Arch Biol Sci. 2019;71(1):83-94 https://doi.org/10.2298/ABS180823050G

Allelopathic potential of selected woody species growing on fly-ash deposits

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Received: August 23, 2018; **Revised**: October 12, 2018; **Accepted**: October 17, 2018; **Published online**: November 7, 2018

Abstract: The objective of this study was to determine the allelopathic potential of *Robinia pseudoacacia* L., *Ailanthus altissima* (Mill.) Swingle and *Amorpha fruticosa* L. that grow on the fly-ash deposits at the "Nikola Tesla – A" thermoelectric power plant in Obrenovac. The chemical characteristics of fly ash, such as pH, electrical conductivity (EC), content of carbon (C) and nitrogen (N), contents of available phosphorus (P_2O_5) and potassium (K_2O), the contents of total and available Fe, Cu, Mn, Ni and Zn as well as of phenolic acids (3,5 dihydroxybenzoic acid (3,5-DHBA) and ferulic acid) and flavonoids (rutin and quercetin) were analyzed in control fly ash (bare zones without vegetation cover) and plant rhizospheric fly ash. In order to determine the allelopathic activity of phenolic compounds in fly ash, modified soil sandwich allelopathic biotests were performed, and *Trifolium pratense* L. (red clover) was used as the indicator species. *A. fruticosa* showed the highest allelopathic activity, followed by *A. altissima* whereas *R. pseudoacacia* showed the lowest allelopathic potential. Negative correlation was noted between radicle and hypocotyl growth inhibition of red clover and the pH of fly ash. Positive correlations were found between radicle growth inhibition and the content of C, P_2O_5, total concentrations of Cu, available concentrations of Mn and Ni, the contents of ferulic acid, 3,5-DHBA, and rutin. Our results indicate that *A. fruticosa* and *A. altissima* increased the content of phenolics in fly ash, which can act as allelochemicals leading to radicle growth inhibition of red clover in the pioneer plant community on fly-ash deposits. These woody species that colonized fly-ash deposits can initiate the beginning of pedogenetic processes altering the ecosystem processes at degraded sites.

Keywords: allelopathy; flavonoids; fly ash; invasive plant species; phenolic acids; radicle growth inhibition

INTRODUCTION

Allelopathy presents both inhibitory and stimulatory interactions between plants through the action of secondary plant metabolites – allelochemicals [1,2]. Allelochemicals are often investigated as compounds that potentially allow the invasion of plant species in new habitats due to the lack of adaptive potential of native species to new allelochemicals originating from introduced species [3]. One of the most studied groups of allelochemicals are phenolic compounds, which in plant tissue can be found in soluble form or bound to the cell wall polysaccharides [4,5].

Phenolic compounds from the plant organism are delivered into soil by leaching from the surface of the plant and fallen leaves, decomposition of litter, and active excretion from roots [6]. Most of the phenolic compounds are soluble in water and rinsed from the surface of the plant body and transferred to the deeper parts of the soil by rainwater [7]. In soil, phenolic compounds represent the second most widespread group of compounds (after cellulose), and occur in three different forms: free, reversibly bound and bound [8]. However, the concentration of phenolic compounds in the soil is much lower than in plant organisms [9].

These compounds have a short lifetime and they are susceptible to degradation by microorganisms, so their detection and identification in soil is much more difficult than in plant tissues [10].

Through allelopathic activity, phenolic compounds have significant effects on the structure and composition of plant communities [11,12]. The phenolic compounds may have direct harmful effects due to their release by donor species or can be degraded or transformed by soil microorganisms. Furthermore, these compounds can have an effect on the physical, chemical and biological soil characteristics or can induce the release of biologically active substances in third plant species [13]. Phenolic compounds in the soil can lead to root and hypocotyl growth inhibition, the limitation of water and mineral uptake, and inhibition of photosynthesis and enzymatic activity in acceptor plants [5,14,15].

Blum et al. [16] indicated that the focus in allelopathic research should be shifted to the soil system since the phytotoxic activity of allelochemicals is a function of the characteristics of plant allelochemicals and soil [17]. Additionally, adsorption and desorption of allelochemicals in soil is a dynamic process and is influenced by various physicochemical factors, such as soil moisture, pH and organic matter content [18,19]. Furthermore, phenolic compounds represent an important link in the dynamics of mineral and organic compounds in the soil due to their effect on the chemical properties of the soil, availability of certain metals and the microorganism community [5,8,15,20,21].

The process of plant colonization on fly-ash deposits (revegetation) is very slow due to unfavorable conditions of substrates [22]. The plants growing on the fly-ash deposits are exposed to a combination of several stress factors, such as high temperatures, increased irradiation, drought and elevated concentrations of some-metal(loid)s [23-25]. Woody plants that have a large cover and great biomass play a prominent role in the process of fly-ash revegetation. Organic matter originating from these plants is of great importance in the process of soil humus formation, which represents a mixture of various organic compounds among which phenolic compounds are very important.

In the majority of studies, the effect of phenolic compounds on vegetation is emphasized, while their effects on substrate characteristics are not fully addressed [26]. In this study, we hypothesized that woody plant species, *Robinia pseudoacacia* L., *Ailanthus altissima* (Mill.) Swingle and *Amorpha fruticosa* L., show a great allelopathic potential due to the production of high amounts of phenolics that are released from these plants in fly ash. It is also assumed that these allelochemicals contribute to the increased availability of certain metals, which can additionally lead to inhibition of seedling (radicle and hypocotyl) growth of the indicator plant species, *Trifolium pratense* L. (red clover). Therefore, the objectives of this study were (i) to compare the chemical characteristics of control and rhizospheric fly ash; (ii) to analyze the total and available metal contents (Fe, Cu, Mn, Ni, Zn) in control and rhizospheric fly ash; (iii) to evaluate the contents of phenolic compounds in control and rhizospheric fly ash; (iv) to determine the allelopathic potential of the selected woody species through screening of seedling (radicle and hypocotyl) growth inhibition of red clover as an indicator plant species.

