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Somaclonal variation at the nucleotide sequence level revealed by RAPD and ISSR markers

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Abstract Genetic diversity is one of the most important factors for crop improvement. On the the other hand, for micropropagation or genetic transformation the most crucial aspect is to retain genetic integrity with respect to the mother plants. In order to select genotypes with in vitro stability or instability we evaluate the regeneration ability of the 30 lines Romanian cultivars and genetic diversity of the regenerants using RAPD and ISSR markers. 3 out of 30 genotypes tested had regeneration ability with a frequency of 1 %. The RAPD primers amplified fragments with the size ranged from 200 bp to 1230 bp. The total number of clear bands obtained from each primer ranged from 4 to 9. The number of polymorphic bands ranged from 2 to 6. The ISSR primers amplified fragments with the size ranged from 130bp to 2420bp. The total number of clear bands obtained from each primer ranged from 10 to 26. There were differences between genotypes regarding the number of polymorphic bands obtained with the same primer. To date, few studies have been conducted to assess the level of variation among somaclones of Medicago sativa using DNA polymorphism.

Key words

genetic diversity, alfalfa, ISSR markers

Tissue culture is a mutagenic technique causes cytogenetic, genetic and epigenetic variation colectivelly referred to as tissue culture - induced or somaclonal variation [7,10]. For this reason, plant regeneration from alfalfa cells cultivated in vitro may be a source of useful variation [17]. However, when in vitro techniques are used for plant propagation and/or genetic modification the most crucial aspect is to retain genetic integrity with respect to mother plants.

Alfalfa is also one of the most frequently studied crops from the point of view of tissue culture-derived embryo production. Plant regeneration in lucerne (Medicago sativa) is highly genotype dependent and inspite of considerable information on in vitro regeneration in this species [4,13].

Inter simple sequence repeats (ISSR) has recently been developed as an anonymous, RAPD – like approach that accesses variation in the numerous microsatellite regions dispersed throughout the various genomes and circumvents the challenge of characterizing individual loci that other molecular approaches require. They are characterized by mono-, di- or multi - nucleotide repeats that have 4 -10 repeat units side-by-side. Extremely high variability combined with greater robustness in repeatability experiments and less prone to changing band patterns with changes in constituent or DNA concentration template make them superior to other readily available marker systems in investigations of genetic variation [3,12]. In the present study, we report the use of molecular marker such as ISSR, for the assessment of genetic diversity of the regenerants compared with mother plants in order to select the most apropiate genotype for the different task of the breeding program. Several strategies are available for detecting somaclonal variation including phenotypic identification, cytological analysis, and molecular methods. Several authors have used molecular markers to monitor genetic stability in *in vitro* culture-derived plants [5, 6, 11, 14, 15, 19].

Randomly amplified polymorphic DNA sequences or RAPD markers are based on the amplification of unknown DNA sequences using single, short and random oligonucleotide primers [18]. RAPD-PCR markers are currently used as genetic markers in breeding programs for the assessment of genetic variability and relationships among alfalfa (Medicago sativa) genotypes [1,9]. RAPD-PCR are currently used as genetic markers quite useful in breeding programs for assessment of genetic variability between genera, species, populations, cultivars and very related lines [8]. The ability of RAPDs to produce multiple bands using a single primer means that a relatively small number of primers can be used to generate a very large number of fragments. These fragments are usually generated from different regions of the genome and hence multiple loci may be examined very quickly.

Material and Method

Plant material. 30 lines and cultivars were involved in this study: Satelit, F 1109-99, F105- 90, Granat, Cosmina, Sigma, F1615-04, F1206-00, F1306-01, F1822-06, Super, F270-91, F1413-02, F1310-01, F1111-99, Pastoral, Magnat, Alina, Selena, Stolo-13, Mf 42-96, Viking, Cristal, F219-91, Coral, Dorina, Saturn, Opal, Venus, F907-97. The seed material was obtained from department working in alfalfa breeding from I.N.C.D.A. Fundulea.

Alfalfa seeds were surface sterilized by immersion in 70% ethanol for 10 sec., and then in 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 3 min, followed by four washes with sterile distilled water. Sterile seeds were inoculated on half-strength MS medium for germination.

The explants (cotyledons, roots, petiole and leaflets) were prepared from plantlets obtained by seed germination in aseptic conditions, three weeks after culture initiation. For callus and embryogenesis induction was used Murashige and Skoog medium added with 2 mg/l 2,4 D and 0,5 mg/l kinetin, 3% sucrose and B5 vitamins, solidified with agar.

Vigorously growing, light-yellow primary callus was transferred on the same basal medium supplemented with 3% (w/v) sucrose, B5 vitamins and solidified with agar for embryos development and plant regeneration. Embryos with cotyledon were transferred to half-strength MS medium added with 4 mg/l GA_3 for germination.

