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Research Article

Androgenic ability and plant regeneration potential in some tomato varieties

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Abstract

Aiming to evaluate the *in vitro* regeneration potential, five varieties of tomatoes (*Solanum lycopersicum*) were studied for their response in anther culture. Anther explants at an early stage of microspore development were inoculated onto three culture media. The first differentiation processes were recorded during the first three weeks of culture, in darkness. The statistical analysis of the data recorded during the anther culture showed significant differences between genotypes regarding their specific response to culture conditions and the significant influence of the initiation medium composition in triggering the differentiation processes. Under the tested conditions were induced: the embryogenic potential in three genotypes ('Ştefăneşti 22', 'Costate 21' and 'Chihlimbar') and the indirect organogenesis in two genotypes ('Argeş 20' and 'Costate 21'). Morphological characteristics of anther-derived plants from 'Argeş 20' variety, grown in greenhouse conditions (growth rate, features of leaf, flower, and fruit), as well as analyses with nine SSR markers (banding patterns, the coefficient of genetic similarity, and the polymorphism information content) in DNA samples from each regenerant and the donor variety, provided clear evidence of the occurrence of spontaneous genetic variation during *in vitro* anther culture, and of the existence of somaclonal variation in regenerated plants. The amplified products obtained with SSR primers revealed a total number of scorable bands of 160 and a mean percentage of polymorphic bands of 21.09%. Two out of the nine tested primers, SSR63 and SLM6-7 proved to be efficient in detecting genetic differences not only among regenerants but also between them and the donor genotype.

Keywords: androgenic induction; molecular markers; phenotypic traits; tissue culture; variability

Introduction

All over the world, tomato (*Solanum lycopersicum*) is considered one of the most important edible crops. Due to its valuable agronomical characteristics, nutritional value, and high demand as a marketable product, tomato plants have been an increasing interest not only from the producers but also from the researchers in conventional and unconventional breeding practices (Fentik 2017; Ahmar *et al.*, 2020). Among the regeneration systems, the biotechnological approach with *in vitro* androgenesis has aroused great interest

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because it is considered a procedure for obtaining haploid and double haploid plants (pure lines) in one single generation (Sánchez *et al.*, 2020).

In comparison with other crops (*Triticum aestivum* L., *Hordeum vulgare* L., × *Triticosecale* Wittm., *Oryza sativa* L., *Brassica* sp., *Solanum melongena* L., *Capsicum annuum* L., and *Nicotiana tabacum* L.) for which doubled haploid technology proved to be efficient for obtaining new commercial varieties (Brew-Appiah *et al.*, 2013), with tomato the results are still poor and need improvements to be applied in tomato breeding programs (Saeed *et al.*, 2019). Under optimal *in vitro* culture conditions, the new anther-derived plants could be regenerated by direct, or indirect embryogenesis, or by indirect organogenesis (Seguí-Simarro and Nuez, 2007; Zhao *et al.*, 2014; Méndez-Hernández *et al.*, 2019; Titeli *et al.*, 2021), the final result being a haploid, or double haploid plant (Seguí-Simarro, 2010). In the case of indirect regeneration by either organogenesis or embryogenesis, is helpful to assess the DNA ploidy level of the calluses (Julião *et al.*, 2015) during the *in vitro* process of regeneration, aiming to predict the possible origin of the regenerants from microspores. A long and sometimes complicated process of *in vitro* regeneration imposes selection of the regenerants for their ploidy either by counting the chromosome number in root tip cells or by measurement of DNA content using flow cytometry (Murovec and Bohanec, 2012).

All the results obtained so far proved that *Solanum lycopersicum* is a recalcitrant species to this type of regeneration, and therefore improvements are needed for regeneration and also for analysis of the new obtained reproductive material (Tradeu De Faria *et al.*, 2002; Prihatna *et al.*, 2019).

Regardless of the regeneration model, the selection of the obtained regenerants is based on the characterization of each new plant with morphological, biochemical, and molecular markers (Cebolla-Cornejo *et al.*, 2013; Prihatna *et al.*, 2019). The plants resulting from plant organs with complex structures and through complicated *in vitro* regeneration systems express often different morphological characteristics in comparison to the donor genotype. In the case of plants obtained by androgenesis, molecular methods, such as SSR and RFLP, are used to prove the genetic uniformity or variability of regenerated plants as a result of the somaclonal variation induced during the regeneration process (Bairu *et al.*, 2011; Krishna *et al.*, 2016; Cao and Deng, 2020).

