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# PROTOCOL FOR *IN VITRO* ANTHER CULTURE IN TOMATO AND THE CHARACTERIZATION OF REGENERATED PLANTS

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## ABSTRACT

*This paper presents a methodology of obtaining whole tomato plants from anther culture and their characterization. Year 1: the initiation of anther cultures and in vitro development of the new regenerated structures. Year 2: phenotypic characterization of plantlets acclimatized to ex vitro conditions, and their molecular analysis with SSR markers. Year 3: morphological characterization of plants obtained by germination of seeds from plants that fruited in the previous year and their molecular analysis with SSR markers. The phenotype descriptors and the SSR markers revealed differences among the regenerants and the donor variety. The uniformity of the genetic profiles with SSR markers in plants obtained from seeds of androgenic plants, but with several differences in comparison to anther-donor plants, indicates that some of the regenerated plantlets may have microspore origin.*

## INTRODUCTION

Besides traditional methods of breeding, the biotechnological approaches are considered very promising alternatives for plant improvement, especially for recalcitrant crops, such as tomatoes (Seguí-Simarro et al. 2011). Despite the economic importance, the technology of double-haploids in species of the *Solanaceae* family is still far from being effectively applied in breeding programs, mainly due to the lack of knowledge about the induction of androgenesis in these species (Julião et al. 2015). The culture of anthers and microspores in tomato represented an important subject of studies for: a) the correlation between the microspore developmental stage and the efficiency of *in vitro* differentiation processes into the anther structures (Dunwell 1986, Summers et al. 1992, Kumar at al. 2020); b) the role of flower bud pretreatments and stress factors induced in *in vitro* culture for the induction of differentiation processes (Zamir et al. 1980, Motallebi-Azar 2020); c) the correlations between genotype and culture media composition for subsequent embryogenic or organogenic processes induction and new plant regeneration (Zagorska et al. 1982, Zhao et al. 2014); d) the assessment of the ploidy level of the regenerative structures during *in vitro* cultures by measurement of DNA content using flow cytometry (Seguí-Simarro & Nuez 2007, Corral-Martínez et al. 2011); e) the possible origin of the regenerated plants by

comparisons between regenerants and donor plants in terms of plant morphological and molecular markers (Murovec & Bohanec 2012, Bădulescu et al. 2022).

Based on our experience, and on the reproducible results obtained in the last years, we proposed a 3 years working protocol for regenerating new tomato plants from anther cultures and characterizing the regenerants in comparison to donor plants through standard descriptors and molecular markers.

## MATERIAL AND METHODS

**Plant material**. As donor plants for tomato anther culture (*Solanum lycopersicum* L.) were selected the Arges 20, Argeș 11, Ștefănești 22, Ștefănești 24 and Costate 21 varieties, all of them obtained and approved at NRDIBH Ștefanesti. Flower buds of proper size (Figure 1a) were harvested from greenhouse-grown plants and kept for 48 hours at 4°C. After that, the floral buds were surface-sterilized as follows: 70% ethanol for 15-20 seconds, 10% sodium hypochlorite for 20 minutes, and then rinsed 3-4 times in sterilized water.

***In vitro* anther culture**. The anthers were excised under sterile conditions and placed in Petri dishes (6 cm diameter) containing initiation medium. Four initiation media were tested, all of them containing MS macro- and microelements (Murashige & Skoog 1962), sucrose (20 g $l^{-1}$) and agar (7 g $l^{-1}$) and supplemented with: 1) 0.5 mg $l^{-1}$ kinetin (Kin) and 0.5 mg $l^{-1}$ of indole-3-acetic acid (IAA); 2) 2.0 mg $l^{-1}$ 6-benzylaminopurine (BAP) and 1.0 mg $l^{-1}$ IAA; 3) 1.0 mg $l^{-1}$ BAP and 1.0 mg $l^{-1}$ of 2,4-dichlorophenoxyacetic acid (2,4-D); 4) 2.0 mg $l^{-1}$ BAP and 2.0 mg $l^{-1}$ 2,4-D. The Petri dishes were placed in darkness at 24°C for two weeks and then transferred in a growth chamber with 16 h photoperiod (24°C during light and 22°C during night periods). The viable anthers and those with organogenic structures were transferred on vessels containing MS induction media supplemented with: 5) 0.5 mg $l^{-1}$ IAA and 0.25 mg $l^{-1}$ zeatin (Z); 6) 2.0 mg $l^{-1}$ BAP and 1.0 mg $l^{-1}$ IAA. For rooting, only normal shoots of 3-4 cm in length were transferred to a hormone-free MS medium.

