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Clonal Variation in Growth, Physiology and Ultrastructure of *Populus alba* L. Seedlings Under NaCl Stress

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Abstract: Afforestation and reforestation (A/R) of non-agricultural and marginal saline lands by promoting fast-growing and salinity-tolerant woody species are crucial strategies to overcome land degradation and vegetation cover scarcity. To obtain basic information before using *Populus alba* clones in such degraded areas, morpho-physiological and cellular responses to salt stress were investigated. The experiment was conducted in a nursery where cuttings of three *P. alba* clones (MA-104, MA-195 and OG) were grown for 90 days in 100 mM NaCl versus a non-saline control. A global approach highlighting clonal differences in terms of dry mass production and plant physiological performance was achieved by comparing plant water status, gas exchange, ionic selectivity, osmotic adjustment and chloroplast ultrastructure under the two treatments. Dry mass production and eco-physiological processes were reduced in response to salt stress, with substantial clonal variation. Clone MA-104 exhibited salinity-tolerant behaviour in contrast to clone MA-195 and OG's medium or sensitive behaviour towards the stress. Tolerance mechanisms may be attributed to enhanced stomatal control and osmotic adjustment, thereby enabling the maintenance of turgor in plants subjected to salt stress. The chloroplast ultrastructure also showed modifications that are often involved in adaptation to salinity stress.

Keywords: *Populus alba*; clonal variation; growth; physiology; salinity tolerance

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1. Introduction

Tunisia, like several countries in the south Mediterranean basin, is facing increased degradation of forest ecosystems, particularly under the combined effects of natural and anthropogenic factors [1]. Overexploitation of natural resources, clearing land for agriculture or grazing and drought are considered to be the main causes of such degradation [2,3]. Climate change is also expected to increase drought frequency, especially in semi-arid regions that are already experiencing substantial water stress and soil salinization [4]. Current estimates based on 73% of the global land surface that has already been mapped indicate that the area of salt-affected soils covers 424 million hectares of topsoil (0–30 cm) and 833 million hectares of subsoil (30–100 cm) worldwide [5]. Salt-affected lands in Tunisia cover around 1.5 million hectares, which is roughly 10% of the country's surface area [6]. Afforestation and reforestation (A/R) through the rehabilitation of non-agricultural and marginal lands by promoting fast-growing and salinity-tolerant woody species have shown

potential for providing soil stabilization and potentially represent a long-term solution to restoring vegetation in sites that are affected by salinity [7,8]. Tree species may provide more effective management of degraded saline areas than herbaceous or shrub species, given that they grow to larger sizes, thereby allowing a larger amount of biomass to accumulate more salts [9]. Yet, tree tolerance to salt stress depends on the adaptive mechanisms that are involved, which may differ depending on the species, provenance, family and clone [10]. Tree species are endowed with phenotypic plasticity, including the adjustment of morphological and physiological traits, which can minimize the effects of extreme environmental conditions, and, thus, maximize their survival and growth [11]. Desert or Euphrates poplar (*Populus euphratica* Oliv.) has a very wide distribution, ranging from North Africa to the Middle East and into Central Asia. It is one of the most salt-tolerant *Populus* species, reportedly up to 450 mM NaCl (about 2.63%) under hydroponic conditions, and showing high recovery efficiency when NaCl is removed from the culture medium [12]. White poplar (*Populus alba* L.) is likewise widely distributed, from the Atlas Mountains (Algeria–Morocco–Tunisia) into southern and central Europe and central Asia. Related to the aspens (*P. Tremula*), *P. alba* is among the fastest growing and ecologically important pioneer tree species that frequently dominates riparian sites in semi-arid environments [13]. Like aspens, with which it can readily hybridize (*P. x canescens*, grey poplar), white poplar can establish itself through root suckering and rapidly forming extensive clonal stands. Various projects have paved the way for establishing *P. alba* in saline soils, both for their use in phytoremediation [9] and for short-rotation coppicing in intensive biomass production [14]. Several clones of this species are endemic or acclimatized to the edapho-climatic conditions of Tunisia and exhibit different levels of resilience to saline conditions [15]. Thus, their use for the rehabilitation of saline marginal lands could contribute to increasing the vegetation cover. Yet, the wide intra-specific variability of *P. alba* in response to salt stress [16,17] requires the prior identification of clones that adapt well to salt exposure.

Salt stress negatively affects the growth and development of crop plants and disrupts their hydro-mineral status [18,19]. Excessive accumulation of Na^+ and Cl^- ions leads to reduced stomatal conductance and transpiration that are associated with decreased photosynthesis [20]. Such a decrease in photosynthesis under osmotic stress can be attributed to both stomatal [21] and non-stomatal limitations [22]. Phenotypic or physiological changes often improve plant performance and confer increased salinity tolerance [21]. Stomatal regulation and osmotic adjustment [23] are among the main strategies for adaptation to salt stress. Furthermore, the maintenance of positive turgor is further enhanced when osmotic adjustment is accompanied by increased cell wall elasticity [24,25]. Na^+ and Cl^- accumulations can disturb the uptake of ions that are essential for plant development, such as K^+ and Ca^{++} [26]. Potassium plays a major role in maintaining cell turgor, stomatal regulation, photosynthesis, enzyme activation and protein synthesis [27,28]. Maintaining pronounced selectivity for K^+ reduces the Na^+ uptake and transport to aerial parts [29]. Moreover, it is often useful to prospect the response of chloroplasts, the centre of photosynthesis and primary metabolism, which can characterize plant tolerance to salt stress [30]. This stress incurs alterations in the chloroplast structure, the severity of which is dependent on several variables, such as the intensity of the applied stress, its duration or the species that is being considered [31].

This study is part of a larger research program that is being carried out by our research group, which focuses on the advancement of knowledge that is related to understanding physiological processes and the selection of clones that are intended for A/R programs specific to saline sites in Mediterranean semi-arid and arid areas [15]. This study focuses on clonal variation in *P. alba* through mechanisms that are involved in salt-stress tolerance at morphological, physiological and cellular levels. This is achieved through the determination

of adaptation mechanisms in response to severe NaCl stress in relation to the plant water status, gas exchange, ionic selectivity, osmotic adjustment and chloroplast ultrastructure.