MATERIALS AND METHODS

Study area

The study was performed on the fly-ash deposit at the "Nikola Tesla – A" thermoelectric power plant (TENT-A) in Obrenovac (N 44°30', E 19°58'), which is located on the right bank of the Sava River 40 km upstream from Belgrade (Serbia) (Supplementary Fig. S1). The climate is semi-continental with local mean annual rainfall and temperature of 647 mm and 11°C, respectively. As the largest producer of electricity in Serbia, southeastern Europe and the Balkans, the six blocks of TENT-A with a total power of 1820 MW annually produce about 8 billion kWh of electricity. Annually, TENT-A burns 12-14 Mt of lignite, which is supplied by the Kolubara-Tamnava surface pits. The chemical analysis of electrofilter ash at TENT-A showed that it consists of SiO_2 (54.21%), Al_2O_3 (24.98%), Fe_2O_3 (6.13%), CaO (5.89%), MgO (3.15%), K_2O (1.12%), Na_2O (0.29%), TiO_2 (0.69%), P_2O_5 (0.07%) and SO_3 (0.96%) (data obtained from the Vinča Institute for Nuclear Sciences, Belgrade, Serbia).

To date, the landfill of TENT-A has disposed of more than 66×106 t of fly ash that occupies 400 ha of agricultural land of fluvisol type. Fly ash is hydraulically transported in a suspension with water at ratios of 1:10

or 1:20. The disposal of fly ash is carried out in three lagoons, one is active (L_2), and the other two are in a temporary mode for the technical consolidation of fly ash and drainage (L_1 and L_3) (Fig. S1C). To reduce the negative impact of fly ash on the environment, TENT-A has provided biological recultivation, i.e. the sowing of legumes (*Medicago sativa* L., *Lotus corniculatus* L., *Vicia villosa* Roth., *Trifolium pratense* L.) and grasses (*Secale cereale* L., *Lolium multiflorum* Lam., *Festuca rubra* L., *Dactylis glomerata* L.), as well as planting of trees (*Robinia pseudoacacia* L., *Ailanthus altissima* (Mill.) Swingle) and shrubs (*Tamarix* sp.). The established herbaceous and woody vegetation cover on fly-ash deposits over time promote natural succession and revegetation. In the flat part of L_1, some spontaneous herbaceous plant species, shrubs and trees were noted: *Calamagrostis epigejos* L., *Oenothera biennis* L., *Sorghum halepense* (L.) Pers., *Erigeron canadensis* L. and *Amorpha fruticosa* L.

Sampling of fly ash

The field research into fly-ash deposits on L_1 was carried out during August 2016. The fly ash was collected at a depth of 0-30 cm on bare zones without vegetation cover represented control fly ash (C_{FA}), whereas the fly ash taken up in the root zone of *R. pseudoacacia, A. altissima* and *A. fruticosa* was marked as plant rhizospheric fly ash ($RP_{FA,}$ $AA_{FA,}$ and AF_{FA}, respectively). Fly-ash samples were collected in the range of 5-10 m from the embankment of L_1. Collected fly ash was packed into plastic bags and brought to the laboratory for analysis. After the removal of visible plant remains, samples were dried at room temperature (25°C) and sifted through a sieve (0.5 mm mesh). For chemical, elemental and biochemical analyses, five composite samples of fly ash were used (n=5).

Chemical analysis of fly ash

Fly-ash pH was measured in water (pH_{H2O}) and 0.1 M KCl solution (pH_{KCl}) with a pH meter (PHT-026 Multi-function meter). Electrical conductivity (EC[dSm^{-1}] in the extract (fly ash:water=1:5) was measured with a conductometer (PHT-026 Multi-function meter). Organic carbon (C) was measured by the method of Tyurin [27], and the total nitrogen content (N) was determined by Kjeldahl digestion [28]. The C/N ratio was calculated. Available forms of phosphorus (P_2O_5) and potassium (K_2O) were analyzed using the standard AL-method [29].

Elemental analysis of fly ash

Total concentrations of chemical elements (Cu, Fe, Mn, Ni, Zn) in fly ash were determined according to the modified method 3051A (EPA SW-846 test methods) [30] as follows: 2-3 g of fly-ash sample were oven-dried for 1 h at 105°C. The sample was dissolved in a mixture of 25 mL of HNO_3 and $HClO_4$ for 12 h at 40°C. Concentrations of available chemical elements in the fly ash were determined according to Zemberyova et al. [31]. Extraction was performed with 0.05 mol/dm^3 EDTA at pH 7. The sample of dried fly ash (2-3 g) was added in 25 mL of 0.05 M EDTA and mixed on a magnetic stirrer for 1 h at room temperature (20±4°C). Flam atomic absorption spectrophotometer (FAAS, "Perkin Elmer 3300") was used for analyzing the concentrations of chemical elements. Standard solutions were used for the preparation of calibrated diagrams. The range of concentrations of test elements of the standard solutions was 0.5-2.0 mg/dm^3 for Cu, Zn and Ni, and 1.0-5.0 mg/dm^3 for Mn and Fe. The measured values of the element contents in fly ash are expressed in μg/g dry matter.

Determination of phenolic acids and flavonoids by HPLC

Phenolic acids and flavonoids were extracted by dissolving 10 g of dry fly ash in 30 mL of pure methanol (99.8%) in an ultrasonic bath for 15 min and then left to dissolve another 24 h. Subsequently, samples were centrifugated 20 min at 10000×g and supernatants were filtered through 0.2-μm cellulose filters (Agilent Technologies, Santa Clara, CA, USA) and stored at 4°C until use.

