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Identification and allelochemical activity of phenolic compounds in extracts from the dominant plant species established in clear-cuts of Scots pine stands

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Dominant plant species established in the understory of clear-cuts may have a strong biochemical influence on pine regeneration process, with important consequences for reforestation management. We evaluated and compared the total phenolic content and the allelopathic activity of acqueous extracts from both roots and shoots of dominant plant species established in 1-yr-old and 2-yr-old clear-cuts of Scots pine (*Pinus sylvestris* L.) stands in Lituania. The highest total content of phenolic compounds was detected in the lingonberry (*Vaccinium vitis-idaea* L.) shoots from 1-yr-old and 2-yr-old clear-cuts, as well as in the common heather (*Calluna vulgaris* [L.[Hull) shoots from 1-yr-old clear-cuts. High-performance liquid chromatography (HPLC) was used to identify and quantify the allelochemicals present in the active fraction to determine their possible role in allelopathy. The highest variety and content of phenolic compounds were observed in shoot extracts of the dominant species from both 1-yr-old and 2-yr-old clear-cuts. Scots pine seed germination and seedling growth were significantly and negatively correlated with *p*-coumaric acid and sinapic acid content, while Scots pine seedling growth was significantly and negatively correlated with ferulic, caffeic and hydroxycinnamic acids contents. The highest contents of these phenolic acids were determined in aqueous extracts of *C. vulgaris* from 1-yr-old clear-cuts and *Rumex acetosella* L. of 2-yr-old clear-cuts, which exerted a strong phytotoxicity on Scots pine seed germination. Moreover, morphometric parameters of Scots pine seedlings were most sensitive to aqueous extracts of *V. vitis-idaea* shoots from both 1-yr-old and 2-yr-old clear-cuts and *R. acetosella* shoots from 2-yr-old clear-cuts.

Keywords: Phenolics Identification, Allelopathy, Dominant Species, Germination

Introduction

Fraenkel (1959) first recognized that secondary plant metabolites are not simply plant waste products, but play an important role in the plant defense against insects and herbivores. Since then, our understanding of the ecological roles of these compounds has greatly increased. Nowadays, it is well known that secondary plant metabolites can provide defence against a broad range of herbivores and pathogens, can mediate the interactions among competitors and mutualists, and may help ameliorating abiotic stress (Iason et al. 2012). Many thousands of plant secondary metabolites have a possible role in allelopathy and may inhibit both seed germination and seedling growth of nearby species (Inderjit & Duke 2003).

The presence of allelochemicals able to affect forest tree growth and development seems to be widespread in all ecosystem types (Van Rooyen et al. 2004). Allelopathy is one of the factors involved in conifer regeneration failure in the presence of dense ericaceous understory as a consequence of forest harvesting or fire (Mallik 2003). The dominant species, mostly ericaceous, in the understory of clear-cuts in Scots pine (*Pinus sylvestris* L.) stands produce and secrete a range of phenolic compounds that are inhibitory to conifer seed germination (mainly primary root growth) and ectomycorrhizal growth. Phenolics are the main allelopathic compounds that inhibit seed germination, plant growth and other physiological processes, thus leading to changes in the floristic composition of plant communities and dominance of one plant species over others (Boudet 2007).

Allelochemicals are released through processes such as volatilization, root exudation, leaching, and decomposition of plant residues, and may be present in all plant organs, including leaves, flowers, fruits, roots, rhizomes, stems and seeds, some of which can store these compounds. However, the quantities and release pathways

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vary across species (Li et al. 2010). Numerous authors have shown that the tissues of herbaceous plants, shrubs and trees of the understory contain different phenolic compounds (Dorning & Cipollini 2006, Li et al. 2010). Typically, the allelopathic inhibitory effect is the result of the concerted action of multiple allelochemicals that mutually affect different physiological processes, thus altering the growth patterns of plants (Parvez et al. 2004, Inderjit & Duke 2003). The action of allelochemicals can affect respiration, photosynthesis, enzyme activity, water regulation, stomatal opening, hormone levels, mineral availability, cell division and elongation, and the structure and permeability of cell membranes and walls (Reigosa et al. 1999, Li et al. 2010). Alterations to cell membranes are one of the primary effects of allelochemicals, and these effects may then exert secondary effects (Barkosky et al. 2000).

