



Expression of triploid heterosis in the biomass productivity of energy willow plants under salinity stress

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ABSTRACT

Bioenergy production from short rotation woody species grown on saline soil provides multiple environmental advantages. Breeding of energy willow for salinity tolerance can support green energy utilization. Here, we quantify triploid heterosis in biomass traits under salt stresses. Crosses between diploid cultivars (Tora and Inger) and our autotetraploid plants (PPE-2/6 and PPE-7) resulted in TH16/24 and TH21/2 triploid hybrid genotypes. Growth characteristics of shoots and roots of triploid hybrids and their parental plants were monitored by pixel-based phenotyping under high salt stress (NaCl 2.0 g kg⁻¹/EC: 8.71 mS cm⁻¹) in greenhouse. Different values of Mid-Parent Heterosis (MPH %) were recorded by comparing these two crossing combinations and top or above views (for TH16/24 plants: 3.66% (side pixels) and 22.25% (above pixels) and for TH21/2 plants: 63.30% (side pixels) and 72.10% (above pixels)). At the end of the growing period, considerable MPH values were detected in biomass parameters of TH16/24 and TH21/2 plants (shoot length: 18.26%/26.28%; green biomass weight: 40.28%/52.40%; stem diameter: 15.40%/13.98%) in soil with 2.0 g NaCl kg⁻¹. The TH21/2 hybrid plants expressed higher hybrid vigor than plants from the TH16/24 genotype. The TH21/2 hybrid plants exhibited better water use efficiency than their parental plants and showed high MPH values for K⁺/Na⁺ ratio in soil containing 2.0 g NaCl kg⁻¹. The presented data support a conclusion that heterosis depending on crossing partners can ensure improvement in salinity tolerance for triploid hybrids. Field experiment has been initiated for the characterization of these hybrids under natural saline soil.

1. Introduction

Humanity faces crucial global environmental issues that have already emerged as climate change causing warming due to CO₂ emissions, water shortage or sea level rise. Another threat, associated with climatic changes and anthropogenic activities is the salinization of agricultural soils. The prominence of salt stress in soil is rising both in irrigated and dryland systems. According to the FAO, the global area of salt-affected soils covers 424 million hectares of topsoil (0–30 cm) and 833 million hectares of subsoil (30–100 cm) (based on 73% of the land mapped so far) [1]. Use of different methods for the reclamation of salt-affected soil is a way to increase agricultural productivity [2].

Santos et al. (2022) reported that cultivation of *Atriplex nummularia* as woody shrub was effective in reducing the sodicity and salinity of saline-sodic soil [3]. Furthermore, salt-affected marginal lands cultivated by short-rotation coppice plantations of energy willow contribute to amending soil quality, extending CO₂ fixation, and producing biomass feedstock to be used in bioenergy generation. In these applications, differences in salt sensitivity between *Salix* species or cultivars should be considered as key limiting factor (see: Major et al., 2017, Huang et al., 2020 [4,5]). Munns and Gilliland [6] have summarized the complexity of adaptive mechanisms in salt tolerance both at cellular and organ levels.

Breeding of improved willow varieties can rely on the genetic

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diversity represented by 330–500 species, and more than 200 hybrids in the *Salix* genus [7]. Energy willow breeding activities are primarily focused on the increase of the biomass yield by interspecific hybridization [8,9]. Previously, we reported the generation of autotetraploid ($2n = 4x = 76$ chromosomes) variants of a highly productive energy willow cultivar ‘Energó’ (*S. viminalis*). The colchicine treatment-induced duplication of the genome size caused enlargement in leaves with increased net photosynthetic CO_2 uptake and higher transpiration rate. Willow plants with $2n = 4x = 76$ chromosome number developed enlarged root system [10]. These plants developed shorter shoots with wider stem diameters. In attempts in further improvement of biomass yield, we carried out a crossing program between two leading Swedish diploid cultivars ($2n = 2x = 38$:Tordis and Inger) and our autotetraploid genotypes ($2n = 4x = 76$) [11]. According to the detailed characterization of these triploid hybrids with three copies of chromosomes ($2n = 3x = 38$), the crossing progenies showed considerable Mid-Parent Heterosis (MPH%) in the growth rate. These triploid hybrids (THs) developed larger leaves than their parental cultivars, and the MPH% for their CO_2 assimilation rate varied between 0.84 and 25.30% as we reported [11].

Increasing demand for reclamation of salt-affected soils urges targeted breeding projects to produce new woody genotypes with improved salt adaptation potential. We can find reports about salt tolerance of tetraploid black locust (*Robinia pseudoacacia* L.) by Meng et al. [12] and by Luo et al. [13], furthermore *Paulownia fortune* polyploids by Deng et al. [14]. In our autopolyploidization project, we have compared salt responses of tetraploid and diploid willow plants [15]. The increased salt adaptation capability of tetraploid plants was indicated by larger biomass, leaf and root weights under salinity stress (1.5 g NaCl kg^{-1} soil, EC: 7.04 mS cm^{-1}) relative to diploids. Biomass weights were significantly higher in plants from one of the tetraploid lines 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 lower in leaves of tetraploids, and increased in their roots, while the K^+ ion content was higher in tetraploid leaves than in diploids.

Further characterization of autotetraploid willow plants under field conditions, especially in severe drought season showed their limitations in stem size, biomass yield. Therefore, we carried out a crossing program to produce triploid hybrids with the expectation for expression and possible utilization of hybrid vigor [11]. In the present study, we provide detailed results about salinity responses of the diploid and the tetraploid parental plants and their triploid progenies under greenhouse conditions. Digital imaging of shoot growth and the final biomass weight values indicated considerable salt tolerance of triploid hybrids, especially in soil containing relatively high dose of NaCl, (2.0 g kg^{-1} /EC: 8.71 mS cm^{-1}). In saline soil, a more optimal physiological status of triploid hybrids was reflected by the K^+ and Na^+ homeostasis. The reduced inhibition of root systems by salinity could also contribute to the better biomass productivity of hybrids under salt stress.

2. Materials and methods

2.1. Plant material

Salinity responses of energy willow plants from six different genotypes were characterized in greenhouse experiments. Two of them (PPE-2/6 and PPE-7) represent autotetraploid genotypes generated previously from the cultivar Energó (*Salix viminalis*) by colchicine treatment in our laboratory [10]. The Tora (*Salix schwerinii* x *viminalis*) and the Inger (*Salix triandra* x *viminalis*) are commercially available diploid cultivars from Sweden. In our breeding program, we have crossed these diploid and the autotetraploid willow plants and the resulted triploid hybrids (TH) were characterized in details [11]. In the present study, the saline tolerance of plants from two triploid hybrid genotypes: TH16/24 (Tora x

PPE-2/6) and TH21/2 (Inger x PPE-7) was quantified at two concentrations of salt in comparison to their parental plants.

