CONSIDERATIONS ON THE RELATIONSHIP BETWEEN CHROMOSOME CONSTITUTION AND BIOCHEMICAL PHENOTYPE IN FIVE ECOTYPES OF SEABUCKTHORN

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Abstract. Seabuckthorn is a small tree showing pronounced morphological, physiological, biochemical and genetic variability, high ecological plasticity and large limits of resistance to unfavourable factors and to phytopathogens. It is largely exploited in biotechnological, nutritional, and pharmaceutical purposes, cosmetics domain and in environmental protective field. The possibility that some karyotype traits of five seabuckthorn ecotypes to be used as markers in relation with some specific biochemical features was discussed in this paper. There is intraspecific chromosome variability; the formula of haploid complement is different concerning the preponderance of chromosome morphotypes. Also a marked chemical heterogeneity was evidenced. At this research stage, the results not allow us to establish a direct relationship between some chromosome characteristics and certain morphological and biochemical parameters.

INTRODUCTION

Hippophaë rhamnoides L. is a dioecious species, with a marked morpho-physiological polymorphism and with the possibility to fix atmospheric nitrogen due to symbiotic mycorrhizal *Frankia* fungus (Kato *et al*., 2007; Kanayama et al., 2008). The seabuckthorn capacity to fix nitrogen is twice higher than that of soybean (Lu, 1992). The average amount of nitrogen fixed in seabuckthorn forest is 30 - 60 kg/ha/year (Zike *et al*., 1999) or 180 kg/ha/year, according to Stobdan *et al.* (2008). Seabuckthorn has been largely used to improve eco-environments.

The special characteristics of this small tree are exploitable in biotechnological, nutritional, and pharmaceutical purposes or in cosmetics domain and in environmental protective field (Raţi and Raţi, 2003). Besides pronounced morphological, physiological, biochemical and genetical variability, seabuckthorn shows a high ecological plasticity and large limits concerning resistance to unfavourable factors and to phytopathogens. This plant was mentioned as medicinal plant in traditional Tibetan pharmacopoeia (Persson, 2001). More than 100 phytonutrients and bioactive substances have been evidenced in seabuckthorn leaves and berries (Ahmad and Kamal, 2002), these organs being rich in carbohydrates, organic acids, carotenoids, tocopherols, sterols and other lipids, tannins, amino acids, vitamins (C, E, B1, B2, F, K, P, provitamin A) etc. A protein content of 30% and the presence of polyphenols, fatty acids, alkaloids, cellulose and microelements (P, Ca, Mg, K, Fe, Na) also amplify the value of seabuckthorn preparations. Vitamin C content largely ranges in berries (Yao and Tigerstedt, 1992; Li and McLoughlin, 1997), it exceeding more times the content of dog rose hips, orange, kiwi, hawthorn, tomato and other berries like strawberry, raspberry and blackberry. Leaves contain 11 to 22% crude protein, 3 to 6% of crude fat and some flavonoids (Lu, 1996).

The complex composition results in utilization of seabuckthorn preparations in prevention and treatment of some diseases such as flu, cardiovascular troubles, gingivitis, mucosa injuries, skin problems (Negi *et al*., 2005; Chauhan *et al*., 2007). The fruits, leaves and juices have protective actions against hypertension and coronary heart disease; also hyperinsulinemia and dyslipidemia can be ameliorated or modulated by seed purified flavones (Pang et al., 2008). The flavonols and triterpenoids have as effect the inhibition of proliferation of some tumour cells (Hibasami et al., 2005; Yasukawa *et al*., 2009; Grey *et al.,* 2010).

It seems that the most of the pharmacological effects of seabuckthorn and its health benefits may be partly attributed to their high content of phenolic compounds, as phenols possess a wide spectrum of properties such as antioxidant, antimicrobial, antimutagenic and anticarcinogenic potential (Negi *et al*., 2005; Ercisli *et al*., 2007; Pang *et al*., 2008). Phenolic fractions are responsible for free-radical scavenging activity and for DNA protection (Goel *et al*., 2005), the predominate polyphenols being represented by flavonols. More probably, the numerous health benefits are the result of synergy among many different bioactive components in the plant parts (Yang, 2009).

