and branch NADP-ME activities were 37 and 47%, respectively, of those in the leaf in the SA soil, while the corresponding values were 96 and 100%, respectively, in the improved soil (Table 2).

The soil improvement caused PPDK to decrease in the bark, branch and leaves by 45, 67, and 89%, respectively (Fig. 4). In the SA soil, PPDK was 4.9–5.6 times higher in the bark and branch than in the leaves, when Chl basis was used, and it was 1.13–1.18 times higher than in the leaves, when FM basis was used (Table 2). In the improved soil, these factors increased to 15.1–28.6 on Chl unit basis and 1.60–2.33 on FM unit basis, respectively (Table 2).

Concerning NADP-MDH in the bark, there was no obvious difference between the SA soil and the improved soil, but it declined in the branches and leaves in the improved soil by 40 and 87%, respectively (Fig. 4). In the SA soils, NADP-MDH activities in the bark and branches were 1.4–1.7 times higher than that in the leaves, when Chl based unit was used, and they were 32–37% of those in the leaves, when FM basis was used. In the improved soil, these factors increased to 6.5–13.7 times and 77–100%, respectively (Table 2).

Intercorrelations: We pooled all data and performed correlation analysis (Table 3). On a unit basis of Chl, R_D declined exponentially with an increase in different C_4 enzymes (NADP-ME, PEPC, PPDK, and NADP-MDH), and the decline was statistically significant ($r^2 > 0.73$, $p<0.05$). However, only one positive correlation between PEPC and R_D was observed ($r^2 = 0.76$, $p<0.05$), when the unit was based on FM (data not shown), showing that FM basis was less reliable for inter-correlation analysis compared with the unit based on Chl. Similarly, all four C_4 enzymatic activities negatively correlated with P_N, when expressed on Chl basis ($r^2 > 0.66$, $p<0.05$) (Table 3), and only one significant relation was found in PEPC in the unit calculated on FM ($y = 5.6673\ x - 2.3068$, $r^2 = 0.82$, $p<0.01$) (data not shown). No significant correlation was found between C_4 enzymes and PSII efficiency ($r^2 < 0.56$, $p>0.05$, data not shown). In contrast, R_D and P_N were positively correlated with Chl content in different organs ($r^2 > 0.84$, $p<0.05$) (Table 3).

When expressed on Chl basis, MDA, CAT, and SOD activities were negatively correlated with R_D ($p<0.05$), but

