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Somaclonal Variation in the Tomato *Lycopersicon peruvianum*

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Abstract: The aim of this study is to optimise the appropriate conditions to initiate the callus and determine the phenotypic and genotypic variation of the somaclones of *L. peruvianum* derived from callus. On the basis of the results one can conclude that the medium hormonal content has significant influence on the initiation of *L. peruvianum* callus and the cotyledone explants are most useful for this. The optimal medium to initiate the *L. peruvianum* callus cultures is MS enriched with 2 mg•dm^{-3} naphtyl-1-acetic acid (NAA) and 1 mg•dm^{-3} benzylaminopurine (BAP). Explants placed on the medium mentioned above gave the highest mass of the callus on average. The addition of BAP cytokinin to initiating medium stimulated the process of organogenesis. Whereas the process of rhizogenesis was observed in the media with the addition of auxins. The ISSR-PCR method applied in this study allowed to identify the differences among the somaclones derived from callus. Such differences can indicate the presence of the genetically determined somaclonal variation. Each tomato somaclone differed from the other in the DNA bands pattern visible on the electrophoregrams. The somaclones obtained from *L. peruvianum* were similar to each other in 9.1 to 66.7 %.

Key words: Tomato · callus · somaclonal variation · genetic differences

INTRODUCTION

The genetic unification of varieties of numerous species of cultivated plants comprises one of the principal reasons for the lowered breeding progress and a lack of new material for breeding. In this situation, a search for sources of genetic variability of economically important traits, as well as their comprehensive examination is becoming a necessity. One of the methods of searching for new initial materials consists of inducing somaclonal variation caused by plant regeneration in *in vitro* cultures [1].

There are many sources of somaclonal variability. An especially large frequency of this type of changes is observed in callus cultures and clones obtained from Karp [2]; and Peschke and Phillips [3]. Its appearance is also to a considerable degree affected by the explant used, age of culture, hormonal composition of the medium used for callus initiation and plant regeneration, as well as conditions in which the culture was maintained.

In the light of the studies conducted hitherto the somaclonal variability is considered a desirable phenomenon, principally in resistance breeding. In plants it may constitute a source of genes responsible for the resistance to diseases and pests, for tolerance to herbicides and unfavourable environmental factors. Moreover, among the somaclones obtained one may search for morphologic changes in valuable plants, principally in horticultural breeding [4].

The changes observed in *in vitro* cultures are often of a physiological character [5] and differentiating them from those determined genetically (spontaneous mutations) is impossible at the culture stage. However, the character of the variability observed in *in vitro* cultures may be determined by various methods. For the determination of the character of somaclonal variability Smulders *et al.* [6] proposes cytological methods, which render possible a precise identification of the type of chromosome aberrations created. For the evaluation of the genome size of plants regenerated on *in vitro* cultures the method of flow cytometry is useful [7]. However, among the methods most often used for the determination of somaclonal variability one should mention the RFLP method (polymorphism of the restriction fragments length), the RAPD-PCR method (randomly amplified DNA polymorphism) and recently the most popular, the ISSR-PCR technique. The essence of this method lies in the determination of differences in the length of DNA fragments contained between microsatellite sequences and amplified by the PCR technique [8].

The presented studies aimed at separating callus cultures of the *L. peruvianum* line, their regeneration and determination of phenotypic and genetic differences,

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the latter on the DNA level using the ISSR-PCR technique.

MATERIAL AND METHODS

The clones of tomato *L. peruvianum* were obtained from a callus tissue grown on cotyledons of 10-day-old seedlings. The seeds of line LA 0462, chosen for the experiment, originated from Chile, from the vicinity of Sabraya, Tarapaca and were obtained from Tomato Genetic Centre gene bank, California. Before germination, the seeds were disinfected twice. The first time - for 70 min, in a 0.1% solution of NaOCl, the second time - for 1 min, in a 5 mM • dm^{-3} solution of $CaSO_4$, after rinsing in distilled water for 30 min. Next, the seeds were placed in 100 mL Erlenmayer flasks (3 per flask). Each of them contained 20 mL of medium MS [9]. The germination was conducted at a temperature of 24°C in white light 40 PAR ($\mu E\ m^{-2}\ s^{-1}$) for 16 h.