During the anther culture, observations were made to evaluate: the viability of the inoculated anthers, the number of anthers that formed callus, and also the differentiation of organogenic structures. The obtained data were subjected to analysis of variance (ANOVA) with Tukey's test and respectively Duncan test.

**Morphological characterization of the donor variety and the anther-derived plants.** The phenotypic characterization of the field-grown plants was recorded in accordance with the international standards for Descriptors for Tomato (*Lycopersicon* spp.) published by IPGRI (International Plant Genetic Resources Institute 1996).

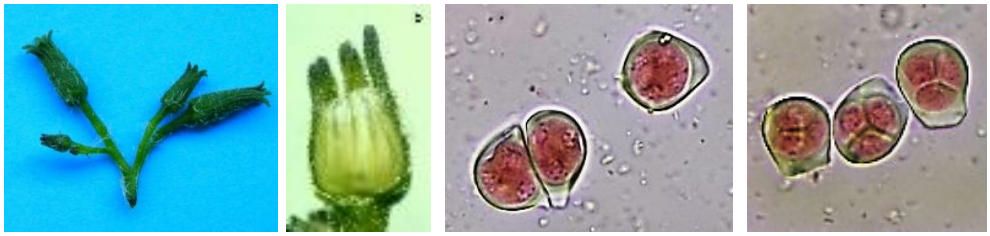
**DNA extraction.** Young leaf tissues from field-growing plants were used for total DNA extraction with Qiagen DNeasy Plant Mini Kit protocol.

**SSR markers and amplification.** PCR analyses were done using 9 SSR markers: SSR 47, SSR 62, SSR 63, SSR 70, SSR 107, SSR 110, SSR 111, SLM 6-7, and SLM 6-12. The amplification and analysis of amplified products followed the same procedures described by Bădulescu et al. 2022.

## RESULTS AND DISCUSSIONS

**Year 1. A. Harvesting of the floral buds at the proper moment**. The morphological aspect of the flower buds differs slightly among the five genotypes tested. The buds of Argeș 20 and Argeș 11 stand out for the length of the perianth elements, the bracts, and sepals of intense green colour, protecting the reproductive

elements: the androecium (all of the anthers) and the gynoecium (ovule). Comparatively, Ștefănești 22, Ștefănești 24 and Costate 21 stand out for the shorter length of the sepals. The appearance of the flower buds (Fig. 1a) and the measurements made on the flower components proved to be very important for establishing the optimal harvesting moment and selection of the flower buds containing anthers in appropriate stages in order to initiate the anther culture. The size of the buds, measured without the elongated terminations of the sepals varied between 4.7 and 8.7 mm, and the anther sizes varied between 2.1 and 6.8 mm. Measurement of stamens length is one of the descriptors (Code 7.2.1.10) used in morphological tomato plant characterization and shows the differences among varieties. Our measurements for the five tomato genotypes and their further evolution on growth media are in accordance with Summers et al. (1992) and Seguí-Simarro & Nuez (2005) who showed that anther length is a good criterion to select the microspores at the appropriate stage of development. Besides these, for the success of the anther culture, it is important to make a correct selection of the anthers having bright yellow colour and translucent aspect at the time of inoculation (Fig. 1b). Three years consecutively of initiating anther cultures with the five tomato genotypes proved that the best results were obtained by using anthers with most of the microspores during the first meiotic division (Fig. 1c), or with many microspores in the tetrad phase (Fig. 1d).



(a) (b) (c) (d)

Figure 1. The optimal time to harvest the flower buds: a) the size and appearance of the buds; b) the proper development stage of the anthers in the flower buds chosen for *in vitro* culture initiation; c) microspore development stages – pollen mother cell and the first meiotic division; d) microspores in the tetrad phase

**B**. ***In vitro*** **anther cultures, de-differentiated processes and plant regeneration.** During the entire period of tissue culture, the ability of the five tomato genotypes to respond and generate any morphogenic differentiation from cultured anthers was assessed. Considering the study of Shariatpanahi et al. (2006), who analysed some physicochemical stress treatments (cold/heat before initiation, or in the first weeks of culture, or poorer components of culture media) and their mechanisms of action in inducing microspore embryogenesis, we also applied a cold treatment of 4°C to the flower buds before anther culture initiation. From our experience with different species and genotypes, it was clear that cold treatment is an inductive factor to trigger cellular de-differentiation into anther structures. We found the same in the case of investigated tomato varieties. The inoculation of anthers, at a stage considered optimal for initiation, immediately after harvesting, resulted in the failure of the culture and complete necrosis of the inoculated anthers.

