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# GENETIC AND PHENOTYPIC STABILITY OF THE TOMATO CULTIVAR 'ARGEȘ 20' REGENERATED VIA *IN VITRO* ANTHER CULTURE USING SSR MARKERS

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***ABSTRACT.*** *Anther culture is essential for obtaining haploid and double-haploid plants, accelerating breeding programs. However, the somaclonal variation associated with the process remains a concern, requiring detailed verification of genetic stability. The present study evaluated the genetic stability of the tomato cultivar` Argeș 20´regenerated by in vitro anther culture over three years using SSR molecular markers, phenotypic, and morphological characteristics. SSR markers were applied to detect genetic polymorphisms between the regenerants and the donor plant, indicating a high level of genetic fidelity. Phenotypic traits, such as fruit size, yield, and morphological characteristics, were analyzed according to international standards. Data collected in three consecutive seasons showed high consistency without significant deviations from the original variety. The results confirm the genetic and phenotypic stability of the regenerants, highlighting the potential of anther culture in ensuring genetic uniformity. The study contributes to understanding tissue culture-induced variation and provides practical support for using the technique in crop breeding programs.*

***KEYWORDS:*** *Solanum lycopersicum, biotechnology, in vitro culture, SSR markers*

## INTRODUCTION

*Solanum lycopersicum* L. (tomato), a species native to the Peru-Ecuador region and a member of the Solanaceae family, is one of the most economically and agronomically essential vegetable crops worldwide (Zhao et al. 2014, Kumar et al. 2023). Despite advances in plant biotechnology, tomato remains recalcitrant to in vitro culture, posing significant challenges to the efficient production of double haploids (Saeed et al. 2019, Marin-Montes et al. 2022). Nevertheless, its potential for regeneration via various in vitro morphogenetic pathways presents valuable opportunities for both fundamental research and applied breeding programs (Seguí-Simarro & Nuez 2007). Accurate determination of the ploidy level in regenerated structures and the identification of the developmental origin of anther-derived plants are critical for validating the outcomes of these techniques. Such analyses can be reliably conducted using high-resolution methodologies, including flow cytometry and cytogenetic approaches (Julião et al. 2015, Sumedrea et al. 2024). Plant regeneration may originate from adult tissues, callus masses, or even single cells, depending on the culture conditions and developmental cues (Long et al. 2022). The foundational concept of plant tissue culture, introduced over a century ago, was aimed at achieving complete plant regeneration from somatic cells cultured in vitro (Long et al. 2022). Complementing conventional breeding strategies, biotechnological approaches offer promising alternatives for crop improvement, particularly in recalcitrant species such as tomato (Seguí-Simarro et al. 2011, Popescu et al. 2022). Initially developed in 1964, the anther culture technique has been extensively employed to obtain haploid plants in a wide range of crop species. The method involves the excision of closed floral buds, followed by surface sterilization and the isolation of anthers containing uninucleate microspores—the developmental stage considered optimal for the induction of androgenesis (Chu 1982, Maheshwari et al. 1982, Bădulescu et al. 2022). The regeneration potential of plants derived from in vitro anther culture is influenced by several critical factors, including: (a) the use of growth regulators and the specific composition of the basal culture medium (Sundararajan et al. 2017, Chimdessa 2020, Gerdakaneh et al. 2020, Bădulescu et al. 2022); (b) the type of explant and its interaction with the culture medium (Zhao et al. 2014, Dhar & Joshi 2005, Minutolo et al. 2020, Long et al. 2022); and (c) pretreatment conditions applied to floral buds and the induction of stress factors, which play a pivotal role in triggering cellular

reprogramming during in vitro androgenesis (Motallebi-Azar 2020, Popescu et al. 2022). In this context, the present study aims to evaluate the genetic stability of the 'Argeș 20' tomato cultivar, regenerated through anther culture over three years. The research focuses on determining the extent to which the androgenic regeneration process influences the plant's genetic characteristics and on identifying potential genetic alterations induced by this method. These findings are intended to enhance the current understanding of genetic stability in in vitro regenerated tomato plants and to support the applicability of anther culture in breeding programs targeting recalcitrant cultivars.

