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The effects of leaf extracts of crack willow (*Salix fragilis*) on the growth of Japanese knotweed (*Fallopia japonica*)

Vpliv listnih izvlečkov krhke vrbe (*Salix fragilis*) na rast japonskega dresnika (*Fallopia japonica*)

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Abstract: Japanese knotweed (*Fallopia japonica*) is one of the most invasive of species in Europe, and can substantially reduce local native biodiversity. In the present study, the allelopathic potential of crack willow (*Salix fragilis*) on growth of Japanese knotweed was investigated. Aqueous extracts of 0.1% and 1% (w/v) were prepared from liophilised willow leaves and used for watering of young knotweed plants. Their growth was monitored for 196 days. Shoot height and leaf number were not affected but the mass of leaves and especially roots was reduced (up to 32%). At the end of experiment, biochemical characteristics related to physiological state (photochemical efficiency of PSII, protein content, enzyme activity of guaiacol peroxidase, lipid peroxidation) were measured. Mostly, they were at control levels, but the activity of guaiacol peroxidase and lipid peroxidation in roots increased. The extracts of crack willow showed moderate inhibitory effect on roots of treated knotweeds while the growth of shoots was unaffected. Given the root reduction described here, further studies with willow extracts and field studies with crack willow and Japanese knotweed plants would be reasonable.

Keywords: *Fallopia japonica, Salix fragilis,* invasive species, leaf extract, allelopathy, growth

Izvleček: Japonski dresnik (*Fallopia japonica*) sodi med najbolj inavzivne tujerodne vrste v Evropi in pomembno vpliva na zmanjševanje lokalne biodiverzitete. V raziskavi smo preučili alelopatski potencial krhke vrbe (*Salix caprea*) na rast japonskega dresnika. Iz liofiliziranih listov vrbe smo pripravili 0,1 % in 1 % vodne izvlečke, s katerimi smo zalivali mlade rastline dresnika in spremljali njihovo rast 196 dni. Višina poganjkov in število listov sta bila podobna kot v kontrolnem tretmaju, medtem ko se je masa listov, predvsem pa korenin, močno zmanjšala (do 32 %). Ob koncu poskusa smo z biokemijskimi analizami (fotokemična učinkovitost FSII, vsebnost beljakovin, encimska aktivnost gvaiakol peroksidaze, lipidna peroksidacija) ocenili še fiziološko stanje rastlin. Večina izmerjenih lastnosti je bila na kontrolni ravni, razen peroksidazne aktivnosti in lipidne peroksidacije, ki sta se pri tretiranih rastlinah povečali. Izvlečki krhke vrbe kažejo zmeren alelopatski učinek na korenine tretiranih dresnikov, medtem ko so poganjki rasli neprizadeto. Glede na opisano zmanjšanje koreninskega sistema predlagamo nadaljnje raziskave z izvlečki vrbe in terenske raziskave o vplivu krhke vrbe na rast japonskega dresnika.

Ključne besede: japonski dresnik, krhka vrba, invazivne vrste, listni izvleček, alelopatija, rast

