THE POTENTIAL OF TWO WILLOWS GENOTYPES FOR RADIONUCLIDE AND HEAVY METALS ACCUMULATION. A CASE STUDY OF ROVINARI COAL ASH POND (GORJ COUNTY, ROMANIA).

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ABSTRACT

Pollution is one of the biggest environmental problems and heavy metals or radionuclides contributed to this. A high amount of heavy metals and radionuclides are found in ash produced by Thermo Electric Power Plants (TEPP). Normally, a mixture made by the ash and water is stored in coal ash pond but sometimes, because the warm weather this is dried and spread by wind it in the surrounded area. One way to manage contaminated land is afforestation. In this respect, experimental trials were established on a coal ash pond, on the prepared and unprepared field. Biological material was represented through common osier willow (cuttings) and white willow (whips and cuttings). Biometrical observations were performed as well as biomass estimation. In order to assess the capacity of willow species (Salix alba and Salix viminalis) to accumulate heavy metals and radionuclides, soil and leaves analyses were performed. The concentration of heavy metals in soil was determined in the rhizosphere horizon (5-20 cm) at the beginning of the experiment and 4 years later, through atomic mass spectrometry, and the radionuclide's activity through gamma spectrometry (Duggan method). The bioactive substances synthesized in leaf cells and the presences of some metabolic structures have been highlighted. Research has shown intense metabolic activity in foliar parenchyma cells and underlines the resistance and adaptation in the presence of a high amount of radionuclides and heavy metals.

INTRODUCTION

Pollution is one of the biggest environmental problems and heavy metals and radionuclides contributed to this. A high amount of heavy metals and radionuclides are found in ash produced by Thermo Electric Power Plants (TEPP). Normally, a mixture made by the ash and water is stored in coal ash pond but sometimes, because the warm weather this is dried and spread by wind it in the surrounded area. One way to manage contaminated land is afforestation. Some heavy metals (cadmium, copper, lead, iron, chromium, mercury a/o) and radionuclides (U235, Th 234 and their decay products) are major environmental pollutants. As result of their accumulation in soil, they exert adverse effects on food safety and marketability, due to their toxicity on environment (air, water and soil). Their toxicity determines an excessive accumulation of reactive oxygen species (ROS), and methyl-glyoxal (MG), which determine the lipids peroxidation, proteins oxidation, enzymes inactivation, DNA damage, as well as damages of the cell organelles. The presence of heavy metals could have different origins: Earth's crust, agriculture and livestock, different industries (extractive, energetic, a/o), anthropogenic activities, a/o (Cheng, 2003, Mukti, 2014). Plants have many genetic, biochemical, and physiological properties that make them ideal agents for phytoremediation after Meagher (2000).

Willow, as a fast-growing woody species has several advantages over herbaceous species, such as a deeper root system, higher productivity and transpiration activity; it represents a promising resource in mitigating impacts of environmental degradation. Remediation by willow plantations can clean or mitigate hazardous waste, stabilize and restore a site and produce wood for fuel and cricket bats. Willows planted as vegetation filters will facilitate excess nutrient uptake, reduce soil erosion, provide habitat for numerous organisms above and below the water level, and enhance a site's visual characteristics (Wani et al. 2011). Marmiroli et al. (2011), consider that in *Salicaceae* family, there are species, which can be used with efficiency in extraction of heavy metals from environment.

