



Increased adaptation of an energy willow cultivar to soil salinity by duplication of its genome size

András Cseri^{a,*}, Péter Borbély^b, Péter Poór^b, Attila Fehér^b, László Sass^a, Mihály Jancsó^c, András Penczi^c, Feríz Rádi^{a,d}, Csaba Gyuricza^c, Tamás Digruber^a, Dénes Dudits^a

^a Institute of Plant Biology, Biological Research Centre, Temesvári Krt. 62, Szeged, 6726, Hungary

^b Department of Plant Biology, University of Szeged, Közép Fásor 52, Szeged, 6726, Hungary

^c National Agricultural Research and Innovation Centre, Anna-liget 35, Szarvas, 5540, Hungary

^d School in Biology of University of Szeged, Hungary

ARTICLE INFO

Keywords:

Autotetraploidy
Saline soil
Digital imaging
Green biomass
Root biomass
CO₂ assimilation rate

ABSTRACT

Soil salinity can limit the use of marginal lands for biomass production based on cultivation of short-rotation woody crops as energy willow. Here, we compare salt stress responses of the diploid, productive cultivar (Energó), and its artificially produced autotetraploid (PPE-2; PPE-7; PPE-13) variants. After pre-testing the effects of various salt concentrations, willow plants with different genome sizes were exposed to 1.5 g NaCl kg⁻¹ soil (electrical conductivity (EC) value: 7.04 mS/cm). Digital imaging of shoot surface area (green pixel) and root surface (white pixel) indicated variable improvements in growth responses of tetraploids relative to diploid ones in saline soils. After nine weeks of salt stress, increase in salt adaptation capability of tetraploid plants was indicated by larger biomass, leaf and root weights under salinity (1.5 g NaCl kg⁻¹ soil) relative to diploids. Biomass weights were significantly higher in the case of tetraploid PPE-2 plants with increased water consumption and leaf water content than of diploid plants. The inhibitory effect of salt stress on photosynthetic assimilation rates was less significant in plants with doubled genome. The Na⁺ accumulation was reduced in leaves of tetraploids and increased in their roots, while the K⁺ ion content was higher in their leaves than in diploids. Tetraploidy improved K⁺/Na⁺ ratio in leaves and roots of willow plants under normal soil condition. This parameter was less reduced in tetraploid leaves exposed to salt stress. The described tetraploid energy willow genotypes with salt tolerance can play a role in the extended use of green energy.

1. Introduction

The use of biomass from the short rotation woody crops, including willow (*Salix* spp.), as renewable energy sources is gaining increasing significance worldwide. Establishment of shrub willow plantations on unproductive lands that are marginal for annual crop production offers an opportunity to realize economic and environmental benefits. Soil salinization is one of the most damaging environmental factors limiting plant productivity. It has been estimated that 20% of total cultivated and 33% of irrigated agricultural lands are affected by high salinity [1]. As reviewed by Mirck and Zalesny [2] the root zone salinity (EC_e: 3–5 dS m⁻¹) can cause 50% yield reduction in the case of willows and poplars. Salt tolerance of willow genotypes is one of the key adaptive traits influencing biomass yield and it can reduce the risk of waterlogging and salinization.

As reviewed by Munns and Gilliam [3], adaptive mechanisms of salt tolerance can function at the cellular as well as tissue or organ levels. Salt load in the soil can reduce energy gain through photosynthesis, and the protective mechanisms generate additional costs for the crop. Considerable salt tolerance of the diploid (2n = 38) *S. interior* plants was revealed by comparison of the survival rate, the biomass yield, and biochemical efficiency parameters for willow plants from three species - *Salix discolor*, *S. eriocephala* and *S. interior*-grown under medium and high salinity [4]. Identification of salt-tolerant variants can be based on the existing genetic variability represented by the collection of willow varieties. A comparison of the relative growth response of 37 native and exotic willow varieties grown in saline soils revealed resistant cultivars for the revitalization of unproductive agricultural lands [5]. The complexity of genetic control of salt responses was shown by identification of more than 200 QTLs in 15 growth-related traits of 195 F1

* Corresponding author.

E-mail address: cseri.andras@brc.hu (A. Cseri).

<https://doi.org/10.1016/j.biombioe.2020.105655>

Received 24 October 2019; Received in revised form 21 April 2020; Accepted 14 June 2020

Available online 11 July 2020

0961-9534/© 2020 The Authors.

Published by Elsevier Ltd.

This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

hybrid offspring of salt-sensitive *S. matsudana* ‘Yanjiang’ × salt-tolerant *S. matsudana* ‘9901’ [6]. As in the case of different agronomic traits, breeding for salt adaptation capability can be based on a variety of techniques as reviewed by Lindegaard and Barker [7] and Karp et al. [8]. Natural and artificial crossings of *Salix* spp. can result in a significant increase in genome size that can be an important source of genetic variability. Allopolyploid triploids from the *S. koriyanagi* × *S. purpurea* × *S. miyabeana* crossing were shown to improve biomass yield [9].

As traditional plant breeding approach, previously we have produced autotetraploid willow plants with $2n = 4x = 76$ chromosomes from the highly productive energy willow cultivar ‘Energo’ (*S. viminalis*). The detailed characterization of these polyploid (PP-E) plants revealed several altered traits that can influence salt stress responses [10]. Recording phenotypic traits showed enlargement of leaves with bigger palisade parenchyma cells. Leaves of these autotetraploid energy willow plants showed higher net photosynthetic CO₂ uptake, and an increased electron transfer rate of PSI and PSII. Tetraploid willow plants transpired more water, as it was shown by the elevated stomatal conductance in leaves. The duplication of the genome size significantly stimulated the root development. Since all these parameters can affect the salt stress reactions of tetraploids, we were urged to characterize the salinity tolerance of these new polyploid genotypes. Our studies were stimulated by publications from Meng et al. [11] and Luo et al. [12] showing improved salt tolerance of tetraploid black locust (*Robinia pseudoacacia* L.) as forest species. Furthermore, in *Paulownia fortune*, polyploidization affected the salt-responsive proteome dynamics patterns, especially the group of ion channel proteins. The autotetraploid seedlings exhibited more salt-responsive proteins than the diploid ones [13].

Postulating positive influence of genome duplication on the stress adaptation potential, we have quantified the relative growth response of diploid and tetraploid willow plants to the inhibitory concentrations of sodium chloride (NaCl kg⁻¹ soil) mixed to soil in plexiglass cylinders. We used digital images of green tissues and roots, and organ weight data as phenotypic markers. We hypothesized that physiological functions can also underline an improved salt adaptation, therefore we have characterized photosynthetic parameters, as well as starch and soluble sugar contents in leaves. Since an optimal K⁺ and Na⁺ ion homeostasis is required for salt tolerance, this physiological status was also used to show improved salt adaptation potential.

2. Materials and methods

2.1. Plant material

Plants of the *Salix viminalis* ‘Energo’ cultivar served as diploid reference genotype with $2n = 2x = 38$ chromosomes. A set of autotetraploid (polyploid Energo [PPE]; $2n = 4x = 76$) genotypes were generated by the following procedure: shoot apical meristems of 8- to 10-cm plantlets were decapitated, and 48 h later, stem sections with axillary buds were placed into sterile colchicine solution (0.05% or 0.1% [w/v]) and incubated for 48 h in dark. Two to 3-cm-long shoots grown from the treated axillary buds were cut and placed in agar-solidified culture medium and used for further *in vitro* propagation. Determination of ploidy levels was conducted by flow cytometry and the genome duplication results were confirmed by chromosome counting using fluorescence microscopy. The detailed characterization of these genotypes in comparison to the Energo plants was published earlier [10].

