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Original Research

Biochemical Impact of Dominant Extracts of Scots Pine Cuttings on Germination

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Abstract

The main objective of this study was to determine the allelopathic impact of dominant species (*Calamagrostis epigeios, Rubus idaeus* and *Chamaenerion angustifolium*) of clear-cuttings of Scots pine (*Pinus sylvestris* L.) forests (*Vaccinio-myrtillo Pinetum*) on test species germination emphasizing the forest ecosystem establishment. Aqueous extracts of roots and shoots were produced at different growth stages and assayed on germination. Additionally, total concentration of phenols was evaluated photo spectrometrically. Extracts of shoots more strongly inhibited germination than those of roots of all investigated species. The strongest inhibitory effect on germination and the highest phenol contents were documented during the flowering stage rather than in spring and autumn. Accordingly, to mean germination data the declining phytotoxicity sequence of the species was determined: *R. idaeus* > *C. epigeios* > *Ch. angustifolium*. Hence this study implied in allelopathic activity of species a potential suppressive factor that could influence germination and forest regeneration, but field condition studies are necessary.

Keywords: *Pinus sylvestris* L., allelopathy, dominants, germination, growth stage

Introduction

Initial recruitment, including germination, seedling growth and establishment, is particularly important in plant communities' development and in forest regeneration as well [1, 2]. These processes can be influenced by several ecological parameters, synergically impacting plant regeneration, but only a few studies deal with plant recruitment and forest regeneration taking into account the concomitant effect of environment indices [3-5]. Studies of biochemical interaction between organisms, or allelopathy, tried to explain the significance of the role of secondary metabolites' diversity in functionality of organisms and ecosystems [6-8]. However, biochemically mediated interferences are those of many interactions (competition, microbial nutrient immobilization, mycorrhizal activity etc.) existing in ecosystems, the distinction of which is difficult if not impossible [6]. It is suggested that allelopathic activity of plants should be the major factor enabling implementation of growth management between neighbours and defence mechanisms developed during long co-evolution with their competitors and enemies [9]. Consequently, biochemical interactions are components with an important ecological role in the maintenance of the ecosystems and biodiversity present in them [10-12]. These interactions include both inhibitory and stimulatory effects of allelochemicals released or incorporated in plants and their debris [13-16]. There are many reports about allelochemical production in many woody species, from *Eucalyptus* sp. forests in Australia [4], to boreal conifer forests [17], tropical forests [18], temperate forests [19], and sub-desert communities [20]. Coder [21] provided a detailed compilation of more than a hundred tree species with allelopathic activity. Problems of conifer regeneration caused by ericaceous understory species have also been described [17].

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Table 1. Descriptions of tested plants' growth stages.

Species	Growth stages	Description of BBCH-scale codes
Red raspberry (*Rubus idaeus* L.)	Stem elongation or rosette growth/shoot development (main shoot proceeded 30% of full length)	3/33
	Flowering (main shoot)	6/69
	Senescence, beginning of dormancy	9/93
Wood small-reed (*Calamagrostis epigeios* (L.) Roth)	Formation of side shoots/tillering	2/26
	Flowering (main shoot)	6/67
	Senescence, beginning of dormancy/stem fully developed, yet green	9/91
Rosebay willow herb (*Chamaenerion angustifolium* (L.) Scop.)	Stem elongation or rosette growth / shoot development (main shoot proceeded 20% of full length)	3/32
	Full flowering (burst 50% blossoms)	6/65
	Senescence, beginning of dormancy/beginning of leaf fall	9/93

In forest ecology and population biology, the inability to germinate and establish seedlings is considered to be a result of competition and aggressiveness of the established plants, but the mechanisms of aggressiveness have not yet been well defined [22, 23]. Many thousands of secondary compounds have the potential to act allelopathically and inhibit or stimulate both seed germination and seedling growth. They have stronger impact on the vicinity of neighbouring species in the invaded range or are negatively affecting the growth of native flora and other organisms [24-27]. Allelopatic research could be widened due to identification, isolation, and characterization of both allelopathic compounds and their role in interference between abiotic and biotic stress factors [28-31]. These studies emphasized the significance of understanding multifunctional aspects of allelopathy in structuring trophical levels, affecting predators and pests, forming symbiotic relations, and mediating competitive circumstances that are crucial for reforestation.

