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Determination of Nuclear DNA Content and Chromosome Number of Verbascum scamandri Murb. (Scrophulariaceae)

Verbascum scamandri Murb. (Scrophulariaceae) Türünün Çekirdek DNA İçeriğinin ve Kromozom Sayısının Belirlemesi

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DETERMINATION OF NUCLEAR DNA CONTENT AND CHROMOSOME NUMBER OF VERBASCUM SCAMANDRI MURB. (SCROPHULARIACEAE)

ABSTRACT

The study aimed to determine the chromosome number and nuclear DNA content of an endemic plant, *Verbascum scamandri,* using 14-week-old plants germinated from seed and to determine the genetic stability based on flow cytometry analyses in callus tissues induced MS medium containing 1 mg/L Kin + 1 mg/L 2,4-D and 9-week-old propagated plants, induced and developed on MS medium containing 2 mg/L Kin + 0.1 mg/L 2,4-D. In the mitotic chromosome counts, it was determined that *V. scamandri* had $2n = 32$ chromosome number. Flow cytometric analysis revealed that 14-week-old *in vitro* grown plants had 0.73 pg/2C ± 0.01, callus tissues had 0.76 pg/2C ± 0.02 and propagated plantlets had 0.79 pg/2C ± 0.01 mean nuclear DNA content. The results proved that propagated plants had similar DNA content to the seed-derived plants which showed analysed plants were genetically stable.

Keywords: Nuclear DNA Content, Chromosome, Tissue Culture, Mullein.

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VERBASCUM SCAMANDRİ MURB. (SCROPHULARİACEAE) TÜRÜNÜN ÇEKİRDEK DNA İÇERİĞİNİN VE KROMOZOM SAYISININ BELİRLEMESİ

ÖZ

Çalışmanın amacı, endemik bir bitki olan *Verbascum scamandri* türünün *in vitro* büyütülen 14 haftalık bitkiler kullanılarak kromozom sayısı ve çekirdek DNA içeriğinin belirlenmesi, 2 mg/L Kin + 0.1 mg/L 2,4-D içeren MS ortamında çoğaltılan 9 haftalık *in vitro* bitkilerde ve 1 mg/L Kin + 1 mg/L 2,4-D içeren MS ortamında indüklenen kallus dokularında flow sitometri analizleri ile genetik kararlılığın tespit edilmesidir. Mitoz kromozom sayımlarında türün kromozom sayısının $2n = 32$ olduğu belirlenmiştir. Flow sitometri analizinde, *in vitro* yetiştirilen bitki örneklerinin 0.73 pg/2C ± 0.01, kallus örneklerinin 0.76 pg/2C ± 0.02, *in vitro* çoğaltılmış bitkiciklerin DNA içeriği ise 0.79 pg/2C ± 0.01 belirlenmiştir. Yapılan flow sitometri analizleri tohumdan gelişen bitkiler ile *in vitro* çoğaltılan bitkilerin benzer çekirdek DNA içeriklerine sahip olduklarını göstermiş ve dolayısıyla genetik stabil oldukları anlaşılmıştır.

Anahtar Kelimeler: Çekirdek DNA İçeriği, Kromozom, Doku Kültürü, Sığır Kuyruğu.