2.2. Plant growth conditions and salt stress treatment

To test salt tolerance of willow genotypes, we used a semi-automated phenotyping platform called complex stress diagnostic system [14]. In winter, one-year-old dormant stems from field-grown willow plants were collected and used for production of 20 cm cuttings. This propagation material was harvested from a five-year-old plantation after

polyploidization, and planted into radio-tagged plexiglass columns with a mixture of 80% Florimo peat soil and 20% sandy soil. The soil was mixed with crystal 1.5 g NaCl kg⁻¹ soil. The electrical conductivity measurements were performed using Orion 4 Star pH-Conductivity benchtop (Thermo Electron Corporation Waltham, MA, USA). Before planting, cuttings were soaked in water for 48 h. At the time of potting, the cuttings were watered with 150 mL per pot to ensure the adaptation of plants. Five plexiglass columns surrounded with polyvinyl chloride tubing were placed on a metal rack. Two racks (10 plants) were used for each treatment and genotype combination with a random arrangement. The growth temperature was in the greenhouse 21 °C. The level of illumination in the greenhouse was approximately 400 mmol photons m⁻² s⁻¹.

2.3. Biomass prediction by digital imaging and organ weight measurements

Quantification of the shoot and root parameters was carried out by using the same protocols as described by Dudits et al. [10]. Previously we showed good correlation ($R^2 = 0.7556$) between pixel numbers and actual organ weight values [15]. Watering and digital imaging of shoot and root system were performed once a week. The analyzed plants were grown under optimal water supply with 60% soil water content throughout the experiment. Water consumption was automatically recorded by the computer program. For imaging, willow shoots were photographed with an Olympus C-7070WZ digital camera from seven different sideways positions, produced by 51.4° step rotation of a pot. In the case shoot (green pixels) we predicted biomass from average of seven different sideways positions. In the case of roots, the plexiglass columns were photographed from four different side positions and from the bottom. The root-related white pixels were identified by subtracting the black soil background from the images. Pixel numbers were converted to millimetres using 65-mm diameter pots captured in the images. To characterize the root area appearing at the surface of the chamber, the metric values of the area of the four side view projections (90° rotation) was summarized and the metric value of the area of the bottom view was added. We used the in house-developed image-analysis software tool to segment green and white pixels [10].

After completion of the nine-week phenotyping experiment, weights of aboveground biomass, separated into leaves and shoots were determined from eight plants/combination. The rooted stems were removed from the soil and the roots were separated from the soil. Root weights were measured immediately after removal from the soil to generate wet weight data.

2.4. Photosynthetic measurements

Stomatal conductance (g_{sw} ; mol H₂O m⁻² s⁻¹) and CO₂ assimilation rate (A_N ; μmol CO₂ m⁻² s⁻¹) were monitored on young, fully expanded leaves with a portable photosynthesis system (LI-6400, LI-COR, Inc., Lincoln, NE, USA), according to Poór et al. [16] with environmental CO₂ source. CO₂ concentration was kept at 400 ppm by soda lime reagent (Sigma-Aldrich ACS reagent. Two cm² areas of a leaf were measured with the controlled CO₂ flow at the concentration of 300 μmol mol⁻¹ under greenhouse conditions at 25 °C.

2.5. Carbohydrate analysis

Carbohydrate (soluble sugars and starch) concentration was determined according to Hansen and Møller [17]. Briefly, 100 mg (FW) of leaf tissues were ground in liquid nitrogen, then soluble sugars were extracted with 1 mL of 80% ethanol at 80 °C for 30 min. The homogenate was centrifuged at 2600 g for 10 min. After a second extraction step, the joined supernatants were used for determination of soluble sugar content at 630 nm after reaction with anthrone (Normapur, VWR Int., Leuven, Belgium) dissolved in 72% sulphuric acid, using glucose

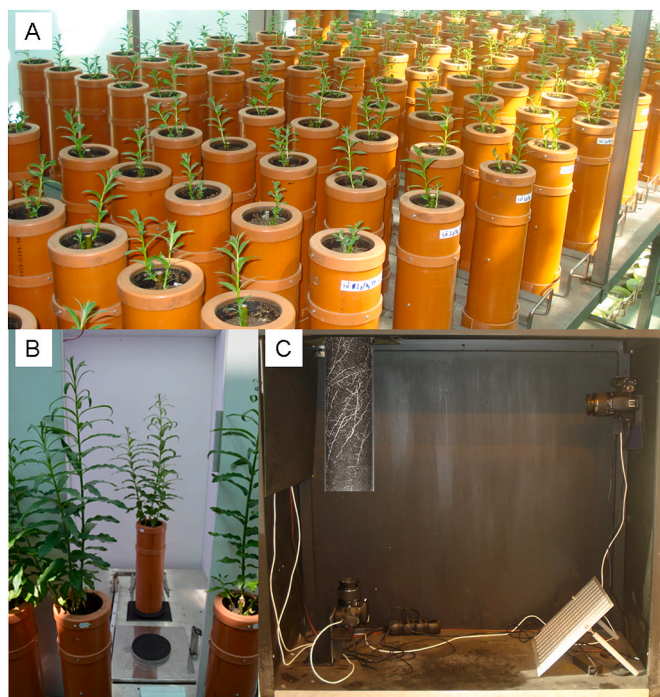


Fig. 1. Energy willow plants grown in soil, filled into plexiglass columns surrounded with polyvinyl chloride tubing under greenhouse conditions (A). Digital imaging of shoots (B) and digital imaging of roots (C) in the semi-automatic phenotyping platform.

(Normapur, VWR Int., Leuven, Belgium) dissolved in 80% ethanol as a standard. The remaining pellet was washed with 1 mL of deionized water, then hydrolyzed by 1 mL of 1.1% HCl at 100 °C for 30 min, and centrifuged for 10 min at 2600 g. Starch concentration was also evaluated using an Uvicon 930 spectrophotometer (KOTRON, Milano, Italy) at 630 nm with anthrone reagent, using starch (Normapur, VWR Int.,

Leuven, Belgium) dissolved in 1.1% HCl as a standard.

2.6. Determination of sodium and potassium by flame atomic absorption spectrometry

The sodium and potassium content of plant samples were analyzed by the Hungarian standard method (MSZ 08 1783–5:1983 Use of high capacity equipments in plant analyses—Quantitative determination of potassium and sodium content of plant materials) using Agilent 240FS Atomic Absorption Spectrometer [18]. For the sample preparation, plants were oven-dried for 24 h at 103 °C. Then a digestion procedure based on the solutions of 6 mL HNO₃ (65%) and 4 mL H₂O₂ (30%) was applied. The digestion was applied for 10 min at 180 °C using a microwave digestion system (Ethos Plus, Milestone). As the last step of sample preparation, 0.03 mol dm⁻³ lithium chloride solution was added to the digested sample solutions and to the standards as ionization buffer. The amount of sodium and potassium in the plant tissues was analyzed by the flame emission method at 589 nm (Na) and 766.5 nm (K).

2.7. Statistical analyses

Statistical analyses were executed by Sigma Plot 11.0 software (Systat Software Inc., Erkrath, Germany) applying one-way analysis of variance (ANOVA). The salt tolerance of different genotypes was compared with the aid of several parameters (previously mentioned), which are appropriate markers for salt adaptation. There was significant difference between group means, therefore after ANOVA, posthoc Duncan's multiple comparisons test was performed, and differences were considered significant if $P \leq 0.05$. Duncan's test minimizes the possibility of type II errors, therefore it is more sensitive to true differences between the examined groups, than other post hoc tests. The number of samples used in each group is given in the caption of each respective Figure as 'n', every sample corresponds to an individual cutting.

