Arch. Biol. Sci., Belgrade, 65 (4), 1651-1667, 2013 DOI:10.2298/ABS1304651G

ECOPHYSIOLOGICAL AND BIOCHEMICAL TRAITS OF THREE HERBACEOUS PLANTS GROWING ON THE DISPOSED COAL COMBUSTION FLY ASH OF DIFFERENT WEATHERING STAGE

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Abstract - The ecophysiological and biochemical traits of *Calamagrostis epigejos* (Roth.) *Festuca rubra* L. and *Oenothera biennis* L. growing on two fly ash lagoons of different weathering stage (L1-3 years and L2–11 years) of the "Nikola Tesla-A" thermoelectric plant (Obrenovac, Serbia) were studied. Species-dependent variations were observed at the L1 lagoon; the greatest vitality (Fv/Fm and Fm/Fo) followed by higher photopigment and total phenolic contents were measured in *O. biennis* in relation to *C. epigejos* ($p<0.001$) and *F. rubra* ($p<0.001$). At the L2 site, higher vitality was found in *O. biennis* ($p<0.001$) and *F. rubra* ($p<0.01$) compared to *C. epigejos*. *O. biennis* had the highest photosynthetic capacity. The results obtained in this study indicate that all examined species maintained a level of photosynthesis that allowed them to survive and grow under the stressful conditions in ash lagoons, albeit with lower than optimal success.

Key words: Fly ash, trace elements, multiple stress, adaptations, chlorophyll fluorescence, photopigments, phenolics

INTRODUCTION

Environmental disturbances resulting from human activities, such as mining and disposal of coal combustion residues (CCRs), greatly modify the dynamics and functioning of natural ecosystems. The cessation of such disturbances generally leads to long-term natural reconstitution of the disturbed ecosystems (Holl, 2002); however, for major disturbances such as soil destruction, the return to a former natural ecosystem is very difficult or even hopeless (Retana et al., 2002). Coal-based thermoelectric plants are the major source of power generation in many countries. The annual worldwide production of fly ash that originates from power stations is estimated to be 550 Mt, with more than 55 Mt produced in European Union countries (ACCA, 2001). In Serbia, eight thermoelectric plants using low-calorie lignite, with a total installed capacity of 5171MW, produce about 5.5 Mt of ash per year, and the "Nikola Tesla-A" power plant in Obrenovac produces 3.6 Mt of ash (EPS, Technical Report 2010), which is disposed on lagoons that occupy 400 ha of arable land.

The disposal and management of coal combustion residues, especially fly ash, remains a major problem to the environment. Ash disposal sites contaminate surface and ground water, degrade agricultural land and have an adverse impact on flora, fauna and human health (Carlson and Adriano, 1993; Tsadilas et al., 2006; Haynes, 2009).

Coal fly and bottom ash are an amorphous mixture of ferroaluminosilicate minerals (Carlson and Adriano, 1993). Physically, fly ash occurs as very fine particles, which can aggregate into spherical parti-

cles (0.01-100 μm size), and these particles can be entrapped into large spheres (Jala and Goyal, 2006). Fly ash particles are hollow, empty spheres (cenospheres) filled with smaller amorphous particles and crystals (plerospheres), and they have high concentrations of macro- and micronutrients (Elseewi et al., 1980). Fine ash particles are subjected to wind erosion which can be a major source of dust in the surrounding area, and, when disposed, cause a serious problem to human and animal health (Page et al., 1979) as well as normal functioning of higher plants (Gupta et al., 2002; Pavlović et al., 2004). Chemically, the pH of fly ash varies from 4.5 to 12, depending mainly on the S content of the parent coal (Adriano and Weber, 2001), with 90-99% of fly ash composed of Si, Al, Fe, Ca, Mg and K (Carlson and Adriano, 1993). Fly ash also contains many essential elements like S, B, Ca, Mg, Fe, Cu, Zn, Mn and P, along with toxic elements, such as As, Ni, Cr, Cd, Hg, Mo (Vassilev and Vassileva, 2005). Numerous studies have shown that coal fly ash typically has high concentrations of toxic elements (Adriano et al., 1980; Carlson and Adriano 1991; Dwivedi et al., 2008).

A vegetative cover is a remedial technique utilized on coal fly ash disposal sites for the stabilization and the physical and chemical immobilization of toxic elements. The use of plants as vegetation cover for the phytostabilization of fly ash disposal sites contaminated by trace elements has considerable potential, although it is an exceptionally slow process due to the unfavorable physical and chemical properties of the ash and the extreme microclimatic conditions at the deposit sites, which are deleterious to plant survival and growth (Townsend and Gillham, 1975; Carlson and Adriano 1993; Pavlović et al., 2004). The selection of plant species is an important factor in determining the success of revegetation, i.e. the restoration of fly ash disposal sites. Many herbaceous plants, primarily grasses that exhibit rapid growth, are moderately resistant to environmental stress, and are therefore often used as cover crops in environmental restoration and remediation projects. A range of surface amelioration treatments for ash lagoons was examined to investigate their ability to overcome the chemical and physical limitations of fly ash for plant growth (Cheung et al., 2000). Many herbaceous plants, for example *Agropyron elongatum* (Host) Beauv., *Festuca arundinacea* L. and *Melilotus officinalis* (L.) Lam., have been found to grow better than many tree species (Mulhern et al., 1989). Likewise, species such as *Mesembryanthemum nodiflorum* L., *Enchylaena tomentosa* R.Br., *Halosarcia halocnemoides* (Nees) Paul G. Wilson, and *H. pergranulata* (P. G. Wilson) are suited for use in revegetation (Carlson and Adriano, 1991; Jusaitis and Pillman, 1997). Due to the large differences in species response to fly ash, more plant species should be tested when selecting the species for restoration of fly ash disposal sites (Cheung et al., 2000).

Revegetation of coal fly ash disposal sites of the "Nikola Tesla-A" power plant is achieved by planting different grass, legume, shrub and tree species (Pavlović et al., 2004; Mitrović et al., 2008). On the lagoons, plants are exposed to multiple stresses due to the synergistic effects of extremely high temperatures, excessive irradiance, lack of water and nutrients, as well as the toxic effects of trace elements (Pavlović et al., 2004; Djurdjević et al., 2006, Mitrović et al., 2008). Stressful conditions result in changes in the metabolic activity of plants and adversely affect their basic physiological (photosynthesis, respiration, water balance) and biochemical (enzyme activity, production of pigments and secondary metabolites) processes (Larcher, 1995; Reid et al., 2004; Moreno-Jiménez et al. 2009; Gajić et al., 2009; Kostić et al. 2012). Namely, toxic elements from ash can inhibit photosynthesis at several physiological levels: pigments, structure and function of chloroplasts (Guidi et al., 2011; Paull et al., 1992; Reid et al., 2004). Long-term exposure to toxic metals affects the synthesis of chlorophyll, and thus directly affects the formation of chloroplasts in young leaves and indirectly inhibits photosynthesis. Research on the effects of heavy metals has shown the great sensitivity of photosystem II (PSII) because they directly affect chlorophyll fluorescence through the inhibition of chlorophyll synthesis and destruction of chloroplast membrane. Indirectly, heavy metals inhibit other physiological processes that in turn affect photosynthesis by changing the amount of

light energy needed for photosynthesis and energy release processes.