Introduction

Japanese knotweed (*Fallopia japonica* (Houtt.) Ronse Decr., *Polygonaceae*) is among the 100 most invasive taxons in the world (Lowe et al. 2000) and is a well known and problematic invasive species in Slovenia, too. It grows in dense populations mainly in ruderal habitats and river banks. The main strategy for its spread in invaded regions is vegetative reproduction with rhizomes and stolons and high level of regeneration (Bailey et al. 2009). Sexual reproduction outside its natural range is limited but seed formation and germination were proved for Slovenian populations (Strgulc Krajšek and Dolenc Koce 2015). So far, the mechanical removal and frequent cutting of knotweed plants have been the most common and efficient ways to limit their growth and spread. For the biological control of invasive plant species, allelopathy could be a potential mean. Allelopathy refers to chemical interactions among plants, including those mediated by microorganisms (Weston and Duke 2003) and is defined as supression of the growth and/or establishment of neighbouring plants by chemicals released from a plant or plant parts (Inderjit et al. 2011). These allelochemicals could be synthesized in all plant tissues and released in environment by leakeage, root exudates, evaporation and degradation of organic material (e.g. decomposed leaf litter). Majority of allelochemicals are secondary compounds; phenolic substances, flavonoids, terpens, alcaloids, steroids and function also in antimicrobial protection. Many plant species, including weeds and crops, have allelopathic activity, the most known and well studied are *Juglans nigra, Ailanthus altissima, Alliaria petiolata, Centaurea maculosa* (Weston and Duke 2003). Allelopathy could also be the mechanism which enables invasive plant species successful colonisation of new habitats; a mechanism known as a novel weapons hypothesis (Bais et al. 2003, Callaway and Aschehough 2000, Callaway et al. 2005, Hierro and Callaway 2003, Inderjit et al. 2011). Some studies on the allelopathic potential of knotweeds have already been carried out and biologically active compounds have been defined in this context (Dommanget et al. 2014, Fan et al. 2010, Gerber et al. 2008, Murrell et al. 2011, Vrchotová et al. 2007).

As previously mentioned, river banks are often overgrown by Japanese knotweed and the same habitat type is characteristic for some species of willow (*Salix*). Willows have high physiological and ecological plasticity, they grow rapidly and can accumulate metals, and for these reasons they are commonly introduced in ecosystems for phytoremediation of degraded habitats (Alvarez et al. 2003, Kuzovkina and Quigley 2005, Prach and Pyšek 2001). However, negative effects of willows on neighbouring plants (inhibited growth) were also reported due to shading, competition for mineral nutrients and allelopathy. At least 12 phenolic allelochemicals were detected in leaves of goat willow (*Salix caprea*) (Ikonen et al. 2002, Hallgren et al. 2003, Moohammadnor et al. 2010). In some areas, crack willow (*Salix fragilis*) is even considered invasive (Cremer 2003). It affects river ecosystems by releasing secondary compunds and organic matter (leaves, cork, fruits), shading, which all changes nutrient cycling, food chains and biodiversity (Groninger and Bohanek 2000, Doody and Benyon 2011). It has high level of regeneration and small fragments of shoots are dispersed by water to new locations where they quickly regenerate and start a new population (Budde et al. 2011). Leaf composition can also account for allelopathic potential of crack willow; leaves are compact and degrade slowly relasing tannins and phenolic compounds for a longer time (Julkunen-Tiitto 1985, Haapala et al. 2001).

The aim of the present study was to evaluate the allelopathic potential of crack willow on invasive Japanese knotweed. Developmental changes during growth of young knotweed plants were observed and their physiological state was determined by measuring photochemical efficiency of PSII, protein content, activity of antioxidative enzyme guaiacol peroxidase and lipid peroxidation.

Materials and methods

Plant materials

The mature leaves of crack willow (*Salix fragilis* L.) were collected in Ljubljana, Slovenia (46° 3′ 55.63″ N, 14° 27′ 42.53″ E) in October. Following their collection, the leaves were lyophilised and stored in dark at room temperature until the extracts were prepared.

Young shoots of Japanese knotweed (*Fallopia japonica* var. *japonica* (Houtt.) Ronse Decr.) were collected in Ljubljana, Slovenia (46° 2′ 59.84″ N, 14° 28′ 28.52″ E) in April of the following year. The collected shoots had approx. 3 cm high aboveground stem and 2 cm long underground rhyzome with at least one stem bud. Each shoot was planted in a separate pot filled with mineral substrat vermiculite. The pots were kept in a climate chamber under control conditions of temperature (25 ±1 °C), humidity (40%), and light/ dark cycle (16h/8h; light intensity 160 µM $m^{-2}s^{-1}$) for 2 months. After that, the plants were transplanted to pots with commercial garden soil for additional 4 months (196 days in total) and grown under laboratory conditions of ambient temperature, humidity and light intensity.