The methanolic extracts were analyzed by a HPLC system (Shimadzu, Kyoto, Japan) which consisted of a degasser DGU-20A3, analytical pumps LC-20AT, 7125 injectors and SPD-M20A diode array detector and CBM-20A system controller. Separation was achieved on a Luna C18 column at 30°C, 250×4.6 mm I.D., 5 μm (Phenomenex, Torrance, CA, USA) with a flow rate of 1.0 mL/min. The injection volume was 20 μL. The

chromatographic data were processed using LC Solution computer software (Shimadzu). Gradient elution was used (5% B 0-5 min, gradient 5-60% B during 5-30 min, 60% B held for 5 min, then ramped from 60% to 90% B for 2-3 min and equilibrated for a further 5 min; mobile phases: A, water acidified with formic acid, pH=3; B, acetonitrile). The identity of compounds was determined by comparing the retention times and absorption maxima of known peaks with pure standards (Sigma) at 290 and 245 nm. Two phenolic acids were used as phenolic acid standards: ferulic and 3,5-dihydroxybenzoic acid (3,5-DHBA); for identification of flavonoids, two different standards were used: rutin and quercetin. The concentrations of phenolic acids and flavonoids are expressed in μg/g of extracts.

Growth inhibition test

The allelopathic activity of selected plant species grown on fly ash was assessed by a modified "soil sandwich method" [32]. In the experiment, 5 mL of agar (0.5%) cooled to 42°C were added into a multi-dish (6 dishes) plate containing 3 g of dried fly ash. After solidification, 3.2 mL of agar (0.5%) was added on a fly ash-agar layer. After 1 h, 5 seeds of *T. pratense* L. were added to the gelled agar culture medium in one dish (30 seeds per multi-dish plate). Control plates contained only agar medium (without fly ash). The multi-dishes were incubated at 25°C in the dark. After 7 days, the lengths of the radicle and hypocotyl were measured and the percentage of growth inhibition was calculated (compared to control). The bioassays were done in 5 replicates (30 seeds per replicate, n=150).

Statistical analyses

Statistical analyses included determination of the mean (M) and standard deviation (SD) for each of the analyzed parameters. Differences between groups in terms of chemical properties of fly ash, total and available concentrations of chemical elements, content of phenolic acids and flavonoids in fly ash as well as the inhibition of radicle and hypocotyl growth of indicator species, were determined by multivariate variance analysis (MANOVA) and Scheffé's post-hoc test. Pearson's correlation coefficients (r) between the allelopathic activity of selected plant species growing on fly ash and the chemical characteristics, total and available concentrations of elements and content of phenolics in fly ash were determined. Statistical analysis was performed using the package Statistica 10.0.

RESULTS

Chemical properties of fly ash

The chemical properties of the control (C_{FA}) and rhizospheric fly ash ($RP_{FA,}$ $AA_{FA,}$ and AF_{FA}) are shown in Table 1. The results showed that the pH (H_2O) and pH (KCl) in RP_{FA} had higher values than the C_{FA} ($p<0.001$), AA_{FA} ($p<0.001$) and AF_{FA} ($p<0.001$). EC values were higher in AF_{FA} compared to C_{FA} ($p<0.001$), RP_{FA} ($p<0.001$) and AA_{FA} ($p<0.001$). In comparison to C_{FA} the results showed statistically significant higher values of C, N and C/N in RP_{FA} ($p<0.01$), AA_{FA} ($p<0.01$) and AF_{FA} ($p<0.01$), except for the values of N in the RP_{FA} and the C/N values in the AA_{FA}. The highest values of C and N were detected in AA_{FA}. Also, statistically significantly higher values ($p<0.001$) for both P_2O_5 and K_2O were detected in all rhizospheric fly ash in comparison to control fly ash. The highest values of P_2O_5 were detected in AA_{FA}, while the highest values of K_2O were detected in the RP_{FA}.

Total and available elements in fly ash

Total and available concentrations of Cu, Fe, Mn, Ni and Zn in C_{FA}, RP_{FA}, AA_{FA} and AF_{FA} are shown in Table 1. The results showed statistically significantly lower concentrations of Cu, Fe and Ni in the C_{FA} compared to all plant rhizospheric fly ash ($p<0.001$), except for Fe and Ni in the AA_{FA}. However, statistically significantly higher concentrations of both Mn and Zn were found in the C_{FA} ($p<0.001$) compared to all other plant rhizospheric fly ash, except for Zn in the AF_{FA}. In the control fly ash and all other rhizospheric fly ash, total metal concentrations decreased in the following order: Fe>Mn>Ni>Cu>Zn.

The available Cu, Fe and Ni concentrations in the C_{FA} were lower in comparison to all plant rhizospheric fly ash ($p<0.001$), except for Fe in the $AA_{FA}$and Ni in the RP_{FA}. However, the available concentrations of Mn and Zn in the C_{FA} were higher versus all the plant rhizospheric fly ash ($p<0.001$). The content of the available

elements in the C_{FA}, RP_{FA} and AF_{FA} showed the same decreasing order: Fe>Mn>Cu>Ni>Zn, whereas the content of available elements in the AA_{FA} decreased in a slightly different order: Fe>Mn>Ni>Cu>Zn. Furthermore, the percentage of available concentrations of Fe, Mn and Ni in fly ash relative to the total content of the elements was the highest in the AA_{FA} (0.42%, 4.07%, 4.39%, respectively). The percentage of available Zn in fly ash relative to the total metal content was the highest in the C_{FA} (11.31%), whereas the highest percentage of available Cu concentrations was detected in AF_{FA} (19.6%).

Table 1. Chemical properties (pH; EC – electrical conductivity, dS/m; C – carbon content, %; N – nitrogen content, %; C/N – ratio of C/N; P_2O_5 – available concentrations of phosphate, mg/100g; K_2O – available concentrations of potassium, mg/100g; total and available element concentrations, µg/g) in control (C_{FA}) and plant rhizospheric fly ash (RP_{FA} – *Robinia pseudoacacia*; AA_{FA} – *Ailanthus altissima*; AF_{FA} – *Amorpha fruticosa*).