The callus tissue was induced on a MS medium supplemented with auxin IAA or NAA, in combination with cytokine BAP or without it (Table 1), in light or in darkness. The callus initiation was accompanied by a process of spontaneous organogenesis (photo 1, 2, 3 and 4). The greatest number of shoots was obtained on mediums containing 1 or 2 mg•dm^{-3} of IAA. In the case of the remaining mediums, the callus forming on cotyledons as a rule showed no totipotent properties. During the subsequent stage of studies as explants were used shoots, roots or callus fragments, generated on a MS medium supplemented with 0.3 mg•dm^{-3} IAA and 3.0 mg•dm^{-3} BAP in temperature and light conditions analogous to that applied in the germination phase. Plants maintained on a MS medium containing no growth regulators were treated as control. In total, from all the experimental combinations (mediums for callus induction) 7 *L. peruvianum* clones were selected. Clones S1, S3 and S5 comprised plants regenerated from a callus tissue grown on cotyledons on a MS medium supplemented with 1 mg•dm^{-3} of IAA and BAP; S2 and S6 - plants regenerated from a callus grown on a medium supplemented with 2 mg•dm^{-3} IAA and 1 mg•dm^{-3} BAP; S4 - plants obtained from a callus grown on cotyledons on a MS medium supplemented with 1 mg•dm^{-3} IAA. Clones S5 and S6 came from a callus induced in darkness, while the remaining lines - from a callus induced in light.

Table 1: Media composition for initiating callus cultures of *L. peruvianum*

Auxin	Quantity [mg•dm^{-3}]	Cytokinin	Quantity [mg•dm^{-3}]
IAA	1	BAP	1
IAA	2	BAP	1
NAA	1	BAP	1
NAA	2	BAP	1
IAA	1	-	-
IAA	2	-	-
NAA	1	-	-
NAA	2	-	-

Control - MS without plant growth regulators

The biometric measurements covered only the height of plants, the number of side shoots (coefficient of multiplication) and roots formed, as well as their length. On this basis phenotypic differences were determined within the selected *L. peruvianum* lines and between them. On the DNA level the differences between lines and the control forms were determined using ISSR markers. DNA was isolated from plantlets, using the Genomic DNA Prep Plus (A&A Biotechnology) kit. Sequences of genomic DNA, contained between microsatellite sequences characterized by a different length and different composition of the repeated motive, were subjected to a molecular analysis. The DNA amplification was conducted according to the method described by Ziêtkiewicz *et al.* [10]. Twenty one primers were used, with sequences given in Table 2.

Table 2: ISSR primers used in the present study

Primer No.	Sequence (5' - 3')	Melting temperature (T_m)	Anneling temperature (T_a)
802	$(AG)_8T$	50°C	48°C
805	$(AC)_8G$	52°C	50°C
812	$(GA)_8C$	52°C	50°C
825	$(AC)_8T$	50°C	48°C
833	$(GT)_8YC$	55°C	53°C
840	$(GA)_8GT$	50°C	48°C
841	$(GA)_8AT$	50°C	48°C
848	$(CA)_8GC$	55°C	53°C
851	$(GT)_8CG$	55°C	53°C
859	$(GT)_8YC$	55°C	53°C

Table 3: Mean values (x) of morphological traits tomato initiated on media with differentiated values of plant growth regulators

		Plant growth regulator [mg dm^{-3}]				
Traits	Ligth conditions	IAA 1, BAP 1	IAA 2, BAP 1	NAA 1, BAP 1	IAA 2	Mean
Plant height	light	2.57 ab	1.57 b	3.81 a	2.21 ab	2.54 a
(cm)	darkness	1.55 ab	3.04 a	0.00 b	0.00 b	1.15 b
	mean	2.06 a	2.31 a	1.91 a	1.11 a	
No. of shoots	light	2.20 a	1.73 ab	2.20 a	1.20 b	1.83 a
axillary	darkness	1.33 a	1.07 a	0.00 b	0.00 b	0.60 b
	mean	1.77 a	1.40 ab	1.10 bc	0.60 c	
No. of roots	light	1.87 b	3.47 a	1.93 ab	0.67 b	1.98 a
	darkness	0.93 a	0.13 a	0.00 a	0.00	0.27 b
	mean	1.40 ab	1.80 a	0.97 ab	0.33 b	
Roots length	light	0.48 b	3.30 a	1.37 b	4.68 a	2.46 a
(cm)	darkness	1.05 a	0.26 a	0.00 a	0.00 a	0.33 b
	mean	0.77 b	1.78 ab	0.69 b	2.34 a	

a, b, ... - mean values followed by the same letters are not significantly different at p = 0.05