Preparation of willow leaf extracts

The lyophilised mature willow leaves were homogenised using a mortar and pestle. Following suspension of the ground leaves in distilled water (5 g leaves in 100 ml distilled water), the extraction was carried out for at least 24 h on a shaker (170 rpm; room temperature). The extracts were then vacuum filtered, once with filter paper for general use and twice with fine filter paper (388 Grade, 84 g/m^2) to provide the 5% (w/v) aqueous extract that was frozen at -20 °C until needed. Defrosted extract was diluted in distilled water to give the final concentration of 0.1% and 1% (w/v). The extracts of final concentration were prepared fresh prior to watering the knotweed plants.

Measuring of Japanese knotweed growth

The knotweed plants were watered with distilled water as the control (N=5), and with the 0.1% and 1% aqueous willow extracts (N=10 for each treatment). In the first two months, the plants were watered with willow extracts once a month and in the following 4 months twice a month because their growth increased substantially. In the interim period, all knotweed plants were watered with distilled water every 3-4 days to prevent dehydration. Every week, the shoot height was measured and leaf number was counted to evaluate the growth dynamics. After 196 days of observation, the photochemical efficiency of the photosystem II was measured, and roots and shoots were separated, weighed, frozen in liquid N_2, and stored at -20 °C prior to biochemical analyses.

Photochemical efficiency

The photochemical efficiency of PSII was measured on the 2nd or 3rd youngest leaf of the same size using modulated fluorometer PAM 2100 (Walz, Germany) according to Germ et al. (2005). After 15 min dark adaptation, leaves were illuminated with a saturating beam of white light (photosynthetic photon flux density = 8000 µmol m^{-2} s^{-1}, 0.8 s) to excite the fluorescence of chlorophyll *a* and the optimal quantum yield (F_v/F_m) was detected. The effective quantum yield of PSII was measured by providing a saturating pulse of white light (PPFD = 9000 µmol m^{-2} s^{-1}, 0.8 s) using a standard 60° angle clip.

Biochemical analyses

Samples of ~100 mg roots and shoots/ leaves were homogenised in 1.5 ml of potassium phosphate buffer (100 mM, pH 7), centrifuged (20 817 g, 20 min, 4 °C) and the resulting supernatants were used to spectrophotometrically (UV-1800 Shimadzu) determine the protein concentration (BCA Protein Assay Kits, Novagen), specific enzyme activity and MDA content (as the measure of lipid peroxidation), as previously described (Dolenc Koce et al. 2014) and as indicated briefly below.

The activity of guaiacol peroxidase (G-POD; EC 1.11.1.7) was measured at 470 nm (e = 26.6 $mM^{-1}\cdot cm^{-1}$). The reaction mixture contained 900 µl potassium phosphate buffer (50 mM, pH 7) with 1% guaiacol and 10 mM H_2O_2, with the addition of 100 µl of the supernatant samples described above.

Lipid peroxidation was evaluated in terms of the content of malondialdehyde (MDA). Here, 200 µl of the supernatant samples obtained for the protein extraction described above was added to 800 µl acetic reagent (0.5% (w/v) thiobarbituric acid in 20% (w/v) trichloroacetic acid). The mixture was incubated for 30 min at 95 °C, and then chilled to stop the reaction. The MDA content was measured spectrophotometrically at 532 nm and 600 nm (Dolenc Koce et al. 2014).

Statistical analysis

Means and standard errors were calculated, and the samples were compared by t-test and ANOVA (Microsoft Excel, GraphPad Prism 3.02). The level of significance was set at $p < 0.05$.