Additionally to these effects and uses, high quality wines, jams, jelly’s, squash, powder juice, butter, ferments, tea and other healthful foods and syrups are prepared form the fruits of seabuckthorn (Lu, 1992; Shigri, 2001).

The aim of this paper is to discuss on the possibility that some karyotype characteristics of five seabuckthorn ecotypes to be used as markers and to be correlated in a reliable manner with some specific chemical phenotypes.

MATERIAL AND METHODS

Seeds from individuals of five *Hippophaë rhamnoides* Romanian ecotypes (noted as HR-L3; HR-L4; HR-B8; HR-Bu2; HR-S16) were used as biological material for cytogenetic investigations. The germination was carried out at 22°C, in dark. At 10-15 mm length, the root tips were pretreated with 8-hydroxyquinoline (0.002 mol/L), for 4 h and were fixed in ethanol-acetic acid mixture (3:l) for 24 h at room temperature. The plant material was stored in refrigerator, at 4^0C, in 70 % alcohol.

In view of analysis, the root tips were hydrolyzed in 50 % hydrochloric acid for 8 minutes. A modified carbol fuchsin solution (Gamborg and Wetter, 1975) was an effective stain for seabuckthorn chromosomes. The squash preparations were obtained in 45 % glacial acetic acid. Microscopic investigation was carried out by a Nikon Eclipse 600 microscope. For morphometric analysis, the cells with well-spread metaphase chromosomes were photographed with digital camera Cool Pix Nikon, 1600x1200 dpi, 100x objective. The images were processed by Adobe Photoshop programmer.

Chromosome measurements included *length of individual chromosomes* (C), *long arm length* (L), *short arm length* (S), *arm ratio*, r (r = L/S), *centromeric index*, CI (CI=S/C x 100), and *the relative length of each chromosome, %* (C/length of haploid complement x 100).

Karyotypes were performed according to Levan *et al*., 1964 nomenclature. The chromosome homology and the establishment of the chromosome types were assigned on the basis of centromere position, respectively on CI and r values: the chromosomes are metacentric (r<1.70, CI=37.5-50.0), submetacentric (r=1.70–2.99, CI=37.5–25.0), subtelocentric (r= 3.00–6.99, CI=25.0–12.5), and telocentric (r=7.00–∞, CI<12.5).

Dry matter was determined by gravimetric method – biologic material is kept at 105°C to constant weight. The results are expressed in g dry matter/100 g fresh biological material.

Reducing carbohydrates were analyzed by method with 3,5-dinitrosalicylic acid based on the capacity of reducing carbohydrates to reduce 3,5-dinitrosalicylic acid to 3-amino, 5-nitrosalicylic acid, orange coloured, that is spectrophotometrically determined (Miller, 1959).

Total lipids are quantified by Soxhlet gravimetric method. The lipids are extracted at heat, by repeated washing (percolation), with specific organic solvents, under reflux in a special glassware. Results are expressed as g biological material/100 g dry matter (Artenie and Tănase, 1981).

Soluble protein content in enzyme extracts was established by Bradford method (Bradford, 1976). Method is based on binding of Coomassie Brilliant Blue G-250 solution to the amino acids radicals and recording of absorbance at λ = 595 nm using UV-VIS 1700 Spectrophotometer PharmaSpec - Shimadzu. Extraction of soluble protein from seabuckthorn fruits was performed using a Tris-HCl buffer containing dithiothreitol, ascorbic acid, EDTA, TRITON X 100 and cysteine.

Carotenoid pigments are determined by spectrophotometric method. Plant material is triturated with a mixture of reagents which retain the coloured compounds – other then carotenoids – and prevent the decomposition of these. Carotenoids are extracted with acetone and then in petrol ether. In final extract, the carotenoids are spectrophotometrically evaluated (Artenie and Tănase, 1981).

Assimilatory pigments content was measured by spectrophotometrical assay, after extraction with acetone in more steps. The chlorophylls were read at wavelengths specific to each pigment type.