Salicaceae family is represented in the classical systematic through about 300 species, spread in the holarctic region, from plain to alpine region (trees, shrubs, and sub-trees). Recent genetics studies expanded the genera number of this family at 55-60 genera (Chase et al. 2016). Willows are important for their biomass accumulation, many species can be used for short rotation coppice (Gomes 2011). Different clones (especially belonging to *Salix viminalis*), are high accumulators of Cd and Zn. After different authors, up to 150 clones (mainly from *S. viminalis,* few from *S. alba* and *S. caprea*) have been screened for uptake, transport of trace elements to shoots, and tolerance to Cd, Zn, and Cu (Landberg and Greger 1994; 1996; Vaculík et al. 2003; Hammer et al., 2003; Meers et al. 2005, Iori V.et al. 2015). In experiments performed with four willow genotypes (two clones of *S. alba*, S. *matsudana* and *S. nigra*), Borišev et al. (2009), established that the ability of these clones to extract and translocate Cd, Ni, and Pb is depending on the quantity of metal content in the nutrient solution and of the willow genotype. Also, the ability of these clones for the Cd accumulation in leaves, is similarly with the values reported by other authors. Thus, studies performed in UK by French et al. (2009), point out that after a period of 20-years the *Salix x calodendron* can reduce Cd and Zn levels by 5.6 and 96 mg kg^{-1}, respectively. In an experiment performed in hydroponic culture, Wenzel et al. (2005) have been analysed the reaction of different trees species (*S. caprea*, *S. fragilis*, *S. smithiana*, *Populus tremula*) towards different concentration of some heavy metals. The plant reaction was established through analysis of the seedling's tolerance to Cd, Cu, Zn, Pb, and biomass accumulation. Experimental results, point out that willow absorbed Cd and Zn from environment. In other experiment, Borowiak et al. (2012), analysed the reaction of seedlings of *Salix viminalis* cv "Cannabina" developed on copper enriched culture media. They established that the ions ratio Ca/Mg are implied in the copper accumulation in seedlings.

The aim of this paper is to evaluate the tolerance of two willows genotypes to heavy metals and radionuclides, their ability to clean up the soil and the modification that occur on ultrastructural level in willows leaves.

MATERIAL AND METHODS

Study area. The study site is located on a closed coal ash pond, near an open one and near a surface mining exploatation, in Rovinari, Gorj County, on South-West of Romania. The field trial was establish in 2009 (Long 44°55'40'', Lat 23°10'08'', Alt 168m, Fig.1).

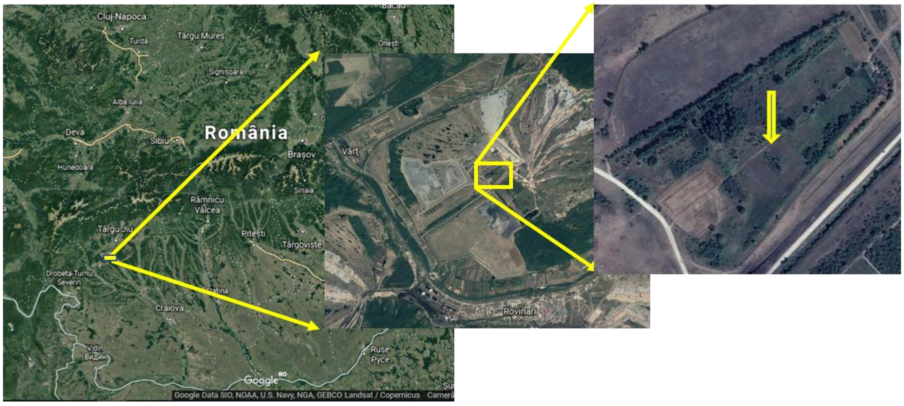

Figure 1 The location of the experimental trial in the Gorj county (Romania)

Biological material. Biological material was represented through common osier willow and white willow cuttings (20 cm length) and white willow whips (1.8-2 m length) produced by forest nursery Zaval, Romania.

Experimental design. The field experiment was established in 2009 on an unprepared field (*S. alba*), and prepared field (*S. alba* and *S. viminalis*). Rectangular tree spacing, 4m x 2m for whips and 1m x 0.5m for cuttings was adopted.

Soil analyses. The amount of heavy metals from soil was determined in the 5-20 cm soil level at the beginning of the experiment and 4 years later, through atomic absorption spectrometry in air-acetylene flame, and the radionuclide's activity through gamma spectrometry (Duggan method, 1989).

Phenotypic and biometrical observations. The observations were performed on survival of whips and cuttings for the both species. Assessment on height and diameter of *S. alba* plants were made. Biomass estimation was performed at the end of the growing seasons by drying material at 105^0C, until constant weight.

Ultrastructural investigations on the mature leaves, were performed with a TEM 1010 JEOL-JEM, the biological samples being prepared after the usually methods: prefixed in a 2.7% glutaraldehyde solution (2 ½ h); fixing in a 1% Millonig solution (1 ½ h), infiltration and embebed in EUPARAL 812. Sections of 89-90 nm were contrasted in uranyl acetate and lead citrate (Electron Microscopy Centre, *Babeş-Bolyai* University).