Results

During the experiment which lasted cca. 6 months, some knotweed plants did not survive and final number of plants for statistical analysis was 4 for control, 8 for 0.1% extract and 10 for 1% extract treated plants. At the end of experiment, the roots of knotweed plants that were treated with willow leaf extracts were more affected than the aboveground stems and leaves. Shoot height and leaf number were at all measuring points similar as in control plants (Fig. 1). In case of 1% extract, the shoots were on average even heigher but the difference was not statistically significant.

The root growth was evaluated at the end of the experiment by weighing the mass of the root system and was 21% and 32% lower in knotweed plants treated with 0.1% extract and 1% extract, respectively (Fig. 2). Despite the substantial decrease, the differences were not statistically significant ($p = 0.148$). Similar, but less inhibitory effect was observed with the total leaf mass which was approx. 20% lower in treated plants ($p = 0.402$).

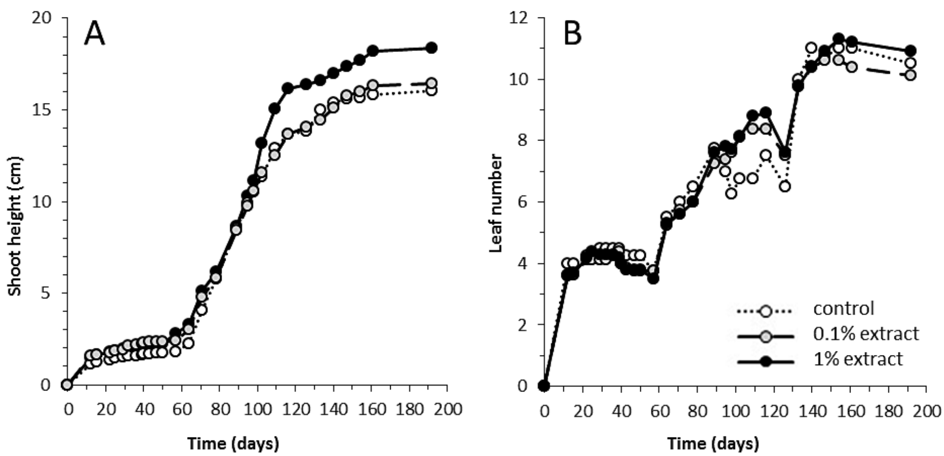

Figure 1: Growth of knotweed plants treated with willow leaf extracts. **A** – shoot height, **B** – leaf number. Data are means (n = 4 for control, 8 for 0.1% extract and 10 for 1% extract treatment).

Slika 1: Rast japonskega dresnika, tretiranega z listnimi izvlečki krhke vrbe. **A** – višina poganjka, **B** – število listov. Prikazane so povprečne vrednosti (n = 4 za kontrolo, 8 za tretma z 0,1 % izvlečkom in 10 za tretma z 1 % izvlečkom).

Long-term treatment with willow extracts showed diverse and mainly statistically insignificant effects at the biochemical level (Tab. 1). Protein content increased up to 23% in roots and decreased up to 21% in leaves, the specific enzyme activity of G-POD incresed in all investigated tissues with maximal (313%) increase in roots, treated with 0.1% extract (ANOVA for G-POD in roots; p = 0.012). Lipid peroxidation, estimated as MDA content, was up to 76% higher in roots (ANOVA; p = 0.119) and up to 15% lower in leaves (ANOVA; p = 0.084). The increase of lipid peroxidation in roots correlates with decreased root biomass caused by treatment with willow extracts (Fig. 3). The negative correlation between these two parameters was higher (Pearson correlation coefficient r = -0.6353 and -0.1669 for 0.1% and 1% treatment) than in roots and leaves of control plants (for all root data r = -0.3562 and for all leaf data r = 0.0285).