Ascorbic acid determination was carried out by titrimetric method. Principle of this method consists in titration of ascorbic acid from plant extract with 2,6-dichlorophenolindophenol (2,6-DCPIP) solution having an accurately known titre. 2,6-dichlorophenolindophenol is reduced to its leucoderivate by ascorbic acid (Artenie and Tănase, 1981).

RESULTS AND DISCUSSIONS

Hippophae rhamnoides L. is an important plant in Romania, although its valences are not yet entirely exploited. In the last years, the research interest was focused on the realization of an inventory of Romanian seabuckthorn resources by their complex phenotypic and genotypic characterization, construction of a large theoretical and practical basis for the selection of valuable genotypes, and establishment of a germplasm national fund.

The high level of variability amplitude is the result of long term evolution and it constitutes the evolutive potential of the species, because it assures the basis for selection and amelioration activities. The marked polymorphism was revealed by a high number of genotypic and phenotypic studies; in last years the modern techniques of molecular biochemistry and

genetics were also used to determine the seabuckthorn genetic variability and to elucidate the genus taxonomy: *RAPD* (Random Amplified Polymorphic DNA) markers (Jeppsson *et al*., 1999; Bartish *et al*., 2000; Chowdhury *et al.*, 2000; Sheng *et al.*, 2006; Sun *et al.*, 2006), *cp DNA* (chloroplast DNA) (Bartish *et al.*, 2002), *ITS* (internal transcribed spacer) (Sun *et al.*, 2003), *AFLP* (Amplified Fragment Length Polymorphism) markers (Ruan and Li, 2005; Ruan, 2006), *ISSR* (inter-simple sequence repeats) markers (Tian *et al*., 2004), *DNA microsatellite loci* (Wang *et al*., 2008), *intron sequences* (chalcone synthase intron – *Chsi*) (Bartish *et al.*, 2006). However, in spite of these numerous studies, there are still undeciphered zones concerning the seabuckthorn classification, genetic constitution and sex determination.

As previously was shown, the phenotypic and genotypic studies revealed a very large heterogeneity in all seabuckthorn provenances; the Romanian seabuckthorn resources are not the exception concerning the high morphological, biochemical and cytogenetic diversity in this species (Olteanu *et al*., 2009; Oprică *et al*., 2009; Truţă *et al.*, 2009; Zamfirache *et al*., 2009). This impressive phenotype heterogeneity depends on seabuckthorn origin, age, harvest moment, phenophase, methods used for extraction and determination, pedoclimatic factors and genotype characteristics (Li and McLoughlin, 1997; Li, 2002; Zeb, 2004).

Morphological and biochemical characterization is a conventional technique used for evaluation of the plant genetic diversity, although the morphological and biochemical traits are limited in number, are modified by the environment and may be controlled by epistatic and pleiotropic gene effects (van Beuningen and Busch, 1997). Despite these limitations, morphological and biochemical traits have been successfully used for genetic diversity analyses. Analysis of biochemical parameters in different seabuckthorn genotypes could therefore result in evaluation of genotype – phenotype relationship and in accumulation of useful information for selection of desired combinations in further breeding studies. A substantial part of the phenotypical variability is associated with the respective genotypes.

If literature regarding morphology and seabuckthorn chemical composition is very rich, there is a paucity of data on the chromosome constitution of this species. The cytogenetic studies establish the chromosome number of a species and help to decipher the morphological particularities of chromosomes and the metric characteristics of these, followed by karyotype construction. The pattern of chromosome formula, the presence of ploidy level, the existence of some chromosome anomalies can be discussed in relation with respective phenotypes and can direct the activities of selection and amelioration.

To conclude if it is possible that some of cytogenetic traits to be considered as markers in identification of one or more phenotype characters of bioproductive interest, we will comment on some aspects concerning the manner in which the chromosome variability reflects in phenotypisation of quantitative traits in analyzed ecotypes.

The detailed analysis of karyotypes evidenced a relatively high degree of intraspecific uniformity for all studied variables in the five studied ecotypes (Table 1). The metaphases of somatic cells displayed 24 chromosomes. 2n=24 is the diploid number repeatedly reported in the literature, the data being quasi-unanimous to sustain the existence of this chromosome number for all studied varieties, independently of their Asian or European provenance. Its widespread occurrence suggests that it is probably the true diploid number of *Hippophaë rhamnoides* species.