Allelopathy is related to the solution of practical problems of chemical interference between seedlings and weeds, toxicity of trees and weeds residues, and/or exudates etc. Nowadays, great attention is paid to the identification of plant bioactive compounds, their production and application as components for bio-preparations: phytoherbicides, phytopesticides etc. [31]. Therefore allelopathy must be considered a part of the biotic resource management strategies in modern forest ecosystems where natural and mixed communities are replaced by a single species [33-35].

Plant allelochemicals have different impacts on neighbour species due to contributed inhibitors (e.g. phenols), promoters (e.g. nitrates) and neutral substances (e.g. glucose) [25, 36-38]. Inhibitory substances are often argued to explain growth pattern, while other substances remain neglected. The overwhelming evidence suggests that plant phenols accumulate up to 1-3% concentration in fresh mass and have the potential to influence germination and growth patterns [8, 11, 28, 39].

The objectives of this research were to evaluate and compare the total concentration of phenolic compounds and allelopathic activity of the aqueous extracts produced from both ground parts and roots of some dominant species of clear-cuttings of pine forests (*Vaccinio-myrtillo Pinetum*): wood small-reed, raspberry and rosebay willow herb at different growth stages. A multifunctional laboratory experiment was designed in order to understand the implication and interaction between allelopathic and plant development parameters (growth stages) on germination and the processes of early recruitment. There are no references about phytotoxicity and allelopathic activity on germination of these plants, so new information will be helpful for understanding the common allelochemical potential of these dominant species, and also their impact on reforestation and on management of forest ecosystems.

Materials and Method

Sampling

In order to evaluate biochemical activity and impact on initial plant growth stage – on germination of typical dominant species in clear-cuttings of Scots pine (*Pinus sylvestris* L.) forests (*Vaccinio-myrtillo Pinetum*), small-reed (*Calamagrostis epigeios* L. Roth), red raspberry (*Rubus idaeus* L.) and rosebay willow herb (*Chamaenerion angustifolium* L. Scop) laboratory trials were carried out in the environment laboratory of the Lithuanian University of Agriculture. These species grow and spread intensively become dominant, and whose impact on germination and forest regeneration is relevant to investigating clear-cuttings [40]. Plants and their parts (roots and shoots) were collected at different growth stages in clear-cuttings of pine forest in Kačerginė (Kaunas district, Lithuania) for producing and bioassay of aqueous extracts (Table 1). BBCH scale was chosen for determining plant growth stages [2].

Determination of Total Phenolic Content

The total concentration (ppm) of phenols as the essential allelopathic characteristic for plant cells was estimated according to the modified Jermakov et al. [34, 41] and K. Slinkard and V. L. Singleton [5] spectrophotometric method using Folin-Ciocalteu (or Folin-Denis) reagent, chlorogenic acid as a standard phenolic compound and 20% Na_2CO_3. Concentrations of phenolic compounds in the plant aqueous extracts were expressed as parts per million (ppm) equivalent to chlorogenic acid.

Bioassay

Biochemical activity of species was estimated on the basis of seed germination bio-screening accordingly to Grodzinsky's method [28]. According to this mode, germination in extracts was recorded as that one of seed with the highest response on growth agents, *i. a*. 50% (G_{50}) germination in distilled water (control). G_{50} rate is equated to 100%. This method enables us to assess not only inhibitory, but also stimulatory effects of extracts. Fast germinating and having a high germination energy rate rape seed (*Brassica napus* L.) cv. *Valesca* was chosen as the receptor plant. One hundred rape seeds were placed on filter paper in each 6-cm diameter Petri dish. Five ml aqueous plant extracts (0; 0.2; 0.1; 0.05 and 0.02% concentration) were added per Petri dish as per treatment. Treatments were replicated four times. Petri dishes were kept at 26°C for 16 h. Seeds sown in distilled water served as control. Germination was considered when radicle emerged from seed coat. Seed germination was used to calculate the allelopathic potential of aqueous extracts in conventional coumarine units (CCU) using Grodzinsky's method [28]. In accordance with this mode, a universal index of allelochemicals activity – CCU, is evaluated by a nomograph composed according to coumarine activity and germination.