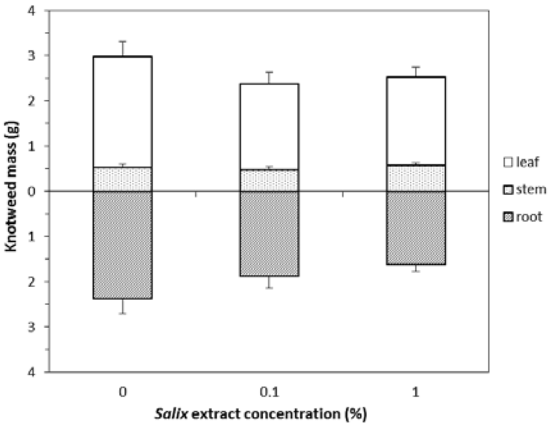

Figure 2: Mass of leaves, stem and roots of knotweed plants treated with willow leaf extracts. Data are means ± SE (n = 4 for control, 8 for 0.1% extract and 10 for 1% extract treatment).

Slika 2: Masa listov, stebla in korenin japonskega dresnika, tretiranega z listnimi izvlečki krhke vrbe. Prikazane so povprečne vrednosti ± SN (n = 4 za kontrolo, 8 za tretma z 0,1 % izvlečkom in 10 za tretma z 1 % izvlečkom).

Relatively good physiological state of leaves was confirmed also by results of photochemical efficiency of PS II, expressed as optimal quantum yield (F_v/F_m) which was not affected when willow extracts were applied (Tab. 1).

Discussion

Biological suppression of growth by allelopathic interactions is a possible mechanism to control and reduce spread of invasive plant species. Among most noxious species is Japanese knotweed which grows over river banks and anthropogenically degraded habitats and reduces biodiversity (Bailey et al. 2009). In the present study, leaf extracts of crack willow were used as a source of potential allelochemicals because both species grow in the same habitat type and share growing conditions. When young knotweed plants were watered with willow extracts of two concentrations (0.1 and 1%), the mass of root system decreased and its physiological state was disturbed which was confirmed by increased biochemical parameters associated with oxidative stress. The activity of guaiacol-peroxidase which is the enzyme involved in the hydrogen peroxide degradation increased for more than 3-fold in roots and the content of malondialdehyde which is a product of peroxidation of membrane lipids increased up to 76%. Nevertheless, the aboveground shoots were less affected; the shoot height, leaf number,

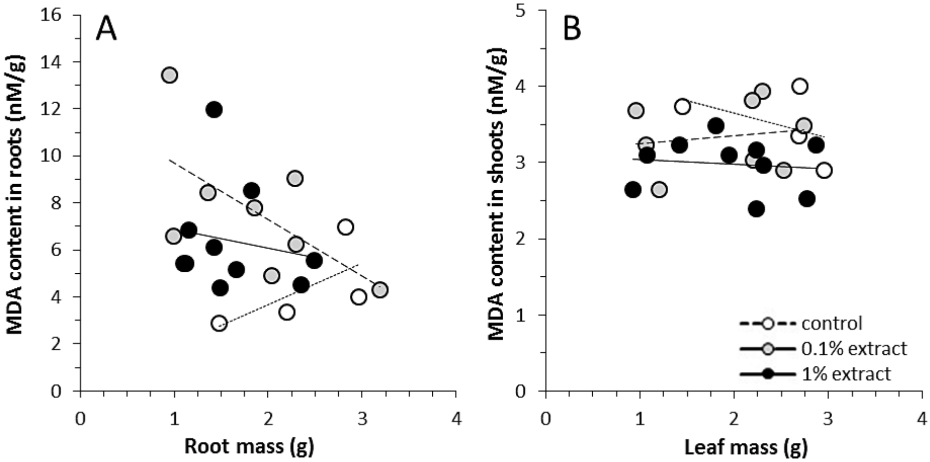

Figure 3: Correlation of malondialdehyde (MDA) content with mass of (A) roots and (B) leaves of knotweed plants treated with willow leaf extracts. Data are individual measurements per treatment (n = 4 for control, 8 for 0.1% extract and 10 for 1% extract treatment). Trendlines are linear regression lines.

