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PHYTOTOXICITY OF SODIUM CHLORIDE TOWARDS COMMON DUCKWEED (*LEMNA MINOR* L.) AND YELLOW LUPIN (*LUPINUS LUTEUS* L.)

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Abstract: Salinity has adverse effects on plants and is one of the causes of environment degradation. Plants have developed many defensive mechanisms, protecting them from sodium chloride (NaCl), including accumulation of osmoprotective compounds, which maintain osmotic balance, protect cell structure and enzymes. In the current study, we investigated the effects of salinity resulting from a range of sodium chloride concentrations (from 0 to 400 mM) on the growth of common duckweed (*Lemna minor* L.) and yellow lupin (*Lupinus luteus* L.). Increasing concentration of sodium chloride decreased the area of common duckweed leaves. At the highest applied salt concentration, the decrease of leaf area was associated with leaf chlorosis. In yellow lupin, the increasing sodium chloride concentration inhibited root and stem elongation. The highest tested NaCl concentration of 400 mM completely stopped elongation of yellow lupin shoots. The content of cyclitols and soluble carbohydrates in plant tissues was evaluated as well. Cyclitols (D-*chiro*-inositol and D-pinitol), as well as soluble carbohydrates (glucose, fructose and sucrose) were detected in common duckweed tissues. Yellow lupin seedlings also contained cyclitols – D-pinitol, *myo*-inositol and D-*chiro*-inositol – and soluble carbohydrates – glucose, galactose and sucrose. The content of osmoprotectants in plant tissues, especially sucrose and cyclitols, increased with increasing concentration of sodium chloride in the soil. The results indicate that the content of cyclitols and soluble carbohydrates in plant tissues can be an indicator of plant response to salinity stress.

INTRODUCTION

Soil salinity adversely affects vegetation, and is one of the factors in degradation of environment. About 77 mln hectares of the world's soils are salinized due to natural factors [7, 23]. Altogether in the EU, there are from 1 to 3 mln hectares of salinized grounds, and eventually their salinity turns them into deserts [37].

It is estimated that about 20% of irrigated areas in the world is affected by salinity [42]. They amount to 15% of the total of agricultural lands [35], out of which 43 mln hectares are definitely salinized, often as a result of sprinkling them with salt water or only partly desalinated water [7]. Inappropriate irrigation and surface runoffs lead to the accumulation of excessive amounts of sodium ions in the environment [42].

Environment salinity is defined as higher than natural content (electroconductivity higher than 4 ds/m) of soluble salts which appear in the environment. Salts are found in soil also due to evaporation, thus the problem of salinity refers to areas characterized by low atmospheric precipitation or high evapotranspiration in coastal regions as well as hard coal and copper mining areas [3]. In saline soils the content of soluble salts exceeds 0.2% and can reach up to 100 cm into the ground which is tantamount to about 40 mM concentration of sodium chloride (NaCl). In order to absorb water from salinized soil, plants have to overcome additional osmotic pressure of 0.2 MPa [23].

In fresh water reservoirs the chlorides are also found [39]. They are especially toxic towards fresh water hydrophytes [21]. Increasing salinity hinders growth and development of fresh water flora and fauna, especially in reservoirs situated lowest in a given area [9].

Apart from climatic, hydrologic, geologic, geographic and weathering related conditions, road works with the use of sodium chloride are reported to be the cause of salinity [39, 19]. When dry, this compound can be carried by winds even as far as 100 meters away from transportation routes [18, 19]. Moreover, sodium chloride remains in the soil throughout the whole vegetation season, impeding water absorption in plants. Sodium chloride is used to de-ice roads and in one season 10 mln tons are applied for this purpose in the USA [39], and 5–15 tons per 1 km in Sweden [18]. Maintaining roads in winter degrades about 250–500 thousand hectares of agricultural lands the world.

Excessive sodium chloride concentration lowers the osmotic potential of the environment, impedes water absorption abilities of plants (the so called physiological drought) [46, 43, 28], disturbs transpiration processes, as well as ionic regulation, etc. [15, 28]. High concentrations of sodium chloride in soil can lead to the death of plants. Yet, it has been reported that small amounts of NaCl can stimulate plant growth [36]. A plant tolerance to sodium chloride depends on plant species. Young plants, especially at the germination and active growth stage, are most sensitive due to rapid cell division processes. Such plants need an appropriate amount of water to grow and develop normally [32]. Plants have developed many resistance mechanisms to NaCl, e.g. accumulation of osmoprotective compounds, which maintain osmotic balance, protect cell structures and enzymes against dehydration. Such compounds are represented among others by some amino acids, soluble carbohydrates and cyclitols.