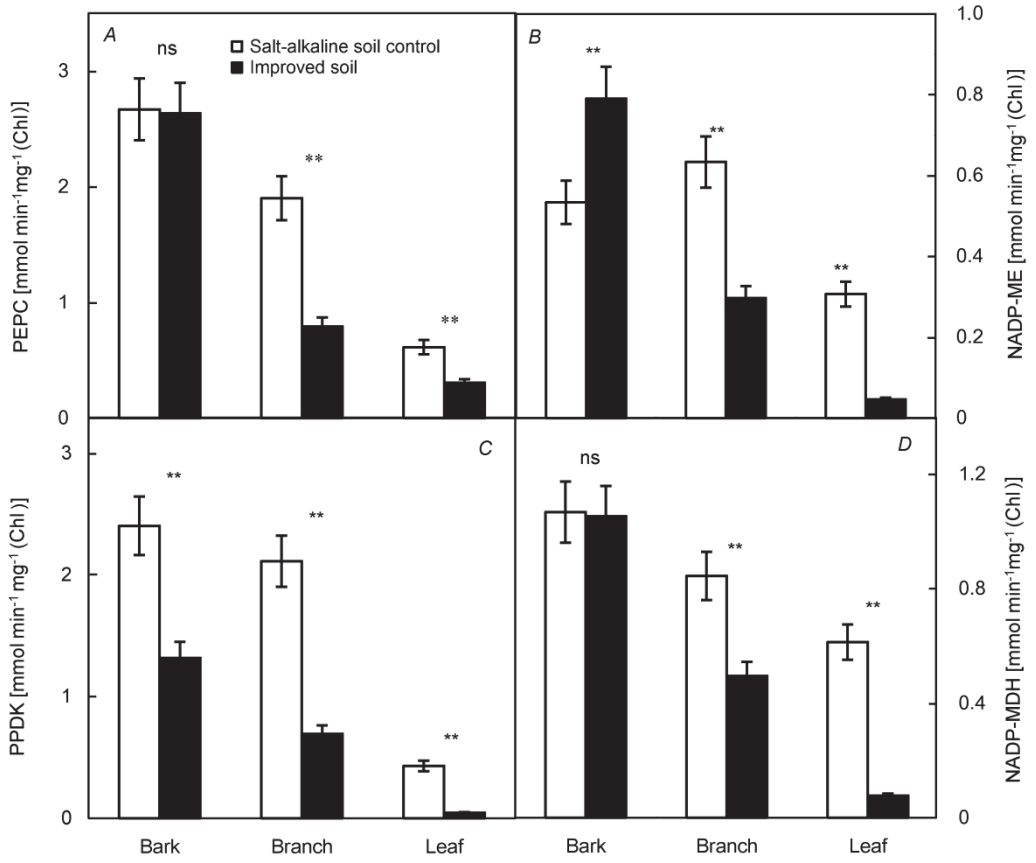

Fig. 4. Effect of soil amelioration on phospho*enol*pyruvate carboxylase (PEPC) (*A*), NADP-dependent malic enzyme (NADP-ME) (*B*), pyruvate orthophosphate dikinase (PPDK) (*C*), and NADP-dependent malate dehydrogenase (NADP-MDH) (*D*) enzyme activities. Means ± SD; $n = 5$. * – significant difference between the control and the improved soil ($p<0.05$);** – the significance level at $p<0.01$; ns – no significant difference ($p>0.05$).

Table 3. Correlation analysis between dark respiration rate (R_D), net photosynthetic rate (P_N) and variable enzymes, malondialdehyde (MDA) and chlorophyll (Chl) in leaf, bark and branch chlorenchyma. $n = 6$. CAT – catalase; FM – fresh mass; GR – glutathione reductase; NADP-MDH – NADP-dependent malate dehydrogenase; NADP-ME – NADP-dependent malic enzyme; PEPC – phospho*enol*pyruvate carboxylase; PPDK – pyruvate orthophosphate dikinase; SOD – superoxide dismutase.

	Correlations	Coefficient r^2	Significance
R_D [y, μmol m^{-2} s^{-1}]			
NADP-ME [x, mmol min^{-1} mg^{-1} Chl (*a*+*b*)]	$y = 3.1186\ e^{-1.219x}$	0.7898	$p<0.05$
PEPC [x, mmol min^{-1} mg^{-1} Chl (*a*+*b*)]	$y = 2.87\ e^{-0.301x}$	0.7323	$p<0.05$
PPDK [x, mmol min^{-1} mg^{-1} Chl (*a*+*b*)]	$y = 2.7578\ e^{-0.349x}$	0.7972	$p<0.05$
NADP-MDH [x, mmol min^{-1} mg^{-1} Chl (*a*+*b*)]	$y = 3.5122\ e^{-0.938x}$	0.9269	$p<0.01$
MDA [x, μmol g^{-1} Chl (*a*+*b*)]	$y = 3.0431\ e^{-0.023x}$	0.8791	$p<0.01$
CAT [x, μmol min^{-1} mg^{-1} Chl (*a*+*b*)]	$y = 2.7648\ e^{-1E-03x}$	0.6930	$p<0.05$
GR [x, μmol min^{-1} mg^{-1} Chl (*a*+*b*)]	$y = 2.5284\ e^{-0.008x}$	0.5218	$p>0.05$
SOD [x, U mg^{-1} Chl (*a*+*b*)]	$y = 2.7134\ e^{-1E-04x}$	0.6935	$p<0.05$
Chl (*a*+*b*) [x, mg g^{-1}(FM)]	$y = 0.6845\ x + 1.39$	0.9128	$p<0.01$
P_N [y, μmol m^{-2} s^{-1}]			
NADP-ME [x, mmol min^{-1} mg^{-1} Chl (*a*+*b*)]	$y = -1.119\ \ln(x) - 0.618$	0.7378	$p<0.05$
PEPC [x, mmol min^{-1} mg^{-1} Chl (*a*+*b*)]	$y = -1.235\ \ln(x) + 0.791$	0.6638	$p<0.05$
PPDK [x, mmol min^{-1} mg^{-1} Chl (*a*+*b*)]	$y = -0.822\ \ln(x) + 0.3216$	0.8497	$p<0.05$
NADP-MDH [x, mmol min^{-1} mg^{-1} Chl (*a*+*b*)]	$y = -1.137\ \ln(x) - 0.0733$	0.7091	$p<0.05$
MDA [x, μmol g^{-1} Chl (*a*+*b*)]	$y = -0.941\ \ln(x) + 3.257$	0.4849	$p>0.05$
CAT [x, μmol min^{-1} mg^{-1} Chl (*a*+*b*)]	$y = -1.097\ \ln(x) + 6.8999$	0.6995	$p<0.05$