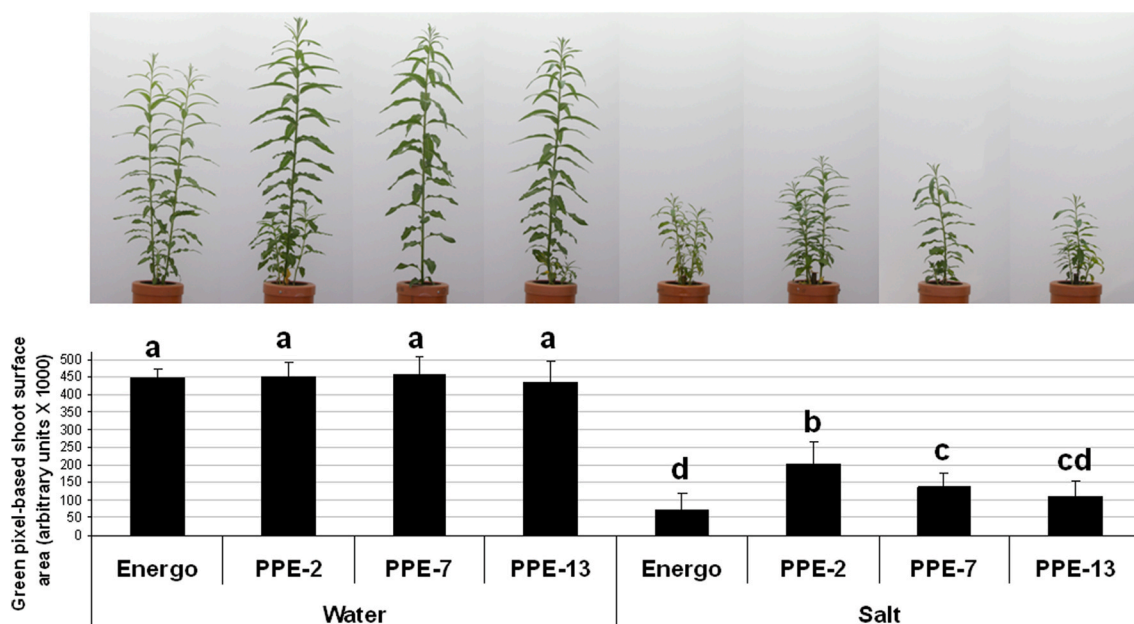


Fig. 2. Autotetraploid energy willow plants (PPE-2; PPE-7; PPE-13) show an improved biomass production in comparison to the diploid Energo plants under salt stress generated by 1.5 g NaCl kg⁻¹ soil. Genotype-dependent variation in shoot phenotypes (A) and in the calculated green pixel-based shoot surface area values (B). Average green pixel data are presented from eight plants at the nine-week time point. Results are means ± SD, n = 8. Values denoted with different letters show significant differences at $P \leq 0.05$ level (Duncan's test). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

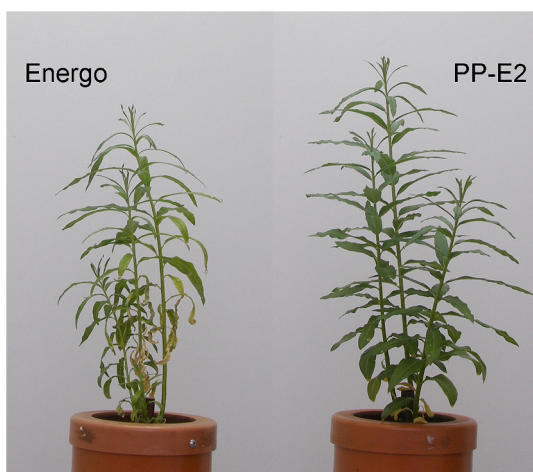


Fig. 3. Representative picture about the tetraploid plant (PPE-2) that developed healthy leaves in contrast to severely damaged leaves of the diploid Energó cultivar after cultivation for eight weeks in soil containing 1.5 g NaCl kg⁻¹ soil (EC:7.04 mS/cm).

3. Results and discussion

3.1. Phenotyping of salt stress adaptation of diploid and tetraploid willow plants by digital imaging of shoot or root surface area under greenhouse conditions

The semi-automatic stress diagnostic systems presented in Fig. 1

generated green or white pixel-based relative quantitative data for characterizing salt stress responses of willow plants with different genome size. After pre-testing various doses of NaCl (1.0–1.5–2.0 g/kg soil) for the detailed characterization of the salt stress adaptation potential, willow plants were grown under salinity stress induced by 1.5 g NaCl kg⁻¹ soil (EC: 7.04 mS/cm) in the further experiments. This value can be considered as high salinity treatment compared to other studies on willow species [4]. Fig. 2 shows representative pictures of willow shoots from the different genotypes and presents biomass values calculated on the bases of imaged green pixel numbers after nine weeks in control or NaCl containing soil. All tetraploid variants represent better growth performance than the reference diploid Energó plants with statistically significant values in the case of PPE-2 and PPE-7 plants. In addition to the growth reduction, in soil containing 1.5 g NaCl kg⁻¹ soil severe symptoms could be recognized on leaves of the Energó plants with large sectors of necrotic tissues, while willow plants with doubled genome size maintained formation of healthy leaves (Fig. 3). Even at a higher dose of NaCl (3 g NaCl kg⁻¹ soil), the Energó plants completely died, while the tetraploids could survive (data not shown). Similar differences of leaf symptoms were observed between diploid and tetraploid black locust (*Robinia pseudoacacia L.*) potted plants after treatment with 250 mM (moderate salinity stress) or 500 mM NaCl (high salinity stress) for 7 days [11,12]. However, the tetraploid leaves showed no serious damage in soil 500 mM NaCl, they maintained mitochondrial structure and triggered different expression patterns of mitochondrial proteins during salinity stress. Meng et al. [11] reported increased H₂O₂ accumulation and simultaneously higher levels of antioxidative enzymes and non-enzymatic antioxidants in tetraploid plants compared with diploid plants under salt stress. In a different experimental system, diploid and