The plantlets regenerated were maintained in aseptic conditions for further growth. For DNA extraction young leaves from in vitro growing plants were sampled. All in vitro cultures were maintained at 24°C, with a 16 h light regime.

DNA extraction. Total genomic DNA of 39 regenerated plants via somatic embryogenesis and from mother plants was extracted using Maxwell™ 16 Instrument from Promega. Purified concentrated products are obtained at high quality and high yield and can be used directly in a variety of downstream applications.

ISSR analysis

Primers and ISSR-PCR assays. The 9 primers were screen for detection of the genetic polymorphism among regenerated plants and mother plants: A2, A3, A7, A10, A12, A13, A17, A21, UBC818) with following sequences: (ACTG)5, (GACA)5, (AG)10T, (CT)10T, (GA)6CC, (GT)6CC, (GTG)3GC, (CA)6AC, (CA)7G [20]. These were based on either di- or multinucleotide repeats that were complementary to microsatellite. The discriminative power of DNA markers used as tool to characterize genetic diversity is very important because they can be used to assess the polymorphism of alfalfa in vitro regenerated plants. The amplifications were carried out in a 25 µl PCR buffer containing: GoTaq® Green Master Mix ready-to-use solutions (GoTaq® DNA Polymerase, dNTP, MgCl2, and reactions buffers at optimal concentrations for efficient amplification of DNA templates), ISSR primers, DNA template and Nuclease-Free Water. GoTaq® Green Master Mix contains two dyes (blue and yellow) that allow monitoring of progress during eletrophoresis. DNA amplification was carried out using a Thermal-cycler by Corbett and reactions were submitted to the following PCR program: preliminary DNA denaturation for 5 min at 94°C, followed by 35 cycles consisting of denaturation (1 min, 94°C), primer annealing (1 min, 46-51°C), and extension (3 min, 72°C). A final extension for 7 min at 72°C was included. The ISSR products were separated by electrophoresis (3V cm-1) in 2% agarose gels, which run with 1xTAE buffer. Photos documentation was performed under UV light using a photo imaging system. Number of bands generated by different pairs of primer used was scored and the frequency of polymorphism was calculated.

RAPD analysis

Primers and RAPD-PCR assays. The 5 primers were screen for detection of the genetic polymorphism among regenerated plantlets and mother plants: G06, G10, G16, L14, L12 with following sequences: GTGCCTAACC, AGGGCCGTCT, AGCGTCCTCC, GTGACAGGCT, GGGCGGTACT [16]. The amplifications were carried out in a 25 µl PCR buffer containing: GoTaq® Green Master Mix ready-to-use solutions (GoTaq® DNA Polymerase, dNTP, MgCl2, and reactions buffers at optimal concentrations for efficient amplification of DNA templates), RAPD primers, DNA template and Nuclease-Free Water. GoTaq® Green Master Mix contains two dyes (blue and yellow) that allow monitoring of progress during eletrophoresis. DNA amplification was carried out using a Thermalcycler by Corbett and reactions were submitted to the following PCR program: : preliminary DNA denaturation for 5 min at 94°C, followed by 45 cycles consisting of denaturation (1 min, 94°C), primer annealing (1 min, 36°C), and extension (2 min, 72°C). A final extension for 3 min at 72°C was included. The RAPD products were separated by 2% agarose gels electrophoresis, which run with 1xTAE buffer. Photo documentation was performed under UV light using a photo imaging system. Number of bands generated by different pairs of primers used was scored and the frequency of polymorphism was calculated.

Data analysis. Data were analyzed by current statistical analysis methods. The amplification bands were scored as (1) and (0) on band (allele) presence and absence, respectively. The frequencies of the ISSR and RAPD fragments were estimated for each of individuals from each genotype. Sizes amplification bands were estimated using PhotoCapt Molecular Weight system.

Results Obtained

Somatic embryogenesis and plant regeneration. All types of explants (cotyledons, petioles, roots and leaflets) produced callus on MS medium supplemented with 2, 4-D and kinetin combination with similar morphological characters. Embryos could not form unless the callus was transferred to a growth regulator-free medium. After 1 month, all the calli were transferred to hormone-free MS medium for embryos development. For plant regeneration and rooting, cotyledonary embryos developed were transferred to half-strength MS medium added with 4mg/l GA3. A few of the embryos developed into plants, the rest were arrested at the globular or torpedo stages.

Almost all cultivars tested produced embryogenic calli but embryos failed to develop. 3 cultivars out of 30 genotypes tested have regeneration ability. The frequency of plants with regeneration ability was 1% in the population of all three cultivars selected for this trait. The results obtained are presented as follow: F105-90 genotype produced 15 regenerants, Sigma - 15 regenerants and Selena 9 regenerants.

