Influence of drought and salt stress on the growth of young *Populus nigra* 'Italica' plants and associated mycorrhizal fungi and non-mycorrhizal fungal endophytes

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Abstract

Populus nigra 'Italica' (Lombardy poplar) is a breeding cultivar of black poplar, widely used as a street tree or windbreak, often exposed to salinity and limited water availability. *Populus* roots can develop dual mycorrhizal associations with ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungi, and with non-mycorrhizal fungal endophytes (FE). The symbiotic fungi may alleviate the effects of adverse environmental conditions. We investigated the performance (growth and symbiotic associations) of one-year-old *Populus nigra* 'Italica' grown from woody cuttings in soil from natural poplar habitat and subjected to water scarcity and soil salinity (50 mM NaCl, 150 mM NaCl, 250 mM NaCl). With increasing soil salinity, a decrease in the growth parameters of the aboveground parts of the poplar plantlets and their fine roots were found; however, the roots were more resistant to the stress factors analyzed than the shoots. ECMF, AMF, and non-mycorrhizal FE were all tolerant to increased salt levels in the soil, and the ECM abundance was significantly higher under conditions of mild salinity (50 mM NaCl, 150 mM NaCl) compared to the control plants and those treated with 250 mM NaCl. Our results indicated that enhanced soil salinity increased the content of sodium and chlorine in leaves, but did not affect significantly the concentrations potassium, magnesium, calcium, phosphorus, or nitrogen. Significant accumulation of proline in leaves suggest salt stress of *P. nigra* 'Italica' treated with 250 mM NaCl and contribution of proline to the plant defense reactions.

Keywords Lombardy poplar · Arbuscular mycorrhiza · Ectomycorrhiza · Nonmycorrhizal endophytes · Proline

Introduction

Soil salinity and drought are among the major abiotic factors that significantly limit plant growth. Saline soils contain various dissolved salts, such as NaCl, Na₂SO₄, MgSO₄, CaSO₄, MgCl₂, KCl, however the most common type of salt that accumulates in the soil

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is sodium chloride (NaCl) (50–80% of the total soluble salts) (He et al. 2021; Munns and Tester 2008; Rengasamy 2002). High soil salinity increases osmotic pressure in the soil solution, causing water deficit. Most plants growing under salt stress accumulate increased concentrations of Na^+ and Cl^- in their tissues (Chen and Polle 2010; Maggio et al. 2007). Accumulation of Na^+ and Cl^- in leaves lead to damages of cellular organelles, reduction of photosynthesis due to chlorophyll degradation (Tavakkoli et al. 2010), and disturbance in cell ionic balance via limited uptake of K^+ , Ca^{2+} , and NO_3^- from the soil (Niu et al. 1995; Munns and Tester 2008).

Urban and roadside soils have been contaminated for decades with de-icing salt application in the winter (Dmuchowski et al. 2021; Gałuszka et al. 2011; Gibbs and Burdekin 1983; Hofstra et al. 1979; Křenová et al. 2018). The salt deicers (usually NaCl) cause excessive soil salinity, increased soil pH, limited availability of water and essential nutrients (Łuczak et al. 2021; Polle and Chen 2015), and has been recognized as the main reason for tree death in urban ecosystems in the Northern Hemisphere (Dmuchowski et al. 2011a, 2011b, 2021). *Populus nigra* ‘Italica’ (Lombardy poplar) is a breeding cultivar of black poplar, a homogeneous male clone, propagated vegetatively. This tall, slender tree, of columnar habit, widespread around the world, is frequently used for windbreaks and screens, and as a roadside tree, where is exposed to salinity and limited water availability (Kogawara et al. 2014; Kratsch et al. 2008).

Effects of excessive soil salinity can be minimized by symbiotic association between tree fine roots and specialized soil fungi: ectomycorrhizal (ECM) (Chen and Polle 2010; Polle and Luo 2014), arbuscular mycorrhizal (AM) (Porrás-Soriano et al. 2009; Wu et al. 2015; 2016) and non-mycorrhizal endophytic fungi (EF) (Mateu et al. 2020). Several studies indicated positive influence of inoculation of young poplar plants with ECM fungi on the growth of trees under salt stress (Chen and Polle 2010; Chen et al. 2014; Luo et al. 2009; Li et al. 2012) and drought (Beniwal et al. 2010; Danielsen and Polle 2014). Mycorrhizal fungi increase uptake of water and mineral nutrients by their plant partners receiving carbon compounds in return, and enhance plant tolerance to environmental stress, biotic and abiotic (Perez-Moreno and Read 2000; Smith and Read 2008). *Populus* spp. can develop dual mycorrhizal associations, with ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungi colonizing the same root system (e.g., Dominik 1956; Gehring et al. 2006; Karliński et al. 2010; Lodge 1989; Tyburska et al. 2013). Several factors that can affect the ratios of ECM/AM in dual mycorrhizal colonization of poplars have been reported: tree genotype (Gehring et al. 2006; Karlinski et al. 2010; Khasa et al. 2002; Tyburska et al. 2013), the age of trees (Dominik 1956; Goncalves et al. 2001), the soil depth (Karliński et al. 2010), and soil moisture (Gehring et al. 2006; Neville et al. 2002; Truszkowska 1953). It has been proven that the degree to which poplar cultivars tolerate salt stress is closely related to the ionic balance of the plants, and that the adaptation of *Populus* species and hybrids to salinity depends on the ability to avoid a high K^+/Na^+ ratio (Chen and Polle 2010; Polle and Chen 2015; Yu et al. 2020).