RESEARCH RESULTS

1. Radionuclide's activity in soil. Soil analysis revealed the presence of natural radionuclides belonging from ash and coal dust, as well as of Cs-137 (artificial radionuclide), of Chernobyl provenance. Radionuclides activity was dependent on the considered site and the distance from the pollution source (Table 1). Radionuclide's content over the normal limits for Romania was recorded for Th-234, Pb-210, Bi-214, Pb-214, U-235, Ac-228, Pb-212, Ra-226 and Cs-137. In time, the radionuclides activity (Bq/kg soil), is reduced, on one hand due to half-life and the other hand, due to the presence of the plant species, that extract pollutant elements from soil. If in the Pb-210, Bi-214 and Pb-214 cases, the radioactivity decrease can be explained by short or very short half-life, for all other radionuclides, decrease in activity is mostly due to the process of phytoextraction.

Unprepared field, *Salix alba*. The radionuclide's activity in the 5-20 cm topsoil have been decresed after for four years of experiment (Table 1). The slightly enhanced of the U-235 radionuclide can be due to their deposit at the soil surface, because the surface coal exploitation, as well as due to strong wind present in this area (similar to some cyclones).

Prepared field, *S. alba* and *S. viminalis* The phytoremediation effect was dependent on the radionuclide type and willows genotypes (Table 1). The significant decreased values (after 4 years), were recorded in the area planted with *S. alba*, for Pb-210. In the area planted with *S. viminalis*, significant decreased values were recorded for Cs-137 and K-40. The both species manifested a strong phytoremediation activity for Th-234/U-238, Ra-226, Pb-210, Bi-214 and Pb-212, reducing their activity in soil with 27.4 – 67.2%. Also, in this case, the U-235 radionuclide recorded an upper activity, probably to the same causes (mining activity at soil surface and strong winds). Higher values were recorded (at the end of the experiment) also for Ac-228/Th-232, especially in area with *S. viminalis*.

2. Heavy metals content in soil

Some heavy metals (cadmium, copper, lead, iron, chromium, mercury a/o), are major environmental pollutant. As a result of their accumulation in soil, they exert adverse effects on food safety and marketability, due to their toxicity on the environment (air, water and soil). Their toxicity, determine an excessive accumulation of reactive oxygen species (ROS), and methyl-glyoxal (MG), which determine the lipids peroxidation, proteins oxidation, enzymes inactivation, DNA damage, as well as damages of the cell organelles (Mukti, 2014). The heavy metals present a different origin: Earth's crust, agriculture and livestock, different industries (extractive, energetic, a/o), anthropogenic activities, a/o. In the present experiment, the amount of heavy metal in soil was recorded different values, depending on the considered metal/element, the soil type (unprepared or prepared field), the genotype culture and the year of experiment (Table 2). In generally, on unprepared field cultivated with *S. alba*, the heavy metal amount in soil recorded higher values at the beginning of the experiment (2009 year) for Zn, Mn, Ni, Co, Pb and Cd, and at the end of experiment (2013 year) for Cu, Fe and Cr (Table 2).

On the prepared field, *S. alba* and *S. viminalis* cultures were established in the year 2009. After 4 years, the soil content in heavy metals was different in the two areas. Differences between the capacities of the two species to absorb heavy metals from soil have been recorded (Table 2). Thus, on the area with *S. alba*, the amounts of Zn and Mn are higher, while in area with *S. viminalis* those of Ni, Co and Pb are. The amounts of Cu, Fe, Cr and Cd are relative similarly (into the normal variation limits). Thus *S. alba* manifested a better phytoremediation capacity for Ni, Co and Pb, Cd, while *S. viminalis* showed a preference for Zn, Mn, Pb and Cd. Cosio et al. (2006), in an experiment performed in a hydroponic culture of *S. viminalis,* with different Cd concentration (0 – 200 mM), established the natural resistance to cadmium of this species. Vaculik et al. (2012), in experiments performed on *S. caprea* point out that Cd and Zn interfere differently. The variation in the root tissue organization, as well as Zn, Cd and other elements, developed apoplastic barriers, being the result of environmental adaptation. They suppose that "the molecular basis of these adaptive traits might help to increase the tolerance and heavy metal accumulation capacities of *S. caprea* for phytoremediation technologies". Regarding the phytoremediation action of different species toward Cu, different authors reported different aspects. Also, different heavy metals interfere particularly between them.

