Heavy Metal-induced Cuticular Alkane Changes of Tall Fescue (*Festuca arundinacea*) Plantlets

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Heavy metal pollution of ecosystems is of great concern, due to the persistence of metals in ecosystem. In this regard, the mountainous pastures of Romanian Carpathians, especially those in the areas of abandoned mines, need particular attention. The aim of this work was to assess the effect of heavy metals and metalloids exposure on cuticular wax composition of tall fescue leaves (Festuca arundinacea). Therefore, we have particularly investigated the variations of long chain hydrocarbon fractions, which are acknowledged to act as a protection for plants especially in toxic environments. The exposure experiments were conducted in triplicates for arsenic and the following metal ions: Ba^{2+}, Cu^{2+}, Fe^{2+} and Pb^{2+}. The 0.5 mM aqueous solutions of their corresponding salts were used as treatment solutions. Triplicate experiments were also performed for control samples using distilled water as exposure media. The plantlets were kept for growing in controlled environment for 15 days followed by n-hexane hydrocarbon fraction extraction from 0.5 g of sampled leaves. The obtained extracts were semi-quantitatively analyzed (identification followed by peak area measurement) through an optimized method based on gas-chromatography coupled with mass spectrometry. We focused on the measurement of several long chain n-alkanes with the following number of carbon atoms: C21-C31. Significant differences were obtained between results performed for Ba^{2+}, Pb^{2+} and Fe^{2+}, when compared to AsO_4^{3-} and Cu^{2+}, which may suggest multiple mechanisms through which the tested plants could develop and adapt when exposed to various chemicals characterized by different degrees of toxicity.

Keywords: heavy metals, Festuca arundinacea, cuticle, n-alkanes, GC-MS

In recent years, heavy metal pollution of terrestrial environments is of great concern, due to the persistence of metals in the ecosystem [1]. The problem of heavy metal pollution is becoming increasingly serious with the rise in industrialization and disturbance of natural biogeochemical cycles [2-6]. The mobilization of heavy metals through extraction from minerals and subsequent processing for various applications has led to the release of these elements into the environment [2]. In this regard, the mountainous pastures of Romanian Carpathians, especially those in the areas of abandoned mines, need particular attention [7-9].

Unlike organic substances, heavy metals are essentially non-biodegradable and therefore accumulate in the environment [10-13]. The accumulation of heavy metals in soil and water constitutes a risk to both human and environmental health [14]. It is well known that there is a good correlation between the heavy metal concentration in surface waters and the heavy metal concentration in soil and vice versa to assess contamination as a result of anthropogenic activities [15-20]. These elements accumulate in the body tissues of living organisms (bioaccumulation) and their concentrations increase as they pass from lower trophic levels to higher trophic levels (a phenomenon known as biomagnification). In the soil, heavy metals cause toxic effects on soil microbial population, which can lead to a decrease in their number and activities [1]. Nevertheless, numerous methods have been advanced to remove heavy metals from the environment [21]. Some authors reported thus on removing efficiency of Mn^{2+}, Ni^{2+}, and Cu^{2+} from the environmental and food samples [22]. For detoxifying water and slurries rich in heavy metal ions (Ni^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+} etc.) a series of purification procedures including precipitation, coagulation and flocculation processes or electrocoagulation ones was advanced [23]. In the case of nickel recovering under the influence of ultrasound, it is possible to produce hard, compact and adherent electrodeposits even using high current densities in the electroplating process [24].

The surface of leaves is known as the cuticle, which is a primary point of contact with pathogens, and regulates water retention [25]. The cuticle is an extracellular matrix of two main components: cutin, which is a simple polymer and waxes. Waxes are secondary metabolites and include alkanes, fatty acids, primary and secondary alcohols, ketones, esters, and aldehydes [26].

Therefore, the aim of this work was to investigate the n-alkane content in the cuticle of tall fescue (*Festuca arundinacea*) plantlets exposed to arsenate and metal cations. Our results showed that copper ions and arsenate change much the pattern of n-alkanes in the cuticle whereas barium, iron and lead ions do not influence the metabolism of the secondary compounds. This relationship between heavy metals in the environment and cuticle alkane composition could be used as a marker to monitor forest areas pollution around the closed mines.

