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# Comparative anatomical, morphological, and physiological parameters controlling photosynthesis in two *Populus*×*euramericana* clones during short-term osmotic treatment

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## Abstract

The relationships between drought response and anatomical/physiological properties were assessed in two poplar clones belonging to the Aigeros section: *Populus*× *euramericana* clone Dorskamp (drought-tolerant) and clone Luisa Avanzo (drought-sensitive). Cuttings of both clones were exposed for 12 h to 0 mM (control), 50 mM (osmotic potential -0.112 MPa), and 150 mM (-0.336 MPa) mannitol. In control, Dorskamp had smaller stomata than Luisa Avanzo, one or two layers of palisade cells, a spongy mesophyll, and high concentrations of antioxidative compounds (ascorbate, glutathione). After exposure to 50 or 150 mM mannitol, both clones closed their stomata: leaf conductance and opening of stomata decreased. When exposed to 50 mM mannitol, net photosynthetic rate ($P_N$) and chlorophyll (Chl) and total solute contents remained stable; ribulose-1,5-bisphosphate carboxylase/-oxygenase activity, Chl synthesis and turn-over, ascorbate peroxidase and glutathione reductase activities were less affected in Dorskamp than in Luisa Avanzo. Following an exposure to 150 mM mannitol, Dorskamp exhibited higher $P_N$ and higher contents of antioxidants (ascorbate, glutathione) and antioxidative enzymes (ascorbate peroxidase, glutathione reductase) than Luisa Avanzo. Hence the drought-tolerant poplar was able to better avoid and tolerate osmotic stress.

*Additional key words*: active oxygen species; aminolevulinate dehydratase; antioxidants; carboxylation; carotenoids; chlorophyll; chlorophyllase; poplar; ribulose-1,5-bisphosphate carboxylase/oxygenase; stomatal conductance.

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*Abbreviations*: AG cycle - ascorbate-glutathione cycle; ALAD - aminolevulinate dehydratase; AP - ascorbate peroxidase; AsA - ascorbic acid; DAsA - dehydroascorbate; DTT - dithiothreitol; EDTA - ethylene diamine tetraacetic acid; $g_s$ - stomatal conductance; GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidized glutathione; $P_N$ - net photosynthetic rate; PMSF - phenylmethylsulfonyl fluoride; PVPP - polyvinylpolypyrrolidone; ROS - reactive $O_2$ species; RuBPCO - ribulose-1,5-bisphosphate carboxylase/oxygenase activity.

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## Introduction

*Populus* spp. displays a wide variability in water-use efficiency and drought-tolerance (Schulte *et al.* 1987, Ceulemans 1990, Tchaplinski and Tuskan 1994). Several studies on anatomical and physiological traits in various poplar clones differing in drought-responses showed that the most tolerant clones were able to osmotically adjust and to maintain a larger carbon allocation to organs (Tchaplinski and Blake 1989, Tchaplinski and Tuskan 1994, Wang *et al.* 1997, Tchaplinski *et al.* 1998). More particularly, the maintenance of net $CO_2$ assimilation rate resulted from avoidance (closure of stomata, thick spongy mesophyll) and tolerance (high carboxylation efficiency of ribulose-1,5-bisphosphate carboxylase/oxygenase, RuBPCO) mechanisms (Schulte *et al.* 1987, Tchaplinski and Blake 1989, Ridolfi and Dreyer 1997).

However, the relationships between drought tolerance and anatomical and physiological properties were assessed comparing poplars that belong to different species or sections; moreover, few data on the reactions taking place in the chloroplasts are available so far. Therefore, to minimize the taxonomic differences and to establish a relevant process for drought tolerance at the cellular level, two *Populus* types originating from the Aigeros section were retained (Brignolas *et al.* 1997, Guerrier *et al.* 1999).

In order to find whether the drought-tolerant poplar sustains photosynthesis under water deficit, several parameters related to stomatal and non-stomatal components were studied: number and aperture/closure of stomata, mesophyll thickness, leaf conductance, chlorophyll synthesis/catabolism, RuBPCO activity, carotenoid and sugar contents, and activities of antioxidative enzymes (linked to the ascorbate-glutathione cycle) and compounds (carotenoids, ascorbate, glutathione) which can prevent the increased ROS production induced by the osmotic shock (Larson 1988, Smirnoff 1993, Foyer *et al.* 1994).

