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CHANGES IN THE CHEMICAL COMPOSITION OF *ACER NEGUNDO* AND *ROBINIA PSEUDOACACIA* GREEN LEAF BIOMASS AFTER PASSIVE COMPOSTING

Acer negundo L. (Sapindaceae) and *Robinia pseudoacacia* L. (Fabaceae) are woody weeds with an expanding secondary range, recognized as invasive species in Belarus and many temperate countries. Due to their high biomass production potential, these plants are being considered for use as organic fertilizer or mulch after mechanical removal. To assess the risk of phytotoxicity from their phytomass to cultivated plants, a study was conducted on the content of organic compounds with presumed allelopathic activity, both before and after 10 months of composting, including during the cold seasons.

The Folin & Ciocalteu's assay, carried out independently in two laboratories, showed a reduction in total phenolic contents after composting, with 5.0—6.7-fold decrease in *A. negundo* leaves and 2.1—4.9-fold decrease in *R. pseudoacacia* leaves. Gas chromatography-mass spectrometry (GC-MS) analysis of water extracts showed disappearance of selected (marker) phenolic compounds in both plants after composting. At the same time, the increase in the levels of organic acids was detected in composted phytomass compared to the non-composted material, especially lactic and phosphoric acids. The presumed decomposition of phenolic compounds reduces the phytotoxicity of leaves after composting, while the presence of allelopathically active organic acids may explain some remaining allelopathic effects in the compost. Both the Folin & Ciocalteu's assay and GC-MS analysis showed no apparent differences in phenolic compounds between *A. negundo* and *R. pseudoacacia* non-composted leaves that were dried in the shade and those dried in the sun.

Key words: *Acer negundo*; allelochemicals; invasive plants; organic acids; phenolic compounds; phytomass utilization; *Robinia pseudoacacia*.

Table 3. Ref.: 35 titles.

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ИЗМЕНЕНИЯ В ХИМИЧЕСКОМ СОСТАВЕ ЗЕЛЕННЫХ ЛИСТЬЕВ *ACER NEGUNDO* И *ROBINIA PSEUDOACACIA* ПОСЛЕ ИХ ПАССИВНОГО КОМПОСТИРОВАНИЯ

Acer negundo L. (Sapindaceae) and *Robinia pseudoacacia* L. (Fabaceae) — древесные сорняки с расширяющимся вторичным ареалом, признанные инвазионными видами в Беларуси и многих странах с умеренным климатом. По причине высокой продуктивности эти растения рассматриваются для использования в качестве органического удобрения или мульчи после механического удаления. Для оценки риска фитотоксичности их фитомассы для культурных растений было проведено исследование содержания органических соединений с предполагаемой аллелопатической активностью как до, так и после 10 месяцев компостирования, которые включали холодный период года.

Анализ методом Фолина—Чокальтеу, проведенный независимо в двух лабораториях, показал снижение общего содержания фенолов после компостирования с 5,0—6,7-кратным снижением в листьях *A. negundo* и 2,1—4,9-кратным снижением в листьях *R. pseudoacacia*. Анализ водных экстрактов методом газовой хроматографии-масс-спектрометрии показал исчезновение выбранных (маркерных) фенольных соединений в обоих растениях после компостирования. В то же время было обнаружено увеличение уровней органических кислот в компостированной фитомассе по сравнению с некомпостированным материалом, особенно молочной и фосфорной кислот. Предполагаемое разложение фенольных соединений снижает фитотоксичность листьев после компос-

тирования, в то время как присутствие аллелопатически активных органических кислот может объяснить некоторые остаточные аллелопатические эффекты в компосте. Как анализ методом Фолина—Чокальтеу, так и анализ методом газовой хроматографии-масс-спектрометрии не выявили очевидных различий по фенольным соединениям между некомпостированными листьями *A. negundo* и *R. pseudoacacia*, высушенными в тени и высушенными на солнце.

Ключевые слова: *Acer negundo*; аллелопатически активные вещества; инвазионные растения; органические кислоты; фенольные соединения; использование фитомассы; *Robinia pseudoacacia*.