According to the values of arm ratios and centromeric indexes, the five karyotypes have exclusively metacentric (m) and submetacentric (sm) chromosomes, the metacentrics being more frequent; some differences are present in the proportion of these chromosome morphotypes (Truţă *et al.*, 2010, *in press*). Our data on the existence in seabuckthorn of only metacentric and

submetacentric chromosome types are in agreement with the reports of Cireaşă and Dascălu, 1983-1984 (2n = 8m + 16sm); Cao and Lu, 1989; Cimpeanu *et al*., 2004 (2n = 20m + 4sm).

The sea buckthorn chromosomes can be considered as small, they having sizes lower than 4 μm. Only HR-L8 ecotype has one chromosome pair exceeding 4 μm, all the other chromosomes having sizes smaller than 3.5 μm. The mean absolute length of individual chromosomes varies between 4.08 μm (HR-L8 ecotype) and 1.05 μm (HR-Bu2 and HR-L4 ecotypes). The length of haploid complement is comprised between 27.87 μm and 21.73 μm. No secondary constrictions and satellites were evidenced.

The results of analyses performed on seabuckthorn Romanian ecotypes confirm the great morphological, biochemical and cytogenetical variability of this species. Large phenotype heterogeneity was evidenced concerning colour (yellow, orange, or red), size and weight, shape (spherical, cylindrical, ovate, ellipsoidal, or irregular) and number of berries, their placement and degree of agglomeration on branch as well as the frequency and shape of leaves and thorns (Table 1). Thorns show large variation in terms of density, shape and sharpness.

Seeds also showed differences between ecotypes regarding their length, width and length/diameter ratio (Fig. 1), although these differences are not pronounced. Letea ecotypes (HR-L3, HR-L4, HR-L8) are close regarding the three parameters, comparatively with the other two analyzed ecotypes.

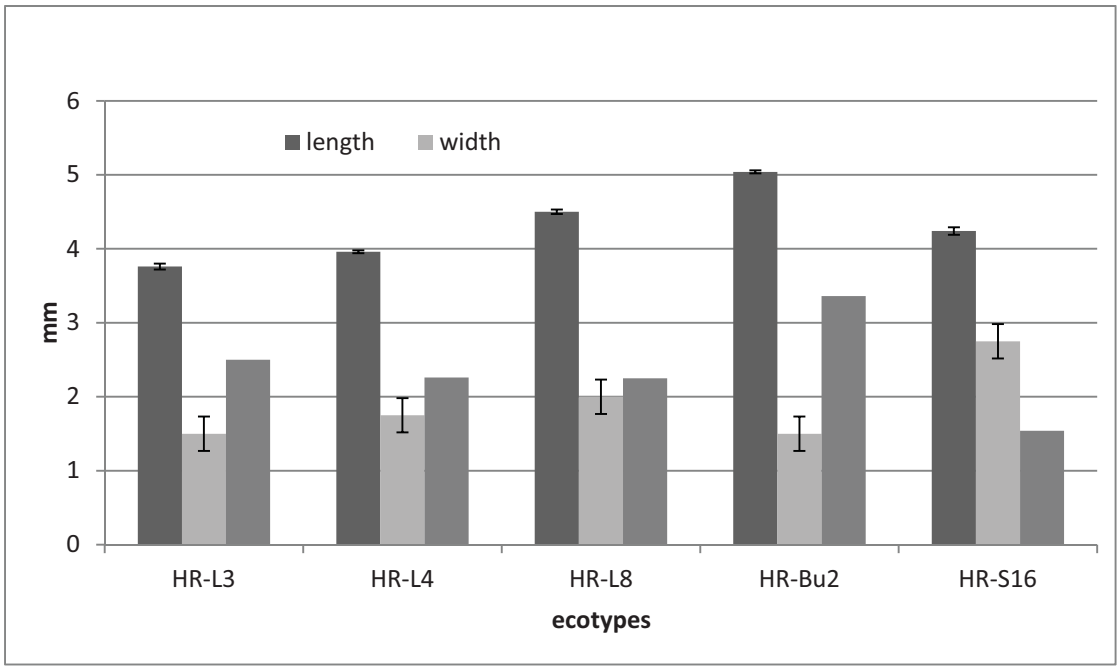

Fig. 1. Graphic representation of metric characters of seeds of *Hippophae rhamnoides* L. ecotypes (x±SE)