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Experimental part

Materials and method

Sampling

In brief, 1 g seeds of *Festuca arundinacea* were distributed in 18 Petri dishes containing double layer of filter paper. The following aqueous solutions, 5 mL for each Petri dish, were used to treat the three replicate samples: Control – distilled water, As(V) -0.5 mM natrium arsenate (AsO_4^{3-}) , Ba^{2+} -0.5 mM barium chloride, Cu^{2+} -0.5 mM copper(II) chloride, Fe^{2+} – 0.5 mM iron(II) sulfate, and Pb^{2+} - 0.5 mM lead (II) acetate.

After 24 days of germination, the aerial part of *F. arundinacea* plantlets was collected. An amount of 0.5 g of each aerial part was taken and immersed in 50 mL n-hexane for 3 minutes. The resulted extract was concentrated using a rotary evaporator (Rotavapor R-100, BÜCHI Labortechnik AG, Switzerland) to a volume of 1 mL. An aliquot of 5µL was injected into the inlet of GC/MS instrument for analysis.

GC/MS analysis

The processed samples were analysed by gas-chromatography coupled with mass spectrometry. GC–MS analysis was performed with an Agilent 6890N gas chromatography instrument coupled to an Agilent 5975 mass spectrometer, operated by the Agilent ChemStation software (Agilent Technologies, Palo Alto, CA). A capillary column (30 m×0.25 mm i.d.) coated with 0.25 µm film 5% phenyl methyl siloxane (DB-5) was used for separation. High purity helium was used as carrier gas with flow-rate at 1.0 mL/min.

The other GC conditions were optimized as follows: inlet mode: splitless (50 mL/min after 2 min); injection temperature: 250°C; separation temperature program: from 40 °C (at 6°C/min) to 280°C (for 5 min); total run time: 78 min; the spectrometer was operated in electron-impact (EI) mode, the scan range was 41–550 amu; the quadrupole and ionization source temperature were 200 and 250°C, respectively.

Statistics

From each sample, multiple determinations were performed, the systematized data representing the mean of these replicas ± the standard deviation. Statistical analyses were performed using the t-Student test. The differences between the control and the exposed samples being considered significant at $p < 0.05$ (***$p < 0.001$ - very significant; ** $0.001 < p < 0.005$ - significant; *$0.01 < p < 0.05$ - less significant; $0.05 < p < 0.5$ - not significant).

Results and discussions

Alkane separation. The GC/MS chromatograms displayed alkane peaks from 48.28 min to 73.03 min as a function of their molecular size (table 1).

Effect of arsenate on alkane cuticular composition

Arsenate treatment has led to a strong inhibition of germination of tall fescue plantlets, as can be seen in figure 1.

Effect of barium ions

Table 2 illustrates the changes in the cuticular alkane composition under the action of $BaCl_2$. Generally, a weak effect of such ions on the higher alkanes of tall fescue was noticed. However, barium ions reduced the content of the entire spectrum of alkanes, although this decrease was not significant. Thus, the heneicosane (C21) content is only 0.25% very close to the control with distilled water (0.31%).

Table 2 shows that the highest values were measured at hentriacontane ($C_{31}H_{64}$) - 3.03%, followed by heptacontane ($C_{27}H_{56}$) – 2.07%. The lowest values were found for docosane ($C_{22}H_{46}$) – 0.21%, heneicosane ($C_{21}H_{44}$) - 0.25% and tetracosane ($C_{24}H_{50}$) - 0.26%. These values were proportional to those in samples subjected to other treatments, resulting in a species-dependent alkane composition rather than treatment-dependent.