2.2. Plant growth conditions and salt stress treatment

As first approach, the salt response of energy willow plants with different genetic background, we used a semi-automated phenotyping platform, its setup was published earlier [16]. During the winter period (December, January, February), one-year-old dormant stems from willow plants grown in the field were collected and used for production of cca. 20 cm cuttings. This propagation material was planted into radio-tagged plexiglass columns with a mixture of 80% Florimo peat soil and 20% sand, and 6 g Substral Osmocote (Evergreen Garden Care, UK) was added to the mixture as nutrient supplement. The soil was mixed with finely ground 1.5 g or 2 g NaCl kg^{-1} soil in the case of the salt-stressed pots. The electrical conductivity measurements were performed using Orion 4 Star pH-Conductivity benchtop (Thermo Electron Corporation Waltham, MA, USA). The cuttings were soaked in water for 48 h before the plantation in the greenhouse. The soil in the columns were water saturated at the time of potting to ensure the proper rooting of the cuttings. Five plexiglass columns surrounded with polyvinyl chloride tubing were placed on a metal rack. Ten plants distributed into two sets (2x5) were used for each treatment and genotype combination with a random arrangement. The growth temperature was set to 21 °C, and the level of illumination during the days was approximately 400 $\text{mmol photons m}^{-2} \text{s}^{-1}$ in the greenhouse. The plants were watered to 60% field capacity of the soil once a week, as a part of the phenotyping process.

2.3. Shoot surface and root density predictions by digital imaging, growth parameters and biomass 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 [16]. Watering and digital imaging of shoot and root system were performed once a week. 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 sideway positions, produced by 51.4° step rotation of a pot, and another camera of the same type was used to make the top-view images. In the case shoots, we calculated the plant surface from the average of the green pixels of the seven different sideway positions. In the case of roots, the plexiglass columns were photographed from four different side positions and from the bottom using two Canon EOS 600D digital cameras. The root-related white pixels were identified by subtracting the black soil background from 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) and the bottom view was summarized. We used an in house-developed image-analysis software to segment green and white pixels [10].

At the end of the nine-week duration of phenotyping experiment, plant height, stem diameter, weights of above-ground biomass, separated into leaves and shoots were determined from the harvested willow plants.

2.4. 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) using Agilent 240FS Atomic Absorption Spectrometer [17]. Plant samples (leaf and root) were oven-dried for 24 h at 103 °C. The digestion procedure - based on the solutions of 6 mL HNO_3 (65%) and 4 mL H_2O_2 (30%) - was applied for 10 min at 180 °C using a microwave digestion system (Ethos Plus, Milestone), followed by the addition of 0.03 mol dm^{-3} LiCl

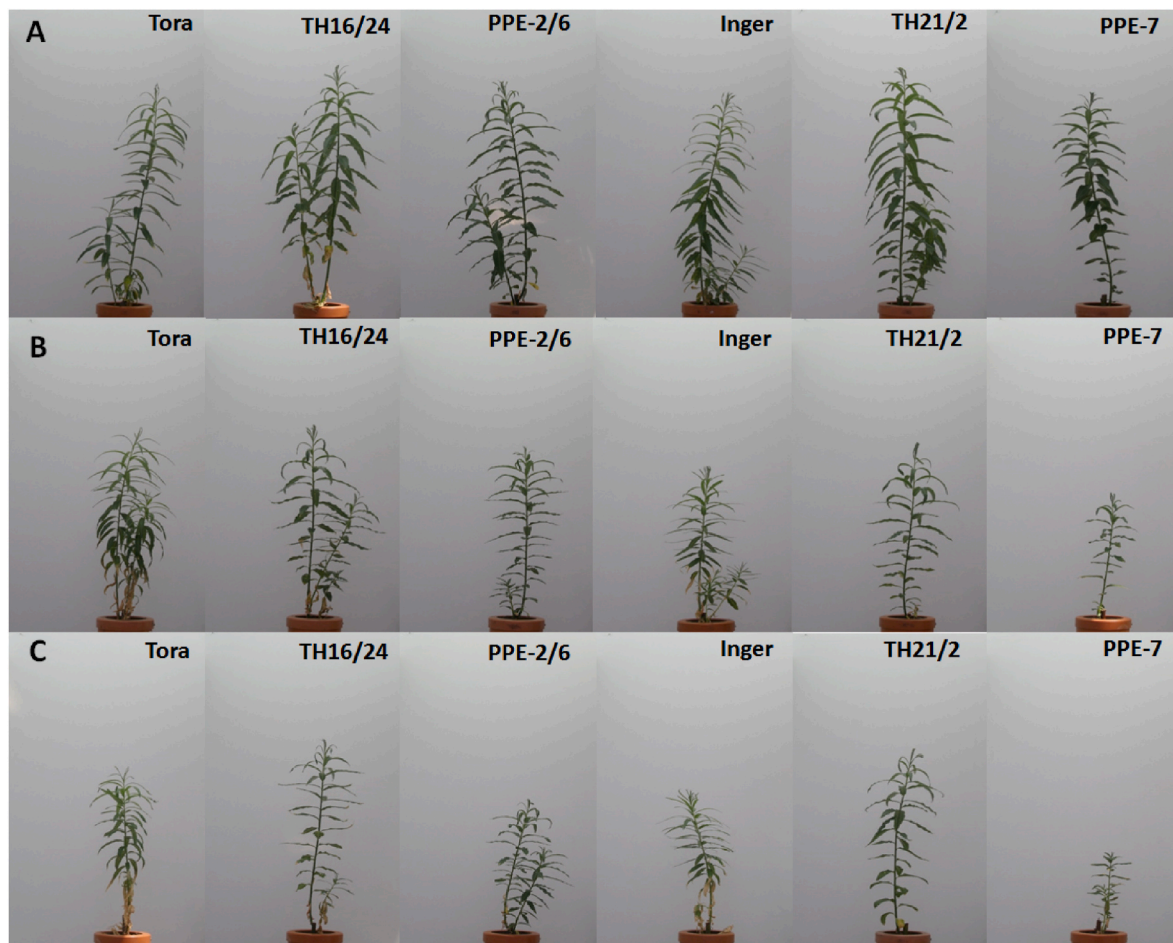


Fig. 1. Representative phenotype of triploid hybrids (TH16/24 and TH21/2) and their parental willow plants. Manifestation of hybrid vigor in green biomass is visible both in the control soil and under salt stress treatment. A: plants grown under control conditions; B: plants from soil with 1.5 g NaCl kg⁻¹; C: plants from soil with 2 g NaCl kg⁻¹. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

solution to the digested samples 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).

2.5. Statistical analysis

The statistical significance of the observed variations in different parameters was determined by Student's T-test using the online software GraphPad. The salt tolerance of different genotypes was compared with the aid of several parameters, which are appropriate markers for salt adaptation. Statistically significant events were related to the diploid parent (*) or to tetraploid the parent (‡) based on the p values calculated from T-values. Differences were considered to be significant at three different levels as $p \leq 0.001$ ***, †††; $p \leq 0.01$ **, †† and $p \leq 0.05$ *, †. 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. The standard deviations in the MPH % values were determined by calculation of the error propagation.