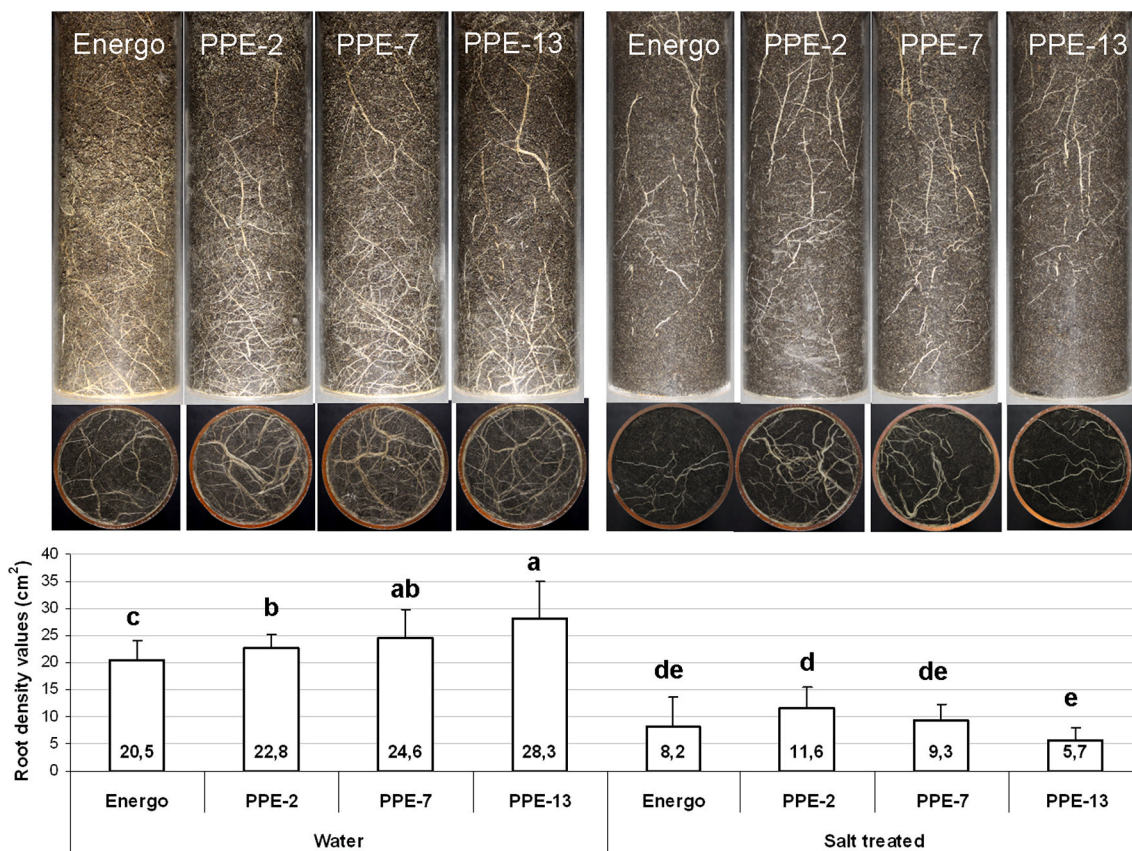


Fig. 4. Autotetraploid energy willow plants (PPE-2; PPE-7; PPE-13) show larger root biomass in the control, water-treated soil. Reduction in the root systems is shown by photos under salt stress of 1.5 g NaCl kg⁻¹ soil (A). The calculated root density values show moderated tolerance for the PPE-2; PPE-7 plants (B). Average root density data are presented from eight plants after a four-week growing period. Results are means ± SD, n = 10. Values denoted with different letters show significant differences at P ≤ 0.05 level (Duncan's test).

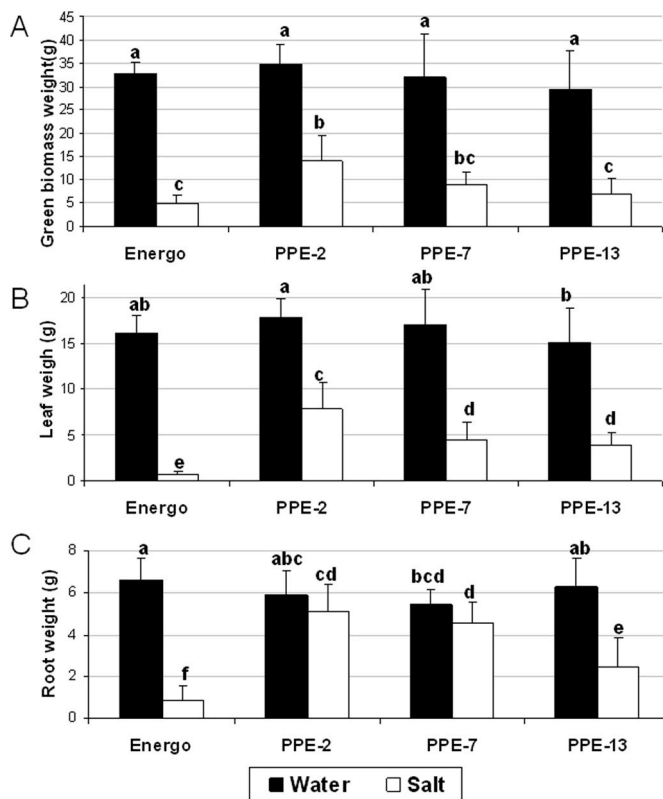


Fig. 5. Variability in improved salt stress tolerance shown by biomass and organ weight data in case of the autotetraploid energy willow plants (PPE-2; PPE-7; PPE-13) in comparing to the diploid plants. (A): above ground biomass of willow plants from different genotypes. (B): leaf weight data indicate salt stress tolerance of tetraploids (C): larger root system of tetraploid plants reflects better salt stress adaptation. Weight data were measured from eight plants at the end of the nine-week cultivation period in soil treated with water or supplemented with 1.5 g NaCl kg⁻¹ soil. Results are means ± SD, n = 8. Values denoted with different letters show significant differences at P ≤ 0.05 level (Duncan's test).

autotetraploid apple (*Malus domestica*) seedlings were treated with 200 mM NaCl for 8 days in 0.5 × Hoagland's nutrient solution. Symptoms of leaf chlorosis and necrosis appeared earlier in the diploid than in tetraploid plants. In addition, the authors recorded higher levels of expression of aquaporin genes in response to salt stress in autotetraploid apples [19].

The stress diagnostic system provided white pixel data for characterization of root growth responses of diploid and tetraploid willow plants in salty soil (Fig. 4). Under normal growing conditions, the tetraploid plants developed enlarged root system as published previously [10]. The salinity stress produced by 1.5 g NaCl kg⁻¹ soil (EC:7.04 mS/cm) retarded root growth in plants from all genotypes, but plants from the two tetraploid lines (PPE-2 and PPE-7) showed better adaptation potential relative to the diploid ones. In the case of PPE-2 plants, the predicted root biomass was reduced only by 50% relative to the water-treated control plants.

3.2. Doubling the genome size of energy willow plants can improve salt stress adaptation reflected by organ size and biomass productivity

Phenotyping with imaging parameters allows only comparative analysis of traits. Biomass prediction of shoots and roots is limited since the pixel values cannot represent the whole organ size, especially in the case of root tissues. Photos are taken only from roots growing at the surface of plexiglass columns. Therefore, the organ biomass from different lines was also quantified at the end of the nine-week cultivation

