

Diploid vs. tetraploid *Centaurium erythraea* Rafn: a comparative study of regenerative *in vitro* potential and biosynthetic capacity

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The impact of ploidy level on both the regenerative potential under *in vitro* conditions and the production of major bioactive specialized metabolites, such as iridoids and xanthenes, was examined in *Centaurium erythraea* Rafn. Shoot regeneration frequency was genotype dependent, but not affected by explant ploidy level. In most cases, the regenerated shoots of autotetraploid (4x) genotypes were more robust than diploid (2x) ones. Regeneration efficiency of root explants declined from the apical to the basal root segment. Shoot and root biomass production of two month-old plants was not significantly different between 2x and 4x genotypes. Both 4x and 2x genotypes were characterized by the predominance of secoiridoid glucoside gentiopicrin in shoots and roots, which is followed by sweroside and swertiamarin. Loganic acid, loganin and secologanin were much less abundant. Methylbelidifolin was the major xanthone in both shoots and roots. Diploid plants showed higher biosynthetic capacity for the production of secoiridoids and xanthenes in both shoots and roots. Results highlight a higher potential of diploid *C. erythraea* genotypes for biotechnology-based sustainable production of secoiridoids in comparison to tetraploid genotypes.

Key words: *Centaurium erythraea*; diploid; autotetraploid; morphogenesis *in vitro*; secoiridoids; xanthenes

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1. INTRODUCTION

Centaurium erythraea Rafn (common centaury), belonging to the family Gentianaceae, is well-known plant for its various important medicinal properties but also as a food or flavor additive. Aerial parts of common centaury are a rich source of different bioactive specialized metabolites, including terpenoids, phenolics, alkaloids and steroids (Šiler and Mišić, 2016). The most abundant bioactive specialized metabolites, secoiridoids and xanthenes, exhibit antidiabetic, antioxidative, hepatoprotective, antibacterial, antifungal, hepatoprotective, gastroprotective, antiulcer, antiinflammatory, diuretic, antitumorogenic, analgesic, antipyretic, anti-inflammatory, antimutagenic, anti-rheumatic activities, and are thus commonly used for treating gastrointestinal disorders, to stimulate appetite, for regulation of digestion issues, against fever, anemia, anorexia, rheumatism, hypertension and many other conditions (reviewed in Šiler and Mišić (2016)). The dominant monoterpenoids of the iridoid type in *C. erythraea* are secoiridoid glycosides sweroside, swertiamarin and gentiopicrin. Accumulation of pharmacologically valuable bioactive

metabolites in plants vary due to a number of factors including changeable environmental parameters, growth conditions, genotype, different plant parts, plant development stages, etc. Polyploidy is recognized as important factor which can affect specialized metabolites production both in terms of diversity and quantities of phytochemicals. Duplication of the entire genomes is a widespread mechanism in plants which promotes evolutionary success, species diversification, genetic variation and genomic complexity. Polyploids may arise through two generally recognized mechanisms: allopolyploidy, which results from interspecific hybridization and subsequent doubling of non-homologous genomes, and autopolyploidy, resulting from doubling of structurally similar, homologous genomes (Parisod et al., 2010). Autopolyploidy is more common in plants than traditionally assumed, but has received little attention compared with allopolyploidy (Parisod et al., 2010). Autopolyploidy can lead to changes in cytological, biochemical, physiological and developmental characteristics in many species, including conspicuous changes in morphology and metabolite production (Lavania, 2005; Sun et al., 2011).

The duplicated gene dosage in polyploids offers the opportunity for increased metabolic activity/gene expression and enhanced concentration of high-value secondary metabolites in tissues and organs (Lavania, 2005). Therefore, manipulation with ploidy, as a biotechnological tool, could be useful in improving pharmaceutical value of medicinal plants, since synthetic autopolyploids usually exhibit increases in bioactive metabolite concentration compared to their diploid progenitors (Javadian et al., 2017; Kong et al., 2017; Lavania et al., 2012; Sadat Noori et al., 2017; Tavan et al., 2015; Xu et al., 2014). However, several studies have shown that polyploidization did not affect the metabolite production (Hull-Sanders et al., 2009; Wohlmuth et al., 2005) or even that the effect of polyploidy on the production of specialized metabolites was negative (Caruso et al., 2013; Cohen et al., 2013; Mansouri and Bagheri, 2017; Tan et al., 2017). Furthermore, in comparison with diploids, polyploids generally produce larger cells which can result in an increased size of plant structures such as leaves, flowers and fruit, increased biomass and often show greater resistance to biotic and abiotic stress (Lavania et al., 2012; Yildiz, 2013). The production of the synthetic polyploids is convenient because they can be compared to the diploid counterparts; however, tetraploid genotypes with natural origin can display a unique genome composition, since they have existed in natural environment for maybe hundreds of generations shaping their genetic background (Orzechowska et al., 2016).