The amplification products (Mastercycler 5333 - Eppendorf) were separated on a Sub Gell GT (Bio-Rad) apparatus, on 2% agarose, under a constant current of 100 V. The products obtained were visualized on a transiluminator UV-21 (Fotodyne) in the presence of ethidium bromide (5 mg•mL^{-1}) and documented (camera Polaroid DS-34). For the analysis of the ISSR amplified products obtained the "Diversity one" 1.3 (Pharmacia LKB) computer software was used. The amplification products were analysed in a binary arrangements (0/1). The overall number of ISSR products was determined including the number of mono- and polymorphic products. A comparison was made between the products specific for the amplification of *L. peruvianum* clones and for control plants.

RESULTS

The culture conditions in which the callus was initiated had a significant effect on the height of the regenerated plants, the multiplication coefficient expressed as the number of side shoots and number and length of roots. The height of plants obtained from a callus culture maintained in light ranged from 1.57 to 3.81 cm, depending on the hormonal composition of the medium (Table 3). The callus initiated in the dark on a medium containing 2 mg•dm^{-3} IAA or NAA and BAP (1 + 1 mg•dm^{-3}) did not regenerate into shoots. In turn, the height of plants regenerated on mediums supplemented with IAA and BAP (1+1 or 2+1 mg•dm^{-3}) reached 1.55 and 3.04 cm, respectively.

The lowest multiplication coefficient (1.07) was observed for plants regenerated from a callus tissue initiated in the dark on a medium containing IAA and BAP (2+1 mg•dm^{-3}), while the highest (2.20) - on mediums maintained in light and containing IAA+BAP (1 + 1 mg•dm^{-3}) or NAA and BAP (1 + 1 mg•dm^{-3}). The subsequent effect of a callus initiating medium was observed also in the case of roots. Plants regenerated from a callus initiated in light formed on an average from 0.67 to 3.47 roots; the least on a medium supplemented with 2 mg • dm^{-3} IAA and the most on a culture with IAA and BAP (2 + 1 mg•dm^{-3}). A lack of rhysogenesis was observed in the case of callus cells obtained from cultures maintained in darkness on mediums containing 2 mg IAA or NAA and BAP (1 + 1 mg•dm^{-3}). In the case of plants obtained from a callus induced in the dark on mediums containing IAA and BAP (1 + 1 i 2 + 1 mg•dm^{-3}) a mean of between 0.93 and 0.13 roots was observed (Table 3).

The hormonal composition of the callus initiating medium also had a significant effect on the length of roots of the regenerated plants. In the case of cultures maintained in light the longest roots were observed for plants obtained from a callus initiated on a medium supplemented with 2 mg•dm^{-3} IAA (4.68 cm) and the shortest (0.48 cm) - on a medium with IAA and BAP (1 + 1 mg•dm^{-3}). Short roots (0.26 cm) were observed in plants obtained from a callus created in the dark on a medium containing IAA and BAP (2 + 1 mg•dm^{-3}). The length of roots of regenerated plants grown from a callus obtained on a medium supplemented with 1 mg•dm^{-3} IAA and 1 mg•dm^{-3} BAP amounted to 1.05 cm (mean for the population).

As result of the ISSR-PCR reaction numerous polymorphic *loci* were amplified, differentiating the genotypes of the selected callus cultures of the *L. peruvianum* line and of control plants (Tables 4 and 5). For somaclone S1 a total of 13 *loci* were amplified.