2. Materials and Methods

2.1. Experimental Design and Growth Conditions

The experiment focused on three *Populus alba* clones, i.e., OG, MA-195 and MA-104. These are derived from stock plants with diverse eco-geographical origins. Clone OG (*Populus alba* L. Dode) grows on the banks of wadi Oued Gherib, which is located in northwestern Tunisia, a region that is characterized as having a subhumid bioclimate. Clones MA-195 and MA-104 (*Populus alba* L. var. *hichkeliana* (Dode) have been imported from Morocco since the 1960s. These 3 clones were selected from among the 31 clones that were tested [32], exhibiting different levels of salt tolerance. They were maintained as stock plants in a regional forest nursery (City of Jendouba, northwest Tunisia; latitude 36°58′31″ N, longitude 9°04′51″ E). Their propagation was continued as cuttings taken from different stock plants per clone. For each clone, cuttings (length: 15 ± 1.4 cm; diameter: 1.8 ± 0.15 cm) were taken during the winter dormancy period at the same branching level and height. They were planted in plastic pots (50 cm in diameter, 60 cm deep) containing inert substrate, i.e., coarse sand. These pots had drains to remove excess water. The experimental design was laid out at the National Research Institute of Rural Engineering, Water and Forests Nursery, with plants grown under a semi-arid bioclimate with mild winter conditions (36°50′ N, 10°14′ E; elevation 3 m). Average annual rainfall is about 475 mm, with average monthly minimum temperatures of 7.2 °C and maxima of 34.8 °C. The saline treatments were commenced once rooted cuttings were acclimated (after 60 days once seedlings of the three clones had reached the same development). The treatment consisted of applying 100 mM NaCl (electrical conductivity: 11.2 dS.m^{-1}). This salinity threshold represents the average NaCl load of surface and irrigation waters in the semi-arid and arid zones of the country, especially during summer [6]. Salt concentration was gradually increased until it reached the threshold of 100 mM NaCl after 12 days. The control (T) consisted of deionized water to which no NaCl was added. For each clone, there were two treatments and four replications with three seedlings per block. A total of 72 seedlings were used in a factorial randomized complete block design (3 seedlings × 3 clones × 2 treatments × 4 blocks). The salt treatment lasted 3 months, i.e., the period from June to August, which corresponds to the vegetation period of the *P. alba* clones. The substrate was enriched once a week with a half-concentrated Hoagland nutrient solution [33]. The salinity of the substrate was maintained by regular monitoring (once a week) of drainage water electrical conductivity using a microprocessor conductivity meter (model Cellox 325, Multiline P3 PH/LF-SET, Xylem Analytics GmbH, Weilheim, Germany).

2.2. Determination of Relative Growth Rate, Gas Exchange and Chlorophyll Concentrations

Shoot and root dry masses were measured on four randomly selected seedlings per clone per treatment at the beginning and end of the treatments after drying at 60 °C to constant mass. The relative growth rate (RGR) of shoot and root dry mass (expressed in g.g^{-1}.d^{-1}) was calculated according to the formula established by [34]:

$$RGRn = (lnW2 - lnW1)/(t2 - t1)$$

where W2 and W1 represent plant shoot or root dry mass at t2 and t1 (90 days), respectively. Transpiration (E), stomatal conductance for CO_2 (Gs), net assimilation (A) and intercellular CO_2 concentration (Ci), were measured using a portable open-mode gas analyzer system (Model LCA-4, Analytical Development Company, Hoddesdon, Herts., UK), with four ran-

domly selected seedlings per clone per treatment. Measurements were made after 90 days of seedling exposure to the salinity treatments. They were taken between 8:00 and 10:00 on whole leaves of rows 5 to 8, the most exposed to solar radiation under the following ambient conditions: leaf area in the cuvette, S = 6.25 cm^2; relative humidity (RH) ranging from 40% to 50%; photosynthetically active radiation (PAR) of 950 ± 52 µmoles.m^{-2} s^{-1}; ambient CO_2 (Ca) concentration of 380 µmoles.mol^{-1}; and leaf temperature of 30 ± 2 °C. Intrinsic water use efficiency (iWUE) was calculated by dividing net photosynthesis by stomatal conductance [35]. The stomatal limitation for CO_2 (Ls) was calculated as 1 − (Ci/Ca), where Ci is the intercellular CO_2 concentration and Ca is the ambient air CO_2 concentration [36]. Total chlorophyll concentration was determined spectrophotometrically on the same seedlings that were used for the gas exchange measurements. One hundred mg fresh mass (FW) of leaf material was ground in a pre-chilled mortar in 8 mL of 80% aqueous acetone (v/v). After complete extraction, the mixture was filtered and the volume adjusted to 10 mL with cold acetone. The absorbance (A) of the extract was read at 645 nm and 663 nm, and pigment concentrations were calculated according to the following formula [37]:

$$chl(total) = ((20.2(A645) + 8.02(A663)) * V)/((1000 * M))$$

where V is the volume of acetone needed for extraction (mL); and M is the mass of the fresh foliar sample (g).

2.3. Determination of Water Relations and Osmotic Adjustment

Water relations variables were determined at the end of the experiment from pressure–volume curves (PVC) using four seedlings that were randomly selected within clone and treatment. Measurements were conducted using simultaneous pressure chambers (model PMS 1000, PMS Instrument Co., Corvallis, OR, USA) and a precision balance (±0.1 mg) to generate the data that were needed to establish pressure–volume curves, according to the method described by [38]. Each PVC allows the determination of a set of variables describing the water relations [39] of the plants, viz., the osmotic potential at full turgor ($\Psi\pi^{100}$), the osmotic potential at loss of turgor ($\Psi\pi^{0}$), the relative water content at loss of turgor (RWC_0), apoplastic water content (AWC), the modulus of elasticity and the osmotic adjustment (OA). The modulus of elasticity (ε_{max}) that is defined by the change in tissue turgor pressure relative to the change in intracellular volume was calculated according to the formula that was established by [40]:

$$\epsilon max = -\Psi 100(1 - AWC)/(1 - RWC0)$$

Osmotic adjustment (OA) was calculated from the difference between the osmotic potential at full turgor $\Psi\pi^{100}$ of the control and salt-stressed seedlings.