Under salt stress ectomycorrhizal symbiosis can increase water transport capacity (Marjanović et al. 2005), preserve water balance at an optimal level (Danielsen and Polle 2014), reduce uptake of Na^+ and Cl^- by root (Chen et al. 2002, 2003), inhibit K^+ loss, and maintain a high K^+/Na^+ balance (Chen and Polle 2010; Chen et al. 2014; Langenfeld-Heyser et al., 2007; Li et al. 2012; Luo et al. 2009; Ma et al. 2014). Different ECM fungal species and strains can vary in tolerance to salinity (e.g. Hryniewicz et al. 2015; Ma et al. 2014; Thiem et al. 2018), and variable efficiencies of different AM fungi in the alleviation of salt stress and drought stress were reported by Porrás-Soriano et al. (2009). Mycorrhizal fungi, native of urban environment, can be effective symbionts of mature *Populus* spp.,

however lower colonization of tree roots by ECM and AM fungi has been found in strongly disturbed urban habitats (i.e. Nielsen and Rasmussen 1999; Bainard et al. 2011; Tyburska et al. 2013). The symbiotic activity of native mycorrhizal fungi in disturbed urban sites can be supported by basic tillage practices, such as enrichment of the soil with organic matter. With a very low content of mycorrhizal propagules in soil, the use of an inoculum of salt tolerant mycorrhizal fungi may be appropriate (Kumar et al. 2007). In response to salinity and water deficit, plants can accumulate proline—osmotically active compatible substance, that is known to increase plant tolerance against salt-induced water loss (Bandurska 1991; Stolarska et al. 2008) and is a sensitive metabolic indicator of water deficit in plants (Chen and Polle 2010; Dar et al. 2016; Stolarska et al. 2008; Verbruggen and Hermans 2008).

Although *P. nigra* 'Italica' is exposed to soil contamination by salt when grown along streets or roads, the tolerance of this cultivar to salinity is unclear. To date the influence of soil salinity and drought on growth of this poplar cultivar and its symbiotic relationships with fungi have not been yet investigated. The aims of this study was to investigate the effect of water deficit and salt stress, using three levels of sodium chloride, on: 1) the growth of young *P. nigra* 'Italica', 2) the degree of root colonization by fungi native to a riparian forest: ectomycorrhizal, arbuscular mycorrhizal (AM), and non-mycorrhizal fungal endophytes (EF), 3) nutritional status of the poplar plants. Concentration of proline in leaves was determined as indicator for water and salt stress. The research was based on pot experiments. The use of the cultivar *P. nigra* 'Italica', which is unified genetic material, allows the impact of genetic diversity on the results to be avoided. We expected that the symbiotic fungi able to form effective associations with the *P. nigra* 'Italica' under water deficit and salinity would have a positive effect on the plant nutrition and growth.

Material and methods

Plant materials and growing conditions

One clone of *Populus nigra* 'Italica' was selected from the poplar collection of the Institute of Dendrology of the Polish Academy of Sciences in Kórnik, Poland (52°14'30''N, 17°05'44''E). One-year-old shoots were harvested in the middle of January and stored in polyethylene bags at 4 °C until planting. In the first week of April (2009 and 2011) uniformly sized cuttings (25 cm long, diameter 15 ± 2 mm, with 4 vegetative buds) were planted singly in 2 L plastic pots ($\varnothing$ 12 cm) in a soil:perlite mixture (3:1, v/v), and maintained under greenhouse conditions. The soil was sampled at a natural poplar stand (willow-poplar riparian forest) near Chełmno, Kujavian-Pomeranian Province, Poland (53°21'25''N 18°24'25''E). Basic soil parameters were as follows: C_{org} 4.14%, N_{tot} 0.37%, P_{og} 281 mg kg⁻¹, P_{Olsen} 4.52 mg kg⁻¹, pH 7.7. From April to July the plants were cultivated under high soil moisture (80% field capacity). Field capacity (FC) was controlled by weight. The potted plants were then divided into five experimental variants (12 pots each): I—control: well-watered (200 mL water every third day), II—drought (200 mL water every sixth day), III—low salinity (200 mL of 50 mM NaCl solution every third day), IV—medium salinity (200 mL of 150 mM NaCl solution every third day), V- high salinity (200 mL of 250 mM NaCl solution every third day). The FC in variants I, III, IV and V was maintained in the range of 60–80%, and in the variant II the FC was 25–30%. The experiment was terminated after six weeks of treatment.