Therefore, the objectives of this present study were: a) to determine differences in the physical-chemical properties of the ash of different weathering stages (L1 – 3-year-old lagoon and L2 – 11-year-old lagoon) in order to identify factors which affect plant function; b) to determine the toxic effects of ash on photosynthetic efficiency, concentration of photopigments and phenolic compounds as key parameters in tolerance to the stressful conditions at ash lagoons of different weathering stage, and c) to provide a comparative analysis of the vitality level of the examined species in order to assess their potential for the establishment of an effective vegetative cover on ash disposal sites. Analysis included the following species: sown *Festuca rubra* L. that was used in the grass-legume mixture for revegetation; *Calamagrostis epigeios* L. (Roth.) and *Oenothera biennis* L. that spontaneously colonized the fly ash lagoons. These findings may contribute to determining the adaptive potential of plants that are capable of colonizing ash disposal sites, which could be important for understanding the mechanisms that contribute to stress tolerance in plants, as well as creating strategies for ecological restoration of ash disposal sites.

MATERIALS AND METHODS

Study site

The thermoelectric plant "Nikola Tesla-A" (TENT-A) is located in the municipality of Obrenovac (Serbia) (Fig. 1). TENT-A combusts low-caloric lignite coal and produces on average 3.6x106 Mt of fly and bottom ash. The ash that is produced is aluminosilicate (approximately 80%) with a significant proportion of Fe, Ca, Mg, K, and Ti oxides. The ash also contains As, B, Ba, Cr, Cu, Cl, F, Ga, Hg, Li, Mn, Mo, Nb, Ni, Pb, Rb, Sc, Sr, V, Zn, Zr and Y (Table 1). The ash is mixed with water and hydraulically transported and deposited on the open lagoons of ash and clay, which occupy an area of 400 ha located on the right bank of the Sava River, and are surrounded by villages and agricultural fields. Ash is disposed on three lagoons, one of which is always active (L0); the other two are temporary inactive lagoons that serve for technical consolidation of ash and drainage, but also in case of accidents or cessation of ash discharge (Fig. 1E). Research was performed on the two inactive lagoons: the first lagoon has been inactive for three years (L1); the second lagoon for eleven years (L2) and a third site was a control site (CS), located on the banks of the River Kolubara, 3 km from the ash deposit site (Fig. 1C, E).

Scanning electron microscopy (SEM) analysis of ash particles

The shape, size and chemical composition of ash particles were determined by SEM microscopy (JEOL, JSM-6460LV), using an energy-dispersive X-ray (EDS) Oxford, INCA microanalytical system and the necessary software for point microanalysis and chemical mapping of the surface under examination. The samples were coated with carbon, using a BALTEC SCD005, Sputter Coater.

Chemical analysis of soil and fly ash

The actual pH was measured potentiometrically with a glass membrane electrode by suspending 10 g of soil and ash in 25 ml H_2O, (n=3). The soluble salt content in the fly ash and soil was measured by assessing the electrical conductivity (EC) of an extract of soil (ash):water (distilled) = 1:5, at a depth of 0-20 cm, with 3 replicates. EC was expressed in dS m^{-1}.

Concentrations of As, B, Mo, Se, Cu, Zn and Mn were measured in the soil from the control site (CS) and in the fly ash from L1 and L2 lagoons (n=3). For trace element analysis, the soil and ash samples (0.5 g) were digested in a microwave (CEM MDS-2000) using 10 ml of concentrated HNO_3. Concentrations of As, Mo, Se, Cu, Zn and Mn were determined through atomic absorption spectrophotometry (Pye Unicam SP9), using a sodium atomic absorption standard solution (Sigma Co.). The analytical procedure was validated using standard reference materials: ash (coal ash CRM 252-BS1) and soil

(grey soil CRM 054-2504-83), obtained by Spex CerpiPrep.Ltd. (Middlesex, UK). Boron concentrations were determined using the spectrophotometric method with the aid of curcumin (Wear, 1965). Concentrations were expressed in μg/g of the dry weight.

Chlorophyll fluorescence measurements

The chlorophyll fluorescence parameters (Fo, initial fluorescence; Fm, maximum fluorescence; Fv = Fm – Fo, variable fluorescence; $t_{1/2}$, half the time required to reach maximum fluorescence from Fo to Fm; photosynthetic efficiency Fv/Fm and ratio Fm/Fo) of intact leaves were measured using a portable Plant Stress Meter (BioMonitor S.C.I. AB, Sweden) according to method described by Krause and Weis (1991). Chlorophyll was excited for 2 s by actinic light with a photon flux density of 200 μmol $m^{-2}s^{-1}$. Before measuring the chlorophyll fluorescence, twenty leaves (n=20) from each site (CS. L1, L2) were adapted to the dark for approximately 30 min in order to maximize the oxidation of the primary quinone electron acceptor pool of PSII and to allow the full relaxation of any rapidly recovering fluorescence quenching. The ratio of variable to maximum fluorescence (Fv/Fm) was used as a measure of the photochemical efficiency of PSII and this ratio correlates with the number of functional PSII reaction centers.

Photosynthetic pigments measurements

One disc (1 cm diameter) per leaf was harvested from leaves from each site (CS, L1, L2), (n=5). Chlorophylls and total carotenoids were extracted with dimethyl sulfoxide (DMSO). The absorbance of extracts was measured at 663 nm, 645 nm and 480 nm with UV-visible recording spectrophotometer (Shimadzu UV-160). The equations of Arnon (1949) were used to calculate chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and chlorophyll *a+b* (Chl *a+b*). The ratio chlorophyll *a/b* (Chl *a/b*) was also determined. Total carotenoids (Tot Carot) were calculated according to Wellburn (1994). Chlorophyll and carotenoid concentrations in the leaves were expressed in mg per gram of the dry leaf weight (mg/g d.w.).

*Phenolics **measurements***

Phenolics were extracted from 5 x 2.0 g of dry plant material with 80% (v/v) boiling aqueous methanol solution followed by ethyl acetate from each site (CS, L1, L2), (n=5). After filtration, pooled methanol and ethyl acetate extracts were evaporated with a rotary evaporator under N_2, the residue was dissolved in 10 ml of distilled water adjusted to 2.0 pH with 2N HCl and phenolics were transferred to ethyl acetate. The ethyl acetate phase was dehydrated with anhydrous Na_2CO_3 and evaporated to dryness in a stream of nitrogen, and the residue dissolved in 80% (v/v) MeOH. Through this procedure, free phenolics (Free Ph, highly soluble fractions) were prepared. Bound phenolics (Bound Ph, fractions of phenols that are either esters, or bound to the polysaccharide matrices of the cell wall or polymerized into lignin) were prepared by boiling the insoluble residue that remained after the first procedure in 2N HCl for 60 min and transferring to ethyl acetate. The ethyl acetate phase was dehydrated with anhydrous Na_2CO_3 and evaporated to dryness in a stream of nitrogen, and the residue dissolved in 80% (v/v) methanol solution. Total phenolics (Tot Ph, free and bound phenolics) were determined according to Djurdjević et al. (2007). The absorbance of free and bound phenolics was measured at 660 nm spectrophotometrically (Shimadzu UV 160 spectrophotometer) according to Feldman and Hanks (1968), with a sensitivity of 0.05 $\mu g g^{-1}$ d.w. A standard curve was constructed with different concentrations of ferulic acid (Serva, Germany).

