Salt stress effect on morphometrical and anatomical leaf traits of promising poplar biotypes

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Abstract. The purpose of the research was to determine the effect of short-term salt stress on the morphometric, anatomical and biochemical traits of fast-growing woody plants and to evaluate the promising poplar genotypes according to their degree of resistance. The experiments were conducted on five annual varieties in containers. The fourth leaf from the top of apical shoot, 80% of which corresponds to a maximum leaf area and high photosynthetic activity, was used in the experiment. The studies showed the nature of changes in the specific surface density and water content of the leaf of annual shoots. A short-term salt stress causes changes in leaf anatomy. Thus, the less stable genotype of poplar ‘Kitayskiy’ responds by the reduction of the leaf thickness. The changes in leaf anatomy of the experimental plants also include a decrease in height of the palisade and spongy parenchyma cells, which leads to the reduction of assimilation surface area and unproductive water loss. The study included analysis of changes in the proline concentration as a result of short-term salinization of the experimental plants. All samples showed a change in proline levels in response to stress, but most intense reaction was shown by genotypes ‘Veduga’ and ‘Hybrid e.s.-38’. In general, greater stability and plasticity at the initial stage of salinization is characteristic of poplars ‘Volosistoplodny’, ‘Sacrau-59’, ‘Hybrid e.s.-38’ and ‘Veduga’, which allows us to recommend them for reforestation and afforestation on irrigated soils.

1. Introduction
Salinity is one of the most significant environmental problems limiting plant productivity [1, 2]. Salinity in irrigation water and in soils is one of the major abiotic constraints on agriculture worldwide and the situation has worsened over the last 20 years due to the increase in irrigation requirements [3, 4]. Soil salinity affects about 800 million hectares of arable lands worldwide. As a consequence, ion toxicity leads to chlorosis and necrosis mainly due to Na⁺ accumulation that interferes with many physiological processes in plants [5].

The harmful effect of salinity can vary depending on climatic conditions, light intensity, plant species or soil conditions. Depending on the ability of plants to grow in saline environments, they are classified as either glycophytes or euhalophytes and their response to salt stress differs [6, 7].

Most salinity adaptive mechanisms in plants are accompanied by certain morphological and anatomical changes [8]. Glycophytes, which include most crops, cannot grow in the presence of high salt levels; their growth is inhibited or even completely prevented by NaCl concentrations of 100–
200 mM, resulting in plant death. Such growth inhibition can even occur in the short term. In contrast, halophytes can survive in the presence of high NaCl concentrations (300–500 mM) because they have developed better salt resistance mechanisms [2].

Euhalophytes can also accumulate salt in their cell sap up to a level at which their osmotic potentials are lower than in the soil solution.

Salt stress is first perceived by the root system and impairs plant growth both in the short term, by inducing osmotic stress caused by reduced water availability, and in the long term, by salt-induced ion toxicity due to nutrient imbalance in the cytosol [9]. Therefore, two main threats imposed by salinity are induced by osmotic stress and ionic toxicity associated with excessive Cl\(^-\) and Na\(^+\) uptake, leading to Ca\(^{2+}\) and K\(^+\) deficiency and to other nutrient imbalances. In addition, salt stress is also manifested as oxidative stress. All these responses to salinity contribute to the deleterious effects on plants [10].

Under saline conditions, plants have to activate different physiological and biochemical mechanisms in order to cope with the resulting stress. Such mechanisms include changes in morphology, anatomy, water relations, photosynthesis, hormonal profile, toxic ion distribution and biochemical adaptation [11, 12].

The amino acid proline is known as a reliable biochemical marker of the stress state of plants. Studies established that the result of the response to abiotic effects (including salinity and freezing), the concentration of proline in shoots and leaves increases [13]. Osmotic and oxidative stress caused by salinization can be smoothed out by the accumulation of compatible solutes and osmoprotectors in a plant. Proline has the function of inert osmolyte. Under the action of stressors it performs a number of other interrelated functions: membrane-protective, chaperone, antioxidant, and also participates in the regulation of the expression of certain genes [14, 15].

