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Research Article

# Assessment of Genetic Stability of Propagated Plants of *Alyssum caricum* Using Flow Cytometry

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## Abstract

This study aimed to investigate the nuclear DNA content of *A. caricum* using *in vitro*-grown plants and to analyse the genetic stability of *in vitro*-propagated plantlets through flow cytometry. The process involved germinating seeds on Murashige and Skoog medium with 3% sucrose and 0.7% phytoagar. The germinated seedlings were then cultured *in vitro* in a controlled environment. For the *in vitro* propagation experiment, nodal explants were cultured on three shoot induction media for 3-4 weeks. Subsequently, the adventitious shoots were transferred to the MS medium with the same formulations as the shoot induction medium to facilitate shoot propagation. The propagated adventitious shoots were then moved to full-strength MS medium with 3% sucrose and 0.7% phytoagar, without any plant growth regulators, to induce root development. The nuclear DNA content was estimated using flow cytometry for both the *in vitro* germinated seedlings and the propagated plantlets. The analyses revealed that *A. caricum* had 1.75 pg/2C±0.01 mean nuclear DNA content. Furthermore, the propagated plants exhibited similar nuclear DNA content. These results suggest that the analysed individuals were genetically stable. However, to obtain more comprehensive information about *A. caricum*, additional studies such as chromosome counting, genetic analyses are necessary.
**Keywords:** *A. caricum*, *In vitro* propagation, Nuclear DNA Content, Genetic stability, Flow cytometry.

## *In vitro* Çoğaltılan Endemik *Alyssum caricum* Bitkilerinin Flow Sitometri ile Genetik Stabilitelerinin Değerlendirilmesi

## Öz

Bu çalışmada, flow sitometri analizi ile *in vitro* yetiştirilmiş *A. caricum* bitkilerinin çekirdek DNA içeriğinin belirlenmesi, ayrıca *in vitro* çoğaltılan bitkilerin genetik stabilitelerinin değerlendirilmesi amaçlanmıştır. Bitkinin tohumları, 3% sukroz ve 0.7% fitoagar içeren Murashige ve Skoog besin ortamında çimlendirilmiştir. Çimlenen fideler kontrollü bir ortamda *in vitro* olarak büyütülmüştür. *In vitro* çoğaltım için nodal eksplantlar üç farklı sürgün indüksiyon ortamında 3-4 hafta kültüre alınmıştır. Adventif sürgünler, sürgün indüksiyon ortamı ile aynı formülasyonlara sahip MS besin ortamına çoğaltılmaları için aktarılmıştır. Çoğaltılan adventif sürgünler, daha sonra bitki büyüme düzenleyicileri içermeyen MS ortamına aktarılmıştır. *In vitro* yetiştirilen bitkiler ve *in vitro* çoğaltılan bitkiler flow sitometri ile analiz edilmiştir. Yapılan flow sitometri analizleri, *in vitro* yetiştirilen bitkilerin 1.75 pg/2C±0.01 DNA içeriğine sahip olduğunu göstermiştir. Sonuçlar, çoğaltılan bitkilerin tohumdan çimlenen bitkiler ile benzer DNA içeriğine sahip olduğunu göstermiştir. Bu sonuçlar, analiz edilen bireylerin genetik olarak stabil olduğunu önermektedir. Bununla birlikte, *A. caricum* hakkında daha kapsamlı bilgilere ulaşmak için kromozom sayımı, genetik analizler başta olmak üzere ek çalışmalar gerekmektedir.
**Anahtar Kelimeler:** *A. caricum*, *In vitro* çoğaltım, Çekirdek DNA içeriği, Genetik stabilite, Flow sitometri.