It was the purpose of this paper to define an impact of sodium chloride in water on the growth and development of common duckweed (*Lemna minor* L.) and the impact of soil NaCl on the growth and development of yellow lupin (*Lupinus luteus* L.). Moreover, the effect of salinity stress on the contents of cyclitols and soluble carbohydrates in plant tissues were assayed.

MATERIAL AND METHODS

Seed germination and root growth test

Seeds of yellow lupin (*Lupinus luteus* L.) cv. Mister were germinated for seven days in Phytotoxkit™ plates (MicroBio Test Inc., Belgium). Germination and seedling growth occurred at 8/16 hours photoperiod, with 140 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} irradiance PAR, and temperatures of 20°C at day and 16 at night in incubator ALL -Round -AL 185 -4.

Ninety ml of soil (sand, vermiculite, peat 1:0.3:1, v/v/v) were placed in plastic microbiotest plates. Germination was scored after 9-d inhibition, when the radicle

had emerged from the testa. The soil was covered with Whatman No.1 filter-paper and watered with 27 ml distilled water supplemented with sodium chloride at final concentrations: 3.91, 7.8, 15.6, 31.25, 62.5, 125, 250, 400 mM. The control plants were watered with pure distilled water. The root and shoot length, and cotyledon area were estimated using Image Tool for Windows. Dry and fresh mass of roots and shoots were determined too.

The toxicity of NaCl to *Lemna minor* L. (common duckweed) was tested according to the draft OECD 221 guidelines for the testing of chemicals [26]. *L. minor* was grown in a plant growth chamber (ALL -Round -AL 185 -4) illuminated with fluorescent lights (140 µmol photon $m^{-2} \cdot s^{-1}$ PAR) in a light-to-dark cycle of 16 h:8 h (mean maximum temperature during daytime was 20°C, and during nighttime 16°C, respectively) for nine days. The response of common duckweed to NaCl concentrations (3.91, 7.8, 15.6, 31.25, 62.5, 125, 250, 400 mM) was determined by the growth rate of frond, leaf area, dry and fresh mass of plants.

Soluble carbohydrates

Monosaccharidres, cyclitols, sucrose and stachyose content in tissues (lupin and common duckweed) were analysed by GC chromatography according to Piotrowicz-Cieślak (2005) [31]. Tissues (10–60 mg fresh mass) were homogenised in ethanol:water, 1:1 (v/v) containing 300 µg phenyl-α-d-glucose as internal standard. The homogenate and the wash were combined in a 1.5 ml microfuge tube, heated at 75°C for 30 min to inactivate endogenous enzymes and centrifuged at 15 000 g for 20 min. The supernatant was passed through a 10 000 MW cut-off filter (Lida, Kenosha, WI USA). Aliquots of 0.3 ml filtrate were transferred to silylation vials and evaporated to dryness. Dry residues were derived with 300 µl of silylation mixture (trimethylsilylimidazole: pyridine, 1:1, v/v) in silylation vials (Thermo Scientific) at 70°C for 30 min, and then cooled at room temperature. One µl carbohydrate extract was injected into a split-mode injector of a Thermo Scientific gas chromatograph equipped with flame ionisation detector. Soluble carbohydrates were analysed on a DB-1 capillary column (15 m length, 0.25 mm ID, 0.25 µm film thickness, J&W Scientific). Soluble carbohydrates were identified with internal standards as present, and concentrations were calculated from the ratios of peak area, for each analysed carbohydrate, to the peak area of respective internal standard. Quantities of soluble carbohydrates were expressed as mean ± SD for 3–5 replications of each treatment.

Statistical analysis

The experiment was conducted in nine replicates. The results were statistically evaluated using analysis of variance (F test) for two factor experiments (split-plot). The mean values of the plots were compared using q SNK test (Student-Newman-Keuls).

RESULTS

The impact of different concentrations of sodium chloride (3.91, 7.8, 15.63, 31.25, 62.5, 125, 250, 400 mM) on germination, root and shoot elongation, fresh and dry mass, and cotyledon area of seedlings of yellow lupin, cv. Mister, as well as on leave growth and fresh and dry mass of common duckweed was analysed in this study.