Nonetheless, little is known about the phenolic compounds of the dominant understory species which are able to influence the process of reforestation in clear-cuts of Scots pine stands in boreal forests.

The objectives of this work were to evaluate and compare under laboratory conditions the identified content of phenolic compounds (IPC) and allelopathic activity of aqueous extracts produced from both shoots and roots of the dominant species growing in 1-yr-old and 2-yr-old clear-cuts of Scots pine stands (*Vaccinio myrtilli-Pinetum*). High-performance liquid chromatography (HPLC) was used to identify and quantify the allelochemicals present so as to ascertain their possible role in allelopathy, specifically in terms of Scots pine seed germination and the morphology of pine seedlings.

Materials and methods

Study area and dominant species record

We studied the phytocenoses of herbaceous successional stages in 1-yr-old and 2-yr-old clear-cut areas of *Vaccinio myrtilli-Pinetum* (Ellenberg 2009) forests during June-July 2010. In total, 17 clear-cuts in Central and South-Eastern Lithuania (forests of Kuras, Ropeja and Zeronys) were included in the survey (Tab. 1). Plant diversity, abundance (%) and projection cover (%) were estimated for herbaceous species in order to identify the dominant species. The analyses were carried out in $1\ m^2$ replicated subplots (n=25) established along transects every 5 m at each site (Braun-Blanquet 1964). Species were classified into a dominant phytosociological group for each site according to the projection coverage based on abundance proportion. The dominant species served as the source of aqueous extracts for assessing the impact of their allelochemicals on seed germination and seedling growth inhibition in Scots pine.

Germination bioassay

Germination tests were carried out in the Laboratory of Ecology at the Institute of Forestry, Lithuanian Research Centre for Agriculture and Forestry.

Allelopathic activity of aqueous extracts of the dominant species was estimated based on seed germination bio-screening (Baležentienė & Šežiene 2010). For the preparation of aqueous extracts, shoots and roots were chopped into 0.5 cm long pieces before extraction. Fifty grams of each piece were immersed in a 15 × 20 × 5 cm plastic tray containing 250 ml of distilled water. Containers were closed with glass plates and kept at 5 °C in an incubator. After 12 h, the aqueous extracts were filtered through Whatman no. 1 filter paper and diluted to 0.2% (w/v) concentration and used for germination assays. The germination of pine seed was assessed according to the ISTA technique (ISTA 2007). One hundred Scots pine seeds were placed on filter paper in each 9-cm diameter Petri dish. Five milliliters of 0.2% extract was added to each Petri dish. Three replications were used per treatment. Seeds sown in distilled water served as a control. All Petri dishes were kept at 20 °C/29 °C and a 16/8-h light/dark photoperiod. Percentage germination and the length of the radicle and hypocotyls were recorded after 21 days. The values were expressed as relative (%) to the control.

Tab. 1 - The location of the studied clear-cuts within Scots pine stands in Lithuania.

Forest district	Year of clear-cut	Forest block no.	GPS Coordinates
Kuras	1	6 (46)	54° 56′ 20.4″ N, 23° 34′ 53.9″ E
Ropeja	1	90 (11)	54° 31′ 29.9″ N, 25° 03′ 38.9″ E
	1	114 (1)	54° 31′ 20.4″ N, 25° 03′ 31.1″ E
Zeronys	1	307 (1)	54° 28′ 10.4″ N, 24° 52′ 27.9″ E
	1	307 (9)	54° 28′ 15.6″ N, 24° 52′ 24.7″ E
	1	343 (3)	54° 26′ 55.4″ N, 24° 48′ 19.4″ E
	1	241 (3)	54° 27′ 41.1″ N, 24° 47′ 38.7″ E
	1	74 (3)	54° 31′ 52.8″ N, 24° 51′ 26.1″ E
Ropeja	2	90 (4)	54° 31′ 44.5″ N, 25° 03′ 52.2″ E
	2	125 (8)	54° 31′ 53.4″ N, 25° 08′ 57.1″ E
	2	125 (12)	54° 31′ 49.9″ N, 25° 08′ 58.6″ E
	2	113 (3)	54° 31′ 18.6″ N, 25° 02′ 56.4″ E
	2	46 (7)	54° 32′ 29.6″ N, 25° 03′ 59.6″ E
	2	47 (14)	54° 32′ 25.5″ N, 25° 04′ 33.8″ E
	2	61 (10)	54° 31′ 42.8″ N, 25° 01′ 23.8″ E
	2	92 (1)	54° 31′ 54.0″ N, 25° 04′ 51.6″ E
	2	145 (4)	54° 31′ 24.5″ N, 25° 07′ 34.3″ E