C. erythraea is commonly found in tetraploid forms in nature (Banjanac et al., 2017; Šiler, 2012). Tetraploid populations are mainly distributed in central and northern Europe while diploid populations occurred in Mediterranean region (Mansion et al., 2005; van der Sluis, 1985). Šiler (2012) reported that among 41 randomly collected centaury populations from the Balkan Peninsula, only two populations were found as diploid. Natural populations of centaury are being disturbed by direct anthropogenic actions due to overharvesting for medicinal purposes. Modern biotechnological approaches such as *in vitro* culture can pose important tools for large scale plant propagation, accumulation of biomass and secondary metabolites as well as for conservation of endangered medicinal plants (Karuppusamy, 2009). Through *in vitro* culture of selected explants, continuous, reliable source of natural products could be provided (Caruso et al., 2013; Karuppusamy, 2009). Although centaury is characterized with extraordinary manageability *in vitro* and great regeneration ability (Filipović et al., 2015), to the best of our knowledge, the information on the effects of gene dosage on regeneration capacity is lacking. The aim of this study was to examine the impact of ploidy level on regenerative *in vitro* potential and biosynthetic capacity of *Centaureum erythraea*.

2. MATERIALS AND METHODS

2.1. Plant material and culture conditions

C. erythraea seeds were collected from two natural populations in locality Tjentište. The seeds of diploid population were sampled in 2007 and seeds of tetraploid population in 2016 and deposited at the Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia, at -20 °C until use. Surface-sterilized seeds were germinated on hormone-free half strength MS (½ MS) medium (Murashige and Skoog, 1962) solidified with 0.7 % agar, supplemented with 3 % sucrose and 100 mg l⁻¹ myo-inositol and adjusted to pH 5.8. Ten genotypes of 2x and 4x centaury plants, with ploidy levels confirmed earlier by flow cytometry as described by Šiler (2012) and Banjanac et al. (2017), were randomly selected from *in vitro* pool of seedlings maintained on ½ MS. For the induction of *in vitro* morphogenesis, root explants, 1.0-1.5

cm in length, were excised either from basal, middle or apical root zone of 3 month-old plants and cultured on ½ MS medium separately, under a 16/8 h light/dark photoperiod. Basal segments were sections in the root zone nearest to the plantlet base, while apical sections contained the root apex. For each genotype, five Petri dishes with 10 root explants each were used. The cultures were maintained at 25 ± 2 °C under fluorescent lamps providing photon flux rate of 47 μmols⁻¹ m⁻² the level of cultures. After 5 weeks of culturing regenerated shoots were transferred onto ½ MS medium for rooting. Following parameters were recorded: the frequency of shoot regeneration (i.e. the percentage of explants forming shoots), the average number of shoots per root explant after 5 weeks of culturing, and shoot and root biomass of 2x and 4x regenerated centaury plants two months after transferring on ½ MS medium for rooting.

2.2. Preparation of plant extracts

For phytochemical extraction, two month-old plantlets (shoots and roots) were ground into fine powder using liquid nitrogen. About 100 mg of plant material was extracted with 1 ml of 99.8% methanol (AppliChem, Cheshire, USA), subsequently vigorously mixed by vortexing for 1 min and stored overnight at 4 °C. After sonication for 20 min (RK100, Bandelin, Berlin, Germany) samples were centrifuged at 8000 g for 15 min. Supernatants were filtered through 0.2 μm cellulose filters (Agilent Technologies, Santa Clara, USA) and stored at 4 °C until use.

2.3. UHPLC/DAD/MS² quantitative analysis of targeted metabolites

Quantification of iridoid glycosides (loganic acid, loganin), secoiridoid glycosides (secologanin, sweroside, swertiamarin, gentiopicrin) and xanthenes (decussatin, eustomin, methylbellidifolin, desmethyleustomin) in methanol extracts of *C. erythraea* was performed using UHPLC/DAD/MS² analysis as previously described by Banjanac et al. (2017). Instrumentation consisted of Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Bremen, Germany) with DAD and a triple quadrupole mass spectrometer (TSQ Quantum access max, Thermo Fisher Scientific, Basel, Switzerland). Samples were chromatographically separated on Hypersil gold C18 column (50 × 2.1 mm) with 1.9 μm particle size (Thermo Fisher Scientific, USA), thermostated at 40 °C. Mobile phase, flow rate and gradient elution were adjusted according to Banjanac et al. (2017), as well as heated electrospray ionization (HESI) source of the mass spectrometer. All solvents were of LC-MS grade (Fisher Scientific UK, Leics, UK), and ultra-pure deionized water was generated using Water Purification System (New Human Power I Integrate, Human Corporation, Republic of Korea). The injection volume was set to 5 μl. All analyses were performed using three biological replicates. Targeted compounds were quantified using the selected reaction monitoring (SRM) mode of the instrument, and by tracking two diagnostic MS² fragments of each compound. SRM chromatograms and MS² spectra of standards are presented in Supplementary Figure 1. External standard method was used for the quantification of iridoids and xanthenes, as described in Banjanac et al. (2017). Calculation of regression for each of the calibration curves showed excellent linearity with correlation coefficients of r = 0.999, p < 0.001. The total concentrations of the analyzed iridoids and xanthenes were obtained by calculating peak areas and are expressed as μg per 100 mg of plant dry weight (μg 100 mg⁻¹ DW).

