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Pigments and genome size variation in *Symphyandra hofmannii* population

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Abstract: The photosynthetic pigments, total anthocyanin content and genome size in six natural populations of endemic species *Symphyandra hofmannii* Pant. (Hoffman's ring bellflower) from Bosnia and Herzegovina were investigated in the present study. The intrapopulation and interpopulation analysis of *Symphyandra hofmannii* growing under different environmental conditions were achieved. The interpopulation analysis of individuals developed under different environmental conditions had shown statistically significant differences for all investigated parameters (chlorophyll *a* and *b*, total chlorophyll, chlorophyll *a*/*b* ratio, carotenoids, anthocyanins and DNA amount). Statistically significant differences between individuals from the same population and different habitats, in chlorophyll *b*, *a/b* ratio and anthocyanins content, were also apparent. Interpopulation pigments' variation could not be related to light as only differential ecological parameter. This variation was depended on combined environmental conditions, such as geological substrate, altitude or anthropogenic factors. The genome size was significantly higher only for one serpentine population (Papratnica).

INTRODUCTION

At present, genus *Symphyandra* A. DC, family Campanulaceae, is represented in Europe by three species (Tutin, 1996): *S. cretica* A. DC distributed in Greece, *S. wanneri* (Rochel) Heuffel in mountains of Bulgaria, Romania and eastern part of ex Yugoslavia and *S. hofmannii* Pant. in Bosnia and Herzegovina.

Symphyandra hofmannii (Hoffman's ring bellflower) was discovered by botanist Sendtner in 1842 and described as *S. wanneri* (Rochel) Heuffel. Four decades later Pantocsek (1881) recognized it as a separate species (Pantocsek, 1881; Fukarek, 1956; Tutin, 1996). Endemic for central Dinaric Alps, this species is distributed in the central part of Bosnia and Herzegovina. Present distribution is restricted to river basins Bosna and Vrbas, including few isolated populations along the river Tinja.

The most of the data about *S. hofmannii* concern morphological (Malý, 1948; Slavnić, 1966), chorological or ecological characteristics (Malý, 1948; Fukarek, 1956; Redžić, 1976). It is on the list for the future "Red book" of Bosnia and Herzegovina described as a rare species (Šilić, 1996, Đug et al. 2013).

Symphyandra hofmannii is biennial species with basal leaves grouped in a rosette and pale yellow pending flowers. Species' morphological features could be distinguished according to different environmental conditions. Short individuals (up to 30 cm) with smaller flowers, including lanceolate, dark green leaves are associated with semi humid and heliophilous habitats. Whereas, tall (up to 50 cm), robust individuals with bigger flowers and light green ovate leaves inhabit humid and sciophilous environments (Slavnić, 1966).

According to different authors *S. hofmannii* is eurivalent species characterized by different environmental conditions (Malý, 1948; Fukarek, 1956; Slavnić, 1966; Redžić, 1976; Šilić, 1990). Its populations can be found at different altitudes (range from 140 m to 900 m),

inclinations, geological substrates (limestone, silicate, serpentine, gabbro, malachite, shale clay), and on shallow or deep soils. This species is also adaptable to different intensity of solar radiation. According to Slavnić (1966), the only common habitat characteristic for all populations is air or soil humidity.

Chlorophyll a and b contents and their ratio have very important role in adaptation of photosynthetic apparatus of higher plants on different light regimes (Porra, 2002). Such analysis can be crucial for investigation of plant's ecological adaptations. In addition, the carotenoids are also associated with the photosynthetic apparatus and their content variation could be an answer to different light regimes as well. Although light intensity was thought as most important factor affecting plant chlorophyll content (Porra, 2002), some other environmental factors could also be included, such as altitude, slope, vegetation density (Boquera et al., 2010), temperature, water stress (Bokhari, 1976) and mineral nutrition (Bokhari, 1976; Bojović and Stojanović, 2005).