Табл. 3. Библиогр: 35 назв.

Introduction. The ash-leaf maple (*Acer negundo* L.) and black locust (*Robinia pseudoacacia* L.) are classified as invasive species or woody weeds in many countries with temperate climates [1—5]. In particular, in Belarus *A. negundo* and *R. pseudoacacia* are ranked among the top invasive trees and shrubs based on the number of populations and area they occupy [6; 7]. The spread of these species results in a decrease in local floristic diversity, as seen with *A. negundo* [8; 9], or a shift in communities towards alien and ruderal species, as observed with *R. pseudoacacia* [10; 11].

While the complete eradication of *A. negundo* and *R. pseudoacacia* in their secondary range may seem unattainable, the mechanical removal of the above-ground part of these plants is a viable method to control their spread. The above-ground phytomass of both species can be quite significant; for example, *A. negundo*, a fast-growing plant, covers over 351 ha in Belarus [7]. Therefore, solutions are needed for the utilization of their phytomass post-removal. A practical approach to managing this phytomass is through composting, with subsequent using leaves or chips as mulch in vegetable and ornamental plantations as well as orchards. The application of mulch using *R. pseudoacacia* leaves was previously suggested and studied [12]. The first author evaluated the use of leaf mass from both tree species as mulch for vegetable crops, aiming to combat weeds and retain soil moisture [13].

Nonetheless, there are evidences of a moderate inhibitory allelopathic effect of *A. negundo* and *R. pseudoacacia* phytomass [14—16]. Therefore, understanding the allelopathic impact of these plants biomasses on crops under different conditions is crucial. Prior to application, fresh leaf mass can undergo various treatments, ranging from short-term outdoor storage to long-term composting. It is anticipated that following such treatments, the levels of allelochemicals will change, influenced by the conditions and duration of exposure of the plant mass.

Previously, the issue of utilizing phytomass from woody weeds was explored using the example of *Lantana camara*, a highly invasive shrub found in many tropical and subtropical regions. The alterations in certain chemical characteristics and the reduction of phytotoxicity in this plant were studied during the composting process [17; 18]. The brief data about chemical composition of recently fallen leaves of *A. negundo* and *R. pseudoacacia*, obtained through GC-MS analysis, were published by Shelepova et al. [19]. However, the dynamics of the chemical composition of *A. negundo* and *R. pseudoacacia* leaf or stem phytomass after passive outdoor treatments have not been examined earlier.

This study aimed to identify differences in the levels of total phenolics and individual allelochemicals in the green leaves of *A. negundo* and *R. pseudoacacia* before and after undergoing outdoor storage for one cold period (composting), as well as before and after sun-drying.

Materials and methods. Plant material as leaves, including petioles, was collected from growing plants. Leaves of *A. negundo* and *R. pseudoacacia* were collected from 3—7 years old plants in synanthropic habitats in the central part of Belarusian Polesie (village Cierabień, northeast of Pinsk district, Belarus). Leaves were selected for the study because they contain significantly more amount of phenolic compounds than stems [20].

Four portions of plant material were prepared as follows: 1) collected in July 2021, dried in the sun, then stored indoor from September 2021 to July 2022; 2) collected in July 2021, dried in the sun, and then composted for 10 months; this material was kept outdoors, above the ground, in wicker plastic bags placed on boards, from September 2021 to July 2022; 3) collected in July 2022,

dried in the sun; 4) collected in July 2022, dried under a shed. The portions 1, 3, and 4 after drying and before powdering were stored in the attic of a barn.

To obtain water extracts, the coarse plant material described was ground into a powder with particle size mostly 0.05—0.50 mm, using an electric coffee mill. This powder was mixed with a specified volume of distilled water preheated to 25 °C and allowed to remain suspended for 24 h at 25 °C in the dark. After this incubation period, the suspension was intensively shaken and filtered first through four layers of gauze and then through Whatman filter paper (100 g / m²).

To obtain the lyophilized water extracts, 7 ml of powdered plant material was mixed with 21 ml of distilled water preheated to 25 °C, suspended, treated as above, and filtered using the same method. The filtered extracts were then freeze-dried with the use of the Christ Alpha 1—4 LDPlus lyophilizer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany).