With the cold-treated anthers, after two weeks of darkness, the percentage of viability of the inoculated anthers was evaluated. The recorded percentages,

between 33.9% (Arges 20) and 47.9% (Arges 11), did not significantly vary neither among varieties nor among the initiation medium with different compositions. Of the total number of viable anthers, some showed de-differentiation processes (ruptures of the anther wall) and the occurrence of calluses on the surface of the anther wall (Fig. 2a). After the first transfer to fresh media with the same composition, the differentiation processes were accelerated. Unlike the anthers inoculated on the media containing 2,4-D, which formed only calli on their surfaces, the viable anthers initiated on media 1 and 2 formed both callus (in proportions varying between 15.3% in Stefanesti 24 and 30.5% in Arges 20) and organogenic structures (Fig. 2b). Although a higher percentage of anthers forming callus was found on media containing 2,4-D, the results obtained by us proved that there are no significant differences among genotypes, or depending on the composition of the medium on which the anthers were initiated (Table 1).

Two types of evolution were noticed after the transfer of the explants on the two differentiation-inducing media: (i) the explants coming from the initiation media 1 and 2, had a spectacular evolution. Thus, after 3 months from the initiation of the anther culture, new organogenic structures were evident in all varieties in proportions varying between 6.9% (Stefanesti 24) and 27.5% (Arges 20) on the medium 5 with 0.5 mg $l^{-1}$ IAA and 0.25 mg $l^{-1}$ Z, and between 0% (Arges 20) and 18.5% (Costate 21) on the medium 6 with 2.0 mg $l^{-1}$ BAP and 1.0 mg $l^{-1}$ IAA (Fig. 2c); (ii) the explants from media 3 and 4 with 2,4-D, formed only calli, most of which proliferated and grew in size, but subsequently, regardless of the composition of the culture medium (variant 5 or 6), they gradually became pale brown and necrosed.

After 5 months from the inoculation of the anthers, normally developed shoots were obtained, which, transferred on a medium without growth regulators, formed roots. With the five tested tomato varieties, after three years of repeated experiments, the following results were obtained: calli with embryogenic potential in Arges 11, Stefanesti 24, calli with roots differentiation in the Ștefănesti 22 variety, and whole plants, with a normal appearance in Arges 20 and Costate 21 (Fig. 2d). Two years consecutively were obtained plants with these two varieties and acclimatized to *ex vitro* conditions. Our results proved the close correlation between the genotype and the composition of the initiation and differentiation media. In the Arges 20 variety, the 10 regenerated plants developed from anthers inoculated on medium variant 1, and the differentiation processes were induced on medium variant 6. With Costate 21 variety, the 36 plants were regenerated from the anthers inoculated on medium 2 of initiation, and the differentiation of new structures was induced on medium variant 5.

**Year 2. A. Phenotypic characterization of the regenerated plants.** With Argeş 20, the main descriptors of the anther-derived plants in comparison to the anther-donor variety were presented in a previous research paper (Bădulescu et al., 2022). The same procedure was applied for the 36 anthers' regenerated plants obtained with Costate 21. Of these, only 20 plants had good vigour for planting in the greenhouse, meaning that 44.4% of regenerants failed to develop into normal plants. The mode of growth, development, and fruiting of each anther-derived plant were analyzed throughout the growing season. Among the greenhouse growing plants, one plant didn't produce flower buds, two plants differentiated flowers with normal appearance, but in which the gynoecium dried up, and only seventeen plants reached fruiting maturity, of which 10 plants produced seeded fruits. Table 2 presents a synthesis of the descriptors for which differences were recorded between

the original variety (Costate 21) and plants regenerated from *in vitro* anther culture. At the end of the growing season, three plants were found to be of high interest: 1) one for a very high foliage density with a semi-erect leaf attitude; 2) one with high growth vigour and high productivity per plant; 3) one with low vigour, low leaf density, and fruits with a special flavor.

(a) (b) (c) (d)

Figure 2. Evolution of the anthers from the initiation to plant regeneration in tomato varieties: (a) After 2 weeks of culture in darkness; (b) After 1 month on the same initiation medium and under 16 h photoperiod; (c) After 3 months from anther culture initiation, calli and organogenic structures; (d) New plant regeneration on the surface of a de-differentiated structure.

Table 1.