In an experiment with *S. viminalis* cv. “Cannabina” performed in a hydroponic culture, Mleczek et al. (2013) reported as in general, take place an increase of Cu accumulation, together with increase of Cu concentration in modified Knop’s culture medium, as well as some significant differences in copper phytoextraction between plants at different Ca/Mg ratio.

3. Willow survival, growth and yield on ash-pond Rovinari.

Plant survival percentage was very good for *S. viminalis*, but not so good for *S. alba*, in both cases, whips and cuttings (Table 3).

Plant height varied for both analysed cultures, the coefficient of variation is higher for whips culture compared with cuttings one. A high value of coefficient of variation was recorded for diameter at breast height too (Table 4).

Table 1

Radionuclids activity in experimental field (ash, 5-20cm) (Bq/kg soil)

	Unprepared filed			Prepared filed					Limits for Romania
	2009	2013	D %	2009	2013	D%	2013	D%	
	Salix alba				*Salix alba*		S. viminalis		
Series U-238									
Th-234 (U-238)	130.1 ± 15.10	90.89 ± 11.89	- 30.1	163.6 ± 17.50	98.26 ± 8.26	- 39.9	102.04 ± 13.84	-37.4	25.0
Ra-226	102.3 ± 33.80	73.50 ± 4.29	- 28.2	229.5 ± 5.10	78.00 ± 8.00	-66.0	75.30 ± 4.60	-67.2	10.0-90.0
Pb-210	127.5 ± 7.95	102.68 ± 11.33	- 19.5	151.1 ± 8.42	109.68 ± 10.18	-27.4	111.52 ± 17.48	-25.5	20.0-40.0
Bi-214	86.8 ± 4.82	70.35 ± 3.39	- 19.0	117.1 ± 4.00	75.16 ± 5.16	-35.8	70.57 ± 4.14	-39.7	20.0-40.0
Pb-214	97.3 ± 5.67	77.10 ± 3.93	- 20.8	142.0 ± 5.35	79.81 ± 6.83	-43.8	88.28 ± 4.34	-37.8	20.0-40.0
Series U-235									
U-235	8.46 ± 1.48	8.96 ± 1.13	+ 5.9	8.99 ± 1.73	8.27 ± 1.00	-8.0	10.53 ± 1.25	+17.1	2.0
Series Th-232									
Ac-228(Th-232)	72.7 ± 7.52	53.11 ± 3.82	- 26.9	88.4 ± 1.73	58.27 ± 1.00	-34.1	50.53 ± 1.25	-42.8	13.0-65.0
Pb-212	90.8 ± 8.47	69.01 ± 5.15	- 24.0	137.8 ± 4.86	72.29 ± 5.97	-47.5	70.25 ± 3.83	-49.0	20.0-50.0
K-40	416.9 ± 38.80	309.82 ± 31.06	- 25.7	519.2 ± 40.90	313.22 ± 28.91	-39.7	278.42 ± 27.67	-46.4	330.0-800.0
Artificial radioactivity									
Cs-137	74.9 ± 4.00	47.44 ± 3.10	-36.7	77.5 ± 3.5	43.57 ± 2.79	-43.8	32.97 ± 2.85	-57.5	0

D% = differences in radionuclides activity between 2013 and 2009 year, reported to 2009

Table 2

Heavy metals content in soil (mg/kg soil)

Metal	Unprepared filed			Prepared field					Normal content in soil	Alert limits
	2009	2013	%	2009	2013	%	2013	%		
	Salix alba				*Salix alba*		*Salix viminalis*			
Zn	122.0	81.0	-33.6	104.0	106.0	+1.9	100.0	-3.8	100	300-700
Cu	46.0	56.90	+23.7	45.0	68.6	+52.4	67,4	+49.8	20	100-250
Fe	26,273	29,948	+14.0	25,111	29,946	+19.3	30,540	+21.6	*	*
Mn	454.0	300.0	-33.9	445.0	339.0	-23.8	315.0	-29.2	900	1500-2000
Ni	63.5	55.0	-13.4	45.0	61.0	+33.6	63.0	+40.0	20	75-200
Cr	46.2	49.0	+6.1	17.10	57.0	+133.3	57.0	+133.3	30	100-300
Co	15.9	12.01	-24.5	15.50	13.75	-11.3	15.33	+1.1	*	*
Pb	30.7	19.40	-36.8	33.0	21.2	-35.8	25.70	-22.1	20	50-250
Cd	1.530	0.265	-82.7	1.370	0.293	-78.6	0.468	-65.8	1.0	3.0-5.0