The total phenolic content in plant extracts was determined colorimetrically by using the Folin & Ciocalteu's assay [21]. To create the first calibration curve, 17 standard solutions of gallic acid were prepared in the experiment with fresh extracts at concentrations of 10, 20, 30, 40, 50, 60, 80, 120, 160, 200, 240, 300, 340, 400, 460, 520, and 580 mg / L. In the second calibration curve, prepared in the experiment with freeze-dried extracts, 6 standard solutions of gallic acid were used at concentrations of 5.5, 11, 44, 88, 132, and 176 mg / L. Linear regression equations were employed to determine the concentrations of total phenolics in the sample solutions, with optical absorbance as the dependent variable. The intercept (a) and slope of the regression (b) were calculated using the LINEAR function in MS Excel. Additionally, the regressions were manually verified by plotting standard points on the paper with a one-millimeter grid and drawing the median line equidistant from all points. Three types of samples, prepared as described above, were tested: 1) water extracts 10 g / L; 2) water extracts 100 g / L; 3) lyophilized water extracts reconstituted in distilled water, 400 mg / L. The 10 g / L concentration was chosen to reflect conditions more commonly found in the field. The experiments with fresh water extracts were conducted at Polessky State University using Folin & Ciocalteu's reagent (Vekton, Russia), gallic acid (Sigma-Aldrich, USA), and a Cary 50 spectrophotometer (Agilent, USA). The experiment with lyophilized water extracts was carried out at Białystok University of Technology using Folin & Ciocalteu's reagent (Chempur, Poland), gallic acid (Pol-Aura, Poland), and an AquaMate Plus spectrophotometer (Thermo Fisher Scientific, USA).

The chemical composition of extracts was studied using gas chromatography combined with mass spectrometry (GC-MS), on an Agilent 7890A chromatograph equipped with an Agilent 7693A automatic sample feeder, and coupled with an Agilent 5975C mass spectrometer, and also on an Agilent 8860 chromatograph equipped with an Agilent 5977B mass spectrometer (Agilent Technologies, USA). Compounds separation was performed on a HP-5MS capillary column (30 m × 0.25 mm inner diameter) with (5 %-phenyl)-methylpolysiloxane as stationary phase (0.25 µm thick film). Helium was used as carrier gas at a constant flow rate of 1 ml / min.

Ten milligrams of each solidified crude extract were dissolved in 1 ml of pyridine mixed with 0.1 ml of *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA); 1 µl of this solution was introduced into the gas chromatograph using an automatic sampler. The derivatization (silylation) process with BSTFA was applied to enhance the volatility and thermostability of the analyzed compounds [22]. The injector operated at a temperature of 300 °C and in a split mode with 1 : 10 ratio. The initial oven temperature was 50 °C, increasing to 325 °C at a rate of 3 °C / min; the final temperature was maintained for 10 min. The total separation time was 100 min. The ion source and quadrupole temperatures were 230 °C and 150 °C, respectively, with an ionization energy of 70 eV. Detection was performed in a full scan mode, covering a range from 41 to 800 a. m. u.

The chromatograms were recorded, analyzed, and compound identified using Enhanced ChemStation E.02.02.1431 and F.01.03.2357 software (Agilent Technologies, Inc., 2011 and 2015, accordingly), supplied with NIST Mass Spectral Library. For chromatogram integration, the Output parameter Minimum peak area was set as 0.1 % of the largest peak, Baseline Preference as Baseline

drop else tangent, and other parameters as default. Following integration, the percentage contribution of each substance to the total ion current (TIC) was calculated based on its peak area, assuming a total integrated peak area is 100 %.

Individual compounds were identified by their experimentally obtained mass spectrum, retention time (RT), and retention index (RI), compared to the mass spectra and RI in NIST library. Temperature programmed retention indices ([23], equation 1) were calculated relatively to the retention times of n-alkanes C10—C36, which were separated as hexane solution under the GC-MS conditions described above. Individual peaks were also compared using the Overlay function in the Enhanced ChemStation software. (+)-Catechin hydrate (Aldrich, USA) and quercetin (Fluorochem Ltd, UK) were analyzed using GC-MS as comparative standards for the subsequent identification of these substances in the extracts. During the naming of substances, trimethylsilyl (TMS) groups were excluded from their formulas to revert to the parent compound groups containing active hydrogen.