$$OA = \Psi\pi 100control - \Psi\pi 100treatment$$

2.4. Determination of Soluble Sugars, Starch and Proline

The leaf concentrations of soluble sugars and starch were determined at the end of the treatments using four randomly selected seedlings per clone per treatment. The method used was that of [41], using pure glucose as the standard. For each plant, 100 mg of finely ground leaf powder was mixed with 10 mL of 80% ethanol. The homogenate was heated in a water bath at 70 °C for 30 min and then centrifuged at 10,000× g for 15 min. The soluble sugars in the supernatant were assayed with 0.2% anthrone in 80% ethanol. The pellet that was recovered from the soluble carbohydrate assay and containing surface starch was taken up in warm 80% ethanol for 5 min, then centrifuged at 10,000× g. The final pellet

was recovered in 1.5 mL of 35% perchloric acid and placed in an ice bath for 15 min for the first and second extractions. The two supernatants that were recovered were mixed to obtain the total starch contained in the extract. Starch determination was performed with anthrone in the presence of 21% perchloric acid. Optical density was measured with a spectrophotometer (Lambda 40 UV/VIS, Perkin Elmer, Waltham, MA, USA) at 640 nm. The concentrations of soluble sugars and starch were expressed as $\mu mol.g^{-1}$ dry matter. The method used for the determination of proline was that of [42], modified by [43] using L-proline as a standard. For each plant, 100 mg of finely ground leaf powder was mixed with 2 mL of 40% methanol. The sample was heated in a water bath at 80 °C for 30 min. After cooling, 2 mL of the extract was added to 2 mL of glacial acetic acid; 25 mg of ninhydrin; and 2 mL of a mixture containing 120 mL of distilled water, 30 mL of acetic acid and 80 mL of absolute ortho-phosphoric acid (density 1.7). The solution was heated in a water bath at 100 °C for 30 min until it gradually turned red. After cooling, 5 mL of toluene was added; the upper phase was recovered and dehydrated with the addition of anhydrous Na_2SO_4. Optical density was measured with a spectrophotometer at 528 nm. L-proline (Sigma Aldrich, Melbourne, VIC, Australia) was used to construct the standard curve. The proline concentration was expressed as $\mu mol.g^{-1}$ dry matter.

2.5. Determination of Na^+, K^+ and Cl^- Concentrations

At the end of the treatments, Na^+, K^+ and Cl^- concentrations were determined in the leaves and roots of the three *P. alba* clones using four randomly selected seedlings per clone per treatment. For each seedling, 500 mg of root and leaf dry mass (DM) was finely ground and placed in pillboxes containing 25 mL of 0.1 N nitric acid. Extraction of the ions took at least 48 h at room temperature. Na^+ and K^+ were determined by flame photometry (Jenway model PFP7, manufactured in the UK by Bibby Scientific Ltd, Dunmow, Essex, UK). The anion Cl^- was determined by coulometry using a chloridometer (Buchler-Cotlove Instruments, New York, NY, USA). The Na/K ratios were determined based on mass concentrations and reported as $mg.g^{-1}$ DM.

2.6. Ultrastructure of Chloroplasts

The chloroplast ultrastructure of *Populus alba* was studied on mature and sunny leaves at the end of the treatments. Sampling was performed on 8 well-developed leaves (i.e., 2 leaves per seedling per clone per treatment), which had been harvested between 8:00 and 10:00. For each treatment, 0.5 cm leaf portions were cut and immediately fixed (3 h at room temperature) in 2% glutaraldehyde solution buffered with 0.1 M sodium cacodylate at pH 7.4 [44]. They were post-fixed with 1% osmium tetroxide (OsO_4), which was similarly buffered for 6 h. This was followed by dehydration by the successive passage of the leaf portions through a series of ethanol baths of increasing concentration, and finally, propylene dioxide. Tissue embedding was performed using Spurr's resin mixture [45]. Ultra-thin sections (70–90 nm thickness) of resin-embedded tissues were made with an ultra-microtome (LKBV), placed in copper mesh grids, and stained with uranyl acetate and lead phosphate. Images were observed and generated using transmission electron microscopy (JEOL JEM-1230, Tokyo, Japan).

2.7. Statistical Analyses

All morphological and physiological data were analyzed according to a factorial randomized complete block design. A two-way analysis of variance (ANOVA) was conducted with salinity treatments (2 levels) and *P. alba* clones (3 levels) as fixed effects in the software package Statistix 8.1 (Analytical Software, Tallahassee, FL, USA). Post-hoc means the separations among different combinations of treatments and clones were tested with

Student–Newman–Keuls (SNK) tests at a significance level of 5%. Values are presented as means ± standard deviations (SD).

3. Results

3.1. Effect of NaCl Salinity on Dry Mass Production

The interaction (treatment x clone) together with the treatment or clone main effects significantly affected dry mass production in *P. alba* clones (Table 1). Under the control conditions, the relative growth rate of shoot dry mass (RGR SDM) in the three clones was 3.53 $mg.mg^{-1}.d^{-1}$ (Figure 1). This rate decreased significantly ($p < 0.05$) under salt stress, but differently, depending upon the clone. Clone MA-104 showed the lowest decrease in dry matter production with a reduction of 26% over the control compared to 50% in MA-195 and 64% in OG. At the root level, RGR RDM decreased by 51% of the control in clone OG versus 36% and 31% in MA-195 and MA-104, respectively.

Table 1. Significance levels of clone effect (C), treatment effects (T) and their interaction (C × T) on *P. alba* performance under saline growing conditions according to two-way ANOVA.