Growth characteristics and measurements of shoots

Ten plants from each variant were taken for the evaluation of growth parameters. The above-ground parts of the plants were separated from the roots, and shoots and leaves were separated from the cuttings. The number and length of shoots, number of leaves, and the fresh masses of shoots and leaves were measured. Shoots and leaves were dried for 48 h and dry weights were recorded. Selected leaves were designated for scanning and chemical analysis immediately after they were separated from the shoots. Fresh leaves (VI, VII, and VIII leaves counted from the apex of the shoot) were scanned using the digiShape 1.9.177 program. The perimeters and surfaces of the leaves were measured.

Determination of proline

Free proline was measured as described by Bates et al. (1973). Proline was determined after its extraction with sulphosalicylic acid, and its reaction with ninhydrin. Absorbance at 520 nm (CECIL spectrophotometer, CE 2011 2000 SERIES) was measured for each sample, using toluene as a reference. A standard curve for proline was used for calibration. Free proline quantity was expressed as $\mu\text{mol}\cdot\text{g}^{-1}$ FW.

Determination of leaf chemical composition

The leaves of the poplar plantlets were washed with demineralized water to remove surface impurities, and dried at 65 °C for 48 h. The total concentration of individual mineral components in the leaves was measured (K, Na, Mg, Ca, P, N, Cl). Chemical analyses were performed in a certified laboratory. The total concentrations of K, Na, Mg, and Ca were determined by atomic absorption spectrophotometry (Varian AA280FS). In order to verify the analyses, certified material was used (M3) (Steinnes et al., 1997). The concentration of phosphorus was determined colorimetrically (HACH LANGE DR 3800 colorimeter). In order to verify the analyses, certified material was used (ISE sample 912). Nitrogen analysis was performed using the Kjeldahl method, with the Kjeltac 2300 Analyzer Unit (FOSS TECATOR), according to the AN 300 application. Verification of the analyses was made using certified material (ISE sample 912). Chloride analysis was performed using ion chromatography (DIONEX ICS 1100, with an AS9-HC column). In order to verify the analyses, certified material was used (IPE sample 993).

Morphometric measurements of the root system

The plantlet root systems were thoroughly cleared from the soil and washed on a sieve under tap water, then rinsed with distilled water. The roots were precisely distributed on the scanner cuvette. If the root was too large, it was divided into two or more trials, each of which were scanned separately, with the results being summed. The computer program radixNova1.0 was used for scanning the following parameters: area of the projection of the whole root system; surface of all roots; volume of all roots; total root length; mean root diameter; number of root tips. Moreover, root surface, root volume, root length, and the number of root tips were analyzed in four thickness classes

(I— $\varnothing \leq 1$ mm, II— $\varnothing = 1\text{--}2$ mm, III— $\varnothing 2\text{--}3$ mm, IV — $\varnothing > 3$ mm). After scanning, the roots were used for the determination of mycorrhizal colonization.

ECM, AM, and endophyte colonization assessment

Fine roots (< 2 mm) were separated from coarse roots under a stereomicroscope (Carl Zeiss Stemi 2000-C). Root samples of 250 mg weight were cut into 1 cm length fragments, then stained according to the modified method of Kormanik and McGraw (1982). Roots were cleared in 10% KOH for 35 min at 95 °C, then bleached for one hour in alkaline H_2O_2 at room temperature and stained with 0.05% trypan blue in lactoglycerol. The stained roots were stored in 1:1:1 (v/v) glycerol:lactic acid:water solution. Mycorrhizal colonization was assessed using the intersection method according to McGonigle et al. (1990), at $200\times$ magnification. Root length colonization (%RLC) by AM symbiotic fungi (arbuscules, vesicles, and internal hyphae), ECM (root tips covered by fungal mantle), and non-mycorrhizal endophytes was determined. In total, approximately 200 cm of fine roots were analyzed per root sample.

Statistical analyses

The study data were the means of two independent experiments carried out in 2009 and 2011. Data were analyzed using STATISTICA version 13.1 (StatSoft®Poland). Statistical differences ($p < 0.05$) in the experimental data were established using one-way analysis of variance (ANOVA). Mean values of parameters were separated using Tukey's honestly significant difference test. In all of the figures, the error bars represent standard deviation ($\pm$ SD) of the means. Before the analyses, data were assessed for normality (Shapiro–Wilk test) and homogeneity (Bartlett's test), and the proportional data were transformed according to the Bliss formula (Snedecor and Cochran 1976): $x = \arcsin \sqrt{(n\%/100) \times 180/\pi}$, where $n\%$ is the percentage value. Figures present non-transformed data.