High light exposure of plants often results in anthocyanins accumulation in leaves. It is supposed that anthocyanins could adjust the amount of the light passing through the leaf and in that way prevent photoinhibition (Gould et al., 1995; Burger and Edwards, 1996).

DNA amount is also one of the biodiversity characters with various practical implications (Bennett and Leitch, 2005). Although the genome size thought to be constant at species rank, there are frequent reports of intraspecific variation (Blondon et al., 1994; Reeves et al., 1998; Moscone et al., 2003). Moreover, recent investigations show the increasing interest for association of genome size variation with geographic and ecological factors (Slovak et al., 2009), that frequently includes detection of differences in DNA content (Doležel, 1991; Price and Johnston, 1996; Doležel and Bartoš, 2005; Siljak-Yakovlev et al., 2008; Muratović et al., 2010; Siljak-Yakovlev et al., 2010; Pustahija et al., 2013).

In the present paper pigments contents and genome sizes were compared among six populations of *Symphyandra hofmannii* with different habitat conditions. This study was particularly focused on inter/intrapopulation variation of studied parameters, as possible consequence of adaptation to different environmental conditions.

EXPERIMENTAL

Plant material

Flowering plants were collected from six *S. hofmannii* natural populations. All specimens from investigated populations were collected at once during the same day in the morning hours. Temperature ranged from 18 to 21 °C for all localities. Investigated populations are characterized by contrasting ecological conditions (e.g. different geological substrate, soil, altitude, and habitat type). All analyses were performed on the same individuals. Within two investigated populations different type of habitat were detected and their specimens are separately analyzed: Bistričak (fringe zone of mixed forest and lowland hay meadow) and Vitovlje (fringe zone of mixed forest and rocky slopes). Provenance of investigated populations, their basic geographic and habitat characteristics are shown in Table 1.

Fresh leaves (from the central part of stem) from 10 individuals per population were collected for chlorophyll a and b and carotenoids contents analysis. To prevent chlorophyll pheophytinization, CaCO3 was added during the leaves sampling. The extractions and analyses were done in the same day. Air-dried lives, from at least two individuals per population were used for anthocyanin determinations. Fresh leaves from at least five individuals from each population were analyzed to estimate the genome size. Vouchers are deposited in the Laboratory for Research and Protection of Endemic Resources, Faculty of Sciences, University of Sarajevo.

Pigments determination

Photosynthetic pigments content: Photosynthetic pigments content was determined spectrophotometrically according to Holm (1954) and Porra et al. (1989). Chlorophylls a and b and carotenoids content was determined by absorbance reading at 663, 645 and 440 nm, respectively, and calculated as mg pigment per g of fresh weight. All experiments were performed in three replicates per sample. Total anthocyanin content: Total anthocyanins were determined using the pH differential method according to Giusti and Wrolstad (2001). Disposable cuvettes were used for spectral measurements at 530 and 700 nm. Pigment content was calculated as cyanidin 3-glucoside (cyd 3-glc), using an extinction coefficient of 26 900 L cm–1 mol–1 and molecular weight of 448.8g/mol All experiments were performed in three replicates per sample.

Flow cytometry

Plant material for flow cytometry was prepared according to Marie and Brown (1993). DNA content of 5000-10000 stained nuclei was determined for each sample using a Flow Cytometer SL3 (Partec, Münster, Germany). *Petunia hybrida* PxPc6 (2C = 2.85 pg) was used as an internal standard. Total 2C DNA value was calculated using the linear relationship between the fluorescent signals from stained nuclei of the unknown specimen and the known internal standard.

Statistical analyses

Data was analyzed by Statistica 7 for Windows, using a one-way ANOVA. The differences were tested using significance at $p<0.01$ and $p<0.05$. To determine the significant differences between group means, after analysis of variance, post hoc Newman-Keuls test was performed.

RESULTS

Effects of the different environmental conditions on some physiological parameters and DNA content in *Symhyandra hofmannii*, an endemic and eurivalent species, were studied. Results of pigments and genome size measurements for investigated species *Symphyandra hofmannii* are presented in the Table 2. Detected variation of these investigated parameters in *S. hofmannii* populations showed different response to diverse environmental conditions.