Taking into account the exciting aspects of plant regeneration from anther culture, and the requirement for a complex analysis of the new tomato varieties, our work had the following objectives: a) testing the *in vitro* regeneration potential of anthers in five tomato varieties; b) characterization of the regenerated plants for their phenotypic traits in comparison with the original variety; c) the use of SSR markers to verify the genetic uniformity of the anther-derived plants or to uncover the somaclonal variation in plants regenerated via indirect organogenesis.

Materials and Methods

Anther-donor plant material and flower bud's sterilization

Five *Solanum lycopersicum* L. varieties with determinate growth ('Argeş 11', 'Argeş 20', 'Chihlimbar') and indeterminate growth ('Costate 21', 'Ştefăneşti 22') were used as donor plants for anther culture. Flower buds of 5-7 mm length were harvested and maintained for 48h in the refrigerator at 4 °C. After this cold treatment, the buds were sterilized with alcohol 70% for 30 sec, sodium hypochlorite 10% for 20 min, followed by 3-4 washes with sterile distilled water.

In vitro anther culture

Under sterile conditions, the anthers were detached from the buds and inoculated onto three initiation media, basal medium MS (Murashige and Skoog, 1962) supplemented with: 1) 0.5 mg l^{-1} kinetin (K) 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) 2.0 mg l^{-1}

BAP and 2.0 mg l^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D). All medium containing sucrose (20 g l^{-1}) and agar (7 g l^{-1}) were adjusted to pH 5.8-6.0 and autoclaved at 121 °C for 20 minutes.

After inoculation of the anthers, the Petri dishes (6 cm diameter) were kept in the growth chamber, in the dark, and at a constant temperature of 22-24 °C for a period of 3 weeks. After this period, only viable anthers and those with callus formation were transferred onto new media for differentiation, or induction, MS medium with 1.0 mg l^{-1} BAP and 1.0 mg l^{-1} of IAA. The culture vessels were maintained under photoperiod conditions of 16 h at 24 °C light/ 8 h at 22 °C dark and the viable new structures were periodically transferred onto fresh media every 3 weeks.

The normal aspect and elongated shoots of 3-4 cm length were transferred to a hormone-free MS medium for roots development.

Ex vitro transfer of regenerated plants and their accommodation

The plantlets with 3-4 roots were taken off from the vessels, washed with water to remove any adherent media, and transferred in Jiffy pots. For hardening, the regenerated plants were covered with plastic transparent bags for another three weeks, kept in a growth chamber at 24 °C with diffuse light, and periodically aired until the new roots were visible coming out from the pots. The robust plants, with new leaves and normal aspects, were transferred in bigger pots with nutrient mixture and maintained in a glasshouse with normal growth conditions.

Data analysis

The anther culture was designed with 3 replications (4 Petri dishes, each one containing 10 cultured anthers, was considered as one replication). All data regarding structures viability, new differentiation processes, or plant regeneration (callogenesis, organogenesis) were regularly recorded and their percentage was subjected to analysis of variance and the mean values were compared with Duncan's test at the significant level of $p \leq 0.05$.

DNA extraction

From anther-derived plants acclimatized to greenhouse conditions were collected young leaves for DNA extraction. It was used the method recommended by Ahmed *et al.* (2009) with modifications tested previously in our laboratory (Bădulescu *et al.*, 2020). The DNA quantity and quality of total DNA were verified with spectrophotometer BioPhotometer plus (Eppendorf). The genomic DNA as eluted solutions from each anther regenerated plant were stored at -20 °C until their further use.

Amplification with SSR markers

The extracted DNA from the ten regenerated plants and from the anther-donor plant were tested with nine SSR primers: SSR T-7 and SSR T-62 tested and recommended by Saravanan *et al.* (2014) and SSR47, SSR63, SSR T70, SSR110, SSR111, SLM6-7, SLM6-12 tested and recommended by Diklesh *et al.* (2016). For amplification were used the following mix: 5 µl of 5x FirePol Master Mix Ready to load (Solis BioDyne, Estonia), 2 µl of forward and 2 µl reverse primers, water to a total volume of 22 µl and 3 µl DNA template. The amplification with each primer for each DNA sample was performed with Techne TC-512 Therman Cycler with cycling amplifications as follows: one cycle for initial denaturation 4 min at 94 °C; 35 cycles with 1 min at 94 °C, 1 min at 55 °C for primers annealing, and 2 min at 72 °C for elongation; the final extension of 7 min at 72 °C, and then maintained at 4 °C. The amplification products with SSR markers were visualized after horizontal electrophoresis in 3.0% agarose gel with TAE buffer and stained with ethidium bromide. After electrophoresis at 75 volts for 1 hour, the amplified products were visualized and photographed with Gene Flash Syngene Bio Imaging system under UV light. As a control for gel migration of tested DNA fragments were used Quick-Load Purple 50 bp DNA Ladder.