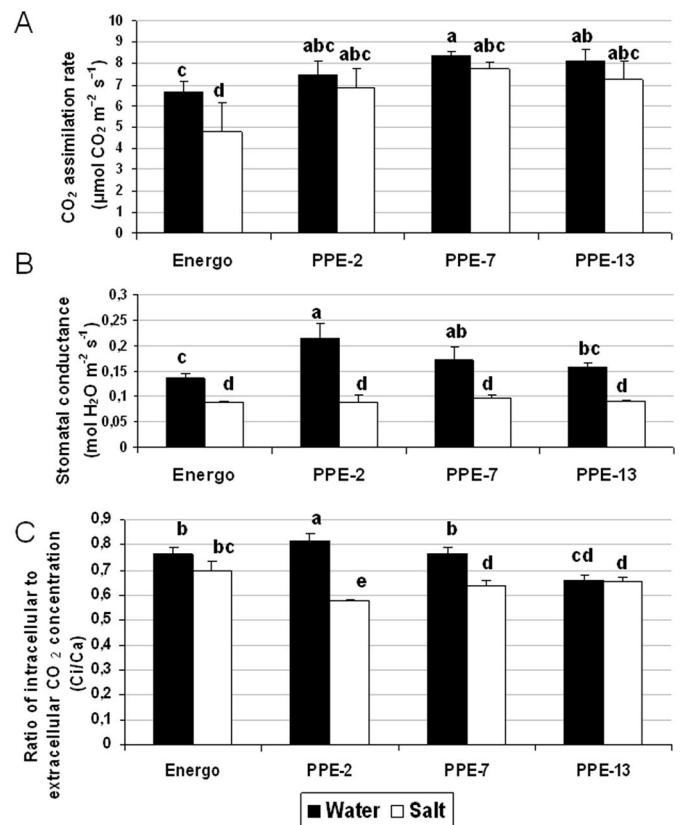


Fig. 6. Photosynthetic parameters indicated improved functionality of polyploid plants. A: CO₂ assimilation rate (A_N ; $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), B: Stomatal conductance to water vapour (g_{SW}) and C: ratio of intracellular to extracellular CO₂ concentration (C_i/C_a) of diploid energy willow cultivar Energo and autotetraploid variants in the presence or absence of 1.5 g/kg soil NaCl. Results are means ± SD, n = 3. Values denoted with different letters show significant differences at P ≤ 0.05 level (Duncan's test).

period in soil supplemented with 1.5 g NaCl kg⁻¹ soil (Fig. 5). As shown by Fig. 5/A, the diploid Energo plants produced only 15.1% of green biomass in comparison to the control, water-treated plants. The tetraploid plants were also inhibited by the presence of NaCl, but to a lesser extent. The tetraploid PPE-2 plants developed three-times more biomass than the diploid Energo plants. This increase in the level of biomass production can have significance in energy crop production in salty soils, especially since here we applied an artificially high dose of salt (EC: 7.04 mS/cm) if we consider the natural situation. In several regions of Hungary, EC: 0.1–0.2 mS/cm values represent the salt contamination [20]. Plants from the PPE-7 tetraploid genotype could also more efficiently adapt to this stress, but with lower productivity than plants from the PPE-2 line. In biomass yield, the leaf parameters play a key role, not only as main components in biomass but also with maintaining functionality (see later). As shown by Fig. 5/B, the Energo plants lost their leaves after the long salt stress exposure, while in the case of tetraploid plants these damages were much more moderated. The diploid plants showed drastic damage to the development of the root system (Fig. 5/C). In this parameter the tetraploid plants (PPE-2 and PPE-7) produced root biomass with only a 12–15% reduction. The PPE-13 plants presented a reduced adaptation potential. In spite of the technical limitations in the quantification of root biomass, both the digital imaging and the tissue weight measurements show similar trends in the responses of the different genotypes.

Based on relative total biomass yields of different native and exotic willow varieties grown for 60 days in severely saline (EC: 8.0 mS/cm) soil [5], the diploid Energo plants can be considered as a salt-sensitive variant. Doubling their genome size essentially improved salt stress

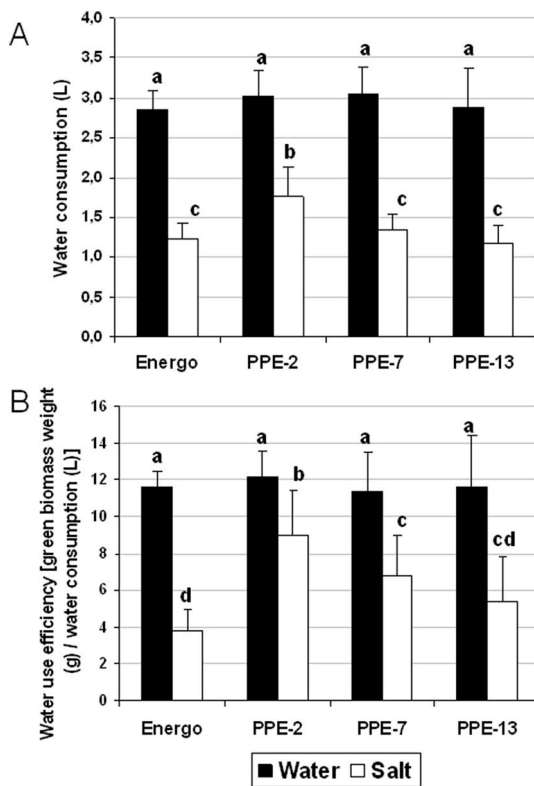


Fig. 7. Variation of water consumption (A) and water use efficiency (B) of diploid and tetraploid genotypes under normal and salt stress conditions (1.5 g NaCl kg⁻¹ soil) after nine weeks. Results are means \pm SD, n = 8. Values denoted with different letters show significant differences at $P \leq 0.05$ level (Duncan's test).

tolerance, but with variable levels for the independent tetraploid lines. This observation emphasizes the need to carry out a selection in polyploid plant populations to identify resistant variants. As shown by Fig. 5, the better salt tolerance of PPE-2 and PPE-7 plants was realized at a higher degree in the root system than in shoot biomass weights. This differential response of underground and aboveground organs to salinity can be characteristic of the tetraploid willow plants while not seen in the diploid Energio plants. After segregation, the offspring population derived from crossing between *Salix matsudana* salt-sensitive and salt-tolerant species, the frequency distribution of total root length parameters were similarly reduced than the fresh leaf weights in a culture solution containing 100 mM NaCl [6]. Salinity-induced alterations in organ size showed a more complex picture in comparison to diploid and tetraploid *Citrus macrophylla* plants [21]. The moderate (40 mM NaCl) treatment stimulated dry weights of diploids and inhibited tetraploid plants, while under high salinity (80 mM NaCl) for 30 days tetraploid plants were found to be more tolerant.

3.3. Improved photosynthetic functions and water status in tetraploid willow leaves under salt stress condition

High salinity emits a mainly dual stress effect onto plants, which consists of an osmotic- and an ionic-component [22]. Salt stress causes stomatal closure [23], which leads to the decrease of photosynthetic efficiency and after prolonged exposure, loss in biomass production [24]. Decreased chlorophyll content [25], inhibition of Calvin-cycle [26], and of the Photosystem II [27] and I [28] can be symptoms of salt stress. In agreement with our previous observation [10] Fig. 6/A shows that the tetraploid plants can exhibit higher photosynthetic efficiency. The prolonged exposure to 1.5 g/kg soil salt significantly decreased CO₂ assimilation rate (A_N ; $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) in diploid

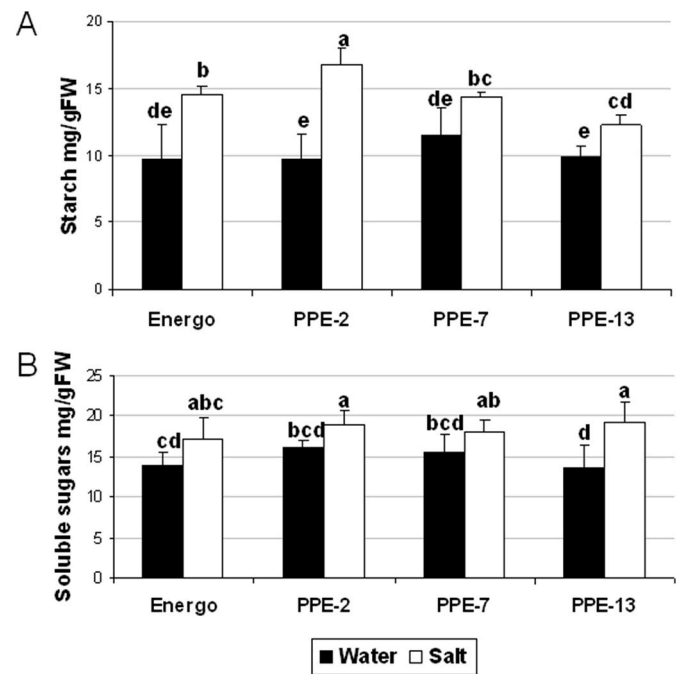


Fig. 8. Starch (A) and soluble sugar (B) content of leaves from the diploid energy willow cultivar Energio and autotetraploid variants in the presence or absence of 1.5 g NaCl kg⁻¹ soil. Results are means \pm SD, n = 3. Values denoted with different letters show significant differences at $P \leq 0.05$ level (Duncan's test).