After 9 days of sodium chloride treatment, seed germination, root and shoots length, and cotyledon area measurements were determined. Lupin seeds which grew in the control soil germinated in 100%, while those growing in the soil with the maximum concentration of NaCl – in 80% (Fig. 1). In contaminated soil lupin roots reached 66 mm length on average. As a result of increasing concentration (from 3.91 to 125 mM), a slight stimulation of root growth appeared, amounting to 9%. The highest concentration (400 mM) inhibited root growth by 96% (Fig. 1). A rapid reduction in root growth occurred at 250 mM NaCl. In shoots, like in roots, growth inhibition occurred as a result of increasing concentrations of NaCl. Lupin grown in the control soil had the longest shoots, while the one grown in soil contaminated with 250 mM NaCl had the shortest shoots (Fig. 1). Lupin grown in soils contaminated with the highest NaCl concentrations did not form any shoots (Fig. 1). The area of cotyledons was affected by soil NaCl similarly to the above described changes in shoot growth. With decreasing concentrations of sodium chloride, the area of cotyledons was systematically increased. At the highest analysed concentrations, the area of lupin cotyledons was equal to the area of cotyledons before germination. Thus seeds at this concentration did not grow at all and did not absorb water (Fig. 1).

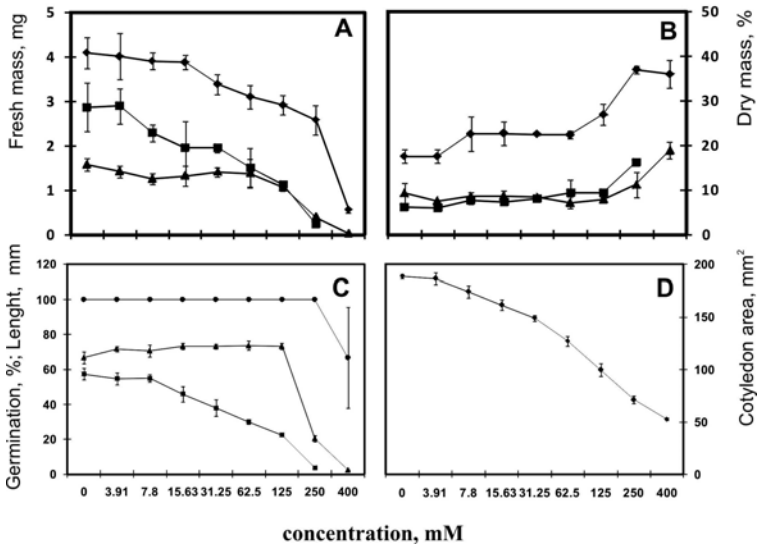

Fig. 1. Roots (■) shoots (▲) and cotyledon (♦) fresh mass (panel A) and dry mass (panel B), and seed (●) germination [%], root (■) and shoot (▲) length [mm] (panel C) and cotyledon area [mm^2] (panel D) of yellow lupin (Lupinus luteus L.)seedlings growing on soil supplemented with different NaCl concentrations (0–400 mM). Data points represent the means ± SD for nine replicate samples.

Contaminating water with duckweed with increasing concentrations of sodium chloride resulted – like in the case of lupin – in reduced growth of leaf area and fresh mass. The area of duckweed leaves ranged from 389 to 89 mm^2, depending on NaCl concentration. Comparing leaf area at the beginning of the experiment and after 9 days of exposure to 250 and 400 mM concentrations negative increments were found (Fig. 2).