Variables	*p*-Values		
	Clone (C)	Treatment (T)	(C) × (T)
Shoot dry mass (SDM)	<0.001	<0.001	<0.001
Root dry mass (RDM)	<0.001	<0.001	<0.001
Net photosynthesis (A)	<0.001	<0.001	<0.001
Stomatal conductance (Gs)	<0.001	<0.001	0.0149
Transpiration (E)	<0.001	<0.001	0.0064
Intercellular CO_2 concentration (Ci)	<0.001	0.0304	0.0089
Chlorophyll concentration (Chl)	0.0279	0.0272	0.0174
Osmotic potential at loss of turgor ($\Psi\pi^0$)	0.006	0.0321	0.0051
Osmotic potential at saturation ($\Psi\pi^{100}$)	<0.001	0.001	0.0065
Relative water content at loss of turgor (RWC_0)	0.0061	0.0321	0.0051
Apoplastic water content (AWC)	0.001	0.001	0.005
Modulus of elasticity (ε_{max})	0.001	0.001	0.002

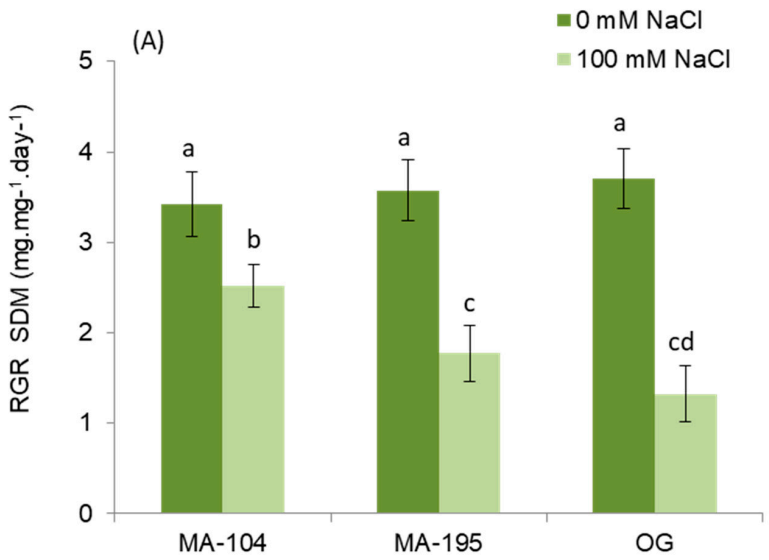

Figure 1. *Cont.*

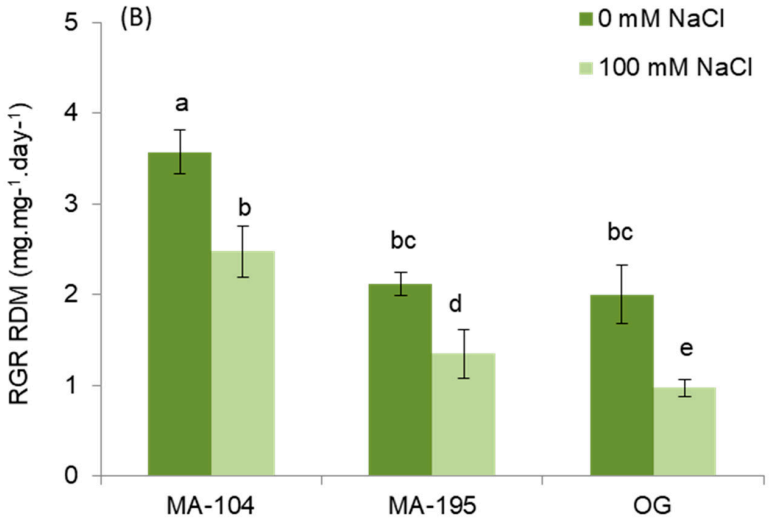

Figure 1. Average values for three clones of *P. alba* under salt treatments (control, and 100 mM NaCl) in relation to their (**A**): relative growth rate (RGR) of shoot dry mass (SDM) and (**B**): relative growth rate (RGR) of root dry mass (RDM). Values followed by the same letter do not significantly differ according to SNK tests ($p < 0.05$) (mean ± standard deviation, n = 4).

3.2. Effect of NaCl Salinity on Gas Exchanges and Chlorophyll Concentration

Significant effects of the clone and treatment ($p < 0.05$) were noted for all measured gas exchange variables (Table 2). The clones showed a significant ($p < 0.05$) decrease in E, Gs, A and Ls under the applied NaCl concentration (Table 2), while intercellular CO_2 concentration (Ci) increased. Compared to the control, MA-104 showed the lowest reduction of (A) and (Gs), which were, respectively, 30% and 46%, compared to 48% and 59% for MA-195, and 52% and 62% for OG. In contrast, a significant increase ($p < 0.05$) in intrinsic water use efficiency (iWUE) was recorded for all clones, which was attributed to a greater decrease in Gs compared to (A). Intercellular CO_2 concentration also increased with a concomitant decrease in the stomatal limitation for CO_2 (Ls). Total chlorophyll concentration increased under salt stress. The highest increase was recorded in OG (31%) compared to 22% and 16% in MA-195 and MA-104, respectively.

Table 2. Mean values for gas exchange variables: transpiration (E), stomatal conductance (Gs), net CO_2 assimilation (A), intrinsic water use efficiency (iWUE) and stomatal limitation (Ls) inferred from gas exchange measurements, intercellular CO_2 concentration (Ci) and chlorophyll concentration for three *P. alba* clones under salt treatments (control, and 100 mM of NaCl) (mean ± standard deviation, n = 4).

Variables	Treatments NaCl (mM)	Clone		
		MA-104	MA-195	OG
E (µmol H_2O)	0	4.32 ± 0.63 a	5.17 ± 0.79 a	4.95 ± 0.77 a
	100	2.81 ± 0.74 b	3.03 ± 0.88 b	2.76 ± 0.72 b
Gs ($mol.m^{-2}.s^{-1}$)	0	0.31 ± 0.06 a	0.37 ± 0.09 a	0.29 ± 0.07 a
	100	0.17 ± 0.04 b	0.15 ± 0.03 b	0.11 ± 0.03 b
A ($\mu mol.m^{-2}.s^{-1}$)	0	11.37 ± 1.54 a	10.94 ± 1.27 a	11.68 ± 1.47 a
	100	8.96 ± 1.07 b	5.72 ± 1.85 c	5.55 ± 0.92 c
iWUE ($\mu mol.mol^{-1}$)	0	36.7 ± 1.5 d	29.6 ± 1.3 e	40.3 ± 2.5 c
	100	46.8 ± 2.1 b	38.1 ± 2.4 cd	50.6 ± 1.3 a