## MATERIALS AND METHODS

### Plant material

The tomato cultivar ´Arges 20´ was obtained through genetic selection in the population of the variety ´Arges 11´, it is a tomato variety with determinate growth with the fruit weighing around 200g and good tolerance to specific pests and diseases - the cultivar was approved at the National Institute for Research and Development in Horticultural Biotechnology Ştefăneşti-Argeş, Romania. Six plants regenerated from *in vitro* and acclimation cultures were morphologically and molecularly analyzed for three growing seasons, starting from the first year of in vitro culture. Tomatoes were grown in an unheated vegetable greenhouse following the cultivation technology (70 cm row spacing and 50 cm plant spacing), and seeds were harvested each year at full maturity (Popescu et al. 2022).

### Morphological characterization of regenerated tomato plants

The morphological and phenotypic characteristics of tomato plants regenerated from *in vitro* culture during three growing seasons were recorded according to the "Descriptors for Tomatoes (*Lycopersicon* spp.)" established by the International Plant Genetic Resources Institute in 1996. The description primarily focused on the fruits, as recommended by the procedure, for the 3rd fruit from the second and/or third ripening stage, when the tomatoes are at full maturity. Data were recorded for 16 morphological descriptors of the fruits: predominant fruit shape (7.2.2.5), fruit size (7.2.2.6), fruit size uniformity (7.2.2.7), fruit weight [g] (7.2.2.8), fruit length [mm] (7.2.2.9), fruit width [mm] (7.2.2.10), exterior color of mature fruit(7.2.2.11), the intensity of the exterior color (7.2.2.12), ribbing at calyx end (7.2.2.14), fruit shoulder shape (7.2.2.16), flesh color of pericarp (interior) (7.2.2.26), flesh color intensity (7.2.2.27), fruit cross-sectional shape (7.2.2.29), number of locules (7.2.2.31), shape of pistil scar (7.2.2.32), fruit blossom end shape (7.2.2.33) and the presence of the

normal seeds.

**DNA extract**

Genomic DNA was extracted from young leaves using the Qiagen DNeasy Plant Mini Kit, following the standardized protocol outlined by Popescu et al. (2022). The extraction process ensured the isolation of high-quality DNA suitable for downstream molecular analyses. To assess the purity and concentration of the extracted DNA, spectrophotometric measurements were performed using a BioPhotometer Plus (Eppendorf). This evaluation provided essential data on DNA integrity, enabling further validation of its suitability for genetic studies. The obtained DNA samples were subsequently stored under appropriate conditions to prevent degradation.

**PCR Amplification Protocol for SSR Markers**

Following the methodologies established by Saravanan et al. (2014), Popescu et al. (2022), and Sumedrea et al. (2024), nine simple sequence repeat (SSR) molecular markers SSR47, SSR63, SSR T7, SSR T62, SSR T70, SSR110, SSR111, SLM6-7, and SLM6-12 were employed for PCR amplification, adhering to the standardized protocol described by Benor et al. (2008). As reported by Sumedrea et al. (2024), the amplification reactions were conducted using a Techne TC-512 Thermal Cycler under specific cycling conditions.

**Visualization of PCR Products**

Electrophoresis was performed on a 3.0% agarose gel (Promega agarose in TAE buffer, with ethidium bromide for DNA staining) at a constant voltage of 75 V for 1 hour. DNA bands were visualized under UV light using the Syngene Bio Gene Flash Imaging System. The size of the PCR products was estimated by comparing them to a 100 bp DNA ladder (Thermo Fisher Scientific), which served as a molecular weight marker. The expected amplicon sizes were determined based on the target gene sequences. The amplified DNA bands were estimated by comparing them to a 50 bp Quick-Load Purple DNA ladder run alongside the samples.

## RESULTS AND DISCUSSION

### Fruit morphology analysis

Tables 1 and 2 present some morphological measurements recorded for each plant analyzed over the three years of study, starting from plants regenerated from anther culture (Figure 1) and continuing with the next two generations.

