

In vitro Üretilen *Digitalis Trojana* Ivanina Bitkilerinin Genetik Kararlılığının Flow Sitometri ve Sitolojik Analizler ile Değerlendirilmesi

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Bu çalışmada, *Digitalis trojana* Ivanina türüne ait dolaylı sürgün organogenezis ile üretilen bitkilerin genetik kararlılığı flow sitometri ve sitolojik analizler ile değerlendirilmiştir. İlk aşamada yaprak eksplantları 3 mg/L BA + 0,1 mg/L NAA içeren MS besin ortamında kültüre alınmıştır. Bu ortamda dolaylı organogenez yoluyla oluşan sürgünler 2 haftada bir altkültüre alınarak çoğaltılmıştır. Bitkiler % 0.1 aktif karbon içeren MS besin ortamında köklendirilmiştir. Üretilen bitkilerin genetik kararlılığı flow sitometri ve sitolojik analizler ile tohumdan yetiştirilen *D. trojana* bitki örnekleri ile karşılaştırılarak değerlendirilmiştir. Flow sitometri analizinde, *in vitro* üretilen bitki örneklerinin DNA içeriği 2.80±0.03 piko gram, tohumdan yetiştirilen bitki örneklerinin DNA içeriği ise 2.80±0.1 piko gram belirlenmiştir. Bitkinin mikroskop ile yapılan mitoz kromozomu sayımlarında kromozom sayısının 56 olduğu ve *in vitro* üretilen bitkilerin kromozom sayısında bir değişiklik olmadığı belirlenmiştir. Yapılan analizler sonucunda 3 mg/L BA + 0,1 mg/L NAA içeren MS besin ortamında üretilen bitkilerde genetik farklılık görülmemiştir. Yapılan bu çalışma ile *D. trojana* türünün ilk kez DNA içeriği ve kromozom sayısı belirlenmiştir. Elde edilen sonuçlar, türün *in vitro* kültüre alınması sırasında kültür koşullarından dolayı meydana gelebilecek olan genetik varyasyonların analizinde flow sitometrinin kullanılabileceğini göstermektedir.

Anahtar kelimeler: C-değeri, kromozom sayısı, flow sitometri, çekirdek DNA içeriği, Yüksük otu, somaklonal varyasyon

Assessment of The Genetic Stability of Indirect Shoot Organogenesis-Derived Plantlets of *Digitalis Trojana* Ivanina by Flow Cytometry and Cytological Analyses

In this study, flow cytometry and cytological analysis was used to evaluate the genetic stability of *Digitalis trojana* Ivanina plants regenerated via indirect shoot organogenesis. For *in vitro* propagation, leaf explants were excised from seedlings grown in sterile conditions and cultured MS medium supplemented with 3.0 mg/L BA + 0.1 mg/L NAA. Shoots and calli were subcultured for a period of 2 weeks for shoot multiplication. For rooting, shoots were separated individually and transferred to MS medium containing 0.1% activated charcoal. Genetic stability of the regenerated plants was assessed by flow cytometry and cytological analyses. Flow cytometric analysis revealed that regenerated plantlets has as 2.80±0.03 pg nuclear DNA (2C) and seed-derived plants has on average 2.80±0.1 pg/2C. Cytological analysis showed that regenerated plantlets have the same number of chromosome with seed-derived plantlets of *D. trojana* (2n=56). Our results have showed that the plantlets propagated in MS medium with 3 mg/L BA + 0.1 mg/L NAA did not differ genetically from donor plants. Therefore, this system can be effective and suitable for clonal propagation of *D. trojana*. Our results also confirmed that flow cytometry is fast, easy, accurate and relatively cheap method to determine ploidy of *in vitro* propagated *D. trojana* plantlets.