3. Results and discussion

3.1. Digital imaging shows hybrid vigor in shoot growth rate of triploid hybrid willow plants grown in saline soil

Physiological damages caused by elevated salt (NaCl) content of soil are expected to alter morphology, size of willow plant organs and

progression in the growth and the development phases. In the present study, we used digital imaging of green shoots to provide integrative information about the leaf and the stem surface area for comparison of parental and hybrid genotypes under normal and salinity soil conditions. As we reported earlier [15] the semi-automatic stress diagnostic system provides photos of individual plants grown in various environment (Fig. 1), furthermore generates green pixel-based relative quantitative data for characterizing salt stress responses of willow plants. This phenotyping approach offers advantage over end-point measurements, since growth dynamics can also be monitored. Fig. 2 and Fig. 3 show representative growing curves for willow plants in control soil (EC: 2.21 mS cm⁻¹) and in soil mixed with 2.0 g NaCl kg⁻¹ (EC: 8.71 mS cm⁻¹). The analyzed pixel values were generated by photography from sideways and above views. In general, the two views present overall similar responses, but the individual pixel values may differ, since the side views provide pixel values primarily determined by stem height, while the green pixel values from above represent mainly leaf areas.

Fig. 2/A, B show growth kinetics presented by pixel-based shoot surface for the TH 16/24 triploid hybrid and its parents (Tora, PPE-2/6) in the control soil. Images from above show a significant growth advantage for the TH16/24 triploid hybrid plants after 6 weeks of cultivation. The hybrid vigor was indicated by the Mid-Parental Heterosis (MPH) values: from side images 23.31% and images from above 37.30% (Table 1). Low growth rate of the Tora plants was also confirmed by biomass weights as shown later by Table 2. These trends were changed by high saline stress (2.0 g NaCl kg⁻¹) as differently shown by the side and the above pixel values. Our photographic system

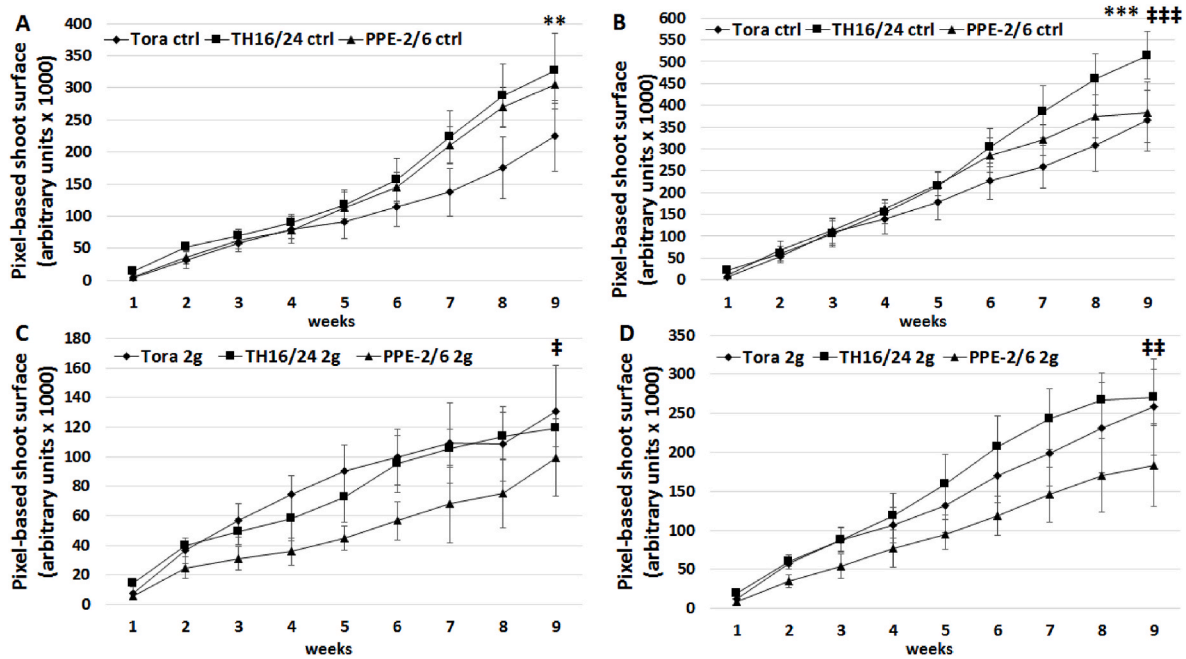


Fig. 2. The pixel-based shoot surface of the TH16/24 genotype and its parental lines Tora and PPE2/6 in the control soil and under salt stress (2 g NaCl kg⁻¹) conditions. A and C: data derived from the side images; B and D: data derived from the above images. The TH16/24 plants exhibited significant hybrid vigor both in control soil and soil supplemented NaCl according to the above images. Statistically significant events compared to the diploid parent (*) or to tetraploid the parent (‡) are indicated a side sample labels as $p \leq 0.001$ ***, †††; $p \leq 0.01$ **, †† and $p \leq 0.05$ *, †. Number of tested plants $n = 10$.

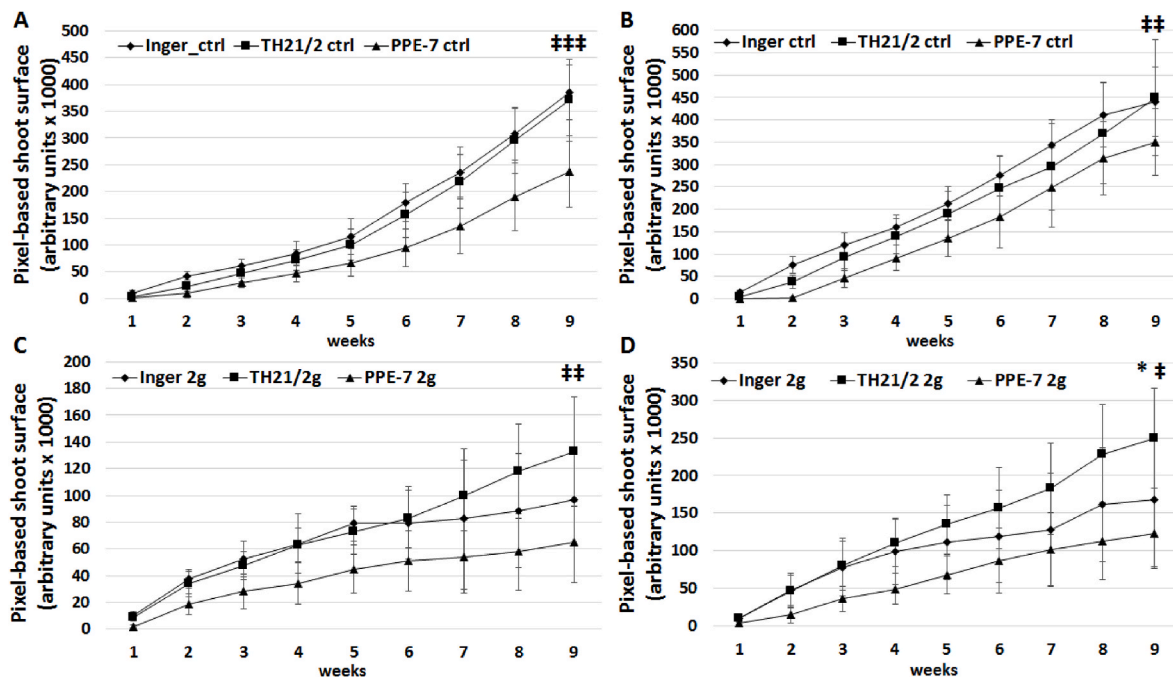


Fig. 3. The pixel-based shoot surface of the TH21/2 genotype and its parental lines Inger and PPE-7 in the control soil and under the salt stress (2 g NaCl kg⁻¹) conditions. A and C: data derived from the side images; B and D: data derived from the above images. The triploid TH21/2 genotype showed largest shoot surface from both views. Statistically significant events compared to the diploid parent (*) or to tetraploid the parent (‡) are indicated a side sample labels as $p \leq 0.001$ ***, †††; $p \leq 0.01$ **, ††, and $p \leq 0.05$ *, †. Number of tested plants $n = 10$.