Results

Growth characteristics and measurements of shoots

The one-way ANOVA showed that drought stress and salt stress significantly influenced the number of leaves, the fresh and dry weights of leaves, and the dry weight of shoots (Table 1). The most sensitive growth parameter was the fresh weight of the leaves ($F=10.32$). A significant reduction in the number of leaves, and in the fresh and dry weights of leaves, was observed in the poplar plantlets treated with increased concentrations of NaCl (150 mM and 250 mM), whereas the lowest concentration of NaCl (50 mM) and the limited watering did not significantly influence the growth parameters of the poplar leaves (Table 1). Mean values of the fresh and dry weights of leaves of the control plants (well-watered), the plants treated with limited watering, and the plants under low salt stress (50 mM NaCl) did not differ significantly (Table 1). Mean leaf perimeter and leaf area were not affected significantly by water limitation and salt stress (Table 1). Mean shoot fresh weight of the poplar plants subjected to drought and salt stress (150 mM NaCl and 250 mM NaCl) was half that of the control plants; however, the differences were not

Table 1 Growth parameters of shoots of one-year-old *Populus nigra* 'Italica' grown under control conditions or after exposure to drought or salt stress: 50 mM NaCl, 150 mM NaCl, 250 mM NaCl. Data are expressed as mean $\pm$ SD ($n = 18$). Different letters in columns indicate significant differences ($p < 0.05$). One-way ANOVA was performed for the shoot traits studied to show significance of differences among the treatments

Growth parameters	Treatment				One-way analysis of variance	
	Control	Drought	50 mM NaCl	150 mM NaCl	250 mM NaCl	<i>F</i> <i>p</i>
Number of shoots	1.64 $\pm$ 0.59	1.89 $\pm$ 0.58	2.00 $\pm$ 0.84	2.00 $\pm$ 0.84	2.17 $\pm$ 0.86	1.2055 0.3145
Length of stems [cm]	43.58 $\pm$ 16.55	33.72 $\pm$ 12.04	36.35 $\pm$ 17.01	36.18 $\pm$ 12.36	31.91 $\pm$ 12.25	1.7557 0.1454
Fresh weight of stems [g]	6.23 $\pm$ 1.69	3.07 $\pm$ 1.14	4.47 $\pm$ 2.53	3.46 $\pm$ 1.23	3.05 $\pm$ 1.25	2.4537 0.0519
Dry weight of stems [g]	1.40 $\pm$ 0.68 ^{ab}	1.16 $\pm$ 0.47 ^{ab}	1.69 $\pm$ 0.95 ^b	1.23 $\pm$ 0.37 ^{ab}	1.11 $\pm$ 0.43 ^a	2.6560 0.0384
Number of leaves	33.67 $\pm$ 10.41 ^b	30.19 $\pm$ 9.27 ^{ab}	33.61 $\pm$ 11.95 ^b	27.17 $\pm$ 10.11 ^{ab}	20.78 $\pm$ 10.24 ^a	4.7721 0.0016
Fresh weight of leaves [g]	6.07 $\pm$ 2.28 ^{cd}	4.83 $\pm$ 1.71 ^c	5.92 $\pm$ 2.49 ^{cd}	3.51 $\pm$ 1.90 ^b	2.50 $\pm$ 1.71 ^{ab}	10.3228 0.0000
Dry weight of leaves [g]	1.35 $\pm$ 0.59 ^b	1.07 $\pm$ 0.32 ^{ab}	1.61 $\pm$ 1.17 ^b	1.02 $\pm$ 0.41 ^{ab}	0.74 $\pm$ 0.37 ^a	4.6266 0.0020
Perimeter [mm]	268.30 $\pm$ 43.84	251.28 $\pm$ 35.95	261.94 $\pm$ 23.44	249.94 $\pm$ 27.71	245.60 $\pm$ 40.24	1.2876 0.2813
Area [mm ²]	2544.50 $\pm$ 635.61	2305.52 $\pm$ 563.60	2496.35 $\pm$ 347.85	2367.60 $\pm$ 407.43	2343.53 $\pm$ 620.56	0.6888 0.6017

statistically significant. The stress of salinity caused statistically significant differences in the dry weight of the annual shoots of the poplars. The plants watered with 50 mM NaCl solution had the highest shoot dry mass (1.69 g), which was significantly greater than the plants treated with 250 mM NaCl (1.11 g). Poplar shoots in the other variants did not differ significantly in terms of dry weight, but with an increase in the concentration of saline solution, a trend of decrease in the mean dry weight of the shoots was observed (Table 1).