Identification and quantification of allelopathic compounds

For the preparation of methanolic extract, 500 mg of dry shoot and root material of each dominant species was soaked in 10 ml of 75% methanol with shaking for 24 h at room temperature using a VWR Mini Shaker (J&M Scientific, JAV). Extracts were filtered through 0.22-µm low-ash filter paper.

Identified phenolics composition (IPC) was carried out using a reversed-phase HPLC. Two chromatograms were recorded simultaneously. The upper chromatogram was obtained by registering the UV absorbance of the effluent at 265 nm prior to the reaction, a mirror chromatogram was obtained by recording the absorbance at 517 nm after reacting the effluent with 2.2-diphenyl-1-picrylhydrazyl radical (DPPH) solution in the reaction coil. The mobile phase was supplied to the column at a flow rate of 0.75 ml min^{-1}. Samples (extracts) of 10 µl were injected onto the HPLC system. A reversed-phase LiChroCart 12.5×0.4 cm column and a 0.5×0.4 cm pre-column filled with LiChroSpher RP-18e 5-µm I.D. packing material was used for separation. DPPH reagent was prepared by dissolving 0.01M DPPH in sodium acetate buffer, pH 7.6, methanol and acetonitrile (50/25/25, v/v). For gradient elution, solutions A (double-distilled water and 0.05% trifluoroacetic acid, TFA) and B (methanol and 0.05% TFA) were used as the component mobile phase. The phenolic compounds were eluted using the following gradient of mobile phase: 10% B at 0 min, 95% B at 30-33 min, 10% B at 37 min, 10% B at 48 min and 10% B at 58 min. Ten µl injection, 0.75 ml min^{-1} flow rate and λ= 254 nm, λ=517 nm detection zone were used. The identified phenolics were valued *via* the linear regression equation of the standards calibration curves.

Statistical analysis

The confidence limits of the data were based on Wilkin's-λ test and Student's theoretical criterion (t). Standard deviations (SD) and Pearson's correlation coefficients (r) were calculated at the level of statistical significance $\alpha = 0.05$. The allelopathic effect of extracts was tested using the package STATISTICA (StatSoft Inc., Tulsa, OK, USA). Cluster analysis was used to group dominant species. Differences in germination values between extract-treated seeds and control seeds were tested using the ANOVA *post-hoc* LSD test, while differences in the lengths of hypocotyls and radicles were tested using the Kruskal-Wallis test.

Results and discussion

Determination of dominant species in clear-cuts

One-year-old clear-cuts

Clear-cutting in pine forests causes drastic abiotic changes (*e.g.*, irradiance, moisture) to the habitat of pine forests, leading to an abrupt change in the abundance of the understory species.

The phytosociological survey carried out in this study allowed the identification of the dominant species in the 1-yr-old clear-cuts, namely the feather moss (*Pleurozium schreberi*) and lingonberry (*Vaccinium vitis-idaea)*, which were detected in 97% and 95% of the experimental plots, respectively. The common heather (*Calluna vulgaris*) was the third most abundant species in the clear-cuts understory (87% of the experimental plots). Numerous studies have been carried out on the natural productivity and medicinal uses of *V. vitis-idaea* and *C. vulgaris* (Kylli et al. 2011, Monschein et al. 2010), and many were focused on the inhibitory effect of the *Vaccinium* canopy on confifer germination and natural regeneration (Mallik & Pellissier 2000, Mallik 2003). Feather moss is an ever-present component of the boreal forest floor (Gundale et al. 2011). While the ecology of this bryophyte within boreal forests has been widely analyzed, its contribution to plant-plant interaction has not been fully studied.