## Materials and methods

**Plants**: Two clones of *Populus*×*euramericana* [*Populus deltoïdes* (Batr.) Marsh × *P. nigra* L.] were selected: the drought-sensitive Luisa Avanzo, and the drought-tolerant Dorskamp (Brignolas *et al.* 1997, Guerrier *et al.* 1999). One-month-old rooted cuttings of both clones (10 cm high, 0.5-0.8 cm stem diameter, with 8-9 fully developped leaves) were water-stressed with mannitol solutions (0, 50, 150 mM corresponding to osmotic potentials of 0, -0.112, and -0.336 MPa) for 12 h. This short-term exposure to osmotic stress (Sibout and Guerrier 1998, Guerrier *et al.* 1999) allows reproducibility for a large number of samples, avoids the reduction in photosynthetic capacity due to a subsequent build-up of saccharides, and does not allow any time for adaptive response. Experiments were carried out at a temperature of 25 °C, a relative humidity of 60-80 %, and an irradiance of 60 W $m^{-2}$. Five or six replicates on independant cuttings were performed; means ± standard errors were given and statistical significances were ascribed using LSD (ANOVA, $p \leq 0.05$).

**Leaf anatomy**: The samples were prepared for light microscopy (*Leitz*, Wetzlar, Germany) as described in Ermel *et al.* (1997). The fully developped leaves were cut in small pieces; the samples were fixed with a solution of 5 % glutaraldehyde and 1 % caffein for 3-4 h, and rinsed with 0.05 M phosphate buffer (pH 7.4). Samples were dehydrated progressively in ethanol, which was thereafter replaced by the embedding medium (*Technovit 7100, Labonord*, Villeneuve d'Asq, France). Sample sections (*ca.* 3-4 μm) were cut with a *Leitz* rotary microtome, and then stained with 0.1 M toluidine blue O.

For the determination of stomatal density, size, and aperture/closure, and of fine vein distances, impressions of 10 abaxial leaf surfaces and 10 adaxial leaf surfaces per clone were made with stainless nail polish (Ceulemans *et al.* 1984).

Stomatal frequency was determined using a *Leitz* microscope at a 400× magnification in 0.15 $mm^2$ area (600 replicates), according to a 3×3 mm grid throughout the leaf, and with a micrometer slide as standard. Stomatal size, stomatal aperture, and fine vein distances were measured (*ca.* 50 replicates) with a calliper square. Epidermal cell frequency was determined on abaxial side in 0.4 $mm^2$ field.

**Leaf conductances** in six plants on both abaxial and adaxial leaf surfaces were estimated using a steady-state diffusion porometer (*Delta-T-Devices*, Cambridge, Great Britain).

**Protein extraction**: Three grams of fresh matter were homogenised with 15 $cm^3$ Tris-HCl 0.05 M buffer (pH 7.4) containing 20 mM $MgSO_4$, 10 mM sodium ascorbate, 5 mM DTT, 1 mM $Na_2$-EDTA, 1 mM PMSF, and 10 % PVPP (m/m). The homogenate was centrifuged at 500×*g* at 4 °C for 4 min. The pellet was discarded and the supernatant was centrifuged again at 2 000×*g* for 15 min. The sedimented chloroplasts were washed gently and resuspended in 4 $cm^3$ Tris-HCl 0.05 M buffer (pH 7.4) containing 20 mM $MgSO_4$, 10 mM sodium ascorbate, 5 mM DTT, 1 mM $Na_2$-EDTA, and 1 % *Triton X-100*. Soluble protein content was measured according to Lowry *et al.* (1951) with bovine serum albumin as standard.

**Enzyme activities**: RuBPCO and glutathione reductase activities were measured spectrophotometrically at 340 nm by coupling the reaction to the oxidation of NADH and NADPH, respectively (Bourgeais-Chaillou *et al.* 1992, Guerrier *et al.* 1999). Aminolevulinate dehydratase was assayed at 555 nm by measuring the condensation of two molecules of aminolevulinate in porphobilinogen—the basic pyrrolic structure of Chl—using an absorption coefficient of 36 000 mol $m^{-3}$ (Prasad and Prasad 1987). According to Mihailovic *et al.* (1997), the assay of chlorophyllase was based on the spectrophotometric measurement, at 652 nm, of the undegraded Chl (from spinach, *Sigma*, St Quentin Fallavier, France). Ascorbate peroxidase was determined at 290 nm with an absorption coefficient of 2.8 mol $m^{-3}$ (Amako *et al.* 1994). Except glycollate oxidase activity, which was measured as dye reduction after addition of dichlorophenolindophenol (Guerrier 1985), all the enzyme activities were expressed per unit of Chl since they are related to the rate of photon capture by the antennae pigments.