Statistical Analysis

The confidence limits of the data were based on Wilkin's-λ test and Student's theoretical criterion. Standard errors (SE) and standard deviation (SD) were calculated at the level of statistical significance $p<0.05$. The results of allelopathic effects were statistically evaluated using the statistical package STATISTICA of Stat Soft for Windows. Results of germination, phenol concentrations and CCU are presented as a mean±SD of 4 independent analyses at the 0.05 probability level.

Results and Discussion

During the experiment the impact of phytotoxicity of produced aqueos extracts was evaluated in dependence on plant species, plant part, growth stage, extract and phenol concentrations (Table 2).

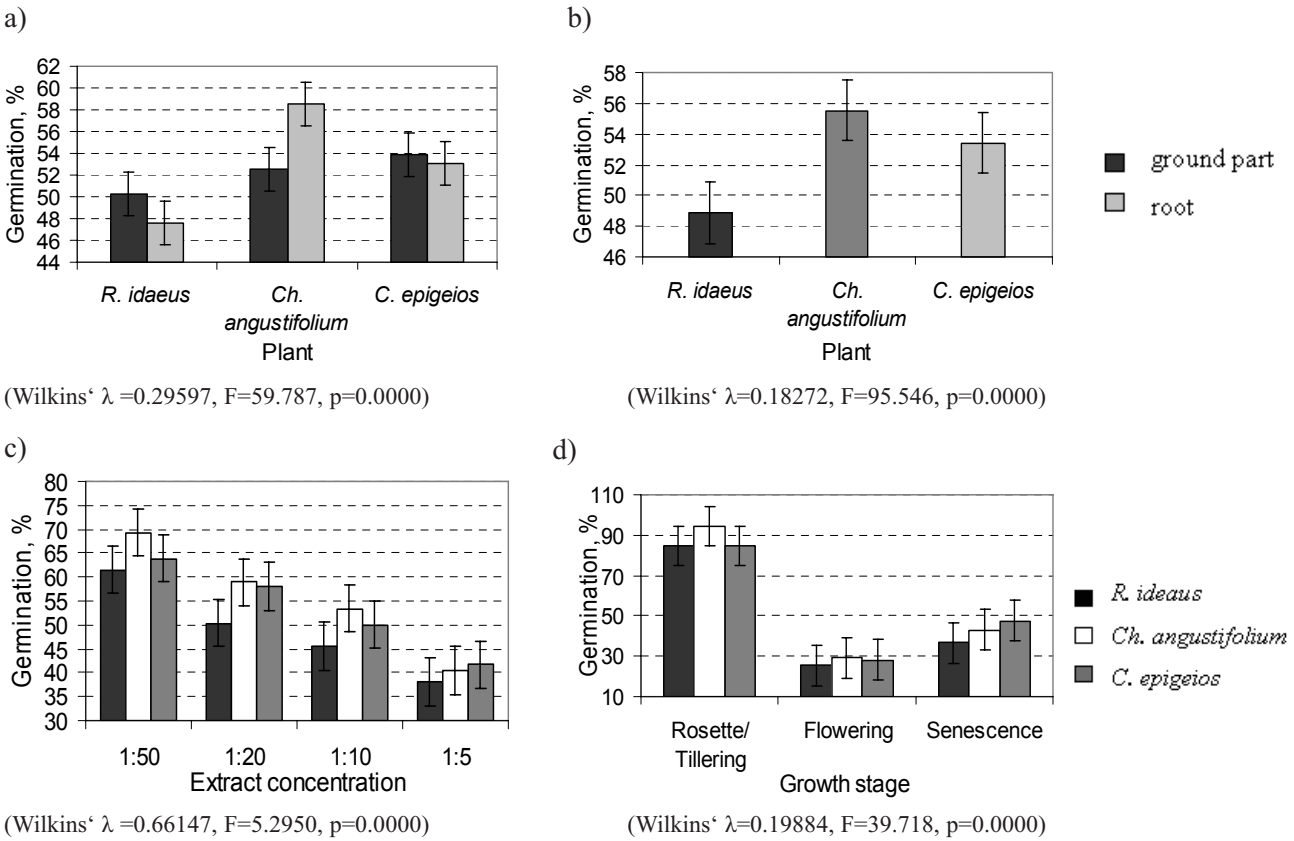

Fig. 1. Impact of plant part (a), plant species (b), extract concentration (c) and growth stage (d) on mean germination (mean±SD).

Table 2. Germination and CCU content in extracts of different plants and plant parts (mean±SE, p<0.05).

Species	Extract concentration							
	0.2%		0.1%		0.05%		0.02%	
	Germination, %	CCU, un.	Germination, %	CCU, un.	Germination, %	CCU, un.	Germination, %	CCU, un.
Extracts of ground part								
Stem elongation or rosette growth/shoot development stage								
R. idaeus	70±2.83	159	85±1.41	115	89±6.36	105	91±3.53	100
Ch. angustifolium	81±12.02	125	96±4.94	92	98±6.36	105	104±0	77
C. epigeios	89±13.43	105	89±9.19	105	98±10.60	88	104±2.82	77
Flowering stage								
R. idaeus	19±2.82	540	25±4.24	425	27±2.82	400	44±1.41	275
Ch. angustifolium	15±6.36	650	23±1.41	460	27±1.41	400	40±7.77	303
C. epigeios	11±4.94	800	15±0	650	36±1.41	332	46±4.24	263
Senescence, beginning of dormancy/beginning of leaf fall stage								
R. idaeus	29±1.41	380	37±2.82	332	38±4.24	315	54±2.82	229