recorded at the 9th week variable difference in the salt inhibition according to the side data (TH16/24: 63.08%; Tora: 57.67%; PPE-2/6: 54.55%) compared to the above view pixel values (TH16/24: 46.00%; Tora: 28.58%; PPE-2/6: 58.00%). These different trends may derive from the morphological differences between the various genotypes. Subsequently, the MPH% values for the TH16/24 plants (Table 1) were

23.31% (side pixels) and 37.30% (above pixels).

As it is expected, we found different growth behavior for TH21/2 hybrid plants and their parents. In these plant populations, the digital imaging recorded similar developmental patterns from the side and the above views (Fig. 3). In the control soil, plants of the leading Swedish cultivar and the triploid hybrid genotype exhibited closely similar

Table 1

Quantification of hybrid vigor expressed in energy willow plants grown in the control soil and under salinity stresses generated by the two doses of NaCl. In untreated soil both hybrid combinations express heterosis, with higher values of TH16/24 genotype. In contrast, higher MPH values were detected in plants of TH21/2 genotype under salt stress (2.0 g NaCl kg⁻¹). The Mid-Parental Heterosis values were obtained from phenotyping pixel data representing the size of organs. The MPH% was calculated as heterosis over Mid-Parent (MP%) = [(F1-MP)/MP x 100], where F1 is the numerical value trait measurement in the hybrid and MP values are the mean values of the parents (P1+P2)/2.

Treatment	Cultivar	Midparent heterosis (MPH %)		Pixel number		Convex hull
		Shoot	Root	Shoot	Root	Shoot
Control	TH16/14	23,31 ± 1,91	37,3 ± 1,80	32,9 ± 3,16	31,9 ± 0,93	
	TH21/2	18,95 ± 1,96	13,7 ± 2,57	-7,7 ± 11,54	2,2 ± 5,45	
1,5 g NaCl kg ⁻¹	TH16/14	-17 ± 1,51	8,46 ± 4,01	4,3 ± 11,02	6,8 ± 4,64	
	TH21/2	28,34 ± 3,23	7,5 ± 5,94	100,5 ± 1,9	6,3 ± 7,09	
2 g NaCl kg ⁻¹	TH16/14	3,66 ± 5,8	22,25 ± 1,76	80,74 ± 2,12	28,9 ± 1,35	
	TH21/2	63,3 ± 1,56	72,1 ± 1,57	-23,16 ± 4,11	58,13 ± 1,41	

Table 2

Quantification of biomass parameters at the end of the growing period for characterization of triploid hybrid and their parental willow plants grown in control soil or under salt stress generated by 1.5 or 2.0 g NaCl kg⁻¹. Results are mean values from 8 to 10 plants. Based on Student's t-test, statistically significant events compared to the diploid parent (*) or to the tetraploid parent (‡) are indicated a side sample labels as p ≤ 0.001 ***, †††, p ≤ 0.01 **, ††, and p ≤ 0.05 *, †. Number of tested plants n = 10.

	Genotype	Plant height (mm)	Biomass (g)	Leaf biomass (g)	Stem diameter (mm)
Control	Tora	638,63	16,36	9,27	4
	TH16/24	679,60 ††	26,17 ***	13,98 ***	5,04 ***
	PPE-2/6	578,3	24,6	12,38	5,25
	Inger	557,4	26,23	14,82	4,77
	TH21/2	691,86 ***	26,93	15,44 †	5,13
	PP-E7	610,3	22,28	11,86	5,18
1,5g NaCl kg ⁻¹	Tora	542,89	15,46	8,95	3,88
	TH16/24	515,00 †	17,53	9,81	4,70 **
	PPE-2/6	437,89	15,08	8,4	4,64
	Inger	442,5	13,63	7,59	4,25
	TH21/2	411,75 †	9,95 †	5,70 †	4,35
	PPE-7	375,67	6,9	3,83	4
2g NaCl kg ⁻¹	Tora	451,4	9,18	6,05	3,62
	TH16/24	480,86 †††	12,46 * †	6,37 †	4,60 ***
	PPE-2/6	301,57	8,58	4,94	4,35
	Inger	343,5	8,94	4,32	4,23
	TH21/2	388,00 †††	9,96 †††	5,70 †††	4,50 †††
	PPE-7	271	4,13	1,94	3,67

growth intensity that exceeded growth characteristics of the tetraploid plants (PPE-7).

After 9 week of growing period, under the higher saline stress (2.0 g NaCl kg⁻¹), resistance of TH 21/2 plants was indicated by lower growth retardation as shown by side pixels (64.62%) and above pixels (44.45%) in comparing to Inger plants (side pixels:79.49%; above pixels:66.67%) as well as to the tetraploid parent, PP-E7 (side pixels:75.52%; above pixels: 71.43%). We may call the attention to the fact that in this analysis the side views present higher inhibition rate than above views.



Fig. 4. Saline tolerance of energy willow plants was tested in soil filled into plexiglass columns surrounded with polyvinyl chloride tubing and supplemented with 2.0 g NaCl kg⁻¹. This greenhouse photo clearly shows that willow plants from the TH16/24 genotype developed the largest shoots under this salt stress.

Table 3

The Mid-Parental Heterosis values calculated from the measured growth parameters. The MPH% was calculated as heterosis over mid-parent (MP%) = [(F1-MP)/MP*100], where F1 is the numerical value trait measurement in the hybrid and MP values are the mean values of the parents (P1+P2)/2.