## Introduction

Various plant species, such as endangered, endemic, threatened, or commercial plants, can be propagated using plant tissue culture methods. The techniques can be utilized to obtain important number of the plants that are genetically similar to a parent plant and each other in a short time and independently of seasonal conditions. However, it should be noted that different key factors, such as plant genotype, donor age, the appropriate culture medium, and physical environmental factors are important for optimizing and enhancing the culture conditions (Hasnain et al., 2022; Çördük et al., 2023). Genetically uniform and equivalent to donor plants are expected for *in vitro* propagated plants. However, different factors such as plant growth regulator, the explant source, subculture number, or additional chemicals can induce environmental stress, commonly triggering genetic or epigenetic variations during the culture, known as somaclonal variation (Leljak-Levanic et al., 2004; Temel et al., 2008; Chinnusamy and Zhu, 2009; Lira-Medeiros et al., 2010). Therefore, *in vitro* propagated plants must be analysed to obtain genetic uniformity of the plants. Since somaclonal variation in plant tissue culture is associated differences in the amount of deoxyribonucleic acid, polyploidy and aneuploidy, evaluating of the genetic stability is significant step (Escobedo-Gracia-Medrano et al., 2018). Nuclear DNA content is fundamental important biological character of the genome which is essential data for many fields of research such as ploidy analysis (Ohri, 1988), breeding (Kron et al., 2007), and in different projects e.g. genome sequencing (Rabinowicz et al., 2006). Flow cytometry has become the common method to estimate nuclear DNA amount and ploidy level because it is reliable, convenient, fast (Dolezel et al., 2007). In plant biotechnology, flow cytometry is an important tool for the screening genetic stability of regenerated plants in the DNA ploidy level and DNA content (Escobedo-Gracia-Medrano et al., 2018).

The genus *Alyssum* (Brassicaceae) is distributed in Asia, Europe, America, and North Africa, and Eastern Mediterranean region which is the centre of diversity (Dudley, 1964; 1965; Li et al., 2014). The *Alyssum* genus is among the largest, with approximately 107 species and subspecies for the flora of Türkiye (Babaoğlu et al., 2006). The genus is known by the public as Kevke or Rabid weed. The rate of endemism is about 55% (Aytaç and Duman, 2000; Orcan and Mısırdalı, 2000; Adıgüzel and Reeves, 2002; Orcan 2002; Orcan, 2006; Orcan and Binzet, 2006; Yılmaz, 2012). *Alyssum* has been reported as a representative of the nickel hyperaccumulator genus with many of its taxa (Babaoğlu et al., 2006; Reeves and Adıgüzel, 2008). One of the nickel-hyperaccumulator *Alyssum* species is *Alyssum caricum* T.R.Dudley & Hub.-Mor. (Adıgüzel and Reeves, 2012). *A. caricum* is a perennial plant which distributed in Muğla and Denizli, Türkiye and shows a semi blush type (Çördük et al., 2023). The species is a serpentine endemic plant in the flora of Türkiye and is classified as an endangered (EN) category by the Red Data Book of Turkish Plants (Ekim et al., 2000; Yeşilyurt and Akaydın, 2012). Researches on *in vitro* propagation (Çördük et al., 2023), identification of the morphological and anatomical features of seeds (Karaismailoglu, 2022), antibacterial, antioxidant, and anti-biofilm activities (Tozyılmaz et al., 2021), determination of proteasome activity (Van Hoewyk et al., 2018), determination of the relative role of aqueous smoke and nitrate on seed germination (Çatav et al., 2015), and adsorption potentials of native and amine-modified plant biomass (Bayramoglu et al., 2013) of *A. caricum* have been reported, but, as far as we know, there has been no research conducted on the assessment of DNA content and genetic stability in *A. caricum* to date. Therefore, we aimed to estimate the nuclear DNA content of *in vitro* growing individuals of *A. caricum* and analyse genetic stability of *in vitro* propagated plantlets by flow cytometry.

## Material and Methods

### Plant Materials

Seeds of *A. caricum*, collected in previous studies (Çördük et al., 2023), were used as plant materials. Seeds were stored at 4 °C until use. All seeds were initially treated with 70% ethanol for 1 min and then sterilized in 5% (v/v) sodium hypochlorite with 0.1% Tween 20 approximately 20 minutes. The seeds were rinsed with sterile water at least five times. The disinfected seeds were cultivated on Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) with 3% (w/v) sucrose and 0.7% (w/v) phytoagar for germination and then transferred to the culture room at a temperature of 25±2°C, 60% relative humidity, and dark conditions. After 7 days, the seedlings were placed in the culture room at 25±2°C under 16 h light/8 h dark photoperiod, 60% humidity, and 72 µmol $m^{-2}s^{-1}$. All chemicals, including MS basal medium, 6-Benzylaminopurine (6-BAP), Kinetin

(KIN), Naphthalene Acetic Acid (NAA), Indole-3-Acetic Acid (IAA), sucrose, and phytoagar, were purchased from Duchefa Biochemie B.V., Haarlem, Netherlands.