Table 4: Polymorphic bands revealer through ISSR fingerprinting

Somaclones	Primers No. and products length in bp
S1	$802_{[1321, 707]}$; $812_{[490, 187, 35]}$; $840_{[1367, 1156, 1012, 797, 671, 533, 378]}$; $859_{[840]}$
S2	$802_{[3000, 1673, 1321, 722]}$; $812_{[490, 187, 35]}$; $833_{[959]}$; $841_{[1423]}$; $848_{[1018]}$; $859_{[840, 610, 503, 268, 166, 78]}$
S3	$802_{[3000, 1321, 722]}$; $812_{[490, 35]}$; $833_{[514, 378]}$; $840_{[873, 671, 378]}$; $841_{[1423]}$; $848_{[1301, 464]}$; $859_{[840, 610, 268]}$
S4	$802_{[1673, 722]}$; $812_{[187, 35]}$; $833_{[959, 763, 514]}$; $840_{[1367, 1012, 873, 671, 352]}$; $848_{[464]}$; $859_{[503, 268, 78]}$
S5	$802_{[1749, 1321]}$; $805_{[1758]}$; $812_{[490, 187, 35]}$; $825_{[1468, 1068]}$; $833_{[1180, 959, 763, 514]}$; $840_{[1599, 1367, 1012, 957, 873, 671, 533, 456]}$; $841_{[1176]}$; $848_{[1301]}$; $859_{[840, 610, 268, 166, 78]}$
S6	$802_{[1749, 1321]}$;,; $805_{[1758]}$; $812_{[490, 187, 35]}$; $825_{[1468, 1068]}$; $833_{[1180, 763, 378]}$; $840_{[1599, 1367, 1156, 1012, 957, 797, 671, 533, 456]}$; $841_{[1176]}$; $848_{[1018]}$; $859_{[840, 610, 503, 78]}$
Control	$802_{[1321, 707]}$; $812_{[490, 187]}$; $840_{[1599, 1367, 1156, 1012, 957, 352]}$

Table 5: Somaclones-specfic bands revealed through ISSR fingerprinting

Clones	Primers No. and products length in bp
S1	$802_{[2551]}$; $812_{[640]}$; $833_{[1557]}$
S2	$833_{[1458, 1155]}$; $848_{[1192]}$
S3	$833_{[974]}$; $840_{[1478]}$; $841_{[1151]}$; $848_{[1213, 983]}$
S4	-
S5	$802_{[972, 737, 559, 422]}$; $805_{[3548, 2579, 2053]}$; $848_{[1421, 1131]}$; $859_{[2236]}$
S6	$802_{[758, 685]}$; $833_{[1013, 493]}$; $840_{[2177]}$; $848_{[1324, 1171]}$
Control	$802_{[2000, 1550, 1095]}$; $840_{[710, 609, 544, 506]}$

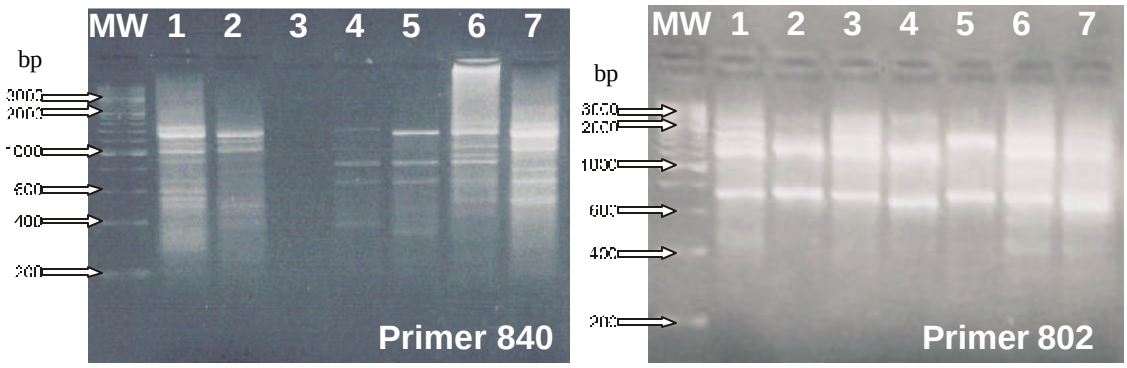
Bands ~2551, ~640 i ~1557 bp in size, amplified by primers 802, 812 and 833, proved to be polymorphic and did not appear either in control plants or in other somaclones (Table 5; photo 1, 2 and 3). For somaclone S2 three specific ISSR products were amplified: ~1458 and ~1155 bp in size, generated by primer 833 and ~1192 bp, amplified by primer 848. For clone S3 5 ISSR products were amplified, specific in relation to other somaclones and control plants: ~974 bp (primer 833), ~1478 bp (840), 1151 bp (841), ~1213 and ~983 bp in size (848). In turn, on the electrophoregrams of clone S4 the presence of 16 polymorphic ISSR products was observed, but no products genetically specific. Clones S5 and S6 were characterised by the highest number of polymorphic ISSR fragments. The genotype of the first differed from the remaining comparable forms by *loci* ~972, ~732, ~559, ~422 ~bp long (primer 802), ~1348, ~2579, ~2053 bp (805), ~1421, ~1131 bp (848) and ~2236 bp (859). In turn, for clone S6 characteristic were ISSR products ~758, ~685 bp, amplified by primer 802, ~1013 and ~493 bp (primer 833), ~2177 bp long (primer 840) and ~1171 and ~132 bp, (primer 848). Polymorphic *loci* for the control plants (~187 to ~1591 bp) were obtained using primer 802, 812 and 840 for DNA amplification (Table 5; photo 1, 2 and 4). ISSR products ~2001, ~1550 and ~1095 bp, amplified by primer 802 as well as ~701, ~609, ~544 and ~506 bp, amplified by primer 840, proved to be specific for control plants. As a rule, in the ISSR reactions conducted, one primer amplified a mean of 19.3 *loci* for the DNA templates examined and the length of the products amplified ranged from ~35 to ~3000 bp in size.