The relationship between the concentration of proline and the reaction to salt stress has been confirmed for many species of plant organisms, including poplar and aspen. Poplar, as a rapidly growing species, is a potential candidate for planting on saline soils. Studies show that, as a result of artificially modeled salt stress on experimental specimens of *Populus tremula*, the levels of proline, spermine, sucrose, mannitol and raffinose increase [16]. The studying of changes in the concentration of osmotic substances of a salt tolerant species of *Populus euphratica* under salt stress showed that the accumulation of proline, glycine, betaine and the amount of soluble sugars increased with increasing salt concentration. The results show that the response of callus of *P. euphratica* to saline stress is similar to that of the whole plant [17].

Thus, many studies have identified a relationship between proline accumulation and plant resistance to stressors. However, the opposite effects have been registered as well. The ambiguity of such a relation can be due to both methodological reasons (different strength of stress effects in different experiments) and the complex interaction of proline with other stress-protector systems, in particular with enzymatic antioxidant [15].

The purpose of our research was to determine the effect of short-term salt stress on the physiological and biochemical characteristics of fast-growing woody plants and to evaluate promising poplar genotypes according to their degree of resistance.

2. Materials and methods

Samples of apical annual shoots of the poplars were selected at the end of January from the collection mother plantation in the forest park area of the Research Institute of Forest Genetics, Breeding and Biotechnology (Voronezh, European part of the Russian Federation). Six apical shoots of each genotype were used for the experiment and they were cut for two stem fragments about 20-25 cm. In total, there were 12 vegetative shoots of each genotype at the age of 3 months and each of the three replicates included four containerized plants. The experiment involved 5 forms and clones of real and white poplars (table 1).
Table 1. Characteristics of the promising biotypes of poplars planted on the territory of the forest park section of the “Research Institute of Forest Genetics, Breeding and Biotechnology”.

| No. | Name               | Inv. No. | Origin of genotype       | Author of hybrid        |
|-----|--------------------|----------|--------------------------|-------------------------|
| I.  | Balsam poplars     |          |                          |                         |
| 1   | ‘Volosistoplodny’  | 45       | *P. trichocarpa* Torr. et. Gray | Natural species [18]    |
| 2   | ‘Kitayskiy’        | 42       | *P. simonii* Carr.       | Yablokov AS [18]        |
| II. | Black poplars with branchy form of crown |          |                          |                         |
| 3   | ‘Sacrau-59’        | 90       | *P. × euroamericana* (Dode) Guinier cv. sacrau | Euro-American Hybrid [18] |
| III. | White poplars with pyramidal crown shape |          |                          |                         |
| 4   | ‘Veduga’           | 26-07    | *P. alba* L. × *P. bolleana* Laurche | Tsarev AP [18]          |
| IV. | Intersectional Hybrids |        |                          |                         |
| 5   | ‘Hybrid e.s.-38’   | 94       | *P. deltoides* Marsh. × *P. balsamifera* L. | Veresin MM [18]         |

The experimental objects were placed in the cassettes of the Swedish company BCC for rooting with a height of 16 cm and a root zone volume of 235 cm³. The bog neutralized peat brand “Agrobalt-N” was used as a substrate.

Cassettes with samples were placed in artificial light conditions at 21±2°C. Throughout the experimental period (until early April), planned maintenance, watering and feeding of all 5 forms of poplars was carried out. A Knopp nutrient solution was used as feed.

Salt stress of the experimental plants was carried out for 7 days by using 0.5M NaCl solution in the root zone to the middle of the cassettes. Feeding of the salt solution from the bottom was performed according to the principle of hydroponics [19]. The frequency of the salt solution feeding was twice a day for 3 hours. Control plants of the poplars were fed accordingly with Knopp solution (figure 1).

![Figure 1. General view of the studied variety samples of poplar after salt stress (a) and without salt stress – control samples (b).](image)

The fourth leaf of the apical shot was used for anatomical, physiological and biochemical studies. It corresponds to 80% of the maximum leaf area of adult leaf and high photosynthetic activity per unit of
leaf area [20].

For measuring the leaf area of each poplar genotype an improved measurement method using a digital camera was used [21].

Specific surface density of the leaf (SSD) which characterizes the ratio of the dry mass of the leaf to its area was determined by weighing each of the 10 even-aged leaves of the apical shoot separately. To get absolutely dry weight, the leaves, after measuring their area, were dried in a drying cabinet of the brand “Binder” (Germany) at 100 ºC.