Energio plants. This effect was absent in autotetraploid plants; furthermore, they showed significantly higher A_N values than salt-treated Energio plants. The stomatal conductance (g_{sw} ; $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) values for the tetraploid plants indicate significantly higher evaporation than in the case of Energio plants (Fig. 6/B). This function was reduced to similar levels under salt exposure in diploid and PP-E genotypes. Under high salinity, the ratio between intracellular and extracellular CO₂ concentration (C_i/C_a) did not change significantly in Energio plants, but in plants from PPE-2 and -7 lines, a significant reduction could be observed during the compared treatment, which was more pronounced in the former (Fig. 6/C). Despite the lower g_{sw} , autotetraploid plants could maintain their photosynthetic activity, which suggests less contribution of non-stomatal photosynthesis-limiting factors in autotetraploid lines.

Variation in salinity tolerance can originate from different capabilities in maintaining ionic homeostasis in the cell cytosol and in establishing water or osmotic homeostasis at both cellular and whole plant level (see reviews: [34–37]). Stomatal closure could help the plant to preserve the available water in an osmotically changed environment [29]. The tetraploid willow plants used in the present study were shown to transpire more water, as indicated by the elevated stomatal conductance values in leaves [10]; (Fig. 6/B). The phenotyping platform allowed to quantify water use for individual plants under control and salinity conditions (Fig. 7/A). The presence of 1.5 g NaCl kg⁻¹ soil reduced the water consumption of willow plants from all genotypes tested. Plants of PPE-2 variant were only capable of ensuring significantly better water status than the diploid ones. A comparison of water use efficiency data indicated significant improvement for both PPE-2 and PPE-7 plants (Fig. 7/B) relative to the diploid Energio plants. This parameter provides the green biomass yield from the use of 1 L water. The better water availability detected in stressed tissues of tetraploid willow plants can also influence both osmotic and ionic homeostasis.

Primary product of photosynthesis is α -glucose and its polymers form starch as insoluble, non-structural carbohydrate. Since starch metabolism can contribute to plant fitness under abiotic stresses and in some

Table 1

Quantitation of Na⁺ (A) and K⁺ (B) contents in leaves and roots of willow plants with different ploidy levels. Results are means ± SD, n = 4. Values denoted with different letters show significant differences at P ≤ 0.05 level (Duncan's test).

A				
Genotype	Leaf Na ⁺ content (g/Kg DW)		Root Na ⁺ content (g/Kg DW)	
	Water	1.5 g/kg NaCl	Water	1.5 g/kg NaCl
Energ	0.35 ± 0.01 e	0.75 ± 0.04 a	4.81 ± 0.94 cd	9.77 ± 1.64 b
PPE-2	0.32 ± 0.01 ef	0.50 ± 0.05 d	3.48 ± 0.61 d	9.86 ± 2.02 b
PPE-7	0.28 ± 0.02 f	0.59 ± 0.09 c	3.60 ± 1.12 d	13.63 ± 2.94 a
PPE-13	0.33 ± 0.02 ef	0.66 ± 0.02 b	3.73 ± 0.66 d	10.62 ± 4.67 c
B				
Genotype	Leaf K ⁺ content (g/Kg DW)		Root K ⁺ content (g/Kg DW)	
	Water	1.5 g/kg NaCl	Water	1.5 g/kg NaCl
Energ	46.17 ± 1.03 a	27.20 ± 2.05 c	25.38 ± 3.76 ab	14.56 ± 4.82 c
PPE-2	48.47 ± 2.87 a	35.11 ± 2.19 b	22.81 ± 3.76 b	12.65 ± 3.03 c
PPE-7	47.49 ± 2.03 a	32.90 ± 2.86 b	26.15 ± 3.76 ab	16.86 ± 5.40 c
PPE-13	47.04 ± 2.66 a	28.61 ± 4.25 c	29.79 ± 3.76 a	10.62 ± 4.67 c

cases starch content increased in response to high salinity [30] we have analyzed this trait (Fig. 8/A). Under prolonged salt stress, an extensive starch accumulation was observed in all genotypes, but only PPE-2 plants showed significant elevation compared to salt-treated Energ plants. However, PPE-13 showed significantly lower starch content at high salinity compared to Energ (Fig. 8/A). Salt treatment caused elevation of soluble sugar levels in leaves of autotetraploid plants compared with salt-treated Energ plants, although only PPE-2 and -13 plants showed significant changes compared with their water-treated plants (Fig. 8/B).

In Energ plants, decreased A_N values are paired with significant starch production. The growth inhibiting effect of salt stress decreases carbon sink strength because of reduced sink tissue growth, and this results in starch accumulation in source tissue [29], which is observed during salt stress in leaves of all energy willow genotypes. Under a steady state condition, the rate of photosynthesis could suffer limitations due to the slowing exchange of cytosolic inorganic phosphate (P_i) with plastidal triose-phosphate. In these circumstances, excess accumulation of photosynthesis in chloroplast and inadequate P_i availability favour internal starch storage at the expense of photosynthetic rate [31]. In

contrast, autotetraploid energy willow variants maintained photosynthetic rate during elevated starch storing under salt stress. The remobilization of starch could have a crucial role to provide carbon to tissues when photosynthesis is suffering limitations. Salinity tolerance mechanisms include the synthesis of compatible solutes (including soluble sugars), which could help maintain optimal water homeostasis of the cells and protect membranes and proteins from harmful stress effects [32]. Although questions as to whether NaCl causes starch accumulation and soluble sugar depletion or vice versa are contradictory, in salt-adapted Eucalyptus shoots, both starch and soluble sugar accumulation were observed [33], as well as in PPE-2. In halophyte common reed (*Phragmites australis*), increased leaf starch production was also detected, but in shoot basis, specific starch grains were synthesized to bind Na⁺, introducing starch as a possible salt tolerance element [34]. However, autotetraploid energy willow variants PPE-2 and 7 exhibited a slower but significant shoot growth rate, healthy water status and good K⁺/Na⁺ (see later) with support from a maintained photosynthetic rate and increased starch and soluble sugar production.