Table 3 (continued)

	Correlations	Coefficient r²	*Significance*
GR [x, μmol min^{-1} mg^{-1} Chl (*a*+*b*)]	y = –0.983 ln(x) + 3.8471	0.8366	$p<0.05$
SOD [x, U mg^{-1} Chl (*a*+*b*)]	y = –1.041 ln(x) + 8.6011	0.7920	$p<0.05$
Chl (*a*+*b*) [x, mg g^{-1}(FM)]	y = 1.0851 x – 0.2437	0.8419	$p<0.05$

no significant relation was found in GR ($p>0.05$) (Table 3). When the unit was based on FM, no significant correlations between MDA, CAT, GR, SOD, and R_D were found (data not shown). In case of P_N, significant negative correlations with CAT, GR, and SOD were observed ($p<0.05$), when the unit was based on Chl, but no significant relation was found in MDA ($p>0.05$) (Table 3). When expressed on FM basis, no significant correlation with P_N was observed except CAT (y = 0.0393 x – 4.6187, $r^2 = 0.7273$, $p<0.05$) (data not shown).

Discussion

Just like MDA, antioxidant enzymes and photosynthetic parameters, our results support the conclusion that changes in C_4 enzyme activities can be used as parameters to indicate SA stress and these changes might favor the acclimatization of plant to the stress. In the field, after more than 1-year stress from the heavy SA soil, the significant increases in MDA content (5-fold) and in activities of different antioxidative enzymes (Fig. 2), such as GR (1.7-fold), CAT (3-fold), and SOD (2.5-fold) indicated that the membrane system was damaged and that enzyme activities had increased in order to protect the photosynthetic apparatus by enhancement of radical scavenging (Munns *et al.* 1986, Parida and Das 2005, Yan *et al.* 2009, Zhang and Yin 2009).

The Car/Chl (*a*+*b*) ratio increase and Chl *a/b* ratio decrease in the SA soil (Fig. 1) were also probably involved in the protection against the photoinhibition. The Chl *a/b* ratio change indicated an increase in the level of the light-harvesting complexes relative to the reaction center cores (which only have Chl *a*). This plus the increased Car contents might be safely dissipating excess energy *via* the xanthophyll cycle (Parida and Das 2005). Similar results have also been found in previous studies (Pan 2004, Yan *et al.* 2009).

Significant increases in various C_4 enzymatic activities were also observed in the SA soil control, *e.g.* leaf PEPC, NADP-ME, PPDK, and NADP-MDH in the SA soil control were 2, 7, 10, and 9 times higher, respectively, than those in the improved soil (Fig. 4). The present study clearly showed that C_4 enzyme activities could be strongly enhanced by soil salinity/alkalinity. C_4 photosynthesis is a more productive and more effective pathway than C_3 photosynthesis, especially in dry or SA lands (Pyankov *et al.* 2000, Tang and Liu 2001, Voznesenskaya *et al.* 2001, Pan 2004, Han *et al.* 2006). The enhancement of C_4 enzymes shows the greater involvement of C_4 photosynthetic pathway in the apparent photosynthesis (Pan 2004). Thus, the marked decrease in the leaf apparent photosynthesis may occur in C_3 rather than C_4 pathway, and as a result, contribution of C_4 photosynthesis to total photosynthetic carbon fixation should be increased in the SA soil. Our findings indicated that more efficient C_4 pathway became more active in the stressful environment, while the C_3 pathway became more dominant under more favourable conditions, *i.e.* in the improved soil.