Anatomical studies were carried out on even-aged leaves of the apical shoot. The cuts were examined on microsamples fixed in glycerol under a light microscope of the brand Variant Jenamed (Carl Zeiss Jena, Germany). The repetition of measurements for each experience option was at least 30 times.

The content of free proline in the green mass of the leaves in this study was determined by direct spectrophotometry. Sample preparation was carried out using an acid ninhydrin reagent (containing 30 ml of glacial acetic acid and 20 ml of 6M orthophosphoric acid, as well as 1.25 g of ninhydrin) according to the method of Bates et al. [22].

A portion of fresh plant leaf tissue (200 mg) was homogenized in a porcelain mortar by adding 2 ml of 3% sulfosalicylic acid, then the homogenate was transferred into test tubes and 1 ml of glacial acetic acid and 1 ml of ninhydrin reagent were added to it. The samples were incubated for 1 hour in a boiling water bath, then rapidly cooled in ice. The color intensity was determined spectrophotometrically at a wavelength of 520 nm on an SF-3 spectrophotometer (Russia). The values of the content of proline were calculated by calibration curve, using the proline from the Serva company for its construction. The determination of the content of proline was performed in 2 biological and 2 analytical replicates.

The obtained data were processed by statistical methods using the software Statistica. For most traits the error of arithmetic means was 5-10%.

3. Results and discussion

Figure 2 shows the results of water content in leaves of annual shoots in various poplar variety samples under influence of short-term salt stress. All studied experimental genotypes of poplars showed the expected decrease of water content in their leaves. However, the degree of water loss in the balsam poplars sample ‘Kitayskiy’ was significantly higher than in other genotypes. The percentage of water content in his leaf tissue was only 51%, which was significantly lower compared to that in control plants – 79%. A comparative analysis of four other varieties of poplar did not reveal significant differences between the control and experimental plants, while water content was about 73-82%.

It is known that plants in saline solution or with insufficient irrigation show weak dehydration, as evidenced by a lower water potential due to more difficult water absorption from the substrate or less available water in the substrate [23, 24].

A similar decrease in water potential under salinization conditions was also observed in lemon trees and in many other plant species [23, 25]. Herewith, the effect of salinization is first perceived by the root system, causing osmotic stress and reducing the availability of water [12].

In general, salt stress causes decrease in an aboveground part of the plant and primarily in the leaf apparatus. This reduction in growth can be considered as a mechanism of minimization of water loss during transpiration.

Figure 3 presents the results of the salt stress influence on the specific surface density of the leaf (SSD) of annual poplar plants. All five genotypes of poplars revealed significant differences in the value of SSD between control and experimental plants. The most significant differences in SSD were observed in balsam poplar ‘Kitayskiy’ and the intersectional hybrid ‘Hybrid e.s.-38’ (they differed by 60% and 47%, respectively).

The balsam poplar ‘Volostistoplodny’ and the Euro-American hybrid ‘Sacrau-59’ only slightly increased the values of SSD in salt stress effect compared to control plants from 2.7 ± 0.22 to 3.1 ±
0.16 and from 3.2 ± 0.24 to 3.9 ± 0.18 mg · cm⁻², respectively. The middle position on the analyzed trait is occupied by the white poplar variety ‘Veduga’. According to Fernández-García et al. [26], who studied henna plants, the ratio of leaf mass to leaf area is a key trait of plant growth and an important indicator of adaptation to salt stress.

**Figure 2.** Leaf water content of annual shoots in various poplar variety samples under salt stress effect (April 2018).

**Figure 3.** Effect of salt stress on the specific surface density of the leaf (SSD) of annual poplar shoots (April 2018).

Since the value of the specific surface density of the leaf is characterized as an integral indicator of the mesostructure of the leaf, it is interesting to analyze its anatomical characteristics of the five poplars genotypes under the effect of salt stress (table 2). The studying of changes in leaf anatomy is an appropriate way to research into abiotic stress situations, including salt stress [2].