3.4. Alterations in K⁺/Na⁺ contents can be responsible for the better salt stress adaptation of tetraploid willow plants

Since ion homeostasis under salinity stress requires the maintenance of stable K⁺ ion acquisition and distribution to balance the toxic effects of Na⁺ accumulation, we quantified the K⁺ and Na⁺ contents in the leaf and root tissues of willow plants grown under control and stressed conditions (Table 1). Under normal growing conditions, the diploid Energ and the tetraploid plants accumulated similar amounts of Na⁺ in their leaves, except PPE-7 plants showing significantly lower value. The presence of 1.5 g/kg NaCl in the soil increased Na⁺ contents in leaves by 1.74–2.22 times relative to the control, water treated leaves. In this parameter the lowest increase was detected in the case of PPE-2 leaves. Na⁺ content values were lower in the untreated roots of tetraploids than in roots of Energ plants. These differences were not significant. The salt stress resulted in 2.0–4.5 times higher Na⁺ contents in roots and the Na⁺ accumulation was significantly higher into roots of PPE-7 and PPE-13 plants. K⁺ homeostasis is vital for the normal functioning of plant cells. K⁺ and Na⁺ are chemically similar and generally present at similar concentrations in non-saline soils. However, high concentrations of Na⁺ in the soil can disturb ionic balance by reducing the K⁺ contents in

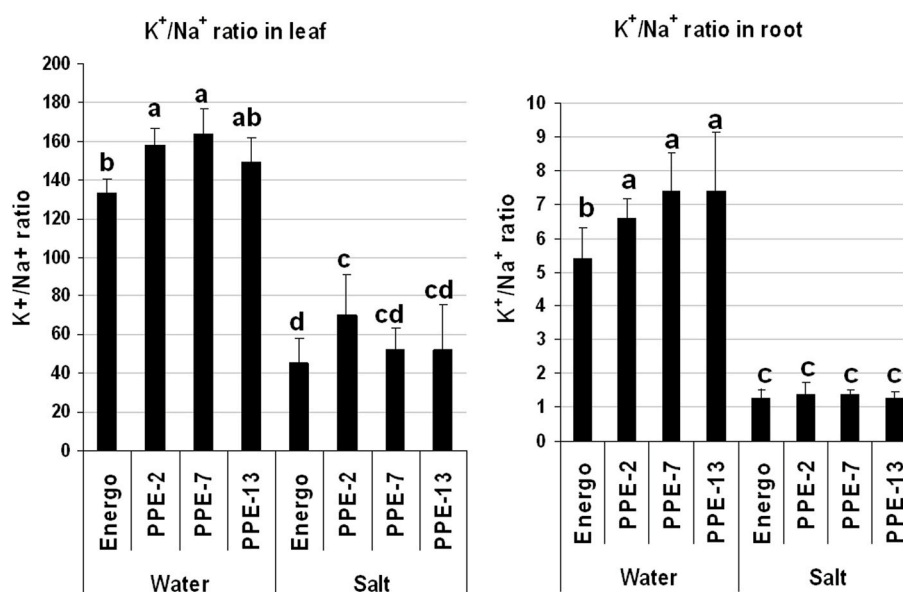


Fig. 9. Doubling genome size can significantly improve ion homeostasis, the Na⁺/K⁺ ratio in leaves of energy willow plants grown under normal conditions. In salt-stressed leaves, reduction in K⁺/Na⁺ ratio was moderate in leaves of PPE-2 plants. Results are means ± SD, n = 4. Values denoted with different letters show significant differences at P ≤ 0.05 level (Duncan's test).

willow tissues as shown by Table 1/B. This reduction was significantly lower in leaves of plants from the PPE-2 and PPE-7 lines than in leaves of Energo plants. This character of the tetraploid plants could contribute to the improved biomass productivity of these genotypes (see Fig. 3). The K^+ content data in salt-treated roots did not present significant differences between genotypes.

Since the K^+ ion content in the tissues relative to that of Na^+ ions may be a contributing factor to salinity tolerance, we determined the K^+/Na^+ ratio in the leaves and roots of willow plants (Fig. 9). Under normal growing conditions, plants from the tetraploid genotypes accumulated significantly higher amounts of potassium ions relative to sodium ions in their tissues than the diploid Energo plants. Similar trends can be seen in root tissues. This is an unexpected finding that can be related to the larger size and increased physiological activities of leaves and roots from the tetraploid plants (see Refs. [10]) The exogenous NaCl lowered this ratio with variable degrees among genotypes. The most salt-tolerant PPE-2 plants (Fig. 5) preserved significantly higher K^+/Na^+ ratio in their leaves as compared to Energo plants at high salinity. The multifunctionality of potassium in several basic biochemical and physiological systems of plants includes activation of the enzymes regulating plant growth, sugar, water and nutrient transport, or protein and starch synthesis (see review by Prajapati and Modi [39]). Salinity stress was shown to reduce drastically K^+/Na^+ ratios in Chinese willow (*Salix matsudana* Koidz.) plants grown in Hoagland nutrient medium containing 100 mM NaCl for 0, 6, 24, 48 and 72 h.

4. Conclusions

Doubling the chromosome number of energy willow plants can generate several beneficial traits contributing to biomass productivity. The present study on the salt stress responses of these novel genotypes extends the list of positive traits with the improved salt stress adaptation of tetraploid variants by a variable degree. The application of artificially high doses of NaCl in the soil of willow plants allowed the identification of the most adaptive polyploid line (PPE-2) on the basis of green and root biomass, high water use efficiency and extensive starch accumulation. The plants of this genotype accumulated the lowest amount of the Na^+ in their leaves with an increase in the K^+ content. All tetraploid genotypes were capable of maintaining the functionality of photosynthesis and developing a root system higher than those of the diploid plants. The presented greenhouse studies provide sufficient basis for testing these willow stocks under natural field conditions with different degrees of salt contamination of soils.

Acknowledgements

This work was funded by grant no. GINOP-2.2.1-15-2017-00081 from the Hungarian government. The authors express their high appreciation to Antal Beck (Beck Antal Erdészeti Csemetekert, Borjád, Hungary) for financial support of this research.