Two-year-old clear-cuts

The average abundance of the dominant species decreased by about 3% in the 2-yr-old clear-cuts, compared to the 1-yr-old clear-cuts, possibly due to the increased light exposure. Nonetheless, the projection cover of *V. vitis-idaea* increased by 2%, up to 12% across the examined 2-yr-old clear-cuts. Mean abundances of 44% and 68% were detected for bushgrass (*C. epigejos*) and sheep's sorrel (*R. acetosella*), respectively, and were classified as dominants due to their vigorous spreading (generative and vegetative), thus structurally altering the phytocenoses in the clear-cut areas. Previous studies showed that the rapid vegetative propagation of *C. epigejos* through stolons favor its rapid population expansion, determining a consumption of nutrients which hampers pine seedling growth in Lithuanian clear-cuts (Karazija 2003). Moreover, its canopy shading suppresses neighboring herbaceous species or tree seedlings. Intensive generative propagation by small and abundant anemophilous seed is a specific characteristic of the acidophilous species *R. acetosella*, providing strong allelopathic potential in clear-cuts.

Inhibition of germination and seedling parameters

The phytotoxicity of aqueous extracts on acceptor-seed germination and hypocotyl and radicle length was dependent on the dominant species, plant part, age of the clear-cut, types of phenolic compounds present, and variation in phenolic concentration. Germination results and seedling parameters relative to control values are presented in Tab. 2.

Extracts of *C. vulgaris* and *R. acetosella* exhibited the strongest phytotoxicity and inhibition on germination of Scots pine seeds, possibly due to differences in accumulation of phenolic compounds. Shoot and root aqueous extracts of *C. vulgaris* from the 1-yr-old clear-cuts reduced the mean seed germination by 78% and 81%, respectively, as compared with control seeds (100%), while shoots and roots extracts of *R. acetosella* of the 2-yr-old clear-cuts reduced mean germination by 70% and 82%, respectively (Tab. 3 and Tab. 4).

The weakest phytotoxicity and inhibition was observed for aqueous extracts of *P.*

Tab. 2 - Effect of the application of shoot and root acqueous extracts from the dominant species in the understory on seed germination and seedling early growth in Scots pine. Data are mean ± standard deviation (n=6) and the proportion relative to controls (=100%) is indicated. (*): p<0.05; (**): p<0.01; (***): p<0.001.

Clear-cuts	Extracts	Dominant species	Germination		Hypocotyl length		Radicle length	
			Initial, %	Relative to Control, %	cm	Relative to Control, %	cm	Relative to Control, %
1-yr-old	Shoot	*Vaccinium vitis-idaea*	68 ± 3	81 ± 3*	1.5 ± 0.1	40 ± 7***	1.7 ± 0.1	35 ± 7
		Calluna vulgaris	66 ± 1	78 ± 5*	1.7 ± 0.1	44 ± 4	1.9 ± 0.1	39 ± 7
		Pleurozium schreberi	70 ± 3	83 ± 2*	1.7 ± 0.0	45 ± 4	2.0 ± 0.6	41 ± 10
	Root	*Vaccinium vitis-idaea*	69 ± 2	82 ± 5*	1.8 ± 0.0	48 ± 6**	2.0 ± 0.6	43 ± 7
		Calluna vulgaris	68 ± 3	81 ± 5*	1.7 ± 0.3	45 ± 4**	1.9 ± 0.0	40 ± 7**
2-yr-old	Shoot	*Vaccinium vitis-idaea*	67 ± 2	80 ± 6*	1.4 ± 0.2	37 ± 6**	1.7 ± 0.0	35 ± 3***
		Calamagrostis epigejos	68 ± 1	81 ± 1*	1.8 ± 0.0	46 ± 6	1.8 ± 0.0	37 ± 13
		Rumex acetosella	59 ± 5	70 ± 12*	1.3 ± 0.1	40 ± 4**	1.5 ± 0.0	28 ± 9*
	Root	*Vaccinium vitis-idaea*	72 ± 2	86 ± 7*	1.8 ± 0.3	48 ± 8*	2.2 ± 0.0	46 ± 5
		Calamagrostis epigejos	71 ± 4	84 ± 7*	1.7 ± 0.2	46 ± 8**	2.0 ± 0.1	41 ± 7**
		Rumex acetosella	69 ± 1	82 ± 5*	1.8 ± 0.1	47 ± 3**	1.9 ± 0.0	40 ± 9**
		Ð¡ontrol	84 ± 2	100	4.8 ± 0.3	100	3.8 ± 0.3	100