Treatment	Cultivar	Plant height	Mid-parent heterosis (MPH%)		
			Biomass (total)	Biomass (leaf)	Stem diameter
Control	TH16/14	11,69 ± 1,77	27,76 ± 1,22	29,12 ± 1,28	8,89 ± 3,07
	TH21/2	18,5 ± 0,89	11,03 ± 3,14	15,78 ± 1,9	3,02 ± 4,20
1,5g NaCl kg ⁻¹	TH16/14	5,02 ± 4,73	14,78 ± 2,97	13,11 ± 2,54	10,36 ± 1,60
	TH21/2	0,65 ± 3,3	-3,09 ± 0,79	-0,14 ± 3,99	5,45 ± 2,45
2g NaCl kg ⁻¹	TH16/14	18,26 ± 1,21	40,28 ± 1,18	15,94 ± 2,76	15,4 ± 1,16
	TH21/2	26,28 ± 0,99	52,4 ± 0,73	82,16 ± 1,49	13,98 ± 1,2

From agronomic point of view, expression of the heterosis under salinity stress is a key characteristics. As a first approach, we calculated the Mid-Parental Heterosis (MPH%) values by using the pixel parameters shown above. The MPH values for the TH16/24 plants in soil supplemented with (2.0 g NaCl kg⁻¹) were 3.66% (side pixels) and 22.25% (top pixels) (Table 1). Comparing to the TH16/24 plants, an increased adaptation potential of the TH21/2 plants can be concluded from the higher MPH values: 63.30% (side pixels) and 72.10% (top pixels). As shown by the MPH values in the control soil, an opposite ranking can be seen, namely for the TH16/24 plants the MPH parameters are the followings: side pixel: 23.31%; top pixel: 37.30% and for the TH21/2 plants the MPH parameters are side pixel: 18.95%; top pixel: 13.70%. These data support a conclusion that levels of the hybrid vigor can be either stimulated or inhibited by salinity. These responses are dependent on the crossing combination and the analyzed traits (see Table 2.)

3.2. Above-ground biomass parameters indicate variable expression of heterosis in salt tolerance in the triploid hybrid plants compared to their parents

In reclamation of saline lands, salinity tolerance of woody species is a key adaptive trait primarily expressed in biomass productivity. Species

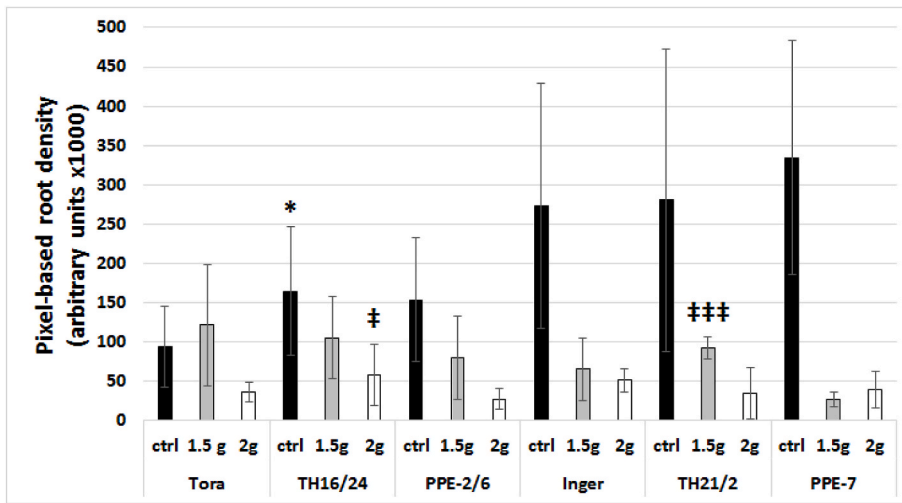


Fig. 5. Size of root systems in the control and the saline soil is differentially determined by genetic factors as ploidy level and heterosis in the various crossing combinations. Development of roots of TH21/2 triploid hybrid plants reflects hybrid vigor in soils supplemented by lower dose of salt (1.5 g NaCl soil kg⁻¹), while plants of the TH16/24 hybrid genotype developed a larger root system than the parental plants in soil containing 2 g NaCl kg⁻¹. Average root density of the energy willow plants in control (black bars), 1.5 g NaCl soil kg⁻¹ (grey bars) and 2 g NaCl soil kg⁻¹ conditions. Statistically significant events compared with diploid parent (*) or with tetraploid parent (‡) are indicated a side sample labels as p ≤ 0.001 ***, †††, p ≤ 0.01 **, ††, and p ≤ 0.05 *, †. Number of tested plants n = 10.

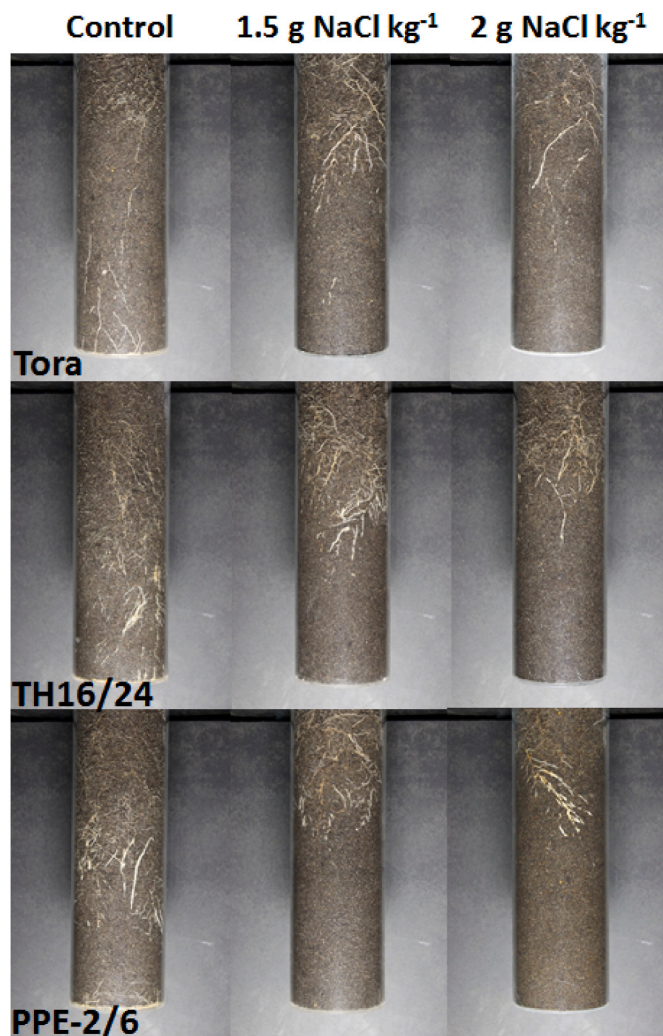


Fig. 6. Similarly to the pixel number values presented in Fig. 5, this figure shows that the root systems of TH16/24 plants developed larger root biomass in comparison to its parental plants in soils exposed to two strengths of the salt stress.

and genotype-dependent differences [10,18,19] in relation to biomass responses to salinity highlight potentials for willow breeding. In these attempts, we have tested a possible contribution of hybrid vigor to adaptation potential of willow genotypes to saline soil. After 9th week, at the end of the growing period, we have quantified biomass parameters of shoots from the two energy willow hybrids and their parental genotypes in control soil and under high saline stress (Table 2). In agreement with the pixel based quantification of biomass, the actual measurement of green shoot weight indicates considerable saline stress tolerance of TH21/2 hybrids even at the higher dose of NaCl.