**Extraction and determination of pigment, soluble sugar, and antioxidant contents**: Pigments were extracted in 80 % acetone (0.5 g fresh mass per 10 $cm^3$ acetone); Chl

and carotenoid contents were calculated spectrophotometrically (Lichtenthaler and Wellburn 1983). Soluble sugars were extracted from 5 g fresh matter with 20 $cm^3$ boiling distilled water; total soluble sugars were determined using anthrone (Morris 1948) with glucose as standard.

Antioxidants were determined according to the methods described in Knörzer *et al.* (1996). One g fresh leaves were homogenized in liquid nitrogen with 3 $cm^3$ of 5 % metaphosphoric acid (m/v). After centrifugation at 15 000×*g* for 15 min at 2 °C, the supernatant was aliquoted and stored at -20 °C. AsA and DAsA were assayed by following the change in $A_{525}$ after the addition of 2,2'-dipyridyl. GSH and GSSG were assayed following the change in $A_{412}$ after the addition of 5,5'-dithio-*bis*(2-nitrobenzoic acid) and glutathione reductase (purchased at *Sigma*).

**$O_2$ evolution** in light ($P_N$) was measured with a Clark type $O_2$ electrode (*Hansatech-CB1D*, Pentney, Great Britain) at a temperature of 25 °C and an irradiance of 750 µmol $m^{-2}$ $s^{-1}$. A constant $CO_2$ concentration (with 350 µM $NaHCO_3$) was provided to foliar disks; the assay medium contained 100 mM sucrose, 50 mM Tris buffer pH 7.5, and 5 mM $MgCl_2$.

## Results

The one month-old rooted cuttings of both clones had 8-9 leaves with similar average leaf area (7.6 $cm^2$ per leaf) and distance between the fine veins (304±14 µm).

On the abaxial side, the stomata length was significantly greater in Luisa Avanzo (26.7±0.6 µm) than in Dorskamp (19.6±0.4 µm); on the adaxial side, stomata size was similar in both clones (27.5±0.5 µm). Stomatal density was very heterogeneous on each leaf side: in average, on the abaxial side 158 ± 27 $mm^{-2}$ in Luisa Avanzo and 139 ± 20 $mm^{-2}$ in Dorskamp; on the adaxial one 52±14 and 62±5 $mm^{-2}$, respectively. Total mesophyll thickness was similar (*ca.* 140-180 µm) in both clones (Fig. 1). Luisa Avanzo exhibited two layers of compact palisade cells (*ca.* 58 µm thick); one or two layers of palisade parenchyma (*ca.* 25 µm thick)—with a large intercellular space between the cells—and a thick spongy mesophyll were found in Dorskamp (Fig. 1). In the latter clone, the air space represented 42.5 % of the mesophyll, while in Luisa Avanzo it was only 26 % of the mesophyll.

After a 12 h exposure to 50 mM mannitol (corresponding to -0.112 MPa), no leaf injury was detected. When both clones were submitted to 150 mM mannitol (-0.336 MPa), leaves were yellowing between the veins; however, neither morphological differences between both clones nor changes in the relative leaf water content (*ca.* 82 %) were observed. After both 50 and 150 mM mannitol treatments, the two clones closed their stomata: the opening fell down from 3.35±0.26 µm (control) to 1.77±0.25 µm in Luisa Avanzo, and from 4.06±0.74 µm to 1.03±0.29 µm in Dorskamp. When exposed to 50 mM mannitol, both clones exhibited a 50 % decrease in leaf conductance ($g_s$), which was measured as the sum of conductances of both leaf sides (Table 1); following a 150 mM mannitol exposure, $g_s$ remained stable in Dorskamp but reached 35 % of the control value in Luisa Avanzo. Therefore, although $g_s$ was two-fold greater in control Luisa Avanzo than in control Dorskamp, similar $g_s$ was

found in both clones submitted to 150 mM. Whereas $g_s$ of the abaxial side represented 80 % of total $g_s$ in both controls, its contribution fell down to 63 % and 40 % of total $g_s$ in Dorskamp submitted to 50 and 150 mM mannitol, respectively, and to 50 % of total $g_s$ in Luisa Avanzo whatever the osmotic treatment.