Tab. 3 - Content of identified phenolic compounds (IPC) in shoot extracts of dominant species in the undestory (mean ± standard deviation, n=6). Different letters in the same column indicate significant differences between means after one-way ANOVA analysis followed by a t criterion (p<0.05).

Clea-cuts	Species	IPC content, mg g^{-1}									
		Gallic acid	Chloro-genic acid	Caffeic acid	Trans p-coumaric acid	Ferulic acid	Trans-sinapic acid	Syringic acid	Hydroxy-cinnamic acid	Couma-rine	Total
1-yr-old	*P. schreberi*	-	-	0.04±0.00^{a}	-	-	0.19±0.02^{b}	0.03±0.01^{a}	0.29±0.05^{b}	0.37±0.03^{b}	0.92±0.15
	V. vitis-idaea	-	3.57±0.16^{b}	6.26±0.53^{c}	0.37±0.01^{a}	0.39±0.03^{a}	0.77±0.01^{a}	1.48±0.25^{a}	9.68±0.03^{e}	8.17±0.10^{d}	30.69±3.74
	C. vulgaris	-	6.68±0.43^{c}	1.78±0.11^{b}	0.10±0.02^{a}	0.18±0.02^{a}	2.00±0.13^{b}	-	2.90±0.26^{b}	8.11±0.48^{d}	21.75±3.12
2-yr-old	*V. vitis-idaea*	-	1.37±0.16^{b}	4.35±0.07^{b}	0.76±0.03^{a}	0.84±0.01^{a}	-	-	6.09±0.15^{d}	6.65±0.13^{d}	20.06±2.70
	C. epigejos	0.23±0.00^{a}	0.43±0.01^{a}	-	-	1.46±0.05^{b}	-	-	-	0.23±0.06^{a}	2.35±0.59
	R. acetosella	-	-	-	1.03±0.05^{b}	0.14±0.01^{a}	4.09±0.20^{c}	-	0.14±0.01^{a}	0.16±0.01^{a}	5.56±1.71

Tab. 4 - Content of identified phenolic compounds (IPC) in root extracts of dominant species in the undestory (mean ± standard deviation, n=6). Different letters in the same column indicate significant differences between means after one-way ANOVA analysis followed by a *t* criterion (p<0.05).

Clear-cuts	Species	IPC content, mg g^{-1}								
		Gallic acid	Chloro-genic acid	Caffeic acid	Trans p-coumaric acid	Trans-sinapic acid	Syringic acid	Hydroxy-cinnamic acid	Couma-rine	Total
1-yr-old	*V. vitis-idaea*	-	0.49±0.13^{c}	0.11±0.07^{a}	0.18±0.13^{a}	-	0.11±0.08^{a}	-	-	0.89±0.18
	C. vulgaris	-	-	-	0.14±0.05^{a}	-	-	0.13±0.04^{a}	0.33±0.08^{a}	0.60±0.11
2-yr-old	*V. vitis-idaea*	-	0.11±0.07^{a}	0.08±0.00^{a}	0.39±0.03^{b}	-	-	-	-	0.58±0.17
	C. epigejos	-	0.34±0.04^{b}	-	0.05±0.01^{a}	-	-	-	-	0.49±0.13
	R. acetosella	0.17±0.00^{a}	-	-	0.31±0.01^{a}	0.19±0.0^{a}	-	0.52±0.13^{a}	-	1.19±0.16

schreberi shoot and *V. vitis-idaea* root sampled from the 1-yr-old clear-cuts, as well as *C. epigejos* shoot and *V. vitis-idaea* root from the 2-yr-old clear-cuts, and may be attributed to different phenolic contents. In general, the observed inhibition of shoot extracts was stronger than that of root extracts, although not statistically significant. This is consistent with previous studies which demonstrated the presence of different allelochemicals and concentrations in the shoots and roots of different dominant species (Dorning & Cipollini 2006).