The analyses and measurements were performed and recorded according to the "Descriptors for tomatoes (*Lycopersicon* spp.) (Sumedrea

et al. 2024). Considering the genetic determinism of the original 'Argeș 20' variety, from which the flower buds were used for *in vitro* culture (determinate growth tomato), no significant fluctuations in plant height (7.1.2.2) were observed during the second and third years.

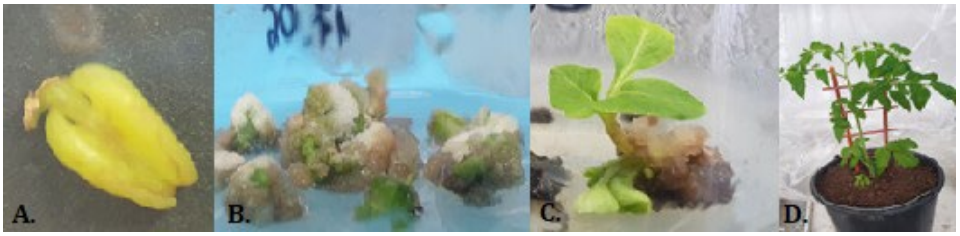


Figure 1. Anther culture and regenerated plants. A) Callus formed from the anther filament; B) Callus formed from the anther filament; C) Indirect organogenesis with differentiated shoots from the callus; D) Regenerated plant from anther culture, in *ex vitro* conditions

The plants measured between 60 and 70 cm in height, with only minimal variation. This suggests that the determinate growth trait specific to the 'Argeș 20' variety is quite stable and was not significantly affected by regeneration through anther culture. However, regarding fruit characteristics, several characteristics exhibited annual morphological variability. The shapes of the fruits (7.2.2.5) ranged from flattened (1) to very round (4), but also with intermediate shapes such as slightly flattened (2) and round (3). The results indicate a significant variability in fruit morphology between the second and third year of cultivation, suggesting that anther culture's regeneration process determined the diversification of the cultivar 'Argeș 20' phenotypic traits. This variability may be influenced by the interaction between the *in vitro* process and the cultivation environment, affecting the expression of the phenotypic characteristics of the plants. This variability was closely correlated with the shape of the fruit cross-section (7.2.2.29). Most plants presented a round profile (1), while others had an angular configuration (2), with the variation being between the two shapes. The diversity of fruit cross-sectional shape indicates that there is variability in the internal structure of the fruit, and this may reflect a genetic factor or an environmental influence on fruit development, suggesting that regeneration by anther culture may lead to some phenotypic variability, even in the case of a variety with well-defined traits (Sahana et al. 2024).

Table 1. Morphological characters (fruit shape, size, uniformity, and weight) of regenerated tomatoes with standard descriptors over the three years of study.