An analysis of the phylogenetic similarity tree for somaclones took into consideration their similarity to the control line. The clones, separated from the callus tissue, were similar in 9.1-66.7% and the greatest genetic similarity (66.7%) was observed between clones S5 and S6. Clones S3 and S4 were similar in 53.1%, while in relation to clone S2 - in 53.3%. The similarity of clones S3 and S2 amounted to 52%. Clones S2, S3, S4, S5 and S6 were similar to the control plants in 44.4%, while to S1 - in 9.1% (Fig. 1).

DISCUSSION

One of the important elements in the process of forming the callus tissue is the hormonal composition of the medium used [4, 11]. The most often used are auxin and cytokine [12]. For the initiation of the callus of tomato *L. esculentum* auxin NAA and cytokine BAP or auxin IAA and kinetin were used [13, 14], for *L. pennelli* and *L. pimpinellifolium* - auxin IBA or picloram together with BAP and also medium MS supplemented only with kinetin [15-18]. Some authors [19] for the tomato callus initiation used auxin: NAA (15 mg • dm^{-3}) or 2,4-D. In the work presented here the callus grew most intensively on a MS medium supplemented with 2 mg•dm^{-3} NAA and 1 mg•dm^{-3} BAP. This callus was characterised by ability for organogenesis, was light green in colour and showed a tubercular structure. The second auxin used (IAA) also was favourable for organogenesis. Light conditions proved to be the second factor, beside the medium, decisive for the success of the culture. In the case of the tomato light clearly stimulated the forming of the callus tissue and had a significant effect on the subsequent regeneration of plants. This is concordant with the results reported by Latkowska and Chmiel [20].

In the work presented the forming of shoots and roots was observed on four of the eight hormonal combinations used in mediums: 1 + 1 mg•dm^{-3} of NAA and BAP, IAA and BAP in a similar concentration of both hormones or with a IAA content increased by 1 mg•dm^{-3} and only 2 mg•dm^{-3} of auxin IAA. On mediums



MW - molecular weight, 1 - control, 2 - somaclone S1, 3 - somaclone S2, 4 - somaclone S3, 5 - somaclone S4, 6 - somaclone S5, 7 - somaclone S6

Fig. 1: Electrophoregrams of ISSR products amplified on DNA templates of seven somaclones of *L. peruvianum* . Arrows mark ISSR polymorphic products

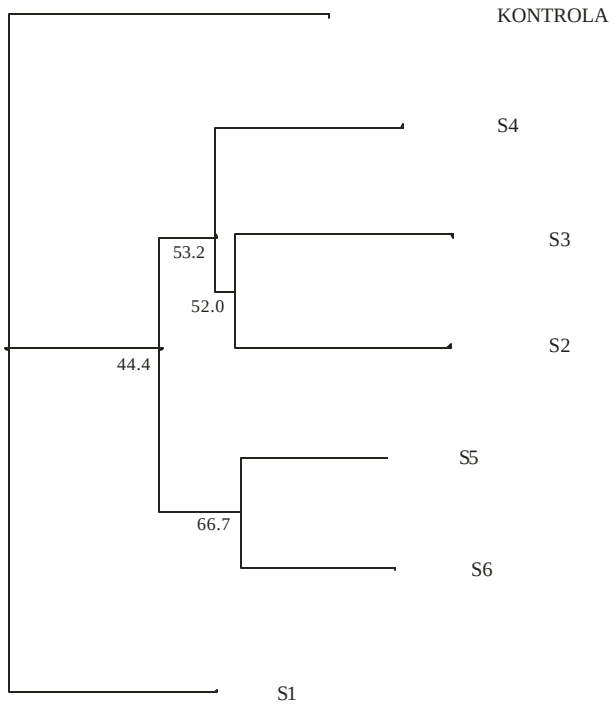


Fig. 2: Dendrogram of seven somacloness based on ISSR markers clustered by UPGMA technique