Table 2. *Cont.*

Variables	Treatments NaCl (mM)	Clone		
		MA-104	MA-195	OG
Ci.µmol CO_2.mol^{-1})	0	94 ± 1.55 d	140 ± 1.25 b	144 ± 1.98 b
	100	119 ± 2.67 c	165 ± 2.35 a	169 ± 2.88 a
Ls	0	0.738 ± 0.018 a	0.610 ± 0.015 a	0.599 ± 0.012 a
	100	0.668 ± 0.011 b	0.540 ± 0.013 b	0.531 ± 0.014 b
Chlorophyll (mg.g^{-1} FM)	0	3.656 ± 0.318 d	4.061 ± 0.323 cd	4.488 ± 0.377 c
	100	4.358 ± 0.212 c	5.225 ± 0.355 b	6.473 ± 0.387 a

For each variable, values that are followed by the same letter do not differ significantly according to the SNK tests ($p \geq 0.05$).

3.3. Effect of NaCl Salinity on Water Relations

Changes in clones water relations were noticeable at the end of the treatment between the control and salt-treated plants. Mean values of $\Psi\pi^0$, $\Psi\pi^{100}$, RWC_0, AWC, εmax and OA are indicated in Table 3. Salt-treated clones had significant decreases in osmotic potential at turgor loss point $\Psi\pi^0$. This potential was more negative under salinity with a decline relative to the control by 9% in both MA-104 and MA-195 clones and 7% in OG. Osmotic potential at full turgor ($\Psi\pi^{100}$) also decreased ($p < 0.005$), indicating the occurrence of osmotic adjustment. A significant difference between clones appeared where the highest adjustment (0.26 MPa) was recorded in MA-104, versus 0.14 and 0.12 MPa in MA–195 and OG, respectively. Apoplastic water content (AWC) increased by 41% and 38% in clones MA-104 and MA-195, respectively, compared to 17% in OG. The bulk modulus of elasticity (ε_{max}) increased significantly ($p < 0.0001$) in all three clones, indicating a substantial increase in tissue rigidity after 90 days of treatment. The greater increase (35% over control) was recorded in MA-104 compared to 20% and 23% in MA-195 and OG, respectively.

Table 3. Mean values for leaf water variables of three *P. alba* clones under salt treatments (control and 100 mM of NaCl): osmotic potential at loss of turgor ($\Psi\pi^0$); osmotic potential at saturation ($\Psi\pi^{100}$); the relative water content at loss of turgor (RWC_0); apoplastic water content (AWC); the modulus of elasticity (ε_{max}); and the osmotic adjustment (OA) (mean ± standard deviation, n = 4).

Variables	Treatments NaCl (mM)	Clone		
		MA-104	MA-195	OG
$\Psi\pi^0$ (MPa)	0	−2.37 ± 0.02 b	−2.34 ± 0.01 b	−2.23 ± 0.02 a
	100	−2.61 ± 0.02 e	−2.56 ± 0.01 d	−2.41 ± 0.01 c
$\Psi\pi^{100}$ (MPa)	0	−1.67 ± 0.03 b	−1.61 ± 0.03 b	−1.52 ± 0.03 a
	100	−1.93 ± 0.02 d	−1.75 ± 0.02 c	−1.64 ± 0.04 b
RWC_0 (%)	0	74.16 ± 1.32 c	74.52 ± 1.28 c	76.31 ± 1.46 b
	100	81.28 ± 1.29 a	80.42 ± 0.92 a	80.75 ± 2.16 a
AWC_0 (%)	0	24.75 ± 1.5 b	17.18 ± 1.24 d	18.32 ± 0.82 d
	100	34.26 ± 1.06 a	24.23 ± 1.81 b	21.53 ± 1.29 c
ε_{max} (MPa)	0	4.33 ± 0.14 c	5.28 ± 0.15 b	5.19 ± 0.62 b
	100	6.7 ± 0.21 a	6.65 ± 0.12 a	6.73 ± 0.88 a
OA (MPa)	100	0.26 ± 0.03 a	0.14 ± 0.03 b	0.12 ± 0.02 b

For each variable, values that are followed by the same letter do not differ significantly according to SNK tests ($p \geq 0.05$).

3.4. Effects of NaCl Salinity on Soluble Sugars, Starch and Proline Accumulation

Soluble sugars as well as proline increased significantly ($p < 0.05$) in leaves in contrast to starch concentration, which decreased under salt stress (Table 4). In comparison to the control, soluble sugars increased 40% in *P. alba* clones. Proline concentration was 2.2 and 1.4 times higher in MA−195 and MA-104 clones, respectively, while no significant change was recorded in OG clone. Leaf starch concentrations decreased by 24%, 32% and 51% in MA-104, MA-195 and OG, respectively.

Table 4. Average values for three *P. alba* clones under salt treatments (control, and 100 mM of NaCl) in relation to their leaf concentration in starch, soluble sugars and proline.

Variables	Treatments NaCl (mM)	Clone		
		MA-104	MA-195	OG
Starch ($mg.g^{-1}$ DM)	0	116.89 ± 3.29 a	117.53 ± 4.44 a	123.59 ± 6.35 a
	100	88.86 ± 3.22 b	78.67 ± 2.35 c	60.19 ± 2.35 d
Soluble sugars ($mg.g^{-1}$ DM)	0	43.21 ± 2.09 b	42.26 ± 3.54 b	39.63 ± 2.52 b
	100	71.06 ± 4.31 a	70.69 ± 3.45 a	66.39 ± 3.84 a
Proline ($\mu mol.g^{-1}$ DM)	0	7.70 ± 0.64 b	4.27 ± 0.62 c	3.95 ± 0.56 c
	100	10.78 ± 0.91 a	9.66 ± 0.84 a	4.45 ± 0.47 c

For each variable, values that are followed by the same letter do not differ significantly according to SNK tests ($p \geq 0.05$).