| Descriptor* / Regenerate code | Year | P1.1 | P4.7 | P4.9 | P4.15 | P4.16 | P6.3 |
|---|---|---|---|---|---|---|---|
| 7.2.2.5 | I | 4 | 2 | 3 | 3 | 2 | 4 |
| | II | 2 | 2 | 2 | 3 | 2 | 2 |
| | III | 3 | 4 | 3 | 3 | 3 | 3 |
| 7.2.2.6 | I | 5 | 3 | 3 | 5 | 5 | 3 |
| | II | 4 | 3 | 3 | 3 | 3 | 3 |
| | III | 5 | 3 | 3 | 4 | 3 | 3 |
| 7.2.2.7 | I | 7 | 7 | 5 | 5 | 5 | 5 |
| | II | 5 | 3 | 3 | 5 | 3 | 3 |
| | III | 3 | 5 | 3 | 5 | 3 | 3 |
| 7.2.2.8 | I | 93.38 ±23.68 | 107.03 ±34.87 | 148.43 ±20.46 | 134.01 ±74.87 | 190.43 ±47.60 | 145.29 ±16.36 |
| | II | 189.94 ±54.89 | 176.78 ±78.85 | 198.86 ±58.55 | 256.74 ±26.20 | 173.02 ±34.01 | 235.63 ±76.29 |
| | III | 180.6 ±88.90 | 181.19 ±10.47 | 189.01 ±39.31 | 224.36 ±10.65 | 167.03 ±10.05 | 232.89 ±10.44 |
| 7.2.2.9 | I | 81.17 ±3.43 | 51.98 ±3.89 | 64.05 ±5.04 | 61.16 ±8.58 | 58.31 ±6.59 | 61.4 ±9.25 |
| | II | 70.53 ±7.99 | 63.21± 4.42 | 56.91 ±3.87 | 66.81 ±12.80 | 62.2 ±16.18 | 75.73 ±11.97 |
| | III | 64.2 ±24.09 | 67.67 ±21.01 | 68.75 ±21.76 | 72.04 ±2.60 | 65.32 ±7.35 | 71.21 ±5.65 |
| 7.2.2.10 | I | 70.95 ±2.89 | 57.35 ±6.07 | 64.72 ±4.74 | 56.44 ±6.34 | 66.18 ±7.20 | 63.64 ±9.66 |
| | II | 70.45 ±7.89 | 77.88 ±17.12 | 77.07 ±7.28 | 83.65 ±18.96 | 64.21 ±18.99 | 85.62 ±9.42 |
| | III | 68.09 ±23.80 | 68.96 ±22.88 | 62.38 ±14.12 | 73.86 ±3.49 | 63.12 ±7.92 | 77.08 ±6.13 |

*Note: fruit shape (7.2.2.5), fruit size (7.2.2.6), fruit size uniformity (7.2.2.7), fruit weight [g] (7.2.2.8), fruit length [mm] (7.2.2.9), fruit width [mm] (7.2.2.10)

The color of the fruits at full maturity expressed (7.2.2.11) was predominantly red (5), except for some plants that showed delayed fruit development, due to biotic and abiotic factors in the vegetable greenhouse,

thus maintaining a green hue and a firm consistency throughout the growing season, they did not reach full maturity and did not produce seed. This suggests that environmental conditions can significantly influence fruit development, and affected plants were unable to complete the normal growth cycle. Also, variations were observed in the weight of the examined tomatoes, the size of the fruits recorded by the codes 7.2.2.8, 7.2.2.9, 7.2.2.1, the number of seminal lodges 7.2.2.31, and the appearance of the pistillate scar 7.2.2.32, which highlighted the morphological diversity of the regenerated tomato fruits from the anther culture in the 2nd and 3rd year. This suggests that regeneration by anther culture resulted in a wide range of fruit phenotypes, which may have implications for selecting and improving fruit characteristics in breeding programs.

The variability of the size and weight of the fruits is illustrated in Table 1, which presents the relationship between their length, diameter, and weight. The data reveal a notable upward trend in weight and size over the three years of study. In the first year, the average fruit weight (7.2.2.8) from regenerated plants was 93.38g, whereas by the third year, the same regenerated plants produced fruits with an impressive average weight of 180.60g for the same sample size. This significant increase in fruit size and weight may suggest an improvement in growing conditions, a more efficient adaptation of regenerated plants, or the influence of genetic and climatic factors, with direct implications for agricultural production and marketing. The evolution of the physical characteristics of autumn fruits is an area of interest in modern agriculture, with implications for both agricultural production and marketing (Khan et al. 2021). The analysis of the evolution of fruit weight (7.2.2.8), length (7.2.2.9), and width (7.2.2.10) aimed to identify the correlations between these characteristics based on fruits harvested in September for two consecutive years. The data obtained highlighted the following average values: fruit weight remained stable over the two years, with average fruit weight in the second year of 205.16 g and 195.85 g in the third year.

Similarly, the average fruit length showed minimal variations, measuring 65.89 mm in the second year and 68.20 mm in the third year, while the width experienced a slight decrease from 76.48 mm in the second year to 68.92 mm in the third year of vegetation. These results suggest that the physical characteristics of the fruit remained relatively constant from one year to the next, indicating a stable growing environment and consistent growing conditions.