Tomato genotypes androgenetic responses and anthers evolution (viability, callus formation and organogenesis processes) on different culture media.

| Tomato variety | Media for initiation | After one month on the initiation media | | After three months |
|---|---|---|---|---|
| | | Viability (%) | Anther with calli (%) | Organogenic structures (%) |
| Argeș 11 | 1 | 50.5 | 28.9 | 19.4 b |
| | 2 | 55.5 | 13.9 | 6.9 d |
| | 3 | 41.2 | 22.5 | 0 |
| | 4 | 42.5 | 38.7 | 0 |
| Argeș 20 | 1 | 35.0 | 17.8 | 27.5 a |
| | 2 | 33.5 | 20.8 | 0 |
| | 3 | 31.7 | 30.0 | 0 |
| | 4 | 35.7 | 33.3 | 0 |
| Ștefănești 22 | 1 | 33.3 | 23.7 | 13.9 c |
| | 2 | 49.5 | 16.7 | 6.9 c |
| | 3 | 37.2 | 24.3 | 0 |
| | 4 | 35.7 | 24.3 | 0 |
| Ștefănești 24 | 1 | 48.6 | 19.4 | 6.9 d |
| | 2 | 34.7 | 8.3 | 18.1 b |
| | 3 | 57.2 | 20.8 | 0 |
| | 4 | 50.0 | 27.8 | 0 |
| Costate 21 | 1 | 36.2 | 26.6 | 13.3 c |
| | 2 | 55.5 | 15.7 | 18.5 b |
| | 3 | 31.4 | 37.0 | 0 |
| | 4 | 63.1 | 38.1 | 0 |
| Mean ± S. deviation | | 42.8 ± 10.95 ns | 24.43 ± 8.28 ns | 6.57 ± 8.74 |

ANOVA analysis of variance and Tukey's test significant at 0.01 level; ns – not significant at 0.05 level. Means±S.dev with different letters are significantly different at the P=0.05 level (Duncan test)

**B. Molecular analyses**. This characterization with standard descriptors revealed the magnitude of variability among the anther-derived plants and also in comparison to the anther-donor variety regarding morphological, agronomical, and yield traits. Because the phenotype is strongly dependent on environmental conditions, molecular analyses with SSR markers were used to obtain the

confirmation of the genetic variability induced during the regeneration processes from anther culture. Were selected 9 SSR markers that had been proven to generate polymorphic banding patterns and to be efficient for detecting differences among tomato genotypes (Bădulescu et al., 2022). With all these markers, different profiles of the distribution of DNA bands were obtained both, among the regenerated plants and between them and the original variety (Table 3). Knowing that the anther has a complex structure, during *in vitro* culture there is the possibility of new structures developing from the anther filament, from the anther wall, or from the de-differentiated callus proliferating from the somatic tissues. In this case, all regenerants would be phenotypically and molecularly identical to the donor plant and the demonstrated differences at the molecular level would not be possible unless mutations occurred during the *in vitro* regeneration processes. Our results proved that the plants regenerated through androgenesis and reached maturity are different both morphologically and molecularly from the original variety and support the idea of their origin from different microspores.

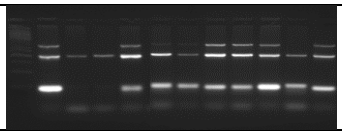
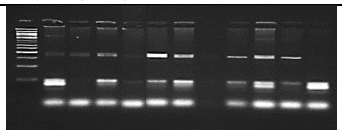
Table 2

The phenotypic differences between anther donor variety (Costate 21) and anther-derived plants (descriptors, registered quotations and their significances).