3.5. Effects of NaCl Salinity on Na^+, K^+ and Cl^- Distribution

Salt stress induced an increase in the Na^+ concentration that was significantly higher in roots compared to leaves with variability between clones. The rates of the Na^+ increase compared to the control were 65%, 69% and 76% in roots versus 9%, 17% and 22% in leaves in clones MA-104, MA-195 and OG, respectively (Figure 2a). An accumulation of Cl^- was also recorded, which was more substantial in leaves (Figure 2b). On the one hand, clone MA-104 recorded the lowest rate of Cl^- increase, which was 25% of the control, followed by clones MA-195 (28%) and OG (33%). On the other hand, the Na^+/K^+ ratio significantly decreased under salt stress in the leaves of clone MA-104, suggesting preferential selectivity for K^+ in this compartment (Figure 2c). In contrast, this ratio varied slightly in MA-195 and OG, reflecting an increase in both Na^+ and K^+ concentrations. Furthermore, the Na^+/K^+ ratio increased strongly in the roots of *P. alba* clones (Figure 2d), indicating higher selectivity for Na^+ in roots. Clone MA-104 exhibited a ratio that was 4.6 times higher than that of the control, against eight-fold higher in MA-195 and 10.5-fold in OG.

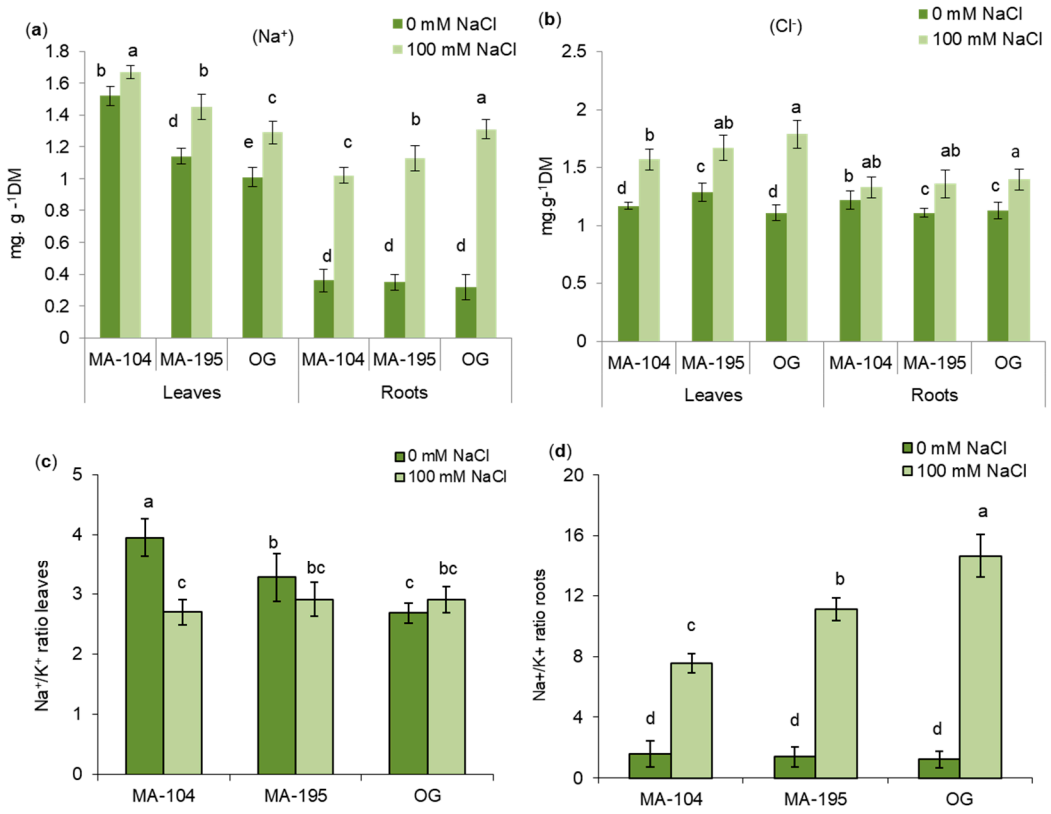

Figure 2. Average values of Na^+ (**a**), Cl^- (**b**) and Na/K ratio in leaf (**c**) and root (**d**) tissues for three *P. alba* clones under salt treatment (control, and 100 mM NaCl). For each plant compartment, values that are followed by the same letter do not significantly differ according to SNK tests ($p \geq 0.05$) (mean ± standard deviation, n = 4).

3.6. Effects of NaCl Salinity on Chloroplast Ultrastructure

The control *P. alba* seedlings exhibited chloroplasts with compactly arranged thylakoids and regularly organized grana stacks with distinct grana lamellae, starch granules and plastid lipid droplets as plastoglobuli (Figure 3A,D,G). Exposure to 100 mM NaCl over 90 days led to several alterations of which the degree of damage to chloroplasts varied, with clones differing in their ability to cope with salinity. Deeper chloroplast lesions were observed in the clones MA-195 and OG, highlighting their increased sensitivity to salinity. These involved alterations to chloroplast integrity, as ruptures of the chloroplast envelope allowed the contents of the stroma to mix with the cytoplasm (Figure 3B,F). Accumulations of plastoglobuli in these clones were also recorded, which were transformed into large lipid vesicles occupying almost the full volume of the stroma (Figure 3B,E). These vesicles lost their membrane integrity under salt stress and were released as lipid droplets into the cytoplasm following the rupture of the chloroplast envelope (Figure 3B,C,E). The chloroplast envelope also showed strong damage, which was characterized by severe membranous invaginations forming vesicles (Figure 3C). The intact chloroplast structure was almost maintained under salinity stress in clone MA-104 with minor changes, such as the swelling of the thylakoids, an increase in the number of plastoglobuli and the degradation of starch granules (Figure 3H). In addition, this clone could be differentiated from MA-195 and OG by the presence of a peripheral chloroplast reticulum in its most elaborate form. This lamellar structure was recognizable by the multiple rows of tubules surrounding the entire periphery of chloroplasts of MA-104 with regular organized grana stacks and dense stromal thylakoid lamellae (Figure 3I).