D% = differences in metal content in soil between 2013 and 2009 year, reported to 2009

Table 3

Willow survival on ash-pit Rovinari

Number of whips/cuttings	Year			
	2009	2010	2011	2013
***Salix alba* whips culture on ash-unprepared field**				
Existing at the beginning of growing season	-	13	113	56
Planted in the year	19	100	-	-
Existing at the end of growing season	13	113	96	-
***Salix alba* cuttings culture on ash-prepared field**				
Existing at the beginning of growing season	-	60	20	14
Planted in the year	100	-	-	
Existing at the end of growing season	60	20	14	
***Salix wiminalis* cuttings culture on ash-prepared field**				
Existing at the beginning of growing season	-	480	480	480
Planted in the year	500	-	-	
Existing at the end of growing season	480	480	480	

Table 4

Willow height and diameter at breast height on ash-pit, Rovinari.

Statistical parameters	*Salix alba* whips culture on ash-unprepared field			*Salix alba* whips cuttings culture on ash-prepared field	
	Height (cm)		Diameter (cm)	Height (cm)	Diameter (mm)
	Year			Year	
	2010	2013	2013	2013	2013
No.	96.00	49.00	49.00	12.00	12.00
Average	248.40	411.32	56.19	421.83	56.86
Minimum	38.00	120.00	11.15	288.00	25.87
Maximum	405.00	754.00	132.14	628.00	103.97
Standard deviation	87.30	152.04	23.16	110.126	23.27
Coefficient of variation (%)	35.14	36.96	41.21	26.11	40.94

After four year of vegetation there has been recorded value of about 300 g dry matter per plant, as in intensive culture reported by Smaliukas (2007) (80-303 g).

Table 5

Biomass yield of willows (kg/plant)

Statistical parameters	Fresh biomass			Dry biomass		
	Year			Year		
	2010	2011	2013	2010	2011	2013
Average	126.20	145.07	578.80	75.12	89.88	299.152
Standard deviation	64.982	28.69	364.869	39.870	17.73	178.727
Coefficient of variation (%)	51.491	19.78	63.67	53.054	19.73	59.745

6. Ultrastructural features of leaf.

***Salix alba* L.** Leaf palisade parenchyma of the shoots (Control), present two regions: near upper epidermis, 2-3 cells layer full with salicylic acid (SA), followed by 2-3 cell layers with many chloroplast parietal disposed, with starch grains. Cells are rich in cytoplasm and cellular organelles: mitochondria, chloroplasts, dyctiosomes. In the palisade parenchyma, the cells are full with lipid drops. The chloroplasts with well developed grana and numerous plastoglobuls, are in active synthesis (being present 4-5 starch grains), and some chloroplasts are transformed in amyloplasts. Lacuna parenchyma is performed by 4-5 cell layers, chloroplasts with starch, SA, electron-dense particles, a/o. In leaf belonging to plants cultivated on ash are synthesized anthocyanin granules, accumulated in epidermis cell vacuole [fig.2 a, arrow], or in parenchyma cells. Anthocyanins (flavonoids) protect the cells from the damage "high-light" by absorbing blue-green and ultraviolet light (high-light stress) (Diaz et al., 1990, Trojak and Skowron 2017). Their synthesis takes place in chloroplast, the molecules being arranged on the thylakoid surface, later on being accumulated in cell vacuole together with other structures. The anthocyanin particles presence stops the toxic action of different substances accumulated in cells (Corneanu et al., 2014). In epidermic and parenchyma cells there are exogenous particles [fig. 2a] originating from environment. Is visible their penetration through cell wall. In some areas from cytoplasm at the endoplasmic reticulum, near chloroplast [fig. 2b, arrow), take place the SA synthesis. This is compacted under amorphous mass shape, and is accumulated in cells from lacuna parenchyma and lower epidermis [fig. 2d]. Also, in palisade parenchyma cells take place the synthesis of other bioactive substances (BAS) probably of lipid nature [fig. 2c]. Ferritin, is a metal-protein of 450 kDal, present in all organisms, rich in iron. The ferritin is associated with redox reactions, protecting the cell against the toxic effects of free iron, serving as a primary antioxidant (Goto et al, 2001). There is synthesized in chloroplast in structures of “crystal like bodies” (Harrison and Arosio 1996), as well as in mitochondria at the crysta level in *Typha latifolia* (Corneanu et al. 2014). In *S. alba* was observed in chloroplast [fig. 2f], being involved in antistress processes.