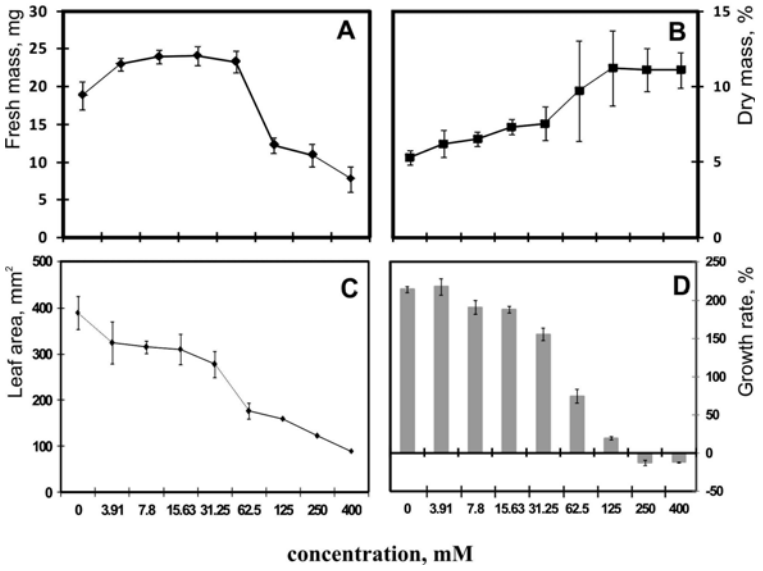

Fig. 2. Fresh mass (panel A), dry mass (panel B), leaf area [mm^2] (panel C) and the growth rate [%] (panel D) of common duckweed (Lemna minor L.) growing in water supplemented with different NaCl concentrations (0–400 mM). Data points represent the means ± SD for nine replicate samples.

The fresh mass of lupin roots, shoots and cotyledons systematically decreased in line with increasing sodium chloride concentrations. The fresh mass of roots which grew in soil contaminated with sodium chloride at the highest concentration was lower by 98% than of those growing in the control soil. The fresh mass of lupin shoots was two times higher than of roots. The fresh mass of shoots in the control soil and in the soil with 250 mM NaCl concentration dropped 10 times. The fresh mass of control cotyledons was 4 g on average, while in the soil with 40 mM NaCl concentration – 0.56 g (Fig. 1).

The dry mass of lupin roots, shoots and cotyledons which was assessed also after 9 days increased (Fig. 1). Similar tendencies of slight but systematic increase in the dry mass in line with increasing sodium chloride concentrations were observed for roots and cotyledons.

The fresh mass of duckweed which grew in the control clear water amounted to 18 g, while at the concentrations from 3.91 to 31.25 mM it increased slightly. A rapid decrease (by 44%) of the fresh mass was observed starting from 62.5 mM NaCl concentration. The dry mass of duckweed increased slightly but systematically and for the control water and the highest concentration amounted to 5 g and 11 g respectively (Fig. 2).

The above results showed different reactions of morphological features of the analysed plants (yellow lupin and common duckweed) to the sodium chloride contamination of soil and fresh water reservoirs. It was proven that low concentrations of sodium chloride (up to 31.25 mM) in fresh water reservoirs are not phytotoxic for common duckweed, even stimulating its growth. The concentration of 250 mM NaCl proved to be phytotoxic to duckweed. For yellow lupin, the phytotoxic NaCl concentration in soil (inhibiting growth

and development) appeared to be at the 400 mM level, since in such a concentration lupin did not grow at all (Figs 1, 2).

The following carbohydrates could be identified on GC chromatograms of plant extracts: cyclitols (*myo*-inositol, D-pinitol and D-*chiro*-inositol), monosaccharides (glucose and galactose), sucrose and stachyose. Sucrose was the main soluble carbohydrate in lupin cotyledons (Fig. 3), while monosaccharides prevailed in common duckweed (Fig. 4). The level of soluble carbohydrates in lupin cotyledons was about four times higher compared to roots and shoots. The content of soluble carbohydrates increased under the influence of increasing NaCl concentration (Figs 3, 4). The results showed the same reaction of soluble carbohydrates content in both analysed plants (yellow lupin and common duckweed) to the sodium chloride contamination of soil and fresh water reservoirs.

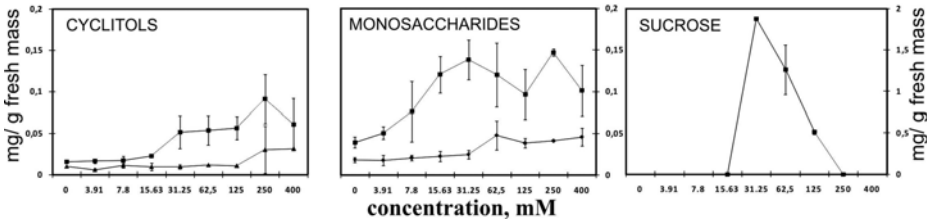

Fig. 3. Cyclitols (D pinitol – ■, D-chiro-inositol – ●, myo-inositol – ▲), monosaccharides (glucose – ■, galactose – ♦) sucrose and stachyose in root, shoot and cotyledon of yellow lupin (Lupinus luteus L.) seedlings growing on soil supplemented with different NaCl concentrations (0–400 mM). Data points represent the means ± SD for four replicate samples.