Interpopulation analysis, considering different life settings (Table 1), showed significantly increased level of chlorophyll a for Papratnica population, as well as significant increase in DNA content at 5% level. Papratnica also had the highest total chlorophylls comparing to the content found in other populations.
Carotenoid content showed statistically significant differences between Papratnica (highest detected value) and Donji Vakuf (lowest detected value), with no significant differences comparing to other populations (Table 2).
Significant differences were also found for chlorophyll b, chlorophyll ratio and anthocyanins (Table 2). The anthocyanins were not detected in all investigated populations (Table 2).

Table 1: Origin of investigated *Symphyandra hofmannii* populations (1– not directly exposed to light, 2 – directly exposed to light)

Localities	Latitude	Longitude	Altitude (m)	Geological substrate	Type of habitat
Bistričak	44°21´16´´ N	17°58´40´´ E	451	Chert, silicate limestone	Fringe zone of mixed forest (1)/lowlend hay meadow (2)
Jajce	44°20´30´´ N	17°16´00´´ E	404	Tufa, limestone conglomerates	Fringe zone of mixed forests near human settlement (1)
Vitovlje	44°20´45´´ N	17°30´04´´ E	841	Limestone shallow soils	Fringe zone of mixed forest (1)/rocky slopes (2)
Vinac	44°16´38´´ N	17°30´04´´ E	397	Limestone	Rocky crevices (2)
Papratnica	44°26´14´´ N	17°58´40´´ E	333	Serpentine	Fringe zone of mixed forest (1)
Donji Vakuf	44°12´29´´ N	17°19´56´´ E	481	Slate, aleurolite	Rocky slopes (2)

Intrapopulation study included only two populations with different types of habitat related to the direct exposure to light or not, Bistričak (fringe zone of mixed forest and lowland hay meadow) and Vitovlje (fringe zone of mixed forest and rocky slopes) (Table 1). Intrapopulation analysis of genome size was excluded from this study, since no significant intrapopulation differences were found. No significant differences among individuals from different types of habitat within one population for chlorophyll *a*, total chlorophylls and carotenoid content were found (Table2). Significant differences were only detected for chlorophyll *b*, *a/b* ratio and anthocyanins content (Table 2).

Table 2. Pigments composition and DNA amount for investigated *Symphyandra hofmannii* populations

Populations	Bistričak		Jajce	Vitovlje		Vinac	Papratnica	Donji Vakuf
Light	1	2	1	1	2	2	1	2
Chlorophyll *a* (mg g^{-1} ±S.D.)	0.12^{b} (±0.05)	0.13^{b} (±0.12)	0.15^{b} (±0.03)	0.09^{b} (±0.02)	0.12^{b} (±0.02)	0.15^{b} (±0.06)	0.21^{a} (±0.06)	0.11^{b} (±0.03)
Chlorophyll *b* (mg g^{-1} ±S.D.)	0.10^{bc} (±0.05)	0.07^{cd} (±0.05)	0.15^{a} (±0.04)	0.11^{b} (±0.02)	0.05^{d} (±0.01)	0.07^{cd} (±0.03)	0.12^{ab} (±0.03)	0.06^{d} (±0.02)
Chlorophyll (*a*+*b*) (mg g^{-1} ±S.D.)	0.27^{b} (±0.11)	0.26^{b} (±0.22)	0.34^{ab} (±0.08)	0.22^{b} (±0.04)	0.23^{b} (±0.03)	0.29^{b} (±0.11)	0.42^{a} (±0.11)	0.22^{b} (±0.06)
a/b ratio	1.20^{c} (±0.06)	1.77^{b} (±0.02)	1.01^{cd} (±0.13)	0.87^{d} (±0.10)	2.35^{a} (±0.39)	2.17^{a} (±0.23)	1.71^{b} (±0.17)	1.82^{b} (±0.06)
Carotenoids (mg g^{-1} ±S.D.)	0.46^{ab} (±0.12)	0.42^{ab} (±0.32)	0.54^{ab} (±0.12)	0.43^{ab} (±0.11)	0.42^{ab} (±0.02)	0.46^{ab} (±0.16)	0.61^{a} (±0.14)	0.38^{b} (±0.09)
Anthocyanins (mg L^{-1} ±S.D.)	0.19^{e} (±0.02)	1.34^{c} (±0.14)	0.23^{e} (±0.16)	0.58^{d} (±0.12)	2.27^{b} (±0.45)	2.65^{a} (±0.41)	nd	0.16^{e} (±0.02)
DNA amount (pg ±S.D.)	3.76^{b} (±0.03)		3.83^{b} (±0.04)	3.84^{b} (±0.02)		3.83^{b} (±0.03)	3.94^{a} (±0.02)	3.78^{b} (±0.01)