1. INTRODUCTION

The genus *Verbascum* L. belongs to the family Scrophulariaceae is commonly known as mullein. The genus *Verbascum* includes about 459 species and is distributed mainly in Asia, Europe, and North America (Heywood, 1993; POWO, 2023). In the flora of Türkiye, 253 natural species and 130 hybrid species are found. Among these species, 198 of them were classified as endemic species. The endemism rate of the genus is about 80% in Türkiye (Huber-Morath, 1978; Karavelioğulları, 2015a; 2015b; Çıngay and Karavelioğulları 2016; Duman et al., 2017; Çıngay et al., 2018). Most *Verbascum* species contain many secondary metabolites including iridoid glycosides, phenylethanoid glycosides, flavonoids, saponins, monoterpenoid glucosides, neolignan glucosides, phenolic acids, steroids, and spermine alkaloids (Tatlı and Akdemir, 2004). These compounds have antioxidant (Mihailović et al., 2016), antiviral (Zanon et al., 1999), antibacterial (Hacıoğlu Doğru et al., 2021), antiinflamatuar (Kupeli et al., 2007), anticancer (Zhao et al., 2013), wound healing (Akdemir et al., 2011), antifibrosis (Wu et al., 2018), neuroprotective (Esposito et al., 2010; Xue et al., 2012), and osteoprotection effects (Young et al., 2017). Due to these effects, *Verbascum* species are used in traditional medicine for some diseases such as respiratory tract diseases, eczema, for the treatment of tumors, asthma, and migraine (Turker and Camper, 2002; Kozan et al., 2011). *Verbascum scamandri* Murb. is a biennial plant species, 50-80 cm high, with shortly and densely stellate-tomentose or glabrescent (Huber-Morath, 1978). This species is distributed on mountain slopes in Northwest Anatolia, Türkiye-Kazdağı, known as "Kazdağı Mullein". The conservation status of this species is declared as endangered (EN, B1-B2a) according to IUCN criteria (IUCN, 2012).

Plant tissue culture is used in plant breeding applications such as haploid plant production, gene transfer, somatic hybridization, species hybridization, somaclonal variation, and in many non-breeding and commercial studies such as synthetic seed, disease-free plant, secondary metabolite production, and micropropagation (Babaoğlu et al., 2001). In addition, it is used for the protection of genetically valuable plant species that are difficult or impossible to reproduce by vegetative and generative means, and plant gene resources that are at a risk of extinction from nature (Mikulík, 1999; Rout et al., 2000). These methods provide the opportunity to multiply plant species in a short time, in a narrow area, regardless of the growing season. However, *in vitro* propagated plants are expected to be genetically uniform and genetically equivalent to donor plants. Callus culture is one of the tissue culture types and is widely used in the production of secondary metabolites of medicinally important plant species. Callus tissues are irregular and undifferentiated parenchymatic cells (Sökmen and Gürel, 2001; Çalışkan et al., 2019). However, many factors such as callus stage, number of subcultures, explant source, plant growth regulators, or applied chemicals may cause environmental stress in culture and induce genetic or epigenetic variations, widely known as somaclonal variation, in

propagated plants during the culture process (Leljak-Levanic et al., 2004; Temel et al., 2008; Chinnusamy and Zhu, 2009; Lira-Medeiros et al., 2010). For this reason, it is necessary to analyse and compare the nuclear DNA content of callus tissues and *in vitro* propagated plants to determine if genetic changes occur during culture (Çördük et al., 2018).

The nuclear DNA content is the total amount of DNA which present in each cell nucleus of a eukaryotic organism. In species with the same ploidy level, nuclear DNA content is mostly constant among cells of an individual and relatively constant among individuals of species (Bennett and Leitch, 1955). Therefore, it is crucial data in genome analysis (Rees and Walters, 1965), ploidy analysis, evolution, taxonomy (Ohri, 1998), and breeding studies (Lee et al., 2020). Nowadays, the flow cytometry method (FCM) has been used commonly to estimate the nuclear genome size which is a convenient, fast, and reliable method (Dolezel et al., 2007; Galbraith, 2009). FCM can also allow detecting DNA amounts from material cultured *in vitro* (Dolezel et al., 1989; Makowczyńska et al., 2008). Somaclonal variation in tissue culture resulted instability in DNA content of plant material (Escobedo-Gracia-Medrano et al., 2018; Sliwinska, 2018) and screening of genetic stability of plants propagated through tissue culture techniques has been analysed successfully using flow cytometry in different plant species (Kubalaková et al., 1996; Kevers et al., 1999; Makowczyńska et al., 2008). Although nuclear genome size is a fundamental biological character, it has been only estimated until now in a limited number of *Verbascum* species e.g. *V. levanticum* (0.75 pg/2C) and *V. virgatum* (1.44 pg/2C) (Castro et al., 2012). In the *Verbascum* genus, chromosome number showed variation among species and even within the species (Benedi et al., 2009). However, there is no information about the nuclear DNA content and chromosome number of *V. scamandri*.