Statistical analysis

One-way analyses of variance (ANOVA) were performed to test the differences in the pH, EC values and trace-metal content on the control site (CS) and L1, L2 fly ash lagoons, as well as the differences between the among the plant species in chlorophyll fluorescence (Fv/Fm and Fm/Fo), photopigments (Chl *a*, Chl *b*, Chl *a+b*, Chl *a/b* and Tot Carot) and phenolics (Free Ph, Bound Ph and Tot Ph). The Canonical Discriminant Analysis (CDA) was used to establish

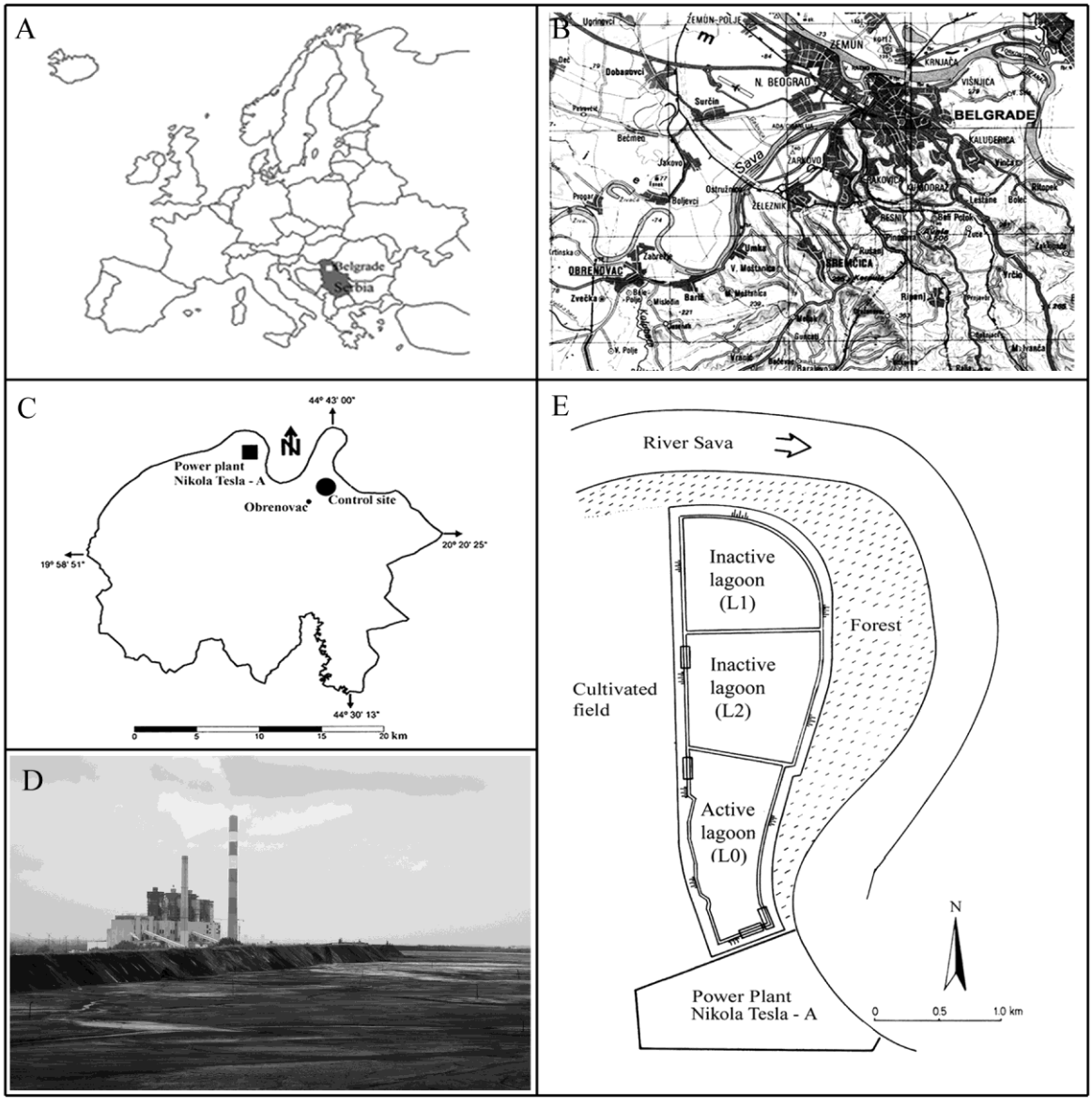

Fig.1. Municipality of Obrenovac (Serbia) (A, B); Study sites: power plant Nikola Tesla – A and the banks of the River Kolubara (control site) (C, D); Plan of the Nikola Tesla-A fly ash lagoons (L0-active lagoon, L1 and L2-inactive lagoons) (E).

possible connections among groups of samples from different sites.

RESULTS

Fly ash particles

Spectral analysis of the chemical composition of the ash particles confirmed the presence of major elements such as Si, Al, Ca, Fe, O, K, Mg and Ti, as presented in Table 1. Analysis also showed a higher fraction of coarse-grained particles of fly ash (63-100 μm and 100-500 μm) and less numerous were fine-grained fractions (<63 μm and <20 μm) with hollow, empty spheres (cenospheres) filled with amorphous particles (plerospheres) (Fig. 2A). Irregular, angular, massive,