For example, they range in length from 3.76±0.04 mm (HR-L3) to 4.5±0.02 mm (HR-L8), with the length/width ratio ranging from 2.25 to 2.50, whereas this parameter is 3.36 for

HR-Bu2, respectively 1.54 for HR-S16. The mean length for HR-Bu2 ecotype is 5.04±0.02 mm and 4.24±0.05 mm for HR-S16 ecotype.

The biochemical results included in this study are an additional proof for the large scale of phenotypisation of studied characters, as reflection of genotype expression of each ecotype, in the specific environmental conditions. Next, a discussion on relation between chromosome constitution and some biochemical and morphological traits will be presented.

Karyotype characteristics of HR-L3 and HR-L8 ecotypes are very close. So, length of haploid complement is 27.87 μm, for HR-L3, respectively 26.98 μm, for HR-L8, whereas the formula of haploid complement is identical – n = 8m + 4sm – for both ecotypes. They are also similar concerning the average chromosome length (2.32±0.19 μm, respectively 2.24±0.23 μm). These features differentiate these ecotypes by HR-L4 which has a smaller length of haploid complement (HCL – 21.73 μm) and a different haploid chromosome formula – n = 11m + 1sm. The mean length of chromosomes is also different (x±Sx = 1.81±0.16 μm).

For these reasons it could be expected that phenotype expression of HR-L3 and HR-L8 ecotypes to be similar. On the contrary, morphological and biochemical indicators are different. Thus, HR-L3 has ovoid, yellow-orange fruits and small thorns (1-2 cm), while HR-L8 ecotype carried light orange, almost round and large sized fruits; rare leaves and 4-5 cm in length thorns. Among pulp chemical compounds, the first ecotype showed 14.84 mg% carotenoid, but the second had only half from this amount.

Also, the other constituents conferring value to seabuckthorn berries – ascorbic acid, carbohydrates, lipids, proteins – show different levels in the two ecotypes. The differences are significant for ascorbic acid (281.40 mg% for HR-L3, 405.20 mg% for HR-L8), pulp lipids (30.35 g% - HR-L3, 25.88 g% - for HR-L8), pulp protein (38.07 mg% – HR-L3, 22.39 mg% - HR-L8).

HR-Bu2 ecotype is characterized by phenotypisation at high levels of ascorbic acid content (698.96 mg%), exceeding 2 times that of HR-L8 (Letea karyotype with the biggest value of ascorbic acid), and of soluble protein (45.35 mg%), surpassing 2 times the protein amount of HR-L8 and HR-S16 ecotypes. However, it not shows – at least in the case of the present used analysis methods – any cytogenetic marker which allows us to establish a direct, definitive and undisputable relationship between genetic background and one trait of interest, in this case one biochemical character.

Although the cytogenetic parameters of HR-L4, HR-Bu2 and HR-S16 ecotypes are relatively similar (chromosomes are smaller than 3 μ, HCL is about 22 μ, two chromosome types are present) their morphological and biochemical parameters are very different. To argue this, the average values of some biochemical determinations are presented, in order for HR-L4, HR-Bu2 and HR-S16 ecotypes:

- ascorbic acid (mg%): 284.85 – 698.96 – 58.34
- lipids in fruit pulp (g%): 39.97 – 19.71 – 32.77
- protein in fruit pulp (mg%): 31.73 – 45.35 – 23.05
-protein in seeds (mg%): 33.28 – 97.00 -8.66.

Carotenoids range between 12.22 - 14.84 mg%, in HR-L4, HR-Bu2 and HR-L3, but show much smaller values in HR-S16 (3.84 mg%) and HR-L8 (7.25 mg%). Concerning carbohydrates, their limits of variability are small (1.47 – 1.66 g%).

The similarity of karyotypes pledges for the idea that the respective ecotypes are the expression of the same genotype constitution; they are in fact representatives of a single species.