Data collection and analysis

For each SSR marker were recorded all scorable bands (the total amplified bands) and calculated the percentages of polymorphic bands. With each used SSR primer was calculated the coefficient of genetic similarity (GS) between anther-derived plant and control using Dice (1945) formula, respectively:

$2Nab/(Na+Nb)$,

where Nab is the number of shared bands (control and regenerated plant), while Na and Nb represent the total number of bands detected in the control plant and each anther-derived plant respectively.

To calculate the polymorphism information content (PIC) was used formula established by Anderson *et al.* (1993), as

$PIC = 1 - \sum pi^2$,

where pi is the frequency of each allele at all locus in the set of eleven tomato genotypes (control and 10 regenerants).

Results and Discussion

In vitro response of anther cultures and plant regeneration

For the initiation of the anther cultures, the harvesting moment of flowering buds was chosen considering the studies carried out by Zhao *et al.* (2014), as well as the conclusions of previous attempts that showed the importance of the stage of anther development at the moment of inoculation for the subsequent induction processes of callogenesis, embryogenesis, or organogenesis (Zamir *et al.*, 1980; Gulshan *et al.*, 1981; Dunwell, 1986). Thus, were selected for sterilization only flowering buds of 3-7 mm length containing anthers of 1-3 mm length, bright yellow in colour, and with a translucent appearance. Anyway, our previous attempts with anthers at a later stage of development were not efficient, all anthers necrosing in a short time after inoculation (data not shown). With anthers in a desired phase of growth were obtained good values of viability after the first three weeks of culture in darkness onto the initiation media (Table 1), varying between 37.1% and 45.4%. Overall, 'Argeş 20' and 'Costate 21' varieties had the best responses for anthers viability on all the three tested media. Similar to Summers et al. (1992) and Seguí-Simarro *et al.* (2011), in our experiment were noticed, in darkness conditions, the first de-differentiation processes and callus formation either from the anther filament or from the anther wall (Figure 1A).

After the transfer of viable anthers onto induction medium and cultured vessels maintenance in photoperiod conditions, were noticed progressively changes into inoculated structures and acceleration of differentiation processes. In the case of cultured anthers from initiation 1 and 2 media were noticed proliferative green and morphogenic structures (Figure 1B) in a high proportion of 24.1% to 25.5% respectively (Table 1). The anthers coming from medium 1 proved to have a good response for all the five tomato varieties, while the anthers initiated on medium 2 showed more efficient proliferation (35.5-48.1%) but only for three tested varieties ('Costate 21', 'Ştefăneşti 22', 'Chihlimbar'). Every three weeks transfer on the same induction medium assured either proliferation and differentiation of new structures coming from the anthers, or their maintenance at the same stage and arresting further on evolution.

It seems that the medium containing BAP and IAA, used as induction medium had a stimulation effect for cell reorganizing into new proliferative nodules and so, were obtained a relative high proportion of structures with regenerative potential. After three months from the anthers' inoculation, were noticed the following significant morphogenic responses: the formation of callus with shoots organogenesis in 'Costate 21' (Figure 2 A), and 'Argeş 20' (Figure 2B) varieties, callus with roots formation in 'Ştefăneşti 22' variety, or proliferation of compact vascular-like structures which arrested their further evolution in 'Chihlimbar' variety. On the same induction medium were obtained only organogenic responses with meristematic structures, shoots, and whole plant regeneration. By detaching the arising shoots (having an apical meristem) from the

basal compact structures were obtained rooted plants only after their transfer into MS medium without growth regulators.