This research aimed to determine the chromosome number and nuclear DNA content of *V. scamandri* species using 14-week-old *in vitro* grown plants by flow cytometry for the first time. Also, the DNA content of 9-week-old propagated plantlets and callus tissues of *V. scamandri* were analysed and compared to determine whether genetic changes occur during *in vitro* culture.

2. MATERIALS AND METHODS

2.1 Plant Material

The studies were carried out using the *in vitro* cultures of *V. scamandri* that had been established by Cambaz (2022). *V. scamandri* seeds were collected from in Çanakkale-Bayramiç, Türkiye during the flowering period in Agust 2021. The taxonomic identification was made according to the genus *Verbascum* L. in Flora

of Turkey and the East Aegean Islands (Huber-Morath, 1978), and checked with reference collection in in Çanakkale Botanic Garden Herbarium (CBB, Çanakkale, Türkiye) by Prof. Dr. Ersin KARABACAK from Çanakkale Onsekiz Mart University, Faculty of Science, Department of Biology. A voucher specimen was recorded in the CBB under the number "CBB00002743".

V. scamandri seeds were sterilized in 5% (v/v) sodium hypochlorite for 20 minutes and then rinsed 4-5 times with sterile distilled water. The seeds were inoculated on Murashige and Skoog basal medium (MS: Murashige and Skoog 1962) containing 3% (w/v) sucrose and 0.7% (w/v) phytoagar. The adventitious shoots were induced from leaf explants (5x5 mm) cultured on MS medium containing 2 mg/L Kinetin (Kin) + 0.1 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D), 3% sucrose, 1 g/L polyvinylpyrrolidone (PVP, Sigma Aldrich), and 0.7% phytoagar. The shoots were propagated and rooted on MS medium without plant growth regulators. Callus induction has occurred from leaf explants (5x5 mm) cultured on MS medium containing 1 mg/L Kin + 1 mg/L 2,4-D, 3% sucrose, 1 g/L PVP, and 0.7% phytoagar. All media were adjusted to pH 5.75 before autoclaving at 121°C for 15 min. All the cultures were kept in the growth chamber at 25 ± 2 °C under 16 h light/8 h dark photoperiod, 50 ± 5% humidity with 72 µmol $m^{-2}s^{-1}$.

2.2. Nuclear DNA Content Estimation

The nuclear DNA content of *V. scamandri* samples was determined by the flow cytometer (Partec, CyFlow® Space Münster, Germany) equipped with a green solid-state laser (Cobolt Samba, 532 nm, 100 mW). *Lycopersicon esculentum* (2C = 1.96 2C/pg) was used as an internal standard. The intact nuclei suspension was prepared from the youngest and healthy leaves of both the *in vitro* grown plants and the propagated plants, as well as from the callus tissues. The intact nuclei suspensions were prepared using commercial kits (CyStain PI absolute P) manufactured by Sysmex Partec GmbH (Münster, Germany). Approximately 20 mg fresh leaf of sample and 40 mg fresh leaf of internal standard was co-chopped into small pieces for approximately 40–60 s using a razor blade in a petri dish containing 500 µl nuclei extraction buffer. The homogenized solution was transferred into a glass tube through a 30 µm filter. A 2 µl of staining buffer (CyStain PI Absolute P) was added to each tube and the samples were incubated at room temperature in the dark for at least 1 h before analysis. 2C nuclear DNA contents of samples were calculated based on the ratios of the G1 peak means of sample and internal standard in three replicates per sample using the following equation: Nuclear DNA content of sample = (mean of sample G1 peak/mean of standard G1 peak) × Known DNA content of standard (pg)

2. 3. Chromosome Preparation

Cytological analysis was done on root tips of 14-week-old *in vitro* grown plants. Chromosome preparations were performed according to the protocol as described by Tsuchiya and Nakamura (1979) with some modifications. Roots, approximately 1.0-2.0 cm long, cut from the plants were immersed in 0.002 M 8-hydroxyquinoline (Sigma, USA) for 2 h at room temperature followed by fixation in ethanol/glacial acetic acid (3:1) and stored at +4°C until use. Hydrolytic maceration was done in 1N HC1 at 60°C for 10 minutes. Root tips were stained with 2% acetocarmine and kept for about 3 or 4 days at 4 °C. The root cap of stained-root tips was removed before squashing and samples were squashed on a glass slide. Chromosome counts were performed under the light microscope (Motic, BA210) for at least five metaphase cells.