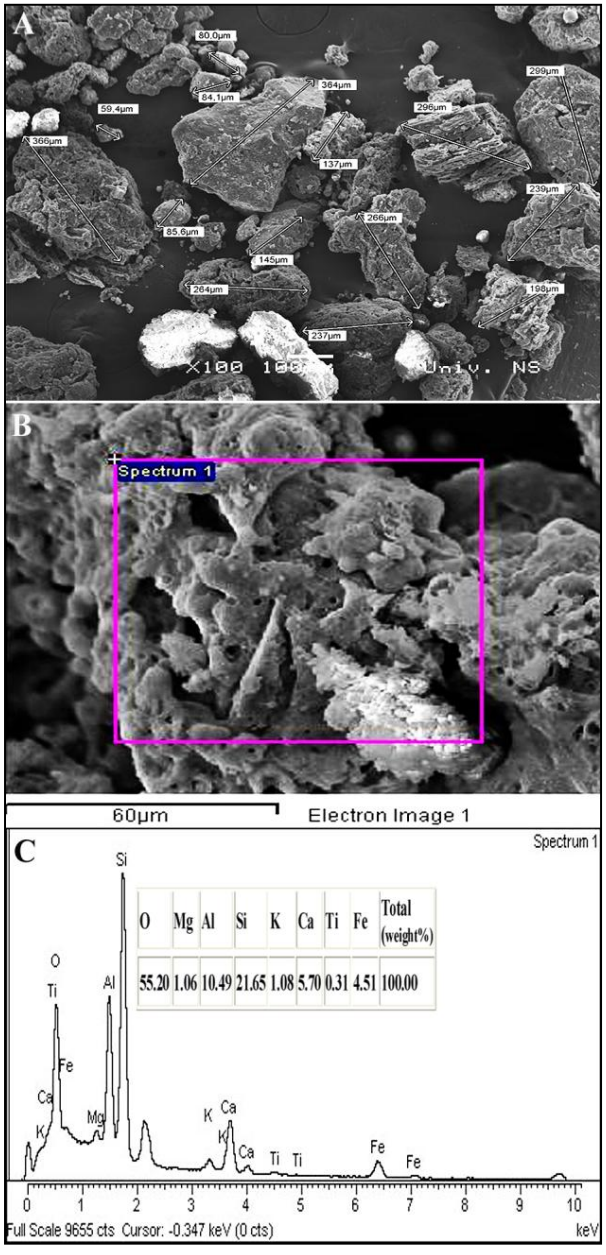

Fig. 2. SEM micrograph of the fly ash particles (A, B), and spectral analysis of the chemical composition of the fly ash particles (C).

hollow, porous, spherical particles and amorphous, vesicular aggregates were also observed (Fig. 2B). SEM analysis of chemical composition of agglomerates showed the presence of elements in the following order: O>Si>Al>Ca>Fe>K>Mg>Ti (Fig. 2C).

Chemical composition of the fly ash and soil

The pH and EC values at each site are given in Table 2. The pH of bare ash from the active lagoon L0 was higher than of soil from CS ($p<0.01$) and ash from L1 ($p<0.01$) and L2 ($p<0.01$). There were no differences in pH values between the control site and the ash lagoons. Electrical conductivity (EC) was higher in L0 in relation to the CS ($p<0.001$), L1 ($p<0.001$) and L2 ($p<0.001$), while there were no differences between CS and L1. At the L2 site, EC had lower values in relation to CS ($p<0.01$), L0 ($p<0.001$) and L1 ($p<0.01$).

Trace element concentrations in the soil and fly ash are given in Table 3. The As concentrations in L1 ($p<0.01$) and L2 ($p<0.001$) sites were significantly higher than at the control site (CS). The As concentrations at L2 were higher than in L1 ($p<0.001$), and far above the normal range for soil (4.4-9.3 µg/g, Kabata-Pendias and Pendias, 2001).

Concentrations of B at the L1 ($p<0.001$) and L2 ($p<0.001$) ash deposit lagoons were higher than at the control site. Boron concentrations at the CS were below the normal range for soils (35 µg/g, Kabata-Pendias and Pendias, 2001), whereas at L1 it was higher than at L2 ($p<0.001$), and far above the normal range for soils.

The Se content at CS was higher than at the L1 and L2 sites. Selenium concentration at the CS was above the normal range for soils (0.33 µg/g, Kabata-Pendias and Pendias, 2001), whereas at L1 and L2 they were below the normal range for soils. The Mo content at L1 ($p<0.001$) and L2 ($p<0.001$) was higher than at the control site (CS). Mo concentration at the CS was below the normal range for soils (1.80 µg/g, Kabata-Pendias and Pendias, 2001). At the L2 site, the Mo content was higher than at L1 ($p<0.01$), and above the normal range for soils.

The Cu content at the L1 and L2 sites was higher than at the CS site ($p<0.001$, $p<0.001$). Cooper concentration at the CS was higher than the normal range for soils (13-24 µg/g, Kabata-Pendias and Pendias, 2001). At the L2 site, the Cu content was higher