Ch. angustifolium	25±2.12	425	39±2.82	315	47±9.19	263	53±7.07	229
C. epigeios	24±3.53	460	38±14.84	315	48±10.60	252	56±9.19	218
Extracts of roots								
Stem elongation or rosette growth/shoot development stage								
R. idaeus	72±12.02	151	79±0	131	91±3.53	100	100±4.24	84
Ch. angustifolium	83±0	120	96±4.94	92	102±4.24	77	109±2.12	71
C. epigeios	62±13.43	192	75±6.36	145	81±19.79	125	85±4.24	115
Flowering stage								
R. idaeus	12±6.36	720	19±5.65	540	23±4.24	460	38±6.36	315
Ch. angustifolium	13±0	720	30±4.24	380	40±12.02	303	53±2.82	229
C. epigeios	23±4.24	460	25±7.07	425	36±6.36	332	40±2.82	303
Senescence, beginning of dormancy/beginning of leaf fall stage								
R. idaeus	26±3.53	425	31±7.07	362	35±13.43	332	46±7.77	263
Ch. angustifolium	28±9.19	400	44±10.60	275	52±5.65	241	62±0	192
C. epigeios	46±1.41	263	60±0	200	56±2.82	218	57±2.82	208

Germination rate (11-109%) and CCU content (71-800) varied accordingly to concentration gradients of all extracts. The majority of aqueous extracts (0.2-0.05% concentration) indicated a suppressive impact on germination, with exception of 0.02% extracts produced from ground part of *C. epigeios* (104%) and 0.02 and 0.05% extracts produced from both ground part and roots of *Ch. angustifolium* (104, 109 and 102%, respectively) at stem elongation/rosette growth, which impacts slightly stimulated germination.

According to earlier studies [9, 29, 42], germination and ecosystem (forest) regeneration are reliant on present species and plant parts. Roots and ground part extracts of *R. idaeus* indicated the strongest inhibitory effect on germination suggested in this study species (Fig. 1a). These results clearified *R. idaeus* as the violent inhibitor that could suppess early species recruitment in forest ecosystems.