2.4. Statistical analysis

Statistical analysis was performed using Statgraphics Centurion XVI. The data regarding *in vitro* regeneration capacity of

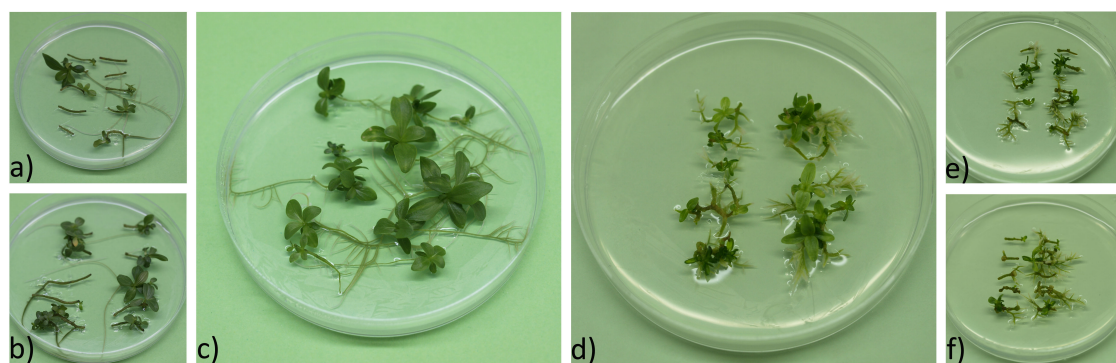


Fig. 1. Induction of morphogenesis *in vitro* in root culture of tetraploid and diploid plants of *C. erythraea* on $\frac{1}{2}$ MS medium: (a), (b), and (c) Regeneration of shoots and roots in root culture of representative tetraploid genotype 4-3; (d), (e) and (f) Regeneration of shoots and roots in root culture of diploid genotype 2-4; Regeneration was achieved using following explants: (c), (d) apical root segment; (a), (e) middle root segment; (b), (f) basal root segment

diploids and tetraploids as well as biomass of 4x and 2x regenerated centaury plants were analyzed by one-way ANOVA, following least significant difference test (LSD) for the confidence level of $P < 0.05$. Statistical analysis of the significant differences between contents of the individual compounds in diploids and tetraploids was done by the Student's t-test.

3. RESULTS AND DISCUSSION

In this study, five *C. erythraea* genotypes of 2x and five of 4x ploidy level were tested for their *in vitro* regeneration ability. The use of root explants for *in vitro* regeneration in centaury, as well as high regeneration potential of the centaury roots was described earlier (Subotić et al., 2004; 2006; Subotić and Grubišić, 2007; Trifunović-Momčilov et al., 2016). The authors showed that morphogenesis *in vitro* in centaury root culture is direct, without callus phase, and spontaneous, on medium without addition of any plant growth regulators (Subotić et al., 2004; Trifunović-Momčilov et al., 2016). Roots provide an excellent source of explants for mass clonal propagation because of their efficiency in the multiplication of a large number of genetically identical plants, as well as due to their easy maintenance and manipulation *in vitro* (Pant et al., 2010; Vinocur et al., 2000). Root cultures have also received a considerable attention due to their stable metabolite production ability (Carvalho and Curtis, 1998).