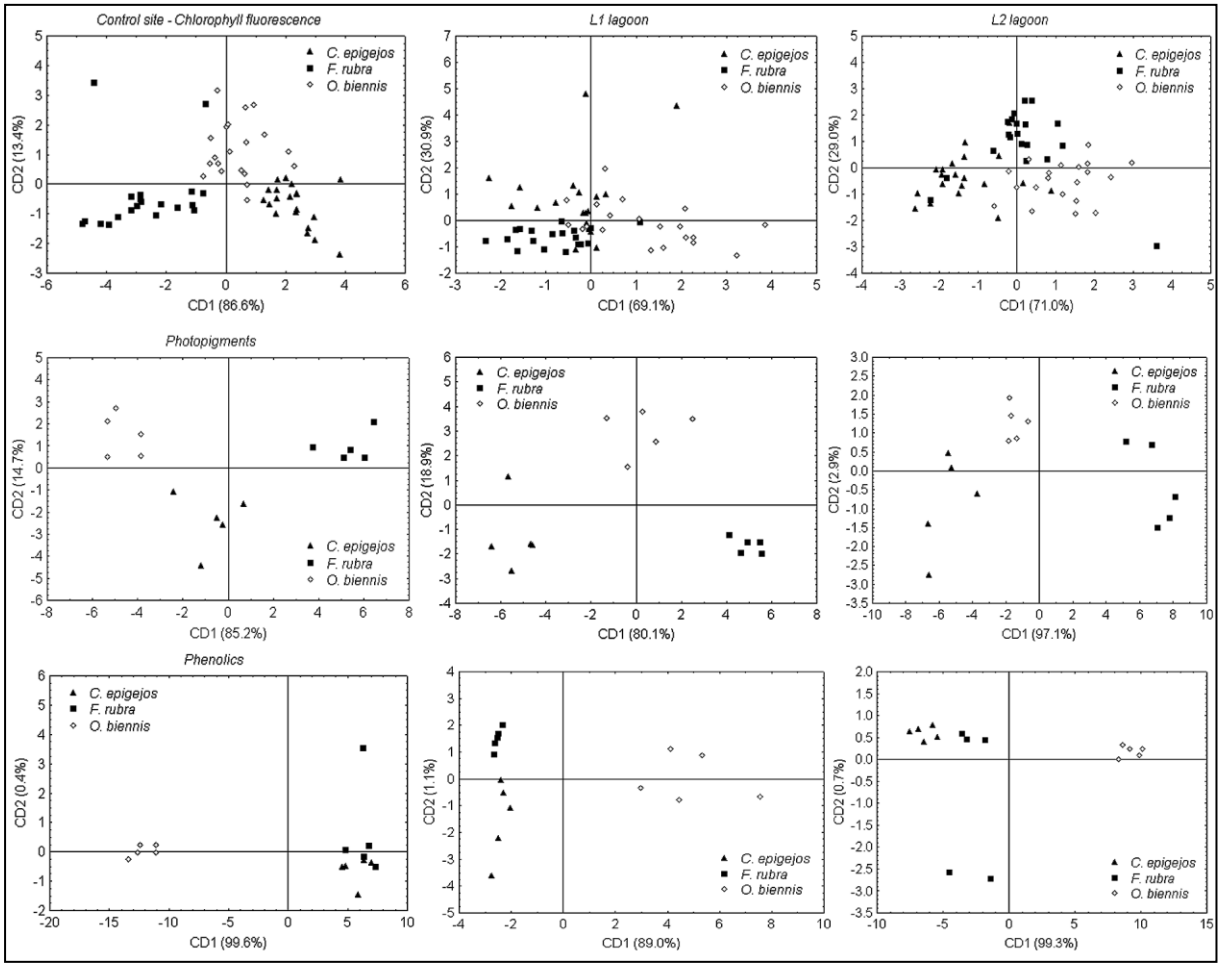

Fig. 3. Canonical Discriminant Analysis (CDA) based on the variations of chlorophyll fluorescence parameters (Fo, Fm, Fv, $t_{1/2}$, Fv/Fm and Fm/Fo), photopigments (Chl *a*, Chl *b*, Chl *a*+*b*, Chl *a/b* and Tot Carot) and phenolics (Free Ph, Bound Ph and Tot Ph) between the *C. epigejos*, *F. rubra* and *O. biennis* at the control site (CS), L1 and L2 fly ash lagoons.

than at L1 ($p<0.001$), and above the normal range for soils. The Zn content at the CS was lower than at L1 ($p<0.05$) and higher than at L2 ($p<0.05$). Zn concentrations at all three sites were below the average concentration in soils (64 μg/g, Kabata-Pendias and Pendias, 2001). At L1, the Zn content was higher than at L2 ($p<0.01$). The Mn concentrations measured at L1 and L2 were significantly lower than at the control site (CS) ($p<0.001$; $p<0.001$). Manganese concentration at the CS was higher than the average concentration in soils (437 μg/g, Kabata-Pendias and Pendias, 2001). At L2, the Mn content was lower than at L1 ($p<0.001$), and within the normal range for soils.

Chlorophyll fluorescence

Photosynthetic efficiency (vitality) of the examined species is given in Table 4. At the control site, the greatest vitality (Fv/Fm and Fm/Fo) was noted for *F. rubra* in relation to the *C. epigejos* ($p<0.001$, $p<0.001$) and *O. biennis* ($p<0.001$, $p<0.001$). At the L1 fly ash deposit lagoon, the greatest vitality (Fv/Fm and Fm/Fo) was noted for *O. biennis* in relation to *C. epigejos* ($p<0.05$, $p<0.01$) and *F. rubra* ($p<0.01$, $p<0.01$). At L2, the mean values of Fv/Fm and Fm/Fo in *F. rubra* ($p<0.01$) and *O. biennis* ($p<0.001$) were higher in relation to *C. epigejos*.

Table 1. Chemical properties of fly ash from the "Nikola Tesla-A" thermoelectric power plant.

Silicate analysis of ash from electrostatic precipitors[a] (%)		Elements	Unweathered ash[b] (μg/g)	Weathered ash[b] (μg/g)
SiO_2	54.21	As	130	119
Al_2O_3	24.98	B	620	516
Fe_2O_3	6.13	Ba	423	392
CaO	5.89	Cr	342	392
MgO	3.15	Cu	102	110
Na_2O	0.29	Cl	<10	<10
K_2O	1.12	F	<1	<1
TiO_2	0.69	Ga	53	40
P_2O_5	0.07	Hg	<0.01	<0.01
SO_3	0.96	Li	55	99
		Mn	293	287
		Mo	<5	<5
		Nb	18	14
		Ni	123	151
		Pb	38	36
		Rb	210	150
		Sc	<1	<1
		Sr	177	190
		V	99	116
		Zn	95	45
		Zr	76	96
		Y	22	30

Source: [a]Vinča Institute for Nuclear Sciences; [b]Holding Institute of General and Physical Chemistry.

Table 2. Electrical conductivity (EC) and pH values of ash and soil at the control site (CS), L0, L1 and L2 fly ash sites.

	EC (dS m^{-1})					pH				
Parameter	M (SD)	CS	L0	L1	L2	M (SD)	CS	L0	L1	L2
CS	0.184 (0.007)	-	***	ns	**	7.54 (0.200)	-	**	ns	ns
L0	0.351 (0.037)	***	-	***	***	8.03 (0.008)	**	-	**	**
L1	0.203 (0.027)	ns	***	-	**	7.78 (0.130)	ns	**	-	ns
L2	0.153 (0.007)	**	***	**	-	7.72 (0.080)	ns	**	ns	-

ANOVA, n=3, values are means (SD), ** p<0.01; *** p<0.001, ns = not significant.

Canonical discriminant analysis (CDA) based on chlorophyll fluorescence parameters (Fo, Fm, Fv, $t_{1/2}$, Fv/Fm and Fm/Fo) showed differences between the examined species at each of the sites (Fig. 3). At the control site (CS), the CD1 axis clearly separates *F. rubra* from *C. epigejos* and this discriminant function explains 86.6% variability. At lagoon L1, CD1 explains 69.1% of variability and separates *O. bien-*

Table 3. Trace metal content in soil from the control site (CS), and in fly ash from L1 and L2 lagoons (μg/g).

Site	As	B	Se	Mo	Cu	Zn	Mn
CS	3.60 (0.360)	22.00 (0.757)	0.53 (0.013)	1.43 (0.045)	63.44 (0.437)	43.24 (0.440)	670.76 (8.103)
L1	84.46 (1.560) a***	450.08 (1.746) a***	<0.1	9.53 (0.665) a***	126.30 (1.555) a***	45.06 (1.204) a*	273.20 (3.928) a***
L2	121.08 (0.485) a***c***	390.26 (2.602) b*** c***	<0.1	12.31(0.292) b*** c**	173.23 (2.236) b*** c***	40.34 (1.825) b*c**	222.18 (3.860) b***c***

ANOVA, n=3, values are means (SD), compared are: CS – L1 (a); CS – L2 (b); L1 – L2 (c); level of significance: *p<0.05; ** p<0.01; *** p<0.001.

Table 4. Chlorophyll fluorescence, photopigments and phenolic content (mg/g) of the examined species at different sites.