A comparative analysis of the leaf thickness of poplar plants showed a significant decrease in the analyzed trait in two genotypes ‘Hybrid e.s.-38’ and poplar ‘Kitayskiy’. Fernandez-García et al. [26] studied the ratio of leaf mass to leaf area in henna plants during prolonged (30 days) salinization. They noticed that high salt levels increased leaf thickness and suggested that the increase in leaf thickness and therefore in SSD in salt-stressed henna plants may imply a greater investment in assimilatory tissue as a strategy to maximise the photosynthesis potential. However, in our research, we studied the
effect of short-term salinization, in which the assimilation apparatus affected a significant water loss, which led to increasing of the SSD. At the same time, the short-term effect of salt stress reduced the size of spongy and palisade mesophyll cells, which, apparently, had a significant influence on the size of leaf thickness. In addition, a decrease in the size of the upper and lower epidermis of genotypes ‘Volosistoplodny’ and ‘Kitayskiy’ was revealed under the salt stress effect. However, a longer effect of salt stress on leaf anatomical traits led to the opposite effect. Thus, the salt-tolerant species Atriplex patula, irrigated by various NaCl solutions, showed a greater thickness of the leaf due to the increase in the thickness of epidermis and mesophyll [27]. According to other researchers, in salted Citrus L. plants, an increase of leaf thickness in combination with several metabolic components, such as chloride overload, low contents of Mg\(^{2+}\), closure of stomata, and loss of chlorophyll, may be a reason of decrease in photosynthesis among other factors [28].

Table 2. Leaf anatomical traits of five poplar genotypes under saline effect.

| Option          | Leaf thickness, \(\mu m\) | Cell size, \(\mu m\) | Upper epidermis, \(\mu m\) | Lower epidermis, \(\mu m\) |
|-----------------|---------------------------|----------------------|-----------------------------|-----------------------------|
|                 |                           | Palisade mesophyll   | Spongy mesophyll            |                             |
| ‘Sacrau-59’     | Ctrl 175.0 ± 4.00         | 77.1 ± 2.56          | 64.1 ± 2.51                 | 17.6 ± 0.57                 | 16.1 ± 0.68                 |
|                 | Exp 166.2 ± 1.66          | 67.6 ± 1.19*         | 64.7 ± 1.28                 | 18.3 ± 0.25                 | 15.6 ± 0.43                 |
| ‘Volostiplodny’ | Ctrl 167.8 ± 3.0          | 60.7 ± 1.10          | 75.9 ± 2.39                 | 16.1 ± 0.41                 | 15.2 ± 0.28                 |
|                 | Exp 163.5 ± 3.7           | 59.2 ± 2.25          | 72.0 ± 2.85                 | 16.3 ± 0.44                 | 16.0 ± 0.59                 |
| ‘Veduga’        | Ctrl 81.7 ± 1.45          | 31.7 ± 0.51          | 24.6 ± 1.11                 | 14.0 ± 0.25                 | 11.5 ± 0.27                 |
|                 | Exp 78.0 ± 0.96           | 31.3 ± 0.62          | 23.9 ± 0.91                 | 12.9 ± 0.32*                | 9.8 ± 0.18*                 |
| ‘Hybrid e.s.-38’| Ctrl 170.4 ± 2.01         | 69.7 ± 1.27          | 67.0 ± 1.64                 | 15.7 ± 0.42                 | 18.0 ± 0.48                 |
|                 | Exp 150.2 ± 2.21*         | 55.8 ± 0.93*         | 61.2 ± 2.21*                | 15.3 ± 0.27                 | 17.8 ± 0.40                 |
| ‘Kitayskiy’     | Ctrl 144 ± 2.09           | 55.9 ± 0.74          | 58.6 ± 1.52                 | 15.3 ± 0.33                 | 14.1 ± 0.25                 |
|                 | Exp 120 ± 3.06*           | 47.1 ± 2.05*         | 46.6 ± 1.17*                | 14.2 ± 0.25*                | 12.1 ± 0.30*                |

Ctrl – control plants, Exp – experimental plants (under salt stress)
* reliably at \(t>t_{a}=2.1\) at a significance level of 0.95

Figure 4 shows the changes of a leaf anatomical structure of two poplars forms belonging to different salt resistance groups. The genotype ‘Kitayskiy’ showed a clear picture of a decrease of the leaf anatomical traits, which is apparently due to their lower resistance to extreme salinity (figure 4d). Another representative of the balsam poplars ‘Volostiplodny’ did not show anatomical rearrangements of the leaf structure in saline stress conditions, and is characterized as more stable form of poplar (figure 4b).