Radicle growth of pine seedlings was more strongly inhibited than their hypocotyl growth by extracts of the tested dominant plants from both 1-yr-old and 2-yr-old clear-cuts. This finding is consistent with those of previous studies on different species, and suggests that seedling roots are more sensitive than shoots to allelochemicals (Parvez et al. 2004, Baležentienė & Renćo 2014).

The analysis of seedling morphology revealed that *V. vitis-idaea* and *C. vulgaris* shoot extracts from the 1-yr-old clear-cuts had a stronger inhibition in term of hypocotyl and radicle growth than their root extracts. Contrastingly, *P. schreberi* shoot extracts and *V. vitis-idaea* L. root extracts from 1-yr-old clear-cuts showed a lesser impact on the same morphological variables (Tab. 2).

Hypocotyl length was similarly strongly supressed by applying *V. vitis-idaea* shoot and *C. epigejos* root extracts from the 2-yr-old clear-cuts. However, it was less inhibited by *C. epigejos* shoot extract compared to *V. vitis-idaea* shoot extract, possibly due to the different identified phenolic content between tested species. Radicle length was severely reduced by *R. acetosella* shoot and root extracts. Nonetheless, it was less suppressed by *C. epigejos* shoot and *V. vitis-idaea* root extracts.

Tab. 5 displays the results of the correlation analysis between the phenolic compounds identified in the extracts and their effects on Scots pine seedling growth. However, it has been proposed that the different responses of seedlings to shoot and root extracts might depend on evolutionary differences in resistance to allelopathic compounds among the acceptor species (Wang et al. 2010).

Content and identified phenolic compounds

Phenolic compounds are the most important and common plant allelochemicals (Fraenkel 1959). Phenolics play a major role in ecosystem functionality as they mediate many interactions between the plant and its biotic and abiotic environments. Moreover, phenolic compounds are a striking example of metabolic plasticity, enabling plants to adapt to changes in their biotic and abiotic environments (Boudet 2007). Additionally, phenolics are particularly important in seed germination, development, and plant resistance to various stresses. Nonetheless, their content and composition are likely to differ depending on plant species, tissues and cells during ontogenesis and under the influence of various environmental stimuli (Inderjit & Duke 2003).

We found that IPC composition and content varied depending on dominant species and cut-age, though in all cases an inhibitory effect on germination and seedling growth was observed. The highest IPC content was found in *V. vitis-idaea* shoot extracts from the 1-yr-old and the 2-yr-old clear-cuts and in *C. vulgaris* shoot extracts of the 1-yr-old clear-cuts (Tab. 3). IPC content was found significantly lower in *P. schreberi* of the 1-yr-old clear-cuts, and in *C. epigejos* and *R. acetosella* shoot extracts of the 2-yr-old clear-cuts, possibly due to species-specific biological peculiarities to synthesize and accumulate phenolics.

The largest content and the largest variety of IPC was determined in *V. vitis-idaea* shoot extracts from both the 1-yr-old (chlorogenic, caffeic, *trans*-*ρ*-coumaric, ferulic, *trans*-sinapic, syringic, hydroxycinnamic acids and coumarin) and the 2-yr-old clear-cuts (all the aforementioned compounds, except *trans*-sinapic and syringic acids). These properties explain the highest phytotoxicity and inhibition of the species extracts on pine seeds and seedlings. Chlorogenic, caffeic, *trans*-*ρ*-cumaric, ferulic, *trans*-sinapic, hydroxycinnamic acids and coumarine were also found in aqueous extracts of *C. vulgaris* from the 1-yr-old clear-cuts.