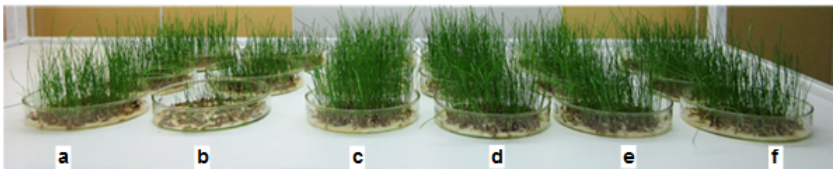

Fig. 1. Seedlings of *F. arundinacea* after 14 days from the treatment: a) Control-distilled water, b) As(V) - 0.5 mM natrium arsenate (AsO_4^{3-}), c) Ba^{2+}-0.5 mM barium chloride, d) Cu^{2+}- 0.5 mM copper(II) chloride, e) Fe^{2+}-0.5 mM iron(II) sulfate, and f) Pb^{2+} - 0.5 mM lead (II) acetate

Table 1
THE EFFECT OF ARSENATE IONS ON CUTICULAR n-ALKANE COMPOSITION AS DETERMINED BY GC/MS

Retention time (min)	n-Alkanes*	Formula	Peak area**		Control	
			$\bar{x}_n$ n = 3	SD	$\bar{x}_n$ n = 2	SD
48.28	Heneicosane	$C_{21}H_{44}$	0.32	0.06	0.31	0.03
51.21	Docosane	$C_{22}H_{46}$	0.27	0.07	0.29	0.02
54.04	Tricosane	$C_{23}H_{48}$	0.40	0.09	0.45	0.04
56.76	Tetracosane	$C_{24}H_{50}$	0.32	0.07	0.40	0.06
59.29	Pentacosane	$C_{25}H_{52}$	0.77	0.14	1.00	0.03
64.24	Heptacosane	$C_{27}H_{56}$	1.75	0.24	2.14	0.08
66.56	Octacosane	$C_{28}H_{58}$	0.49	0.12	0.66	0.16
70.91	Triacontane	$C_{30}H_{62}$	1.18	0.55	1.25	0.16
73.03	Hentriacontane	$C_{31}H_{64}$	4.47	1.73	5.13	0.43

*hexacosane, $C_{26}H_{54}$, and nonacosane, $C_{29}H_{60}$, were not identified;
**expressed as area percent of major peak.

RT (min)	n-Alkane	Formula	Peak area		Control	
			$\bar{x}_n$ n = 3	SD	$\bar{x}_n$ n = 2	SD
48.28	Heneicosane	$C_{21}H_{44}$	0.25	0.06	0.31	0.03
51.21	Docosane	$C_{22}H_{46}$	0.21	0.04	0.29	0.02
54.04	Tricosane	$C_{23}H_{48}$	0.35	0.05	0.45	0.04
56.76	Tetracosane	$C_{24}H_{50}$	0.26	0.03	0.40	0.06
59.29	Pentacosane	$C_{25}H_{52}$	0.80	0.16	1.00	0.03
64.24	Heptacosane	$C_{27}H_{56}$	2.07	0.23	2.14	0.08
66.56	Octacosane	$C_{28}H_{58}$	0.56	0.12	0.66	0.16
70.91	Triacontane	$C_{30}H_{62}$	0.76	0.29	1.25	0.16
73.03	Hentriacontane	$C_{31}H_{64}$	3.03	0.32	5.13	0.43

Table 2
THE EFFECT OF BARIUM IONS ON CUTICULAR n-ALKANE COMPOSITION AS DETERMINED BY GC/MS

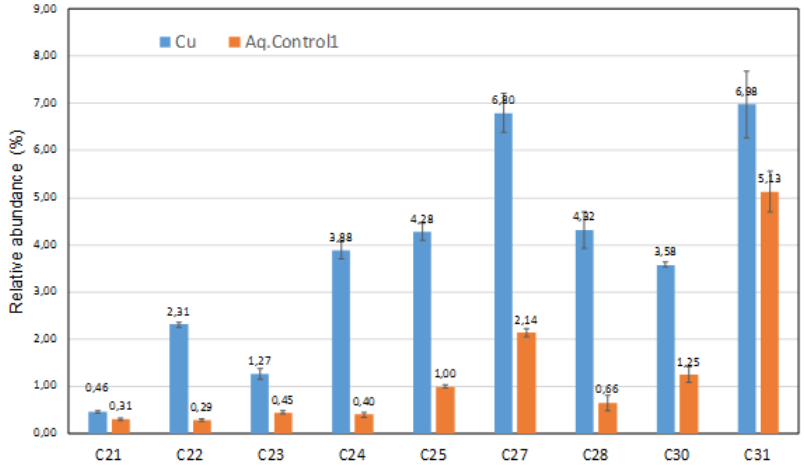

Fig. 2. Effect of Cu^{2+} ions on alkane composition of cuticle of the tall fescue plantlets: C21-C31 means alkane chain length Cn