Parameters	C_{FA}	RP_{FA}	AA_{FA}	AF_{FA}
Chemical properties of fly ash				
pH (H_2O)	6.45 (0.01) $a^{***}b^{ns}c^{***}$	8.1 (0.08) $d^{***}e^{***}$	6.34 (0.01) f^{ns}	6.32 (0.03)
pH (KCl)	5.28 (0.02) $a^{***}b^{***}c^{***}$	7.98 (0.04) $d^{***}e^{***}$	5.86 (0.05) f^{**}	5.41 (0.02)
EC (dS/m)	0.103 (0.00064) $a^{***}b^{***}c^{***}$	0.18 (0.002) $d^{***}e^{***}$	0.07 (0.001) f^{**}	0.307 (0.007)
C (%)	1.44 (0.02) $a^{***}b^{***}c^{***}$	3.47 (0.06) $d^{***}e^{***}$	4.5 (0.08) f^{ns}	4.43 (0.01)
N (%)	0.11 (0.011) $a^{ns}b^{***}c^{***}$	0.13 (0.0057) $d^{***}e^{***}$	0.36 (0.02) f^{**}	0.21 (0.01)
C/N	26.69 (0.46) $a^{***}b^{***}c^{***}$	12.51 (0.47) $d^{***}e^{***}$	21.12 (0.95) f^{**}	12.72 (0.65)
P_2O_5 (mg/100 g)	18.26 (0.02) $a^{***}b^{***}c^{***}$	22.11 (0.02) $d^{***}e^{***}$	27.6 (0.03) f^{**}	49.12 (0.02)
K_2O (mg/100 g)	10 (0.2) $a^{***}b^{***}c^{***}$	16.8 (0.03) $d^{***}e^{***}$	31.9 (0.02) f^{**}	14.6 (0.03)
Total element concentrations				
Cu	15.6 (0.33) $a^{***}b^{***}c^{***}$	18.32 (0.3) $d^{ns}e^{***}$	18.5 (0.35) f^{**}	21.3 (0.450)
Fe	22015.34 (413.42) $a^{***}b^{***}c^{***}$	23640.74 (190.98) $d^{***}e^{***}$	20689.66 (160.21) f^{**}	25252.58 (1429.660)
Mn	176.46 (0.64) $a^{***}b^{***}c^{***}$	142.12 (2.05) $d^{***}e^{***}$	149.44 (0.73) f^{**}	167.52 (1.81)
Ni	77.76 (0.57) $a^{***}b^{***}c^{***}$	85.7 (2.6) $d^{***}e^{***}$	67.34 (0.32) f^{**}	104.36 (0.72)
Zn	15.54 (0.38) $a^{***}b^{***}c^{ns}$	11.38 (0.37) $d^{***}e^{***}$	12.44 (0.36) f^{**}	14.48 (0.370)
Available element concentrations				
Cu	2.43 (0.016) $a^{***}b^{***}c^{***}$	3.412 (0.019) $d^{***}e^{***}$	2.744 (0.022) f^{**}	4.174 (0.017)
Fe	77.08 (0.240) $a^{***}b^{***}c^{***}$	79.28 (0.277) $d^{***}e^{***}$	88.04 (0.207) f^{**}	71.7 (0.274)
Mn	6.83 (0.030) $a^{***}b^{***}c^{***}$	4.35 (0.030) $d^{***}e^{***}$	6.084 (0.011) f^{**}	6.428 (0.033)
Ni	2.08 (0.019) $a^{***}b^{***}c^{***}$	1.42 (0.021) $d^{***}e^{***}$	2.956 (0.019) f^{**}	2.532 (0.016)
Zn	1.758 (0.023) $a^{***}b^{***}c^{***}$	1.158 (0.008) $d^{***}e^{***}$	1.248 (0.016) f^{**}	1.352 (0.022)

ANOVA, data represent the means±SD (n=5); (a) C_{FA}-RP_{FA}; (b) C_{FA}-AA_{FA} (b); (c) C_{FA}-AF_{FA}; (d) RP_{FA}-AA_{FA}; (e) RP_{FA}-AF_{FA}; (f) AA_{FA}-AF_{FA}; ***p<0.001, ns=not significant.

Table 2. Phenolic acid and flavonoid contents (μg/g) in control (C_{FA}) and plant rhizospheric fly ash (RP_{FA} – *Robinia pseudoacacia*; AA_{FA} – *Ailanthus altissima*; AF_{FA} – *Amorpha fruticosa*) and radicle and hypocotyl growth inhibition of red clover (%).

Parameters	C_{FA}	RP_{FA}	AA_{FA}	AF_{FA}
Phenolic acids				
3,5-DHBA	0.67 (0.03) a^{***} b^{***} c^{***}	0.19 (0.04) d^{ns} e^{***}	0.18 (0.04) f^{**}	4.12 (0.08)
Ferulic acid	0.02 (0.001) a^{***} b^{***} c^{***}	0.46 (0.02) d^{***} e^{***}	0.7 (0.2) f^{**}	4.11 (0.08)
Flavonoids				
Rutin	0.09 (0.01) a^{***} b^{***} c^{***}	0.33(0.02) d^{***} e^{***}	0.08 (0.02) f^{**}	3.3 (0.2)
Quercetin	54.4 (0.5) a^{ns} $b^{***}c^{ns}$	55 (7) $d^{***}e^{ns}$	60 (10) f^{**}	55 (7)
Seedling growth inhibition				
Radicle growth inhibition of red clover	31.07 (10.87) $a^{ns}b^{ns}$ c^{**}	23.79 (9.28) d^{**} e^{***}	40.93(6.26) f^{ns}	50.45 (8.89)
Hypocotyl growth inhibition of red clover	32.83 (7.703) $a^{***}b^{**}c^{***}$	3.11 (0.903) d^{**} e^{ns}	17.99 (1.585) f^{ns}	4.90 (0.983)

ANOVA, data represent the means±SD (n=5 for phenolic compounds; n=150 for growth inhibition test); (a) C_{FA}-RP_{FA}; (b) C_{FA}-AA_{FA}; (c) C_{FA}-AF_{FA}; (d) RP_{FA}-AA_{FA}; (e) RP_{FA}-AF_{FA}; (f) AA_{FA}-AF_{FA}; **p<0.01; ***p<0.001, ns=not significant.