In accordance with our previous studies [11], the present crossing combinations resulted in triploid hybrids with significantly higher shoot length in control soil. Under this environment, the TH16/24 plants exceeded plants from the Tora cultivar in total and leaf biomass, furthermore in stem diameter at significant level. These traits of hybrids resemble to the characteristics of the tetraploid (PP-E 2/6) parental plants. Similar trend was not recorded in characterization of the hybrid TH21/2 plants in comparing with the parental Inger plants. As shown by Table 1, we have analyzed responses of plants from the various genotypes to saline stresses, when the cultivation soils were supplemented by two doses of salt (1.5 g NaCl kg⁻¹ - EC: 7.04 mS cm⁻¹ or 2.0 g NaCl kg⁻¹ -EC: 8.71 mS cm⁻¹). Application of these doses represents high salinity treatment [18]. Regarding the total biomass yield per plant, the TH16/24 hybrids represented the most productive genotype at both NaCl doses relative to the control plants. Improved adaptive capability of these plants was clearly visible even in soil supplemented by 2.0 g NaCl kg⁻¹ (see Fig. 4). The TH16/24 hybrid plants are progenies of two parents with different sensitivity (inhibition: Tora (43.89%); PPE-2/6 (65.13%). Hangs et al. [18] reported responses of 37 native and exotic willow cultivars to severe saline stress (EC: 8.00 mS cm⁻¹). The mean value of inhibition was 61.67% being relative to the control total biomass. Out of 37 genotypes, 4 variants failed to show biomass reduction. In the present analysis, 52.39% reduction was detected for triploid hybrid plants (TH16/24) under similar salt stress.

Considering the phenotyping data, it was essential to confirm these findings by measuring leaf biomass (Table 1). In saline soils with both concentrations of NaCl, willow plants of TH16/24 hybrid genotype developed the highest leaf biomass (9.81 g and 6.37 g) per plants. These plants are expected to be able for increased CO₂ fixation with positive environmental impact. Stem diameter can essentially determine wood yield in the use for bioenergy production. In the control soil (Table 1), the triploid hybrids and the tetraploid parental plants (PPE-2/6, PPE-7) produced stems being wider than 5 mm. Previously, we published that autotetraploidy slowed primary growth and increased shoot diameter (a parameter of secondary growth) of willow plants [10]. In soils

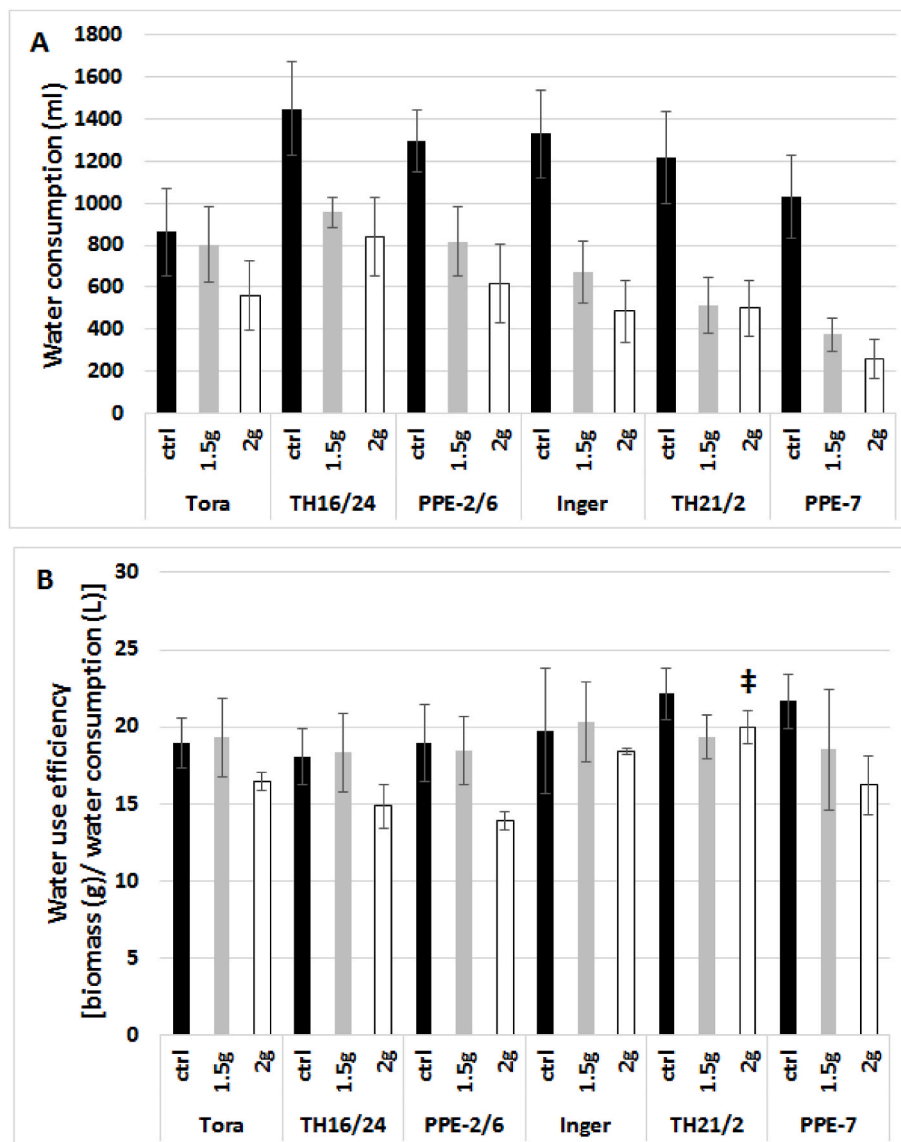


Fig. 7. Variation of water consumption (A) and water use efficiency (B) of diploid, triploid and tetraploid plants under normal and salt stress conditions (1.5 g and 2 g NaCl kg⁻¹ soil) after nine weeks. Statistically significant events compared with diploid parent (*) or with tetraploid parent (‡) are indicated a side sample labels as p < 0.001 ***, †††, p < 0.01 **, ††, and p < 0.05 *, †. Number of tested plants n = 10.

containing 2.0 g NaCl kg⁻¹, reduction in stem diameters was only moderate (9–13%) in plants of the tested genotypes.

Further analysis of hybrid vigor in saline environment was based on biomass productivity components by quantifying end-products after nine week treatment (Table 2). As a general trend, the Mid-Parent Heterosis values for both triploid hybrids were the highest in soil with 2.0 g NaCl kg⁻¹ (EC: 8.71 mS cm⁻¹) dose of salt (Table 3). In agreement with the pixel-based MPH values, the TH21/2 hybrid combination expressed higher heterosis response, than plants from the TH16/24 genotype. An unaccepted finding is that MPH values of triploid hybrid plants grown in soil with 1.5 g NaCl kg⁻¹ dose of salt are lower even in comparison to plants grown in control soil. If we exclude any technical problem, one may postulate that the higher NaCl dose can activate an additional defense system affecting also a heterosis mechanism. Nan et al. [19] reported that treatment of seedlings from the F1 hybrid varieties of *Physocarpus amurensis* X *P. opulifolius* species with elevated NaCl concentration resulted in increase of non-photochemical quenching (NPQ). van Amerongen and Chmeliov [20] discussed that switching between the light-harvesting and photoprotective mode requires time to de-activate NPQ. The relatively slow switching has a negative effect on

photosynthetic efficiency, while the faster switching rates can lead to increased crop productivity. Faster relaxation of NPQ and recovery of CO₂ fixation rate in transgenic tobacco plants over-expressing zeaxanthin epoxidase can cause an higher leaf carbon dioxide uptake and increasing plant dry matter productivity by about 15% [21].