		Control site (CS)			L1 – 4 years old lagoon				L2 – 12 years old lagoon			
Species	M (SD)	*C. epigejos*	*F. rubra*	*O. biennis*	M (SD)	*C. epigejos*	*F. rubra*	*O. biennis*	M (SD)	*C. epigejos*	*F. rubra*	*O. biennis*
						Fv/Fm						
C. epigejos	0.664 (0.030)	-	***	**	0.594 (0.051)	-	ns	*	0.590 (0.070)	-	**	***
F. rubra	0.800 (0.023)	***	-	***	0.588 (0.045)	ns	-	*	0.660 (0.057)	**	-	ns
O. biennis	0.707 (0.052)	**	***	-	0.629 (0.091)	*	*	-	0.683 (0.074)	***	ns	-
						Fm/Fo						
C. epigejos	3.0 (0.267)	-	***	***	2.5 (0.351)	-	ns	**	2.5 (0.426)	-	**	***
F. rubra	5.0 (0.582)	***	-	***	2.5 (0.296)	ns	-	**	3.0 (0.507)	**	-	ns
O. biennis	3.5 (0.530)	***	***	-	2.9 (0.738)	**	**	-	3.3 (0.759)	***	ns	-
						Chl a						
C. epigejos	3.5 (0.482)	-	**	ns	2.1 (0.518)	-	ns	***	2.3 (0.496)	-	*	ns
F. rubra	5.3 (1.049)	**	-	**	2.4 (0.089)	ns	-	***	2.9 (0.279)	*	-	**
O. biennis	3.5 (0.386)	ns	**	-	3.7 (0.253)	***	***	-	2.2 (0.256)	ns	**	-
						Chl b						
C. epigejos	1.3 (0.306)	-	***	ns	0.6 (0.150)	-	**	***	0.5 (0.170)	-	***	*
F. rubra	2.2 (0.122)	***	-	***	1.5 (0.418)	**	-	ns	1.8 (0.428)	***	-	***
O. biennis	1.1 (0.257)	ns	***	-	1.2 (0.110)	***	ns	-	0.7 (0.098)	*	***	-
						Chl a+b						
C. epigejos	4.8 (.760)	-	**	ns	2.7 (0.660)	-	**	***	2.9 (0.653)	-	***	ns
F. rubra	7.5 (1.141)	**	-	***	3.9 (0.495)	**	-	**	4.8 (0.532)	***	-	***
O. biennis	4.6 (0.623)	ns	***	-	4.9 (0.303)	***	**	-	2.9 (0.349)	ns	***	-

Table 4. *Continued*

Species	Control site (CS)				L1 – 4 years old lagoon				L2 – 12 years old lagoon			
	M (SD)	*C. epigejos*	*F. rubra*	*O. biennis*	M (SD)	*C. epigejos*	*F. rubra*	*O. biennis*	M (SD)	*C. epigejos*	*F. rubra*	*O. biennis*
Chl a/b												
C. epigejos	2.7 (0.325)	-	ns	*	3.7 (0.362)	-	***	**	4.5 (0.861)	-	***	**
F. rubra	2.3 (0.395)	ns	-	*	1.7 (0.367)	***	-	***	1.7 (0.382)	***	-	***
O. biennis	3.1 (0.357)	*	*	-	3.0 (0.298)	**	***	-	3.2 (0.17)	**	***	-
Tot Carot												
C. epigejos	1.1 (0.181)	-	**	ns	0.8 (0.156)	-	ns	**	0.9 (0.201)	-	ns	ns
F. rubra	1.6 (0.169)	**	-	**	0.9 (0.136)	ns	-	***	1.0 (0.130)	ns	-	**
O. biennis	1.3 (0.214)	ns	**	-	1.3 (0.069)	***	***	-	0.8 (0.080)	ns	**	-
Free Ph												
C. epigejos	11.6 (1.377)	-	ns	*	17.3 (5.090)	-	**	*	15.6 (4.436)	-	ns	*
F. rubra	9.6 (3.874)	ns	-	**	6.8 (1.467)	**	-	**	16.9 (2.088)	ns	-	*
O. biennis	14.6 (2.407)	*	**	-	11.0 (3.129)	*	**	-	21.8 (3.973)	*	*	-
Bound Ph												
C. epigejos	13.2 (2.378)	-	ns	***	19.6 (3.700)	-	*	***	25.8 (2.509)	-	**	***
F. rubra	12.9 (2.742)	ns	-	***	14.9 (2.209)	*	-	***	34.1 (4.224)	**	-	***
O. biennis	55.2 (2.261)	***	***	-	158.1 (33.074)	***	***	-	67.6 (2.016)	***	***	-
Tot Ph												
C. epigejos	24.8 (3.574)	-	ns	***	36.9 (3.708)	-	**	***	41.4 (6.113)	-	**	***
F. rubra	22.5 (2.258)	ns	-	***	21.7 (7.110)	**	-	***	51.0 (0.608)	**	-	***
O. biennis	69.8 (4.229)	***	***	-	169.1 (33.685)	***	***	-	89.4 (4.935)	***	***	-

ANOVA, values are means (SD), n=20 (Fv/Fm, Fm/Fo), n=5 (Chl *a*, Chl *b*, Chl *a+b*, Chl *a/b*, Tot Carot, Free Ph, Bound Ph, Tot Ph), Levels of significance: *p<0.05, **p<0.01, ***p<0.001, ns = not significant

nis from the other two species, whereas at L2 lagoon, CD1 is 71.0% and discriminates *C. epigejos* from *F. rubra* and *O. biennis*.

Photopigments

Concentrations of photopigments of the examined species are given in Table 4. At the control site, the highest Chl *a*, Chl *b*, Chl *a+b* and Tot Carot content was measured in *F. rubra* in relation to *C. epigejos* ($p<0.01$, $p<0.001$, $p<0.01$, $p<0.01$) and *O. biennis* ($p<0.01$, $p<0.001$, $p<0.001$, $p<0.01$). However, the highest Chl *a/b* ratio was found in *O. biennis* in relation to *C. epigejos* ($p<0.05$) and *F. rubra* ($p<0.05$).

At the L1 lagoon, the highest Chl *a*, Chl *a+b* and Tot Carot content were measured in *O. biennis* in relation to *C. epigejos* ($p<0.001$, $p<0.001$, $p<0.001$) and *F. rubra* ($p<0.001$, $p<0.01$, $p<0.001$). The lowest Chl *b* content and highest Chl *a/b* ratio were found in *C. epigejos* in relation to *F. rubra* ($p<0.01$, $p<0.001$) and *O. biennis* ($p<0.001$, $p<0.01$).

At the L2 lagoon, the highest Chl *a*, Chl *b*, Chl *a+b* and lowest Chl *a/b* ratio was found in *F. rubra* in comparison to *C. epigejos* ($p<0.05$, $p<0.001$, $p<0.001$, and $p<0.001$) and *O. biennis* ($p<0.01$, $p<0.001$, $p<0.001$ and $p<0.001$). However, a higher carotenoid content was measured in *F. rubra* than in *O. biennis* ($p<0.01$).

Canonical discriminant analysis (CDA) based on photopigments (Chl *a*, Chl *b*, Chl *a+b*, Chl *a/b* and Tot Carot) showed differences between the examined species at each of the sites (Fig. 3). At control site CS, the CD1 axis clearly separates *F. rubra* from *C. epigejos* and this discriminant function explains 85.2% variability. At L1 lagoon, CD1 explains 80.1% of variability and separates *F. rubra* from *C. epigejos*, whereas the CD1 of 97.1% clearly discriminates *F. rubra* from *F. rubra* and *O. biennis* at L2.

Phenolics

The phenolic contents in the examined species at each site are given in Table 4. At the control site, the highest Free Ph, Bound and Tot Ph content was measured in *O. biennis* in comparison to *C. epigejos* ($p<0.05$, $p<0.001$, $p<0.001$) and *F. rubra* ($p<0.01$, $p<0.001$, $p<0.001$).