Formation of shoots on apical root explants was recorded after 5 weeks in culture (Figure 1), when 4x genotypes displayed regeneration frequencies ranging between 30% and 100%, while in 2x genotypes this frequency was between 40% and 86% (Figure 2a). The highest shoot regeneration percentage (100%) and average shoot number per apical segment (1.7) were recorded in the tetraploid genotype 4-3. In 2x genotype 2-4 these parameters reached 86% and 1.6. Regeneration of shoots on middle root segments varied between 14% and 72% in 4x genotypes, and between 30% and 66% in 2x genotypes, while basal root segments achieved regeneration frequencies between 20% and 74% in 4x genotypes, and between 16% and 44% in 2x genotypes. Our results indicate that regeneration frequency of centaury root explants might rather be genotype dependent (Figure 2a, 2c), but not affected by explant ploidy level (Figure 2b, 2d). This is in agreement with results of Fras and Maluszynska (2004), since calli lines, derived from the same type of explants of diploid or autotetraploid *Arabidopsis thaliana* plants, showed very similar regeneration ability. On the contrary, in some other species, differences in regeneration capacity under *in vitro* conditions between diploids and tetraploids were observed. In *Dendrobium officinale* regeneration ability of synthetic tetraploid cultivars was lower compared to diploid ones (Pham et al., 2019). In the last men-

tioned study, the protocorm-like bodie (PLB) induction rates from the stem node of the tetraploid cultivars were significantly higher than those of diploid, but PLB proliferation of tetraploids and the mean number of plantlets regenerated from tetraploids was lower than that of the diploid. (Sun et al., 2011) suggested that polyploidization caused the decrease in or loss of *in vitro* organogenic potential, since regeneration frequencies of shoots from leaf explants of most of the induced neopolyploids of *Pyrus communis* were significantly decreased compared to the original diploid control. On the contrary, few other studies showed that regeneration ability of tetraploids was increased. For example, the highest shoot regeneration percentage and shoot number per explant were obtained from petiole explants of the tetraploid line of sugar beet compared to diploid lines (Yıldız, 2013). In comparison with diploid Columbia plants, regeneration capacity of a natural tetraploid ecotype of *A. thaliana* (Warschau) was found higher under *in vitro* conditions (Orzechowska et al., 2016). Within the present study, clone 2-4 among diploid genotypes and clone 4-3 among tetraploid ones stand out for their regenerative potential. However, statistically significant differences between these two genotypes were observed regarding middle and basal root segments.

Obtained results showed that regeneration potential of centaury root explants was affected by the position of the isolated segment on the root (Figures 1, 2). All three types of root sections regenerated shoots, however regeneration efficiency generally declined from the apical to the basal root segment. Some genotypes were exceptions, e.g. genotype 4-1, 4-3 and 2-2 (Figures 2a, 2c). On the contrary, in *Svertia hirata* apical root segments remained nonresponsive, while basal and middle segments exhibited similar shoot regeneration (Pant et al., 2010). Basal segment showed the best result for producing the highest number of buds per explant in *Populus tremula* (Vinocur et al., 2000), middle segment was the most efficient in oriental lily cultivar "Rosato" (Kumar et al., 2008), while basal and apical segments in *Brassica campestris* showed the best regeneration potential (Paul and Sikdar, 2005). It is likely that centaury root explants from actively growing meristematic apical zone are better source of competent cells for shoot regeneration than sections from more differentiated root zones. Furthermore, differences in regeneration capacity may have originated from various levels of endogenous hormones in centaury root sections.

Differences in leaf size and quality of shoots regenerated from root explants of diploid and tetraploid centaury genotypes were noted. The most often regenerated shoots of 4x genotypes were more robust than 2x ones which also displayed hyperhydration symptoms (Figure 1). Shoots regenerated from