3. RESULT AND DISCUSSION

The seeds of *V. scamandri* were successfully germinated on MS medium and the seedlings were healthy grown *in vitro* for 14 weeks (Figure 1a). In this work, the adventitious shoot induction has occurred from leaf explants on MS medium containing 2 mg/L Kin and 0.1 mg/L 2,4-D. The shoots were propagated and rooted on MS medium without plant growth regulators. The propagated plants were grown for 9 weeks (Figure 1b). Callus induction was achieved by culturing leaf explants on MS medium containing 1 mg/L Kin, 1 mg/L 2,4-D within 3-4 weeks of culture (Figure 1c). 14-week-old *in vitro* grown plants, 9-week-old propagated plantlets, and callus tissues of *V. scamandri* were used for analyses.

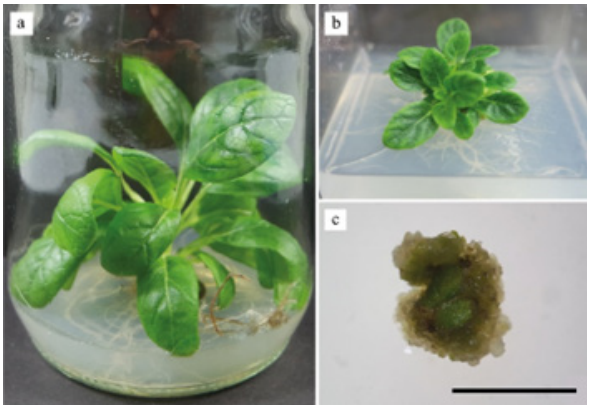

Figure 1. 14-week-old *in vitro* seedling (a), 9-week-old propagated plantlet of *V. scamandri* (b), callus tissue induced from leaf explants cultured on MS medium with 1 mg/L Kin + 1 mg/L 2,4-D (c) (bar = 5mm)

The nuclear DNA content of *V. scamandri* was estimated using flow cytometry with *L. esculentum* (1.96 pg/2C) as an internal standard plant. *L. esculentum* was excellent as an internal standard for *V. scamandri* since *V. scamandri* G1 peak was distinguishable from the *L. esculentum* G1 peak (Figure 2). Based on the flow cytometric analysis of nuclear DNA content, the *in vitro* grown plants, propagated plantlets, and callus tissues all have very similar amounts of DNA. The mean nuclear content of seed derived plants of *V. scamandri* was determined as 0.73 pg/2C ± 0.01, while callus tissues and the leaf of propagated plantlets had a slightly higher DNA content with 0.76 pg/2C ± 0.02 and 0.79 pg/2C ± 0.01, respectively. The flow cytometry analysis indicated that nuclear DNA content remained stable during the successive subcultures.

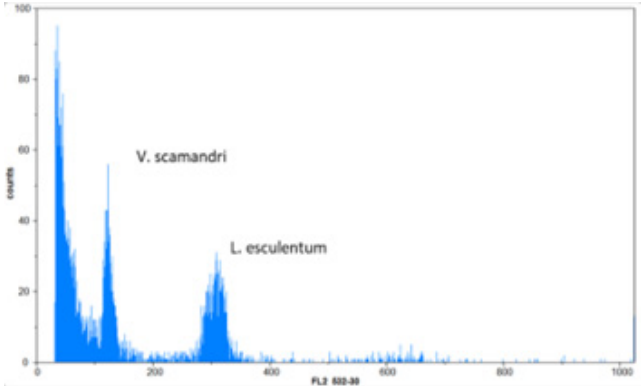

Figure 2. Relative positions of G1 peaks of *V. scamandri* and standard

The 2C DNA content of some *Verbascum* species previously was reported. Based on the results of the previous study using flow cytometry, the nuclear DNA contents of *V. levanticum, V. litigiosum*, and *V. pulverulentum* were reported as 0.75 2C/pg, 0.76 2C/pg, and 0.78 2C/pg, respectively. The previously analysed nuclear DNA content of species indicated similar results obtained in the present study. On the other hand, the nuclear DNA content of *V. virgatum* was reported as 1.44 2C/pg, which is approximately two-fold of other analysed species (Castro et al., 2012).