Key words: C-value, chromosome number, flow cytometry, nuclear DNA content, foxglove, somaclonal variation

Introduction

The genus *Digitalis* L., commonly known as the foxglove, is a member of the Plantaginaceae and encompasses approximately 20 species (. All

Digitalis species are biennial or perennial herbs, rarely small shrubs with simple, alternate leaves, which are often crowded in basal rosettes (Bräuchler et al 2004; Albachten et al 2005). The

genus *Digitalis* is medicinally important group because of these cardenolides applied in human medicine. They have been used therapeutically for the treatment of cardiac insufficiency for more than 1500 years (Herrera et al 1990; Haux 1999), moreover several interesting anticancer effects have been observed in *Digitalis* (Winnicka et al 2006). This genus is native to western and southwestern Europe, western and central Asia, Australasia and northwestern Africa. *Digitalis* species is represented by eight species and two subspecies, including four endemic, in Turkey. Among these species, *Digitalis trojana* Ivanina is an endemic and distributed in Kazdağı (Mt. Ida), Balıkesir-Canakkale, Turkey (Uysal and Öztürk 1991). This plant has been marked as a vulnerable (VU) in Red Data Book of Turkish Plants (Ekim et al 2000). Lungeanu (1973), Yakar Tan (1979) and Baltisberger (1991) were reported chromosome number of *D. trojana* as $2n=56$. In recent years, some researchers have been reported about *in vitro* studies and cardenolides content of *D. trojana* (Çördük and Akı 2010; 2013; 2014, Verma et al 2012).

Plant tissue culture is a technique of culturing plant cells, tissues and organs on artificial plant growth medium under aseptic controlled environmental conditions such as light, temperature and humidity to produce new cells, tissues, organs or whole plants. *In vitro* culture technologies are now essential component of plant genetic resources management and they are becoming increasingly important for the *ex situ* conservation of rare and endangered plants (Benson et al 2000). Micropropagation is one of the tissue culture techniques that refer to the process of asexual reproduction by multiplication of genetically identical copies of individual plants. When plants have cultured for propagation, propagated plants are expect to be genetically uniform and genetically same as donor plants. However, tissue culture conditions such as plant growth regulators (PGRs) and other applied chemicals, callus stage, number of subculture, explant source may cause environmental stress and induce genetic and epigenetic variations in regenerated plants (Bairu et al 2011). In consequence, genetic or epigenetic modifications may be spontaneously occurred in regenerated plants during the culture process. These modifications have widely known as somaclonal variation. These variations are frequently epigenetic and unstable and as such not inherited but when they are genetic and stably inherited. As

a result of genetic variation, regenerated plants derived from organ cultures, calli and somatic embryos can be varied from the donor plants in phenotypic, physiologic, cytological and molecular characteristics (Sangthong et al 2005). Similar variations have been observed in many studies (Larkin and Scowcroft 1981; Vazquez 2001; Orbovic et al 2008; Rodriguez-Enriquez et al. 2011; Neelakandan and Wang 2012; Wang and Wang 2012; Slazak et al 2015).

Somaclonal variation is unexpected and mostly undesired phenomenon in propagation. Therefore, *in vitro* propagated plants have to be screened to ensure genetic uniformity of the plants. There are a wide variety of molecular methods available to characterize plant genomes (Henry 1998). Most of them have potential for the analysis of somaclonal variation. There have been numerous attempts to detect somaclonal variations by using cytogenetic analysis including chromosome counting and/or flow cytometry and molecular markers such as isozymes, restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) simple sequence repeat (SSR) and inter-simple sequence repeat (ISSR) (Orbovic et al 2008; Leva et al 2012). Among these attempts, flow cytometry has been shown to be an easy, quick, accurate and relatively economic method to screen genetic stability of plants propagated through tissue culture techniques (Johnston et al 1999; Bennet et al 2000; Thiem and Sliwinska 2003; Dolezel and Bartos 2005; Ochatt et al 2011; Pasqual et al 2012; Singh et al 2013; Thiem et al 2013; Nybom et al 2014).