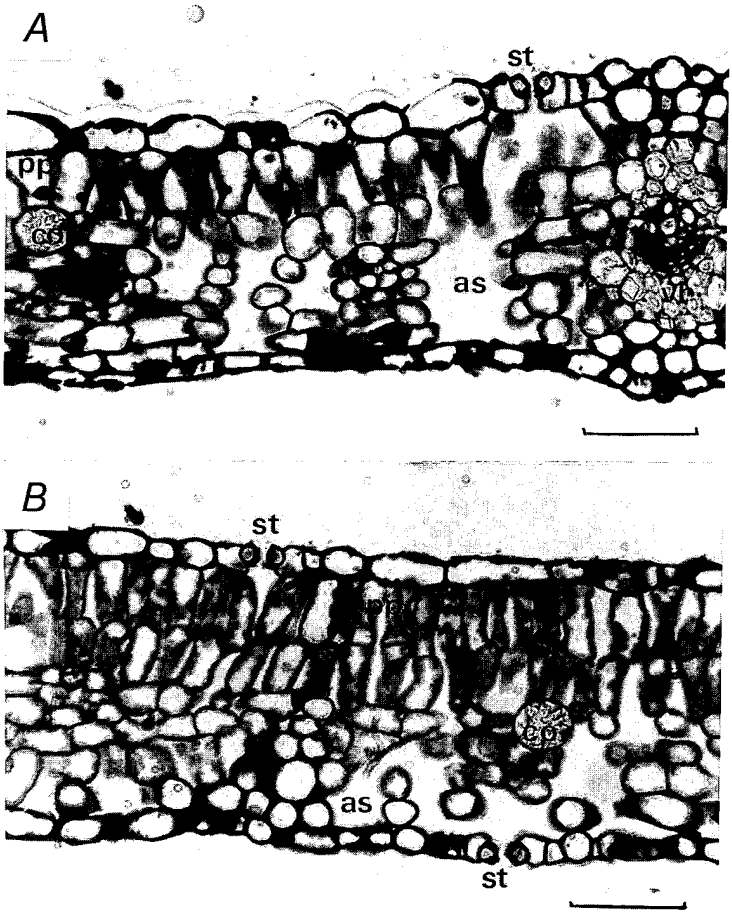


Fig. 1. Cross-sections (bar = 60 μm) of Dorskamp (*A*) and Luisa Avanzo (*B*) lamina at right angle. Stomata (*st*) and oxalate crystals (*co*) are present in both clones. (*A*) One or two layers of palisade parenchyma (*pp*), large air spaces (*as*), and vascular bundle (*vb*). (*B*) A compact palisade parenchyma two cells deep (*pp*) and small air spaces (*as*).

Both clones exhibited a similar $P_N$ in control conditions (Table 1). In Dorskamp cuttings, $P_N$ was unaffected by the osmotic treatments; it significantly fell down in Luisa Avanzo exposed to 150 mM mannitol. In Luisa Avanzo, a 50 mM mannitol treatment resulted in a 3-fold drop in RuBPCO activity (Table 1), but RuBPCO activity was slightly affected (*ca.* 20 %) in 50 mM mannitol treated Dorskamp. Under a 150 mM mannitol treatment, the decrease in RuBPCO was also the highest

in Luisa Avanzo. Total soluble sugar contents were equal in both clones [535 mmol $kg^{-1}$(dry m.)] and did not change under stress.

Table 1. Net photosynthetic rate, $P_N$ [μmol($O_2$) $kg^{-1}$(fr.m.) $s^{-1}$], leaf conductance, $g_s$ [mmol $m^{-3}$ $s^{-1}$], chlorophyll (Chl) content [g $kg^{-1}$(d.m.)], and activities of aminolevulinate dehydratase, ALAD [mmol(porphobilinogen produced) $kg^{-1}$(Chl) $s^{-1}$], chlorophyllase [g(Chl degraded) $kg^{-1}$(Chl) $s^{-1}$], and ribulose-1,5-bisphosphate carboxylase/oxygenase, RuBPCO [mmol(NADH oxidized) $kg^{-1}$(Chl) $s^{-1}$] in leaves of the drought-tolerant Dorskamp and the drought-sensitive Luisa Avanzo exposed for 12 h to 0, 50, and 150 mM mannitol. Mean ± standard error ($n$ = 5).