Slika 3: Korelacija med vsebnostjo malondialdehida (MDA) in maso (A) korenin in (B) listov pri japonskem dresniku, tretiranem z listnimi izvlečki krhke vrbe. Prikazane so posamezne meritve za tretma (n = 4 za kontrolo, 8 za tretma z 0,1 % izvlečkom in 10 za tretma z 1 % izvlečkom). Trendne črte so linearne regresijske premice.

Table 1: Biochemical analysis and photochemical efficiency of knotweed plants treated with willow leaf extracts.

Tabela 1: Biokemijska analiza in fotokemična učinkovitost japonskega dresnika, tretiranega z listnimi izvlečki krhke vrbe.

	Control	0.1% extract	1% extract
Roots			
Protein concentration (μg/ml)	896.78 ± 168.87	982.94 ± 112.19	1102.68 ± 119.34
G-POD (μM/min*mg)	29.26 ± 3.22	**120.96 ± 22.58***	63.35 ± 10.35
MDA (nM/g)	4.31 ± 0.92	7.60 ± 1.01	6.39 ± 0.73
Leaves			
Protein concentration (μg/ml)	2086.26 ± 343.73	1739.17 ± 259.02	1639.57 ± 205.50
G-POD (μM/min*mg)	85.68 ± 19.67	151.35 ± 24.02	127.93 ± 14.00
MDA (nM/g)	3.50 ± 0.24	3.34 ± 0.16	2.98 ± 0.11
F_v/F_m	0.82 ± 0.01	0.81 ± 0.01	0.81 ± 0.00

Data are means ± SE (n = 4 for control, 8 for 0.1% extract and 10 for 1% extract)

G-POD, specific enzyme activity of guaiacol peroxidase; MDA, malondialdehyde content; F_v/F_m, optimal quantum yield
Bold *, statistically significant difference between treated and control plants (t-test; $p < 0.05$).

photochemical efficiency and lipid peroxidation were at control levels. On the other hand, the mass of leaves decreased and the activity of G-POD increased but the differences were not statistically significant. Different effects of *Salix caprea* leaf extracts were reported previously. The reduction of root mass was proved for seedlings of *Picea abies* treated with *Salix caprea* extracts (Schütt and Blaschke 1980). Also decreased germination of *Arrhenaterum elatius, Lotus corniculatus* and *Plantago lanceolata* was reported when seeds were directly treated with leaf litter of *S. caprea* but the effect was less inhibitory or even stimulatory when litter was added to substrate improving its quaility (Mudrak and Frouz 2012).

The inhibition of growth can be related to some methodological problems as well. It was shown that type of substrate can influence the plant growth (Mudrak and Frouz 2012); when seedlings of tested plant species grew in sand, the allelopathic effects of willow were more pronounced because the sand has lower absorption capability and chemical interactions between compounds in plant extracts and sand are less intense than in soil. The same effect was observed in our study; when knotweed plants grew in mineral substrate vermiculite first 2 months, their growth was slow. When they were transferred to soil, their growth increased but the change was also related to different temperature, humidity and light conditions. To prove allelopathic potential of an extract, activated carbon can be added to the substrate to absorb chemicals (Prati and Bossdorf 2004). Also the use of distilled water for control could have negative effects on plants. The practical experiences show that watering with distilled water results in weaker growth than watering with tap water which contains higher level of minerals.

The reduction of the underground tissues of Japanese knotweed could be important mechanism for limiting its growth and spread because rhyzome and stolons are the prime structures that enable knotweed successful vegetative reproduction (Bailey et al. 2009). To prove allelopathic potential of crack willow for biocontrol of Japanese knotweed further studies are necessary using willow extracts of higher concentrations, root extracts, more frequent watering with extracts and field studies with co-growth of knotweed and willow plants in the same substrate. The latter is of special importance because in natural conditions other factors can influence allelopathic potential of certain plant species. Soil biota and chemistry, abiotic factors related to seasonal differences and neighbouring organisms (Inderjit et al. 2011) affect interactions among organisms therefore appropriate methodolgy for integrating chemically mediated interaction into ecology is crucial (Inderjit and Callaway 2003). In case of interactions between willows and knotweeds the allolopathic potential of knotweed to reduce willow growth should also be considered and tested.