Concentration of proline

The concentration of proline in leaves of the poplar plantlets varied from $0.35 \mu\text{mol g FW}^{-1}$ in the control plants, up to $2.6 \mu\text{mol g FW}^{-1}$ in plants treated with the solution with the highest concentration of NaCl (250 mM) (Fig. 1). Proline concentration in the leaves of plants under water deficit conditions was higher than in the control plants ($0.4 \mu\text{mol gFW}^{-1}$), but this was not significant (Fig. 1). An upward trend in the concentration of proline in poplar leaves occurred with increasing salt level in the watering solution. The treatment with 50 mM NaCl solution resulted in a slightly higher proline concentration in the leaves ($0.45 \mu\text{mol g FW}^{-1}$). Proline concentration in the leaves of plants treated with 150 mM NaCl was three-fold that of the control ($1.3 \mu\text{mol g FW}^{-1}$), and seven-fold that of leaves from plants watered with 250 mM NaCl.

Results of chemical analysis of leaves

The contents of selected elements in leaves of poplars under drought stress and different levels of salinity stress are shown in Table 2. Salinity stress increased the content of sodium and chloride in leaves, but did not affect the concentrations of potassium, magnesium, calcium, phosphorus, or nitrogen. A higher level of sodium in leaves (nine-fold and 13-fold compared to the control) was recorded in the plants treated with 150 mM and 250 mM NaCl,

Fig. 1 The concentration of proline in the leaves of one-year-old *Populus nigra* 'Italica' seedlings under control conditions, or after exposure to drought or salt stress: 50 mM NaCl, 150 mM NaCl, 250 mM NaCl. Data are expressed in $\mu\text{moles per gram of fresh leaf weight}$ (mean $\pm$ SD) ($n = 12$). Different letters indicate significant differences (Tukey's tests, $p < 0.05$)

Table 2 The concentration of selected elements (K, Na, Mg, Ca, organic P, organic N, Cl) in the dry mass of leaves of one-year-old *Populus nigra* 'Italica' under control conditions or after exposure to drought or salt stress: 50 mM NaCl, 150 mM NaCl, 250 mM NaCl. Data are expressed as mean $\pm$ SD ($n = 12$). Different letters in columns indicate significant differences ($p < 0.05$). One-way ANOVA was performed for the elements analyzed to show significance of differences among the treatments

Elements	Treatment					One-way analysis of variance	
	Control	Drought	50 mM NaCl	150 mM NaCl	250 mM NaCl	F	p
K [g/kg]	17.857 $\pm$ 1.474	13.793 $\pm$ 8.828	15.249 $\pm$ 7.364	16.114 $\pm$ 8.016	16.841 $\pm$ 6.907	0.0969	0.9790
Mg [g/kg]	4.222 $\pm$ 0.664	3.821 $\pm$ 0.204	4.685 $\pm$ 0.870	4.226 $\pm$ 0.238	4.070 $\pm$ 0.272	0.7251	0.6111
Ca [g/kg]	27.574 $\pm$ 4.098	25.395 $\pm$ 3.311	35.604 $\pm$ 6.970	33.631 $\pm$ 3.78	30.031 $\pm$ 2.695	2.1101	0.2169
P [g/kg]	0.422 $\pm$ 0.057	0.358 $\pm$ 0.092	0.328 $\pm$ 0.060	0.310 $\pm$ 0.053	0.347 $\pm$ 0.202	0.3109	0.8597
N [%]	2.86 $\pm$ 0.38	2.91 $\pm$ 0.06	2.51 $\pm$ 0.27	2.34 $\pm$ 0.05	2.49 $\pm$ 0.11	2.6421	0.1577
Na [%]	0.064 $\pm$ 0.010 ^a	0.062 $\pm$ 0.022 ^a	0.074 $\pm$ 0.040 ^a	0.602 $\pm$ 0.312 ^b	0.845 $\pm$ 0.587 ^b	427.3976	0.0000
Cl [%]	0.79 $\pm$ 0.20 ^a	0.67 $\pm$ 0.12 ^a	3.16 $\pm$ 0.34 ^b	6.68 $\pm$ 0.46 ^c	6.84 $\pm$ 0.10 ^c	233.7180	0.0000
K/Na	279.06	222.42	206.08	26.77	19.93		
Mg/Na	65.969	61.629	63.311	6.105	4.816		
Ca/Na	430.84	488.36	481.13	55.86	35.54		

respectively; this was statistically significant ($p < 0.05$). The leaf concentration of chlorine was significantly higher ($p < 0.05$) in plants under salt stress: four times higher in plants treated with 50 mM NaCl solution, and eight times higher in plants treated with 150 mM NaCl and 250 mM NaCl, compared to the control plants. In the case of drought stress, a slight decrease in the concentrations of sodium and chlorine in leaves was recorded (Table 2).