*In vitro* propagation cultures of *A. caricum* were established according to the protocol optimized in a previous study (Çördük et. al., 2023). The nodal explants (ca. 5mm long) isolated aseptically from *in vitro* grown seedlings were transferred onto three shoot induction media (Medium A: full-strength MS medium containing 2 mg/L BAP+0.1 mg/L NAA, Medium B: full-strength MS medium containing 2 mg/L KIN+0.1 mg/L IAA, and Medium C: half-strength MS medium containing 2 mg/L BAP+0.1 mg/L NAA). The prepared cultures were maintained in the plant culture room (25±2°C, 16/8 h photoperiod with a light intensity of 72 µmol $m^{-2}$ $s^{-1}$). 3% (w/v) sucrose and 0.7% (w/v) phytoagar were added to all media. The pH of media was adjusted to 5.75, and the media were autoclaved at 121°C for 20 min. All cultures were retained for four weeks, then the adventitious shoots (ca. 1 cm in length) were sub-cultured onto the MS medium with the same formulations as the shoot induction medium to facilitate shoot propagation within eight weeks of culture. The propagated adventitious shoots (2-3 cm) were transferred onto full-strength MS with 3% sucrose and 0.7% phytoagar without plant growth regulators for root induction.

**Nuclear DNA Content Estimation**

The nuclear DNA content of *A. caricum* samples was determined by the flow cytometer (Partec, CyFlow® Space Münster, Germany). *Vicia sativa* (3.95 2C/pg) used as an internal standard. The intact nuclei suspension was prepared from the young and healthy leaves of propagated plants and *in vitro* grown plants. The intact nuclei suspensions were prepared using commercial kits manufactured by Sysmex Partec GmbH. The healthy and fresh leaf of sample (20 mg) and leaf tissue from standard (50 mg) 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 prepared homogenized solution was transferred into a glass tube through a 30 µm filter. A 2 µl of staining buffer (CyStain PI Absolute P) was transferred to each tube. Before analysis, the prepared samples were kept at room temperature in the dark for approximately 1 hour. 2C nuclear DNA content of analysed samples were calculated using the ratios of the G1 peak means of the sample and the internal standard with three replicates per sample using the following equation: Nuclear DNA content of sample = (Fluorescence intensity of sample (mean of G1 peak) / Fluorescence intensity of standard (mean of G1 peak) × Known DNA content of standard (pg).

**Results and Discussion**

The seeds were successfully germinated on MS medium. The *in vitro* germinated seedlings were healthy and grown *in vitro* conditions for 23 weeks (Figure 1). Shoot induction occurred on nodal explants in all shoot induction media in 3-4 weeks of culture via indirect organogenesis. The adventitious shoots were successfully propagated on MS medium containing the same plant growth regulators as the shoot induction medium. The shoots obtained via multiple shoot induction and organogenesis were successfully developed and rooted on full-strength MS medium without plant growth regulators (Figure 2).