In *V. scamandri* genome, analyses of leaves from *in vitro* grown plants and *in vitro* propagated plants had similar nuclear DNA content. Similarly, the *in vitro* culture of *Plantago asiatica* produced genetically stable material. The nuclear DNA content of the leaves of the following seedling and plant materials of *P. asiatica* cultured *in vitro* ranged from 2.97 to 3.45 pg/2C using flow cytometry. It was reported that *in vitro* culture material produces genetically stable material since the nuclear DNA content of the samples was similar to the source of the material (Makowczyńska et al., 2008). Çördük et al. (2017) reported DNA ploidy levels remained

stable *in vitro* cultures cloned of *Digitalis trojana* since regenerated plantlets and seed derived plants had 2.80±0.03 pg and nuclear DNA 2.80±0.1 pg/2C nuclear DNA content with same chromosome number (2*n*=56) respectively. On the other hand, somaclonal variation had been determined in different species such as *Plumbago zeylanica* (Sivanesan, 2007) and rice cultivar (Araújo et al., 2001). It has been reported that somaclonal variation is particularly common in plants regenerated from callus (Bhatia and Sharma, 2015; Çördük et al., 2017).

Based on cytological investigations and mean nuclear DNA content analyses chromosome number of the *in vitro* grown plants was determined 2*n* = 32 with 0.73 pg/2C ± 0.01 (Figure 3). In genus *Verbascum* wide range of chromosome number variation is reported for example, 2n = 18, 24, 28, 30, 32, 34, 36, 40, 48, 44, 52, 58 (Benedi et al., 2009; Petrova and Vladimirov 2020). Dysploidy was suggested as the possible reason for variability in chromosome number in *Verbascum* (Castro et al., 2012). Numerical and structural changes in chromosomes are important mechanisms that can drive speciation and diversification in plants (Lysak and Weiss-Schneeweiss, 2021).

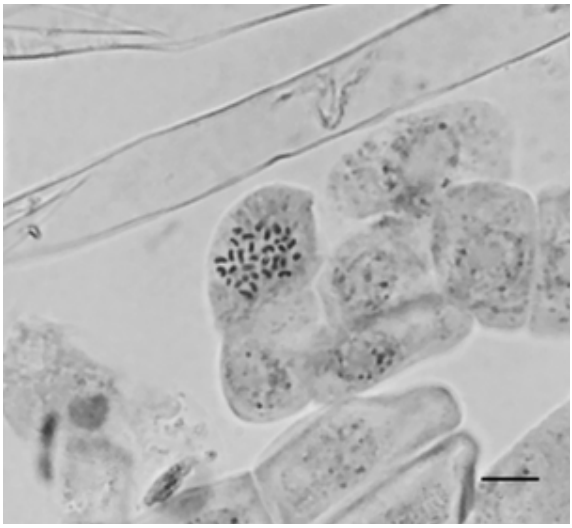

Figure 3. The mitotic chromosomes of *V. scamandri*, 2*n* = 32 (scale bar =10 µm)

4. CONCLUSION

In conclusion, chromosome number of endemic *V. scamandri* was determined 2*n* = 32. The mean nuclear DNA content of *V. scamandri* was determined 0.73 pg/2C. In addition to that analysis of *V. scamandri* using flow cytometry has proven that flow cytometry is a rapid and simple technique to estimate nuclear DNA con-

tent in plant genome analyses. It was determined that the culture conditions were suitable for *in vitro* propagation of this species since no somaclonal variation was occurred during the culture. According to the flow cytometry results, regenerated plants had similar nuclear DNA content to the source of the material. Additionally, this allowed us to control the nuclear DNA content stability during *in vitro* culture.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethics

This study does not require ethics committee approval.

Author Contribution Rates

Design of Study: NÇ(%80), GY(%20)

Data Acquisition: EC(%60), NÇ(%20), GY(%20)

Data Analysis: EC(%60), NÇ(%20), GY(%20)

Writing Up: NÇ(%50), GY(%40), EC(%10)

Submission and Revision: NÇ(%60), GY(%40)

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