At the L1 lagoon, the highest Free Ph content was measured in *C. epigejos* in relation to *F. rubra* ($p<0.01$) and *O. biennis* ($p<0.05$). The concentrations of Bound Ph and Tot Ph in *O. biennis* were higher in relation to *C. epigejos* ($p<0.001$, $p<0.001$) and *F. rubra* ($p<0.001$, $p<0.001$).

At the L2 lagoon, higher Free Ph, Bound and Tot Ph content were found in *O. biennis* in relation to *C. epigejos* ($p<0.05$, $p<0.001$, $p<0.001$) and *F. rubra* ($p<0.05$, $p<0.001$, $p<0.001$).

Canonical discriminant analysis (CDA) based on phenolic (Free Ph, Bound Ph and Tot Ph) revealed differences between the examined species at each of the site (Fig. 3). At the examined sites, the CDI axis clearly separates *O. biennis* from *F. rubra* and *C. epigejos*: at control site, CD1 is 99.6% of variability, at L1 lagoon CD1 is 89.0% of variability and at L2 lagoon CD1 is 99.3% of variability.

DISCUSSION

Fly ash particles

In this study, spectral analysis of the fly ash particles showed the presence of major elements such as Si, Al, Ca, Fe, O, K, Mg and Ti, which are usually covalently and ionically bound in organometallic compounds (Huggins et al., 1997). The concentrations of O, Si, Al, Fe and Ca indicate high-calcium Class C fly ash type, produced from the burning of lignite at the thermoelectric plant "Nikola Tesla-A". Analysis also showed a higher fraction of coarse-grained particles of ash with diameters ranging from 59.4 μm to 366.0 μm, while smaller particles were less numerous. Similar results were obtained by Vassilev et al. (2005) for fly and bottom ash produced in the Soma thermoelectric power station in Turkey, which indicates the similar properties of lignite coals on Balkan Peninsula. Fly ash particles can cause both physiological and morphological damage to plants. The deposition of ash particles on the leaves inhibits photosynthesis and transpiration processes because thick layers of fly ash interfere with the light required for photosynthesis and thereby reduce the photosynthetic rate. Leaves covered with fly ash absorb heat more effectively and, consequently, the increased leaf temperature results in increased transpiration rates (Gupta et al., 2002; Hirano et al., 1995; Naidoo and Chirkoot, 2004).

Chemical composition of the fly ash and soil

Coal lignite used in the thermal electric plant Nikola Tesla – A tends to be higher in Ca, thereby producing alkaline ashes. In this study, the pH indicated a moderate to sub-alkaline character of the fly ash

with the highest values measured in unweathered ash. Likewise, the highest electrical conductivity was measured in unweathered ash, indicating higher levels of Ca, Mg, Na and B soluble salt concentrations in relation to soil and weathered ash. The concentration of overall soluble salts in the ash is very low (in bare ash EC=0.351 dS m^{-1} with a tendency to be reduced in weathered ash). The growth of most plants, including agronomic crops, is adversely affected by EC values of ≥4 dS m^{-1} (Mass 1990); thus, our findings on the EC values of the weathered ash compared to the given range suggest that salts have no negative effects on vegetation because the weathering of ash results in substantial declines in soluble salt levels as they are progressively leached away (Adriano et al., 1980). Hence, the lower EC values at L2 compared to the L1 site indicate a continuous weathering process of fly ash over a period of several years that could be linked to soluble salts and B leaching, as well as formation of an environment that is more suitable for plant growth.

In the present study, the As concentrations in the fly ash lagoons were in an excessive range for soils due its increased extractability at higher pH levels (pH 7-9), because anionic As species have no free metal ions that would cause them to precipitate (Theis and Wirth, 1977). Therefore, the bioaccumulation of soluble arsenic forms could be toxic for plants (Kabata-Pendias and Pendias, 2001). The higher As concentrations in the L2 ash lagoon than in L1 could be a result of lower phosphate content. It is well known that phosphate and arsenate compete for the same sorption sites in media surrounding a plant and within the plant because both are taken up by the same transport system (Marschner, 1997). Wang et al. (2002) found that phosphate starvation resulted in a 2.5-fold increase in As net uptake. The available arsenic levels in media slowly decrease with time, although no data is available to predict how long would be required for the As to decrease to a background level (Walsh et al., 1977).

As with As, B concentrations in ash were in the excessive range. The higher B concentrations in both lagoons could be a result of the higher pH and EC of the ash. The high B content in L1 lagoon suggests its potential toxicity to plants (Purves and Mackenzie, 1974). The lower B content in L2 indicates its gradual decrease with time, as leaching occurs due to the process of weathering (Carlson and Adriano, 1993). Approximately 17-64 % of B is immediately soluble in water (James et al., 1982), but a further 2 to 3 years is required for the amount of B to decrease to a concentration which plants can tolerate (Carlson and Adriano, 1993).

In this study, Mo concentrations in the L1 and L2 lagoons were above the normal range for soils. Namely, Mo is readily mobilized in the alkaline reaction of the fly ash and the solubility as well as availability of Mo to plants is highly dependent on pH (Kabata-Pendias and Pendias, 2001). Molybdenum concentrations in the L1 lagoon were lower than in the L2 lagoon, suggesting possible interaction with other elements such as B (Kabata-Pendias and Pendias, 2001). Likewise, elevated sulfate in the rooting zone strongly depresses Mo uptake in medium with toxic levels of Mo, and also sulfate combined together with superphosphate reduces Mo uptake (Marschner, 1997; Kabata-Pendias and Pendias, 2001). The Se concentrations at both lagoons were lower than the average Se concentrations in soils (Kabata-Pendias and Pendias, 2001), which means that this element is not a potential threat to plants.

The higher Cu content in the L1 and L2 lagoons than at the CS indicate possible high Cu accumulation and potential toxicity to plants. Grupe and Kuntze (1988) observed that anthropogenic Cu oxide is more available to plants than that of pedogenic origin. Many antagonistic interactions of Cu with other elements, for example B, Mo, Se, Zn, Mn and P in a nutrient solution, as well as in the external root media and plant tissue, are commonly observed, and apparently related to their physiological mechanisms and tolerance (Kabata-Pendias and Pendias, 2001). Thus, high Cu content in the L1 and L2 lagoons may be due to high B and Mo content and low content of Se, Zn and Mn in the ash.

Zn concentrations were below the normal range for soils at all sites, which indicates a Zn deficiency in the soil and fly ash and possibly in plant tissues. Zn deficiency in the CS may be due to high Se, Cu and Mn contents in the soil (Kabata-Pendias and Pendias, 2001). At L1 and L2, Zn deficiency may be associated with high As and B supplies (Graham et al., 1987; Lonergan et al., 1979). The lower Mn content in fly ash than in soil could be explained by high contents of As, B, Mo and Cu and low contents of Se and Zn (Kabata-Pendias and Pendias, 2001). A Mn deficit for plants growing on fly ash has been previously observed (Adriano et al., 2002; Pavlović et al., 2004; Mitrović et al., 2008).