Results and discussion. The regression equation derived from the first calibration set (gallic acid concentrations ranging from 10 to 580 mg / L) is as follows: $A_{765} = 0.0058c + 0.0797$, where A_{765} — absorbance at 765 nm, as measured by spectrophotometer, c — gallic acid concentration, mg / L. The regression equation obtained from the second calibration set (gallic acid concentrations ranging from 5.5 to 176 mg / L) is: $A_{765} = 0.0025c + 0.0152$. The total phenolic content for the extracts, determined from the A_{765} values using these regression equations, is presented in Table 1. The extracts from powdered leaves 100 g / L demonstrated excessively dark (bluish black) solutions following the Folin & Ciocalteu's reaction, so they were excluded from the measurements.

Т а б л и ц а 1. — Total phenolic content, expressed as gallic acid equivalents, in water extracts from the leaves of invasive plants subjected to various treatments

Т а б л и ц а 1. — Общее содержание фенольных соединений, выраженное в эквивалентах галловой кислоты, в водных вытяжках из листьев инвазионных растений, подготовленных различными способами

Plant species	Sample no.	Leaf mass preparation	Total phenolic content	
			in water extracts, % to the dry mass, suspended for extraction	in lyophilized water extracts, repeatedly dissolved in water, % to the dry mass of extract
<i>Acer negundo</i>	1	Collected in 2021, non-composted	2.56	5.40
	2	Collected in 2021, composted from September 2021 to July 2022	0.51	0.81
	4	Collected in 2022, dried in the shade	1.94	n. d.
	3	Collected in 2022, dried in the sun	2.61	n. d.
<i>Robinia pseudoacacia</i>	5	Collected in 2021, non-composted	3.27	3.49
	6	Collected in 2021, composted from September 2021 to July 2022	0.67	1.67
	8	Collected in 2022, dried in the shade	3.72	n. d.
	7	Collected in 2022, dried in the sun	2.74	n. d.

Note — n. d. — determination was not carried out.

The application of GC-MS revealed that water extracts from the leaves of both *A. negundo* and *R. pseudoacacia* predominantly contained mono- and disaccharides, organic acids (including fat acids), polyols, and lactones, with carbohydrates being the most abundant class of compounds. Only a few substances were chosen for quantitative characterization based on their established or presumed allelopathic activity. Specifically, certain organic acids and phenolic compounds were identified in the chromatograms as markers for assessing the dynamics of the chemical composition (Table 2). Our study confirmed the presence of caffeic acid and catechin in *A. negundo* leaves, as previously documented by Barrales-Cureño et al. [20].

Table 2. — Quantitative changes in the chemical composition of invasive tree leaf phytomass after passive composting, as determined through GC-MS profiling

Таблица 2. — Количественные изменения химического состава фитомассы листьев инвазивных деревьев после пассивного компостирования, выраженные с помощью газовой хроматографии-масс-спектрометрии