| Mannitol [mM] | Dorskamp | | | Luisa Avanzo | | |
|---|---|---|---|---|---|---|
| | 0 | 50 | 150 | 0 | 50 | 150 |
| $P_N$ | 0.46±0.14 | 0.34±0.09 | 0.41±0.06 | 0.52±0.21 | 0.44±0.14 | 0.11±0.08 |
| $g_s$ | 3.13±0.27 | 1.89±0.06 | 2.11±0.10 | 6.02±0.27 | 3.15±0.13 | 2.17±0.20 |
| RuBPCO | 0.25±0.02 | 0.19±0.02 | 0.09±0.02 | 0.07±0.00 | 0.02±0.00 | 0.01±0.00 |
| Chl | 9.73±2.27 | 13.10±1.44 | 12.50±1.75 | 13.20±1.54 | 15.50±1.80 | 14.90±1.12 |
| ALAD | 20.10±2.54 | 16.80±2.76 | 6.18±1.49 | 3.98±1.01 | 2.35±0.66 | 1.29±0.17 |
| Chlorophyllase | 1.24±0.09 | 1.11±0.11 | 0.68±0.04 | 0.34±0.02 | 0.26±0.03 | 0.25±0.01 |

The total Chl content did not change in both clones irrespective of the treatment (Table 1); Chl *a/b* ratio increased in Dorskamp exposed to 150 mM mannitol from 0.446 to 0.557, but remained stable in Luisa Avanzo. Under 50 mM mannitol, the ALAD and chlorophyllase activities did not significantly change in Dorskamp; conversely, both activities decreased in Luisa Avanzo (Table 1). Following a 150 mM mannitol treatment, a 3-fold decrease in ALAD activity occurred in both clones, while the percent reduction in the turn-over of Chl mediated through chlorophyllase was greater in Dorskamp than in Luisa Avanzo.

Table 2. Activities of ascorbate peroxidase, AP [mol(ascorbate oxidized) $kg^{-1}$(Chl) $s^{-1}$] and glutathione reductase, GR [mol(NADPH oxidized) $kg^{-1}$(Chl) $s^{-1}$], and contents of carotenoids [mmol $kg^{-1}$(d.m.)], ascorbate, AsA [mmol $kg^{-1}$(Chl)], and glutathione, GSH [mmol $kg^{-1}$(Chl)] in chloroplasts of Dorskamp and Luisa Avanzo exposed for 12 h to 0, 50, and 150 mM mannitol. Mean ± standard error ($n$ = 5).

| Mannitol [mM] | Dorskamp | | | Luisa Avanzo | | |
|---|---|---|---|---|---|---|
| | 0 | 50 | 150 | 0 | 50 | 150 |
| AP | 0.19±0.03 | 0.15±0.02 | 0.16±0.03 | 0.13±0.02 | 0.20±0.02 | 0.07±0.01 |
| GR | 0.14±0.02 | 0.15±0.02 | 0.17±0.02 | 0.10±0.01 | 0.08±0.01 | 0.03±0.00 |
| Carotenoids | 2.00±0.28 | 2.34±0.22 | 3.18±0.22 | 3.02±0.26 | 3.26±0.15 | 3.47±0.37 |
| Total ascorbate | 2.51±0.41 | 2.70±0.37 | 2.42±0.40 | 1.91±0.32 | 1.98±0.28 | 1.62±0.31 |
| AsA [% in the reduced form] | 92.30±4.80 | 95.60±5.90 | 95.40±5.30 | 91.00±6.20 | 94.40±5.20 | 97.10±4.80 |
| Total glutathione | 49.20±6.80 | 54.40±7.20 | 50.70±6.70 | 34.10±3.51 | 26.10±3.27 | 25.50±2.78 |
| GSH [% in the reduced form] | 92.50±4.90 | 89.80±5.70 | 87.60±4.50 | 91.20±5.80 | 87.80±3.80 | 84.20±3.50 |

The photorespiratory capacity of crude leaf extracts of Luisa Avanzo cuttings remained stable [57 mmol(glycolate oxidized) $kg^{-1}$(protein) $s^{-1}$] whatever the osmotic treatment; it increased in 150 mM treated Dorskamp [47 mmol(glycolate oxidized) *vs.* 31 mmol in control]. In Dorskamp, none of the osmotic treatments led to significant changes in AP and GR activities (Table 2). Enhanced AP activity and decreased GR activity were observed in 50 mM mannitol-treated Luisa Avanzo; a 150 mM mannitol treatment decreased both activities, but more GR than AP.

Carotenoid content remained stable in Luisa Avanzo, but was significantly enhanced (*ca.* 50 %) in 150 mM stressed Dorskamp (Table 2); more particularly, a significant enhancement in the β-carotene content occurred in 150 mM mannitol-treated Dorskamp [42 μmol $kg^{-1}$(d.m.)] *vs.* 30 μmol $kg^{-1}$(d.m.) in control. Total AsA and GSH contents, as well as the percent of their reduced forms, remained stable in Dorskamp. However, when exposed to 150 mM mannitol, the total GSH pool and the percent of GSH in the reduced form decreased in Luisa Avanzo; under these conditions, the amount of total GSH pool was twice less than in Dorskamp.