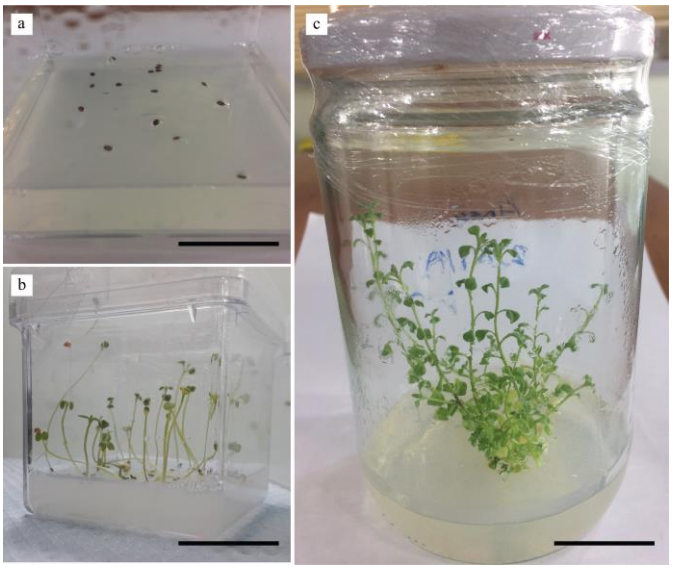


**Figure 1. a)** *A. caricum* seeds on MS medium for germination; **b)** germinated seedlings of *A. caricum*; **c)** 23-week-old plants derived from *in vitro* germinated seeds (bar=1 cm).

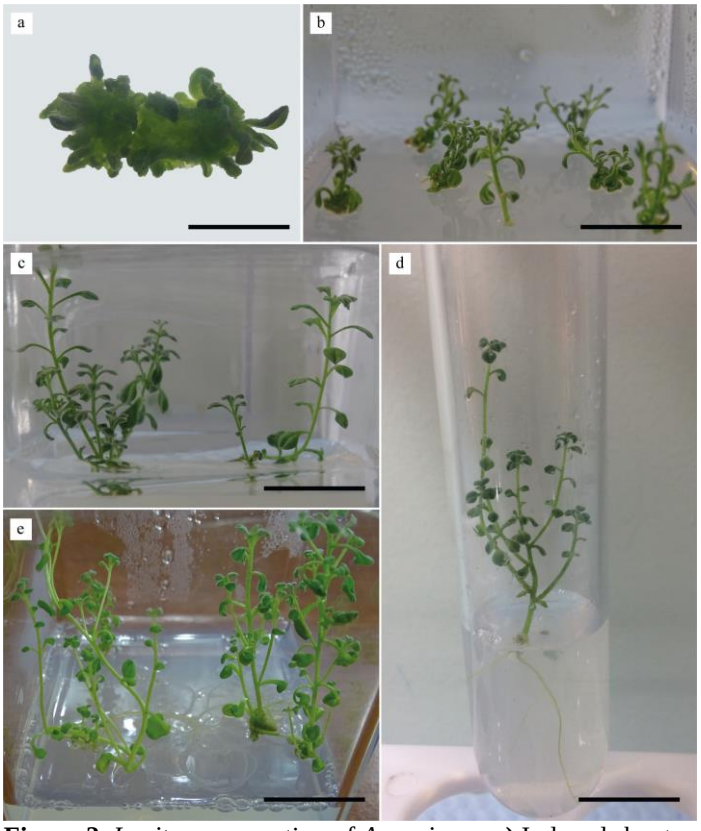


**Figure 2.** *In vitro* propagation of *A. caricum*. **a)** Induced shoots on nodal segment in 4 weeks of culture; **b-c)** The adventitious shoots on MS medium containing PGRs in in 12 weeks of culture; **d)** Rooted plants on MS medium without plant growth regulators **e)** propagated plantlets (bar=1 cm).

The *in vitro* germinated seedlings and the propagated plantlets were used to estimate the mean nuclear DNA contents using flow cytometry. Technically, the analyses of the nuclear DNA content were efficient in *in vitro* grown and propagated individuals of *A. caricum*. Flow cytometric analysis resulted in high resolution histograms for each analysed individual plant (Figure 3). The mean nuclear DNA content of *A. caricum* was detected using flow cytometry with *V. sativa* (3.65 pg/2C) as an internal standard plant. *V. sativa* was excellent as an internal standard for *A. caricum* nuclear DNA

content analyses since analysed plant G1 peak was clearly distinguishable from the standard plant G1 peak. According to the flow cytometric analysis, *in vitro* grown plants and propagated plantlets have similar amounts of nuclear DNA. Flow cytometry results indicated that *in vitro* grown plants (23-week-old) had 1.75 pg/2C ± 0.01 mean nuclear DNA content, whereas propagated plants had (Medium A) 1.65 pg/2C± 0.01, (Medium B)1.67 pg/2C ± 0.1, and (Medium C) 1.69 pg/2C ± 0.1, which were slightly lower than seed derived plantlets.