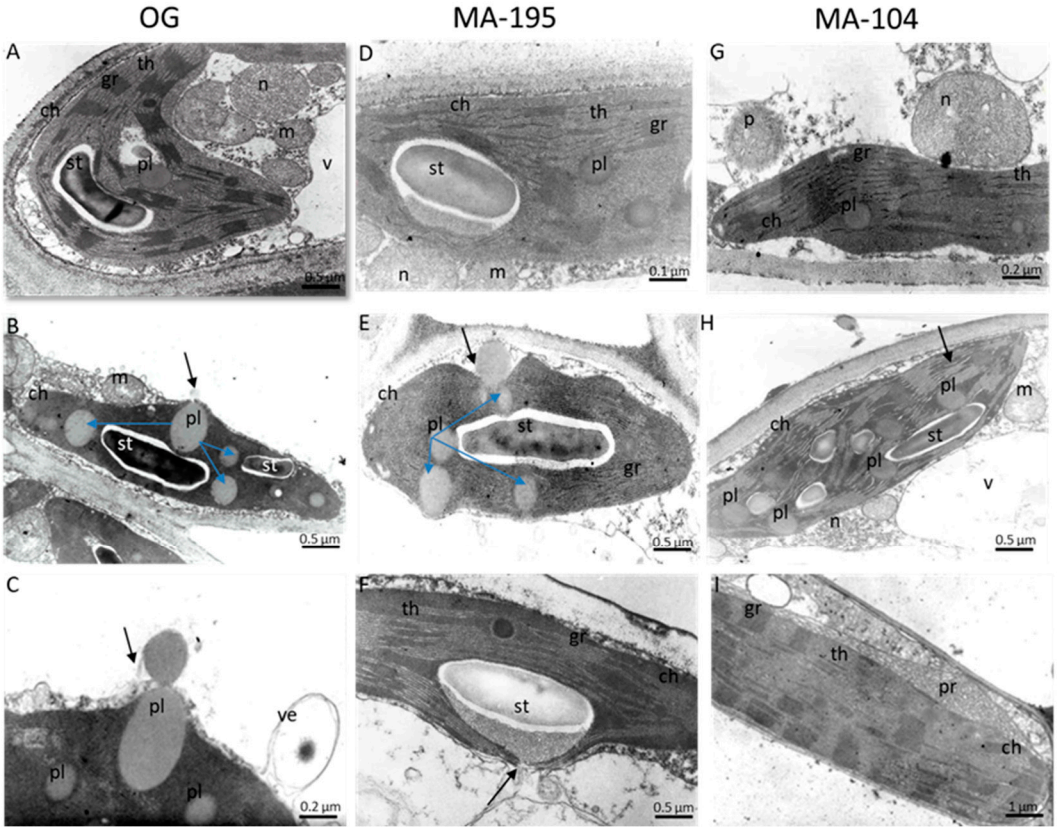

Figure 3. Ultrastructural changes under salt stress of chloroplasts in mesophyll cells of *Populus alba* clones. (**A**,**D**,**G**): Chloroplasts of control clones with compactly arranged thylakoids and well compartmentalized grana stacks, starch granules and plastoglobuli. (**B**,**C**,**E**,**F**,**H**,**I**): Ninety days of salinity stress. Clone OG; (**B**): Lipid accumulation (colored arrows), disruption of chloroplast envelope and plastoglobuli membrane (black arrow). (**C**): Migration of plastoglobuli into the cytoplasm (black arrow) and chloroplast envelope invagination, forming vesicles. Clone MA-195; (**E**): Plastoglobuli accumulation (colored arrows) and lipid deposition into cytoplasm (black arrow). (**F**): Chloroplast envelope rupture and degradation of starch granules (black arrow). Clone MA-104; (**H**): Swelling of the thylakoids (black arrow), accumulation of plastoglobuli and degradation of starch granules (**I**): Compactly arranged thylakoids and well compartmentalized grana stacks, chloroplast peripheral reticulum. Note: ch, chloroplast; gr, grana; pl, plastoglobuli; st, starch granules; th, thylakoids; pr, peripheral reticulum; ve, vesicles; m, mitochondria; n, nucleus; p, peroxysome; v, vacuole.

4. Discussion

4.1. Clonal Variation in Dry Mass Production and Na^+, K^+ and Cl^- Distribution

Salt treatment leads to a significant decrease in total dry mass in *P. alba* clones, which is a characteristic response of glycophytes to salinity [46]. This implies that salt stress slows down the metabolic mechanisms leading to decreased growth, and, consequently, to the reduction in biomass [47]. Clonal variability in response to salt stress has been demonstrated where lower dry mass loss was recorded in clone MA-104 compared to MA-195 and OG, suggesting the lower susceptibility of MA-104 to salt stress. Furthermore, the ability of *P. alba* clones to sustain their growth under high NaCl concentrations could be governed by the compartmentalization of Na^+ ions in their roots and the ion's exclusion from leaf tissues. This is considered as a mechanism of salinity tolerance in *Populus* sp. [9,48] and in woody halophytes [49]. The potential of the *P. alba* clones to exclude salt seems to depend upon the adaptability mechanism that limits the transport of Na^+ to the aerial parts. Our results showed that MA-104, which is differentiated by higher dry mass production, was most efficient in limiting the foliar Na^+ uptake compared to clones MA-195 and OG. In contrast,

salinity tolerance in woody species is closely related to their capacity to exclude Na^+ or Cl^- ions [50]. The preferential accumulation of Cl^- in aerial parts can cause leaf stomatal damage, leaf necrosis and early leaf senescence [32,51]. In this study, the accumulation of salt ions in *P. alba* plants did not seem to have reached toxic levels, given that symptoms of phytotoxicity were not observed. The accumulation of Na^+ leads to the competitive inhibition of K^+ uptake [52] and essential mineral nutrients [53]. This plant ion imbalance was characterized by an increase in the Na^+/K^+ ratio in roots versus a decrease in this ratio within the leaves of the clones that were tested. Under salt stress, the foliar Na^+/K^+ ratio generally increases in various *Eucalyptus*, *Populus* and *Acacia* genotypes [14,54]. As a consequence of NaCl stress, potassium deficiency may occur [17,55]. In contrast, our results showed high ion selectivity for K^+ ions, particularly in clone MA-104, that was due to its greater ability to tolerate salinity. Indeed, selective Na^+/K^+ substitution resulting in K^+ accumulation can be considered as a criterion for salinity tolerance [26]. This has been demonstrated in salt-tolerant *Eucalyptus* species and can be used as a criterion for tolerance to K^+ selectivity relative to Na^+ [56]. The foliar accumulation of K^+ was accompanied by a depletion of this element in the roots of *P. alba* plants, which resulted in an increase in the foliar Na^+/K^+ ratio. This antagonism was thought to be a consequence of a greater rate of translocation of K^+ to leaves, which may contribute to the maintenance of turgor in leaf cells through osmotic adjustment [57,58]. Furthermore, clone MA-104 developed the lowest root Na^+/K^+ ratio, reflecting a greater ability to control K^+ transport under salt stress.