Morphometric measurements of the root system

The one-way ANOVA showed that drought stress and salt stress influenced the total number of root tips; this was statistically significant. The number of root tips $\phi \leq 1$ mm, and the growth parameters of the roots of thickness > 3 mm (surface, volume, length) are shown in Table 3 (Supplementary Information). The most sensitive growth parameter was the number of tips of the thinnest roots ($\phi \leq 1$) ($F = 4.28$). Overall, the morphometric parameters of the roots decreased with increasing salt concentration, and the adverse effects of drought stress were similar to the effects of high salinity (250 mM NaCl). Water deficit and the highest salinity treatment (250 mM NaCl) resulted in a statistically significant decrease (twofold) in the number of root tips $\phi \leq 1$ mm. The finest roots ($\phi \leq 1$ mm) had the largest surface area among all thickness classes (468 cm²). In the samples tested, as their root diameter increased, their surface area decreased. The results show that drought stress and high salt concentrations (250 mM NaCl) resulted in reduced root surface area, volume, and length in all thickness classes, although the parameters were statistically different only in the thickness class $\phi > 3$ mm.

Colonization by AM, ECM, and non-mycorrhizal endophytes

Dual mycorrhizal colonization of poplar fine roots in all variants of the experiment were found (Fig. 2). The ANOVA showed a statistically significant effect of drought stress and

Fig. 2 Mycorrhizal colonization of the roots of one-year-old *Populus nigra* ‘Italica’ seedlings under control conditions or after exposure to drought or salt stress: 50 mM NaCl, 150 mM NaCl, 250 mM NaCl. Root length colonization (%RLC): ECM—ectomycorrhiza; AM—arbuscular mycorrhiza; E—endophytes. Data are expressed as %mean $\pm$ SD (n = 10). Different letters indicate significant differences (Tukey’s tests, $p < 0.05$)

Fig. 3 The proportion of arbuscular mycorrhiza structures in the root colonization of one-year-old *Populus nigra* ‘Italica’ seedlings under control conditions, or after exposure to drought or salt stress: 50 mM NaCl, 150 mM NaCl, 250 mM NaCl. Root length colonization (%RLC): A—arbuscules; V—vesicles; H—hyphae. Data are expressed as %mean $\pm$ SD (n = 10). Different letters indicate significant differences (Tukey’s tests, $p < 0.05$)

salt stress on the degree of ectomycorrhizal colonization (% ECM) and on the proportion of arbuscules (% A) in root colonization (Fig. 3). The highest percentage of the total AM colonization was found in roots of the control, well-watered plants (25% RLC). The level of AM colonization was greater than that of ECM colonization in the roots of the control plants, and also in plants under the influence of drought (AM: ECM=2.0) (Fig. 2). AM colonization had a tendency to decrease in the roots of plants influenced by drought and salt stress; however, the mean degree of AM colonization did not differ significantly between the treatments (Fig. 2). At the low and the medium salt treatments (50 mM and 150 mM NaCl, respectively), ECM colonization of the roots of plants influenced by drought and by the highest salt stress (250 mM NaCl) was significantly higher compared to the well-watered control plants, and dominated over AM colonization (Fig. 2). Drought stress and salt stress did not affect the degree of root colonization by non-mycorrhizal endophytic fungi (Fig. 2). Although soil water deficiency and increased salinity did not

cause statistically significant changes in the total colonization of plant roots by AM fungi (Fig. 2), a statistically significant reduction in the proportion of arbuscules in the roots of plants treated with drought and salinity stress was observed (Fig. 3).

Discussion

This study indicated a significant degree of accumulation of Na^+ and Cl^- in the leaves of *P. nigra* ‘Italica’ with increasing NaCl concentration in the soil (Table 2), after six weeks of the treatment with 250 mM NaCl. Osmotic stress due to salinity is a major cause of decreased plant growth and vegetative development. The first symptoms of salt stress that we noticed in this study were the edges of the leaf blade drying out, and older leaves dropping. Mao et al. (2010) also observed drying of the tips and edges of the leaf blade, and a significant loss of leaves (up to 50%) of two-year-old black poplar plants treated for four weeks with 100 mM NaCl solution. Significant differences in susceptibility to salt stress were found in various species of poplars, in the form of growth inhibition and leaf damage, as well as physiological disorders related to metabolic functions (Chen and Polle 2010; Polle and Chen 2015; Sixto et al. 2005).