***Salix viminalis* L.** The leaves from the Control, had normal ultrastructural features [fig. 3a]. This species is a hyperaccumulator for some heavy metals, like Cu, Ni, Cd, Zn (Borišev et al., 2009; Cosio et al, 2006; Marmiroli et al., 2011, a/o). The different exogenous particles, that penetrated the leaf were observed on tonoplast surface and in vacuole (Fig.3e), in plants cultivated on ash. In mitochondria from the leaf palisade parenchyma take place ferritin synthesis, as well as in chloroplast, where are also synthesized different BAS. The aging organelles are transformed in myelinic structures (red arrow, fig. 3b), present in vacuole. Single or together in vacuole are present also multivesicular bodies (MVB-s; arrow), responsible of membranes, implied in the exocytosis processes. The membranes formed by MVB, are implied in coating of the starch granules released from chloroplast (Fig. 3 b). The MVB are present also in the ligneous vessels from circulating system (Fig. 3c). The endodermis cells from central cylinder, situated near conducting vessels, are functioning as deposit cells (Fig. 3e). The cellular residues and exogenous matter accumulated in deposit cells (situated near conducting vessels) was described previously in *Thypha latipholia* (Corneanu et al., 2014) or in *Phragmites australis* (Corneanu et al., 2018). The leaf cells are in an intense metabolic activity. Thus, the cells from the fundamental parenchyma from central cylinder, are in metabolic activity, some cells in mitotic activity (prophase, pro-metaphase) or in endomitosis (Figs. 3d). The intense metabolic activity of the cells, is underlined through different synthesis activities: ferritin synthesis in mitochondria (Fig. 3f) and chloroplast; lipid droplets in chloroplast, a/o). Similar results were obtained by Hakmaoui et al. 2007 in *S. purpurea*.