Effect of copper(II) ions

Copper ions (Cu^{2+}) induce a significant effect on the variation in the composition of the higher alkanes. Figure 2 clearly demonstrates that copper ions cause a dramatic increase in the content of higher alkanes as compared to the control. Indeed, the highest increases in the peak intensities were found for tetracosane (from 0.40 to 3.88%), docosane (from 0.29 to 2.31%) and octacosane (from 0.66 to 4.32%). Generally, a three-time increase was spotted for tricosane (from 0.45 to 1.27%), heptacosane (from 2.14 to 6.80%) and triacontane (from 1.25 to 3.58%), whereas the smallest increase was noticed for heneicosane (from 0.31 to only 0.46%) and hentriacontane (from 5.13 to 6.98%).

The influence of ferrous ions

Unlike copper ions, iron has had opposite effects, although the decrease in alkane content was relatively low. Thus, the largest decrease was observed in the case of triacontane, $C_{30}H_{62}$, being 65% compared to control and pentacosane, $C_{25}H_{52}$, which was 79%. A medium decrease for docosane, $C_{22}H_{46}$, with 86%, tetracosane, $C_{24}H_{50}$, with 88%, octacosane, $C_{28}H_{58}$, with 89% was noticed. The lowest decrease was observed for heptacosane, $C_{27}H_{56}$, with 94% of control, whereas tricosane, $C_{23}H_{48}$, was 96% of the control, hentriacontan, $C_{31}H_{64}$, 97% and heneicosane, $C_{21}H_{44}$, 99%.

Lead effect

Pb^{2+} displayed an insignificant effect on the ratio of alkanes in the cuticle of tall fescue. Generally, there was a slight decrease in the alkanes content of cuticle when compared with the control (table 3). Only content of triacontane was significantly dropped.

Our data showed for the first time the strong effect of some arsenate and heavy metal ions on alkane composition in the cuticle of *F. arundinacea*. However,

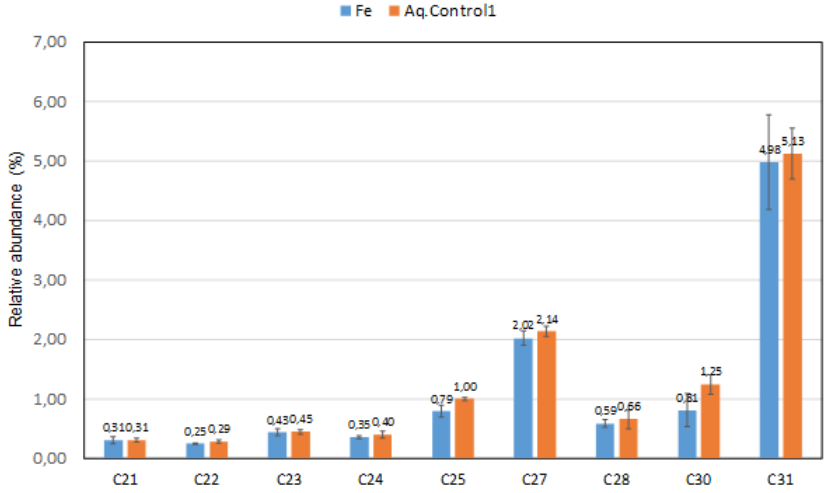

Fig. 3. Effect of Fe^{2+} ions on alkane composition of cuticle of *F. arundinacea* plantlets: C21-C31 means alkane chain length Cn; iron ions reduced slightly the concentrations of all alkanes.