A plant nuclear DNA content refers to the total amount of DNA in nucleus. It is commonly measured as the C value and 1C represents the amount of DNA contained within a haploid nucleus. Genome DNA content remains constant in both different cells of the same individual and different individuals of a species. At the molecular level, variations in tissue culture-derived plants arise from changes in chromosome number or structure, or from more subtle changes in the DNA. Flow cytometry has been used to determine genome size and polyploidy levels of *in vitro* regenerated plants in various plant species (Sangthong et al 2005; Orbovic et al 2008; Leva et al 2012; Pasqual et al 2012; Stanisic et al 2015). The main objective of present study is to evaluate the genetic stability of *Digitalis trojana* Ivanina

plants regenerated via indirect shoot organogenesis by flow cytometry and cytological analysis.

Materials and methods

Plant materials

Plant samples and seeds of *Digitalis trojana* were collected from the Kazdağı National Park, Turkey. Plant samples were prepared as herbarium materials and voucher specimens were deposited in the Herbarium of Canakkale West Anatolia (CBB, Canakkale, Turkey).

Propagation of *Digitalis trojana*

Propagation of *Digitalis trojana* was achieved as previously described by Çördük and Akı (2010). *D. trojana* seeds were sterilized with 3% sodium hypochlorite and 0.1% Tween 20 for 20 min and then rinsed with sterile dH₂O. After surface sterilization, seeds were germinated aseptically on MS (Murashige and Skoog, 1962) medium (M0222, Duchefa Biochemie B.V., Haarlem, Netherlands) supplemented with 3% (w/v) sucrose (S0809, Duchefa) and 0.7% (w/v) agar (P1001, Duchefa). The pH of medium was adjusted 5.75 before adding agar and then autoclaved at 121°C for 15 min. For *in vitro* propagation via organogenesis, leaf explants were excised from seedlings grown in sterile conditions and cultured MS medium supplemented with 3.0 mg/l 6-benzylaminopurine (BA; B0904, Duchefa), 0.1 mg/L α -naphthaleneacetic acid (NAA; N0903, Duchefa), 3% (w/v) sucrose, 0.7% (w/v) agar and 2 mg/L (w/v) polyvinylpyrrolidone (PVP; Sigma N0640) at a density of ten explants per plate. Shoots were subcultured for a period of two weeks and subdivided repeatedly for shoot multiplication. For rooting, shoots were separated individually and transferred to MS medium containing 0.1% (w/v) activated charcoal. Each treatment had ten plates with ten explants per plate. All cultures were maintained in growth chambers at 25±2°C under 16/8 h photoperiod with 72µmol m⁻²s⁻¹.

Flow cytometry analysis

Flow cytometric analyses were carried on fresh leaf tissues from randomly chosen healthy and fully developed plantlets by using flow cytometry

(Partec CyFlowR Space) in Laboratory of Plant Genetics and Cytogenetics at Namık Kemal University. A Partec commercial kit (CyStain PI absolute P) was used in isolation of nuclei. Propidium iodide was used as fluorescent dye. *Vicia sativa*, which has 3.65 pg/2C DNA content, was used as internal standard. Briefly, the procedure is as below: approximately 20 mg fresh leaf of sample (*D. trojana*) and 40 mg fresh leaf of internal standard were placed into a petri dish including 500 µl of extraction buffer. The tissues were chopped with a razor blade into small pieces for 30-40 seconds. The solution was transferred into a glass tube through a filter. 2000 µl staining buffer was added to the glass tube and incubated for 1 h. The absolute DNA content of *D. trojana* plants were calculated based on ratios of the G1 peak means of sample and internal standard in three replicates per samples. The mean DNA content per plant was based on the 2000 scanned nuclei.

Mitotic metaphase chromosomes counting

Cytological analysis was done on root tips of *in vitro* regenerated plants. Chromosome counts were done on slides made according to acetocarmine squash protocol as described by Tsuchiya and Nakamura (1979) with some modifications. Root tips, approximately 1.5 cm in length, were cut and immediately pre-treated with 8-hydroxyquinoline (Sigma, USA) for 4 h at room temperature and then fixed in Farmer's solution (3:1 absolute ethanol:glacial acetic acid) and stored at 4°C for at least 24 h. For mitotic analysis, root tips were stained with 2% acetocarmine and kept 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 under 45% acetic acid. The chromosome numbers and structures were observed under the light microscope to count chromosome number and determine ploidy level at least five metaphase cells in each samples