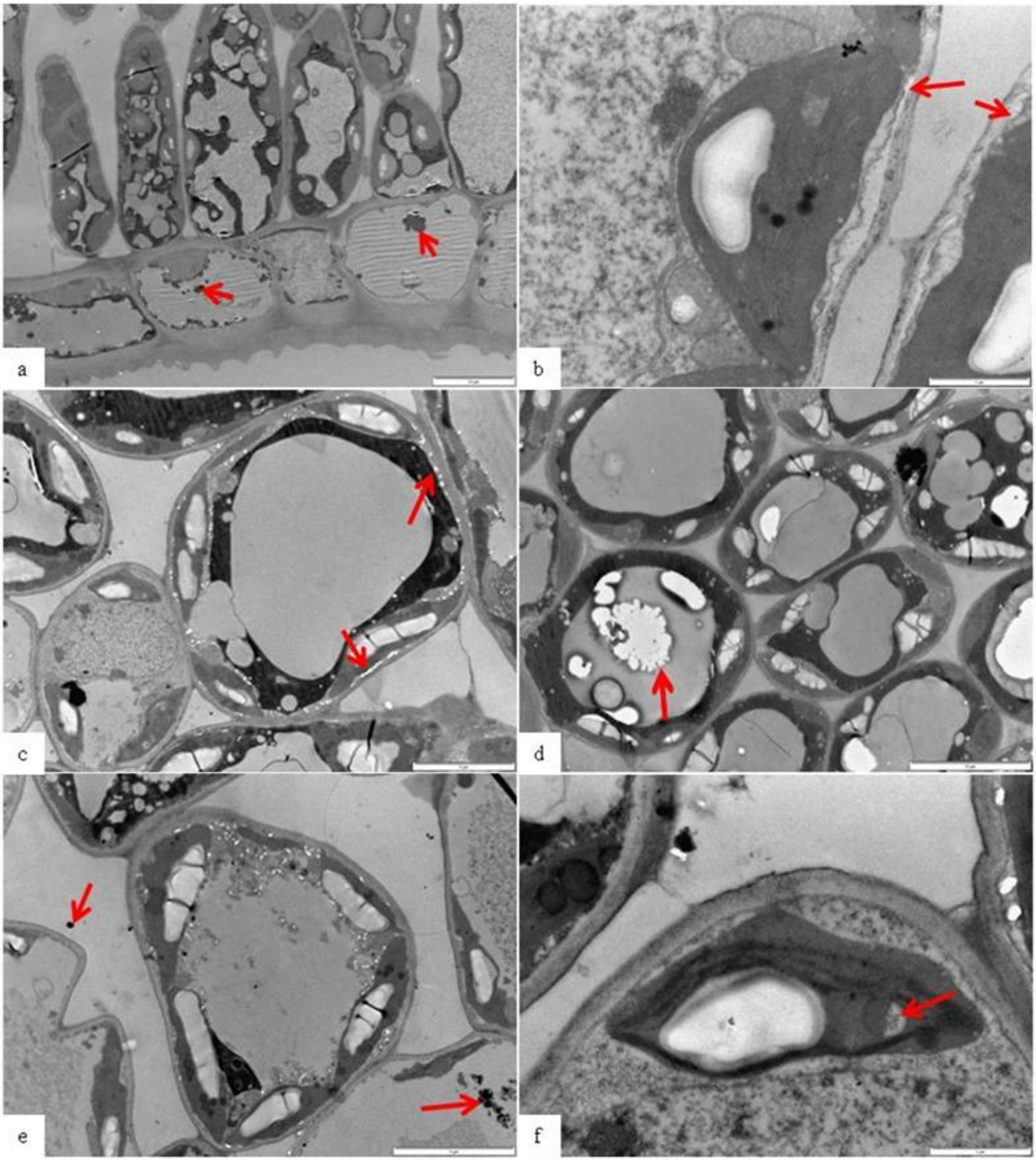

Fig.2 *Salix alba* leaf cells features.
a - upper epidermis and palisade parenchyma ; anthocyanin granules (arrow) in epidermis vacuole (barr 10 µm); b – SA synthesis (arrow) in an area near chloroplast and endoplasmic reticulum (barr 1 µm); c - palysade parenchima cell with salycillic acid synthesis in cytoplasm, near chloroplast, and lipid drops in chloroplast (arrow) (barr 5 µm); d – amorphous blocks of salycillic acid (arrow) in parenchyma cell (barr 10 µm); e – anthocyanin granules in cell vacuole and in intercellulare space (arrow) (barr 5 µm); f – ferritin synthesis in „like-crystalloid body" from chloroplast (arrow) (barr 1 µm).