RT (min)	n-Alkane	Formula	Peak area		Control	
			$\overline{x}_n$ n = 3	SD	$\overline{x}_n$ n = 2	SD
48.28	Heneicosane	$C_{21}H_{44}$	0.32	0.01	0.31	0.03
51.21	Docosane	$C_{22}H_{46}$	0.22	0.01	0.29	0.02
54.04	Tricosane	$C_{23}H_{48}$	0.41	0.01	0.45	0.04
56.76	Tetracosane	$C_{24}H_{50}$	0.30	0.03	0.40	0.06
59.29	Pentacosane	$C_{25}H_{52}$	0.73	0.06	1.00	0.03
64.24	Heptacosane	$C_{27}H_{56}$	1.74	0.11	2.14	0.08
66.56	Octacosane	$C_{28}H_{58}$	0.44	0.07	0.66	0.16
70.91	Triacontane	$C_{30}H_{62}$	0.56	0.02	1.25	0.16
73.03	Hentriacontane	$C_{31}H_{64}$	3.21	0.55	5.13	0.43

Table 3
THE EFFECT OF Pb^{2+} ON CUTICULAR n-ALKANE COMPOSITION AS DETERMINED BY GC/MS

some other metal ions like iron, lead and barium had almost no influence on alkane composition. Previously, the pollution with heavy metals and metalloids of Tarniţa area around a barite closed mine has been studied [8, 21]. Their interaction with living organisms is found to generate ROS, which are able to cause major imbalance of metabolism by destroying the antioxidant potential of the plant body [27].

As for the role of heavy metals in biological systems, they have been classified as essential and non-essential. Essential heavy metal ions are those that are necessary for living organisms in small amounts for vital physiological and biochemical functions. Examples of essential heavy metals ions are Fe^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, and Ni^{2+} [28, 29]. Non-essential heavy metals are those that are not needed by living organisms for any physiological and biochemicals function. Examples of non-essential heavy metal ions are Cd^{2+}, Pb^{2+} (AsO_4^{3}), Hg^{2+}, and Cr^{6+} [30-36]. Heavy metal concentrations above the threshold limits have adverse health effects because they interfere with the regular processes of living organisms.

High levels of heavy metal ions such as Fe^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Cd^{2+} and Pb^{2+} were found by other authors in vegetables like parsley, carrot, onion, lettuce, cucumber and green beans grown in contaminated mining areas compared with those grown in reference [37]. Others have evaluated the effects of different polyphenol-rich medicinal herbs (Lemon balm, Sage, St. John's wort and Small-flowered Willowherb) used as dietary supplements on bioaccumulation of some essential metals like Fe, Mn, Zn and Cu in different chicken meats [38]. Thus, the polyphenol-rich medicinal herbs influenced much the accumulation of metals in the liver, legs and breast chicken by comparison with the group that received a diet supplemented with metal salts solely. Heavy metal ions seem to interfere with various cellular enzymes and even are bound to proteins and peptides [39, 40]. They also affect the antioxidant capacity of plants, especially of those rich in polyphenols and other secondary metabolites [41]. Besides, they are considered as risk factors in cancer and other degenerative diseases [42, 43].

Conclusions

Here, we have investigated the effect of arsenate and some heavy metal ions on the alkane composition of the cuticle of *Festuca arundinacea*. Our results highlight unambiguously the effect of Ba^{2+}, Fe^{2+}, Cu^{2+}, Pb^{2+} and AsO_4^{3-} ions in the polluted area around closed mines on the cuticular alkane content of tall fescue plantlets. Thus, arsenate treatment has led to a strong inhibition of germination of the seedlings, as opposed to the metal ions treatment which had a limited effect on the growth of tall fescue. Copper(II) ions induced a dramatic increase in the content of higher alkanes as compared to the control. In contrast, the metal ions Ba^{2+}, Fe^{2+} and Pb^{2+} had a weak effect on the higher alkanes of tall fescue. However, these ions reduced the content of the entire spectrum of alkanes, although this decrease was not significant, resulting in a species-dependent alkane composition rather than treatment-dependent.

Patterns of alkane distribution could be used as a potential fingerprint analysis and identification of metal ion pollution of mountainous wild pasture.

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