There were more dramatic changes in C_4 enzymes between the leaf and the young woody chlorenchyma than the old woody chlorenchyma in the bark, and similar tendencies were observed in antioxidant enzymes, pigments and photosynthetic parameters. Eleven out of the 15 parameters [except Chl *a/b*, Car/Chl (*a*+*b*), PPDK, and NADP-ME] for the bark chlorenchyma and 3 out of the 15 parameters (PSII, P_N, and Car) for the branch chlorenchyma showed no significant differences between the improved soil and the SA soil control, while all the tested parameters for the leaf showed significant differences ($p<0.05$). The C_4 enzymatic ratios between the nonleaf chlorenchyma and the leaf are usually used to discern the degree of C_4 photosynthetic pathway involvement (Hibberd and Quick 2002, Berveiller and Damesin 2008, Brown *et al.* 2010). This type of organ-dependent enzymatic changes resulted in salinity/alkalinity-dependent changes in ratios of C_4 to antioxidant enzyme activities (Table 2). Thus, soil salinity/alkalinity should be taken into account, when the ratios are used to discern the involvement of the C_4 photosynthetic pathway.

Moreover, unit differences (FM *vs*. Chl basis) could also strongly affect these C_4 enzymatic ratios. When expressed on a total Chl basis, our results were the same as referred in the previous studies (Hibberd and Quick 2002, Berveiller and Damesin 2008). For example, the bark/leaf ratios for PEPC, NADP-ME, PPDK, and NADP-MDH were 8.58, 17.16, 28.61, and 13.73, respectively, in the improved soil, while the corresponding value for the branch/leaf was 2.07, 6.45, 15.05, and 6.45, respectively (Table 2). However, contrary to the results on Chl basis, 3 out of the 4 enzymes (PEPC, NADP-ME, NADP-MDH) in the woody chlorenchyma of branch and bark were lower than those in the leaf blades on FM basis

(Table 2). Thus, the C_4 enzymatic ratio between the woody chlorenchyma and the leaf blade was unit-dependent (Table 2). C_4 enzymatic activity per one unit of Chl was equal to enzymatic activity per one unit of FM divided by the corresponding Chl content. Much lower Chl content in the branch and bark relative to the leaf (Fig. 1) was responsible for the high PEPC, NADP-ME, and NADP-MDH activities, when expressed on Chl basis (Table 2).

The differences in Chl content also resulted in the observation of significant inter-correlations among different enzymes (Table 3). Because Chl content was positively correlated with R_D and P_N (slope > 0.68, $r^2 > 0.84$, $p<0.05$), negative correlations between various enzymes calculated on the Chl basis (NADP-ME, PEPC, NADP-MDH) and gas exchanges (R_D and P_N) were observed (Table 3). Actually, most of the correlations were not significant on FM basis (data not shown). This unit-dependent enzymatic difference should be used cautiously in a future data analysis, although it should be noted that most published papers favor the Chl unit basis instead of FM (Hibberd and Quick 2002, Berveiller and Damesin 2008, Brown *et al*. 2010).

In conclusion, similar to changes in pigments, antioxidant enzymes, and photosynthetic and respiratory activity during an acclimation to the SA soil, changes of C_4 enzymatic activities might improve the plant fitness in acclimation to stress. Because of the dependence on the degree of soil salinity/alkalinity as well as unit-expression, we suggest a caution, when using C_4 enzymatic ratios between the woody chlorenchyma and the leaf blade to discern, how active is the C_4 photosynthetic pathway in nonleaf organs.

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