Quantitative and qualitative differences in allelochemicals may explain the different extent of suppressive allelopathic effects among dominant species in the understory. In order to identify the underlying patterns of species allelopathic effects, the dominant species were clustered on the basis of their chemical properties. As the lowest content of IPC was found in the roots, cluster analysis was applied on data from the shoots of the dominant species. Two main groups of dominant species were identified based on their shoot IPC content. According to the dendrogram reported in Fig. 1, *V. vitis-idaea* and *C. vulgaris* clustered

Tab. 5 - Effect of the phenolic compounds identified in acqueous extracts of dominant species in the understory on early growth parameters of Scots pine. Pearson's correlation coefficients are shown (n="?). (*): p<0.05.

Parameter	Total phenolic content	Trans p-coumaric acid	Ferulic acid	Chloro-genic acid	Gallic acid	Caffeic acid	Trans-sinapic acid	Syringic acid	Hydroxy-cinnamic acid	Couma-rine
Seed germination	0.3	-0.4*	0.1	0.2	0.01	0.3	-0.5*	0.2	0.3	0.3
Seedling radicle length	-0.01	-0.4*	-0.1	0.1	0.1	-0.02	-0.3*	-0.04	-0.02	0.1
Seedling hypocotyl length	-0.3*	-0.5*	-0.5*	0.04	0.3	-0.4*	-0.1	-0.2	-0.3*	-0.2

together in the group showing the strongest inhibition effect (Group 2), whereas *P. schreberi, C. epigejos* and *R. acetosella* grouped to a different group (Group 1) characterized by weaker suppression allelochemical activity on Scots pine seedlings.

All the phenolic compounds identified in this study have been considered as potential allelopathic agents in previous studies (Macias et al. 2007, Li et al. 2010). According to Boudet (2007), the lower variety in allelochemicals identified in plant roots might be related to the lower content of phenolic compounds in shoots, as opposed to that in roots. Our results confirmed the higher IPC in shoot than in root in all the dominant species examined (Tab. 3, Tab. 4). The highest content in IPC was observed in *V. vitis-idaea* L. shoot from 1-yr-old and the 2-yr-old cuts. The highest root IPC was determined in *R. acetosella* from the 2-yr-old clear-cuts (1.19 ± 0.16 mg g^{-1}) and in *V. vitis-idaea* and *C. vulgaris* extracts from the 1-yr-old clear-cuts.

In general, we found a significant correlation between the identified phenolics in extracts of dominant species and their inhibitory effect on seed germination in Scots pine (Tab. 5), though such effect varied across different phenolic compounds. Consistently with previous reports (Djurdjevic et al. 2004, Khan et al. 2009), a significant negative correlation ($r = -0.39$, $p<0.05$) was detected between *trans*-ρ-coumaric acid and seed germination, as well as between the above compound and seedling parameters, namely radicle length ($r = -0.43$, $p<0.05$) and hypocotyl length ($r = -0.45$, $p<0.05$). Therefore, our results confirmed that the tested dominant species may influences Scots pine recruitment in clear-cuts through their phenolic compounds, which inhibit seed germination and seedling growth.

Various phenolic compounds have been experimentally proven to have allelopathic activity, such as ferulic acid (Chung et al. 2002), caffeic acid (Li et al. 2010), *p*-hydroxybenzoic and cinnamic acids (Abe et al. 2012, Heidarzadeh et al. 2012). In this study, a significant negative correlation of the *trans*-sinapic acid content was observed with seed germination ($r = -0.47$, $p<0.05$), and seedling radicle growth ($r = -0.34$, $p<0.05$). Noteworthy, a similar allelochemical effect of *trans*-sinapic acid was previously reported by Chung et al. (2002). Furthermore, a significant negative correlation was observed between the length of Scots pine seedling hypocotyls and ferulic acid ($r = 0.45$, $p<0.05$), caffeic acid ($r = 0.35$, $p<0.05$) and hydroxycinnamic acid ($r = 0.33$, $p<0.05$). The highest contents of ferulic, caffeic and hydroxycinnamic acids were detected in *V. vitis-idaea* extracts from both the 1-yr-old and 2-yr-old clear-cuts. The highest content of *trans*-sinapic acid was measured in *R. acetosella* extracts from the 2-yr-old clear-cuts (4.09 ± 0.20 mg g^{-1}) and in *C. vulgaris* aqueous extracts from the 1-yr-old clear-cuts (2.00 ± 0.13 mg g^{-1}).