Ground part of *C. epigeios* and roots *Ch. angustifolium* showed the weakest suppressive effect on germination (mean value 54 and 59% respectively). Stonger inhibitory effect was documented of ground part extracts (mean germination 52%) than of roots' (mean germination 53%). These data may indicate the presense of different allelochemicals or their concentrations in ground part and roots, therefore the shoots of researched plants are the maine source of their allelochemicals. This phenomenon of differ-

Table 3. Total phenols content (ppm) in different plant part extracts (mean±SE, $p<0.05$).

Extracts of ground part				
Species	Extract concentration			
	0.2%	0.1%	0.05%	0.02%
Stem elongation or rosette growth/shoot development stage				
R. idaeus	0.1059±0.0043	0.0585±0.0036	0.0463±0.0027	0.0362±0.0019
Ch. angustifolium	0.0382±0.0009	0.0363±0.0001	0.0196±0.0009	0.0173±0.0015
C. epigeios	0.0234±0.0021	0.0161±0.0008	0.0139±0.0029	0.0182±0.0002
Flowering stage				
R. idaeus	0.5900±0.0049	0.4350±0.0363	0.2820±0.0059	0.1650±0.0132
Ch. angustifolium	0.5810±0.0041	0.3580±0.0038	0.2400±0.0049	0.1634±0.0532
C. epigeios	0.2110±0.0053	0.1210±0.0148	0.0610 ±0.0093	0.0200±0.0018
Senescence, beginning of dormancy/beginning of leaf fall stage				
R. idaeus	0.0960±0.0912	0.0310±7.0711	0.0270±0.0064	0.0240±0.0103
Ch. angustifolium	0.0375±0.0035	0.0250±0.0135	0.0200±0.0158	0.0050±0.0031
C. epigeios	0.0310±0	0.0240±0.0095	0.0240±0.0106	0.0130±0.0016
Extracts of roots				
Stem elongation or rosette growth/shoot development stage				
R. idaeus	0.1395±0.0205	0.0797±0.0021	0.0777±0.0052	0.0341±0.0134
Ch. angustifolium	0.0317±0.0006	0.0219±0.0003	0.0163±0.0005	0.0158±0.0008
C. epigeios	0.0242±0.0001	0.0209±0.0003	0.0162±0.0023	0.0145±0.0009
Flowering stage				
R. idaeus	0.3770±0.0394	0.2760±0.0489	0.1600±0.0600	0.1310±0.0849
Ch. angustifolium	0.1890±0.0008	0.0960±0.0042	0.0390±0.0015	0.0220±7.0710
C. epigeios	0.3730±0.0532	0.1740±0.0382	0.1020±0.0721	0.0910±0.0643
Senescence, beginning of dormancy/beginning of leaf fall stage				
R. idaeus	0.0407±0.0065	0.0300±0.0009	0.0200±0.0067	0.0230±0.0046
Ch. angustifolium	0.0310±7.0710	0.0260±0.0072	0.0250±0.0093	0.0180±0.0025
C. epigeios	0.0270±0.0055	0.0270±0.0063	0.0220±0.0030	0.0210±0

ent impact of plant ground part and roots has already been observed in several references [43-45].

In general, accordingly to mean germination data, the declining phytotoxicity sequence of plant species was determined: *R. idaeus* > *C. epigeios* > *Ch. angustifolium* (Fig. 1b). These results suggest that *R. idaeus* has the strongest potential to impact initial stages of forest ecosystem regeneration, but further research is needed in this regard *in situ*.

Strong negative correlation (0.6 and 0.7) confirmed the significant impact of extract concentration (Fig. 1b) and growth stage on germination (Fig. 1d). Both ground part and root extracts expressed the strongest phytoinhibitory effect on seed germination at flowering stage (11-53%), and in autumn (24-57%) as distinct from extracts occurring at the beginning of vegetation plants (Table 2). In agreement with these results, shoot extracts of other plants at the reproductive stage acted more suppressive than at vegetative stages [15, 22, 26, 42, 46].

This germination evidence indicates that allelopathic potential could be regulated by plant species, age and ground plant part would be the main source of allelochemicals [1, 22, 47].