Results

In vitro culture

Induction of indirect shoot regeneration of *D. trojana* were occurred via culturing on MS medium containing 3 mg/L BA and 0.1 mg/L NAA. Leaf explants were cultured on MS medium containing 3 mg/L BA and 0.1 mg/L NAA. This treatment promoted callus induction within 7d in

culture. Compact green calli were appeared at the cut ends of leaf explants within 2 weeks. During the following week, dark green regions appeared on the calli surfaces and became organogenic. Adventitious shoots began to develop from these green regions after 3-4 weeks in culture. Adventitious shoots occurred in 100 % of explants on MS medium with PGRs. All adventitious shoots obtained after 4 weeks in culture were propagated on MS medium with 3.0 mg/L BA + 0.1 mg/L NAA. Shoots and calli were subcultured for a period of 2 weeks for shoot multiplication on MS

medium with 3.0 mg/L BA + 0.1 mg/L NAA. All elongated shoots (2-3 cm long) were transferred to MS medium with 0.1% activated charcoal for root induction. However root initiation occurred longer than 2 weeks at 100 % of adventitious shoots (Fig 1). At the end of the experiment, regenerated plantlets developed fully and looked healthy. When regenerated plants were compare with control plants, anatomic and morphologic differences were not observed between regenerated plants and control plants.

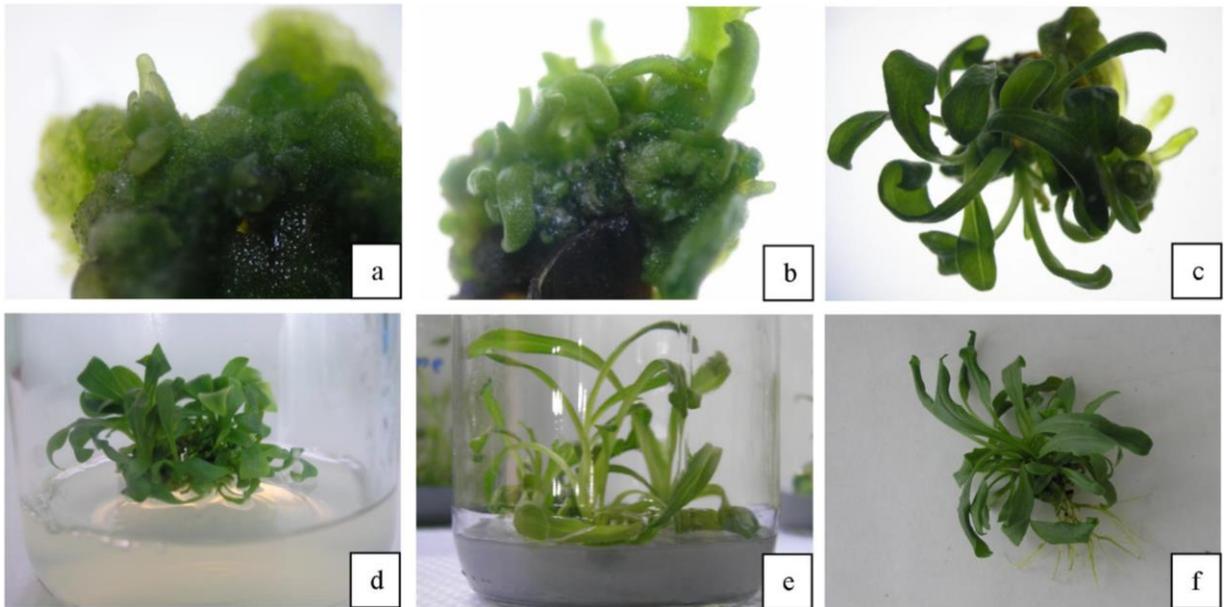


Fig 1. In vitro propagation of *D. trojana* via indirect shoot organogenesis in MS medium containing 3 mg/L BA and 0.1 mg/L NAA a) induction of adventitious shoots from leaf explants b) adventitious shoots c) propagation of shoots d) elongated shoots e) root induction on MS medium with 0.1% activated charcoal f) regenerated whole plantlets.