## Discussion

There are wide differences in anatomical and morphological traits between *Populus* genotypes (Pallardy and Kozlowski 1979, Ceulemans 1990). This is also the case for the two model clones which were selected. With regard to leaf anatomy, the most drought-sensitive clone (Luisa Avanzo) exhibited weak intercellular spaces of the palisade cells, and a thin spongy mesophyll which must limit the normal path for gas flow within the leaf (Levitt 1980). Conversely, Dorskamp showed a spongy mesophyll and a great volume occupied by air spaces while the thickness of the palisade parenchyma was twice less than in Luisa Avanzo. Stomata of Dorskamp appeared peculiarly drought-responsive, since their closure (indicated by stomata opening and $g_S$) occurred at higher leaf water potentials. Together with leaf anatomy and stomata morphology (*i.e.*, small stomata), Dorskamp could avoid drought and behave as sun plants that possess a high drought-resistance (Levitt 1980). As observed in *Vigna* (Cruz de Carvalho *et al.* 1998), change in $g_s$ was not related to the relative leaf water content, suggesting the involvement of a trigger signal originated from roots and translocated *via* the xylem to stomatal guard cells.

Despite this adaptive stomatal closure that could limit net influx of nutrients and $CO_2$ into the chloroplast, $P_N$ and saccharide status were not altered in Dorskamp; moreover, the carboxylation process in the drought-tolerant poplar was less susceptible to osmotic shock than in Luisa Avanzo. Such a loss in RuBPCO activity which was also the first drought syndrome in *Populus nigra* (Liu and Dickmann 1993), lead to excess of reductants since they were less consumed by the Calvin cycle. When NADP is limiting, oxygen can serve as final acceptor of electron in a pseudocyclic phosphorylation process that results in an increased production of ROS (Smirnoff 1993, Foyer *et al.* 1994).

To avoid subsequent damages to the photosynthetic apparatus, plants have evolved antioxidative systems that compete with ROS produced in chloroplast. Enhanced

photorespiratory capacity, which uses substrates accumulated during photochemical conversion of radiant energy, sustained chloroplastic AG cycle, enhancement of carotenoid pool size, high constitutive levels in AsA and GSH were indicative of such an antioxidative system and of the capacity of Dorskamp to cope with ROS (Larson 1988, Foyer *et al.* 1994). Such adaptive mechanisms, which were also observed in $O_3$-treated *Populus deltoides*×*Populus caudina* and *Populus maximowizii* ×*trichocarpa* (Sen Gupta *et al.* 1991, Brendley and Pell 1998), constitute a mean to tolerate stress (Sairam *et al.* 1998): the 50 mM mannitol stressed Dorskamp cuttings exhibited the lesser changes in synthesis and turn-over of Chl.

When Luisa Avanzo was exposed to 150 mM mannitol, $P_N$, GR activity, and GSH content drastically decreased. Although the redox status was not affected over the exposure period, one must assume that the drought-sensitive Luisa Avanzo was less able to counteract with the oxidative stress induced by the osmotic stress. Nevertheless, the contribution of the cytosolic AG cycle (Foyer *et al.* 1994, Zhang and Kirkham 1996)—which implies the movement of ROS between chloroplastic and cytosolic compartments and the existence of protection of chloroplast membranes—remains to be studied.

Chl content remained stable regardless of the treatment, due to the decreases in their synthesis and turn-over. The rapid yellowing observed in leaves of 150 mM-treated cuttings could possibly result, at least in part in Dorskamp, from synthesis of carotenoids or xantophylls which function as quenchers (Larson 1988).

Dorskamp, which exhibited the better capacity to avoid and tolerate osmotic stress, would be more productive under water deficit environment. However, can the previously defined correlation between hybrid poplar productivity and photosynthesis (Schulte *et al.* 1987) be reasonably retained for predicting the yield of such agri-forestry systems? More field experiments are required for providing some insights into (*1*) the ability of Dorskamp and Luisa Avanzo to retain green leaves late and have photosynthetic production in autumn contributing to a late season growth; (*2*) the interactive effects among environmental factors exerted on stomatal responses; and (*3*) the physiological changes affecting the distribution of photosynthates during the growing season.

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