Phenolic content in fly ash

The content of phenolic acids and flavonoids in C_{FA}, RP_{FA}, AA_{FA}, and AF_{FA} is presented in Table 2. The content of 3,5-DHBA in C_{FA} was higher, whereas the content of ferulic acids was lower compared to the RP_{FA} and AA_{FA} ($p<0.001$). Results in this study showed the highest content of these phenolic acids in the AA_{FA}. The rutin content in C_{FA} was lower in relation to the RP_{FA} and AF_{FA} ($p<0.001$), while the quercetin content in C_{FA} was lower in comparison to the AA_{FA} ($p<0.001$). The highest content of rutin was noted in the AF_{FA}, whereas the highest content of quercetin was found in the AA_{FA}. The phenolic acid and flavonoid contents in the C_{FA} showed the following decreasing order: quercetin>3,5-DHBA>rutin>ferulic acid. In the RP_{FA} and AA_{FA}, the order of phenolic content was as follows: quercetin>ferulic acid>rutin>3,5-DHBA. However, the content of phenolic acids and flavonoids in the AF_{FA} showed a different decreasing order: quercetin>3,5-DHBA>ferulic acid>rutin.

Table 3. Pearson's correlation coefficients (r) among the contents of phenolic acids (3,5-DHBA and ferulic acid) and flavonoids (rutin and quercetin) in fly ash and chemical parameters, total and available concentration of elements in fly ash.

Parameters	3,5-DHBA					Ferulic acid					Rutin					Quercetin				
pH (H_2O)	r	=	-	0.414	ns	r	=	-	0.346	ns	r	=	-	0.299	ns	r	=	-	0.378	ns
pH (KCl)	r	=	-	0.452	ns	r	=	-	0.324	ns	r	=	-	0.315	ns	r	=	-	0.157	ns
EC	r	=	+	0.787	**	r	=	+	0.875	***	r	=	+	0.877	***	r	=	+	0.239	ns
C	r	=	+	0.348	ns	r	=	+	0.585	*	r	=	+	0.462	ns	r	=	-	0.580	*
N	r	=	-	0.029	ns	r	=	+	0.161	ns	r	=	+	0.004	ns	r	=	+	0.945	***
C/N	r	=	+	0.219	ns	r	=	+	0.296	ns	r	=	+	0.345	ns	r	=	-	0.489	ns
P_2O_5	r	=	+	0.925	***	r	=	+	0.990	***	r	=	+	0.955	***	r	=	+	.0470	ns
K_2O	r	=	-	0.346	ns	r	=	-	0.121	ns	r	=	-	0.277	ns	r	=	-	0.953	***
Cu_{total}	r	=	+	0.706	*	r	=	+	0.864	***	r	=	+	0.798	**	r	=	+	0.199	ns
Fe_{total}	r	=	+	0.717	**	r	=	+	0.705	*	r	=	+	0.777	**	r	=	-	0.639	*
Mn_{total}	r	=	+	0.467	ns	r	=	+	0.232	ns	r	=	+	0.316	ns	r	=	-	0.405	ns
Ni_{total}	r	=	+	0.855	***	r	=	+	0.818	**	r	=	+	0.888	***	r	=	-	0.603	*
Zn_{total}	r	=	+	0.519	ns	r	=	+	0.304	ns	r	=	+	0.372	ns	r	=	-	0.331	ns
$Cu_{available}$	r	=	+	0.791	**	r	=	+	0.872	***	r	=	+	0.878	***	r	=	-	0.267	ns
$Fe_{available}$	r	=	-	0.768	**	r	=	-	0.614	*	r	=	-	0.733	**	r	=	+	0.818	**
$Mn_{available}$	r	=	+	0.387	ns	r	=	+	0.235	ns	r	=	+	0.239	ns	r	=	+	0.092	ns
$Ni_{available}$	r	=	+	0.276	ns	r	=	+	0.349	ns	r	=	+	0.231	ns	r	=	+	0.755	**
$Zn_{available}$	r	=	+	0.046	ns	r	=	-	0.206	ns	r	=	-	0.116	ns	r	=	-	0.372	ns
3,5-DHBA	r	=	+	1.000	ns	r	=	+	0.963	***	r	=	+	0.986	***	r	=	-	0.275	ns
Ferulic acid	r	=	+	0.963	***	r	=	+	1.000	ns	r	=	+	0.987	***	r	=	-	0.091	ns
Rutin	r	=	+	0.986	***	r	=	+	0.987	***	r	=	+	1.000	ns	r	=	-	0.246	ns
Quercetin	r	=	-	0.275	ns	r	=	-	0.091	ns	r	=	-	0.246	ns	r	=	+	1.000	ns

p<0.05**; **p<0.01; ***p<0.001; ns=not significant

In this study, the relationship between chemical properties and the phenolic content in fly ash is presented in Table 3. Positive correlations were noted between EC, the P_2O_5 content, the total and available concentrations of Cu, total concentrations of Fe and Ni and 3,5-DHBA, ferulic acid and rutin ($p<0.05$, $p<0.01$, $p<0.001$), and between the C content and ferulic acid ($p<0.001$), the N content, the available concentrations of Fe and Ni and quercetin ($p<0.01$). However, negative correlations were found between the C and K_2O contents, the total concentrations of Fe, the available concentrations of Ni and quercetin in fly ash ($0<0.05$, $p<0.01$, $p<0.001$).