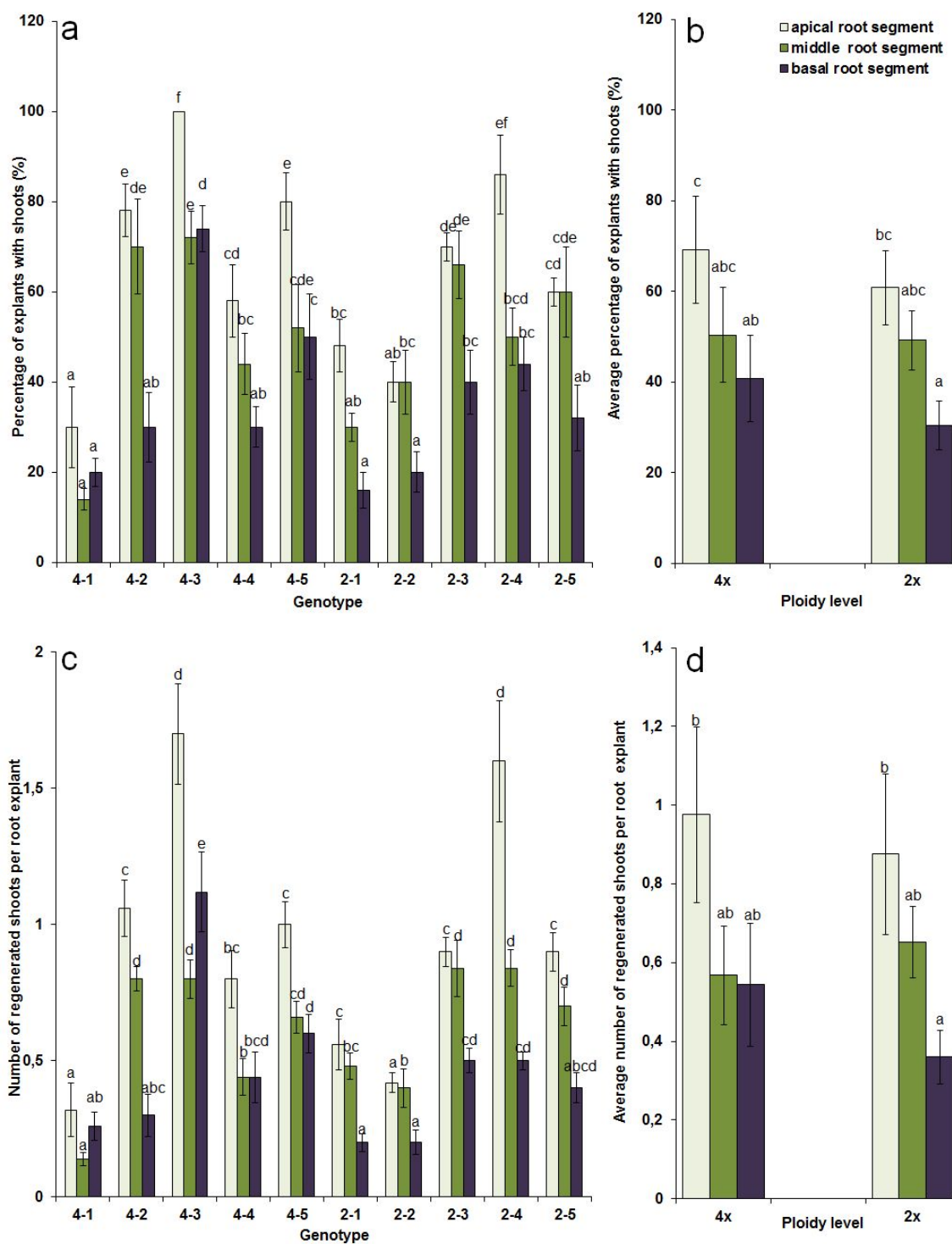


Fig. 2. Regeneration of shoots from root explants of 4x (4-1 to 4-5) and 2x (2-1 to 2-5) centaury genotypes recorded 5 weeks after culture initiation on $\frac{1}{2}$ MS hormone-free medium; (a) Percentage of explants with shoots and (c) number of regenerated shoots per explant for each genotype independently, and separately for apical (light green bars), middle (green) or basal (purple) root explants; Average values for the percentage of explants with shoots (b) and average number of regenerated shoots per root explant (d) were compared between 4x and 2x genotypes. The experiment with 10 explants per Petri dish was repeated five times ($n=50$) and results are presented as means \pm SE. Letters above the bars indicate if the values are statistically different at the $P<0.05$ level according to the LSD test, independently for each type of root segments

root explants of 2x and 4x genotypes were successfully rooted on $\frac{1}{2}$ MS medium (Figures 3a, 3b, 3c). After two months in culture, 2x genotypes produced higher shoot and root biomass than 4x genotypes, however this difference was not statistically significant (Figure 3d). Several studies reported that tetraploid plants generally have larger organs such as leaves, flowers and fruits and higher biomass than diploids (Javadian et al., 2017; Sadat Noori et al., 2017; Xu et al., 2014). In contrast, autotetraploidy did not lead to increase in total biomass

production in maize despite its positive effect on leaf size and stem thickness (Sokness and Dudley, 1989). The overall effect of polyploidy on total biomass production is varying in different species (Abel and Becker, 2007). Biomass production of the tetraploids was even reduced in *Spathiphyllum wallisii* (Van Laere et al., 2011). Similarly, the effect of polyploidization on vegetative biomass production in *B. rapa* and *B. oleracea* was negative (Abel and Becker, 2007). Lavania et al. (2012) suggested that negative impact of polyploidy on biomass may