CCU content, as a universal index of extract bioactivity, and germination exhibited the same tendencies of variation. The biggest amount of CCU (800 and 720 CCU) was determined in 0.2% extracts at the flowering stage of *C. epigeios* ground part, also of *R. idaeus*, and *Ch. angustifolium* roots due to the highest phenol content. 0.02% extracts of ground part of *Ch. angustifolium* (3/32 BBCH)

and *C. epigeios* (2/26 BBCH) with the stimulating effect had the least content of CCU content (77 and 71 respectively). Strong negative correlation (0.8-0.9) was determined between germination and CCU.

Phytotoxicity of aqueous extracts depended not only on plant age and part, but also on phenol content, which decreased accordingly to extract concentration gradients (Table 3). Many authors have pointed out the phytotoxic and inhibitory effect of phenols, especially on germination through impact on mitotic activity [12, 17, 46]. In this study strong negative correlation (0.5-0.6) was observed between germination and phenol concentrations and confirms the significantly negative role of phenols on germination. The highest phenol concentrations (0.59 and 0.3770 ppm) were determined in *R. idaeus* ground part and roots 0.2% extracts at flowering stage. Due to the highest phenols content, these extracts had the strongest phytotoxicity and demonstrated the strongest inhibitory effect on germination. Phenols concentration was insignificant and bioactivity of extracts was appointed negligible at shooting (0.0139-0.0585 ppm) and at senescence (0.0050-0.0960 ppm) stages of both the ground part and roots of all plants (Fig. 2a).

As a result of such variation of phenols concentration, the least phytotoxicity and inhibitory impact on germination were determined at the beginning and the end of plant vegetation. In autumn mean phenol concentration of *R. idaeus* and *C. epigeos* decreased by 9 times and that of *Ch. angustifolium* – by 2 times compared with plants at flowering stage. Mean values of phenol concentrations in ground part extracts was approximately two times higher and explained its stronger suppressive activity compared with roots (Fig. 2b). Exhibited tendencies of stronger inhibition on germination of tested plant ground part correspond with similar observations made with *Medicago sativa* L. [26].

Phytotoxicity of produced aqueous extracts was expressed as CCU. The highest phenols concentration was determined in extracts of the highest concentration, in which the least mean germination (40%) and the highest mean allelophatic activity (220 CCU) were recorded (Fig. 2c). The weakest impact of the least concentration (0.02%) extracts on mean germination (60%) could be explained due to the lowest phenols concentration and CCU content (60 CCU).