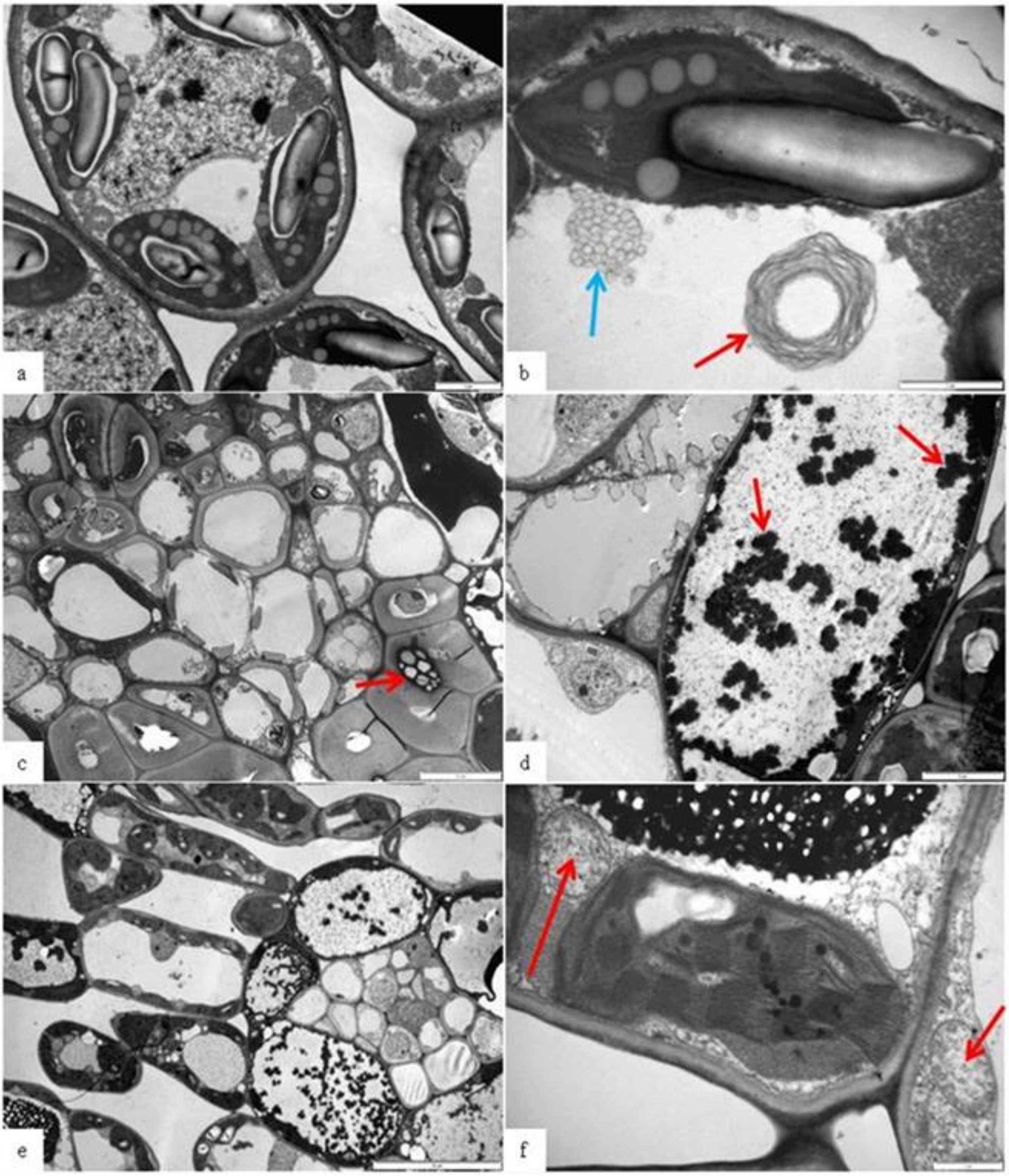

Fig. 3 *Salix viminalis* leaf cells features.
a - palisade parenchyma cell with normal features (barr 2 µm); b – vacuole with multivesicular body's (MVB's -blue arrow) and a mielinic structure (red arrow); a starch granule released by chloroplast (barr 1 µm) ; c – MVB in ligneous cell (barr 10 µm); d – endomitosis (prometaphase) in a fundamental parenchyma cell (barr 1 µm); e – endodermis cell function as „deposit cell" (barr 20 µm); f – ferritin synthesis on the crista level in mitochondria (arrow) (barr 1 µm).

CONCLUSIONS

- Soil analysis revealed the presence of natural radionuclides, belonging from ash and coal dust, as well as of Cs-137 (artificial radionuclide), of Chernobal provenance. During experiment (4 years), the radionuclides activity (Bq/kg soil), has been decreased, on one hand due to its half–life and on the other hand due to the phytoremediatory species, that extracted pollutants elements from soil.
- In term of willow survival, best results have been recorded for *S. viminalis* compared with *S. alba;* differences have been observed between white willow whips and cuttings culture, the superiority of whips has been highlighted.
- Exogenous particles from environment which penetrate leaf tissue, are spreaded through plasmodesmata and conducting vessels system. Exogenous matter and cellular debris are accumulated in endodermic cells (which function as "deposit cells"), then being removed through exocytosis.
- The presence of a higher amount of radionuclides and heavy metals stimulate the metabolic activity of the leaf parenchymatic cells (active mitotic divisions, ferritin synthesis in mitochondria and chloroplasts, anthocyan and SA synthesis).
- The ultrastructural features of the mature leaves from *S. alba* and *S. viminalis*, underlined their resistance and adaptation at a great amount of heavy metals and radionuclide's activity by: (i) vacuole compartmentation; (ii) multivesicular bodies (MVB's) present in vacuole; (iv) ferritin synthesis in mitochondria, near crysta and like-crystaloid area in chloroplast; (v) SA and other BAS synthesis in cytoplasm; (vi) deposit cells for accumulation of different matter.

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