1 – individuals/population not directly exposed to light; 2 – directly exposed to light; (±S.D.) – data represents average values; nd – not detected; individuals/populations not shearing the same letter within one parameter differ significantly at $p \leq 0.05$ level.

DISCUSSION

Investigated variation of pigments in *Symphyandra hofmannii* showed different response to diverse environmental conditions. Although many authors confirmed that chlorophyll content was higher in leaves directly exposed to sun instead to those from shade (Castrillo et al., 2001; Medvegy et al., 2005), opposite findings also exist (Johnson et al., 1982; Sarracino et al., 1992; Chartzoulakis et al., 1995; Souza and Válio, 2003; Dias et al., 2007). There is still no consensus about increasing or decreasing of chlorophyll *a* and *b*, total chlorophylls, carotenoids or chlorophyll *a*/*b* ratio in correlation to different light conditions (Johnson et al., 1982; Sarracino et al., 1992; Chartzoulakis et al., 1995; Souza and Válio, 2003; Dias et al., 2007). This study showed that direct exposure to light (Populations Bistričak 2, Vitovlje 2, Vinac 2 and Donji Vakuf 2) is not exclusively the cause of the chlorophyll content increase, however other ecological factors also have their influence. For instance, population Papratnica had the highest value of chlorophyll *a*, total chlorophylls and carotenoids due to the stressful conditions caused by serpentine geological substrate. Furthermore, increasing of chlorophyll content under stress conditions was reported by different authors (Jamil et al., 2007; Pinheiro et al., 2008; Mafakheri et al., 2010; Rahdari et al., 2012).

Investigated parameter chlorophyll *a/b* ratio gave clearer picture where its increase is significantly associated with direct exposure to light in populations Vitovlje 2 and Vinac, followed by Bistričak 2 and Donji Vakuf, with exception of population Papratnica. Despite the fact that decrease of chlorophyll *a/b* ratio is related to drop in light intensity (Beneragama and Goto, 2010; Biswal et al., 2012) our results showed that it might not be always a rule, especially when stressful conditions such as serpentine substrate are implicated (Table 2).

Although the anthocyanins were not identified in all investigated populations (Table 2), significantly higher anthocyanins values were always found in populations/individuals that were directly exposed to the light, with exception of population Donji Vakuf. Absence of anthocyanins in Papratnica population characterized by extremely dense zone of mixed forest directly showed dependence of their concentration to light intensity. Contrary to this, very low concentration of anthocyanins content in open habitats, like recorded in D. Vakuf suggesting that some other factor can affect anthocyanins content (Neufeld et al., 2011)