| Descriptor code | Donor variety | Anther-derived plants with different notations |
|---|---|---|
| 7.1.2.2 Plant size | 7 large | 3 small - 4 plants; 5 intermediate - 11 plants; 7 large - 5 plants |
| 7.1.2.3 Vine lenght | 250 cm | under 140 cm - 4 plants; taller than 260 cm - 2 plants |
| 7.1.2.5 Stem internode length | 5 intermediate | 3 short - 9 plants; 5 intermediate - 10 plants; 7 long - 1 plant |
| 7.1.2.6 Foliage density | 7 dense | 3 sparse - 3 plants; 5 intermediate - 8 plants; 7 dense - 9 plants |
| 7.1.2.8 Leaf attitude | 7 drooping | 3 semi-erect - 1 plant; 5 horizontal - 8 plants; 7 dropping - 11 plants |
| 7.1.2.9 Leaf type | 4 peruvianum | 1 dwarf - 1 plant; 3 standard - 6 plants; 4 peruvianum - 13 plants |
| 7.2.1.7 Style position | 3 slightly exserted | 1 inserted - 6 plants; 2 same level as stamen - 5 plants; 3 slightly exserted - 3 plant; 4 highly exserted - 3 plants |
| 7.2.1.8 Style shape | 2 fasciated | 1 simple - 3 plants; 2 fasciated - 13 plants; 3 divided - 1 plant |
| 7.2.2.5 Predominant fruit shape | 1 flattened (oblate) | 1 flattened - 1 plant; 2 slightly flattened - 8 plants; 3 rounded - 7 plants; 4 high rounded - 1 plant |
| 7.2.2.6 Fruit size | 3 intermediate (5.1 - 8 cm) | 1 very small < 3.0 cm - 2 plants; 2 small 3.1 - 5.0 cm - 8 plants; 3 intermediate 5.1 - 8 cm - 7 plants |
| 7.2.2.7 Fruit size homogeneity | 3 low | 3 low - 11 plants; 5 intermediate - 3 plants; 7 high - 3 plants |
| 7.2.2.14 Ribbing at calyx end | 7 strong | 1 very weak - 4 plants; 3 weak - 10 plants; 5 intermediate - 2 plants; 7 strong - 1 plant |
| 7.2.2.16 Fruit shoulder shape | 3 slightly depressed | 1 flat - 7 plants; 3 slightly depressed - 8 plants; 5 moderately depressed - 2 plants |
| 7.2.2.27 Flesh colour intensity | 5 intermediate | 3 light - 2 plants; 5 intermediate - 11 plants; 7 dark - 4 plants |
| 7.2.2.28 Colour of core | 5 intermediate | 3 light - 1 plant; 5 intermediate - 13 plants; 7 dark - 3 plants |
| 7.2.2.29 Fruit cross-section shape | 3 irregular | 1 round - 11 plants; 2 angular - 6 plants |

Tabel 3

SSR markers used to detect the genetic variability among donor variety and anther regenerated plants in Costate 21.

| SSR marker | No. of alleles in donor variety | No. of alleles in anther-derived plants | Polymorphic pattern with SLM 6-12 |
|---|---|---|---|
| SSR 47 | 4 bands - 70-230 bp | 3 to 5 bands - 70-250 bp |  |
| SSR 62 | 4 bands - 100-300 bp | 1 to 5 bands - 100-350 bp | |
| SSR 63 | 1 - 70 bp | 1 to 3 bands - 70-250 bp | |
| SSR 70 | 6 bands - 70-550 bp | 5 to 7 bands - 70-550 bp | |
| SSR 107 | 2 bands - 100 and 270 bp | 1 or 2 bands - 100 and 270 bp | Polymorphic pattern with SSR 111 |
| SSR 110 | 4 bands - 100-250 bp | 3 to 6 bands - 100-250 bp |  |
| SSR 111 | 3 bands - 70-100 bp | 1 to 5 bands - 70-250 bp | |
| SLM 6-7 | 1 band - 70 bp | 1 to 5 bands - 70-500 bp | |
| SLM 6-12 | 3 bands - 100-350 bp | 1 to 3 bands - 70-350 bp | |

**Year 3**. Because the plants that reached fruiting maturity were characterized by variability at the morphological and molecular level, it was important to determine to what extent these differences are preserved in the offspring. The same molecular markers were used for the analysis of total DNA samples extracted from plants obtained by seeds germination from plants derived from anthers. In all cases, we obtained the same distribution of alleles in the electrophoresis gel for the plants of the second generation, compared to the profiles of the plants from which the seeds came, specific for cultivated tomato, which is an autogamous plant and implicitly has a high level of homozygosity.

These results reveal the genetic stability of anther-derived plants regenerated in the previous year. Plants from the second generation can be considered new genotypes and valuable genetic resources for breeding purposes.

## CONCLUSIONS

The optimum stage to initiate anther culture in tomato is chosen depending on the length of anthers at the moment for flower buds harvesting. More responsive anthers proved to be those with a high proportion of pollen mother cells and microspores in the early prophase of meiosis.

The working method presented with the tomato varieties Argeș 11, Argeș 20, Ștefănești 22, Ștefănești 24, and Costate 21, showed that the morphological uniformity of the anthers at the moment of inoculation determines a similar response among genotypes, without significant differences regarding the viability of the anthers, the frequency of callus formation, or structures with organogenic potential. The appearance of newly formed structures and shoots depends on the interaction between the genotype and the composition of the differentiation media.

The phenotypic and molecular differences between the plants regenerated from anther culture, as well as between them and the donor variety, proved the possibility of using androgenesis as an alternative to exploit genetic variability in *Solanum* sp. This protocol could be considered a biotechnology approach for obtaining and analyzing the newly regenerated genotypes, valuable in breeding programs for genetic improvement in tomatoes.

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