Allelopathic effects of plants growing on fly ash deposits

Higher inhibition of radicle growth of red clover was noted in AF_{FA} in comparison to the C_{FA} and RP_{FA} ($p<0.01$) (Fig. 1). Higher inhibition of red clover radicle growth was found in the AA_{FA} versus RP_{FA} ($p<0.001$). The inhibition of radicle elongation decreased in the following order: $AF_{FA}>AA_{FA}>C_{FA}>RP_{FA}$. Hypocotyl growth inhibition in red clover was significantly lower in the rhizospheric fly ash of all plant species compared to the C_{FA} ($p<0.01$, $p<0.001$), as well as in RP_{FA} compared to AA_{FA} ($p<0.01$) (Fig. 2). The inhibition of red clover hypocotyl growth decreased in the following order: $C_{FA}>AA_{FA}>AF_{FA}>RP_{FA}$.

Negative correlations were detected between radicle growth inhibition in red clover and fly ash pH (H_2O) ($p<0.01$) and pH (KCl) ($p<0.05$) (Table 4). Positive correlations were found between radicle growth inhibition in red clover and C content ($p<0.05$), P_2O_5 content ($p<0.001$), the total concentrations of Cu ($p<0.05$), available concentrations of Mn ($p<0.05$), available concentrations of Ni ($p<0.01$), 3,5-DHBA content ($p<0.01$), ferulic acid ($p<0.001$) and rutin ($p<0.01$) in fly ash (Table 4). However, red clover hypocotyl growth inhibition was negatively correlated with pH (KCl), EC, C contents, C/N ratio, total concentrations of Cu, Fe, Mn, the available concentrations of Cu ($p<0.05$, $p<0.01$, $p<0.001$) in fly ash, whereas it positively correlated with available concentrations of Mn ($p<0.05$) and Zn ($p<0.001$) (Table 4).

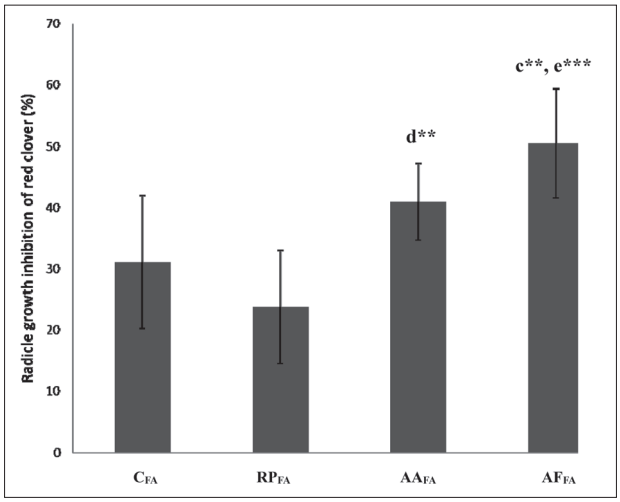

Fig. 1. Radicle growth inhibition of red clover in control (C_{FA}) and plant rhizospheric fly ash (RP_{FA} – *Robinia pseudoacacia*; AA_{FA} – *Ailanthus altissima*; AF_{FA} – *Amorpha fruticosa*). ANOVA; data represent the means±SD (n=150); (c) C_{FA}-AF_{FA}; (d) RP_{FA}-AA_{FA}; (e) RP_{FA}-AF_{FA}; **p<0.01; ***p<0.001.

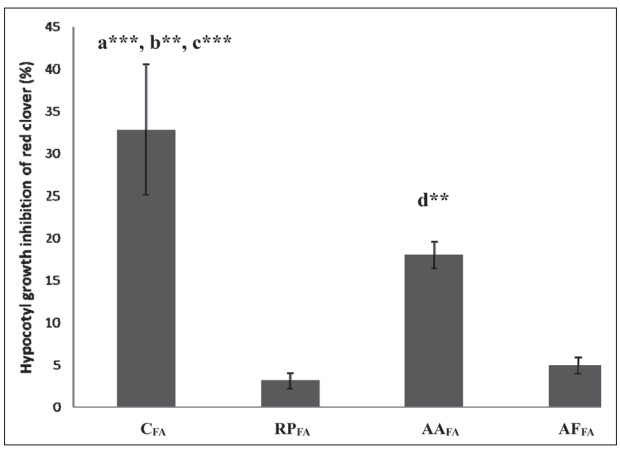

Fig. 2. Hypocotyl growth inhibition of red clover in control (C_{FA}) and plant rhizospheric fly ash (RP_{FA} – *Robinia pseudoacacia*; AA_{FA} – *Ailanthus altissima*; AF_{FA} – *Amorpha fruticosa*). ANOVA; data represent the means±SD (n=150); (a) C_{FA}-RP_{FA}; (b) C_{FA}-AA_{FA}; (c) C_{FA}-AF_{FA}; (d) RP_{FA}-AA_{FA}; **p<0.01; ***p<0.001.

DISCUSSION

The phytotoxic activity of allelochemicals in soils is a function of the interaction of the allelochemicals from plants and soil characteristics [17]. Allelochemicals primarily affect the soil pH, aggregation and aeration, content of N and C, plant-water relations, nutrient ion uptake, degree of decomposition, composition and activity of soil microbiocenosis [5]. Phenolic compounds

Table 4. Pearson's correlation coefficients (r) between red clover radicle and hypocotyl growth inhibition and chemical parameters, total and available concentration of elements and phenolic contents in fly ash.

Parameters	Radicle growth inhibition of red clover					Hypocotyl growth inhibition of red clover				
pH (H_2O)	r	=	-	0.770	**	r	=	-	0.512	ns
pH (KCl)	r	=	-	0.687	*	r	=	-	0.591	*
EC	r	=	+	0.486	ns	r	=	-	0.868	***
C	r	=	+	0.582	*	r	=	-	0.721	**
N	r	=	+	0.559	ns	r	=	-	0.063	ns
C/N	r	=	-	0.268	ns	r	=	-	0.858	**
P_2O_5	r	=	+	0.875	***	r	=	-	0.556	ns
K_2O	r	=	+	0.280	ns	r	=	-	0.124	ns
Cu_{total}	r	=	+	0.692	*	r	=	-	0.799	**
Fe_{total}	r	=	+	0.195	ns	r	=	-	0.716	**
Mn_{total}	r	=	+	0.344	ns	r	=	+	0.596	*
Ni_{total}	r	=	+	0.375	ns	r	=	-	0.623	*
Zn_{total}	r	=	+	0.444	ns	r	=	+	0.563	ns
$Cu_{available}$	r	=	+	0.474	ns	r	=	-	0.863	***
$Fe_{available}$	r	=	-	0.233	ns	r	=	+	0.224	ns
$Mn_{available}$	r	=	+	0.596	*	r	=	+	0.672	*
$Ni_{available}$	r	=	+	0.810	**	r	=	+	0.223	ns
$Zn_{available}$	r	=	-	0.014	ns	r	=	+	0.849	***
3,5-DHBA	r	=	+	0.783	**	r	=	-	0.383	ns
Ferulic acid	r	=	+	0.826	***	r	=	-	0.557	ns
Rutin	r	=	+	0.757	**	r	=	-	0.530	ns
Quercetin	r	=	+	0.347	ns	r	=	+	0.067	ns