In Medicago sativa regeneration is genotype specific and only a few genotypes in some cultivars have been isolated for their ability to regenerate plants from explants-derived calli. The ability of alfalfa culture to regenerate plants is under genetic control and occurs with a frequency of 1 to 10% in most cultivars [2]. The variable response within a cultivar reflects the facts that alfalfa is an open-pollinated species and each cultivar is actually a heterogeneous mixture of genotypes.

Somaclonal variations at molecular level.

The 9 primers were screened for their ability to generate ISSR polymorphic DNA bands using the accessions total DNA. Only 5 produced reproducible fragments. The data revealed that the di- and tri-nucleotid are more informative than tetranucleotide. The number of total bands, their polymorphism, the percentage of polymorphism and genetic similarity coeficient are presented in table 1.

Table 1

Polymorphism rate for the regenarants of F 105-90 line using ISSR and RAPD primers

No. crt.	Primer	Nucleotide sequence	Bands No.		Polymor-ph %	Bands (pb)	PIC $\bar{x} \pm s_{\bar{x}}$	PI	Genetic similarity coef.	
			Total	Polymorph					$\bar{x} \pm s_{\bar{x}}$	$s_{\%}$
1	A-12	(GA)6CC	26	17	65,38	330-1730	0,321±0,038	5,453	0,699±0,038	21,85
2	A-13	(GT)6CC	21	14	66,66	220-1660	0,286±0,037	4,008	0,791±0,036	18,06
3	UBC-818	(CA)7G	15	7	46,66	310-1660	0,334±0,055	2,336	0,759±0,048	25,53
4	G -06	GTGCCTAACC	8	6	75,00	300-830	0,298±0,053	1,789	0,594±0,040	27,12
5	G -10	AGGGCCGTCT	4	3	75,00	240-330	0,242±0,085	0,727	0,479±0,061	51,09
6	G -16	AGCGTCCTCC	8	4	50,00	430-650	0,229±0,066	0,914	0,734±0,043	23,15
7	L -14	GTGACAGGCT	8	4	50,00	200-400	0,189±0,045	0,758	0,734±0,036	19,53

The total polymorphism generated by a certain primer (PIC), which indicates its discriminating power, presented reduced amplitude of variation, with values comprised between 0.286 in A-13 and 0.334 in UBC-818, with a mean of 0.313. The discriminating index (PI), which proves the efficiency of a certain primer in polymorphism detection, had values comprised between 5.453 for the primer A-12 and 2.336 for the primer UBC-818. The results prove that, among the regenerants of the F 105-90 line, the primer A-12 has the highest polymorphic capacity.

Between regenerants and the line F 105-90, we recorded differences at the level of alleles of the locus A-12, with a frequency of 30%, beside a high intrapopulational variability of 21.85 %.

Only four of the five primers **RAPD** tested generated polymorphism in the regenerators of the line **F 105-90**. The polymorphism rate presented values of 50 %, in the case of the G-16 and L-14, primers and 75%, in the case of the G-06 and G-10 primers (table 1).

The discriminating power of the primers analyzed ranged between 0.189, in the L-14 primer, and 0.298, in the G-06 primer, the mean being 0.239.

The discriminating index had values between 0.727, in the case of the G-10 primer, and 1.789, in the case of the G-06 primer, which proved the highest polymorphic capacity in the regenerants of the F 105-90 line.

Between regenerants and the F 105-90 line, we recorded differences at the level of alleles of the G-06 locus, which had a frequency of 52%, associated with a very high intrapopulational variability. On the contrary, in the case of the alleles of loci G-16 and L-14, we recorded the highest genetic similarity (73.40%), on the background of a high interindividual variability. The high discriminating capacity of the G-06 primer was associated with a high polymorphism rate and with an average genetic similarity.

Table 2

Polymorphism rate for the regenarants of Sigma cultivar using ISSR and RAPD primers

No. crt.	Primer	Nucleotide sequence(5'-3')	Bands No.		Polymor-ph %	Bands (pb)	PIC $\bar{x} \pm s_{\bar{x}}$	PI	Genetic similarity coef.	
			Total	Polymorph					$\bar{x} \pm s_{\bar{x}}$	$s_{\%}$
1	A-12	(GA)6CC	10	6	60,00	350-1150	0,476±0,067	2,857	0,644±0,049	28,24
2	A-13	(GT)6CC	13	5	38,46	350-750	0,173±0,041	0,867	0,900±0,028	11,53
3	L -12	GGGCGGTACT	6	5	83,33	300-1200	0,269±0,048	1,347	0,771±0,046	22,41

The results of the assessment of regenerants originating in explants derived from the **Sigma** variety showed that only A12 (60%) and A13 (38.46%) primers have generated a polymorphism rate that allowed the assessment of the genetic variability between the regenerants obtained by *in vitro* culture.