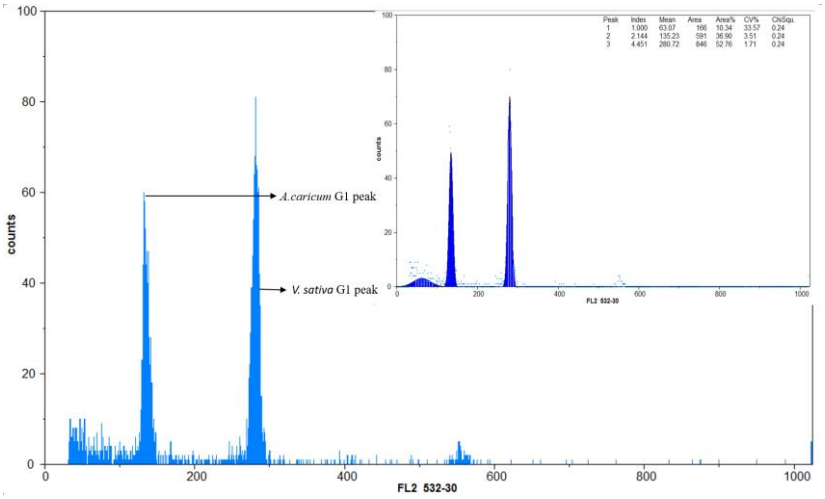


**Figure 3.** Relative positions of the G1 peaks of *A. caricum* L. and internal standard (*Vicia sativa*).

It was previously reported that, analyses of materials from *in vitro* grown plants and propagated plants had similar nuclear DNA content, e.g., *Digitalis trojana* (about 2.80 pg/2C; Çördük et al., 2017) and *Verbascum scamandri* (from 0.73 pg/2C to 0.79 pg/2C; Yücel et al., 2023). In another studies, *in vitro* cultures of *Plantago asiatica* (Makowczynska et al., 2008) and *Silene vulgaris* (Çördük et al., 2018), showed that produce genetically stable material since the 2C DNA content of *in vitro* cultures was similar to the source of the material. Mirzaei et al. (2021) reported that since the genetic stability of olive during *in vitro* culture is important for elite olive genotype selection the genetic stability of the propagated materials was evaluated based on flow cytometry analyses. *Salix myrtilloides* has been reported as a relict species which is extinction in some Central European countries. The genetic and flow cytometry analyses with tissue culture materials of *S. myrtilloides* indicated that plants are genetically identical to the mother ones, and conducted research was suggested to use the studied protocol for propagate *S. myrtilloides* (Parzymies et al., 2023). The stability of the ploidy level in regenerated *Cucumis melo* was verified by a flow cytometry. In addition, the combination of BAP and NOA (Naphthoxyacetic Acid) suggested more convenient for the tissue culture of *C. melo* (Raji and Farajpour, 2021). The flow cytometry is used to detect DNA content and ploidy level also in regenerated plant to verifying the stability in different plants e.g. *Musa acuminata* cv. Grand Naine (Escobedo-Gracia-Medrano et al., 2018). As we can see from the previous studies, genetic stability is important from different perspectives, which makes it necessary character to detect it. In the present study, flow cytometry was used successfully to analyse the mean nuclear DNA content of *A. caricum* and evaluate its genetic stability as well.

## Conclusion

In conclusion, *Alyssum caricum* had 1.75 pg/2C±0.01 mean nuclear DNA content. The mean nuclear DNA content was similar during the successive subcultures which may suggest that the culture conditions were suitable for *in vitro* propagation of this species since no variation based on flow cytometric analyses occurred during the culture. This confirms that tissue culture methods and conditions can be useful for *A. caricum* regeneration. However, more comprehensive studies, e.g., chromosome counting, different tissue culture conditions, genetic analyses and more sample analyses are necessary to have comprehensive information about *A. caricum.*

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## Authors' Contributions

The authors declare that they have contributed equally to the article.

## Conflicts of Interest Statement

The Authors declare that they have no conflict of interest.

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