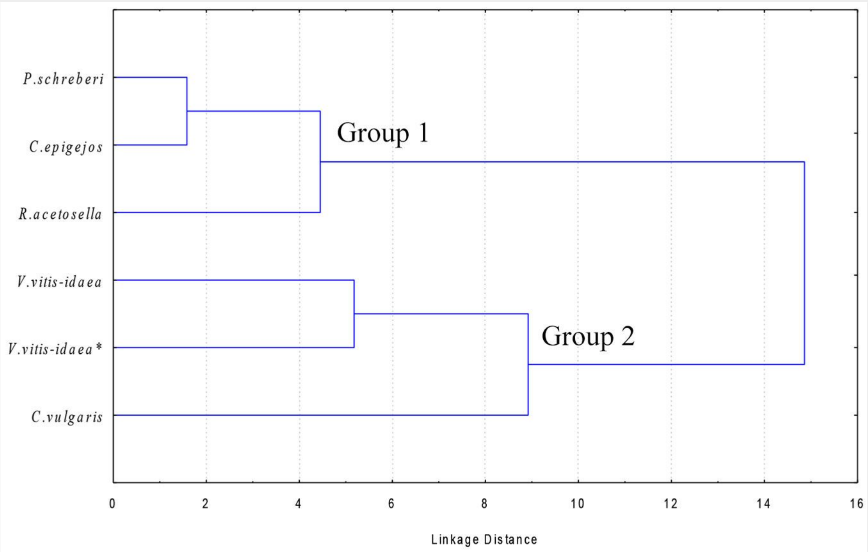

Fig. 1 - Cluster analysis of the dominant species in the understory of clear-cuts in Scots pine stands, based on the identified phenolic compounds in their shoot extracts.

The highest content of *trans*-ρ-coumaric acid was found in *R. acetosella* and *V. vitis-idaea* extracts in the 2-yr-old clear-cuts, as well as in *V. vitis-idaea* and *C. vulgaris* extracts from the 1-yr-old clear-cuts. Our findings are in line with those of Mallik (2003), who demonstrated that the accumulated litter of ericaceous plants (*Vaccinium, Calluna* spp.) contains an array of phenolic compounds that is inhibitory to conifer seed germination, primary root growth, and ectomycorrhizal growth. Moreover, many of these phenolic compounds might provoke soil nutrient imbalance through the reduction of available nitrogen (by forming protein-phenol complexes), thus leading to long-term site degradation (Inderjit & Duke 2003).

Based on the results of this investigation, the accumulation of high amounts of allelochemicals by species such as *C. vulgaris* and *V. vitis-idaea* could be hypothesized to be one of the primary reasons for conifer regeneration failure after forest disturbance in Lithuania. The negative effect of dominant understory species on Scots pine early growth might have practical significance in the forest management, *e.g.*, reforestation might be preferred to natural regeneration in presence of such negative impact.

Conclusions

The inhibitory allelopathic effect of aqueous extracts depends on the dominant species in clear-cuts, the plant part, the quantity and composition of phenolic compounds, and cut's age. The aqueous extracts of *C. vulgaris* of 1-yr-old clear-cuts and *R. acetosella* of 2-yr-old clear-cuts exhibited the strongest phytotoxicity, inhibiting Scots pine seed germination due to their highest phenolics accumulation. Our results showed that shoot extracts has higher content and variety of phenolic compounds compared to root extracts. The highest content of phenolic compounds was obtained in extracts of *V. vitis-idaea* (both from 1-yr-old and 2-yr-old clear-cuts), *R. acetosella* (2-yr-old clear-cuts) and *C. vulgaris* (1-yr-old clear-cuts). Moreover, strong negative correlations between the content in phenolic compounds of extracts and both seed germination and seedling growth were observed.

Species such as *V. vitis-idaea, C. vulgaris* and *R. acetosella* can negatively affect the process of natural reforestation due to high contents and variety of phenolic compounds in their biomass or in plant debris. Furthermore, the knowledge of allelopathic activity of dominant species in the understory of clear-cuts might help choosing the best strategies to mitigate the negative phytotoxic impact of herbaceous species on early pine growth.

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