References

- P. Shrivastava, R. Kumar, Soil salinity: a serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation, *Saudi J. Biol. Sci.* 22 (2015) 123–131.
- J. Mirck, R.S. Zalesny, Mini-review of knowledge gaps in salt tolerance of plants applied to willows and poplars, *Int. J. Phytoremediation* 17 (7) (2015) 640–650.
- R. Munns, M. Gilliland, Salinity tolerance of crops what is the cost? *New Phytol.* 208 (2015) 668–673.
- J.E. Major, A. Mosseler, J.W. Malcolm, S. Heartz, Salinity tolerance of three *Salix* species: survival, biomass yield and allocation, and biochemical efficiencies, *Biomass Bioenergy* 105 (2017) 10–22.
- R.D. Hangs, J.J. Schoenau, K.C.J. Van Rees, H. Steppuhn, Examining the salt tolerance of willow (*Salix* spp.) bioenergy species for use on salt-affected agricultural lands, *Can. J. Plant Sci.* 91 (2011) 509–517.
- J. Zhang, H. Yuan, Q. Yang, M. Li, Y. Wang, Y. Li, X. Ma, F. Tan, R. Wu, The genetic architecture of growth traits in *Salix matsudana* under salt stress, *Horticult. Res.* 4 (2017) 17024.
- K.N. Lindegaard, J.H.A. Barker, Breeding willows for biomass, *Aspect Appl. Biol.* (1997) 49.
- A. Karp, S.J. Hanley, S.O. Trybush, W. Macalpine, M. Pei, I. Shield, Genetic improvement of willow for bioenergy and biofuels, *J. Integr. Plant Biol.* 53 (2011) 151–165.
- M.J. Serapiglia, F.E. Gouker, L.B. Smart, Early selection of novel triploid hybrids of shrub willow with improved biomass yield relative to diploids, *BMC Plant Biol.* 14 (2014) 74.
- D. Dudits, K. Török, A. Cseri, K. Paul, A.V. Nagy, B. Nagy, L. Sass, Gy Ferenc, R. Vankova, P. Dobrev, I. Vass, F. Ayaydin, Response of organ structure and physiology to autotetraploidization in early development of energy willow *Salix viminalis*, *Plant Physiol.* 170 (2016) 1504–1523.
- F. Meng, Q. Luo, Q. Wang, X. Zhang, Z. Qi, F. Xu, X. Lei, Y. Cao, W.S. Chow, G. Sun, Physiological and proteomic responses to salt stress in chloroplasts of diploid and tetraploid black locust (*Robinia pseudoacacia* L.), *Sci. Rep.* 6 (2016) 23098.
- Q. Luo, M. Peng, X. Zhang, P. Lei, X. Ji, W. Chow, F. Meng, G. Sun, Comparative mitochondrial proteomic, physiological, biochemical and ultrastructural profiling reveal factors underpinning salt tolerance in tetraploid black locust (*Robinia pseudoacacia* L.), *BMC Genom.* 18 (2017) 648.
- M. Deng, Y. Dong, Z. Zhao, Y. Li, G. Fan, Dissecting the proteome dynamics of the salt stress induced changes in the leaf of diploid and autotetraploid *Paulownia fortunei*, *PLoS One* 12 (7) (2017), e0181937.
- A. Cseri, L. Sass, O. Török, J. Pauk, I. Vass, D. Dudits, Monitoring drought responses of barley genotypes with semi-robotic phenotyping platform and association analysis between recorded traits and allelic variants of some stress genes, *Aust. J. Crop. Sci.* 7 (10) (2013) 1560–1570.
- E. Fehér-Juhász, P. Majer, L. Sass, Cs Lantos, J. Csizsár, Z. Turóczy, R. Mihály, A. Mai, G.V. Horváth, I. Vass, D. Dudits, János Pauk, phenotyping shows improved physiological traits and seed yield of transgenic wheat plants expressing the alfalfa aldose reductase under permanent drought stress, *Acta Physiol. Plant.* 36 (2014) 663–673.
- P. Poór, K. Gémes, F. Horváth, A. Szepesi, M.L. Simon, I. Tari, Salicylic acid treatment via the rooting medium interferes with stomatal response, CO₂ fixation rate and carbohydrate metabolism in tomato, and decreases harmful effects of subsequent salt stress, *Plant Biol.* 13 (2011) 105–114.
- J. Hansen, I.B. Møller, Percolation of starch and soluble carbohydrates from plant tissue for quantitative determination with anthrone, *Anal. Biochem.* 68 (1975) 87–94.
- D.A. Horneck, D. Hanson, Determination of potassium and sodium by flame emission spectrophotometry, in: Y.P. Kalra (Ed.), *Handbook of Methods for Plant Analysis*, CRC Press, 1998, pp. 153–156.
- H. Xue, F. Zhang, Z.-H. Zhang, J.-F. Fu, F. Wang, B. Zhang, Y. Ma, Differences in salt tolerance between diploid and autotetraploid apple seedlings exposed to salt stress, *Sci. Hortic.* 190 (2015) 24–30.
- T. Tóth, K. Balog, A. Szabó, L. Pásztor, E.G. Jobbágy, M.D. Noretto, Z. Gribovszki, Influence of lowland forests on subsurface salt accumulation in shallow groundwater areas, *AoB PLANTS* 6 (2014) plu054, <https://doi.org/10.1093/aobpla/plu054>.
- M. Ruiz, A. Quinones, B. Martínez-Alcántara, P. Aleza, R. Morillon, L. Navarro, E. Primo-Millo, M.-R. Martínez-Cuenca, Effects of salinity on diploid (2x) and doubled diploid (4x) *Citrusmacrophylla* genotypes *Scientia, Horticulturae* 207 (2016) 33–40.
- F. Kaleem, G. Shabir, K. Aslam, S. Rasul, H. Manzoor, S.M. Shah, A.R. Khan, An overview of the genetics of plant response to salt stress: present status and the way forward, *Appl. Biochem. Biotechnol.* 186 (2018) 306–334.
- L. Taiz, E. Zeiger, fourth ed., Sinauer Associates, Inc., Sunderland Plant physiology, 2006.
- F. García-Sánchez, V. Martínez, J. Jifon, J.P. Syvertsen, J.W. Grosser, Salinity reduces growth, gas exchange, chlorophyll and nutrient concentrations in diploid sour orange and related allotetraploid somatic hybrids, *J. Hortic. Sci. Biotechnol.* 77 (2002) 379–386.
- M.C. Martínez-Ballesta, V. Martínez, M. Carvajal, Osmotic adjustment, water relations and gas exchange in pepper plants grown under NaCl or KCl, *Environ. Exp. Bot.* 52 (2004) 161–174.
- C.E. Lovelock, M.C. Ball, Influence of salinity on photosynthesis of halophytes. *Salinity: Environment-Plants-Molecules*, Springer, Dordrecht, 2002, pp. 315–339.
- P. Mehta, A. Jajoo, S. Mathur, S. Bharti, Chlorophyll a fluorescence study revealing effects of high salt stress on Photosystem II in wheat leaves, *Plant Physiol. Biochem.* 48 (2010) 16–20.
- M. Zaghdoudi, N. Msilini, S. Govindachary, M. Lachaál, Z. Ouerghi, R. Carpentier, Inhibition of photosystems I and II activities in salt stress exposed Fenugreek (*Trigonella foenum graecum*), *J. Photochem. Photobiol., B* 105 (2011) 14–20.
- A. Parida, A. Das, Salt tolerance and salinity effects on plants: a review, *Ecotoxicol. Environ. Saf.* 60 (2005) 324–349.
- M. Thalmann, D. Santelia, Starch as a determinant of plant fitness under abiotic stress, *New Phytol.* 214 (3) (2017) 943–951.
- G.J. MacNeill, S. Mehrpouyan, M.A.A. Minow, J.A. Patterson, I.J. Tetlow, M. J. Emes, Starch as a source, starch as a sink: the bifunctional role of starch in carbon allocation, *J. Exp. Bot.* 68 (2017) 4433–4453.
- J. Krasensky, C. Jonak, Drought, Salt, and Temperature Stress-Induced Metabolic Rearrangements and Regulatory Networks, 2012, pp. 1–16.
- F.J. Keiper, D.M. Chen, L.F. De Filippis, Respiratory, Photosynthetic and ultrastructural changes accompanying salt adaptation in culture of eucalyptus microcyrs, *J. Plant Physiol.* 152 (2007) 564–573.

- [34] M. Kanai, K. Higuchi, T. Hagihara, T. Konishi, T. Ishii, N. Fujita, Y. Nakamura, Y. Maeda, M. Yoshida, T. Tadano, Common reed produces starch granules at the shoot base in response to salt stress, *New Phytol.* 176 (2007) 572–580.
- [35] J.-K. Zhu, Plant salt tolerance, *Trends Plant Sci.* 6 (2001). No.2 February.
- [36] R. Munns, M. Tester, Mechanisms of salinity tolerance, *Annu. Rev. Plant Biol.* (2008) 651–681, 59:1.
- [37] U. Deinlein, A.B. Stephan, T. Horie, W. Luo, G. Xu, J.I. Schroeder, Plant salt-tolerance mechanisms, *Trends Plant Sci.* 19 (6) (2014 June) 371–379.
- [39] K. Prajapati¹, H.A. Modi, The importance of potassium in plant growth - a review (Online) An Online International Journal Available at: *Indian J. Plant Sci.* 1 (2012) 177–186 (02-03) Jul.-Sept. & Oct.-Dec. ISSN: 2319-3824, <http://www.cibtech.org/jps.htm>. /Prajapati and Modi.