Table 2. Morphological characters of regenerated tomatoes with standard descriptors over three years: color, ribbing, shoulder shape, locules, pistil scar, blossom end shape, and seed presence

| Descriptor* / Regenerate code | Year | P1.1 | P4.7 | P4.9 | P4.15 | P4.16 | P6.3 |
|---|---|---|---|---|---|---|---|
| 7.2.2.11 | I | 5 | 5 | 5 | 5 | 5 | 5 |
| | II | 5 | 5 | 3 | 5 | 5 | 3 |
| | III | 5 | 3 | 3 | 5 | 5 | 3 |
| 7.2.2.12 | I | 5 | 5 | 5 | 3 | 5 | 5 |
| | II | 5 | 5 | 5 | 5 | 3 | 5 |
| | III | 5 | 5 | 5 | 5 | 5 | 3 |
| 7.2.2.14 | I | 3 | 3 | 3 | 5 | 5 | 3 |
| | II | 3 | 5 | 5 | 5 | 7 | 5 |
| | III | 3 | 5 | 3 | 3 | 5 | 3 |
| 7.2.2.16 | I | 5 | 5 | 5 | 5 | 7 | 5 |
| | II | 5 | 5 | 3 | 3 | 5 | 5 |
| | III | 5 | 5 | 3 | 3 | 5 | 3 |
| 7.2.2.26 | I | 5 | 5 | 5 | 5 | 5 | 5 |
| | II | 5 | 5 | 5 | 5 | 5 | 5 |
| | III | 5 | 5 | 5 | 5 | 5 | 3 |
| 7.2.2.27 | I | 5 | 5 | 5 | 5 | 5 | 5 |
| | II | 5 | 5 | 5 | 5 | 5 | 5 |
| | III | 5 | 5 | 5 | 3 | 3 | 3 |
| 7.2.2.28 | I | 5 | 5 | 5 | 5 | 5 | 5 |
| | II | 5 | 5 | 5 | 5 | 5 | 5 |
| | III | 5 | 5 | 5 | 5 | 5 | 5 |
| 7.2.2.29 | I | 1 | 2 | 3 | 3 | 3 | 4 |
| | II | 3 | 3 | 2 | 2 | 1 | 3 |
| | III | 3 | 1 | 3 | 2 | 1 | 3 |
| 7.2.2.32 | I | 2 | 2 | 4 | 4 | 4 | 2 |
| | II | 4 | 2 | 3 | 4 | 4 | 4 |
| | III | 2 | 2 | 4 | 4 | 4 | 4 |
| 7.2.2.33 | I | 2 | 2 | 2 | 2 | 2 | 2 |
| | II | 2 | 2 | 2 | 2 | 2 | 2 |
| | III | 2 | 2 | 2 | 2 | 2 | 2 |
| seed presence | I | yes | yes | yes | yes | yes | yes |
| | II | yes | yes | yes | yes | yes | yes |
| | III | yes | yes | yes | yes | yes | yes |

*Note: exterior color of mature fruit (7.2.2.11), the intensity of the exterior color (7.2.2.12), ribbing at calyx end (7.2.2.14), fruit shoulder shape (7.2.2.16), flesh color of pericarp (interior) (7.2.2.26), flesh color intensity (7.2.2.27), fruit cross-sectional shape (7.2.2.29), number of locules (7.2.2.31), shape of pistil scar (7.2.2.32), fruit blossom end shape (7.2.2.33) and the presence of the normal seeds.

The slight decrease in width could be attributed to natural variability, environmental factors, or minor differences in agricultural practices. Still, the overall uniformity implies that external changes did not significantly influence the fruit development process. Concluding, regarding the characteristics of the fruits, the measurable fruit oscillations from one year to another were insignificant, demonstrating the uniformity of the genetic material under study. The strong correlations between weight and length, observed in both periods, confirm the coherence in fruit development. Although there were small differences in width, these were considered minor and did not significantly affect the overall profile of fruit dimensions.