3.3. Manifestation of triploid hybrid vigor in responses of root system to salinity can provide genetic basis for salt tolerance breeding

In saline soils, essential functions of roots are to insure normal plants growth by encountering insufficient water absorption and by reducing influx of toxic ions such as Na⁺ and Cl⁻, furthermore restricting Na⁺ transfer from the roots to shoots (see review by Karahara and Horie, [22]). Under saline stress, the length of wheat roots served as indicator of heterosis [23]. In the present study, we used digital imaging of willow root systems to predict root biomass of the triploid hybrids and their parents. Fig. 5 presents average pixel numbers determined by side photography after 9 weeks, at the end of the growing period.

In normal soil, plants of the Inger x PPE-7 crossing combination developed three times larger root system than plants from the Tora x

Table 4

Genotype-dependent variation of sodium and potassium contents in the leaf and the root tissues of the investigated genotypes under control and salt stress conditions. Statistically significant events compared with diploid parent (*) or with tetraploid parent (‡) are indicated a side sample labels as $p \leq 0.001$ ***, †††, $p \leq 0.01$ **, ††, and $p \leq 0.05$ *, †. Number of tested plants $n = 3$.

Na ⁺ content	Leaf (g kg ⁻¹)		Root (g kg ⁻¹)	
	Control	2g NaCl kg ⁻¹	Control	2g NaCl kg ⁻¹
Tora	0,06 ± 0,002	0,08 ± 0,013	1,04 ± 0,07	4,32 ± 0,61
TH16/24	0,13 ± 0,02 **	0,31 ± 0,078 ** †	1,1 ± 0,09	5,32 ± 0,31 * ‡‡
PPE-2/6	0,12 ± 0,03	0,18 ± 0,02	1,11 ± 0,27	4,23 ± 0,34
Inger	0,08 ± 0,005	0,2 ± 0,006	1,2 ± 0,1	4,81 ± 0,5
TH21/2	0,08 ± 0,006	0,14 ± 0,002	1,09 ± 0,12	3,71 ± 0,36
PPE-7	0,13 ± 0,018	0,81 ± 0,08	0,91 ± 0,018	3,81 ± 0,008
K ⁺ content	Leaf (g kg ⁻¹)		Root (g kg ⁻¹)	
	Control	2g NaCl kg ⁻¹	Control	2g NaCl kg ⁻¹
Tora	26,12 ± 1	27,39 ± 3,8	3,89 ± 0,54	3,55 ± 0,74
TH16/24	36,11 ± 3,77 ** ‡‡	24,0 ± 0,63	4,22 ± 0,28	3,79 ± 0,004 ‡‡‡
PPE-2/6	22,64 ± 0,06	22,41 ± 0,23	4,22 ± 0,55	3,1 ± 0,14
Inger	31,4 ± 4,48	21,75 ± 1,72	4,35 ± 0,61	3,41 ± 0,16
TH21/2	31,94 ± 2,57	24,26 ± 0,25 *	6,02 ± 0,51	2,57 ± 0,19
PPE-7	30,94 ± 1,04	26,66 ± 0,88	4,6 ± 0,48	2,78 ± 0,14

PPE-2/6 crossing. At the same time, as a general trend we can conclude that roots of genotypes from the Tora x PPE-2/6 crossing are less sensitive to saline stress than those from the Inger x PPE-7 crossing (inhibition by 2.0 g NaCl kg⁻¹: Tora: 60.99%; TH16/24: 64.69%; PPE-2/6: 81.18%; Inger: 81.18%; TH21/2: 87.53%; PPE-7: 88.16%). Furthermore, the Mid-Parental Heterosis value for the root density of the TH16/24 plants was 80.74% in the soil supplemented with 2.0 g NaCl kg⁻¹ (Table 1.) Fig. 6 presents representative photos of root systems developed under different soil environments.

Both pixel data presented in Fig. 5 and pictures in Fig. 6 indicate a stimulatory effect of the low NaCl dose (1.5 g NaCl soil kg⁻¹) in Tora roots. Similar effect of the lower salt concentration could not be observed in plants from the other genotypes. Therefore, we can postulate that this stimulation response is genotype-dependent. Hongqiao et al. [24] have reported that the improvement of carbon and sulfur assimilation in *Arabidopsis thaliana* plants could be responsible for growth stimulation by low concentration of NaCl.

3.4. Genotype-dependent water status of energy willow plants in the control and the saline soil

In the multicomponent regulation of water use, both the size of root system and the transpiration rate of leaves play a pivotal role. Since these traits are damaged by saline stress, plants grown in saline soil consume less water [15,25,26]. This water deficit is primarily responsible for reduction in biomass production. Therefore, in the present study we have determined water consumption and water use efficiency values in the case of triploid hybrids and their parental plants (Fig. 7/A and Fig. 7/B). As it is expected, willow plants with various genetic background consumes more water in the control soil as compared to those grown in saline soil. In the Tora x PPE-2/6 crossing combination, plants of TH16/24 genotype showed increased water uptake than the parental plants at both salt concentrations. In the Inger x PPE-7 crossing combination, the triploid hybrid plants (TH21/2) used smaller amounts of water under the three soil conditions as the Inger plants with exception in soil with 2.0 g NaCl soil kg⁻¹.

The genotype-dependent biomass productivity (Table 2) altered the ranking of genotypes according to the water consumption (Fig. 7/B). In the Tora x PPE-2/6 crossing combination, the higher dose of salt lowered the water use efficiency values. In contrary, the TH21/2 hybrid plants present significantly better water use efficiency than the parental plants

in soil with 2.0 g NaCl soil kg⁻¹.

3.5. Salinity responses of energy willow genotypes are reflected by differential accumulation of Na⁺ and of K⁺ as well as by variation in K⁺/Na⁺ ratio in leaves and roots

In interpreting salinity reactions of willow triploid hybrids especially that are expressed in biomass productivity, we have to consider alterations of the ionic homeostasis. Insufficient availability of K⁺ as consequence of K⁺ and Na⁺ antagonism can cause serious damage in plant functions [27,28]. As presented by values in Table 4., both the leaves and the roots of TH16/24 and PPE-7 plants accumulated considerable amounts of sodium. Regarding these hybrid plants, their Na⁺ contents were significantly higher than in organs of its parental plants. As the potassium contents are considered, leaves of the TH16/24 plants contained significantly more K⁺ than the parental leaves under normal soil condition. Based on all data, the presence of 2 g NaCl soil kg⁻¹ caused in average 18.22% reduction in potassium accumulation in leaves. This reduction was 29.68% in root tissues.

Importantly, roots of TH16/24 hybrid plants accumulated significantly more potassium as their parental plant roots.