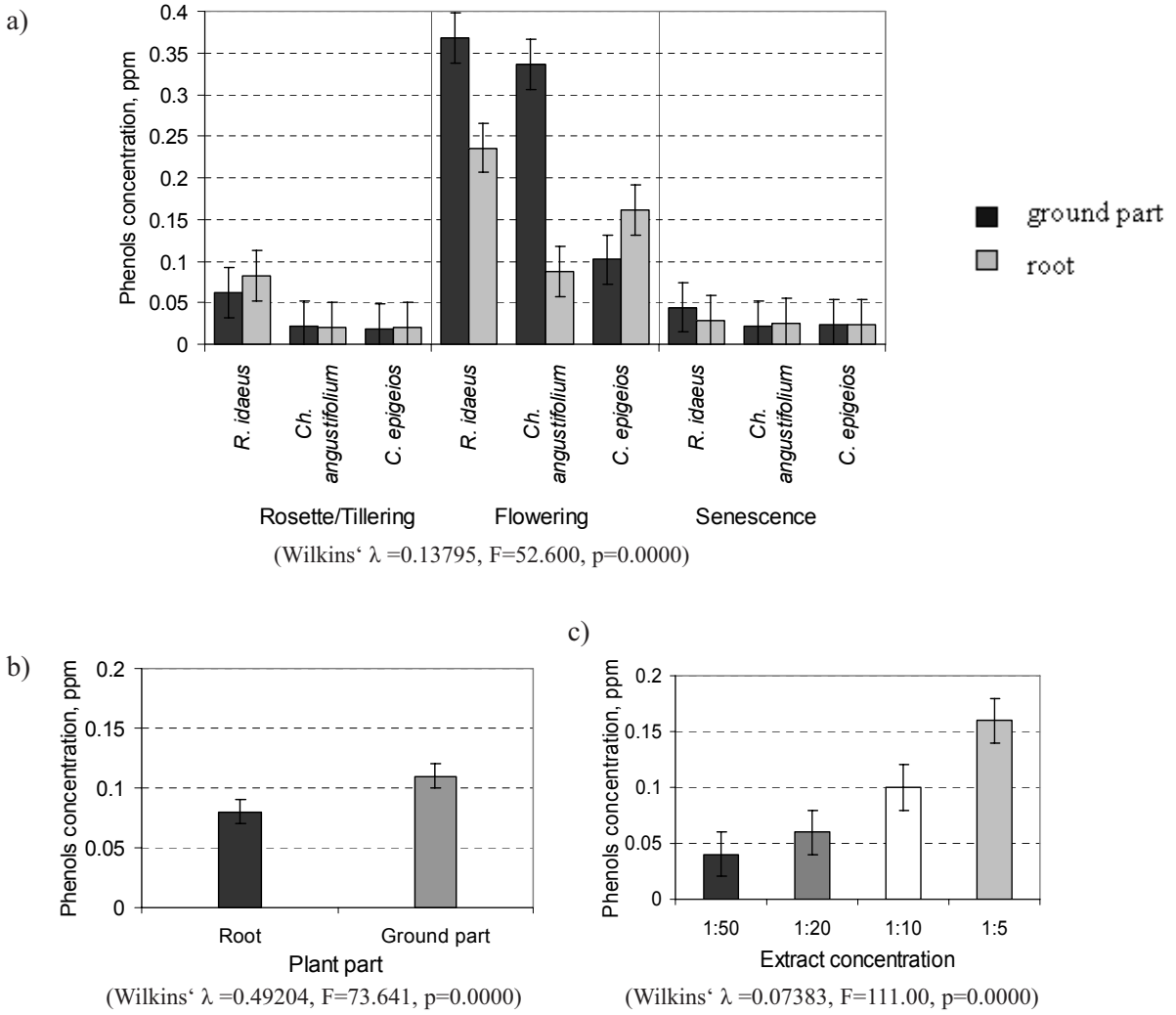

Fig. 2. Impact of plant, plant part and growth stage on phenolic content (a) and comparison of phenols mean concentration in different plant parts (b) and aqueous extracts (c) (mean± SD).

These results suggest that release of phenols in the environment inhibited germination and could impact natural forest regeneration and reforestation. However, more detailed studies estimating the impact of these compounds on recipient species *in situ* are necessary.

Conclusions

Different allelopathic effects of aqueous extracts produced from ground parts and roots of dominants of pine forests' clear-cuttings was documented in this study. The character of allelopathic activity (inhibitory, neutral or stimulatory) on germination depended on extract concentrations, plant species and growth stage.

The extracts of ground parts of all species were more toxic than root extracts, and had the strongest suppressive effect on germination due higher phenolic content. Nonetheless, the presence of other bioactive metabolites (flavonoids) cannot be excluded. Germination was suppressed at all growth stages in extracts of the highest concentration (0.2-0.05%) of plant ground part and roots, while it was stimulated weakly at initial growth stages in the extracts (root and ground part of *Ch. angustifolium* and ground part of *C. epigeios*) of the least concentration (0.02%). All tested extracts at flowering stage had the strongest inhibitory effect on germination compared with those prepared in spring and autumn due to decreased content of CCU and dissolved phenol concentrations. Fluctuation of total (unidentified) content of dissolved phenols in the extracts was similar to that of CCU in individual species, plant part and growth stage.

Allelochemical activity and phytotoxic impact of treated dominant species are based on dissolved phenols and may be substantial for solving practical problems of chemical interference (allelopathy) between theses species and germination, seedling growth and reforestation by regulating inter and intraspecific interactions. But more detailed investigation of germination *in situ* and identification of phenols composition is required for evaluating these factors' impact on natural forest regeneration or reforestation.

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