Table 1. Evolution of androgenic responses of the five tomato varieties in anther culture initiated onto three media

Quantified parameter	Viability (%) of anthers – after 3 weeks on initiation media			Anthers with callus formation (%) – after 3 weeks on inductive medium			Anthers with organogenic structures (%) – after 3 weeks on inductive medium			Organigenic structures with multiplication (%) - 12 weeks from anther initiation			Regenerated plants (%) out from the number of inoculated anthers		
Initiation media	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
'Ştefăneşti 22'	32.5b	37.2c	35.7b	23.7b	24.3a	24.3c	28.3b	43.7a	0	10.3c	0	-	-	-	-
'Costate 21'	35.6b	44.0b	54.2a	26.6b	22.1a	33.3a	23.3c	35.5b	0	0	21.9a	-	-	10.83	-
'Chihlimbar'	43.1a	46.3b	33.7b	14.7c	23.3a	37.5a	16.6d	48.1a	0	22.2b	25.0a	-	-	-	-
'Argeş 20'	42.0a	58.3a	37.2b	33.3a	20.0b	28.8b	35.5a	0	0	81.8a	0	-	8.33	-	-
'Argeş 11'	32.2b	41.2b	42.5b	15.6c	22.5a	28.7b	16.7d	0	0	0	0	-	-	-	-
Mean ±S dev.	37.1±5.2	45.4±7.9	40.7±8.2	22.8±7.8	22.4±1.6	30.5±5.0	24.1±8.0	25.5±23.7	0	22.9±34.2	9.4±12.9	-			

Mean values within a column followed by the same letters are not significantly different at $p < 0.05$ according to Duncan's multiple range test.

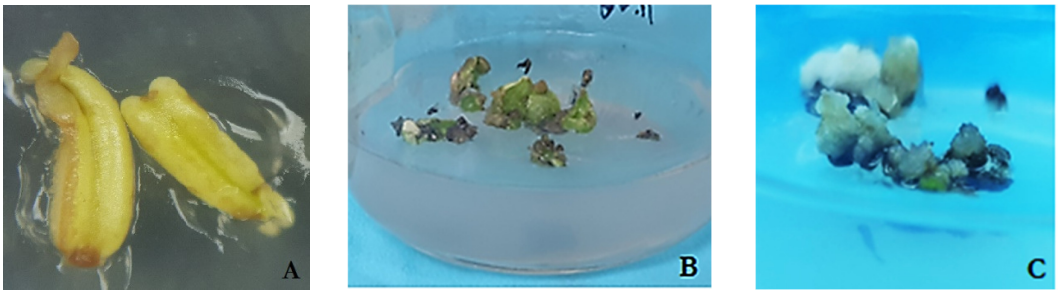

Figure 1. Evolution of anthers during the first three weeks of *in vitro* culture: (A) Callus formation from anther' filament and wall ('Argeş 11') on initiation medium; (B) Callus and morphogenic structures ('Argeş 20'); (C) Callus with embryogenic potential ('Costate 21')

Figure 2. Plant regeneration from anther culture: (A) Indirect organogenesis with shoots growth; (B) *In vitro* regenerated and rooted plants; (C) Anther-derived plants, accommodated to *ex vitro* conditions, obtained in 'Argeş 20' variety

Starting from anthers culture, we succeeded in obtaining normal plants with two out of the five tested varieties initiated on different media: in a proportion of 8.33% from the anthers inoculated on medium 1 for 'Argeş 20', and in a proportion of 10.83 for 'Costate 21' from anthers inoculated on medium 2. Thus, our results further strengthen the arguments in support of the close correlation between genotype, the stage of development of flower buds at the moment of initiation, and the composition of the initiation medium.

On the contrary, with transferred structures from the initiation medium 3 (containing 2,4-D) onto the induction medium was induced only callus formation (Figure 1C) in a percentage varying between 24.3 ('Ştefăneşti 22') and 37.5 ('Chihlimbar'). As long as the cultures were kept in the dark on the initiation medium, the calluses formed were mostly white and translucent. After the first transfer onto fresh medium and

light/dark photoperiod, most of these calluses proliferated, increased in size, and became greenish in color, while others gradually became pale brown and soon died. By repeated transfers, in the calluses with embryogenic potential could be initiated processes of formation of somatic pro-embryos, from which could be generated somatic embryos germinating into plantlets (Murovec and Bohanec, 2012). Our observations revealed the possibility of continuing the processes of differentiation and development of structures with embryogenic potential (Figure 3A and 3B). Normally, this type of structure had cell totipotency for plant regeneration. In our case, with tested varieties and induction medium composition (MS with BAP and IAA), such structures had not the ability to continue the embryo development. With tomatoes, we consider regeneration via embryogenesis starting from the anthers easy to be induced, but this does not guarantee subsequent development into new plantlets. Many other research papers presented the efforts to achieve regeneration via embryogenesis in tomatoes, but the results were poor and very often not reproducible (Saeed *et al.* 2019).