Substance	Retention parameters, stated in this research		Before composting		After composting	
	Retention time, min	Retention index	Peak area, $\times 10^6$	% of the total peaks area	Peak area, $\times 10^6$	% of the total peaks area
<i>Acer negundo</i> , water extract						
α -Lactic acid	12.45	1 067	0.83	0.04	126.07	59.49
Glycolic acid	13.07	1 082	1.13	0.05	0.92	0.43
β -Lactic acid	16.05	1 152	0.39	0.02	0.22	0.10
Malonic acid	18.77	1 216	traces		—	—
Phosphoric acid	22.02	1 290	83.15	3.94	29.75	14.04
Glyceric acid	24.62	1 351	4.22	0.20	0.32	0.15
Malic acid	31.22	1 510	19.68	0.93	0.35	0.17
2,3,4-trihydroxybutyric acid	33.71	1 575	0.90	0.04	—	—
Fructose	43.72	1 854	298.90	14.18	—	—
Caffeic acid	53.00	2 153	1.44	0.07	—	—
Catechin	72.77	2 936	2.77	0.13	—	—
Acacetin	73.96	2 990	7.62	0.36	—	—
Quercetin	78.62	3 214	traces		—	—
Neochlorogenic acid	79.71	3 268	15.99	0.76	—	—
<i>Robinia pseudoacacia</i> , water extract						
Phenol	11.83	1 052	traces		—	—
α -Lactic acid	12.51	1 068	2.16	0.09	302.47	32.24
Hexanoic acid	12.75	1 074	traces		traces	
β -Lactic acid	16.09	1 153	1.27	0.06	0.36	0.04
3-hydroxybutyric acid	16.83	1 170	0.41	0.02	0.96	0.10
4-vinylphenol	21.54	1 279	0.96	0.04	—	—
Phosphoric acid	22.04	1 291	57.17	2.50	102.33	10.91
Succinic acid	23.51	1 325	0.91	0.04	3.37	0.07
Glyceric acid	24.63	1 351	4.90	0.21	0.68	0.07
3,4-dihydroxybutanoic acid	28.95	1 454	0.42	0.02	traces	
Fructose	43.71	1 854	159.10	6.96	—	—
Quinic acid	45.28	1 898	195.30	8.54	—	—
Catechin	72.78	2 937	0.99	0.04	—	—

Note — “—”the substance was not detected.

Short-term composting of *Acer negundo* resulted in the complete disappearance of several phenolic compounds, including caffeic acid, catechin, acacetin, quercetin, and neochlorogenic acid. Additionally, there was a decrease in the chromatographic peak area corresponding to glycolic, phosphoric, glyceric, and malic acids. Conversely, the level of α -lactic acid increased significantly (Table 2).

In the case of *R. pseudoacacia*, a similar pattern was observed regarding the disappearance of phenolic compounds, including phenol, 4-vinylphenol, quinic acid, and catechin. The increase in α -lactic acid followed a pattern akin to that observed in *A. negundo*. Furthermore, there were notable increases in the signals for phosphoric, succinic, and hydroxybutyric acids after composting, while the amount of glyceric and 3,4-dihydroxybutanoic acids decreased (Table 2).

Table 3 shows the quantitative differences in some phenolic compounds in *A. negundo* and *R. pseudoacacia* samples exposed to shade-drying and sun-drying. Overall, there were no significant differences in the general patterns of the GC-MS profiles between the phytomass prepared using these two methods.

Table 3. — Quantitative characteristics of some phenolic compound in leaves dried in the shade and in the sun, as determined through GC-MS profiling

Таблица 3. — Количественные характеристики некоторых фенольных соединений в листьях, высушенных в тени и на солнце, выраженные с помощью газовой хроматографии-масс-спектрометрии

Species	Substance	Leaves dried in the shade		Leaves dried on the sun	
		Peak area, $\times 10^6$	% of the total peaks area	Peak area, $\times 10^6$	% of the total peaks area
<i>Acer negundo</i>	Catechin*	1.80	0.10	3.01	0.20
	Neochlorogenic acid	12.05	0.68	52.81	3.48
<i>Robinia pseudoacacia</i>	Quinic acid	111.09	5.62	107.59	4.78
	Catechin**	4.62	0.23	3.44	0.15

Note — * — isomer with RT = 72.19 min and RI = 2910; ** — isomer with RT = 72.78 min and RI = 2 937/

The debris of invasive trees can come from branches, trunks, and woody roots. However, green leaves are considered the most common type of soft material that accumulates after the mechanical cutting of these trees. Leaves are particularly promising for short-term composting.

It is widely recognized that phenolic compounds are significant components in allelopathic interactions [24–26]. The primary hypothesis regarding the allelopathic action of fallen leaves posits that substances, including phenolics, migrate from leaf litter into the soil [27]. Analyzing and experimentally applying crude water extracts is the most effective method for studying allelopathy. This approach simulates the effects of rain and other natural waters on litter, i.e. leaching of phenols from leaves [28], even though the extraction of phenolic compounds from tissues by water is not entirely complete.