*p<0.05**; **p<0.01; ***p<0.001; ns=not significant

have an effect on the accumulation and availability of soil nutrients, which in turn have a great influence on plant growth [17]. However, phenolics can also play an important role in inhibiting the nitrification that affects a plant's nutrient status [1]. Recent studies are more focused on the environmental impact of allelochemicals on ecosystems than on plant-plant interactions [11], indicating a great influence of allelochemicals on the turnover of inorganic and organic compounds in soil [33].

In this study, the pH values of fly ash ranged from 6.32-8.1, which is similar to results obtained by other authors (8.85 [34], 7.95 [25]). According to Whitehead et al. [35], soil pH can affect the concentration of phenolic compounds in the soil solution. Our research showed that as the pH of fly ash increases, the inhibition of radicle and hypocotyl growth of red clover is less pronounced. A high pH value (8.1) and low nitrogen content (0.13%) in the rhizospheric fly ash of *R. pseudoacacia* (RP_{FA}) was noted, although this is a nitrogen-fixing species. The cause of this phenomenon may be the detrimental effect of high pH on the microorganisms involved in nitrogen fixation [36]. Generally, microorganisms can reduce the allelochemical activity of phenolics in the rhizosphere and reduce the inhibition of plant growth due to rapid mineralization of phenolic compounds [15]. The limitation of phytotoxic activity of active substances released from the donor plants could occur through sorption to organic matter which is greatest under neutral to slightly alkaline conditions [21]. However, high inhibition of red clover radicle and hypocotyl growth could be attributed to the lower pH values of the AA_{FA} and AF_{FA} (6.34 and 6.32, respectively), which can modify the nutrient status and element availability in fly ash [21].

In the present study, the C content in plant rhizospheric fly ash (RP_{FA}, AA_{FA} and AF_{FA}) was greater than in the control fly ash (C_{FA}) (2.41, 3.12 and 3.07 times, respectively), and this might be due to its origin from humus that contains humic acids, peptides and carbohydrates as compared to control fly ash where the C is derived from coal [37]. Our findings show that phenolics in the AA_{FA} and AF_{FA} had a more negative impact on red clover radicle growth with an increasing C content in fly ash compared to phenolics in the RP_{FA} and C_{FA}. The C pool in soils depends on its input through leaf and root litter and root exudates and decomposition of organic matter by microorganisms [38]. The high inhibitory effect of *A. altissima* and *A. fruticosa* on red clover radicle growth could be related to the high content of phenolics in fly ash which, being C-rich compounds, are released from plants to fly ash and contribute to the C-stock in fly ash. The positive correlation between ferulic acids and carbon content in fly ash (r=+0.585) points to their high content in the AF_{FA} (4.11 μg/g, 4.43%) and AA_{FA} (0.7 μg/g, 4.5%). Hence, it was assumed that some phenolics from these plants were less susceptible to microbial decomposition and had a high allelopathic effect.

The N content in the AA_{FA} and AF_{FA} was higher than in C_{FA} (3.27 and 1.90 times, respectively) and in RP_{FA} (2.77 and 1.61 times, respectively). The high N content in rhizospheric fly ash reveals the ability of these plant species to increase the amount of nitrate in the soil where the net nitrification rate in N-deficient

substrates, such as fly ash, depends on the quality of litter [39]. Furthermore, in N-limited soils, the high content of phenolics inhibits N mineralization, leads to the release of dissolved organic N from leaf litter and reduces overall ecosystem N loss [40]. In our study, the highest content of quercetin (60 μg/g) and N (0.36%) in the AA_{FA} and the positive correlation between quercetin content and N in fly ash (r =+0.945) suggest that the high polyphenol production from this plant species may present an advantage to increased plant uptake of organic N [40].

The content of available P was significantly higher in plant rhizospheric fly ash (RP_{FA}, AA_{FA} and AF_{FA}) than in the C_{FA} (1.21, 1.51 and 2.69 times, respectively). Herr et al. [41] noted that the rhizospheric soil of *Solidago gigantea* had a lower pH value and a higher content of available P than surrounding soils without this species, which is similar to our observations in *A. altissima* and *A. fruticosa*. Our results showed significant inhibition of red clover radicle growth in response to the high available content of P in fly ash. According to Kafkafi et al. [42], phenolic compounds can compete with P for sorption sites on mineral surfaces and may form complexes with Al and Fe. The high content of phenolic allelochemicals (3.5-DHBA, ferulic acids and rutin) that are released from plants can increase P availability (r=+0.925, r=+0.990, r=+0.955, respectively), desorbing previously bound P, which might be related to our results obtained for *A. fruticosa*. The low content of P in the RP_{FA} can be related to the inhibition of N fixation [43] as well as with the low content of phenolic acids, which can reduce solubility and availability of P [5].