In our study, water deficit and salt stress did not have a significant influence on the concentrations of macronutrients (N, P, K, Mg, Ca) in the leaves of *P. nigra* ‘Italica’, however some slight increases in Ca^{2+} concentration in the leaves of plants exposed to salinity stress were found (9–29% compared to the control plants), and only a slight decrease in the K^+ concentrations (7.5–15%) was recorded with the accumulation of Na^+ in leaves (Table 2). Moreover, this study showed dual mycorrhizal colonization of *P. nigra* ‘Italica’ roots by ECM and AM fungi, and by non-mycorrhizal endophytic fungi, and indicated that symbiotic fungi occurring in the soil from natural habitat of poplars, were effective symbionts for young poplar plants, able to survive under water deficit and salt stress (Figs. 2, 3). The relationship between Na^+ accumulation in the leaves, and its negative effect on leaf health and shoot growth, has been confirmed in several poplar species (Chen et al. 2001, 2002, 2003). Salt-sensitive poplars (*P. tomentosa*, *P. popularis*, *P. × euramericana* cv. I-214) had higher rates of Na^+ and Cl^- uptake and transport to shoots than salt-tolerant *Populus euphratica*, a species that can restrict radial salt transport in its roots by accumulating salt ions in its cortical vacuoles, and is able to maintain stable K^+ concentrations in plant tissues in the presence of high external Na^+ concentrations Chen et al. (2002), Chen et al. (2003).

Our results showed that drought and increased soil salinity (150 mM and 250 mM NaCl in the watering solution) reduced the shoot and root growth of *P. nigra* ‘Italica’ seedlings, but the reduction of plant growth was statistically significant only at the highest salt concentration (250 mM NaCl). At a low NaCl concentration (50 mM), the growth parameters of the poplars were equal to, or even significantly greater, compared to the control and to plants exposed to drought (Tables 1, 3). Similarly, Bolu and Polle (2004) found that young salt-sensitive *Populus x canescens*, exposed to low saline concentration (25 mM NaCl) in hydroponic culture had a higher biomass increment compared to the control plants. A stimulation of growth of *P. nigra* ‘Italica’ under low salinity observed in our study may be related with the increased concentration of Ca^{2+} ions and $\text{Ca}^{2+}/\text{Na}^+$ ratio in the leaves (Table 2). Calcium is known to ameliorate Na^+ toxicity (Chen and Polle 2010). Under NaCl stress Ca^{2+} improves absorption of ions across cell membranes, influence the transfer of sugars, and inhibits K^+ loss. Therefore Ca^{2+} is important for the maintenance of K^+/Na^+ balance in plants and is a crucial regulator of growth and development in plants (Chen

and Polle 2010; Hepler 2005; Shabala et al. 2006; Zhao et al. 2021). In poplars after exposure to salt, initially increased Ca^{2+} uptake was observed, both in the salt-tolerant species *P. euphratica* (Chen et al., 2001), and in the salt-sensitive hybrid *P. x canescens* (Ottow et al. 2005; Ma et al. 2014). However, decreases in Ca^{2+} concentration was found after prolonged salt treatment (Ottow et al. 2005) and after exposure to high NaCl (Ma et al. 2014). Selected ECM fungi associated with poplar roots have been shown to release Ca^{2+} , and promote the establishment of K^+/Na^+ homeostasis under salt treatment (Sun et al. 2009; Li et al. 2012).

In our study, the most sensitive indices of the responses of *P. nigra* ‘Italica’ to drought and salt stress were the biomasses of shoots and leaves (Table 1), the length, surface, and volume of fine roots ($\phi \leq 2$ mm), and the number of the total and the finest root tips ($\phi \leq 1$ mm), potentially mycorrhizal (Table 3 – Supplementary Information). These growth parameters decreased in plants grown under water deficit and at high levels of salt in the soil (250 mM NaCl). In the current work the salt and water stresses did not cause statistically significant changes in the degree of total colonization of the *P. nigra* ‘Italica’ roots by AM fungi. In contrast, a considerable decrease in the AM colonization in plant roots under salt stress has been reported by Ye et al. (2019). Contrary to our results, a significantly lower degree of AM was found in very dry and in flooded soils compared to wet soils, both in *Populus* and *Salix* trees (Lodge 1989) and in grasses (Miller 2000). In our study only the contribution of arbuscules was significantly lower in the roots of plants treated with drought and salinity than in the control roots (Figs. 2, 3). Although both ECM and AM symbionts of *P. nigra* ‘Italica’ were able to survive under drought and salt stress, AM clearly dominated over ectomycorrhiza in well-watered control, in plants grown under drought stress, and in plants under the highest salinity treatment (Fig. 2). ECM colonization significantly increased in *P. nigra* ‘Italica’ plants treated with the low and medium salinity (50 and 150 mM NaCl), proving that the ECMF were more tolerant to salinity than AMF (Fig. 2). Ectomycorrhiza affects water-transporting proteins (aquaporins) in the fine roots of the host (Landhäusser et al. 2002; Muhsin and Zwiazek 2002a, Marjanović et al. 2005); however, the mechanisms regulating the flow of water in root cells colonized by mycorrhizal fungi remain unclear and subject to discussion.

Our results indicated tolerance non-mycorrhizal endophytic fungi colonizing roots of *P. nigra* ‘Italica’ to drought and salt stress. The degree of the colonization did not change with salinity of soil (Fig. 2). Similarly, previous studies have shown high tolerance of non-mycorrhizal endophytic fungi associated with the roots of *Populus* spp. to elevated concentrations of toxic heavy metals in soil and can compete with AM fungi for poplar root colonization under stress conditions (Karliński et al. 2010). Endophytes may direct plant metabolism towards the secretion of soluble sugars, amino acids such as proline and polyols, and alkaloids, all of which confer wall elasticity and osmotic adjustment during drought conditions (Mengistu 2020).