containing IAA and BAP, irrespectively of the concentration, the plant regeneration started both in light and In dark, while on mediums containing NAA + BAP or exclusively IAA - only from callus cultures maintained in light. Tomato shoots in callus cultures maintained in light, in the presence of the plant hormones tested in the present study (IAA and BAP), though in a different concentration (0.5 mg•dm^{-3} IAA i 5 mg•dm^{-3} BAP), were obtained also by de Faria *et al.* [15], while in the presence of cytokine BAP alone (4 mg•dm^{-3}) - by Sonyia *et al.* [18].

The investigations demonstrated that light had a significant effect on the number and height of tomato shoots forming on callus cultures as also on the development of roots. His is concordant with the results reported by other authors [15, 18, 21]. According to Pugliesi *et al.* [21] the forming of shoots in callus cultures is possible only In the case of cultures maintained in light. In the studies presented here on cultures maintained in light the regeneration of shoots was not observed in the case of two (out of four) medium combinations (with 2 mg IAA or with 1 + 1 mg•dm^{-3} NAA and BAP). On mediums containing IAA and BAP (1 + 1 or 2 + 1 mg•dm^{-3}) the forming of shoots and roots was observed, but their development was much poorer than on cultures maintained in light.

The evaluation of genetic differences between the callus cultures of *L. peruvianum* clones was based on the ISSR-PCR technique. According to numerous authors [22-24], it combines the simplicity of the RAPD technique with the repeatability of the AFLP technique. For this reason it is increasingly often used for the evaluation of polymorphism within the DNA sequences. Kochieva *et al.* [23], for the evaluation of genetic differences between cultivated and wild forms of the tomato used 14 ISSR primers, Smolik *et al.* [25] - 18 primers, Leroy *et al.* [24] determined the genetic differences between 38 cauliflower lines using only 4 microsatellite primers for the DNA amplification. In the present studies 21 microsatellite primers were used. Only 10 of them generated products visible on the electrophoregramme. For the genotypes examined a total of 193 ISSR products were amplified (~35 to ~3548 bp in size). The *L. peruvianum* clones differed in the number of both mono- and polymorphic DNA products and they created band patterns specific for each genotype. In total, 35 genetically specific products were identified (from ~422 to ~3548 bp in size). Within only Polish tomato varieties, using 18 ISSR primers, 136 ISSR products were amplified, of which 34 proved to be polymorphic and 12 - monomorphic [25]. The length of the products obtained ranged from ~3711 to ~229 bp. Basing on the results cited it was ascertained that the varieties obtained were to a considerable degree genetically uniform - the genetic similarity ranged from 80 to 94.4%.

In the studies presented here the genetic differences between the *L. peruvianum* lines grown from callus cultures were considerable. This is testified by the variability ranging from 9.1 to 66.7%. The smallest genetic similarity in relation to the original (control) form was demonstrated by clone S3 (9.1%), while the most similar phylogenetically were clones - S5 and S6 (66.7%).

The results obtained demonstrated that the callus cultures of the *L. peruvianum* tomato may be successfully used when searching for genetic variability, necessary in the breeding of new varieties of this plant. They constitute an introduction to further studies aiming at the search for genotypes determining the tolerance of the tomato to soil salinity.

CONCLUSIONS

The medium composition and culture conditions at the stage of forming the callus tissue have a significant effect on the regeneration and subsequent growth of the tomato *L. peruvianum.* Callus cultures, maintained in light demonstrated an ability to organogenesis, irrespectively of the medium composition. Plants regenerated from callus cells grown on a MS medium supplemented with NAA and BAP (1 + 1 mg•dm^{-3}) were characterised by the highest shoots, while those regenerated from a callus induced on a medium containing IAA and BAP (2 + 1 mg•dm^{-3}) - the best developed roots.

The *L. peruvianum* clones grown from callus cultures were characterised by a considerable variability, determined at the DNA level (9.1-66.7%). The electrophoregrammes obtained for a majority of the clones examined demonstrated the presence of ISSR products characteristic exclusively for the given clone and control sample. The results obtained render it possible to state that the differences observed between clones are determined genetically.

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