4.2. Osmotic Adjustment and Water Relations

The P–V curves in *P. alba* clones indicated active osmotic adjustment (OA) in response to NaCl, which was considered an important tolerance index [28]. Clone MA-104 developed an OA almost two-fold higher than values in MA-195 and OG, reflecting its increased tolerance towards salt stress. The results indicate the salt-induced accumulation of soluble sugars and proline in *P. alba* plants, a response which could be involved in the OA. Indeed, these osmolytes contributed to the growth support of *P. alba* seedlings and functioned as osmoprotectors to mitigate the negative effects of stress [58]. The accumulation of soluble sugars and proline was accompanied by starch hydrolysis in all *P. alba* clones, which is a response that has been correlated with improved tolerance to salt or drought stress [59]. In fact, starch remobilization could partially buffer fluctuations in sugar concentrations when photosynthetic activity is limited [60]. The modulus of elasticity (ε) increased in the *P. alba* clones, but significantly so in MA-104. This increase under salt stress leads to the preservation of a positive water balance in alligator weed (*Alternanthera philoxeroides* [Mart.] Griseb.), an invasive, clonal herb [61]. Such a mechanism prepares the cell to undergo large variations in apoplast water content without affecting the dynamic structure of the cell wall [62]. This was confirmed by the significant increase in the apoplast water content of *P. alba* clones compared to the controls, especially in clone MA-104. The higher water reserve in the apoplast could help stimulate the passive concentration of solutes that allowed the maintenance of turgor in the plants under salt stress [63,64].

4.3. Gas Exchange, Chlorophyll Concentration and Chloroplast Ultrastructure

Salt stress caused a reduction in the leaf gas exchange parameters in *P. alba*. Stomatal conductance decreases would preserve water by limiting its loss through transpiration. This was considered to be a promising strategy for improving plant salt-stress tolerance by controlling the toxic concentrations of Na^+ and Cl^-, and their translocation to the aerial parts [52,65]. Furthermore, salt stress affected the mesophyll conductance in *P. alba* clones, expressed by an increase in intercellular CO_2 (Ci). This can be attributed to either stomatal or non-stomatal limitations that impair the photosynthetic process [35], and which may

operate successively or jointly depending upon the intensity and duration of salt stress [66]. Total chlorophyll concentration increased in the three *P. alba* clones under salinity, in contrast to studies indicating a decrease, rather than an increase, in such parameters, related to a decline in photosynthetic activity in fast-growing species [67]. Such increases, which were more pronounced in clone OG, are controversial, considering the reductions in net photosynthesis that we recorded under salt treatment. Several studies have demonstrated that, despite net photosynthetic decline under salt stress, an increase in total chlorophyll content was recorded in the fast-growing species *Populus euphratica* [68], *Eucalyptus* and *Populus* [14]. Furthermore, this response has been linked to the enhancement of leaf thickness [69]. In this context, our previous research is in agreement with these results, in which leaf thickening was demonstrated in *P. alba* under increasing NaCl concentrations, especially in clone OG [70].

Several modifications have been associated with the chloroplast ultrastructure in response to long-term salt stress. As these organelles are the sites of the biochemical and biophysical processes of photosynthesis, any perturbation of the chloroplast function will impair plant growth and development as well as biomass production [71]. The degree of damage to chloroplasts depended upon the ability of the *P. alba* clones to cope with salinity. The deeper chloroplast damages that were recorded in clones MA-195 and OG, as manifested in the destruction of the chloroplast envelope membrane, or its invagination, are evidence of the strong plasmolysis of cells under salt stress, highlighting their vulnerability to such constraints. These chloroplast membrane lesions would be attributed to the loss of cell turgor under osmotic stress-induced Na^+ and Cl^- ion toxicity [72], as much as to the accumulation of reactive oxygen species (ROS) [73]. By contrast, clone MA-104 exhibited salt tolerance by maintaining the chloroplast ultrastructure and function with changes that are often associated with chloroplast adaptation to salinity stress. These include the degradation of starch granules, together with the accumulation of plastoglobuli [74]. The chloroplast peripheral reticulum that was also observed in clone MA-104 was believed to have contributed to the better performance of this clone under salt stress. This complex membrane structure is a system of tubes and vesicles that is continuous with the chloroplast inner membrane, and which would be a form of adaptation to salinity insofar as the larger exchange surface that it confers to the chloroplast membrane ensures the active and rapid transport of metabolites inside or outside the chloroplast [75,76].

5. Conclusions

Our findings demonstrate the existence of substantial clonal variation in *Populus alba* in response to salinity stress. This study has revealed an enhanced tolerance to salt stress in clone MA-104. This clone can elicit several mechanisms that are involved in the adaptive response to salinity stress, including the protection of the chloroplast ultrastructure and biochemical defence reactions. These results highlight the importance of conducting eco-physiological measurements that are coupled with chloroplast ultrastructure assessments to better understand the genetic variability of *P. alba* clones to salt tolerance. Thus, the use of relevant eco-physiological traits (stomatal behaviour, osmotic adjustment, Na/K ratio and chloroplast ultrastructure) to select appropriate salt-tolerant clones represents a key tool in ensuring the success of A/R projects, especially those in marginal saline areas. Furthermore, this study provides a practical framework for integrating MA-104 into saline agroforestry systems, leveraging its adaptive traits to improve productivity in degraded saline soils.

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Z.B., M.S.L. and D.K.; supervision, Z.B., M.S.L. and D.K. All authors have read and agreed to the published version of the manuscript.

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