Conclusions

Long-term experiments are important way to study allelopathic effects because they simulate conditions in natural environment. Our study shows moderate allelopathic effect of leaf extracts of crack willow to roots of Japanese knotweed while shoots developed unaffected. In roots, biomass decreased over 6 months of growth in the presence of willow extracts and biochemical characteristics related to oxidative stress elevated.

Povzetek

Japonski dresnik (*Fallopia japonica*) sodi med 100 najhujših invazivk v svetovnem merilu (Lowe et al. 2000). Njegova hitra rast in vegetativno razmnoževanje s korenikami in stoloni mu omogočajo uspešno naseljevanje novih habitatov, kjer zaradi svoje invazivnosti izpodriva avtohtone vrste in spreminja ekosisteme ter povzroča gospodarsko škodo. Njegovo odstranjevanje je večinoma kemično, kar lahko povzroča negativne posledice na ostale rastline in okolje na splošno, in mehansko. Eden od možnih biotičnih načinov zatiranja bi lahko bila alelopatija, tj. negativen vpliv ene rastline na drugo preko delovanja alelopatskih spojin, ki jih rastlina izloča ali sprošča v okolje in vključuje tudi delovanje preko mikroorganizmov, povezanih z rastlinami.

V raziskavi smo želeli ugotoviti, ali vodni izvlečki iz listov krhke vrbe (*Salix fragilis*) vplivajo na rast in razvoj japonskega dresnika. Vrbe so vrste, ki se pogosto uporabljajo v poskusih stabilizacije in obnove uničenih ekosistemov. Poleg vlagoljubnih vrst najdemo med njimi tudi hitro rastoče pionirske rastline zgodnjih sukcesijskih faz, ki naseljujejo nove, s pomočjo človeka nastale odprte površine, torej podobna rastišča kot japonski dresnik. Poleg tega je zanje značilen tudi alelopatski potencial, ki skupaj s senčenjem in tekmovanjem za hranila zavira rast podrasti.

Iz liofiliziranih listov krhke vrbe smo pripravili 0,1 in 1 % vodni izvleček, s katerim smo 1 do 2-krat mesečno zalivali mlade rastline japonskega dresnika, ki smo jih posadili v vermikulit in zemljo. Njihovo rast in razvoj smo spremljali 196 dni.

Ugotovili smo, da so izvlečki zavrli predvsem razvoj korenin, kar se je pokazalo z zmanjšanjem njihove mase, povečale pa so se značilnosti, povezane z oksidativnim stresom, tj. lipidna peroksidacija, ocenjena kot vsebnost malondialdehida, in aktivnost antioksidativnega encima gvajakol peroksidaze. Poganjki so bili manj prizadeti, saj so bili višina poganjka, število listov, fotokemična učinkovitost FS II in lipidna peroskidacija na kontrolnih vrednostih. Zmanjšala se je masa listov, povečala pa aktivnost gvajakol peroksidaze, vendar so bile razlike statistično neznačilne.

Slabša rast korenin zaradi delovanja izvlečkov iz listov krhke vrbe predstavlja temelj za nadaljnje raziskave, v katerih bi bilo potrebno preučiti delovanje koreninskih izvlečkov, izvlečkov z višjo koncentracijo, pogostejše zalivanje z izvlečki, ter ugotoviti kako vplivajo na rast japonskega dresnika vrbe, ki bi rastle skupaj z njimi v istem substratu. S tem bi lahko potrdili alelokemijsko delovanje krhke vrbe in njen potencial za biološko kontrolo invazivnih tujerodnih rastlin.

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