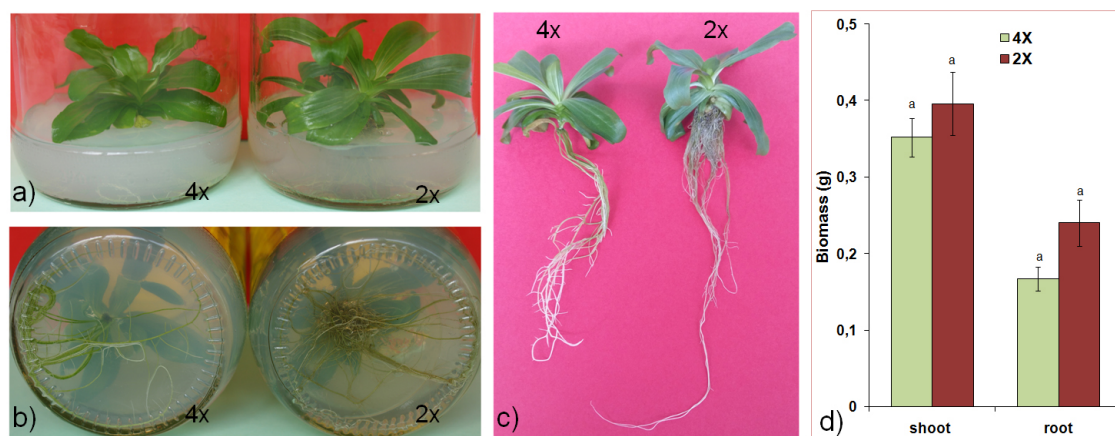


Fig. 3. Shoots (a) and roots (b) of two month-old 4x and 2x *C. erythraea* plants were removed from culture medium (c). Mean biomass (d) of shoots and roots was measured for 4x and 2x genotypes. Same letters above the vertical bars indicate that differences between 4x and 2x plants are not statistically significant at the $P < 0.05$ level according to the LSD test, for each plant part independently.

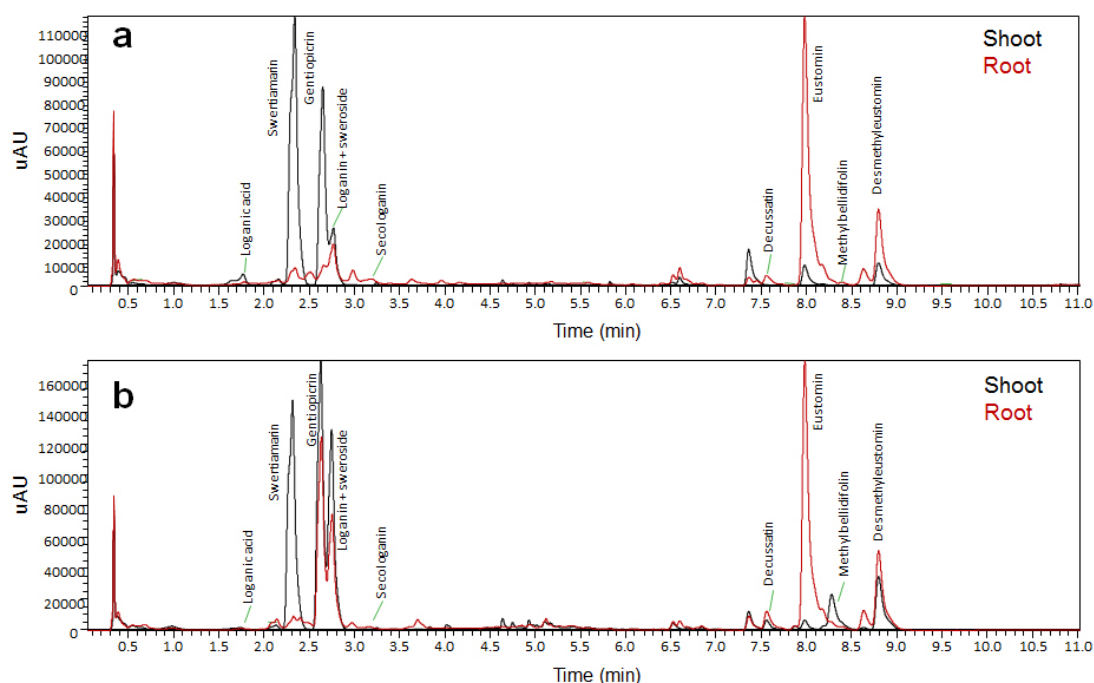


Fig. 4. UHPLC/DAD chromatograms of 4x (a) and 2x (b) *C. erythraea* genotypes, presenting major iridoids (loganic acid, loganin, secologanin, sweroside, swertiamarin and gentiopicrin) and xanthenes (decussatin, eustomin, methylbellidifolin and desmethylleustomin) in shoots (black line) and roots (red line), acquired at $\lambda = 260$ nm.

depend on the energy cost of produced metabolite i.e. longer the metabolic pathway larger is the negative effect of polyploidy on biomass production.

UHPLC/DAD/MS² analysis of *C. erythraea* shoots and roots methanol extracts (Figure 4) was targeted towards two iridoid glycosides (loganic acid, loganin), four secoiridoid glycosides (secologanin, sweroside, swertiamarin, gentiopicrin) and four xanthenes (decussatin, eustomin, methylbellidifolin, desmethylleustomin).