The genome size was significantly higher only for serpentine population Papratnica (Table 2). Possible explanation for recorded increase of DNA amount is a result either of B chromosomes presence or increasing of GC bands of individuals in serpentine substrates (Pustahija et al., 2013). Namely, serpentine soils produce extreme ecological conditions for plant development (Riter-Studnicka, 1963; Kruckeberg, 1984; Proctor and Nagy, 1992; Baker et al., 1993; Brady et al., 2005). These soils contain low concentration of plant nutrients, low Ca/Mg ratio and high concentration of heavy metals: Cr, Ni, Co and Mn (Kruckeberg, 1992; Proctor, 1999; Oze et al., 2004). In addition, serpentine soils are usually shallow and permeable for water, which induced for *S. hofmannii* unfavorable dry habitats. But, this population exists in habitat with higher aerial humidity which, probably, allowed its surviving on serpentine soil. *Symphyandra hofmannii* is known as eurivalent species for different habitats (Malý, 1948; Fukarek, 1956; Slavnić, 1966; Redžić, 1976; Šilić, 1990) with air or soil humidity as the only common characteristic (Slavnić, 1966). Additionally, it is known that plant adaptations on such atypical geological substrates can be recognized as individual morphological and physiological changes (Riter-Studnicka, 1963; Iturralde, 2001; Muratović et al., 2005). Moreover, the collected individuals from Papratnica population differed in their size from all others populations. Instead to be up to 50 cm in height, what is a maximum for humid and habitats not directly exposed to the light (Slavnić, 1966), these individuals were over 100 cm high.

Intrapopulation investigation of *S. hofmannii* included two populations, Bistričak and Vitovlje (Tables 1 and 2). This analysis (where all other ecological factors except exposure to the light were excluded) confirmed that higher light intensity is not solely in positive correlation with chlorophyll content. Therefore, no significant differences among individuals from different light regimes within one population for chlorophyll *a*, total chlorophylls and carotenoid content were found (Table 2).

Our results confirmed that from all analyzed parameters only chlorophyll *b*, *a/b* ratio and anthocyanins content were directly related to light regime (Table 2). When other ecological factors are included these findings could deviate and such results might give an explanation for the opposite findings by different authors (Johnson et al., 1982; Sarracino et al., 1992; Chartzoulakis et al., 1995; Castrillo et al., 2001; Souza and Válio, 2003; Medvegy et al., 2005; Dias et al., 2007).

Interpopulation analyses gave no clear picture about the effect of exposure to the light since exclusion of other ecological factors was not possible. Such findings confirmed that intrapopulation analyses are unavoidable part of investigation dealing with the effect of light regimes on photosynthetic pigments. Intrapopulation analyses clarified that chlorophyll *b*, *a/b* ratio and anthocyanin content are the only two parameters directly dependent upon light regime. Given that *S. hofmannii* is rare, endemic but also very attractive horticultural species, further analysis of its photosynthetic light response, leaf nitrogen content, stomata conductance, leaf area index and other anatomical features should be enlightening for its planting and expansion.

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Summary/Sažetak

U ovom istraživanju ispitivan je sadržaj fotosintetskih pigmenata, ukupnih antocijanina i veličina genoma u šest prirodnih populacija endemične vrste *Symphyandra hofmannii* Pant. (Bosanska zvončika ili Hofmanova zvončika) iz Bosne i Hercegovine. Izvršena je intrapopulacijska i interpopulacijska analiza vrste *Symphyandra hofmannii* koja je rasla u različitim stanišnim uvjetima. Interpopulacijska analiza individua koje su se razvijale u različitim ekološkim uvjetima pokazala je statistički značajne razlike za sve ispitivane parametre (hlorofil *a* i *b*, ukupni hlorofili, odnos hlorofila *a*/*b*, antocijanina, karotenoida i količini DNK). Uočene su i statistički značajne razlike između individua iz iste populacije ali različitih habitata u sadržaju hlorofila b, odnosu hlorofila *a*/*b* i antocijana. Interpopulacijska varijabilnost u sadržaju pigmenata nije se mogla pripisati svjetlosti kao jedinom diferencijalnim ekološkom parametru. Ova varijabilnost je bila uslovljena kombinacijom sredinskih faktora kao što su geološka podloga, nadmorska visina ili antropogeni faktori. Veličina genoma signifikantno je odstupala samo kod jedine serpentniske populacije (Papratnica).