Chlorophyll fluorescence

In the present study, the values of Fv/Fm and Fm/Fo parameters in leaves of *C. epigejos*, *F. rubra* and *O. biennis* at the ash disposal sites were below the optimum range for plants (Fv/Fm about 0.750-0.850 and Fm/Fo about 5.0-6.0) obtained by Björkman and Demmig (1987). The decrease in photosynthetic efficiency reflects the photoinhibition of PSII of plants growing on the fly ash. Our results confirmed earlier findings for *C. epigejos* and *F. rubra* (Mitrović et al., 2008) and other herbaceous plants, grasses, as well as for trees and shrubs growing on the fly ash of TENT-A (Kostić et al., 2012; Pavlović et al., 2004; Pavlović et al., 2007). Overall, lower photosynthetic efficiency occured due to the stressful effects of high temperatures, high radiation, drought and toxicity of the ash. Toxicity of trace elements such as B and Cu, and deficiency of Mn, can modify the structure and electron transport rate of the photosynthetic apparatus (Landi et al., 2012; Marschner, 1997; Yruela et al., 1996). Other elements, such as As, Mo and Zn, can also affect the PSII apparatus by the synergistic activities of different factors, such as inhibition of chlorophylls and carotenoid formation, oxidative load (an elevated ROS production) and decrease in photosynthetic enzymatic activity (Marschner, 1997; Miteva and Merakchiyska, 2002). The results obtained in this study indicate that all of the examined species maintained the level of photosynthesis, which allowed them to survive under stressful conditions on ash lagoons, albeit with lower than optimal success, The planted *F. rubra* had the highest photosynthetic capacity to grow under the stressful environmental conditions at ash deposit together with naturally colonized species *O. biennis.*

Photopigments

In our study, reduced concentrations of Chl *a*, Chl *b*, Chl *a+b* and Tot Carot at the fly ash lagoons were measured. The reduced levels of photopigments in *C. epigejos* and *F. rubra* species indicate changes in the main pigment of the photosystem II reaction center core (Chl *a*) and the main components of the light harvesting protein complex (LHCP) (Chl *b* and carotenoids). However, an increase in Chl *a/b* in *C. epigejos* at the fly ash lagoons suggests that this species has protective mechanisms for maintaining stability of photosynthetic efficiency. Similarly, a significant reduction in chlorophylls and carotenoids is observed for plant species that grow on fly ash compared to those that grow on regular soil (Rai et al., 2004; Techer et al., 2012; Kostić et al. 2012). Stressful conditions, such as excessive irradiance, high temperatures and drought can cause the destruction of photopigments (Munne-Bosh and Alegre, 2000). Photopigments are also known to be the most sensitive to heavy metal toxicity (Vajpayee et al., 2000). Reduction of the chlorophyll content, which is the result of inhibition of the biosynthesis of pigments in different species of plants that grow in conditions of high concentrations of B and As, was previously observed (Gupta et al., 2002; Apostol and Zwiazek, 2004; Moreno-Jiménez 2008; Yunusa et al., 2009; Kostić et al. 2012). A decrease in chlorophyll and carotenoid content in plants may be due either to reduced synthesis of photopigments, or to their accelerated degradation. For example, it was noted that Cu readily displaces Mg from the chlorophyll molecule (Küpper et al., 2002). Likewise, a deficiency of Mn-dependent enzymes leads to the deterioration of the biosynthetic pathway of isoprenoids that produce carotenoids (Wilkinson and Ohki, 1988).

A species-dependent tolerance of stress was found. Namely, *O. biennis* at the L1 and L2 sites

showed the high photosynthetic potential and a high tolerance of photopigments in fly ash conditions. However, *C. epigejos* has the highest Chl *a/b* ratio in relation to *F. rubra* and *O. biennis*, which suggests the stability of its physiological activity on the fly ash lagoons.

Phenolics

Higher levels of phenolics in the examined species were observed in samples from the L1 and L2 sites than in samples from the CS, which indicates their increased phenolic production under the adverse environmental conditions on fly ash disposal sites. Similarly, higher levels of phenolics have been observed by Singh et al. (2008) in *Beta vulgaris* L. growing in fly ash-amended soil. Elevated phenolic compounds have been associated with environmental stress, such as UV radiation, drought, salinity, pathogen attack, heavy metals, as they act as antioxidants against oxidative stress (Grace, 2005). In general, the higher Free Ph content compared with Bound Ph content in the leaves of the examined plants at all sites, except for *O. biennis* at L1, indicates that an increased level of soluble phenolics could play a main role in the scavenging of ROS and protection of the photosynthetic apparatus from photoinhibition, thereby maintaining a satisfactory level of photosynthesis (Close and McArthur, 2002). Phenolics can function as metal chelators and/or can participate in ROS scavenging through peroxidases (Grace, 2005). At the L1 and L2 fly ash sites, plants grow under excess supply of Cu and B, which proves earlier findings on increased phenolic content in plants growing under excess B conditions (Chamacho-Cristobal et al., 2002), indicating that the formation of borate complex with certain phenols is probably involved in the regulation of the free phenolic level (Pilbeam and Kirkby, 1983).

The responses of phenolic metabolism are species-specific and metal-specific (Kováčik and Klejdus, 2008). According to Marschner (1997), phenolics dominate in dicotyledonous species in comparison to graminaceous species, and between these groups of plants, there are differences in element demand, phenol metabolism and in the pathway of lignin biosynthesis. Thus, species with a high demand for B might also have a higher capacity to sequester B in the cell wall (Marschner, 1997). In dycotyledons, some *o*-diphenols, which are precursors of lignin biosynthesis, possess the *cis*-diol configuration and hence form stable borate complexes (McClure, 1976). Results indicate that *O. biennis* at all sites has enhanced accumulation of phenolics, especially Bound Ph, suggesting that the synergistic effects of excessive irradiance, drought and toxicity of the ash may increase lignification as a mechanism for tolerance, probably by maintaining metal in the cell wall fraction.

In conclusion, all the examined species were affected by the stressful conditions at both of fly ash deposit lagoons with a different weathering stage and different chemical properties. The results obtained in this study showed site-dependent and species-dependent variations for all the parameters examined. At the fly ash lagoons, concentrations of As, B, Mo and Cu were in excessive range, whereas Se, Mn, and Zn were in deficiency range. Stressful conditions at the fly ash lagoons induced a reduction of photosynthetic efficiency and photopigment content in plants, pointing out the sensitivity of the photosynthetic apparatus on the one hand, while an increase of phenolics indicates high antioxidant activity and tolerance mechanisms on the other. According to the overall plant response, we can conclude that all three species possess high potential to survive on fly ash, which is very important for successful reclamation, i.e. for the long-term sustainable management of such sites. However, naturally colonized *O. biennis* showed the higher potential for revegetation of the fly ash deposit lagoons, especially in the early phase of the colonization process, whereas *F. rubra* should be planted on weathered ash.

Acknowledgments - This work was supported by the Ministry of Education, Science and Technological Development of Serbia, Grant No. 173018.

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