DISCUSSION

Organisms react to environmental contamination by distorted biochemical or physiological functions in cells and tissues. Biotic and abiotic stresses affect plant distribution, growth, development, and productivity. Sodium chloride used on purpose during winter remains in soil during the whole vegetation period, inhibiting seeds germination and water uptake in plants. With increasing salinity, growth and development of flora and fauna in fresh water reservoirs are limited, especially in those situated lowest in a given area [40, 9]. Plant sensitivity to environment contamination is often used to estimate the degree of environment degradation. Plants respond in different manners to many kinds of toxic substances. The phytotoxic effect is a result of interaction between the compound and the plant in given environmental conditions. The symptoms include morphological deformations and changes in plant biochemistry [28, 29]. Excessive sodium chloride concentration diminishes the osmotic potential of the environment and impedes plants capacity for water uptake [46, 43, 28]. Plants use different strategies to deal with high soil salinity. One strategy is activation of pathways that allow the plant to export or compartmentalise salt. Relying on their phenotypic plasticity, plants can also adjust their root system architecture and the direction of root growth to avoid locally high salt concentrations [8].

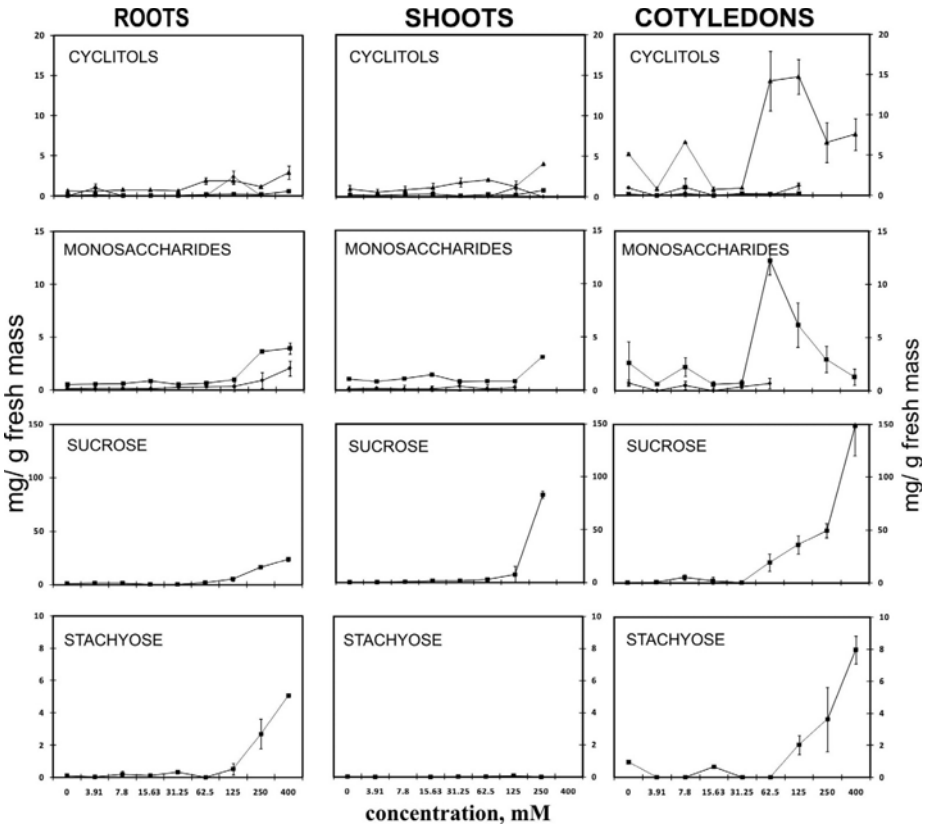

Fig. 4. Cyclitols (D pinitol – ■, D-chiro-inositol – ●), monosaccharides (glucose – ■, galactose – ♦) and sucrose in common duckweed (*Lemna minor* L.) growing in water supplemented with different NaCl concentrations (0–400 mM). Data points represent the means ± SD for four replicate samples.