In the case of anther culture, the regeneration process can be followed, but the origin of each regenerated plant (from microspores, or anther's wall) remains uncertain. Either is a regeneration process by embryogenesis, or by organogenesis, in the case of anther culture any new regenerative structure could appear from anther walls, connective, filament tissues, or from interlocular septa that separate the two pollen sacs (Corral-Martínez *et al.*, 2011). If the aim is to obtain haploid, or dihaploid plants, these aspects are undesirable and impose to analyze every single regenerant for its origin and ploidy (Seguí-Simarro and Nuez, 2007).

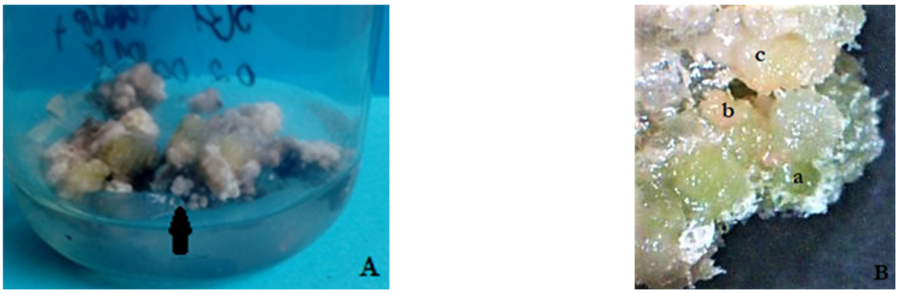

Figure 3. Somatic embryogenesis in tomato anther-derived callus. (A) Somatic embryos (arrow) obtained in 'Ştefăneşti 22' variety; (B) Globular-shaped embryo (a), Heart-shaped embryo (b) and torpedo-shaped embryo (c) arising on the surface of the callus in 'Chihlimbar' variety

Morphological aspects, comparisons between regenerated and donor plants

Between the 5th and the 8th month from the initiation of anther culture, ten plantlets with well-developed root system were gradually transferred in *ex vitro* conditions, in three series (in November: plants 1, 2 and 3; in December: plants 4, 5, 6 and 7; in January: plants 8, 9 and 10). Simultaneously, these anther-derived plants (Figure 2C) and the seedlings obtained by seed-germination with 'Argeş 20' variety (control) were transferred in the greenhouse to grow under normal conditions. At the end of the growing season, after evaluation of all morphological and agronomic traits of the plants, were noticed the following: one anther-derived plant (the 7th one) grew slowly and didn't produce flowers, other four plants (number 2, 3, 9 and 10) grew slowly and formed flowers which failed to produce fruits, and only five plants had a normal morphological appearance (plants number 1, 4, 5, 6 and 8), with relatively good growth, produced normal flowers that fruited (Table 2).

Although at the moment of transfer in the greenhouse, the anther derived plants had normal aspect and proper size, similarly to the control variety plants, we noticed a direct correlation between growing parameters of each plant and the duration of the tissue culture necessary until *ex vitro* transfer. The later the plantlets were acclimatized, the slower their growth and the lower their height. Also, the tomato fruit size was in all cases smaller, with a lower number of fruits/plant and lower homogeneity of their size and shape. All these morphological features (growth rate, leaf type and its attitude, flower characteristics, sterility, fruit features)

were noticed with different plant crops in regenerated plants from anther culture (Kozik *et al.*, 2002; Zagorska *et al.*, 2004; Guo *et al.*, 2005) and were associated with to changes in chromosome number, cytoplasmic changes or mutations induced during *in vitro* regeneration process.