Thus, a comparative analysis of the morphometric and anatomical leaf traits of the studied poplars showed that the balsam poplar ‘Kitayskiy’ was the least resistant to the short-term effects of extreme environmental factors. The intersectional hybrid ‘Hybrid e.s.-38’ is intermediate among the analyzed poplars. Greater resistance is characteristic of the three forms of poplars ‘Volostiplodny’, ‘Sacrau-59’ and ‘Veduga’, which makes it possible to recommend their use in reforestation and afforestation on soils exposed to salinization.

The results of measuring the content of free proline in poplar control and saline stress samples show an unambiguous trend: as a result of salt stress, the concentration of proline increases, but the quantitative characteristics of this process vary in different species and varieties (figure 5). The initial levels of proline observed in control samples vary slightly in different varieties and species. At the same time, the increase in the concentration of proline after the exposure of saline solution is different for the studied representatives: for the sample ‘Sacrau-59’, the concentration of proline in the test sample increases in 2.1 times, compared to the control, balsam poplar ‘Volostiplodny’ – in 1.2 times, in the variety Veduga – 6.4 times, in the hybrid ‘Hybrid e.s.-38’ – 4 times, in the form of the poplar ‘Kitayskiy’ – 2.6 times. Thus, the obtained data showed that the most intense reaction to salt stress in terms of changes in the concentration of proline was shown by the
samples ‘Veduga’ and ‘Hybrid e.s.-38’.

Figure 4. Effect of salt stress on the leaf anatomy of various poplar varieties:
a - ‘Volosistoplodny’ control plants, b - ‘Volosistoplodny’ experimental plants, c - ‘Kitayskiy’ control plants, d - ‘Kitayskiy’ experimental plants.

Figure 5. Proline levels in control and salt-stressed poplar samples.

Other studies confirm the trend we have identified. Thus, in experiments evaluating the effect of salt stress on the concentration of osmolytes conducted on aspen, it was found that the effect of a solution of 150 mM NaCl led to a significant increase in the endogenous level of free proline after 7 days of experiment (an increase of 2-2.5 times was recorded) [16].

A study of the effects of salt stress on callus cultures of Euphrates poplar showed that after 15 days an increase in proline levels by 4-8 times was observed in different salt solutions (from 50 to 250 mmol^{-1}NaCl) [17].
A strong reaction to stress, characterized by a significant change in the level of biochemical markers, may indicate the sensitivity of the species or varieties to the studied stress factor. On the other hand, enhanced synthesis and accumulation of substances such as proline is a defensive reaction, and plant’s ability to respond to salt stress by increasing its concentration indicates adaptive plasticity, allowing it to adapt to extreme environmental conditions.

Thus, the results of biochemical studies of the proline content in poplar leaves under the effect of short-term salinity allow us to characterize the intersectional hybrid ‘Hybrid e.s.-38’ and the white poplar variety ‘Veduga’ as more stable and plastic forms.

4. Conclusion
The stressful effect of short-term salinization on various genotypes of poplars activates physiological and biochemical adaptation mechanisms. Short-term salt stress causes changes in the leaf anatomy. Thus, the less stable genotype of poplar ‘Kitayskiy’ responds by reducing the leaf thickness. Anatomical changes in the leaves of the experimental plants also include a decrease of palisade and spongy parenchyma cells height, which leads to the reduction of the assimilation surface area and unproductive water loss. Comparison of proline concentration in control and experimental plants showed that all of the studied samples increase proline accumulation in response to salinization, but the most intense reaction to salt stress was shown by the samples ‘Veduga’ and ‘Hybrid e.s.-38’. In this case, an increase in endogenous proline levels can be regarded as an adaptive reaction. In general, greater stability and plasticity in the initial stage of salinization is characteristic of the poplars ‘Volosistoplodny’, ‘Sacrau-59’, ‘Hybrid e.s.-38’ and ‘Veduga’, which allows us to recommend them for reforestation and afforestation on irrigated soils.

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