Table 1. Karyotype and phenotype variability in five *Hyppophae rhamnoides* L. ecotypes (HCL=haploid complement length; CL=mean chromosome length; m=metacentric chromosomes; sm=submetacentric chromosomes; x±SE = mean±standard error)

ecotype	karyotype			morphological traits	chemical phenotype									
	2n	HCL (µm)	CL(µm) x±SE		ascorbic acid (mg%)	glucides in pulp (g%)	lipids (g%)		soluble protein (mg%)		carotenoids in pulp (mg%)	chlorophyll in leaf (mg/g)		dry matter –pulp- (g%)	water –pulp- (g%)
							seed	pulp	seed	pulp		a	b		
HR-L3	16m+8sm	27.87	2.32±0.19	ovoid, yellow-orange fruits; 1-2 cm thorns	281.40	1.55	30.35	21.23	38.07	33.35	14.84	4.18	1.46	19.13	80.87
HR-L4	22m+2sm	21.73	1.81±0.16	relatively small, orange fruits; long, thin thorns (8-9 cm)	284.85	1.47	39.97	17.33	31.73	33.28	12.22	4.78	2.29	19.24	80.76
HR-L8	16m+8sm	26.98	2.24±0.23	light orange, almost round and large sized fruits; rare leaves; thorns 4-5 cm	405.20	1.66	25.88	18.02	22.39	34.75	7.25	3.96	1.42	15.97	84.03
HR-Bu2	18m+6sm	22.57	1.88±0.17	numerous, intensely orange oblong fruits; discrete thorns; rare leaves	698.96	1.53	19.71	18.92	45.35	97.00	13.17	4.57	2.00	15.35	84.65
IIR-S16	18m+6sm	22.20	1.85±0.16	round, yellow fruits	58.34	-	32.77	17.83	23.05	8.66	3.84	-	-	20.04	79.96

*Except lipids, the other results are reported to fresh plant material

In this study, some differences in morphotype or chromosome size can be result from various factors such as the high chromosome stickiness and the reduced size of chromosomes which can generate errors in cytogenetic determinations. Because of the small and very small sizes of chromosomes, it is somewhat difficult to make a very exact determination of centromere position especially for the chromosomes smaller than 2 μm where the details are few distinguishable.

The phenotype heterogeneity both at morphological and biochemical level could be the consequence of a differentiated gene expression in some environmental conditions, specific for each ecotype; it is known that any phenotype character is a resultant of the circulation of hereditary information on genetic channel, in concrete environmental conditions.

This kind of approaches resuming to the analysis of morphological and biochemical characters not always provides clear responses in relation to genotype features.

For this reason, sooner or later, thoroughgoing molecular approach becomes necessary and obligatory to be made in order to identify some specific markers allowing the deciphering of still unsolved problems, although relatively recent studies have demonstrated that even relationships established on molecular markers do not always accurately agree with the phenotype reality (Fufa et al., 2005; Li et al., 2009).

For example, till now, utilization of molecular markers - especially RAPD - closely linked to sex determination is irrelevant. It is known that in seabuckthorn, it is very important to determine the plant sex before anthesis. For this reason, the identification of one specific genetic marker to allow early identification and removal of superfluous male plants may be helpful in seabuckthorn breeding programmes. But, in the research of Persson and Nybom, 1998, although in the F1 descendance of one cross, the RAPD marker was present both in male parental and in all male descendants and was absent in all female individuals, it can not be considered universal, because in F1 progenies of another cross it was present in only one of the males.

CONCLUSIONS

Analysis and interpretation of cytogenetic results in relation with morphological and biochemical traits of studied seabuckthorn ecotypes led to the following conclusions:

The five studied ecotypes have 2n=2x=24 chromosomes; chromosomes have small sizes (<4 μm), and karyotypes are symmetric.

There is intraspecific chromosome variability. Although two types of chromosomes are present (metacentric, with median placed centromere, and submetacentric, with submedian placed centromere), the formula of haploid complement is different concerning their preponderance.

Currently the results not allow us to establish a direct and reliable relationship between cytogenetical characteristics and certain morphological and biochemical parameters; approaches of molecular biology are necessary to be used to evidence specific markers.

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