Table 3 showed higher MPH values in the above-ground parameters of triploid willow plants from the TH21/2 genotype in comparison to the TH16/14 plants in soil supplemented with 2.0 g NaCl kg⁻¹. This difference may relate to the lower Na⁺ content in leaves of TH21/2 triploid plants (Table 4). In K⁺ contents, characteristic differences cannot be seen between the genotypes analyzed. The pivotal role of root functions under salinity stress is well documented (see review by Karahara and Horie [23]). Beyond the observed differences in Na⁺ contents in leaves, the differential accumulation of Na⁺ in roots can be an influential factor. Roots of triploid plants from the TH24/16 genotype contained significant amount of Na, 5.32 g/kg. Unexpectedly the K⁺ content was also high in these roots.

Furthermore, taking into account the close correlation between the tissue concentration of K⁺ and plant growth (see review by Ragel et al. [29]), we related MPH% in potassium content in leaf tissue samples of non-stress willow plants (Table 4) to MPH% in the leaf weights. As shown in Table 3, triploid hybrid plants from the TH16/24 genotype showed MPH 29.12%, while this parameter was only 3.02% for plants of the other hybrid (TH21/2) in leaf biomass. In agreement with this ranking, the MPH in leaf potassium content was 48.11% in TH16/24 hybrids, while in the other hybrid combination (TH21/2) of willow plants presented 4.19% heterosis. Similar parallelism cannot be seen in root samples. However, the high accumulation of potassium in roots of TH21/2 willow plants may have physiological importance. Since an increase in the K⁺ content can stimulate plant organ development, Mo et al. have analyzed the transcriptional background of significant heterosis in K⁺ content of tobacco leaves [30].

Resistance of willow plants against salinity stress essentially depends on the maintenance high K⁺/Na⁺ ratio in the cytosol (see review by Almeida et al. [31]). Fig. 8 presents results from analysis of this parameter in leaves of various energy willow genotypes that shows considerable MPH values for the TH21/2 hybrids in both the control (MPH%: 35.59) and the saline soil (MPH%: 93.93). In roots exposed to 2.0 g NaCl kg⁻¹ the ration between K⁺ and Na⁺ was strongly reduced, independently from the genetic background.

4. Conclusions

Acknowledging the increase in green bioenergy demand, the extended use of marginal lands for cultivation of short rotation woody crops requires priority in environmental policies. In addition to the selection of proper wood species, targeted breeding programs for improvement of salinity tolerance can support the utilization of saline soils. Since, we have already produced energy willow triploid hybrids showing hybrid vigor in biomass parameters, in the present work we

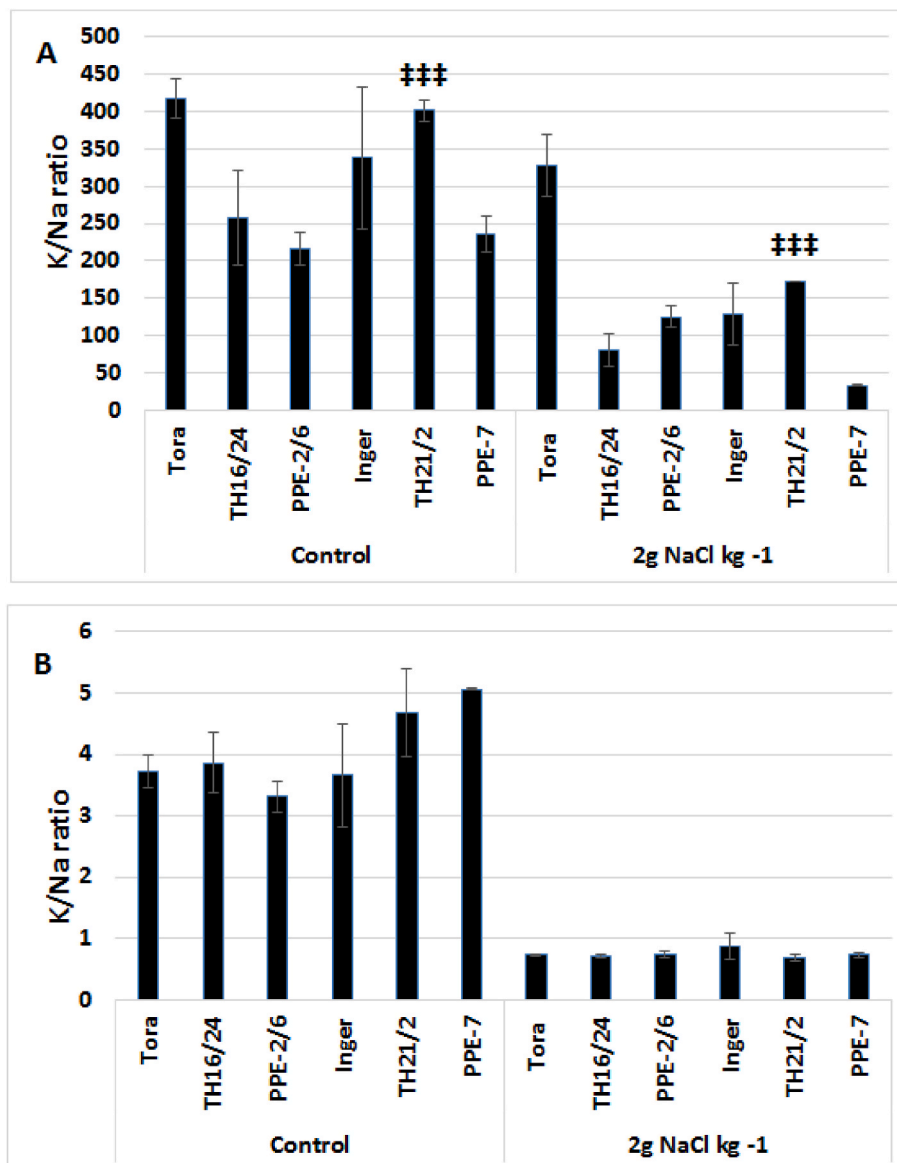


Fig. 8. The ratio of the K and Na ions in the leaf (A) and in the root (B) samples. Statistically significant events compared with diploid parent (*) or with tetraploid parent (†) are indicated a side sample labels as $p \leq 0.001$ †††, †††, $p \leq 0.01$ ††, ††, and $p \leq 0.05$ *, †.

have tested their salt tolerance in comparison to the parental diploid and the autotetraploid plants. The Mid-Parent-Heterosis (%) was also detected at high level in willow plants exposed to the stronger salt stress ($2.0 \text{ g NaCl kg}^{-1}$ (EC: 8.71 mS cm^{-1}). Despite of the fact that triploid hybrid plants from the two crossing combinations differed in the hybrid vigor levels, the presented experiments support a conclusion that heterosis breeding can insure an improved salinity tolerance for utilization of saline soil even for biomass production.

The additive interaction between salt and drought stresses required the characterization of water use efficiency of willow plants. Here, we present triploid willow hybrid genotype with significantly better water use efficiency than the parental plants. Monitoring leaf biomass weights and potassium contents, we found parallelism between these traits in comparison of triploid hybrids. The high K^+/Na^+ ratio in leaves can be a sign for saline tolerance in a genotype-dependent manner. Based on the presented results field trials has been initiated to validate salt tolerance of triploid hybrid energy willow plants.

Data availability

Data will be made available on request.

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