P. nigra ‘Italica’ influenced by an enhanced concentration of NaCl (250 mM) accumulated in leaves similar concentrations of Na^+ as salt sensitive *P. popularis* and *P. euramericana* cv. I-214 (Chen et al. 2002; 2003), and approximately two-fold higher Na^+ concentration when compared to *P. euphratica*, a species that is tolerant to enhanced salinity, as analyzed by Chen et al. (2002). Cl^- toxicity is considered to be more harmful to some woody plants than Na^+ toxicity (Munns and Tester 2008). In sensitive poplar cultivars, excessive Cl^- accumulation causes damage to plant leaves, and can reduce plant growth (Chen et al. 2001, 2002, 2003). An increase in the accumulation of Cl^- ions in the leaves of salt-treated *P. nigra* ‘Italica’ (Table 2), fourfold higher than in salt-tolerant *P. euphratica* and 1.7–1.9-fold higher than in the salinity-sensitive poplar cultivars, *P. popularis* and *P.*

euramericana cv. I-214 as reported by Chen et al. (2002). The capacity of *Populus* plants for the exclusion of Na^+ and Cl^- is an important mechanism of salinity tolerance (Chen et al. 2001). Plants activate also other defense mechanisms that involve the production of specific defense compounds.

Under stress conditions, salt-tolerant plants accumulate more free proline in the leaves than do susceptible cultivars, including *Populus cathayana* (Yang et al. 2009), and *Salix viminalis* (Stolarska et al. 2008). In our study, increases in proline concentration were found in the leaves of *P. nigra* ‘Italica’ treated with 150 mM and 250 mM NaCl solution (3.7-fold and 7.4-fold over the control plants, respectively) (Fig. 1). Similar increase in proline content was found by Watanabe et al. (2000) in young leaves of tolerant to salt stress *Populus euphratica*, treated with 250 mM NaCl, (eightfold compared to the control). The similarity in proline accumulation between salt-tolerant *P. euphratica* and *P. nigra* ‘Italica’ suggests that enhanced proline accumulation contributes to the osmotic adjustment of the both poplar cultivars under salt stress. Proline is often reported to accumulate in mycorrhizal plants exposed to various types of abiotic stresses (e.g. Sharifi et al. 2007; Xia 2021), but some studies have shown a slightly higher amount of proline in non-mycorrhizal than in mycorrhizal plants grown under stress (Bhosale and Shinde 2011; Jahromi et al. 2008). In our study proline accumulation is due to salinity stress but not to abundance of ECM and AM colonization (Figs. 1, 2). The concentration of proline can also be increased by drought stress, and accumulates more in drought-tolerant than in drought-sensitive varieties poplars. The water deficit applied in our work did not increase the proline concentration in *P. nigra* ‘Italica’ leaves (Fig. 1).

Only a few published works on the influence of salinity on woody plants have included *P. nigra* ‘Italica’ as a study species. *P. nigra* ‘Italica’ has been indicated as a salt-tolerant species (Lumis et al. 1973), or as a poorly salt-tolerant cultivar (Carpenter 1970; Pellett 1972). Similarly, the salt sensitivity of *P. nigra* ‘Italica’ has been reported by Kratsch et al. (2008), and Kogawara et al. (2014), who demonstrated that *P. nigra* ‘Italica’ has a low degree of tolerance both, to salinity and drought. The results of our research indicated that young plants of *P. nigra* ‘Italica’, supported by ECMF, AMF and non-mycorrhizal FE, can to maintain the ionic balance necessary for protection against stress and grow well under drought and mild and medium salinity.

Conclusion

Our results showed that under elevated levels of NaCl, the leaves of *Populus nigra* ‘Italica’ accumulated high amounts of salt ions (Na^+ , Cl^-), similar to the poplar species and hybrids that are susceptible to salt stress. However, *P. nigra* ‘Italica’ is able to activate some defense mechanisms in response to stress conditions, such as increasing its Ca^{2+} concentration, and enhancing the production of proline at levels that are similar to that recorded in poplars that are tolerant to salinity. These results indicated that well-developed mycorrhizal associations of *P. nigra* ‘Italica’ with efficient mycorrhizal fungi increased the salt tolerance of this poplar cultivar.

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Drafting the article and designing of the figures was done by Magdalena Kulczyk-Skrzeszewska. Critical revision of the article was performed by Barbara Kieliszewska-Rokicka. Both authors contributed to the final version of the manuscript.

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Availability of data and materials The datasets generated and analyzed during the current study are available from the first author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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