In both 4x and 2x genotypes of *C. erythraea*, the major site of targeted compounds accumulation were aerial parts (Figure 5). These results are consistent with previous findings regarding *C. erythraea* (Matekalo et al., 2018; Šiler et al., 2012). Both 4x and 2x genotypes of *C. erythraea* are characterized by the predominance of gentiopicrin in shoots (Figure 5a) and roots (Figure 5b). The second and third most abundant iridoids were sweroside and swertiamarin. Loganic acid, loganin and secologanin were much less abundant. Previous

reports showed that the dominant secoiridoid glucoside of *C. erythraea* in shoots is swertiamarin (Matekalo et al., 2018; Šiler et al., 2012). Methylbellidifolin was the major xanthone in shoots and roots of both 4x and 2x genotypes.

In our study, diploid plants showed higher biosynthetic capacity for the production of iridoids and xanthenes in both shoots and roots (Figure 5). The results indicated that the shoots of diploid plants had about 2 times higher content of gentiopicrin and sweroside than tetraploid plants (Figure 5a). Interestingly, the content of secologanin and swertiamarin were higher in shoots of tetraploid plants (9.48 – fold and 1.49 – fold, respectively). Similar trend was observed in roots, since the amount of gentiopicrin and sweroside were up to 1.97 times and 3.29 times higher in diploids than in tetraploids (Figure 5b). Amount of secologanin was 4.9 – fold higher in 4x than in 2x roots. Overall, the content of total iridoids in shoots was significantly higher in diploids, but in roots the differences were not statistically significant (Figure 5c).