Table 2. The descriptors for tomatoes with distinct differences between donor plant and the anther-derived regenerants (the cod of descriptor, its quotation, or mean value, and its significance)

Descriptor for tomato	Donor plant	Number of anther-derived plant									
	'Argeș 20'	1	2	3	4	5	6	7	8	9	10
7.1.2.3 Vine length [cm]	49	144	113	103	85	97	88	21	60	55	50
7.2.2.5 Predominant fruit shape	3/ Rounded	2/ Slightly flattened	-	-	4/ High rounded	3/ Rounded	2/ Slightly flattened	-	4/ High rounded	-	-
7.2.2.6 Fruit size	5/ Very large	2/ Small	-	-	3/ Intermediate	2/ Small	2/ Small	-	2/ Small	-	-
7.2.2.9 Fruit length [mm]	66.6	34.21	-	-	62.55	39.33	33.24	-	41.85	-	-
7.2.2.10 Fruit width [mm]	64.4	40.43	-	-	50.51	43.06	37.54	-	39.94	-	-
7.2.2.32 Shape of pistil scar	2/Stellate	4/Irregular	-	-	1/Dot	1/Dot	1/Dot	-	4/Irregular	-	-

Genetic diversity with SSR markers

A new regenerant, obtained via *in vitro* culture and expressing phenotypic characteristics different in comparison to the donor plant, represents a somaclonal variation, or a somaclone (Larkin and Scowcroft, 1981). In the case of anther culture is difficult to establish the origin of the new regenerant, but is possible to detect and evaluate certain genetic modifications induced into the new derived plant during the process of regeneration and to use it further on. Taking into consideration our previous experience with SSR markers and the advantages of their use (they can easily be amplified, have a high level of allelic diversity at different loci, are very efficient to prove the differences among different genotypes), we chose to test this type of markers with our regenerants.

The nine tested SSR markers were selected for their ability to produce polymorphic patterns as was shown by Saravanan *et al.* (2014) and Diklesh *et al.* (2016) with tomato genotypes. With these markers were scored amplified products with most of the DNA samples and the number of bands/markers varied between two (SSR T-7) and seven (SSR T-62). A total of 160 reliable bands were observed, of which 33 bands were polymorphic (Table 3).

Table 3. SSR primers with the detected bands, and their banding performance expressed by the coefficient of genetic similarity and polymorphism information content, recorded with donor plant and regenerants from anther culture

Primer	Scored bands	Percentage of polymorphic bands	Coefficient of genetic similarity (GS)	The polymorphism information content (PIC)
SSR T-7	11	18.18	0.50	0.415
SSR T-62	35	20.0	0.477	0.08
SSR T-70	13	15.4	0.448	0.297
SSR47	22	22.7	0.558	0.125
SSR63	12	25.0	0.481	0.579
SSR110	21	14.29	0.575	0.01
SSR111	16	31.25	0.480	0.406
SLM6-7	11	27.28	0.398	0.628
SLM6-12	19	15.79	0.519	0.091
Total	160	-	-	-
Average	17.8	21.09	0.493	0.292

The coefficient of genetic similarity (GS) is considered as a degree of homology between two genotypes and its value could vary between 1 (meaning identity of the two analysed genotypes) and 0 (meaning no relatedness between the two genotypes, or individuals). With all tested SSR markers, the average of detected homology between the control variety and the 10 regenerants was 0.493. Among markers, the highest value of similarity was found with SSR 110 (0.575) which was correlated with the lowest value of the PIC and the highest number of in pair-correlation (control-regenerant) with identity. On the contrary, with SLM6-7 marker was obtain the lowest value for genetic similarity, which was correlated with the highest value of the polymorphism information content. The average value of PIC for all tested SSRs of 0.292, ranging between 0.001 (for SSR110) and 0.628 (for SLM6-7) and could be considered good enough to investigate the differences among donor plant and each of the anther-derived regenerants.