In the population of Sigma regenerants, the A-12 locus' alleles expressed a higher genetic diversity (25.6%) than the donor plant, associated with high intrapopulational variability as well (28.24%). On the contrary, the alleles of the locus A-13 manifested a high genetic similarity (90%), on the background of a medium interindividual variability (table 2).

In the population of the 14 regenerants originating in explants derived from the **Sigma** variety, the polymorphism was generated only as results of the amplification with L-12 primer.

The polymorphism rate was 83.33%, the discriminating capacity 0.269 and the polymorphic index 1,347. Compared with the donor plant, the regenerants' genetic similarity, at the level of locus L-12, was about 77.10% (table 2).

Table 3

Polymorphism rate for the regenarants of Selena cultivar using ISSR and RAPD primers

No. crt.	Primer	Nucleotide sequence	Bands No.		Polymor-ph %	Bands (pb)	PIC $\bar{x} \pm s_{\bar{x}}$	PI	Genetic similarity coef.	
			Total	Polymorph					$\bar{x} \pm s_{\bar{x}}$	$s_{\%}$
1	A-12	(GA)6CC	16	12	75,00	320-1200	0,321±0,034	3,852	0,481±0,039	24,27
2	A-13	(GT)6CC	19	12	63,15	350-750	0,370±0,034	4,444	0,641±0,037	17,15
3	UBC-818	(CA)7G	19	9	47,36	330-1550	0,373±0,047	3,358	0,693±0,048	20,96
1	G -06	GTGCCTAACC	6	3	50,00	550-620	0,181±0,087	0,543	0,111±0,059	76,12
2	G -10	AGGGCCGTCT	5	4	80,00	400-980	0,259±0,062	1,037	0,750±0,049	23,57
3	G -16	AGCGTCCTCC	5	3	60,00	230-380	0,379±0,092	1,136	0,668±0,079	35,48
4	L -14	GTGACAGGCT	9	5	55,55	200-350	0,267±0,091	1,333	0,511±0,048	28,43

In order to assess the variability of the **Selena** regenerants, we used ISSR primers: A-12, A-13 and UBC-818. The polymorphism rates had values between 47.36%, in the case of the UBC-818 primer, and 75%, in the case of the A-12 primer (table 3). The discriminating power of the analyzed primers had much reduced amplitude of variation, with values comprised between 0.321 in A-12 and 0.373 in UBC-818, mean of 0.354. The discriminating index recorded values between 3.358, for the UBC-818 primer, and 4.444, for the A-13 primer, which had the highest polymorphic capacity in the regenerants of the Selena cultivar.

Between regenerants and the donor plant, we recorded differences at the level of alleles of the A-12 locus with a frequency of about 52%, on the background of a high intrapopulational variability of 24.27%. On the contrary, at the level of alleles of the locus UBC-818, the genetic similarity had high values (69.30%), beside an interindividual variability that was more reduced than for the previous locus. We may notice that the efficiency of the primer A-13 in the localization of variability among regenerants is associated with high levels of genetic similarity and total polymorphism.

In the case of *RAPD primers*, the rate of the polymorphism generated with the tested primers for the assessment of the genetic variability induced by *in vitro* culture had values between 50%, in the case of the G-06 primer, and 80%, in the case of the G-10 primer.

The highest value of the total polymorphism generated by a certain primer (PIC) was recorded in the case of the G-16 primer (0.379), while the discriminating power of the G-06 primer was more reduced (0.181) (table 3).

Conclusions

The results obtained proved a genetic polymorphism that was relatively higher at the level of the alfalfa regenerants studied and a good discriminating power of the RAPD and ISSR techniques. The RAPD and ISSR analyses may be used in the establishment of genetic differences, but polymorphism absence may be associated only with genetic stability.

The most conclusions could be drawn during this abstract. Our results proved a relatively high ADN polymorphism of the alfalfa studied regenerants and a better discriminating power of the ISSR marker system compared with RAPD.

Conclusions regarding the assessment of regenerants variability using ISSR and RAPD molecular markers:

- The results of polymorphism assessment at ADN level in somaclones and donor plants, with **ISSR** primers, showed major genotypic differences; the Sigma cultivar manifested the highest stability in the *in vitro* culture.
- Of the three ISSR primers studied, three generated polymorphism in the somaclones derived from the F 105-90 line and Selena cultivar (A-12, A-13, UBC-818) and two primers (A-12, UBC-818) in the somaclones of the Sigma cultivar.
- Of the five **RAPD** primers used, four generated polymorphism in the somaclones derived from the F 105-90 line and Selena cultivar (G06, G10, G16, L14), and only one primer (L12) in the somaclones of the Sigma cultivar.

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