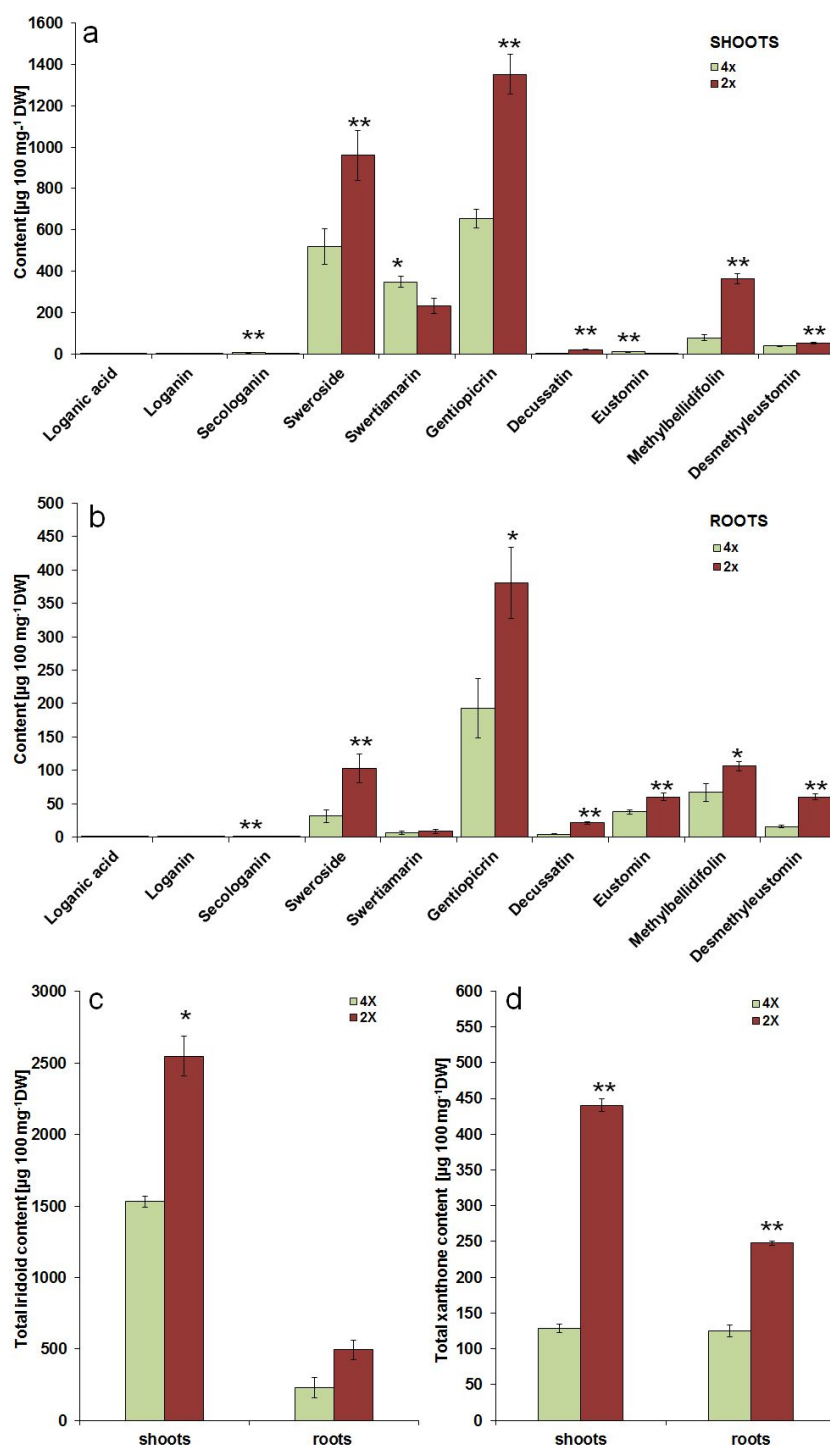


Fig. 5. UHPLC/DAD/MS² quantification of targeted metabolites content in shoots (a) and roots (b) of 4x and 2x regenerated centaury plants. Asterisks above the bars indicate if the values are statistically different at the P<0.05 (*) and at P<0.01 (**) levels according to the t-test, for each compound independently. Total amount of monoterpene iridoids (c) and xanthones (d) differ significantly between 4x and 2x plants. Asterisks above the bars indicate if the values are statistically different at the P<0.05 (*) and at P<0.01 (**) levels according to the t-test, for each plant part independently. Abbreviations: DW – dry weight.

The content of total xanthones in shoots and roots of diploid plants was significantly higher than in tetraploid plants (Figure 5d). Higher content of decussatin, methylbellidifolin and desmethyleustomin were observed in shoots of diploid plants than in tetraploid plants. The exception was eustomin, with content 2.3 – fold higher in 4x than in 2x plants (Figure 5a). The content of decussatin, eustomin, methylbellidifolin and desmethyleustomin in 2x roots were 4.8 – fold, 1.6 – fold, 1.6 – fold and 3.78 – fold higher compared to those of 4x roots, respectively (Figure 5b). Literature survey pointed to the studies which explained that polyploidization in plants results either in increases or decreases in specialized metabolites production,

depending on the species in question. For example, concentrations of gingerol were lower for the tetraploid clones than for the parent diploid *Zingiber officinale*, although the differences were not statistically significant (Wohlmuth et al., 2005). Betacyanins, the major fruit pigments in fruit of diploid *Hylocereus monacanthus* and synthetic allotriploid, decreased following genome doubling (Cohen et al., 2013). Similarly, tetraploid genotypes of *Solanum bulbocastanum* displayed either similar or lower amounts of phenylpropanoids, tryptophan, tyrosine and α -chaconine compared to diploids (Caruso et al., 2013). Furthermore, non-targeted and targeted metabolic profiling of mature leaves from three doubled diploids of citrus and their

diploid controls showed that primary metabolism takes priority over specialized metabolism in doubled diploid plants, since the levels of primary metabolites tended to increase and the levels of secondary metabolites tended to decrease (Tan et al., 2017). As can be observed, there is a lack of linearity between the increase in genetic material (ploidy level) and the production rate of specialized metabolites in different medicinal and aromatic plants (Iannicelli et al., 2020). Changes in the metabolic profile of autopolyploids were interpreted as being the result of an alteration in the mechanism(s) regulating the biosynthesis of individual compounds (Fasano et al., 2016). Naturally occurring polyploids have been under pressures of the natural selection for a long period of time after polyploidization and therefore differences in metabolite concentration between diploid and polyploid genotypes may be due to selection. In the Balkan Peninsula tetraploid populations of *C. erythraea* dominate over diploid ones, which are recorded in only two locations (Šiler et al., 2012). The wide distribution of tetraploids in common centaury implies that this genome status has an adaptive significance and that it is selected positively. Secondary metabolites are often produced to protect plants against biotic and abiotic stresses and require a high investment in energy and carbon (Tan et al., 2017). Taking into account that *C. erythraea* plants within the present study were grown under experimental *in vitro* conditions where 2x and 4x genotypes were exposed to the same environment, the differences in specialized metabolites production may be attributed to genetic differences between the two groups of samples, and to their metabolic capacity under these specific conditions.

CONCLUSION

Our results highlighted a great potential of diploid *C. erythraea* genotypes for biotechnology-based sustainable production of secoiridoids. Higher production of secoiridoids and xanthones in shoots and roots of diploid centaury plants regenerated *in vitro*, as well as their successful regeneration from root explants, indicate a great potential of *in vitro* cultivation of diploid centaury plants for obtaining valuable bioactive metabolites on a large scale. Such cultures may therefore provide an alternative to naturally grown plants as a potential biomass source for the production of bioactive compounds. Further studies are conducted towards elucidation of molecular background of secoiridoid glycosides production as influenced by ploidy level.

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