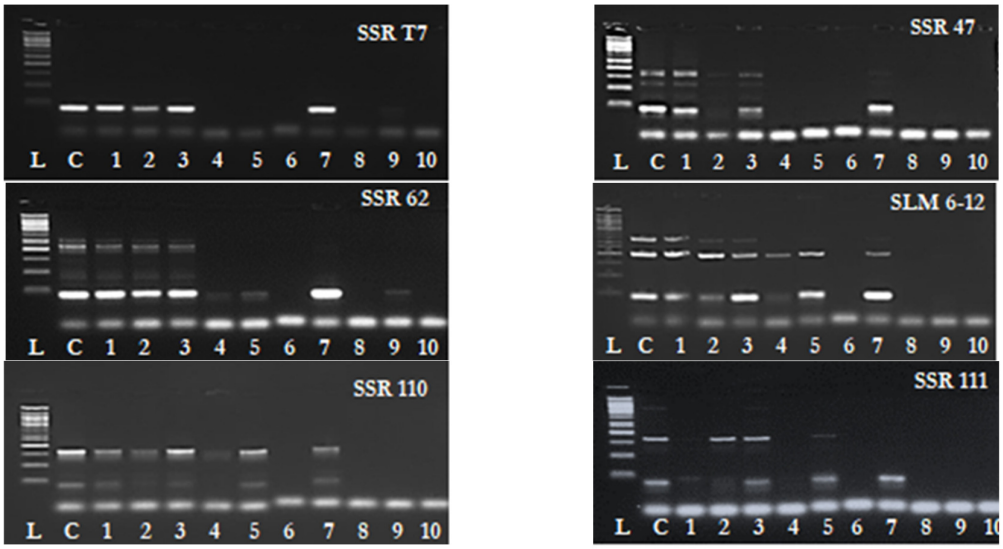

Figure 4. Banding patterns for six tested SSR markers in 'Argeş 20' variety and the ten tomato plants regenerated from anther culture. L - DNA ladder: 50 bp DNA; C- DNA of 'Argeş 20' (control variety); 1,2, ...10 – DNA of each regenerated plant

Similar to Diklesh *et al.* (2016), we scored all amplified products, including shadow and faint bands. With all SSR markers was obtained a lower number of detectable bands, sometimes weak, or no bands for the last three plants (number 8, 9 and 10) acclimatized to *ex vitro* conditions (Figure 4). SSRs have been the most widely used markers for genotyping plants over the past 25 years because they are highly informative, codominant, multi-allele genetic markers that are experimentally reproducible and transferable among related species (Gheorghe *et al.*, 2010; Shirasawa *et al.*, 2014; Aguirre *et al.*, 2017; Al-Shammari and Hamdi 2021). However, when using this type of molecular marker to distinguish between various genotypes, we must consider that SSRs have a high mutation rate. Several studies highlighted that as the result of selection pressure against mutations that alter the reading frame, their occurrence in gene regions is lower, and it was shown that in coding regions there is a predominance of SSRs with gene motifs of the tri- and hexanucleotide type (Zhang *et al.*, 2004; Xu *et al.*, 2013; Vieira *et al.*, 2016). This can be an explanation for the lack of DNA bands or the weak bands we can see in electrophoresis gels for the tomato plants developed from regenerants originated from calluses after a longer period of culture (8 months). Besides, in our experiment, the lack of DNA bands or weak bands cannot be considered as resulting from errors of DNA extraction or purification, as this was performed at the same time, using the same protocol for all the tomato plants derived from anther culture.

Conclusions

The results of our studies confirmed that the main factors involved in plant regeneration from anther culture are the genotype, the stage of development of the microspores at the moment of harvest, and the composition of the initiation medium. Were observed two regeneration patterns: either by embryogenesis, or by indirect organogenesis in the proliferative calluses dedifferentiated from the somatic tissues of the anthers. The regenerants obtained from 'Argeş 20' variety, acclimatized to *ex vitro* and grown in greenhouse conditions were analysed in comparison to the control genotype. The morphological characterization of the ten regenerants showed phenotypic variation in comparison to control related to the growth rate of the plants, traits of leaves, flowers, and fruits.

SSRs analysis aiming to reveal variation among anther-derived tomato plants, which was considered likely because the plant regeneration from *in vitro* anther cultures involved an intermediary callus phase, showed that out of the nine SSR primers tested, at least two primers (SSR63 and SLM6-7) proved to be very efficient in detecting genetic differences not only among regenerants, but also between them and the donor genotype ('Argeş 20').

Due to difficulties in establishing an efficient and reliable protocol for plant regeneration starting from *in vitro* anther culture, tomato is still considered a recalcitrant crop. Before becoming a reliable and optimized protocol, more investigations are necessary to improve the efficiency of regeneration starting from anthers. Besides the morphological variability, more complex investigations are necessary in order to detect and select valuable somaclones. The fruits harvested from anther-derived plants and the assessment of the uniformity/or variability among the second-generation plants obtained from seed germination will give us more answers and will provide additional tools for the selection of new tomato genotypes for breeding purposes.

Authors' Contributions

AB and CFP: conceived and designed the experiments; AB, CFP, AM and AMD: methodology; DIS: formal analysis; AB, CFP, AEM and AMD: investigation; AB, CFP and AMD: data curation; CFP and AEM: writing-review and editing; AB: visualization; DIS: supervision; CFP: project administration; CFP and AB: funding acquisition. All authors read and approved the final manuscript

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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