Flow cytometry analysis

Based on the flow cytometric analysis, nuclear DNA content of both regenerated plantlets and seed derived plants were very similar while Nuclear DNA content of *in vitro* propagated *D. trojana*

plantlets and seed-derived *D. trojana* plantlets determined as 2.80 ± 0.03 pg/2C and 2.80 ± 0.1 pg/2C respectively (Fig 2). The flow cytometry analysis have showed that the DNA ploidy level remained stable in all cloned *D. trojana* plantlets during the successive subcultures in the multiplication medium.

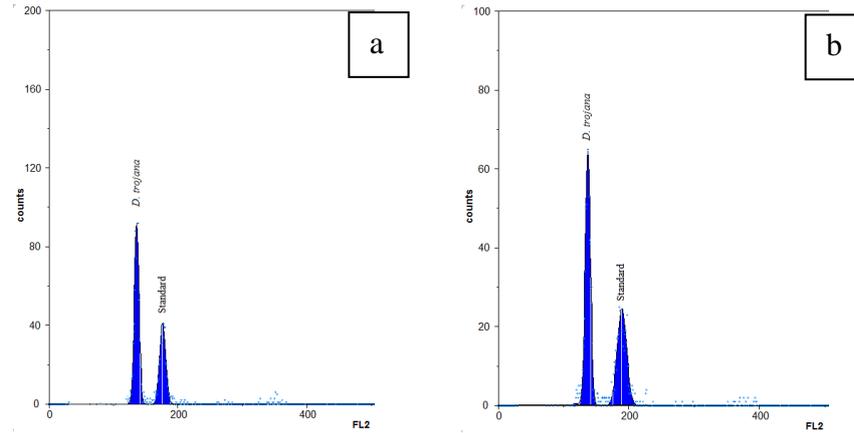


Fig 2. Flow cytometry analysis. a) Relative positions of G1 peaks of regenerated *D. trojana* (first peak) and internal control of *Vicia sativa* (second peak) b) Relative positions of G1 peaks of *D. trojana* (first peak) and internal control of *Vicia sativa* (second peak).

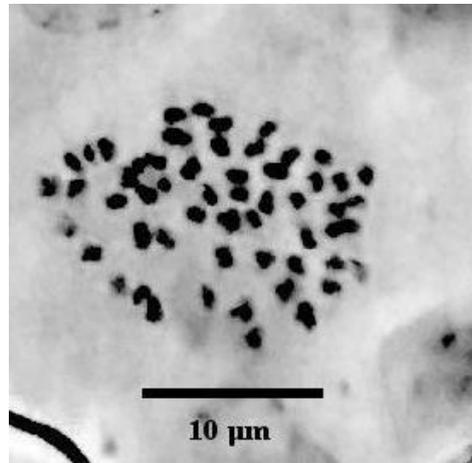


Fig 3. Mitotic metaphase chromosomes of in vitro propagated *Digitalis trojana* plants ($2n=56$)

Mitotic metaphase chromosomes counting

The mitotic metaphase chromosomes of *in vitro* propagated *D. trojana* was count in root-tip cells and compared with seed-derived plantlets ($2n=56$). Based on the mitotic metaphase chromosomes counting results, chromosome number variations did not observe at *in vitro* propagated *D. trojana* (Fig 3).

Discussion

In our study, we obtained a highly efficient and rapid indirect shoot regeneration protocol developed and optimized for *D. trojana*. Our results showed that any of the regenerated plantlets of *D. trojana* did not have genomic alterations and different ploidy level compared to seed-derived plants of *D. trojana*.

There are numbers of factors which determine the frequency of variation during *in vitro* culture. Our results showed that tissue culture conditions used in this study such as explant tissue source, type and concentration of plant growth regulators, media components, and number of subcultures may provide genomic stability of propagated plantlets of *D. trojana*.