The transport of sucrose is necessary to meet the cellular energy demands and also for osmoprotectant activities during drought and salinity stresses [13]. Osmotic adjustment is revealed by the accumulation of inorganic ions (Na^+), and organic osmolytes (proline, glycine betaine, and total soluble sugars) in *in vitro* grown cells of *Sesuvium portulacastrum* L. on a medium containing different levels of salt (0–400 mM NaCl) [16]. Salinity regulates the sucrose synthase activity by controlling its gene expression. But the effects of salinity treatment on sucrose phosphate synthase activities is weak under the condition of salt stress [17]. Salinity stress decreases starch content but increases sucrose content in four barley cultivars [2]. The amount of soluble carbohydrates in the roots and shoots sharply increased at higher salinities (more than 125 mM NaCl, Figs 3 and 4). The *myo*-inositol is commonly considered a stress metabolite in plants, stimulated by drought and salinity [24, 20]. The level of *myo*-inositol in plants treated with NaCl increased indeed, however, only when the NaCl had been used at very high concentrations (above 62.4 mM). The content of free *myo*-inositol in plant roots is generally relatively low. In roots of Japanese persimmon (*Diospyroskaki*) *myo*-inositol

could be found in amounts ranging from 0.9 to 1.2 mg/g d.m. (compared to 50 m/g d.m. in *Eucalyptus* seedlings growing in xeric ecosystems) [5, 20]. Furthermore plant roots contain a transport system protecting them from high *myo*-inositol content by exuding it to the soil [41]. The plant response to salinity consists of numerous processes that must function in coordination to alleviate both cellular hyperosmolarity and ion disequilibrium [44]. Salt tolerance requires that compatible solutes accumulate in the cytosol and organelles where these function in osmotic adjustment and osmoprotection [34]. It is believed that two factors are important for osmoprotective functions of carbohydrates: high number of hydroxyl groups and molecular mass (the higher, the better protection). The hydroxyl groups of carbohydrates bind polar groups of lipids and dissociate the intermolecular van der Vaals bonds and separate lipid chains. As a result, they maintain cell membrane fluidity even at a very low level of hydration and low temperature [4].

Lupin seeds growing in the soil with maximum NaCl concentration germinated in 80% (Fig. 1). Lower germination rates were also observed for other seeds growing in soils containing NaCl, e.g. canola, cabbage, cauliflower and other vegetable species [14, 22].

Roots, similarly to seeds, have a direct contact with the contaminated soil while absorbing water which is later delivered to the developing seedlings. At low NaCl concentrations (from 3.91 to 125 mM) a slight stimulation of root growth appeared (Figs 1 and 2). It is known that short-term NaCl stress produces reversible effects on growth, leaf water relations and on superoxide dismutase and ascorbate peroxidase activities [10].

Rapid inhibition of root elongation occurred in lupin at 250 mM of NaCl. According to Zidan et al. [47], inhibition of root growth in maize under salinity is due to reduction in the similar tendency. The highest NaCl concentration (400 mM) reduced root growth by 96% (Fig. 1). Salinity also inhibits shoot elongation due to limited water absorption by roots [25]. Lupin in the control soil had the longest shoots (57 mm), while the shortest ones (30 mm) appeared in soil contaminated with 250 mM sodium chloride concentration. In our research, lupin which grew in soil contaminated with the highest concentration (400 mM) did not form any shoots (Fig. 1). The field soil salinity can reach the level of 150 mM and can reduce shoot growth by 50% or more [33]. Salinity rapidly inhibits root growth and hence capacity of water uptake and essential mineral nutrition from soil [25]. In maize plants, roots are more salt sensitive than shoots [38], since they have direct (6-weeks) contact with the toxic substance.

Like in the case of lupin, a systematic decrease in the leaf area due to increasing concentrations of NaCl appears in cabbage, cauliflower, and canola [14]. The leaf area of flag leaf decreases also in *Sesbania grandiflora*, in line with increasing osmotic pressure in cell sap as a result of increasing NaCl concentrations (from 10 to 50 mM) [5]. Water contamination with increasing concentrations of sodium chloride resulted in a decreasing growth of leaf area and fresh mass of common duckweed (Fig. 2), just as it was in the case of lupin in our experiments (Fig. 1). The area of duckweed leaves ranged from 214 to 19 mm^2, depending on NaCl concentration. Gain in the leaf area determined by comparing the rate of growth at the beginning of the experiment and after 9 days of exposure to 250 and 400 mM concentrations was negative. The isosmotic potential of NaCl in these concentrations facilitated leaf shrinkage and water removal from duckweed tissues into the environment.