**Germination of seeds and obtaining new plants**

To maintain rigorous continuity and optimal genetic homogeneity in the study, tomato seeds were harvested annually from selected plants that had fruited and formed normal seeds, considered representative of the target characteristics. The seeds were sown, respecting the specific tomato cultivation indications, for two consecutive years, under controlled conditions, conducive to the production of seedlings (temperature and humidity). In the second and third years, the germination process recorded a percentage of 72%. The seedlings obtained presented phenotypic traits following the reference standards. This experimental approach, by generating two consecutive generations from the initially collected seed material, allowed the maintenance of a stable genetic line, as well as the comparative evaluation of germination capacity and plant viability, providing a solid basis for analyzing the potential effects of environmental factors or other variables (Vivek & Duraisamy 2017).

**Genetic evaluation of regenerated tomato plants**

Research on the genetic diversity and stability of the tomato cultivar' Argeș 20´ began with other cultures that led to the regeneration of morphologically and genetically distinct plants. In the initial stage, SSR molecular markers were used to assess the genetic variability among the regenerated plants and their differences from the original donor cultivar from which the anthers were collected (Popescu et al. 2022, Sumedrea et al. 2024). Electrophoresis analysis revealed distinct DNA banding patterns in the first year after *in vitro* culture, indicating diversity among the regenerated individuals (Figure 2a). These findings suggest that the plants originated from different microspores rather than somatic tissues, confirming the genetic variability induced by *in*

*vitro* androgenesis (Bădulescu et al. 2022, Bull & Michelmore 2022). A two-year evaluation was performed to evaluate the stability of these new genotypes, combining morphological and phenological observations with SSR marker analysis. Electrophoresis profiles of plants obtained from seeds of androgenetic individuals showed identical allele distributions, confirming genetic uniformity across generations (Figure 2b).

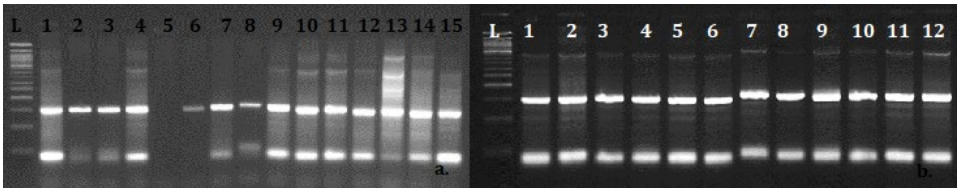


Figure 2. Profiles of DNA bands obtained after amplification in the presence of primers SSR 6-7 (left) and SSR 110 (right). L = molecular weight marker; 1a. 1-15 - the first year with different regenerated plants from *in vitro* anther culture and 1b. 1-12 – the third year with different plants obtained from the seed of *in vitro* regenerated plants.

Figures 2a and 2b illustrate the two key aspects of this study: the initial variability among regenerated plants and the genetic stability maintained in subsequent generations. The presence of distinct DNA banding patterns in the first year confirms that the plants originated from different microspores, demonstrating the effectiveness of androgenesis in generating genetic variability. Meanwhile, the identical allele distributions in later generations indicate that the selected genotypes remained stable over time, ensuring their reliability for further cultivation. These results emphasize the potential of androgenesis as a valuable tool for breeding programs, enabling the development of improved and genetically stable tomato varieties, such as ‘Argeș 20’, with desirable agronomic traits.

## CONCLUSIONS

The study concludes that plants regenerated through in vitro anther culture of the ´Argeș 20´ cultivar exhibit a high degree of phenotypic and genetic uniformity, maintained over three years of evaluation. The androgenesis process proved to be stable and effective in producing genetically uniform

plants without compromising the genetic integrity of the donor material. Molecular analyses using SSR markers confirmed the strong genetic similarity between regenerants and the original cultivar, demonstrating the reliability of this method for obtaining high-quality, consistent plant material in breeding programs for *Solanum* sp.

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