The concentration of Cu, Mn, Ni, and Zn in fly ash can be 30 times higher than in coal, making fly ash the main threat for the surrounding environment and human health [44]. In our study, the total concentrations of Ni in all rhizospheric fly ash samples were toxic (12-34 μg/g, [45]), the concentrations of Cu were within the normal range (13-24 μg/g, [45]) whereas the concentrations of Mn and Zn were deficient (270-525 μg/g and 45-100 μg/g, respectively [45]). The availability of Cu, Fe, Mn, Ni and Zn in control and plant rhizospheric fly ash samples was relatively low. In addition, the concentrations of total and available Mn and Zn in the rhizospheric fly ash of all woody species were lower than in control fly ash. Higher Cu availability in AF_{FA} in relation to the C_{FA} can be due to low pH values (6.3), as in slightly acidic conditions Cu is more available [46]. However, red clover radicle growth inhibition is positively correlated with total concentrations of Cu and the available content of Mn and Ni in fly ash. The content of heavy metals in soil can be crucial for the persistence and activity of allelochemicals [47] and may affect the production and release of secondary metabolites from the plant roots, which in turn increase the availability of nutrients or form chelates with toxic metal in the soil [48].

Phenolic compounds can increase the availability of Cu, Fe and Mn by forming organic complexes that enhance the uptake of these elements by plants [5,21]. The high concentrations of 3.5-DHBA, ferulic acid and rutin in fly ash were related to the high total content of Cu (r=+0.706, r=+0.863, r=+0.798, respectively), Fe (r=+0.717, r=+0.705, r=+0.777, respectively) and Ni (r=+0.8555, r=+0.818, r=+0.888, respectively), which coincides mostly with the results obtained in *A. fruticosa*. The available content of Cu increased and the available content of Fe decreased with the higher contents of 3.5-DHBA (r=+0.791, r=-0.768, respectively), ferulic acid (r=+0.872, r=-0.616, respectively) and rutin (r=+0.878, r=-0.733, respectively) in fly ash mostly found in *A. fruticosa*, whereas the highest content of quercetin in fly ash coincided with the highest available content of Ni (r=+0.755) in the AA_{FA}. The decrease of hypocotyl growth of red clover was lower with increasing total concentrations of Cu, Fe, Mn and Ni and with decreasing available concentrations of Mn and Zn in fly ash. Since there are no significant correlations between hypocotyl growth inhibition of red clover and phenolic compounds in fly ash, it can be assumed that these phenolics do not express an allelopathic effect on red clover hypocotyl growth.

The results of this study showed that the contents of 3,5-DHBA, ferulic acid and rutin were greatest in the AF_{FA} where they expressed the strongest inhibitory potential on red clover radicle growth. The cause of this phenomenon may be the increased allelopathic activity of several different phenolic compounds in the combination [49]. Thus, the additive and synergistic effects of several different phenolic compounds become significant and decisive in allelopathic inhibition of various ecophysiological processes in the acceptor

plant [5,8]. Csizar [50] showed that *A. fruticosa* had stronger allelopathic potential than *A. altissima* and *R. pseudoacacia*, which is in line with our results. Generally, different allelopathic compounds were found in *R. pseudoacacia* and *A. altissima* [51,52], but the data on the allelopathic activity of these woody species grown on fly-ash deposits are still lacking.

In this study, the presence of phenolic compounds in all plant rhizospheric fly ash samples indicates that *R. pseudoacacia, A. altissima* and *A. fruticosa* can affect the beginning of pedogenetic processes of a sterile substrate, such as fly ash. Phenolic compounds, as the most prevalent allelochemicals in plants, enter the soil by leaching from the surface of the plant body, decomposition of litter and active secretion from roots [6,53]. Free phenolics are very important in the interactions between donor and acceptor plants because they are first available to the acceptor plant when they reach the litter and the soil [23]. Therefore, the detection of allelochemicals in the rhizosphere of the donor species is very important for allelopathy [54]. Further studies will determine the seasonal dynamics of phenolic compounds in rhizospheric fly ash and leaf litter of investigated plant species, the contents of these allelochemicals in roots and aboveground plant parts, as well as the effects of these compounds on germination and the seedling growth of the other herbaceous plant species that grow on fly-ash deposits.

CONCLUSIONS

The allelopathic activity of phenolic compounds in rhizospheric fly ash is strongly influenced by pH, content of C and available P, as well as the total content of Cu and the available contents of Mn and Ni in fly ash. In addition, the unfavorable conditions that prevail on fly-ash deposits promote the high production of phenolic acids and flavonoids in woody plant species such as *A. fruticosa*, *A. altissima* and *R. pseudoacacia*. These phenolic compounds have the properties of allelochemicals in fly ash and can lead to the inhibition of growth of *T. pratense* as the understory herbaceous species present in the fly-ash plant community. These woody species that colonized the fly-ash deposits may act as promotors of pedogenetic processes on fly ash, altering the ecosystem processes in anthropogenically degraded sites.

Funding: This work was supported by the Ministry of Education, Science and Technological Development, Grants III41010, OI173018 and TR31070.

Acknowledgments: We would like to thank Zoran Bajić and Isidora Glišić from the Service for Environmental Control and Protection of the of "Nikola Tesla – A" thermal power plant, for their assistance during field work, the possibility to access the fly ash deposits and for the expert information they provided to us.

Author contributions: Filip Grbović was the main contributor who performed the work; he was responsible for field work, conducting the experiments and for writing the manuscript. Gordana Gajić helped with the data analysis, data interpretation and explanation, and manuscript writing. Snežana Branković helped with the allelopathic bioassays and data interpretation. Zoran Simić performed the determination of total and available elements on FAAS. Andrija Ćirić conducted the HPLC analysis. Ljubinko Rakonjac contributed to the evaluation of chemical analysis of fly ash. Pavle Pavlović and Marina Topuzović contributed to the evaluation and interpretation of the results, participated in designing the experiments and supervised the data interpretation and manuscript writing.

Conflict of interest disclosure: All authors confirm no actual or potential conflicts of interest.

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Supplementary Data

Supplementary Fig. S1.
Available at: http://serbiosoc.org.rs/NewUploads/Uploads/Grbovic%20et%20al_3353_Supplementary%20Fig.%20S1.pdf