The working hypothesis of this research posited that a part of allelopathically active substances in the leaves of *A. negundo* and *R. pseudoacacia* degrade after a period of composting. Our measurements of the total phenolic content revealed a significant decrease of these compounds in both plants after composting: a reduction of fivefold in *A. negundo* and 4.9-fold in *R. pseudoacacia*, as indicated by fresh water extracts (Table 1). Analysis of dried water extracts further demonstrated that the phenolic content in the plant material of *A. negundo* decreased by a factor of 6.7, while *R. pseudoacacia* it decreased by 2.1 (Table 1).

In addition to the dynamics of composting, there are notable differences in the total phenolic content between two invasive plant species, as observed in the initial phytomass. This content for

the 2021 harvest was 28 % higher in *R. pseudoacacia* compared to *A. negundo*. In the 2022 harvest, *R. pseudoacacia* showed 92 % higher level of phenolics than in *A. negundo* (Table 1).

Folin & Ciocalteu's assay is widely recognized; however, two important points should be emphasized. First, this method quantifies phenolic compounds in terms of gallic acid equivalents, rather than measuring the mass or concentration of total phenolic compounds. The second is that this assay can yield positive results also for reducing sugars [29] in their open-chain forms [30].

The GC-MS analysis of the substances extracted by water from the studied plants had two limitations regarding phenolics. The first limitation is the low solubility of many phenolic compounds in water. For instance, the water solubility of quercetin is 2.15 mg / L [31], and (+)-catechin is 450 mg / L at room temperature [32]. The second limitation is the poor separation of compounds with a molecular weight above 300.

Our previous biotest research on germinating seeds [33] demonstrated that the inhibitory effect of extracts from *A. negundo* reliably decreases after composting of the leaves, particularly at high extract concentrations. Consequently, the biotest results for *A. negundo* align with the reduction of phenolic compounds observed in this study. In contrast, similar biotests revealed that the inhibitory effect of high concentrations of extract from *R. pseudoacacia* leaves increases following the composting of the plant material [33]. The latter effect can be explained supposedly by organic acids accumulation. The allelopathic activity is known for lactic, glycolic, malic, and succinic acids [34; 35], but has not yet been documented for phosphoric acid.

Research conducted by other authors on the composting of leaves from allelopathically active weeds has demonstrated that plant material can partially or completely lose its allelopathic properties [17]. In particular, composting has been proposed as a viable method for managing the biomass of the aggressive and toxic shrub *Lantana camara*, which can subsequently be used for soil fertilization [18].

The influence of sunlight on the total phenolic content in dead plant tissue is not clearly established in this study. *Acer negundo* exhibited a total phenolic content that was 1.3 times higher in sun-dried material compared to the control sample dried in the shade. Conversely, *R. pseudoacacia* leaves showed a 1.3-times higher phenolic content when dried in the shade compared to those dried in sunlight. The GC-MS analysis of selected phenolic compounds in leaves prepared in the shade and in the sun yielded results similar to those obtained by using the Folin & Ciocalteu method (Table 3).

Conclusion. A reduction of phenolic compounds in *A. negundo* and *R. pseudoacacia* leaves, even after one year of plant material exposure to wind, rain, snow, frost, and some sunlight, was confirmed through experiments in two separate laboratories. The decrease is likely due to chemical transformations into other organic compounds, mineralization into inorganic forms, and leaching caused by precipitation. The trend in phenolic content dynamics observed through GC-MS after composting was consistent with the results of Folin & Ciocalteu's assay. In addition to the degradation of phenolic compounds, two other key trends are observable during composting through GC-MS analysis. The first is the decrease or complete disappearance of sugars, such as fructose, which is attributed to their consumption by fungi and bacteria. The second trend is the accumulation of low molecular weight organic acids, especially lactic acid, leading to acidification. This study, in conjunction with previous research [13; 33], suggests the potential use of leaf debris from *A. negundo* and *R. pseudoacacia* as a fertilizer or mulch for cultivated plants, especially after an extended composting period.

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