The fresh mass of lupin roots, shoots, and cotyledons systematically decreased in line with the rise of sodium chloride concentrations. The fresh mass of roots which grew in the soil contaminated with sodium chloride at the highest concentration (400 mM) was lower by 98% than of those growing in the control soil. The fresh mass in lupin shoots was two times higher than in roots. The fresh mass of shoots, as well as of roots, decreased in line with increasing NaCl concentrations in soil. At 100 mM NaCl concentration, in two eggplant cultivars, Bemisal and Dilnasheen, there occurs a decrease of these parameters [1].

The dry mass of lupin roots, shoots and cotyledons showed similar tendencies of slight but systematic increase. It confirms the reaction to salinity described for pea. Growth of pea (*P. sativum*) plants, estimated as shoot fresh and dry weight, was not affected by 0.07 M or 0.09 M NaCl in the nutrient medium but was considerably reduced (about 40%) in the presence of 0.110 M [11].

The same reaction to soil salinity, namely decreasing leaf area, occurred in six genotypes of cotton – 200 mM NaCl treatment reduced cotton plant height, leaf area, and dry weight of total leaves per plant and biomass accumulation [12]. Common duckweed root apex is known to be a sensitive site for the NaCl-salinity induced oxidative damage and a coordinated antioxidant defence mechanism is involved as a response to salt stress tolerance [27].

Environmental toxicity determination is usually done with the aid of phytotests according to OECD norms (2006) [26], mainly in relation to pesticide [29] and veterinary medicine contamination [29, 42]. Biotests, in contrast to instrumental (chemical) methods, allow for simple and inexpensive detection of very low levels of active substances in soil that can be phytotoxic to crop plants [40]. In the present study, phytotests were applied to assess phytotoxicity of sodium chloride in water and soil environment on the example of common duckweed and yellow lupin. Plant growth rate and the levels of cyclitols and soluble carbohydrates can be used as indicators of the burden of stress imposed by the environmental NaCl upon organisms.

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FITOTOKSYCZNE DZIAŁANIE CHLORKU SODU WOBEC ŁUBINU ŻÓŁTEGO (*LUPINUS LUTEUS* L.) ORAZ RZĘSY DROBNEJ (*LEMNA MINOR* L.)

Zasolenie wpływa niekorzystnie na roślinność i stanowi jedną z przyczyn degradacji środowiska wodnego i glebowego. Rośliny wykształciły wiele mechanizmów odporności na NaCl, jednym z nich może być akumulacja związków osmoprotekcyjnych, utrzymujących równowagę osmotyczną, chroniących struktury komórkowe i enzymy. W pracy badano wpływ zasolenia wywołanego różnymi stężeniami chlorku sodu (od 0 do 400 mM) na tempo wzrostu rzęsy drobnej (*Lemna minor* L.) i łubinu żółtego (*Lupinus luteus* L.). Ponadto w tkankach roślin oceniano zawartość cyklitoli i węglowodanów rozpuszczalnych. Wzrastające stężenie chlorku sodu zmniejszało powierzchnię liści rzęsy drobnej. W najwyższym z zastosowanych stężeń obok redukcji pola powierzchni liści obserwowano również intensywną chlorozę liści. Wzrastające stężenie chlorku sodu hamowało wzrost elongacyjny korzeni i łodyg łubinu żółtego. Najwyższe z badanych stężeń

NaCl całkowicie hamowało wzrost elongacyjny łodyg łubinu żółtego. W tkankach rzęsy drobnej występowały cyklitole (D-*chiro*-inozytol i D-pinitol) oraz węglowodany rozpuszczalne (glukoza, fruktoza i sacharoza). Natomiast w siewkach łubinu żółtego występowały cyklitole (D-pinitol, *myo*-inozytol i D-*chiro*-inozytol) oraz węglowodany rozpuszczalne (glukoza, fruktoza, galaktoza i sacharoza). Wykazano, że wraz ze wzrostem stężenia chlorku sodu w podłożu wzrastała zawartość osmoprotektantów (cyklitoli i sacharozy) w tkankach. Badania wykazały, że cyklitole i węglowodany rozpuszczalne obecne w tkankach łubinu żółtego i rzęsy drobnej są dobrymi biomarkerami środowiska zanieczyszczonego chlorkiem sodu.