It has been reported that explant types are important for prevention somaclonal variations. In this study, although we used leaf explants for propagation of *D. trojana*, any variation was not observed in propagated plants. It was reported that the use of highly differentiated tissues such as leaves, stems, and roots as starting material for tissue culture generally produce more variants than explants from axillary buds and shoot tips

which have pre-existing meristem (Kunitake et al 1995; Sahijram et al 2003; Sharma et al 2007; Leva et al 2012).

In this study, we carried out propagation of *D. trojana* via indirect shoot organogenesis. Indirect organogenesis means that adventitious shoots are induced after callus phase. Callus is defined as an unorganized cell mass growing on explant. When unorganized cell mass growing is occurred in culture, somaclonal variation may be occurred in these cells and regenerated plants from callus can be genetically different from donor plant (Karp 1994; Araújo et al 2001; Cooper et al 2006; Sivanesan 2007). Some reports have carried out that probability of occurrence of somaclonal variation is high at callus stage. We think that not to enhance duration of callus stage may be preventing variation in this study. We didn't extend callus stage for a long time and this duration callus stage in culture may be effective for genetic stability of regenerated plants of *D. trojana*.

In this study, a high rate of propagation of *D. trojana* is achieved in relatively shorter periods and leads to more frequent subculturing. This may be preventing somaclonal variation in culture. It is reported that number of subcultures is another factor for somaclonal variation. Some reports have demonstrated that callus induction stage in indirect organogenesis or number of subcultures especially can be caused somaclonal variation and regenerated plants from callus can be genetically different from donor plant as a result of somaclonal variation.

We used plant growth regulators in 0.1 mg/L (NAA) and 3.0 mg/L (BA) concentration. According to our results, plant growth regulators used 3.0 mg/L BA + 0.1 mg/L NAA did not reveal genetic alterations at any regenerated plantlets. Another factor for somaclonal variation is concentration of plant growth regulators. We think that plant growth regulators used in culture were not too high and it is also lead genetic stability. It is reported that higher concentration growth regulators may be lead genomic alterations. The presence of a relatively high concentration (15 mg/L) of BA was implicated the increase in chromosome number in a somaclonal variant. The genetic composition of a cell population can be influenced by the relative levels of both auxins and cytokinins (D'amato 1975). It was reported that micropropagation from preformed meristem is a common method of propagation for

conservation purposes, due to the low probability of somaclonal variation, although the use of auxin and cytokinin, usually essential for micropropagation, together with the pressure of the *in vitro* culture itself, may increase genetic instability (George 1993; Mallón et al 2010).

The cause of tissue-culture induced variation is cannot be controlled or predicted because it is not fully understood. Therefore, a reliable and quick method is needed for screening ploidy stability of the plants. Flow cytometry and cytological analyses can be used to screen ploidy level of regenerated plants. Based on the results of this study, flow cytometry is fast, easy, accurate and relatively cheap method to determine ploidy of *in vitro* propagated *D. trojana* plantlets. The determination of DNA content by flow cytometry requires that appropriate standard be included in the experimental design. The genome size should be considered for the choice and handling of internal standards. Peaks of internal standard and samples should not overlap. Furthermore it should not be too big difference between genome size of the sample and the standard to be selected. The difference between genome sizes should not be more than 3 times since the sensitivity decreases as the difference increases. Therefore we used *Vicia sativa* (3.65 pg/2C) as internal standard.

In conclusion, DNA content of *D. trojana* was determined for the first time in this study. Nuclear DNA content information is very useful information to study taxonomy, genetics and evolution of the plant species. The results of both flow cytometry and karyotype analysis demonstrate that the *in vitro* propagation protocol developed apparently did not induce any genetic changes in the regenerants. Results of this study confirmed that flow cytometry is a quick and effective method of estimating the ploidy and DNA content of propagated plants is needed to ensure that regenerated plants have a stable ploidy level.

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NÇ designed and supervised the study. NÇ performed the *in vitro* regeneration studies. GY and MT performed flow cytometry analyses. NA performed karyotype analyses.

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